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Single-Molecule Studies of DNA Motor Proteins Using Dual-Trap Optical Tweezers

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Single-Molecule Studies of DNA Motor Proteins Using Dual-Trap Optical Tweezers

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Physics

by

Damian Jorge delToro

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2015
The Dissertation of Damian Jorge delToro is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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__________________________________________

Chair

University of California, San Diego

2015
DEDICATION

I dedicate this dissertation to my family.
“…since men almost always walk the paths beaten by others and go about their actions by imitation, unable either wholly to keep the ways of others or to add to the virtue of those whom you imitate, a prudent man must always enter by the paths beaten by great men and imitate those who have been most excellent, so that, if his own virtue does not reach it, at least it might be able to yield some of its scent: and do like prudent archers who, the place where they intend to wound seeming too far, and knowing how far the virtue of their bow reaches, aim much higher than the destined place, not to reach such height with their arrow, but in order to be able to attain their design with the aid of such high aim.”

--- Niccolò Machiavelli, *The Prince (as translated by Angelo M. Codevilla)*

“I don't know anything, but I do know that everything is interesting if you go into it deeply enough.”

--- Richard Feynman
TABLE OF CONTENTS

Signature Page ........................................................................................................ iii
Dedication ............................................................................................................... iv
Epigraph ................................................................................................................ v
Table of Contents ................................................................................................ vi
List of Figures ....................................................................................................... xii
Acknowledgements ............................................................................................ xvii
Vita ....................................................................................................................... xix
Abstract of the dissertation ................................................................................. xx

Chapter 1  Introduction to Dual-Trap Optical Tweezers and Bacteriophages .......... 1
  1.1  Single Molecule Research and the Advantage of Optical Tweezers .......... 1
     1.1.1  Double-stranded DNA Bacteriophage Basics ................................. 3
     1.1.2  Lambda Phage Basics ................................................................. 6
  1.2  Optical Trapping Fundamentals and Typical Instrument Setup ................. 8
  1.3  Typical Experimental Procedures ........................................................... 11
  1.4  Outline of Research ................................................................................ 13
  1.5  References for Chapter 1 ......................................................................... 15

Chapter 2  Walker-A P-loop coordinates ATP hydrolysis with motor output in viral DNA packaging ................................................................. 21
  2.1  Abstract .................................................................................................... 21
  2.2  Introduction .............................................................................................. 22
      2.2.1  Genetic analysis ............................................................................ 28
      2.2.2  Biochemical Analyses .................................................................... 33
          2.2.2.1  Folding and Self-Assembly of the Mutant Terminase Enzymes . 34
          2.2.2.2  DNA Maturation Activities ....................................................... 36
          2.2.3  Solution Ensemble DNA Packaging and ATPase Activities ...... 40
  2.3  Results and Discussion of Single-molecule Analyses ............................... 45
      2.3.1  Comparison with genetic findings .................................................. 46
      2.3.2  Residues involved in ATP binding ................................................ 47
      2.3.3  Implications of motor pausing and slipping .................................... 48
      2.3.4  Residues involved in mechanochemical coupling ......................... 53
      2.3.5  Motor processivity ........................................................................ 56
      2.3.6  Motor pausing ................................................................................ 56
      2.3.7  Initiation of packaging .................................................................... 58
  2.4  Final Discussion/Conclusion ...................................................................... 60
  2.5  Acknowledgements ................................................................................... 68
  2.6  Materials and Methods (Biophysical) ....................................................... 68
      2.6.1  Reagents ....................................................................................... 68
      2.6.2  Optical Tweezers Measurements .................................................. 69
      2.6.3  Analysis ......................................................................................... 70
  2.7  Appendix (microbiology and biochemical methods) ................................. 73
Chapter 3  Mechanism of Termination of Bacteriophage DNA Packaging Investigated with Optical Tweezers  ................................................... 82
3.1  Abstract ........................................................................ 82
3.2  Introduction .................................................................... 82
3.2.1  Lambda Phage DNA Packaging Phase ......................... 83
3.3  Results and Discussion .................................................. 84
3.3.1  Motivation and Results of lambda Pre-stall Packaging Assay 84
3.3.2  The Three Models and Experimental Hurdles ................. 91
3.3.2.1  DNA Construct Designs ........................................... 93
3.3.3  Control Experiments .................................................. 95
3.3.3.1  cos\(^{021}\) Termination Control Experiment .................. 95
3.3.4  Testing the Velocity Model........................................... 97
3.3.4.1  Results ................................................................ 99
3.3.5  Testing the Energy-Monitor Model .............................. 100
3.3.6  Testing the Capsid-Filling Model ................................. 101
3.4  Conclusion ................................................................. 102
3.5  Acknowledgements ...................................................... 103
3.6  Materials and Methods .................................................. 104
3.6.1  Optical Tweezers Instrument ...................................... 104
3.6.2  Production of lambda Terminase and Procapsids ............ 104
3.6.3  DNA Constructs ....................................................... 104
3.6.3.1  10kb lambda Packaging Construct ............................ 104
3.6.3.2  Lambda Termination Construct ................................. 104
3.6.4  Pre-Stalled Packaging Protocol .................................... 106
3.6.5  Tweezer Assay for Pre-Stalled Phage Complexes ............ 106
3.6.6  Identification of the cos\(^{021}\) Site Position in the Tweezers Assay 107
3.7  References for Chapter 3 .............................................. 108

Chapter 4  Building High Resolution Dual-Optical Trap Tweezers .............. 112
4.1  Abstract ................................................................. 112
4.2  Introduction ............................................................. 112
4.3  Experimental and System Design Background and Implementation of Design 114
4.3.1  Background: Dual-trap Optical Tweezers in a Nutshell .......... 114
4.3.2  Building Details of the Higher Resolution Dual-Trap Optical Tweezers ... 115
4.3.3  Background: Environmental Noise Considerations .............. 116
4.3.4  Accounting for Environmental Noise/Drift ...................... 117
4.3.5  Background: Measurement Noise Considerations ............. 119
4.3.6  Accounting for Measurement Noise Considerations .......... 122
4.3.7  Other Measures Taken to Improve the System Design .......... 124
4.4  Results ................................................................. 125
4.4.1  Reduced Noise for New Single Detection System ............... 126
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4.2</td>
<td>Greater Resolution with Dual Detection</td>
<td>128</td>
</tr>
<tr>
<td>4.5</td>
<td>Conclusion</td>
<td>131</td>
</tr>
<tr>
<td>4.6</td>
<td>Acknowledgements</td>
<td>132</td>
</tr>
<tr>
<td>4.7</td>
<td>Materials and Methods</td>
<td>132</td>
</tr>
<tr>
<td>4.7.1</td>
<td>Measuring system noise</td>
<td>133</td>
</tr>
<tr>
<td>4.7.2</td>
<td>Dual detection tests</td>
<td>133</td>
</tr>
<tr>
<td>4.8</td>
<td>References for Chapter 4</td>
<td>134</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>Accurate measurement of force and displacement with optical tweezers using DNA molecules as metrology standards</td>
<td>137</td>
</tr>
<tr>
<td>5.1</td>
<td>Abstract</td>
<td>137</td>
</tr>
<tr>
<td>5.2</td>
<td>Motivation in the Context of Dissertation Research</td>
<td>137</td>
</tr>
<tr>
<td>5.3</td>
<td>Introduction</td>
<td>138</td>
</tr>
<tr>
<td>5.4</td>
<td>Investigating Original DNA Metrology Calibration Method</td>
<td>140</td>
</tr>
<tr>
<td>5.5</td>
<td>Methods and Results of New DNA Metrology Calibration Technique</td>
<td>143</td>
</tr>
<tr>
<td>5.6</td>
<td>Conclusion</td>
<td>149</td>
</tr>
<tr>
<td>5.7</td>
<td>Acknowledgements</td>
<td>149</td>
</tr>
<tr>
<td>5.8</td>
<td>References for Chapter 5</td>
<td>149</td>
</tr>
<tr>
<td>Chapter 6</td>
<td>Bacteriophage Lambda DNA Packaging Step Size</td>
<td>152</td>
</tr>
<tr>
<td>6.1</td>
<td>Abstract</td>
<td>152</td>
</tr>
<tr>
<td>6.2</td>
<td>Introduction</td>
<td>152</td>
</tr>
<tr>
<td>6.3</td>
<td>Results and Discussion</td>
<td>155</td>
</tr>
<tr>
<td>6.4</td>
<td>Conclusion</td>
<td>162</td>
</tr>
<tr>
<td>6.5</td>
<td>Acknowledgements</td>
<td>162</td>
</tr>
<tr>
<td>6.6</td>
<td>Materials and Methods</td>
<td>162</td>
</tr>
<tr>
<td>6.7</td>
<td>References for Chapter 6</td>
<td>163</td>
</tr>
<tr>
<td>Chapter 7</td>
<td>Dynamics of DNA Reannealing Protein HARP</td>
<td>166</td>
</tr>
<tr>
<td>7.1</td>
<td>Abstract</td>
<td>166</td>
</tr>
<tr>
<td>7.2</td>
<td>Introduction</td>
<td>167</td>
</tr>
<tr>
<td>7.3</td>
<td>Results and Discussion</td>
<td>171</td>
</tr>
<tr>
<td>7.3.1</td>
<td>Novel Single-Molecule DNA Unzipping Construct</td>
<td>171</td>
</tr>
<tr>
<td>7.3.1.1</td>
<td>Construction and Results</td>
<td>171</td>
</tr>
<tr>
<td>7.3.1.2</td>
<td>Limitations</td>
<td>179</td>
</tr>
<tr>
<td>7.3.1.3</td>
<td>Generalizability of Method</td>
<td>181</td>
</tr>
<tr>
<td>7.3.2</td>
<td>HARP Acting on Bare Forked DNA</td>
<td>182</td>
</tr>
<tr>
<td>7.3.2.1</td>
<td>Evidence that HARP Impedes Unzipping</td>
<td>182</td>
</tr>
<tr>
<td>7.3.2.2</td>
<td>Evidence that HARP Resists and Temporarily Sustains High Forces</td>
<td>184</td>
</tr>
<tr>
<td>7.3.2.3</td>
<td>Evidence of Forcible Rezipping by HARP</td>
<td>186</td>
</tr>
<tr>
<td>7.3.2.4</td>
<td>Evidence that HARP Slowly Rezips DNA</td>
<td>188</td>
</tr>
<tr>
<td>7.3.2.5</td>
<td>Slow Rezipping Character in ATP</td>
<td>192</td>
</tr>
<tr>
<td>7.3.2.6</td>
<td>Slow Rezipping Character in UTP</td>
<td>195</td>
</tr>
<tr>
<td>7.3.3</td>
<td>RPA Acting on Forked DNA</td>
<td>197</td>
</tr>
<tr>
<td>7.3.3.1</td>
<td>RPA Coats ssDNA Rapidly</td>
<td>198</td>
</tr>
<tr>
<td>7.3.3.2</td>
<td>Force Assisted Unzipping by RPA</td>
<td>200</td>
</tr>
<tr>
<td>7.3.4</td>
<td>HARP and RPA Acting Together on Forked DNA</td>
<td>203</td>
</tr>
</tbody>
</table>
Chapter 8 Appendix ........................................................................................................... 216

8.1 Section 1: Overview and Design, Requirements, Rational for Chosen Equipment and Optics, and Construction Notes ........................................................................ 216

8.1.1 Overview and Design ............................................................................................ 216
8.1.1.1 Basic Design ...................................................................................................... 216
8.1.1.2 Basic Experiment .............................................................................................. 217
8.1.2 Important Requirements ...................................................................................... 217
8.1.2.1 Beam Transmission Criteria ........................................................................... 217
8.1.2.2 Lens Orientation Criteria ................................................................................. 217
8.1.2.3 Beamsplitter Criteria ...................................................................................... 217
8.1.3 Optical Height ........................................................................................................ 217
8.1.4 Mounting Pedestals .............................................................................................. 218
8.1.4.1 Custom Pedestals ............................................................................................ 218
8.1.4.2 Other Pedestals .............................................................................................. 218
8.1.4.3 Mount Spacers ............................................................................................... 219
8.1.5 Optical Mounts/Stages ......................................................................................... 219
8.1.5.1 Lens Mounts .................................................................................................... 219
8.1.5.2 Mirror Mounts ............................................................................................... 219
8.1.5.3 Polarizing Beam Splitting Cube Mounts ......................................................... 220
8.1.5.4 PSD Mounts ................................................................................................... 220
8.1.5.5 Objective Lens Stages .................................................................................... 220
8.1.6 Magnification, Objectives, & Operating Range .................................................... 221
8.1.6.1 Magnification .................................................................................................. 221
8.1.6.2 Objectives ...................................................................................................... 222
8.1.6.3 Back Focal Plane ............................................................................................ 225
8.1.6.4 Approximate Lateral Working Range within Flow Cell ................................. 227
8.1.7 Lenses .................................................................................................................. 228
8.1.7.1 Telescope Lenses ............................................................................................ 229
8.1.7.2 Specimen Imaging Lenses .............................................................................. 229
8.1.7.3 Bead Deflection Interference Pattern Imaging Lens ...................................... 230
8.1.8 Mirrors .................................................................................................................. 230
8.1.8.1 Movable Mirror ............................................................................................. 230
8.1.8.2 Stationary Mirror Opposing Movable Mirror .............................................. 230
8.1.8.3 Dichroic Mirrors ............................................................................................. 231
8.1.8.4 Imaging Mirrors .............................................................................................. 231
8.1.9 Polarizing Beam Splitters ...................................................................................... 231
8.1.10 Attenuator and Filters ................................................................. 232
8.1.11 Optical Table................................................................................. 233
8.1.12 Beam Confinement Tubing............................................................. 233
8.1.13 Custom Enclosures....................................................................... 233
8.1.14 Other Equipment and Construction Notes ................................. 234
  8.1.14.1 Equipment Table .................................................................... 234
  8.1.14.2 Lab Counter Adjacent to Equipment table................................. 234
  8.1.14.3 Laser and Laser Settings .......................................................... 235
  8.1.14.4 Laser Water Cooler ................................................................. 235
  8.1.14.5 Opto-Isolator ......................................................................... 235
  8.1.14.6 Low Pass Filter ....................................................................... 236
  8.1.14.7 Power Conditioner ................................................................. 236
  8.1.14.8 Beam Steering ......................................................................... 237
  8.1.14.9 Imaging LED ........................................................................... 238
  8.1.14.10 Imaging Camera ..................................................................... 238
  8.1.14.11 Television ............................................................................. 238
8.1.15 Position Sensing Detector (PSD) ............................................... 238
8.1.16 Fluidics ....................................................................................... 239
8.1.17 Flow Cell .................................................................................... 240
8.2 Section 2: Construction Procedure, Methods, and Details .......... 240
  8.2.1 Initial Comments ......................................................................... 240
  8.2.2 Building Procedure .................................................................... 241
    8.2.2.1 Laser Mounting and Initial Alignment .................................... 241
    8.2.2.2 Positioning the opto-isolator ................................................. 243
    8.2.2.3 Positioning and Alignment of Telescope #1 Lenses............... 244
    8.2.2.4 Positioning and Aligning the First Beam Splitter ................. 246
    8.2.2.5 Positioning and Aligning of the MCL Stage Mirror ............ 247
    8.2.2.6 Positioning and Aligning the Second Beam Splitter .......... 248
    8.2.2.7 Positioning and Aligning the Mirror for the Stationary Trap .. 248
    8.2.2.8 Readjusting the Height of the Movable Beam ................. 249
    8.2.2.9 Double Checking the Lateral Alignment of the Stationary Mirror .... 250
    8.2.2.10 Positioning and Alignment of Dichroic Mirror #1 ............ 250
    8.2.2.11 Positioning and Alignment of Telescope #2 Lenses .......... 251
    8.2.2.12 Positioning and Aligning Objective Stage #1 .................. 252
    8.2.2.13 Checking the Trapping Ability and Strength ................... 255
    8.2.2.14 Positioning and Aligning Objective Stage #2 ............... 257
    8.2.2.15 Collimating the Beam after Stage #2 ............................. 258
    8.2.2.16 Positioning and Aligning Dichroic Mirror #2 and the Imaging Optics 259
    8.2.2.17 Positioning and Aligning the Last PBSC ........................ 263
    8.2.2.18 Positioning and Aligning the Last Lens (Imaging BFP of Objective #2) 263
    8.2.2.19 Positioning and Aligning the PSD ..................................... 264
    8.2.2.20 Final Comments ................................................................. 264
8.3 Addendum – Installation of Second PSD for Differential Detection .... 266
8.3.1 Components and Instrumentation ................................................................. 266
  8.3.1.1 Position Sensing Detector (PSD), Attenuator, and Filter ................. 266
  8.3.1.2 Mirrors, Mounts, and Pedestals ......................................................... 267
  8.3.1.3 PSD Wiring To DAQ Card .................................................................. 267
8.3.2 Building Procedure ....................................................................................... 267
  8.3.2.1 Positioning and Aligning PBSC ......................................................... 267
  8.3.2.2 Positioning and Aligning the Imaging Lens and PSD for Stationary
          Trap ........................................................................................................ 269
  8.3.2.3 Positioning and Aligning the Mirror for Movable Trap ...................... 269
  8.3.2.4 Positioning and Aligning the Imaging Lens for Movable Trap ......... 269
  8.3.2.5 Positioning and Aligning PSD for Movable Trap .............................. 270
8.4 Additional Figures ......................................................................................... 270
8.5 References for Chapter 8 ............................................................................... 313
LIST OF FIGURES

Figure 2.1: Basic summary diagram of lambda bacteriophage DNA packaging maturation pathways from our collaborators .......................................................... 23

Figure 2.2: Impairments in viral assembly caused by residue changes in the putative Walker A motif in the DNA translocation ATPase of the phage lambda large terminase subunit ...................................................................................................... 30

Figure 2.3: Assessment of Thermal Stability, Folding and Self-Assembly ................................. 35

Figure 2.5: Kinetic Analysis of Packaging and ATPase Activities ........................................ 41

Figure 2.6: Translocation dynamics. Measurements of length of DNA packaged vs. time for WT and mutant terminases under a 5 pN applied load at low procapsid filling ........ 50

Figure 2.7: Average DNA translocation rates ........................................................................ 52

Figure 2.8: Frequencies of pausing and slipping .................................................................. 53

Figure 2.9: Mean pause durations ......................................................................................... 57

Figure 2.10: Efficiencies of initiation of packaging ................................................................ 59

Figure 2.11: Proposed TerL DNA Translocation Hydrolysis Cycle ........................................ 62

Figure 2.12: A homology model of the λ TerL, N-terminal, packaging ATPase site was created with I-TASSER ......................................................................................... 67

Figure 3.1: A cartoon schematic of the lambda DNA phage cos site ........................................ 84

Figure 3.2: Schematics of phage packaging tweezer assays for a dual optical trapping system ................................................................................................................................. 86

Figure 3.3: Example packaging traces using pre-stalled assay and the short lambda termination construct .......................................................................................................................... 88

Figure 3.4: Cartoons of the lambda Termination DNA Constructs .......................................... 94

Figure 3.5: Examples of slowed lambda packaging traces (tether length in kilo base pairs Vs time in seconds) resulting from the use of the lambda termination construct ....... 100

Figure 4.4.1: The new higher resolution dual-trap optical tweezers setup with single trap detection only .............................................................................................................................. 126

Figure 4.2: Displacement measurement of a 2µm diameter bead in the stationary trap of our old and new single detection dual-trap optical tweezers systems ...................................... 127

Figure 4.3: Resolution comparison of our new laser tweezers using single detection (red traces) vs dual detection (blue traces) methodology ................................................................. 130

Figure 5.1: Experimental geometry for DNA force-extension measurement in a dual optical trap system ................................................................................................................................. 140

Figure 5.2: Dependence on measurement parameters of the quality of fits of the WLC model to averaged force-extension measurements with 10.1 and 13.7 kbp DNA molecules ................................................................................................................................. 142
Figure 5.3 .................................................................................................................................................. 144
Figure 5.4 .................................................................................................................................................. 147
Figure 6.1: Comparison of lambda DNA packaging sections that do and do not exhibit stepping behavior .......................................................................................................................... 157
Figure 6.2: Examples of λ phage DNA translocation that occurs in ~5bp steps. .................. 159
Figure 7.1: Cartoon of the dual labeled general unzipping construct designed for single-molecule experiments ........................................................................................................................................ 172
Figure 7.2: Procedure for unzipping DNA using dual-trap optical tweezers and the force vs. trap displacement trace resulting from completely unzipping a 10.7kb unzipping construct .................................................................................................................................. 175
Figure 7.3: Comparison of a force-displacement 10.7 kb unzipping trace with a 10kb dsDNA stretching trace .......................................................................................................................................................................................... 178
Figure 7.4: Force vs. trap displacement trace of unzipping DNA in the presence of HARP .................................................................................................................................................................................. 183
Figure 7.5: Force vs. Time trace of an unzipping construct exhibiting impeded unzipping in the presence of HARP .............................................................................................................................................. 185
Figure 7.6: Example force vs trap displacement and force vs time traces for a test trial of forcible rezipping by HARP .......................................................................................................................................................... 186
Figure 7.7: Force vs trap displacement plots of unzipping and rezipping DNA in the presence of 80nM HARP and 1.5mM ATP ........................................................................................................................................................................ 189
Figure 7.8: Force vs time example trace of slow rezipping in the presence of HARP (80nM) and ATP (1.5nM) ........................................................................................................................................................................... 192
Figure 7.9: Force vs time trace of one event collected in which the slow force rise, measured during step 4, rose above the unzipping plateau .......................................................................................... 195
Figure 7.10: Force vs time example traces of all 5 steps of the measurement mode used in this section ....................................................................................................................................................................... 196
Figure 7.11: Unzipping DNA in the presence of RPA (3nM). .................................................................. 199
Figure 7.12: Force vs trap displacement of unexpected unzipping impedance observed during unzipping/rezipping/unzipping DNA measurement mode in the presence of RPA. .............................................................................................................. 202
Figure 7.13: Cartoon of a proposed RPA-Forked DNA structure that might explain the occasional unexpected impeded unzipping observations ........................................................................ 203
Figure 7.14: Force vs trap displacement traces from a combined RPA & HARP experimental procedure ........................................................................................................................................................................ 205
Figure 8.1: Laser beam incident on an angled mirror ......................................................................... 221
Figure 8.2: Diagram showing the position of the back focal plane for the Olympus objectives used in this dissertation ........................................................................................................................................... 224
Figure 8.3: Diagram showing that rotations of a beam at the back focal plane result in linear translations of the focal point of the objective focused beam ........................................... 226

Figure 8.4: Diagram showing that mirror rotations cause reflected light to rotate by twice that of the mirror rotation angle .......................................................... 227

Figure 8.5: View of PSD housing from above with indication of the position of the detector surface ........................................................................................................... 239

Figure 8.6: Simple cartoon image of optical tweezers layout used here (without second detector) ........................................................................................................... 271

Figure 8.7: Detailed cartoon image of optical tweezers layout used here (without second detector) ........................................................................................................... 272

Figure 8.8: Custom mount design for MCL mirror stage ........................................... 273

Figure 8.9: Custom mount design for opto-isolator .................................................. 274

Figure 8.10: Custom V-block mount design for clamping the laser head .................. 275

Figure 8.11: Additional view of custom V-block mount design for mounting the laser head ........................................................................................................... 276

Figure 8.12: Foam board tool. This tool is used for aligning vertical and lateral positions of the laser beam .................................................................................................. 276

Figure 8.13: Diagram for building step 1 of section 8.2.2 ........................................... 277

Figure 8.14: Methods for rotating and translocating the laser head V-block mount for alignment ........................................................................................................... 278

Figure 8.15: Diagram for building step 2 of section 8.2.2 ........................................... 279

Figure 8.16: Custom pedestal alignment tool ............................................................ 280

Figure 8.17: Diagram for building step 3 of section 8.2.2 ........................................... 281

Figure 8.18: 2nd diagram for building step 3 of section 8.2.2 ..................................... 282

Figure 8.19: Diagram for building step 4 of section 8.2.2 ........................................... 283

Figure 8.20: Diagram for building step 5 of section 8.2.2 ........................................... 284

Figure 8.21: 2nd diagram for building step 5 of section 8.2.2 ..................................... 285

Figure 8.22: Diagram for building step 6 of section 8.2.2 ........................................... 286

Figure 8.23: Diagram for building step 7 of section 8.2.2 ........................................... 287

Figure 8.24: 2nd diagram for building step 7 of section 8.2.2 ..................................... 288

Figure 8.25: 3rd diagram for building step 7 of section 8.2.2 ...................................... 289

Figure 8.26: Diagram for building step 8 of section 8.2.2 ........................................... 290

Figure 8.27: Diagram for building step 9 of section 8.2.2 ........................................... 291

Figure 8.28: Diagram for building step 10 of section 8.2.2 ....................................... 292

Figure 8.29: Diagram for building step 11 of section 8.2.2 ....................................... 293
Figure 8.30: 2nd diagram for building step 11 of section 8.2.2 ........................................ 294
Figure 8.31: Diagram for building step 12 of section 8.2.2 ........................................ 295
Figure 8.32: 2nd diagram for building step 12 of section 8.2.2 ........................................ 296
Figure 8.33: Custom stage alignment tool ................................................................. 297
Figure 8.34: 3rd diagram for building step 12 of section 8.2.2 ................................. 298
Figure 8.35: 4th diagram for building step 12 of section 8.2.2 ................................. 299
Figure 8.36: Diagram for building step 13 of section 8.2.2 ........................................ 300
Figure 8.37: Diagram for building step 14 of section 8.2.2 ........................................ 301
Figure 8.38: Diagram for building step 16 of section 8.2.2 ........................................ 302
Figure 8.39: 2nd diagram for building step 16 of section 8.2.2 ................................. 303
Figure 8.40: 3rd diagram for building step 16 of section 8.2.2 ........................................ 304
Figure 8.41: Diagram for building step 17 of section 8.2.2 ........................................ 305
Figure 8.42: Diagram for building step 18 of section 8.2.2 ........................................ 306
Figure 8.43: Diagram for building step 19 of section 8.2.2 ........................................ 307
Figure 8.44: Simple cartoon image of optical tweezers layout used here (with second detector) ................................................................. 308
Figure 8.45: Detailed cartoon image of optical tweezers layout used here (with second detector). ................................................................. 309
Figure 8.46: Diagram for building step 1 of section 8.3.2 ........................................ 310
Figure 8.47: Diagram for building step 3 of section 8.3.2 ........................................ 311
Figure 8.48: Diagram for building step 4 of section 8.3.2 ........................................ 312
LIST OF TABLES

Table 2.1: Examples of known and putative WA motif sequences for the translocation ATPase in viral packaging motor proteins................................................................. 26
Table 2.2: Metrics of DNA translocation activity .......................................................... 33
Table 2.3: Model Parameters for Single Turnover Kinetic Data .................................. 38
Table 2.4: DNA Maturation and Genome Packaging Activity Relative to WT .......... 43
Table 2.5: Steady State ATPase Activity...................................................................... 45
Table 7.1: Length details of four unzipping constructs prepared for single-molecule experiments by our new method. ................................................................. 173
Table 8.1: Details of Olympus objectives used in this system ................................. 222
Table 8.2: How to calculate the diameter of the back aperture of an Olympus objective. ......................................................................................................................... 223
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ABSTRACT OF THE DISSERTATION

Single-Molecule Studies of DNA Motor Proteins Using Dual-Trap Optical Tweezers

by

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In this dissertation we take advantage of the real-time manipulating, high force generating, and small displacement measuring abilities of dual-trap optical tweezers to investigate properties and mechanisms of motor enzymes that act on DNA, including the DNA translocation motor of bacteriophage lambda and the proposed ATP-driven annealing helicase HARP. Through a combined approach of genetic mutagenesis,
biochemical analysis, and single-molecule analysis we reveal potential functional roles of the residues residing in the ATP phosphate binding motif (Walker A) of phage lambda’s large terminase subunit. Direct DNA packaging measurements of phages with mutated residues within this motif reveal a range of impaired translocation phenotypes. Analysis of the slipping and pausing exhibited by mutant enzymes suggests that residues A78, R79, and V80 mediate coupling between ATP binding/hydrolysis and DNA binding/translocation while implicating residues A78, R79, V80, and G81 in proper ATP alignment for hydrolysis. The combined findings of each analysis also implicate residue R79 in ADP release and triggering ATP hydrolysis. In other studies, initial investigation into the mechanism of termination of DNA packaging in phage lambda provides evidence against a velocity-monitoring model. A higher resolution optical tweezers system was designed and constructed and a new and improved calibration method was developed and applied to initial high-resolution measurements of DNA translocation. The use of environmental and measurement noise reduction techniques and improved optical alignment procedures have yielded tweezers with approximately nanometer spatial resolution. The calibration technique, which optimizes fits of the worm-like chain model to DNA force-extension curves, yields calibration with at least a 7-fold improvement in accuracy over our prior method. The use of these tools in measuring lambda packaging yield preliminary evidence for discrete translocation steps. Lastly, tweezer measurements of HARP interactions with forked DNA provide supporting evidence that HARP is a forked-DNA binding protein that resists DNA unzipping and is not an unwinding helicase. In addition preliminary measurements suggest that this protein forcibly reanneals DNA, prevents reannealing, resists high forces, slows reannealing in an ATP
dependent manner, and is recruited to bind to forked DNA with replication protein A coated single-stranded DNA.
Chapter 1

Introduction to Dual-Trap Optical Tweezers and Bacteriophages

1.1 Single Molecule Research and the Advantage of Optical Tweezers

Single-molecule biophysics research is a relatively small field that resulted from the advent of numerous investigative tools that are capable of probing biological systems one molecule at a time with small scale forces and distances. The method of optical trapping was first developed into a tool for biological research nearly 30 years ago. The groundbreaking results from the use of this tool has motivated innovation that has led to great advancements in optical trapping, development of single-molecule assays for atomic force microscopy (AFM), and the creation of many other single-molecule probing tools and techniques, which include (among others): single-trap and dual-trap optical tweezers, magnetic tweezers, and fluorescence resonance energy transfer (FRET). Over years of development, each of these tools and their assays have been designed and refined to measure and apply physical quantities (e.g. forces and distances), thus making them optimal for investigating the physics of biological molecules.

Because single-molecule research has contributed significantly to the fundamental understanding of various biological systems, these techniques have become widely known among much of the scientific community. However, the expense of the equipment as well as the difficulty in building the instrumentation, developing working \textit{in vitro} protocols, performing experiments, and analyzing the data has prevented such techniques from becoming ubiquitous.
One advantage these tools offer is the ability to observe and measure the behavior and activity of a single molecule whereas conventional biological bulk techniques yield the average behavior of an ensemble of molecules. Modern versions of these tools are capable of performing for a single molecule, in real time: displacement measurements, force measurements, force applications, structure manipulations, or some combination thereof\textsuperscript{10,11}. Such functionality has led scientists to uncover mechanical properties and mechanisms of proteins such as the transport motor myosin\textsuperscript{12,13}; the rotary motor F\textsubscript{1}-ATPase\textsuperscript{14}; dsDNA translocation motors like lambda, T4, and φ29 bacteriophage terminases\textsuperscript{15-18}; and topoisomerases\textsuperscript{19,20}. Moreover, these capabilities have also led to findings about the folding and stretching response of molecules such as RNA, DNA, and various proteins\textsuperscript{21-27}.

For the work presented in this dissertation, we use dual-trap optical tweezers, which has the advantage of being able to perform \textit{all} the functions and measurements listed in the previous paragraph. In particular, dual-trap optical tweezers have the ability to perform, in real time, molecular manipulations (e.g. on DNA and proteins) while applying and/or measuring piconewton forces and resolving tens of nanometer displacements. While AFMs and magnetic tweezers are also capable of performing similar operations, dual-trap optical tweezers are better suited for our experiments because they can probe smaller length scales; measure and apply forces common for a range of molecules (especially those studied here); resolve smaller displacements; utilize either active or passive force clamps (versus, for example, constant applied force by magnetic tweezers); be more specific about molecular attachments; and perform better molecular manipulations. Also, dual-trap optical tweezers have a well-defined
Here, we take advantage of these dual-trap optical tweezers operations and abilities to investigate properties and mechanisms of motor enzymes that act on DNA, including the proposed ATP-driven annealing helicase HARP and the DNA translocation terminase motor of bacteriophage lambda.

1.1.1 Double-stranded DNA Bacteriophage Basics

With an estimated $10^{31}$ viruses in the oceans alone, most of which are bacteriophages (bacteria viruses), viruses are recognized as the most populous biological entity on the planet. Many bacteriophages replicate by way of the lytic cycle wherein a phage binds (often via a tail) to specific receptors of a bacterial host and then injects its genome into the cell. Generally speaking, the DNA then takes control of the cell’s machinery and forces production of all the necessary viral components for creating new viruses. Subsequently viruses begin assembling from this soup of proteins and once the cell becomes full (typically containing ~100 - ~200 phages per cell), it is triggered to burst or lyse, thus releasing offspring capable of infecting other cells.

Many double-stranded DNA bacteriophages such as φ29, T4, and λ as well as human adenoviruses and herpesviruses proceed along an unusual assembly pathway during this lytic cycle. Specifically, such viruses use ATP to actively package their long genomes into a preformed, tightly confined, protein shell (procapsid), ultimately achieving a near-crystalline density. Such a procedure is particularly unusual because it requires packaging DNA against huge resistive forces: repulsive electrostatic forces (since DNA is negatively charged); forces resulting from a resistance to DNA bending (bending rigidity); and resistance against decreasing entropy (DNA prior to packaging is in a loose randomly coiled state of much higher entropy than when confined to the
capsid). This seemingly unfavorable pathway results in internal capsid pressures of several tens of atmospheres, which surpasses typical bacteria cytoplasmic pressures of a few atmospheres. It is suspected that such a process evolved, in part, to provide these viruses with sufficient pressure to drive their genome out of the capsid and into the cytoplasm of a host bacterium\textsuperscript{32-35}. Notably, such viruses have been found to package their genomes at rates of several hundreds of base pairs per second even under high external loads of several tens of piconewtons\textsuperscript{34-35}. This amounts to a motor with a power density that has been estimated to be greater than a typical automobile engine\textsuperscript{17}. In some cases, where viral DNA is replicated in the cell as a chain of multiple genomes (a concatemer)\textsuperscript{32}, it has been proposed that the buildup of the capsid pressure “informs” the motor complex how much DNA has been packaged; and consequently translocation is triggered to terminate and the motor enzyme switches into a DNA-cleaving mode when the pressure corresponding to capsid filling by a single genome length of DNA has been achieved\textsuperscript{36,37}.

Ultimately, it has been found that the motors that drive DNA into these small procapsids actually perform numerous functions which can include DNA binding, cleavage, and translocation; ATP hydrolysis; helicase-like activity; and force generation. Such functions are common among biological molecules and are often the subject of study in the field of molecular biology\textsuperscript{32-35}. The use of many biochemical analysis methods have helped uncovered numerous properties and functions of such molecules, however, they are often limited by their inability to synchronize reactions. Typical biochemical techniques consist of bulk experiments in which certain properties or behaviors are quantified from the activity of an ensemble of molecules. However,
initiation of their activity is stochastic and thus measurements from these experiments can only ever represent averages. Furthermore, biochemical techniques are unable to directly measure physical quantities such as displacements and forces.

Over the past 15 years single-molecule techniques have been used to investigate this viral DNA packaging process and have shed tremendous light on these motors and the mechanisms by which they work. Beyond this, these investigations have also revealed other aspects of phage assembly, such as DNA confinement and capsid expansion, and have added to the general understandings of viral evolution. Use of optical tweezers have allowed for direct real-time monitoring of viral DNA translocation. Such experiments with phages φ29, T4, and λ have demonstrated that viral packaging is dynamic, exhibiting pauses and slips during translocation, and varies not only between virus types but also between individual viral complexes. Such information has helped elucidate a wealth of detailed information about phage motors and their physical character, such as: force generating abilities; translocation rates; slipping and pausing behavior; translocation mechanisms and subunit coordination; step sizes; relationships between functional motifs; ATPase activity; DNA conformations inside procapsids; and capsid expansion (among many other features).\textsuperscript{15-18, 38-42} Not only do we learn from these studies how these particular phages assemble, but we also gain insight into how other viruses function as well as a better general understanding of how these functions are performed among all biological molecules. While the relatively small body of single-molecule bacteriophage work has greatly advanced our understanding of phage DNA packaging and viral assembly, there are still many unanswered questions. For example,
how do different virus types vary in their packaging and assembly mechanisms? How do motor subunits work together to translocate DNA and to generate such outstanding forces? How do different virus types trigger the completion of packaging? Or how do the ATPases of these motors translate chemical energy into mechanical motion? In this dissertation we present work performed using optical tweezers and phage λ in effort to answer some of these questions.

1.1.2 Lambda Phage Basics

The lambda (λ) virus is a double-stranded DNA (dsDNA) tailed bacteriophage that can replicate by either the lytic or lysogenic life cycle. It has an icosahedral prohead with a diameter of ~50nm prior to expansion and ~64nm after expansion. It has a linear genome that is 48,502 base pairs (bp) long with complementary 12-base single-stranded DNA (ssDNA) tails on either end. During replication, the injected λ genome first circularizes by annealing these tails and then uses the cell’s ligase proteins to seal the nicks. Afterward, utilizing the host’s machinery, the DNA is copied by rolling circle replication, which yields a concatemer of λ genomes. At the junction between each genome is a sequence called the cos site, which extends over ~200bp and is composed of three subsites: cosQ, cosN, and cosB. The first, cosQ, exists at the end of the genome (i.e. where packaging terminates) and is a site that, when recognized by the phage’s motor, triggers the arrest of packaging and cleavage of the DNA from the concatemer. cosN is the nicking site and is symmetrically positioned between genomes. During initiation or termination of packaging, the motor complex produces staggered nicks in the DNA on either strand at this site producing 12-base ssDNA 5’-overhang tails on the two genomes when separated. The cosB site comes just after
the \textit{cos}N site at the beginning of the genome (i.e. where packaging first begins) and is a site where the DNA packaging motor binds (typically during initiation)\textsuperscript{43,48}.

The λ DNA packaging motor is referred to as a terminase and is a multimeric protein composed of multiple subunits called protomers where each protomer is composed of one gpA protein and two gp\textit{v\textsubscript{1}} proteins\textsuperscript{43}. Before packaging initiates, these protomers typically assemble around the \textit{cos} site via interactions between the gp\textit{v\textsubscript{1}} subunits and the \textit{cos}B site. The resulting complex is a ringed structure that is wrapped around the DNA. This ring has been proposed to contain four protomer subunits but has also been suspected to have five, based on studies of the phi29 and T4 systems. This ring assembly is the terminase, which is known to perform many functions, including DNA binding, nicking, and translocation; ATP hydrolysis; and complementary strand separation activity\textsuperscript{43,49,50}. After the terminase forms at the \textit{cos} site, the gpA subunits nick the DNA at the \textit{cos}N site and separate the strands while remaining bound to the strand containing the \textit{cos}B site\textsuperscript{43,48}. A simple diagram of the \textit{cos} site can be found in chapter 3, Fig. 3.1. The ring of gpA subunits then bind to the portal proteins that are attached to a preformed capsid shell. ATP binds to the gpA subunits, and upon hydrolysis, the DNA is translocated into the prohead\textsuperscript{32,33,43,44}. The average motor velocity of translocation has been measured to be 600 bp/s\textsuperscript{16}. As packaging proceeds, the pressure builds within the capsid and consequently the packaging speed reduces\textsuperscript{16}. At the end of packaging, when the terminase recognizes the \textit{cos}Q site, packaging is arrested and the terminase is triggered to cleave the DNA at the \textit{cos}N site, after which the remaining terminase-DNA complex detaches from the filled prohead\textsuperscript{43-47}. This final step is referred to as termination. Assembly from here continues as broadly outlined in the previous section.
1.2 Optical Trapping Fundamentals and Typical Instrument Setup

Relatively shortly after the invention of the first laser, it was demonstrated that radiation pressure of a laser will impose a force on dielectric particles. This foundational work led to the development of the first single-beam gradient force optical trap in 1986, which utilized the radiation pressure of a tightly focused laser beam to impose focal-point directed forces on a dielectric particle, thus allowing the capture of such particles (Fig. 1.1a). This behavior is typically understood by two explanations, which depend on the length of the trapped object in relation to the laser wavelength. In the regime where the size of the trapped object is much greater than the laser wavelength, trapping is explained by momentum conservation (Fig. 1.1b). The laser light, which has momentum, is refracted as it passes through a dielectric particle and the refracted photons impart a momentum on the particle such that the net momentum is conserved. In other words, when a dielectric bead is displaced from the center of a trap by an external force, the light that passes through is deflected (as a result of refraction) and the resulting change in momentum of the light imposes a countering force (change in momentum over time) on the bead that is directed toward the focal point of the trap. Now, in the regime where the size of the trapped object is much less than the wavelength of the laser light, trapping is explained by gradient forces of the beam on the dielectric particle. A laser beam with a Gaussian intensity distribution will induce a dipole moment in a dielectric particle and consequently apply a focal point directed force on the dipole moment of this particle that is proportional to the gradient of the beam’s intensity distribution, i.e. $F \sim \alpha \nabla I$ (Fig. 1.1c). Here $I$ is the intensity distribution of the laser in the specimen plane of the experimental chamber and $\alpha$ is the polarizability of the
dielectric particle. This relationship can be found by noting that the Lorentz force on a dipole moment $p$ in external electric and magnetic fields $E$ & $B$ is given by

$$F = (p \cdot \nabla)E + \frac{1}{c} \frac{dp}{dt} \times B$$

where $c$ is the speed of light. For a dielectric bead that is displaced from the center of a trap, the electric field of the Gaussian laser beam can induce a dipole moment within this bead (with polarizability $\alpha$) that points in the same direction as the electric field, which we express as $p = \alpha E$. For a continuous wave laser, $E$ and $B$ are unchanging in time, thus the cross product term is eliminated, leaving $F = (p \cdot \nabla)E = \nabla(p \cdot E) = \alpha \nabla E^2$. Since intensity goes like the time average of $E^2$, the time average of this results in a force imposed on the bead by the laser that is proportional to $\alpha \nabla I$, which results in the bead being pulled toward the region of highest laser intensity.

In order to minimize photo induced damage to the biological molecules being investigated, optical traps typically use near-infrared wavelengths (0.74 - 1.4 μm). Since these wavelengths are similar to the microsphere diameters typically used in optical trapping (~ 0.8 – 3 μm), the explanation for trapping must therefore be some combination of or balance between light scattering forces and gradient forces. However, the simple view of optical trapping is that a trapped microsphere behaves like a bead with a three dimensional spring restoring force.
Figure 1.1: Forces on a dielectric microsphere in an optical trap. a) Simple diagram of a dielectric microsphere displaced from the center of an optical trap. The arrow indicates the restoring force that is imposed on the bead by the trap and is directed toward the focal point. b) Example diagram of a dielectric microsphere that is laterally displaced from the focal point and the forces imposed by light that has been refracted as it passes through the microsphere. Rays A and B show examples paths photons take as they refract through a displaced bead. The forces imparted on the bead due to the change in momentum of these photons are indicated by $F_A$ and $F_B$. The sum of these forces yield a net restoring force $F_{\text{restore}}$ that forces the bead left toward the focal point. c) Diagram demonstrating forces imposed on dielectric microspheres due to gradient forces. The Gaussian distribution represents the intensity distribution of the laser light at the focal point. Here a bead is displaced to the right of the trap center. The electric field $E$ of the laser induces a dipole moment in the bead (in the same direction as $E$ for the conditions here). Consequently, the gradient of the intensity of the beam, which points toward the distribution center, imposes a force $F$ in the same direction.

A typical dual-optical tweezers instrument consists of a near-infrared (1064nm in our work) continuous laser whose beam is first split into two polarizations. The polarizations themselves are not important for trapping, but they allow two beams that originate from the same laser to be independently manipulated and recombined. One such beam is either reflected off a movable mirror or passed through an acousto-optic deflector (AOD) for beam steering. After the beams have been expanded, passed through
a beam steering device, and recombined, they are sent through a microscope objective
and focused within the experimental flow chamber (see section 1.3 and Fig. 1.2) to form
two optical traps. The chosen beam steering device can be used to translate the movable
traps relative to the stationary trap. The outgoing beam is then recaptured by another
microscope lens, which recollimates the beams. By imaging the back focal plane of the
downstream objective onto a position sensing detector (PSD), angular deflections of the
laser light due to displacements of a bead in the stationary trap can be measured and
subsequently converted with the proper calibration into bead displacement distances\textsuperscript{16-}
\textsuperscript{18,54-55}. Such displacements can result from the surrounding fluid or from some activity
occurring by a biological molecule/macromolecule that is tethered between beads in the
two traps. Moreover, these displacements can also be used to evaluate the forces the trap
is applying to the trapped bead when it is displaced. Additional spatial resolution can be
acquired if displacements of a bead in the movable trap are also measured by a second
PSD\textsuperscript{56}. Thorough descriptions of one dual-trap tweezers instrument used in this
dissertation can be found in chapter 4 and the dissertation appendix.

1.3 Typical Experimental Procedures

Experiments with a dual-trap optical tweezers instrument is typically performed
by first coating polystyrene microspheres with either a molecule to be investigated or
with a label for binding a given molecule. These microspheres are injected into the top
and bottom channels of an experimental flow cell chamber, which is a sandwich of two
thin glass microscope slides surrounding a layer of solid thermosetting epoxy. The epoxy
is cut such that, after assembly, three thin chambers are formed that can receive liquid
and reagents. Two incorporated capillary tubes (with inner diameters of approximately
25μm) are positioned to allow a stream of liquid and reagents from the top and bottom channels into the approximately 5mm wide central chamber (Fig. 1.2). The coated microspheres are injected into the top and bottom channels of this flow cell. Within the central chamber, in which a slow steady flow of buffer is running, the optical traps are moved relative to the flow cell to the spouts of the capillary tubes where microspheres are then captured by the traps. Once a single bead from each channel is acquired by the traps, the two beads are moved to an upstream location in the chamber, away from interference with the flow of injected beads.

**Figure 1.2:** Diagram of the chamber (Flow Cell) in which the experiments are conducted. This diagram is not to scale. It consists of two thin microscope slides sandwiching a layer of epoxy. Buffers are flowed into the holes on the left. In this diagram, streptavidin (SA) beads with an attached biotin and digoxigenin labeled DNA molecule are flowed into the top channel (The end of the DNA labeled with a biotin attaches to the SA bead.). Anti-Digoxigenin (AD) coated beads are flowed into the bottom channel. Thin capillary tubes connect the top and bottom channels to the central chamber, which direct beads into this central channel. Once beads are captured in the appropriate optical traps, they are moved to the left of the capillary tubes where the experiment is then performed.
Here, using a movable mirror or acousto-optic deflector, one of the traps is moved toward the other until a single molecule (or a single molecular complex) being investigated becomes tethered between the two trapped beads. Once done, the traps may be separated from one another to apply a stretching force to the tethered molecule or their positions can be held fixed so forces applied by the molecules can be measured. These are two simple measurement modes that can be performed. Additional measurement modes are discussed in the chapters that follow. Examples configurations used in this dissertation are (1) a biotin and digoxigenin labeled DNA strand that is tethered between a streptavidin coated and anti-digoxigenin coated microsphere, which bind to these DNA labels respectively (Fig. 1.2, Fig. 5.1 in chapter 5), (2) a bacteriophage prohead-motor-DNA(biotin labeled) complex that is tethered between an anti-prohead coated and a streptavidin coated microsphere (see Fig. 3.2 in chapter 3).

1.4 Outline of Research

The work in chapter 2 is the result of a collaboration between the labs of Professors Doug Smith, Michael Feiss, and Carlos Catalano. In this chapter we have used genetic, biochemical, and single-molecule assays to survey the residues within the Walker A site of the N-terminal subdomain in the λ phage terminase gpA protein subunit. This Walker A motif is responsible for binding the phosphate of an ATP, which when hydrolyzed, provides the terminase with energy to translocate DNA into the prohead. Through this work we identify and affirm roles that the residues within this site play in binding and hydrolyzing ATP as well as in coupling ATP binding to DNA binding.

Chapter 3 presents an improved optical tweezers assay used for experiments with the λ phage, which is a stalled pre-packaged assay. In addition, this chapter discusses a
project in which the mechanism of termination by phage λ is investigated. This project is a collaboration with the lab of Prof. Michael Feiss. Because the completion of this project awaits the development by the Feiss lab of a special DNA construct with a unique packaging initiation and termination sites, this chapter presents preliminary results and a thorough outline for how the study will be completed. In addition, since this experiment required especially accurate tracking of position along the genome during packaging, we were motivated to build a new tweezer instrument and create an improved calibration method that could allow us to more accurately and precisely determine positions, displacements, and forces, work that is described in chapters 4, 5, and the dissertation appendix.

Chapter 4 and the dissertation appendix describe a project to build a high resolution, dual detection, dual-trap optical tweezers instrument. Chapter 4 provides a broad overview of the methods and techniques implemented in building the instrument as well as some basic comparisons between this newly built system and the previous, lower resolution system. The dissertation appendix is a very thorough description of how we built this particular instrument and is intended to serve as a lab reference and guide for future students/users. It includes details about the equipment and rational used in building as well a description of the building procedure. This project was performed in collaboration with fellow lab graduate student Nicholas Keller.

Chapter 5 presents a novel, improved method we developed for calibrating the dual-trap optical tweezer instruments that was motivated by our discovery that the previous method had limited accuracy and that the studies described in chapters 3, 6, and 7 demanded an improved method.
After having built and calibrated the new instrument presented in chapter 4, we performed preliminary experiments aimed at determining the step size of the λ phage as a test of the new instrument and data analysis methods needed for the dual-trap detection system. These results are presented in chapter 6 and show that the system indeed has improved resolution.

Chapter 7 presents work performed as a "side project" in collaboration with the lab of Prof. James Kadonaga to explore whether single-molecule techniques could be applied to study the proteins known as HARP, reported to be an ATP-driven annealing helicase, and replication protein A (RPA). In this chapter we present (1) a novel, high yielding, and nearly generalizable method for constructing an unzipping DNA construct for use in single-molecule assays and (2) our preliminary findings from measurements with HARP and RPA acting on this unzipping construct. The initial findings indicated a need for an instrument with higher spatial/force resolution as well as one that exhibits lower drift, leading to the work described in chapters 4, 5, 6, and the dissertation appendix. The preliminary results obtained in these exploratory experiments suggest promising avenues for future studies, but pursuing these further was beyond the scope of this dissertation, the primary focus of which was studies of the mechanism of the phage lambda viral DNA packaging motor.

1.5 References for Chapter 1


Chapter 2

Walker-A P-loop coordinates ATP hydrolysis with motor output in viral DNA packaging

2.1 Abstract

During assembly of many viruses a powerful ATP-driven motor translocates DNA into a preformed procapsid. A Walker-A P-loop is proposed to coordinate ATP binding and hydrolysis with DNA translocation. We use a combination of genetic, biochemical, and biophysical techniques to survey the roles of Walker-A residues in the motor function of bacteriophage lambda. We identify 58 amino acid substitution mutations that abolish virus yield and 30 that cause varying reductions in yield. Almost all changes in predicted conserved residues K76, R79, G81, and S83 abolish virus yield. Biochemical analyses show that mutants R79A and S83A fold, assemble, and display genome maturation activity similar to wild-type, but exhibit little or no ATPase or DNA packaging activity. Kinetic DNA cleavage and ATPase assays suggest that change R79A also slows initial DNA binding. Single-molecule measurements detected no DNA translocation for K76A&R, while G81A and S83A exhibited strong impairments consistent with their predicted important roles in phosphate binding. We identified eight mutants with varying partly-impaired translocation phenotypes, which show that Walker-A residues play important roles in determining motor velocity, processivity, and pausing. The frequent longer pauses exhibited by A78V, R79K, V80A, and G81A suggests that these residues are important for proper ATP alignment for hydrolysis. Frequent slipping exhibited by A78V, R79K, and V80A suggests that these residues also mediate coupling
of ATP binding to DNA binding. Our findings support recent structural models implicating the Walker-A arginine and adjacent residues in mechanochemical coupling. An additional finding of this study is that the efficiency of initiation of packaging correlates strongly with motor velocity.

### 2.2 Introduction

Many double-stranded DNA viruses utilize a molecular motor during assembly to package their genomes into preformed procapsid shells. The genome is recognized, cut from a polymeric precursor, and translocated into the shell to a density approximating that of crystalline DNA. Measurements of single DNA molecule packaging with optical tweezers showed that these motors generate very high forces (>50 pN) and translocate DNA rapidly with high processivity (typically <1 slip per kbp packaged). Average translocation rates ranged from ~150 bp/s for phage phi29 up to ~700 bp/s for phage T4 (measured at low prohead filling, low force, saturating ATP, and room temperature). The phi29 motor was shown to translocate in coordinated bursts of four 2.5 bp steps coincident with phosphate release. These motors also exhibit occasional pauses, which are typically a few seconds in duration and occur roughly once per kbp of DNA packaged.

While much is understood about the function of these motors, their structural and biochemical mechanisms are not completely understood, and a number of different models have been proposed. The DNA translocase activity resides in the viral terminase enzyme. Terminases generally are heterooligomers of a small subunit (TerS) involved in genome recognition and a large subunit (TerL) with an N-terminal ATPase domain and C-terminal endonuclease domain. In the present work we study phage lambda. Its
terminase proteins assemble into protomers containing two TerS (gpNu1) and one TerL (gpA) subunits\textsuperscript{11}. In solution the protomer is in slow equilibrium with a tetramer of protomers that assemble into a ring-like complex\textsuperscript{12}. However, in the presence of IHF, four protomers will assemble at the lambda DNA \textit{cos} site and “mature” the genome end (i.e. cleaves and separates the DNA – complex I in Fig. 2.1) in preparation for binding to the portal vertex of a procapsid (complex II in Fig. 2.1), which is followed by DNA translocation. The tetrameric stoichiometry is retained in the post-cleavage complex (complex I) and is likely maintained in the packaging motor complex\textsuperscript{13,14} (Fig. 2.1).

**Figure 2.1:** Basic summary diagram of lambda bacteriophage DNA packaging maturation pathways from our collaborators

Atomic structures of the phage T4, Sf6, and P74-26 large subunits\textsuperscript{15-17} show that their ATPase center’s fold belongs to a large group of oligomeric translocases, the ASCE (Additional Strand Catalytic Glutamate) superfamily\textsuperscript{18,19}. This superfamily includes many cellular ATPases having diverse functions including protein unfolding and degradation; protein transport and translocation; ATP synthesis; and DNA recombination, unzipping,
transport, and translocation. Studies of viral terminases may therefore provide information that is broadly relevant to understanding many ATP-powered machines.

The core of the ATPase center is a five-stranded parallel β-sheet with α-helical segments connecting the β-strands. Two ATP-interacting amino acid segments, the Walker A (P-loop) and B segments, have characteristic sequences. The classical Walker A signature sequence is (G/A)XXXXGK(T/S)\textsuperscript{20}. Extensive studies show that the serine hydroxyl coordinates the Mg\textsuperscript{++} of Mg\textsuperscript{++}-ATP, and the lysine’s ε-amino group coordinates the β- and γ-phosphates of ATP, positioning them for hydrolytic attack\textsuperscript{21-28}. The Walker B segment, whose signature sequence is HHHHD, has four hydrophobic residues followed by an aspartate carboxyl group that also coordinates the Mg\textsuperscript{++} of the Mg\textsuperscript{++}-ATP complex\textsuperscript{20}. Together, the Walker A and B segments position the β- and γ-phosphates for nucleophilic attack by an activated water molecule provided by a glutamate carboxylate group. In terminases, evidence suggests that this glutamate is adjacent to the Walker B aspartate.

Upstream of Walker A are residues forming the adenosine binding pocket\textsuperscript{29}. For phage λ’s terminase, crosslinking studies by Hang et al (ref. [30]) identified an ATP-binding center in the N-terminus of the large subunit, gpA, by showing that residues Y46 and K84 were crosslinked by 8-azido-ATP. Non-conservative changes in these residues yielded no detectible DNA packaging or ATPase activity. Subsequent work further showed that mutant terminase with the conservative Y46F change showed only mild impairments of DNA packaging and packaging ATPase activities, indicating a strong coupling of the high-affinity ATPase activity to DNA translocation\textsuperscript{31}. Further evidence
for an N-terminal ATP-binding domain in lambda gpA was provided by a genetic screen for missense mutations producing endonuclease-competent and DNA packaging defective mutants. This screen identified a set of 10 mutations affecting this domain\textsuperscript{32}, one of which (K76R) is a predicted Walker-A residue.

Genetic and bioinformatic work by Rao and co-workers identified key components of the ATPase center of T4’s terminase subunit, gp17, including the adenine binding, Walker A and Walker B segments\textsuperscript{33,34}. Sequence alignments enabled prediction of the locations of these centers in the terminases of other DNA bacteriophages and identified an unusual Walker A sequence for \(\lambda\) and some other viral terminases, being 76-KSARVGYS-83 in the case of lambda (Table 2.1). Notably, this segment lacks the C-terminal lysine in the classical Walker A "GK(T/S)" sequence normally implicated in phosphate binding but has a lysine at the N-terminus of the P-loop that appears to be conserved among a subset of viral terminases (termed "Deviant I" or "N-lys" Walker A motifs)\textsuperscript{34}. Structural modeling lead Mitchell and Rao (refs. 33 & 34) to propose that this lysine, instead of the one in the classical position, coordinates the \(\beta\)- and \(\gamma\)-phosphates and that S83 (in the classical motif position) coordinates the Mg\textsuperscript{++} ion.
Table 2.1: Examples of known and putative WA motif sequences for the translocation ATPase in viral packaging motor proteins. The first four are examples assigned to a “Deviant I” Walker-A-like motif subfamily (ref. 34). The others are representative examples in viruses that have been extensively studied\textsuperscript{33,35,36}. X-ray structures including the ATP binding domain have been published for T4, Sf6, and P74\textsuperscript{26-28}. In vitro DNA packaging assays using purified terminase proteins and single-molecule optical tweezers assays have been established for lambda, T4, and phi29.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Lambda</td>
<td>76-KSARVGY-83</td>
<td>Mitchell 2004</td>
</tr>
<tr>
<td>21</td>
<td>76-KSARVGY-83</td>
<td>Mitchell 2004</td>
</tr>
<tr>
<td>P2</td>
<td>166-KSROIQGAT-173</td>
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<tr>
<td>Mu</td>
<td>64-KSRRRTGLT-71</td>
<td>Mitchell 2004</td>
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<td>160-LSRQLGKT-167</td>
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</tr>
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<td>62-VSRRVGKS-69</td>
<td>Rao 2008</td>
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<tr>
<td>P74-26</td>
<td>37-LGRQSGKS-44</td>
<td>Kelch 2015, PDB</td>
</tr>
<tr>
<td>Sf6</td>
<td>22-GGRGSGKS-29</td>
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<td>Phi29</td>
<td>24-GARGIGKS-31</td>
<td>Rao 2008</td>
</tr>
<tr>
<td>SPP1</td>
<td>33-GGRGSAKS-40</td>
<td>Mitchell 2006, Mitchell 2004</td>
</tr>
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<td>HSV-1</td>
<td>258-VPRRHGKT-265</td>
<td>Mitchell 2006, Rao/Feiss 2008, medically relevant</td>
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</table>

Sequence alignments identified that most terminase Walker A motifs also contain an arginine not present in the classical motif, corresponding to R79 in lambda gpA\textsuperscript{33-35}. All tested residue changes of this arginine in phage T4 terminase were found to be lethal\textsuperscript{37}. Various critical roles have been proposed for this arginine based on recent structural studies. Studies of T4 terminase suggests it is an "arginine finger" that triggers hydrolysis in response to a conformational change induced by DNA interaction\textsuperscript{16}. Data
on Sf6 terminase, on the other hand, suggests it has a distinct role in mechanochemical coupling in addition to triggering ATP hydrolysis\textsuperscript{15}. The arginine's side chain is proposed to interact with a "linker" domain between N- and C-terminal domains to couple hydrolysis to DNA translocation. Studies of P74-26 terminase suggest that the Walker A arginine is involved in mechanochemical coupling but not in triggering ATP hydrolysis. The arginine and an adjacent residue are proposed to interact with a C-terminal "lid" domain and cause the N-terminal domain to rotate and translocate DNA\textsuperscript{17}.

Here, we use a combination of genetic, biochemical, and biophysical methods to investigate the role of Walker A residues in motor function. Initially we sought to identify mutants exhibiting altered DNA translocation phenotypes to serve as tools for studying the motor mechanism. To this end, we first used a genetic assay to survey effects on phage yield of 88 single residue changes spanning the motif. A subset of these mutants that exhibited varying levels of impairment was chosen for further study by biochemical and biophysical assays. These studies identified three mutants that exhibited no DNA translocation and eight with varying partially-impaired translocation phenotypes. Our initial characterizations of these mutants show that Walker A residues play important roles in determining motor velocity, processivity, and pausing. Our results support the prediction that conserved residues K76, G81, and S83 play critical roles in phosphate/Mg\textsuperscript{++} binding. They also support recent models which predict that the conserved Walker-A arginine and an adjacent residue play important roles in coupling ATP binding to DNA binding and ATP hydrolysis/product release to DNA translocation.
2.2.1 Genetic analysis

To investigate the roles of gpA’s identified Walker A residues, our collaborators mutagenized individual codons of the 76-KSARVGYSK-84 segment to create a collection of missense mutants. In total our collaborators produced 88 different mutants with residue changes spanning the Walker A motif. Although K84 is not considered part of the motif, it was included because the crosslinking results suggested it is ATP-interacting. To ascertain the level of terminase function, a complementation assay was used. A complementing plasmid was used to supply mutant gpA to an inducible prophage carrying two A amber mutations. When wild type (WT) and viable A mutant plasmids provide functional gpA, the lysate from a prophage induction contains a level of λ.Aam indicating that the plasmid supplied functional gpA. The yield of λ.Aam is determined by titrating the lysate on a host containing supF, the appropriate amber suppressor. If a mutant plasmid fails to supply functional gpA, the yield of λ.Aam (Yₐ) will be profoundly reduced, to a level about \(10^{-7}\) of that found with the A⁺ plasmid, \(Y_{\text{min}}\). Mutants expressing partially active gpA will show intermediate yields of λ.Aam, > \(Y_{\text{min}}\), reflecting the level of functional gpA.

These assays revealed significant impairments in response to all changes across every identified Walker A residue (Fig. 2.2). All changes in conserved residues K76, R79, G81, and S83, except chemically-conservative changes G81A and S83T, reduced assembly to undetectable levels (\(Y_{\lambda} < Y_{\text{min}}\)). These findings are consistent with the predicted crucial roles of these residues in coordinating the β- and γ-phosphates, coordinating the Mg\(^{2+}\) of ATP, triggering hydrolysis, and mediating mechanochemical coupling. In contrast, non-conserved residues S77, A78, V80, and Y82 were more
tolerant to changes in that they caused lower levels of impairment and tolerated more substitutions, both conservative and non-conservative. K84, just outside the identified motif, was the most tolerant to substitutions. Altogether, these results strongly support the Walker A motif assignment.
A.

<table>
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<th>Φ yield (pfu/cell) (Relative to WT)</th>
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<td>&lt; 0.3 x 10^{-7}</td>
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B.

<table>
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<tr>
<th>K_{76}</th>
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</table>

**Figure 2.2:** Impairments in viral assembly caused by residue changes in the putative Walker A motif in the DNA translocation ATPase of the phage lambda large terminase subunit. (A) Range of detected degrees of impairment expressed in terms of phage yield in plaque forming units per cell relative to WT (where the WT activity is defined to be 1). (B) Chart listing measured activity levels in response to each single residue change tested. The 1st row lists the residues that were changed and the other rows list the substituted residue and the color of each indicates the activity level. The underlined residues are those observed to be highly conserved in a “Deviant I” Walker A-like motif subfamily [ref. 34]. Note that K84 lies just outside the predicted motif; to indicate this we have shaded its box white.
Our collaborators note that mutants with greater than ~90% reduction in burst size are unable to form plaques, so these changes are "lethal" from a genetic point of view (those labeled non-green in Fig. 2.2), although this does not imply there is no DNA translocation activity. Our assay can provide evidence for viral assembly, and hence translocation activity, even for lethal changes. This approach is advantageous over previous methods used to screen phage T4 Walker-A mutants\textsuperscript{37}, which only distinguished lethal vs. non-lethal changes. Altogether our collaborators identified 58 mutants exhibiting $Y_\lambda < Y_{\text{min}}$ (those labeled grey in Fig. 2.2) and 30 mutants with partial activity (non-grey labels). The latter mutants are good candidates for biochemical and biophysical characterization as they may have impaired DNA translocation. To our knowledge this is the most extensive collection of such mutants available for any Walker A motif.

The strength of the genetic mutagenesis studies is in the ability to evaluate the overall impairment on viral assembly over a broad range of mutations. However, there can potentially be many different causes of the impairments. For example, a mutation could affect not only translocation but also folding of the TerL protein, multimeric assembly with TerS, DNA binding and cleavage, docking with the procapsids, etc. Even if translocation is primarily affected, it can be affected in many different possible ways and for many possible reasons. For example, there may be no translocation at all, or partly impaired translocation, such as slow translocation, more frequent slips and/or pauses. Such defects may potentially be caused by factors such as impaired ATP binding, hydrolysis, and product release, and/or problems with mechanochemical coupling. Importantly, a mutant may be very strongly or even completely impaired in viral
assembly yet still exhibit DNA translocation activity with phenotypic impairments that can shed light on the roles of residues in motor function.

To investigate the nature of the impairments, further biochemical and biophysical studies were conducted. A small subset of the 88 identified mutants for initial characterization were selected. Specifically, twelve mutants (Table 2.2) were chosen for study with single-molecule assays to directly assess DNA translocation dynamics. From this set we successfully identified eight mutants that exhibited partially-impaired translocation and four with no detectible translocation. Two mutants with changes in residues predicted to be especially crucial for phosphate binding, and that exhibited no detectible yield and little or no DNA translocation in the single-molecule assay (S83A and R79A), were also chosen for more extensive biochemical analysis.
Table 2.2: Metrics of DNA translocation activity. These metrics were determined by analysis of the optical tweezers measurements. The first column reports the number of detected packaging events. For each mutant several hundred trials were performed to acquire these results. The other columns are reported as mean values of measured quantities, except for the last column which reports viral assembly activity (phage yield per cell) as a fraction of WT (the color codes in this column are the same as used in Fig. 2.2). All measurements were done with saturating ATP (0.5 mM) except for that labeled “WT Low ATP” which reports wildtype measurements with 2.5 μM ATP. Uncertainties are expressed as standard error in the mean.

<table>
<thead>
<tr>
<th>Terminase</th>
<th># of Events</th>
<th>Motor Velocity (bp/s)</th>
<th>Packaging Rate (bp/s)</th>
<th>Initiation Efficiency (% of WT)</th>
<th>Pauses per kbp DNA packaged</th>
<th>Pause Duration (s)</th>
<th>Slips per kbp DNA packaged</th>
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<tr>
<td>WT</td>
<td>53</td>
<td>400 ± 20</td>
<td>350 ± 20</td>
<td>100 ± 10</td>
<td>1.2 ± 0.4</td>
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<td>S83T</td>
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<td>340 ± 10</td>
<td>300 ± 20</td>
<td>60 ± 20</td>
<td>0.5 ± 0.1</td>
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<td>K84A</td>
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<td>270 ± 20</td>
<td>220 ± 20</td>
<td>60 ± 10</td>
<td>1.3 ± 0.3</td>
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<td>V80A</td>
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<td>230 ± 10</td>
<td>160 ± 20</td>
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<td>A78V</td>
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<td>173 ± 8</td>
<td>36 ± 7</td>
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<td>G81A</td>
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2.2.2 Biochemical Analyses

The genetic data presented above demonstrate that mutation of virtually any of the predicted Walker A residues afford a lethal phenotype. Most notably, K76, R79 and S83 mutations are universally lethal, with the exception of the chemically conservative change S83T (Fig. 2.2). Our collaborators previously characterized the K76R mutant terminase and demonstrated that this mutation abrogates DNA packaging activity in
vitro\textsuperscript{38}, commensurate with the lethal phenotype \textit{in vivo}. Interestingly, however, the ATPase activity of the enzyme is little affected. This indicates that while the ATP binding and hydrolysis steps remain intact, mechanochemical coupling to the DNA translocation domain of the enzyme is severely impaired. Here our collaborators utilize a similar biochemical approach to examine two additional critical Walker A residues, R79 and S83, by studying the genetically lethal mutant enzymes gpA-R79A and gpA-S83A.

\subsection*{2.2.2.1 Folding and Self-Assembly of the Mutant Terminase Enzymes}

To clearly define the role of key Walker A residues on ATP hydrolysis and DNA, mutations introduced into the terminase must be "surgical" in that they must only affect ATPase and/or translocation activities of the enzyme; they must not affect folding or assembly of the protomer nor the assembly of the protomer into a functional ring tetramer. Expression and purification of the mutant proteins gpA-R79A and gpA-S83A was uneventful, an indication that the mutations did not grossly affect the structures of the enzymes (data not shown). To confirm this presumption, our collaborators employed circular dichroism (CD) spectroscopy to ensure that the mutations did not perturb protein folding or stability. Neither mutation was found to affect the far-UV CD spectrum (Fig. 2.3A), an indication that the secondary structures of the mutant proteins remain intact. Moreover, the thermal stabilities of the mutant proteins are virtually identical to that of wild type enzyme (Fig. 2.3B).
Figure 2.3: Assessment of Thermal Stability, Folding and Self-Assembly. (A) CD spectra measurements show that secondary structure compositions for R79A and S83A are similar to WT [~35% α-helical, ~20% β-sheet]. (B) CD thermal melt curve measurements show that the mutants have thermal stability similar to WT ($T_m$=46.5 ± 0.2, 46.4 ± 0.2, 49.5 ± 0.2 °C for WT, R79A, S83A, respectively). (C) SV-AUC analysis indicates the Walker A mutants have no major assembly defects; they self-assemble normally to form monomers and tetramers, albeit at different ratios than WT (62% protomer for WT vs. 45% and 80% for R79A and S83A, respectively). This variability is consistent with prep-to-prep variability in WT enzyme.

Our collaborators note that both mutant enzymes retain a stable heterotrimeric TerL$_1$•TerS$_2$ stoichiometry throughout purification and during prolonged storage, confirming that the mutations do not affect quaternary interactions required for protomer assembly. As described above, the functional enzyme requires further assembly into a
catalytically-competent ring tetramer\textsuperscript{11-13}. Therefore, our collaborators next confirmed that neither mutation affects the protomer-tetramer equilibrium using sedimentation velocity analytical ultracentrifugation (SV-AUC). Consistent with published results, the WT-terminase protomer establishes an equilibrium between the protomer (5S) and the assembled ring tetramer (~15S) (Fig. 2.3C). The SV-AUC data demonstrate that both mutant enzymes similarly establish this slow, but dynamic equilibrium.

### 2.2.2.2 DNA Maturation Activities

Because residue changes R79A and S83A fall within the Walker A motif, which resides in the N-terminal domain of TerL, the presumption is that their effect will be limited to ATP binding and/or hydrolysis by the packaging ATPase site. To further confirm that these mutations are “surgical”, our collaborators examined their effect on the DNA maturation activities of the enzyme. The maturation catalytic sites are centered in the C-terminal domain of TerL and thus should not be affected by these residue changes. DNA maturation includes two steps; (1) nicking of the duplex at the \textit{cos}N subsite and (2) separation of the nicked, annealed strands to afford complex I (Figure 2.1). The implementation of a \textit{cos}-cleavage assay demonstrated that residue change S83A little affects duplex nicking relative to WT and that the observed nicking kinetics of both data sets are well described by the following monophasic exponential time course (Fig. 2.4A).

\[
F_{DP} = A \times [1 - \exp(k_{obs} \times \tau)]
\]  

where \(F_{DP}\) is the fraction of DNA cleaved at time \((\tau)\), \(A\) is the reaction extent at infinite time, and \(k_{obs}\) is the observed rate constant for the reaction. Bear in mind, this reaction has been modeled as irreversible because both the pre-nicking complex and complex I
(Figure 2.1) are extremely stable. Fits to the data show that S83A only has ~1.5-fold reduced $k_{\text{obs}}$ versus WT ($k_{\text{obs}} = 0.01 \pm 0.001$ s$^{-1}$ and $0.015 \pm 0.001$ s$^{-1}$, respectively).

In contrast, the R79A mutant shows a prominent lag and is better described by a model that includes a slow reversible step prior to catalysis (Eq. 2, Table 2.3, Fig. 2.4A).

\[
P + \text{cosDNA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P_4\text{cosDNA} \underset{k_2}{\rightarrow} P_4\text{D}_L\text{D}_R
\]

where $P$ represents the protomer in solution, $P_4\text{cosDNA}$ represents pre-nicking complex composed of four protomers assembled on the cosDNA substrate, $k_1$ is the rate constant for complex assembly, $k_{-1}$ is the rate constant for complex disassembly, $k_2$ is the rate constant for the irreversible chemical step (duplex nicking), and $D_L\cdot D_R$ represent the cleaved duplex products. Since these maturation assays were initiated by adding terminase protomers to the reaction mixture, successful cos-cleavage requires (i) assembly of a catalytically-competent complex at the cos-site and (ii) subsequent nicking of the duplex (Eq. 2 and Fig. 2.1). Slowing of either step could introduce a lag into the kinetic time course. Note, as discussed earlier, the pre-nicking complex is very stable and so here the assembly reaction was modeled as a reversible step where $k_{-1}$ is extremely slow. Thus, when $k_1$ is fast, the model degenerates to the simple monophasic model (Eq. 1).
To test the terminase-\textit{cos} assembly mechanism (stage (i)), our collaborators pre-incubated the protomer with \textit{cos}-DNA for 15 minutes to allow terminase to assemble at the \textit{cos} site and then our collaborators initiated the reaction with the addition of Mg$^{2+}$. Under these conditions, the lag disappears and the resulting kinetic time course is then well described by a monophasic exponential (Fig. 2.4A), which yields a $k_{\text{obs}}$ (0.009 ± 0.001 s$^{-1}$) similar to that of S83A and only ~1.5 fold less than that of WT. These data indicate that change R79A does not significantly affect the nuclease activity of the enzyme, but rather that the protomer possesses a mild DNA binding defect.
Figure 2.4: Kinetic Analysis of Maturation Activities. (A) cos-Cleavage activity. Those for WT and S83A kinetic time courses are well described by a monophasic exponential, and indicate that S83A has ~1.5-fold reduced k\textsubscript{obs} versus WT (k\textsubscript{obs} =0.01 ± 0.001 s\textsuperscript{-1} and 0.015 ± 0.001 s\textsuperscript{-1}, respectively). R79A displays a significant lag phase and is better described by a 3-state model that includes a slow step prior to catalysis. To investigate the nature of the lag, R79A was pre-incubated with cosDNA (dashed line) as described in Methods to form Complex I and the reaction was initiated with Mg\textsuperscript{2+}. This eliminates the lag phase and produces kinetic data well described by a monophasic exponential, indicating R79A has a k\textsubscript{obs} similar to S83A (k\textsubscript{obs} =0.009 ± 0.001 s\textsuperscript{-1}).

(B) Strand-Separation Activity. R79A and S83A strand-separate ~15-20% less DNA than WT, indicating mild defects (n≥3). All error bars indicate standard errors in the mean.

The second step in DNA maturation is separation of the nicked, annealed duplex strands to afford Complex I. As seen in figure 2.4B, mutant enzymes R79A and S83A exhibit only ~15-20% less strand separation activity than WT, thus demonstrating that neither mutation significantly affects this so-called “helicase” activity of terminase\textsuperscript{footnote1}.

In summary, the R79A and S83A mutant enzymes are natively folded, stable, and retain wild-type quaternary interactions required to assemble the protomer and to further assemble into a functional ring-tetramer complex in solution. Neither mutation

\textsuperscript{1} Initially, one would expect that the DNA binding defect observed with the gpA-R79A mutant would also affect the strand separation time course. Our collaborators note, however, that the rate of strand separation is slow (k\textsubscript{obs}= 0.25 min\textsuperscript{-1}) relative to the cos-cleavage reaction (k\textsubscript{obs}= 2.1 min\textsuperscript{-1}) (Andrews, 2012 #641). This suggests that while gpA-R79A protomer assembly on DNA is partially rate limiting in the faster cos-cleavage time course (lag observed), it does not appear in the slower strand separation reaction time course.
significantly affects DNA maturation by the enzyme, catalytic activities that are centered in the C-terminal domain of the TerL subunit. It is of interest that the R79A mutant possess a modest DNA binding defect that affects the rate of complex assembly on DNA, but that does not affect complex assembly in solution or the catalytic activity of the maturation complex once assembled at \textit{cos}. This subtle defect has consequences on the DNA packaging activity of the mutant, which is discussed further below.

2.2.2.3 Solution Ensemble DNA Packaging and ATPase Activities

The data presented above suggest that the lethal phenotypes of the R79A and S83A terminases are related to functional defects in DNA packaging. To directly test this hypothesis, genome packaging by the enzymes was investigated using a DNase protection assay as described in Materials and Methods. As anticipated, the packaging activities of the mutant enzymes are severely impaired (Fig. 2.5A); R79A and S83A terminases package only (3.6 ± 1.4)\% and (1.6 ± 0.8)\% of the input DNA relative to WT, respectively. Thus, the data directly demonstrate that these residues are indeed crucial for DNA packaging.

DNA packaging requires that DNA translocation by the ring complex is directly linked to ATP hydrolysis by TerL. Since the residues we have mutated here are critical Walker A residues at the packaging ATPase site, it is reasonable to expect these mutant enzymes to severely impair ATP hydrolysis as well as packaging. To confirm this presumption, steady-state ATP hydrolysis by R79A and S83A terminases was examined as described in Materials and Methods. The steady-state ATPase activity of both enzymes was found to be strongly impaired with only (1.5 ± 0.1)\% and (0.7 ± 0.1)\% activity relative to WT for changes R79A and S83A respectively (Fig. 2.5A). These impairments
can account for the severe defects in the DNA packaging activities of the mutants and are consistent with their predicted crucial roles in coordinating ATP binding and/or hydrolysis.

**Figure 2.5:** Kinetic Analysis of Packaging and ATPase Activities. (A) Packaging and steady-state ATPase activities were sharply reduced for both mutants (n≥3). (B) Single turnover ATPase reactions were initiated by the addition of enzyme. R79A and S83A display significant lag phases and are well described by a 3-state model that includes a slow step prior to hydrolysis. Pre-incubation of R79A with DNA (dashed line) greatly reduces the lag phase, suggesting DNA interactions are impaired. Parallel experiments with WT showed no effect on kinetics (data not shown).

To further probe the mechanism responsible for impaired ATP hydrolysis by the mutant enzymes, we performed single turnover kinetic measurements. Unlike the steady state conditions employed above, single-turnover studies employ an enzyme concentration that is in *excess* of the substrate (ATP). This design focuses on catalytic steps leading up to and including the chemical step of the enzyme-catalyzed reaction (i.e., hydrolysis, Fig. 2.5B). Similar to the cos-cleavage assay, this reaction was initiated with the addition of terminase protomers. Under these conditions ATP hydrolysis by wild-type terminase was found to be well described by a monophasic exponential time course (with
the same form as Eq. 1) where \( k_{\text{obs}} = (0.123 \pm 0.001) \text{ sec}^{-1} \) (Fig. 2.5B). As anticipated, single turnover ATP hydrolysis by the S83A mutant is severely impaired (with a monophasic exponential fit yielding \( k_{\text{obs}} = (0.18 \pm 0.4) \times 10^{-4} \text{ sec}^{-1} \)), consistent with the steady state kinetic data (Table 2.3 & 2.5). ATP hydrolysis by the R79A mutant is also impaired, albeit less than for S83A, and its reaction time course shows a clear lag in product formation. These data fit poorly to a simple exponential time course and we therefore considered a model that incorporates a slow reversible step prior to hydrolysis.

\[
P + \text{DNA} + \text{ATP} \quad \xrightarrow{k_1} \quad P_4\cdot\text{DNA}\cdot\text{ATP} \quad \xrightarrow{k_2} \quad P_4\cdot\text{DNA}\cdot\text{ADP}\cdot\text{P}_i
\]

where \( P \) represents the protomer in solution, \( P_4\cdot\text{DNA}\cdot\text{ATP} \) represents the catalytically competent tetramer assembled on DNA, \( k_1 \) is the rate constant for complex assembly, \( k_{-1} \) is the rate constant for complex disassembly, and \( k_2 \) is the rate constant for the irreversible chemical step (ATP hydrolysis). Note that this model degenerates to the simple monophasic model when \( k_1 \) is fast. The R79A data in figure 2.5B is fit according to such a model. As a check, we first analyzed the kinetic data for wild-type terminase as described in Materials and Methods, holding \( k_2 \) to the experimentally determined value of \( k_{\text{obs}} = 0.123 \text{ sec}^{-1} \) and allowed \( k_1 \) to float during the fit. This affords a value \( k_1 = (1.0 \pm 0.65) \times 10^6 \text{ M}^{-1}\text{s}^{-1} \), consistent with a diffusion controlled fast step prior to chemical hydrolysis \(^*\text{ref.} \). We next analyzed the data for the R79A mutant according to this model allowing both parameters to float; this affords \( k_1 = (13.2 \pm 9) \times 10^3 \text{ M}^{-1}\text{s}^{-1} \) and \( k_2 = (5.3 \pm 2.9) \times 10^{-3} \text{ sec}^{-1} \), respectively. This analysis indicates that both steps are significantly impaired, with the first step being \(~75\)-fold slower (Table 2.3).
Table 2.4: DNA Maturation and Genome Packaging Activity Relative to WT

<table>
<thead>
<tr>
<th></th>
<th>R79A</th>
<th>S83A</th>
</tr>
</thead>
<tbody>
<tr>
<td>cos-Cleavage (+ DNA)</td>
<td>60%</td>
<td>67%</td>
</tr>
<tr>
<td>Strand Separation</td>
<td>90%</td>
<td>90%</td>
</tr>
<tr>
<td>Packaging</td>
<td>(3.6 ± 1.5) %</td>
<td>(1.6 ± 0.8) %</td>
</tr>
</tbody>
</table>

What is the nature of the slow kinetic step prior to ATP hydrolysis for the R79A mutant enzyme? It was previously reported that the isolated terminase protomer subunit possesses little to no catalytic activity; however, the packaging ATPase activity of the enzyme is activated by assembly of the ring tetramer in solution\textsuperscript{11,12} as well as by assembly on non-specific DNA duplexes (nsDNA). Together these results suggest that activation of the packaging ATPase activity of terminase is directly related to assembly of the ring tetramer complex\textsuperscript{2}. As discussed above, R79A possesses a modest DNA binding defect that introduces a lag in the kinetic time course for the cos-cleavage reaction (Fig. 2.4A). And since the packaging ATPase activity of terminase requires

\textsuperscript{2} This could represent an allosteric activation induced by oligomerization. However, because a number of ATPase proteins from the ASCE family (such as some helicases, proteases, phages within the terminase subfamily, etc.) form oligomeric complexes whose nucleotide-binding pockets are assembled at the interface of adjacent protomers we posit that lambda terminase similarly assembles where catalytically competent ATP binding sites only become complete when TerL subunits bind to one another. Thus, the formation of a ring terminase complex would mean all the ATP binding sites at each TerL-TerL interface are catalytically competent.
protomer assembly, our collaborators considered the possibility that this slow step might similarly be associated with impaired DNA binding. To test this hypothesis, R79A was pre-incubated with DNA for 15 minutes to allow complex assembly and the reaction was then initiated with Mg$^{2+}$. The data presented in Figure 2.5C demonstrate that, under these conditions, the kinetic lag is absent and the data are well described by a simple monophasic time course, which affords $k_{obs} = \left(4.9 \pm 0.6\right) \times 10^{-3}$ sec$^{-1}$. Our collaborators note that this rate is in agreement with the fast rate obtained when this mutant enzyme is not pre-incubated with DNA ($k_2$, vide supra). Our collaborators interpret the ensemble of data to indicate that the R79A mutant protomer has a DNA binding defect that impairs protomer assembly on DNA, which is required for efficient cos-cleavage and activation of packaging ATPase activity. In addition, the rate of chemical hydrolysis by both mutant enzymes is strongly impaired, consistent with the observed defects in both steady-state ATP hydrolysis and DNA packaging activities. These findings are consistent with the predicted critical role of R79 in triggering ATP hydrolysis and mediating mechanochemical coupling. Finally, our collaborators note that pre-incubation of R79A with ATP did not affect the reaction time courses, indicating that slow productive ATP binding is not responsible for the observed kinetic defects in either mutant.
Table 2.5: Steady State ATPase Activity

<table>
<thead>
<tr>
<th></th>
<th>Steady State Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>$k_{obs} = (0.457 \pm 0.022) \text{ sec}^{-1}$</td>
</tr>
<tr>
<td>R79A</td>
<td>$k_{obs} = (7.0 \pm 0.3) \times 10^{-3} \text{ sec}^{-1}$</td>
</tr>
<tr>
<td></td>
<td>(1.5%; 65 fold decrease)</td>
</tr>
<tr>
<td>S83A</td>
<td>$k_{obs} = (3.2 \pm 0.2) \times 10^{-3} \text{ sec}^{-1}$</td>
</tr>
<tr>
<td></td>
<td>(0.7%; 142 fold decrease)</td>
</tr>
</tbody>
</table>

2.3 Results and Discussion of Single-molecule Analyses

We screened many of the partly-impaired mutants identified in the genetic studies with an optical tweezers assay that can directly measure the DNA translocation dynamics of single motor complexes (see Methods). We sought to identify altered translocation DNA phenotypes that could give further insights on the motor mechanism. Importantly, the biochemical and single molecule approaches employed in this study are complementary because they allow us to mechanistically interrogate both deleted and partly-impaired functions, respectively.

Specifically, we selected a subset of twelve mutant terminase enzymes for study with the single-molecule assays, including at least one having a point mutation at each of the Walker A residues (Table 2.2). The measurements focused on motor function in the condition of saturating ATP, low load force, and low capsid filling. We first chose enzymes exhibiting intermediate reductions in virus yield (those labeled orange in Fig. 2.2), where available, or else the only ones with detectible yield (e.g., G81A and S83T). For positions where all tested changes resulted in no detectible yield we tested changes to the relatively inert alanine and also the most chemically-conservative change (e.g.,
From this set we successfully identified eight mutant enzymes that exhibited partially-impaired translocation dynamics and four with no detectible activity. The eight with partial activity revealed a wide range of translocation phenotypes with differences in efficiency of initiation of packaging, packaging rates, pausing, and slipping (Table 2.2, Figs. 2.7-9). Initiation efficiencies ranged from 5% to 90% of that for WT and average packaging rates ranged from 13 to 300 bp/s vs. 350 bp/s for WT (Table 2.2 and Figs. 2.7&8).

2.3.1 Comparison with genetic findings

Consistent with the genetic studies, the DNA translocation impairments were in general greater in response to changes in conserved residues vs. non-conserved ones, implying that the impairments in phage yield can be attributed, at least in part, to defects in translocation. Changes to predicted conserved residues K76, R79, G81, and S83 (except for highly conservative change S83T) showed either no packaging events or very few with slow rates (Table 2.2). These findings are consistent with the biochemical results presented above showing strongly impaired packaging and ATPase activities for mutants R79A and S83A, and with our prior demonstration that K76R does not package DNA (though ATP hydrolysis remains intact). In contrast, smaller impairments were observed in response to changes in non-conserved residues A78, V80, and Y82, and residue K84 just outside the motif.

While the single-molecule and genetic findings are generally consistent from the perspective of packaging rate and initiation efficiency, observed differences suggests possible further insights about the roles of certain residues. Since no translocation was detected for mutant S77V but a small phage yield was observed (Table 2.2), this implies
that S77 plays an important role in determining initiation efficiency, and that change S77V reduces efficiency to a level below that which can be detected by the optical-tweezers assay. The non-zero phage yield demonstrates that packaging and viral assembly can proceed to completion, albeit inefficiently, which suggests that S77 is not absolutely critical for completion of packaging. In contrast mutants R79K and S83A showed no detectable phage yield but some DNA translocation activity. Conservative change S83T also caused a large (~10^4 -fold) reduction in phage yield but only a small (~14%) reduction in packaging rate. These findings show that packaging can initiate but fails to complete, suggesting that residues R79 and S83 are critical for completion of packaging. One possibility is that packaging may fail to complete if the mutation affects the motor's force-generating capability, since the force resisting DNA confinement increases during packaging. To investigate this, we tested mutant S83T with a 5× higher load (25 pN) and found that its packaging rate indeed decreases more sharply with increasing load compared with WT (Fig. 2.7 inset).

2.3.2 Residues involved in ATP binding

Since the polar side chain of the classic Walker A conserved serine/threonine is implicated in coordinating the Mg^{2+} of ATP, the mild translocation impairments due to the chemically conservative change S83T support the prediction that S83 plays this same role in Mg^{2+} coordination. However, surprisingly, change S83A, which removes the polar side chain, does not abolish translocation activity. Large reductions in initiation efficiency and packaging rate were observed, but still two events were recorded, showing that the S83 is not absolutely essential for translocation (but still important). The conserved glycine conceivably also plays an indirect role in ATP binding as it is located
at the apex of the P-loop and likely important in establishing the overall loop shape. Residue change K84A, just outside the identified Walker A motif, causes only very minor impairments in translocation (Table 2.2). The presence of a conserved lysine is critical for phosphate binding in many ATPases, although lambda gpA and a subset of other viral terminases conspicuously do not have a lysine in the classical “GK(T/S)” position (Table 2.1). Our finding that changes K76A and K76R result in no measurable translocation while K84A causes only minor impairment supports the prediction that K76 is the phosphate binding lysine, despite being farther in sequence from the classical position than K84. Surprisingly, change S83A, which eliminates the polar side chain implicated in coordinating the Mg$^{2+}$ of ATP$^{21-28}$, does not abolish translocation activity. Large reductions in initiation efficiency and packaging rate were observed, but two events were recorded, showing that the S83 is not absolutely essential for translocation. Together these findings would imply that other residues, presumably including K76, are sufficient to bind ATP strongly enough to enable some translocation.

2.3.3 Implications of motor pausing and slipping

Occasionally for wild type, DNA translocation is interrupted by: (1) a temporary arrest of packaging that lasts for several hundred milliseconds to several seconds, which we refer to as a "pause", or (2) an increase in tether length resulting from a partial release of DNA from the capsid, which we refer to as a "slip". We found that some of the mutant enzymes cause significant increases in pausing and slipping during translocation, which decrease the average packaging rate (Fig. 2.6 and Table 2.2). We define "motor velocity" to be the average rate of packaging not including pauses and slips. Studies suggest that DNA translocation during sections of continuous packaging is tightly coupled to ATP
hydrolysis and motor velocity is directly proportional to the rate of hydrolysis\textsuperscript{39}. The mean motor velocities for the mutant terminases ranged from 42 to 340 bp/s vs. 400 bp/s for WT (Fig. 2.7). In several cases where mutants exhibited frequent pausing and slipping (e.g. changes A78V and G81A), packaging rate was significantly lower than motor velocity.
Figure 2.6: Translocation dynamics. Measurements of length of DNA packaged vs. time for WT and mutant terminases under a 5 pN applied load at low procapsid filling (<10% of genome length packaged). Each plot shows ~10 typical examples of packaging events (or fewer for mutants that initiated very inefficiently for which fewer than 10 events were recorded). The upper left plot shows WT with saturating ATP (0.5 mM) and the upper right one shows WT with low ATP (2.5 μM). All of the mutants were measured with 0.5 mM ATP. All plots have the same x- and y-axis scale sizes.

Since one of the expected roles of the P-loop is to mediate phosphate binding we considered whether the decreases in motor velocity for all the mutant enzymes might primarily be due to impairments in achieving a productive ATP binding state. Studies of the phi29 motor indicate that after ATP initially associates (a diffusion controlled process
dependent on ATP concentration) the motor protein must also undergo a conformational change that facilitates subsequent hydrolysis, henceforth referred to as "productive ATP binding" (also referred to as "tight ATP binding" in the phi29 studies)\(^{39}\). Impaired productive ATP binding would increase the time it takes to attain a stably bound ATP hydrolyzable state during each iteration of the hydrolysis cycle, and consequently slow DNA translocation. All of the measurements on mutants were done with saturating ATP concentration (500 uM), but to investigate the effect of reducing the rate of productive ATP binding, we also conducted some WT measurements with sufficiently low ATP concentrations to make productive ATP binding rate limited. With 5 and 2.5 μM ATP we measured motor velocities of 130 and 79 bp/s, respectively, compared with 400 bp/s with saturating ATP (Fig. 2.7).
Figure 2.7: Average DNA translocation rates. Darker bars indicate mean packaging rate relative to WT (including pauses and slips; i.e. net change in tether length divided by the total event time.) and lighter bars indicate mean motor velocity relative to WT (packaging rate not including pauses and slips; i.e., total length of DNA translocated during the measurement divided by total time spent translocating). Error bars indicate standard errors in the means. The bar chart is arranged so that the terminases are listed from left to right in order of decreasing motor velocity—the same organization is used in the following figures. Inset: Bar chart showing ratio of mean motor velocity measured with higher applied load (25 pN) to that measured with lower applied load (5 pN) for WT and mutant S83T under saturating ATP conditions.

We found that slowing the productive ATP binding rate with low [ATP] also caused a dramatic increase in the frequency of pausing and slipping (Table 2.2 and Fig. 2.8) and 2.5 μM ATP caused more pausing and slipping than 5 μM ATP. Thus pausing and slipping increases with decreasing [ATP] or decreasing motor velocity. Consistent with this observation, three of the mutants (V80A, G81A, S83A) exhibited slipping frequencies consistent with the ATP-dependent slipping vs. velocity trend observed for WT. For these three, the impairments are consistent with impaired productive ATP binding; however, the frequencies of pausing and slipping did not always increase with
decreasing motor velocity (Fig. 2.8). For example, mutant A78V has significantly higher velocity than that measured for WT with 2.5 μM ATP, yet pauses and slips much more frequently. Conversely, Y82A had lower velocity than WT with 5 μM ATP, but exhibited significantly less slipping and pausing. In yet other cases, S83T, K84A, A78V, Y82A, R79K, impaired productive ATP binding cannot account for the observed impairments.

![Figure 2.8: Frequencies of pausing and slipping. Darker bars indicate mean number of pauses per kbp packaged relative to WT and lighter bars indicate mean number of slips per kbp packaged relative to WT. Error bars indicate standard errors in the means. Terminases are listed from left to right in order of decreasing mean motor velocity.](image)

2.3.4 Residues involved in mechanochemical coupling

Strikingly, changes in three adjacent residues A78V, R79K, and V80A cause the most significant increases in slipping (Table 2.2 and Fig. 2.8). The considerations discussed above suggest that this slipping is not due to impaired productive ATP binding.
Rather, the findings suggest that the coupling between ATP binding and DNA binding has been perturbed, supporting recent structure-based models which implicate the arginine and an adjacent residue in mechanochemical coupling. Conformational changes in the P-loop are proposed to mediate interactions with residues in other subdomains of TerL that result in a "lever"-like motion to translocate the DNA. ATP binding causes the lever to "cock" and then grip DNA. After hydrolysis and product release TerL is proposed to return to its initial conformation, resulting in DNA translocation. In Sf6 terminase the P-loop arginine appears to interact with a residue in a linker region between the globular N- and C-terminal subdomains, and this linker and the C-terminal domain are proposed to interact with DNA. In P74-26 terminase P-loop residues R39 and Q40 appear to interact with residues in a “lid” domain sandwiched between the N- and C-terminal subdomains. A rotation of the lid with respect to an N-terminal DNA binding region is proposed to drive translocation. Our finding that changes in the P-loop arginine and adjacent residues cause slipping support these models.

Previously, our collaborators implicated residue K76 in mechanochemical coupling because change K76R hydrolyzed ATP at nearly WT levels but exhibited no packaging activity in bulk assays. Here, we also find that K76A and S77V exhibited no translocation activity in the single-molecule measurements. Together, these results suggest that residues spanning from K76-V80 may be involved in mechanochemical coupling.

Although changes A78V, R79K, and V80A cause significant increases in slipping, slips still only occur at most once per ~500 bp of DNA packaged, on average. Since DNA is translocated in steps of only a few bp per ATP, this infrequent slipping
would imply only an intermittent failure in coupling between ATP binding and DNA binding, i.e. that slipping is an “off pathway” course from the main hydrolysis-translocation cycle. Besides causing frequent slipping these residue changes also cause significant reductions in motor velocity during translocation (Table 2.2 and Fig. 2.7), implying that an “on pathway” transition in the hydrolysis cycle is also slowed. Since we already concluded that the impairments are likely not due to impaired productive ATP binding, the slow motor velocity is likely due to slowed hydrolysis or ADP release. Since our single-turnover and steady state ATPase measurements for R79A indicate that both ADP release and a step prior to ADP release are slowed, we favor the interpretation that both ATP hydrolysis and ADP release are slowed. Thus, our findings suggest that the residues involved in coupling are also involved in controlling the rate of DNA translocation.

Recall that structural studies of the terminases of phages T4 and Sf6 suggest the P-loop arginine acts as an “arginine finger” to trigger ATP hydrolysis by interacting with the β- and γ-phosphates to stabilize the transition state. Our findings are consistent with this, as changes in the adjacent residues A78 or V80 likely perturb the arginine’s position, while changing the arginine to lysine may slow hydrolysis as such an effect has been observed in other ATPases that utilize an arginine finger. On the other hand, changing these residues may slow hydrolysis by perturbing the P-loop even if the arginine is not specifically acting as an arginine finger. Notably, studies of P74-26 terminase find that the P-loop arginine is critical for ATPase activity, but present evidence that a different arginine, inserted into the ATPase center in trans- from an adjacent subunit, acts as the arginine finger to trigger hydrolysis.17
2.3.5 Motor processivity

While A78V, R79K, and V80A exhibited more frequent slipping than WT, mutants Y82A and S83T actually exhibited less frequent slipping than WT. Y82A exhibited a rather unique phenotype in that it exhibited ~3-fold lower motor velocity than WT but also ~5× less frequent slipping (Table 2.2 and Figs. 2.7&9). This finding suggests that the reduced motor velocity is not due to impaired productive ATP binding. Rather the residue change may be increasing the rate of achieving the productive ATP binding state, resulting in a lower fraction of time in the apo state where we have shown that the motor is prone to slip (unpublished data, M. Ordyan and D. Smith). Slowed ADP release could account for the slow motor velocity. On the other hand, mutant S83T also slips also ~5× less frequently than WT, but its motor velocity is only ~15% lower than WT. The large increase in processivity could be regarded as a "gain in function" caused by the residue change. However, both WT and S83T have such high processivities on an absolute scale that slipping does not significantly affect the packaging rate of either. The S83T change may decrease slipping by increasing the rate of achieving the productive ATP binding state, while not significantly slowing ADP release.

2.3.6 Motor pausing

Four residue changes (V80A, A78V, G81A, and R79K) caused significant increases in both the frequency and duration of pauses in translocation (Table 2.2 and Fig. 2.9). Although our measurements with WT show that decreasing [ATP] increases pausing, the data for these mutant enzymes are not consistent with impaired productive ATP binding. First, mutant A78V (measured with 0.5 mM ATP) exhibited more frequent pausing yet higher motor velocity than WT with 5 μM ATP (Table 2.2 and Fig. 2.8).
Second, as discussed above, the slipping behavior exhibited by A78V and R79K suggests that they are not impaired in productive ATP binding. Third, the pause frequencies and durations imply that the pausing is an off-pathway state from the main hydrolysis cycle. For example, mutant V80A has a motor velocity of 58 bp/s. If ~2.5 bp are translocated per ATP hydrolyzed, each iteration of the hydrolysis cycle, including ATP binding, takes only ~0.04 s on average. In contrast, the average pause duration is ~100× longer, so it is implausible that pauses are due to slow productive ATP binding (if the binding rate is the same during both translocation and pausing, which is expected if the ATP binds in the same manner). Rather, the long pauses suggest that the motor has entered an inactive state which cannot translocate.

![Figure 2.9: Mean pause durations. Pause durations plotted relative to WT. Error bars indicate standard errors in the means. Terminases are listed from left to right in order of decreasing mean motor velocity.](image)

We found that the frequency of pausing of the WT motor is dramatically increased when packaging in a mixture of γ-S-ATP (a non- or slowly hydrolyzed analog)
and ATP, and these pauses have similar duration to those caused by the mutations. A similar effect was observed in studies of phage phi29 and shown to be due to binding of a single γ-S-ATP (with similar affinity as ATP) to a single motor subunit, which arrests packaging until the γ-S-ATP dissociates and is replaced by ATP. The pausing which occurs under normal packaging conditions, and is exacerbated by the residue changes, may be due to binding of ATP in a misaligned orientation such that it cannot be hydrolyzed and the TerL subunit must return to a weak ATP bound state to realign or dissociate the ATP before packaging can proceed. In this case, the ~2× higher average duration of the pauses exhibited by V80A, A78V, G81A, and R79K would imply that these residue changes also modestly increase the affinity of the ATP binding in the misaligned orientation. Overall, these findings suggest that residues A78, R79, V80, and G81 are important for proper ATP alignment for hydrolysis.

2.3.7 Initiation of packaging

Our measurements on the eight partly-impaired mutant terminases and WT at 0.5 mM ATP revealed a strong and statistically significant correlation between efficiency of initiation of packaging and motor velocity (Pearson correlation coefficient R=0.73, P=0.03) (Table 2.2 and Fig. 2.10). Our WT measurements with lower [ATP] also showed lowered initiation efficiency. The implication is that high motor velocity is important for efficient initiation, possibly to avoid DNA release that might occur when only a small length of DNA is packaged. Studies of phi29 and SPP1 show that the portal channel though which the motor pushes DNA into the procapsid has a highly electronegative inner surface. During initiation, this charge may inhibit the DNA from initially entering the channel. A similar conclusion was reached based on recent studies of phage
T4 in which fluorescence was used to measure packaging of multiple short (45 bp) DNA molecules. Packaging was found to occur in bursts separated by periods of inactivity, but the average time between initiation events decreased with decreasing [ATP]. A mutant with ~10-fold reduced ATP hydrolysis rate also exhibited a ~10-fold increase in initiation time, suggesting that rapid succession of ATP hydrolysis is essential for initiation. It was speculated that this effect may serve to regulate packaging to limit initiation under suboptimal nutritional conditions. Our findings provide broader support for these hypotheses.

![Figure 2.10: Efficiencies of initiation of packaging. Relative efficiencies for mutant terminases relative to WT, determined as described in the methods section. Error bars indicate standard errors in the means. Terminases are listed from left to right in order of decreasing mean motor velocity.](image)

On the other hand, the correlation we measure between initiation efficiency and motor velocity is not strict, which implies that motor velocity is not the only factor...
governing initiation. A notable exception to the overall trend is mutant Y82A, which exhibits low motor velocity but high initiation efficiency (Table 2.2 and Fig. 2.7&10). That Y82A also exhibits very low slipping (~5-fold lower than WT), indicates that high processivity may compensate for low motor velocity in initiation. Conversely, mutants A78V and V80A have significantly lower processivity and significantly higher motor velocity than Y82A, but also exhibit similarly high initiation efficiencies. Our finding that conservative change R79K causes frequent slipping implicated R79 in mechanochemical coupling. This change also severely reduced motor velocity and initiation efficiency. The likely reason that non-conservative change R79A resulted in no detected packaging activity is that either coupling is fatally impaired or the slipping is so frequent that it is incapable of initiating.

2.4 Final Discussion/Conclusion

Sequence alignments of the lambda TerL Walker A motif identified residues K76, R79, G81, and S83 as critical (Table 1). Supporting this assignment, we found that changes to these residues are least tolerated in our genetic assays and also caused the most significant impairments in translocation and ATPase activities.

Our demonstration that diverse translocation phenotypes are caused by residue changes in this motif sheds light on the roles of both conserved and non-conserved residues and suggests connections between ATP binding/hydrolysis states and DNA binding/translocation states of gpA. The conclusions suggested by our findings in the results and discussion section are summarized by the model for the ATPase/translocation cycle shown in Fig. 11. Since studies have shown that the subunits are tightly coordinated\textsuperscript{40}, we assume for simplicity that they translocate DNA successively one-at-a-
time. The states labeled 1-5 (shaded grey) represent the main hydrolysis/translocation cycle followed by each single WT TerL subunit. Initially, TerL begins in the Apo (empty) state with DNA weakly bound, from which slipping tends to occur (state 1). ATP then diffuses into the ATP binding pocket, but neither it nor the DNA are strongly bound and slipping still tends to occur (state 2). Interactions with ATP cause TerL to undergo a conformational change which results in ATP being tightly bound and properly positioned for hydrolysis, which we refer to as "productive ATP binding", and DNA being tightly bound (state 3). The ATP is then hydrolyzed, and TerL temporarily retains both ADP and P\(_i\) (state 4). Based on studies of the related phage phi29 motor we assume that the DNA is translocated after phosphate release, though this remains an open question for phage lambda. TerL ends in a state with ADP and DNA bound (state 5) and, after ADP release, it returns to state 1.

Three off-pathway states were suggested by the pausing and slipping behavior exhibited by the WT and mutant motors. In state P1 (pause state 1), occurring for both WT and some mutants, TerL grips DNA tightly but does not undergo the conformational change that allows it to rapidly hydrolyze ATP. Translocation pauses until an ATP binds tightly and hydrolyzes. In state P2 (pause state 2), occurring for some mutants, TerL binds ATP tightly but in a misaligned orientation that does not lead to hydrolysis. Translocation pauses until the ATP binding pocket returns to a loose binding state so that ATP can correctly align, after which the main cycle can proceed. In state DC (decoupling state), occurring for some mutants, a defect in the coupling between ATP binding and DNA binding leads to slipping.
Figure 2.11: Proposed TerL DNA Translocation Hydrolysis Cycle. The dotted ATP in state P1 indicates that ATP need not necessarily be present in the ATP binding pocket for the motor to occupy this state.

This model accounts for the observed pausing and slipping as follows. At low [ATP], transition rate $k_{1\rightarrow2}$ is decreased and the motor spends more time in state 1, which decreases motor velocity, increases the frequency of slipping, and increases the frequency of pauses caused by transitions to state P1. We find that the residue changes S83A and G81A decrease some combination of the transition rates $k_{2\rightarrow3}$, $k_{2\rightarrow DC}$, and $k_{2\rightarrow P2}$ (and/or increase $k_{3\rightarrow2}$, $k_{DC\rightarrow2}$, $k_{P2\rightarrow2}$) such that the time the motor spends in state 2 is increased, which, with decreased transitions into states 3, P2, and DC, means the motor will transition into state P1 about as much as WT under low ATP conditions. Consequently, these motors then exhibit decreased motor velocity and increased pausing and slipping frequencies that are consistent with that of WT in low ATP. In contrast, residue changes Y82A and S83T increase $k_{2\rightarrow3}$ (and/or decrease $k_{3\rightarrow2}$) such that the time the motor spends in state 2 is reduced, which decreases the frequency of slips and pauses, while also
decreasing $k_{4 \rightarrow 5}$ (mild decrease for S83T but significant decrease for Y82A), which decreases motor velocity. Residue changes A78V, R79K, V80A, G81A increase $k_{2 \rightarrow P_2}$, increasing the frequency of longer-duration pauses. Of these, residue changes A78V, R79K, and V80A increase $k_{2 \rightarrow DC}$ (and/or decrease $k_{DC \rightarrow P_2}$), which increases frequency of slipping associated with failed coupling, and exhibit reduced motor velocity due to reduced $k_{3 \rightarrow 4}$ (hydrolysis) or $k_{4 \rightarrow 5}$ (ADP release) transition rates.

The agreement between the steady state and single-turnover ATPase kinetic rates for mutant enzyme S83A suggested that the observed kinetic impairments are due to slowed ATP binding or hydrolysis and not impaired ADP release. However, the single molecule slipping observations narrowed the suspected impairment to productive ATP binding. On the other hand, the enhanced processivity of the mutant enzyme S83T suggested that the productive ATP bound state is more quickly achieved by this particular mutation, which would imply its slightly impaired motor velocity is a consequence of impaired hydrolysis or ADP release. If the impairments caused by change S83A result from a decreased rate of achieving the productive ATP bound state rather than decreased rate of hydrolysis, we suspect that translocation impairments from change S83T also are not because of faulty hydrolysis. By exclusion, the results suggest that the translocation impairments of S83T are due to slowed ADP release. Thus the impaired ATP binding by mutant enzyme S83A and impaired ADP release by mutant enzyme S83T implicate residue S83 in ATP/ADP binding, which is consistent with the suspected role of this residue (i.e. coordinating the Mg$^{2+}$ of ATP for ATP binding).

The analysis of the single-molecule and biochemical experiments with R79K and R79A, respectively, concludes that the observed translocation and ATPase impairments
are not due to any defect in productive ATP binding but rather are likely due to both slowed hydrolysis and ADP release. Since DNA binding is directly related to ATP binding/hydrolysis, the identified DNA binding/ring assembly defect for mutant enzyme R79A support the single molecule findings that R79 participates in communicating between the p-loop motif and the DNA binding motif (i.e. mechanochemical coupling). Lastly, the long pause durations exhibited by R79K suggest that residue R79 also plays a role in proper ATP alignment (state P2 – Fig. 11). As mentioned earlier, studies of Walker A motif’s for phages T4, Sf6, and P74-26 have separately identified some of the functions just described as roles of the Walker A conserved arginine. It is notable that our findings suggest that residue R79 appears to be involved in ATP alignment, triggering ATP hydrolysis, ADP release, as well as coupling between ATP binding and DNA binding. These multiple roles emphasize that this arginine is essential, a conclusion supported also by our genetic assays which found that no other residue at this position was tolerated; not even the chemically conservative change R79K.

Of the structurally similar subfamilies of the ASCE superfamily, the AAA+ subfamily of proteins contain a domain (sensor II) with a conserved arginine that has been found to interact with the gamma-phosphate of bound ATP, is associated with nucleotide binding/hydrolysis, and is thought to coordinate a protein conformational change with ATP hydrolysis. Since the terminase ATPase subfamily is also included within the ASCE superfamily (thus structurally relating terminase and AAA+ ATPases), the functional similarities between the sensor II arginine and the lambda TerL arginine in the Walker A motif support the proposal by Kelch and coworkers that this arginine is analogous to the sensor II arginine of AAA+ proteins.
In this study, R79 and S83 were the only residues we studied using both biochemical and single-molecule methods. Results from our previous biochemical studies with K76R implicated residue K76 with coupling between ATP and DNA binding. And since our experiments with changes K76A and K76R resulted in no translocation events, it is possible that K76 is involved in ATP phosphate binding, a role originally proposed by Rao and coworkers (2004). Similarly, change S77V yielded no translocation events. However, its moderate reduction in phage yield activity suggests S77 plays an important role in initiating packaging. Since the neighboring residues appear to be involved in mechanochemical coupling, we propose that S77 may also be involved in coupling. On this note, we also concluded that residues A78 and V80 play a large role in mechanochemical coupling based on their atypical frequent slipping character. These results, taken together with the observed frequent pausing, suggest that these residues are also involved in proper ATP alignment for hydrolysis and ADP release. However, we suspect these residues (A78 and V80) are only indirectly involved in these functions by way of their interactions with residue R79, which we previously reasoned is likely involved in these functions directly. Residue G81 is also suspected, based on the frequent slipping and pausing exhibited by change G81A, to play an indirect role in proper ATP alignment and achieving a productive ATP binding state. The absence of a side chain at this site and its placement between residues that interact with ATP directly (R79 and S83) suggests that the role of G81 is structural. This residue likely allows the p-loop to bend such that the residues that interact with ATP directly are optimally oriented relative to the ATP phosphates for ATP binding and alignment. Lastly, single-molecule experiments with residue change Y82A resulted in reduced motor velocity but
greater processivity relative to WT, which implies that Y82 may be involved in binding ATP/ADP tightly. We suspect that Y82 also plays a structural role in ATP binding and possibly in a similar manner as G81.

Notably, changes in non-conserved Walker A residues V80A, A78V, Y82A, and K84A adjacent to the motif resulted in modest reductions in motor velocity (35-68% of WT) and modest reductions in initiation efficiency (54-85% of WT). We also recall that motor velocities measured with saturating ATP at room temperature for different phage terminases vary considerably (from ~100 bp/s on average for phi29, which packages a 19.3 kbp genome, to ~700 bp/s for T4, which packages a much longer ~171 kbp genome). It is possible that Walker A mutations can compensate for, or act synergistically with, changes occurring in other regions of the motor protein that may affect DNA translocation activity. During natural evolution changes in non-conserved Walker A residues may have served to “tune” motor velocity according to the needs of different viruses.

As a final note, homology modeling based on the Sf6 structure predicts a lambda TerL Walker A motif with critical residues appropriately oriented for ATP phosphate binding and hydrolysis (Fig. 12). In general, residues 76-83 exhibit a loop structure common of p-loop motifs where the orientation of the residues produces an empty pocket that is available for the ATP phosphates to reside. Also, the Serine from the Walker A sequence and the aspartate and glutamate residues from the Walker B motif appropriately point toward the phosphates for ATP binding and hydrolysis. Residue R79, on the other hand, points away from the ATP phosphates, but can be envisioned to rotate toward these
phosphates, which would cause a conformational change to TerL as proposed by the P74-studies.

**Figure 2.12:** A homology model of the λ TerL, N-terminal, packaging ATPase site was created with I-TASSER. The Walker A motif is highlighted in cyan.

Future studies of the remaining 18 other mutants identified in our genetic studies as impaired but retaining measurable phage production may yield different or similar translocation phenotypes to those observed in this study. Such results can be useful in affirming roles proposed in this study and/or potentially revealing unknown character of the residues within the phosphate binding motif. Naturally, as was demonstrated here, more detailed biochemical analysis can also be performed on any unstudied mutant terminase to provide greater insight into the role of these residues.
2.5 Acknowledgements

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Chapter 2, in part, contains material currently being prepared for submission for publication: Authors include: delToro, Damian, Ortiz, David, Ordyan, Mariam, Keller, Nicholas, Sippy, Jean, Oh, ChoonSeok, Catalano, Carlos E., Feiss, Michael, Smith, Douglas E. Working title is: “Walker-A P-loop coordinates ATP hydrolysis with motor output in viral DNA packaging”. The dissertation author is a primary investigator and author of this paper.

2.6 Materials and Methods (Biophysical)

2.6.1 Reagents

DNA

For the optical tweezers measurements we used a 13,747 bp DNA construct prepared by PCR from a 13,881 bp Lambda Cos containing cosmid, pJM1, using the primers 5’-TCGATAATCGTGAAGAGTCGGCGAGCCTGGTTAG-3’ and Biotin-5’-TACGTCGAAGTGACCAACTAGGCGGAATCGGTAG-3’ and the 5Prime PCR Extender System. We used the standard (non-high fidelity) protocol with 20ng of pJM1 template DNA per 50ul PCR reaction. Primers were chosen such that, after initial DNA cleavage at the CosN site, the resulting packable DNA length was 10,052 bp and that packaging would proceed from the Cos site toward the product DNA’s biotinylated end.

Pre-stalled complexes/method and experimental buffers
The complexes are prepared at room temperature with a total final sample volume 5-12μl (depending on the terminase used-mutant preparations required greater volumes for packaging to be observed). The final concentrations/quantities of the reagents were 25mM Tris-HCl pH 7.5, 5 mM MgCl₂, ~10-25 ng/μl of the 13.7 kbp PCRed DNA, ~0.75-7 μl of crude extract terminase, ~0.2-0.5 μl of 5 mM ATP, ~0.2-0.5 μl of 5 mM γ-S-ATP, and ~6-19 nM of procapsids. First, water, Tris-HCl, DNA, terminase, and ATP are combined and incubated for 5 minutes. Then γ-S-ATP is added and the mixture is incubated for 1 minute. Last, procapsids are added, the mixture is incubated for another 5 minutes, then the complexes are added to ~2 μm streptavidin (SA) microspheres that have been washed 2 times in TM Buffer (25mM Tris-HCl pH 7.5, 5mM Mg²⁺). The bead-complex mixture is rotated and incubated for at least 20 minutes. The quantity of complexes to streptavidin beads is titrated until ~1 tether is acquired during each tweezers trial (see tweezers measurements below). Anti-procapsid beads are prepared by first washing ~2 μm protein G coated polystyrene beads twice with 1xPBS. Lambda antiserum is mixed with these beads and incubated for 30 minutes while rotating. Lastly, the mixture is washed twice in 1xPBS and twice in TM buffer. Prior to injection into the flow cell, the SA-complex beads are diluted in a 1xTM buffer solution while the anti-Lambda procapsid beads are diluted in a 5mM γ-S-ATP and 1xTM solution. The experiments are performed in a 1xTM, 0.5 mM ATP, and 20mg/ml BSA buffer.

2.6.2 Optical Tweezers Measurements

We used dual-trap optical tweezers, as previously described (ref. 6) and calibrated (ref. 46). The data was collected at 1 kHz after filtering with an 8-pole Bessel, 333Hz corner frequency low pass filter. Experiments were done as described previously (ref. 6)
with the following modifications. The streptavidin beads were coated with pre-stalled DNA/terminase/prohead complexes and measurements were done with 500 μM ATP for all terminase experiments except WT low ATP experiments, which used 2.5 μM ATP. Once packaging resumed, a force feedback program adjusted the position of the movable trap to maintain a 5 pN force clamp throughout the duration of packaging. After the prohead was 10% filled (i.e. ~5 kb DNA packaged) or stopped packaging for longer than ~1 minute, the measurement was ended. To obtain a force baseline, the beads were pulled apart until the tether ruptured and then moved toward one another until the beads bumped. The high force clamp measurements followed this same procedure except that after the complex packaged ~1 kbp of DNA (~2% capsid filling) at 5 pN, the movable trap was rapidly moved away from the stationary trap until a force of either 20, 25, or 30 pN was achieved. This high force was maintained until 10% of the capsid was filled or packaging stopped for longer than ~1 minute.

2.6.3 Analysis

Data Cropping and Pause Slip Detection

Packaging traces were cropped to begin at the point where the γ-S-ATP stalled complexes reinitiate packaging in the presence of ATP and cropped to end at the last point of packaging beyond the 10% capsid filling point. For complexes that did not manage to package beyond this 10% filling point, the traces were cropped to end at the onset of a terminal packaging stage/pause event. These cropped traces were then processed by a program that automatically and systematically scores all pauses and slips. The program initially determines the displacement difference from adjacent points of a smoothed trace. Potential pauses and slips are identified as these displacement
differences transition from non-zero values toward zero values where adjacent
differences of zero represent a pause while adjacent differences that pass through zero
represent transitions between packaging and slipping. The beginning and ending of these
pause and slip events are scored based on an empirical based criteria. These potentially
identified pauses and slips are subsequently passed through filters that assess whether
these are correctly determined pauses and slips based on our resolution as well as whether
a series of identified nearby short pauses or slips should in fact be combined. No pauses
or slips with durations less than 300ms were accepted and accepted slips lengths were
based on an empirically determined criteria.

**Motor Velocity Calcs: high and low clamp experiments**

Motor velocity for each trace under a given force clamp force was calculated by
first using the packaging sections of a given trace under the force clamp of interest to
evaluate the total length of DNA translocated and the total time spent translocating DNA.
These two values are then divided by one another to determine the motor velocity per
trace. These motor velocities are then averaged to acquire the mean motor velocity. For
the high force clamp experiments, the motor velocity ratios were determined by first
averaging together the mean motor velocities determined from the 20, 25, and 30pN force
clamped events. The mean motor velocity at 5pN is then divided by this averaged high
force clamped mean motor velocity and multiplied by 100 to represent the ratio as a
percent. A packaging rate for each trace is determined by evaluating the tether length
difference between the beginning and ending points of a given cropped trace and dividing
this length by the total event time of the cropped trace. These packaging rates are then
averaged over all traces to acquire the mean packaging rate presented in the results of this letter.

Note: Technically a line is fit to each packaging section within a given trace and the duration of the packaging section is multiplied by the fit to determine the packaging length of this section. The packaging lengths for all the packaging sections within a given trace are then added up and divided by the total time spent translocating. This determines the motor velocity for a given trace.

**Other Single-molecule Metrics**

Initiation rate for a given day of experiments was determined by dividing the total number of successful packaging events acquired by the total time spent performing experiments. These initiation rates were then averaged over all days spent collecting data for each terminase studied. Pause and slip frequency per length DNA packaged for a particular trace were determined by dividing the total number of pauses or slips observed in a given trace by the total length of DNA translocated in units of kbp and 10kbp respectively. These frequencies were then averaged over all traces for each terminase studied. Mean pause durations are determined by averaging all pause durations per trace and then averaging these means over all traces collected for each terminase studied.

**Correlation Calculations**

Correlation between two metrics, \{X,Y\}, with uncertainties, \{\sigma_Y, \sigma_X\}, was determined by generating 10000 subsets of paired values resampled from the original set \{X,Y\}. Each subset is generated by adding a random normally distributed deviation (based on the uncertainties obtained for each terminase within a given metric) to each value within a metric. A correlation coefficient, r, is calculated for each paired subset of
values using the Matlab function ‘corrcoef’ where each point (x,y) are weighted according to the area of uncertainty determined from their error bars, $A_{\text{uncer}} = \sigma_x \sigma_y$. The smaller the $A_{\text{uncer}}$, the larger the weight associated with the point (x,y). The set of 10000 weighted correlation coefficients are then averaged together. The Matlab ‘corrcoef’ p-value subroutine is then used to evaluate the p-value corresponding to the mean r value.

2.7 Appendix (microbiology and biochemical methods)

The following is a thorough discussion of the methods the genetic and biochemical experiments performed by our collaborators.

2.7.1 Microbiological methods

Media

LB, LB agar, tryptone broth (TB) and TB soft agar (TBSA) were prepared as described in ref. 47. When required, antibiotics were added at 50 ug/ml for kanamycin and 100 ug/ml for ampicillin. Bacteria and phages dilutions were done in 0.01 M MgSO$_4$.

Microbiological methods and strains used

Standard protocols were used for growing bacteria and phages (ref. 47).

Mutagenesis

We used overlap extension to generate DNA segments containing mutated codons (ref. 48). The polymerase chain reaction steps were carried out using Taq DNA polymerase (Invitrogen, Inc.) and λ DNA as template. The two destinations for inserts with Walker A mutations were a complementation vector, pJM5, and an expression vector, pQH101alt. pJM5 is a derivative of pJM1 (ref. 49) with an introduced a Sall site, created using same-sense mutations changing the gene A sequence 1224-GTGGAT-1229 to GTGCAC. pQH101alt is a derivative of pQH101, a holoterminase expression vector that produces enzyme tagged at the C-terminus of gpA with six histidines (ref. 50). The following same-sense mutations were introduced into the pQH101 A gene to create pQH101alt: 781-AGGCCG-786 to AGGCCT (StuI); 899-CGATGG-894 to CCATGG (NcoI); 957-TTCCAA-962 to TTCGAA (BstBI); 1224-GTGGAT-1229-GTCGAC (Sall); 1246-GAACCT-1251 to GAGCTC (SacI); 1318-GGCTCG-1323 to GGATCC (BamHI) and 1366-GGCACC-1371 to GGTACC (KpnI). Control experiments showed pJM5 was as effective as pJM1 at complementing a λ Aam mutant, and that pQH101alt expressed similar levels of fully active gpA as pQH101. Walker A mutations in PCR-generated segments extending from the Sall site at λ bp 1220 to the native SphI site at λ bp 2212 were introduced into pJM5 and pQH101alt. For all constructs, the presence of desired Walker A mutations and the absence of extraneous mutations were verified by DNA sequencing. For pJM5 and pQH101derivatives, DH5α and OR1265, respectively, were used as transformation recipients.

Complementation
Testing the effects of gpA changes on terminase function by complementation assay. Derivatives of pJM5 carrying Walker A mutations were used to transform MF3537. pJM5 contains a segment of the λ chromosome extending from the HindIII site at 44141 across cos to the BamHI site at 5505. This λ segment contains the late gene promoter, the lysis genes, the terminase genes Nul and A. When present in a cell with induced λ prophage, the plasmid’s late genes are activated at the normal time by the phage’s gpQ late gene activator. Previous work shows that the level of terminase gene expression is sufficient to support the growth of a λ mutant with a terminase defect51,52 (Frackman et al., 1984, Frackman et al., 1985). The heat-inducible prophage in MF3537 is λ Aam11 Aam32 red3 cI857. The host bacterium MF3537 is a RecA− derivative of the E. coli C strain C1a53, and lacks a nonsense suppressor, so the prophage, with two amber mutations in A, is unable to produce gpA. For the prophage to produce viable progeny phages, functional gpA expression from the pJM5 plasmid is essential. After induction and growth of the λ Aam11Aam32 red3 cI857 prophage, the ability of the plasmid’s A gene to provide functional gpA is assessed by titering the yield of the induced prophage on strain MF3679, which carries supF, a tyrosine-inserting suppressor.

Initial in vitro screening of Walker A mutants

Mutant terminases were overexpressed and characterized for gpA expression by SDS-PAGE electrophoresis, and for in vitro cos cleavage activity as previously described30,31.

2.7.2 Biochemical Methods

cos-Cleavage and Strand Separation Assays

The cos-cleavage and strand separation reactions were performed as described previously54. Briefly, the reaction mixtures (20 μL) contained 5nM ScaI-linearized pCT-λ DNA, 1mM ATP and 50nM IHF in 50mM Tris buffer, pH 8, containing 10mM MgCl2, 15mM NaCl, 7mM β-ME, and 0.5% glycerol. The reactions were initiated with the addition of terminase (100nM), allowed to proceed for the indicated times at 30°C, and quenched with 45mM EDTA. The extent of strand separation was determined by analysis of the products by 0.6% agarose gel. Alternatively, the extent of cos-cleavage (duplex nicking) was analyzed by heating the quenched reaction mixture at 75 °C for 30 sec prior to loading onto a 0.6% agarose gel. The kinetic data are analyzed according to the simple monophasic exponential time course in equation 1. The experimental data were fit using non-linear regression analysis using Mathematica® allowing all of the parameters to float to their minimized values. In those cases where a lag was observed in the cos-cleavage reaction time course, the kinetic data were analyzed according to a three state kinetic model composed of a slow nucleoprotein complex assembly step followed by an irreversible chemical step (duplex nicking) (Eq. 2). The kinetic data were analyzed according to this model using the COPASI® software package. Where indicated, the order of addition was altered such that terminase was pre-incubated with DNA but without MgCl2 for 15 minutes to allow the formation of Complex I, and cos-cleavage was then initiated with MgCl2.

Circular Dichroism
Far UV CD spectra were obtained for WT and mutant terminases (1μM) in 20mM NaPO₄ buffer, pH 6.8 containing 100mM NaCl, 10% glycerol and 7mM β-ME at 4°C in a 1mm cuvette using an Aviv 420 CD spectrometer. The mean averaged signals were converted to molar ellipticity (θ) and the data analyzed by the K2D3 secondary structure prediction server. Thermal melt curves were performed as above; absorbance was measured at 222nm between 4°C and 95°C in 1°C increments. The mean averaged signals were converted to Δε and fit to a sigmoidal curve in Graphpad.

**Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC)**

Terminase enzymes were dialyzed into 20mM Tris buffer, pH 8 containing 100mM NaCl, 10mM MgCl₂, 5% glycerol and 1mM TCEP-HCl. WT, R79A and S83A were diluted with dialysate to final concentrations of 0.9, 1.3 and 0.7 μM, respectively, and were each loaded into two-sector epon-charcoal ultracentrifugation cells. The samples were placed in a Beckman XLA analytical ultracentrifuge and equilibrated at 4°C at least 1.5 hours, then spun at 42 K rpm and 4°C. Sedimentation was monitored by absorption at 280nm. A c(s) analysis of the SV-AUC data was performed with SEDFIT and the data were normalized so that area under the curve = 1.

**Packaging Activity Assays**

Packaging activity assays were performed as described previously with slight modifications. The maturation reactions are allowed to proceed as described above for 2 min at 37°C to form Complex I. Procapsid (40nM) and ATP (1mM) are added to form Complex II and initiate packaging; the reaction is allowed to proceed for 25 minutes at 25°C. DNAse (0.2 mg/ml) is added to digest unpackaged DNA and allowed to proceed for 5 min at 25°C before quenching with EDTA. Packaged DNA is then extracted and measured by gel electrophoresis.

**Steady State ATPase Assays**

Steady state ATPase assays were performed as described previously. Briefly, the reactions mixtures (20 μL) contained 10μM ATP, 1.5nM mature λ DNA in 50mM Tris buffer, pH 8, containing 30mM NaCl, 10mM MgCl₂, 7mM β-ME and 5% glycerol. Reactions were initiated by the addition of terminase (100nM) and allowed to proceed for 3 minutes at 30°C. The reactions were quenched in the linear phase with stop solution (2.5mM ATP, 2.5mM ADP, 50mM EDTA) and analyzed by thin layer chromatography as previously described *ref. ATPase activities are presented in μM/min and normalized to WT activity.

**Single Turnover ATPase Assays**

Single turnover ATPase activity was assessed for WT and mutant terminases as described for the steady-state assays above, except a 5-fold excess of enzyme over substrate (500nM terminase/100nM ATP) and 2.5 nM mature λ DNA were included. The reactions were initiated by the addition of terminase and were allowed to proceed at 30°C for 20 minutes and quenched at the indicated time points. The much slower S83A was allowed to proceed for 1 hour. The single turnover kinetic data were fit to a monophasic exponential time course of the same form as in equation 1. The experimental data were fit using non-linear regression analysis using Mathematica® allowing all of the parameters to float to their minimized values. In those cases where a lag was observed in the ATPase reaction time course, the kinetic data were analyzed according to a three state kinetic model that includes a slow reversible step prior to an irreversible catalytic step.
(hydrolysis) (Eq. 3). The kinetic data were analyzed according to this model using COPASI® software package. Where indicated, the order of addition was altered such that terminase was pre-incubated in the reaction mixture in the absence of ATP for 15 min, then the reaction was initiated by the addition of ATP.

2.8 References for Chapter 2


Chapter 3

Mechanism of Termination of Bacteriophage DNA Packaging Investigated with Optical Tweezers

3.1 Abstract

The genomes of many dsDNA viruses are replicated by a mechanism that produces a long concatemer of multiple genomes. These viruses utilize multifunctional molecular motor complexes referred to as "terminases" that can excise a unit genome length of DNA and package it into preformed viral shells. Remarkably, the terminase motor can initiate packaging at the appropriate start point, translocate DNA, sense when a sufficient length has been packaged, and then switch into a mode where it arrests and cleaves the DNA to release a filled virus particle. In this chapter, in addition to presenting studies investigating potential methods of termination, we describe an improved method we have developed to measure single phage lambda DNA packaging using dual-trap optical tweezers and pre-stalled motor-DNA-procapsid complexes. We are applying this method to test proposed mechanisms for the sensor that triggers termination; specifically a velocity-monitor model vs. energy-monitor model vs. capsid-filling monitor model. Preliminary tests for termination under slow packaging rate conditions (low ATP or slow-packaging mutant motors) support that termination does not occur by way of a velocity-monitor mechanism.

3.2 Introduction

Many dsDNA bacteriophages (bacterial viruses) have a packaging phase in which a multifunctional protein motor packages concatemerized dsDNA into a preformed
hollow protein capsid (procapsid). Once a single genome has been packaged into the procapsid, the motor terminates packaging and cleaves the DNA\textsuperscript{1,2}. But, how do such viruses ‘know’ that they have packaged a full genome? What trigger or sensing mechanisms do they have that cause them to terminate packaging and cleave the DNA at the appropriate length? We are using a dual optical trapping system and the well-characterized lambda phage virus to test three proposed mechanisms of termination: a velocity-monitor model, an energy-monitor model, and a capsid-filling monitor model, described below. Moreover, to better test these models, we have developed an improved method for conducting single-molecule lambda phage packaging experiments.

3.2.1 Lambda Phage DNA Packaging Phase

The lambda virus has a 48,502bp genome that initiates and terminates packaging at a particular, ~200bp, nucleotide sequence known as the cos (cohesive end sequence) site\textsuperscript{3-8}. In its concatemeric form, the lambda DNA cos site comprises of three subsites: cosQ, cosN, cosB. cosQ is a 7bp segment that is 17bp upstream from cosN, which is a 22bp segment, while cosB is a 114bp segment that is 35bp downstream from cosN\textsuperscript{5,6}. Just prior to the packaging phase, multiple terminase subunits bind to the cosB site on the concatemeric lambda DNA and, in the presence of ATP (or ADP, personal communication, Prof. Carlos Catalano), introduce staggered nicks at the cosN site such that the genome has a twelve base 5’ overhang (Fig. 3.1)\textsuperscript{3,4,7,9}. The terminase then utilizes helicase-like activity to separate the two DNA strands resulting in a stable complex in which the terminase motor complex is bound to the left 5’-end of the lambda genome (known as complex I). The terminase in complex I subsequently binds to the portal proteins of an empty lambda procapsid thus forming complex II\textsuperscript{3,9,10}. Then, in the
presence of ATP, terminase will translocate the DNA into the procapsid until it reaches the cosQ subsite (downstream of the next cosN site) at the end of the genome. Upon arriving at cosQ, the terminase arrests packaging and cleaves the concatemeric DNA at the cosN site in the same staggered fashion as during initiation. However, as will be discussed in section 3.3.2, this final step in packaging (i.e. termination) only occurs when the capsid becomes sufficiently full and the motor identifies the cosQ site. After termination, the filled capsid then is released from the remaining concatemeric DNA and continues with the next phase of virus assembly5, 11-14.

**Figure 3.1:** A cartoon schematic of the lambda DNA phage cos site, which includes three main subsites: cosQ is the site that the terminase recognizes as the end of the genome. When this site is reached in normal in vivo conditions, the terminase terminates packaging and nicks the DNA at cosN; cosN is the subsite where the concatemeric DNA is nicked during initiation and termination such that twelve base 5’ overhangs are produced; cosB promotes the initiation of packaging by providing a site for terminase to initially bind, after which, in the presence of ATP, the terminase nicks the DNA at cosN, releases the DNA to the left using helicase activity, and binds to a procapsid. Note that lambda packaging is polar in that the terminase always translocates to the right given the orientation of this cartoon (that is, it moves in the direction of cosN -> cosB).

### 3.3 Results and Discussion

#### 3.3.1 Motivation and Results of lambda Pre-stall Packaging Assay

Previously we conducted lambda packaging with our dual-optical tweezers using an in situ method in which a complex I-tethered polystyrene bead is trapped in one optical trap while a different polystyrene bead coated with lambda procapsids is trapped
in another optical trap. These beads were then brought together in the presence of ATP to allow the terminase of a single complex I to bind to a procapsid on the opposing bead and begin packaging (Fig. 3.2a)\textsuperscript{15,16}. One difficulty with this method is that the efficiency for measuring packaging events is relatively low and seemingly not always correlated with efficiency measured from \textit{in vitro} bulk assays for a given batch of terminase and proheads. While it is possible to proceed with low event frequency, nearly all experiments \textit{besides} standard wild type (WT) packaging experiments become unfeasible. Conditions and cases in which such a low event frequency makes the experiment unfeasible or extremely cumbersome and time consuming include: low ATP concentrations; mutant terminases whose packaging efficiencies and rates are reduced from that of WT; low concentrated DNA; and/or the use of DNA preps with a low concentration of labelled DNA. In most cases under such conditions, it could take many months to collect enough data by one measurement mode (such as force feedback measurements or fixed position measurements) to yield results with reasonable statistical strength. As such, we endeavored to increase the event frequency since we would be utilizing the conditions described above in order to conduct this experiment as well as many other lambda experiments in our lab. After a tremendous amount of troubleshooting and reagent testing, we discovered we could significantly increase the number of packaging events that could be collected per hour by using a \textit{pre-stalled} packaging assay. This method first involves initiating DNA packaging in bulk and then quickly stalling translocation by introducing $\gamma$S-ATP (a nonhydrolyzable analog of ATP). After attaching these complexes to polystyrene beads, these coated beads are injected into the laser tweezers flow cell, which contains a buffer solution with ATP that stimulates the
restart of packaging (Fig. 3.2b). See Materials and Methods for additional details of the

*pre-stalled* method.

**Figure 3.2:** Schematics of phage packaging tweezer assays for a dual optical trapping system. **A)** The *in situ* assay previously used in our lab. During the third step, the microspheres are separated until a 5pN force is achieved. **B)** Our *pre-stalled* phage packaging tweezer assay. In this case a prepackaged and stalled DNA/terminase/procapsid complex is tethered to an optically trapped microsphere, while an anti-procapsid microsphere is trapped in another optical trap. The beads are brought together to allow the procapsid to bind to the anti-procapsid bead and then they are separated until 5pN is achieved between them. In the presence of ATP, γS-ATP will dissociate from the terminase, ATP will associate, and packaging will resume.
A benefit of the *pre-stalled* packaging assay is that it seems to better correlate with efficiencies obtained in bulk packaging reactions. For example, with a particular batch of wild type (WT) lambda terminase proteins and procapsids that successfully packaged DNA in bulk, we observed *no* packaging events in the tweezers using a short 10kb lambda DNA construct over a 3 hour testing period via the *in situ* assay whereas the *pre-stalled* assay yielded ~ 15 packaging events/hour over a 4.5 hour testing period. And given that a similar number of trials per hour were performed for each of these experiments, these results demonstrate the increased event efficiency and utility of this new *pre-stalled* assay. As previously mentioned, utilizing such a method will allow us to collect more data, which will yield stronger statistics, when testing the three proposed termination mechanisms (explained below). Figure 3.3a shows some WT lambda packaging traces collected in saturating ATP conditions when using the *pre-stalled* assay. These packaging traces show packaging rates of ~600bp/s, which is consistent with previously reported results using the *in situ* assay\(^{15,16}\). These traces also exhibit varying delay times for the reinitiation of packaging. Such delay times could be due to the dissociation/association kinetics of γS-ATP/ATP and/or a number of other factors. However, because such delay times are not relevant to focus of this study, we will not discuss them here.
**Figure 3.3:** Example packaging traces using pre-stalled assay and the short lambda termination construct. **A)** Some example packaging traces using our lambda phage *pre-stalled* packaging assay and a PCR generated lambda DNA construct with 10kb packable length. Each of these events completely packaged the DNA construct, however small variations in bead diameters were not taken into account in this simple analysis when determining tether lengths, so the completion of packaging does not always occur at a tether length of 0kb. **B)** Example packaging traces where our lambda termination construct (11kb packaging length) was used with wild type terminase. The initial delay in initiation (as seen in many traces in plot A have been cropped out from these traces. Also, the individual traces have each been offset by 3 seconds for display purposes. In each of these cases, packaging proceeds through the ectopic *cos* site, which is represented by the horizontal dotted line at 8kb tether length.

One limitation of the *pre-stalled* approach is that it does not allow us to measure the earliest stages of packaging since it is partly prepackaged before we begin measuring packaging within the tweezers. On average, it was observed that ~2.8kbp DNA was prepackaged before the phages reinitiated packaging in the tweezers. Nonetheless, we can still use this method to study termination and can minimize the time we allow for the initial packaging in bulk before we stall it with γS-ATP.
Alternatively, we have also developed and employed a modified pre-stalled tweezer protocol in effort to reduce the amount of pre-packaged DNA. However, before discussing this protocol, we outline here for comparison, the basic pre-stalled protocol, which is as follows:

1. A lambda DNA substrate, lambda terminase, packaging buffer, and lambda proheads are combined and incubated at room temperature (RT) for 5 minutes.
2. ATP is then added and incubated for 1 minute.
3. Immediately after, γS-ATP is added (in the same quantity as the ATP just added). The mixture is then incubated for 5 minutes at RT to ensure that the mixture has become uniform and that the complexes have had sufficient time to stall as a result of binding γS-ATP.
4. The pre-stalled complexes are then added to polystyrene beads.

Note that, under the conditions of equal quantities of ATP and γS-ATP, it is still possible for DNA to occasionally be packaged. The rate, however, would be significantly slowed. Furthermore, the idea behind using equal quantities of ATP and γS-ATP in the protocol was that these conditions would stall the motors as desired for our experiments while also making it easier for the motors to reinitiate. That is, under such conditions, we would expect that only half of the terminase subunits would contain γS-ATP, and thus the rate of reinitiation would be faster since fewer subunits need to be reactivated by exchanging γS-ATP with ATP. We note that experiments by Andrews and Catalano studying coordination of motor subunits suggest that stalling one motor subunit is sufficient to stall the whole motor. Also, given that we used crude extract terminase in our experiments, which contains ATP, this protocol could potentially be redesigned to yield less prepackaged DNA if the ATP is added before the proheads and the mixture is
incubated for 5 minutes at RT before adding the proheads. This however was taken into account when devising the modified pre-stalled protocol.

The modified pre-stalled protocol is as follows:

1. Lambda DNA substrate, lambda terminase, packaging buffer, and ATP are combined. This mixture is then incubated for 5 minutes at RT so that the terminase can bind and cut the DNA at the cosN sites.

2. γS-ATP is added in the same quantity as the previously added ATP and the sample is incubated for 1 minute at RT.

3. Lambda proheads are added and the sample is incubated for 5 minutes at RT before adding these pre-stalled complexes to the polystyrene beads.

By this method, on average, ~ 1.5kbp of DNA was prepackaged before the phages were reinitiated within the tweezers. This is significantly less than the basic pre-stalled protocol. Consequently this protocol was chosen to be used for the lambda termination experiments since one of our lambda termination DNA constructs contained the ectopic termination cos site at ~ 3kb downstream from the initial cos site (see the discussion below). Notably, this amount of prepackaged DNA was more than initially expected. This suggests that this half-and-half mixture of γS-ATP and ATP still permits the phages to package before they are added to the polystyrene beads. As such, if it is desired to have even less DNA prepackaged prior to reinitiation, one could increase the ratio of γS-ATP to ATP in this protocol or simply reduce the 5 minute incubation period prior to adding the complexes to the polystyrene beads. However, in the case of the increased γS-ATP/ATP ratio, it may also take longer for the complexes to reinitiate packaging. Lastly, it has been suggested that just after binding to cosB, terminase can be triggered by ADP or ATP to nick cosN. If true, this could be used to potentially yield assembled prohead/terminase/DNA complexes that have not prepackaged any DNA but simply have
the prohead/terminase complex bound to the initial cosB site waiting for the introduction of ATP so that packaging can be initiated. A protocol (similar to the standard pre-stalled protocol) using this idea has been tested in preliminary trials but did not immediately yield the expected results. Short tethers were observed, indicating that these complexes had prepackaged some DNA, and some packaging events were also obtained, though infrequently compared to the standard wild type protocol. It was later discovered that this result was likely due to the presence of ATP in the terminase extract used. Therefore, additional testing would still need to be performed in order to ascertain whether this concept can work. Such experiments could include using this same protocol but with an overwhelming concentration of ADP or using purified terminase, which would not contain any ATP.

3.3.2 The Three Models and Experimental Hurdles

The velocity model predicts that termination (the point at which translocation ceases and terminase cleaves the DNA) occurs once the motor has slowed sufficiently that it can recognize cosQ, which signals the end of the genome. Whereas the energy-monitor model conjectures that termination occurs when terminase senses that the amount of work per packaging step, or force since \( \text{work} = \text{force} \times \text{distance} \) and the step distance is assumed to be fixed, has achieved a certain threshold value. Lastly, the capsid-filling model proposes that the terminase can directly sense the amount of packaged DNA, or perhaps "DNA pressure" within the capsid, and that termination is triggered when a threshold filling or “pressure” is reached. However, it must also be noted that some viruses terminate packaging at a special DNA sequence. Such is the case with lambda phage and its cos site. It was previously found that DNA constructs with
initial and terminal $cos$ site spacings of 0.75, 0.78, and 0.85 \( \lambda \) genome length (36,377bp, 37,832bp, and 41,227bp respectively) terminated with a frequency of 4\%, 19\%, and 34\% respectively\(^{21-24}\). Consequently, the probability of termination is extremely unlikely if a $cos$ site is placed at a point less than 75\% of the full lambda genome length.

Furthermore, in 2007, Fuller et al. reported that, at long lengths, lambda phage packaging undergoes slowed packaging while the motor exerts high forces (up to 25pN at 90\% of genome packaged) to overcome the forces resisting DNA confinements\(^{28}\). This implies that viruses with DNA termination sequences not only require such a site for termination, but also another mechanism of sensing of the extent of packaging: either slowed packaging, high resistance force, high capsid pressure, or some combination of these three conditions. It is this requirement that will help us discern which model may be the correct one.

With wild type lambda DNA, the near terminal packaging stage is convoluted by low packaging velocity, high capsid pressure, and high resistance force. As such, how do we independently test for velocity, pressure, or force dependent termination? This is where we take advantage of the lambda $cos$ sequence. During the early stage of lambda packaging (less than 20\% of the genome being packaged), packaging is fast and relatively constant while capsid pressure and resistance force are essentially zero\(^{28}\). In other words, velocity, pressure, and force are not convoluted during early stage packaging. With this knowledge, we have designed an experiment where we can test these models by utilizing the unique features of our tweezer assay as well as a specially designed DNA construct that has an ectopic $cos$ site placed \( \sim 6\% \) of the lambda genome length downstream from
the initiation site. The details of these tests will be described below. Also, we will henceforth refer to this special DNA construct as our short termination construct.

### 3.3.2.1 DNA Construct Designs

In designing this termination construct, we needed to account for the fact that terminase will bind to all available *cos* sites present in a given strand of DNA. If a biotinylated DNA construct with two lambda *cos* sites was used in our tweezer assay, both sites would be cleaved, leaving two segments of packable DNA, but only one would have a biotin label. Therefore, we would only ever measure packaging of the biotinylated segment that now no longer contains an internal *cos* site. Given that our tweezer assay cannot limit terminase binding and cleavage to the first *cos* site only, we needed to design a special DNA construct that contained an internal ectopic *cos* site where terminase could terminate packaging but not initiate (i.e. bind to *cosB* and nick at *cosN* just prior to packaging). A *cos* site with such properties had previously been discovered by Feiss and Winder. They showed that lambda terminase can terminate but not initiate at the Lambdoid phage 21 *cos* site, which we denote as *cos*φ21. With this at hand, in order to proceed with our termination model testing proposal, we now needed to create a DNA construct containing a lambda *cos* site followed by a *cos*φ21 site, which proved to be much more difficult than initially expected. In this study we prepared two termination constructs by entirely different methods. The first construct was created, with great difficulty, by ligating two PCR generated segments of DNA together where one segment contained the lambda *cos* site while the other segment was biotinylated and contained the *cos*φ21 site. This construct’s full length is 14,954bp with a packable length of 11,084bp and a distance of 3,085bp (6.4% lambda genome length) between the *cos*3 and *cos*φ21 sites.
(see Fig. 3.4a). Unfortunately, this method yielded low quantities of the desired product. Thus, developing an alternative, higher yielding method was undertaken by our collaborators (Feiss Lab, U. of Iowa). Nonetheless, this construct (made by the ligation method just described) was still used in our initial control experiments. The second method, which also took an unexpectedly long time to develop, inserted a vector containing a $cos^3$ site and another vector containing a $cos^{p21}$ site into a plasmid such that the distance between these $cos$ sites was 3,736bp (7.7% lambda genome length) (see Fig. 3.4b). The resulting plasmid was grown up in bacteria, extracted, and used to create a PCR generated and biotin labelled termination construct. Ultimately this protocol yielded high quantities of DNA that was then used for all subsequent experiments. This construct has a full length of 13,424bp with a packable length of 13,077bp (see Fig. 3.4b). See methods for additional details of these protocols.

**Figure 3.4:** Cartoons of the lambda Termination DNA Constructs used in this study. The biotin at the right is used to tether the DNA to the streptavidin beads in the tweezer assay. A) First developed termination DNA construct (ligation method) used for initial control experiment – see Fig. 3.3b. B) Second developed termination DNA construct (plasmid method) used for all experiments after initial control experiments – see Fig. 3.4a&b.
3.3.3 Control Experiments

As an initial control, we packaged the short termination construct (ligation method construct) with wild type terminase via our pre-stalled assay. In all instances (>10 trials) where packaging initiated before the ectopic cos site, which occurs ~8kb upstream from the polystyrene bead (i.e. the horizontal dotted line in figure 3.3b, ~3kb downstream of the initiation site), packaging proceeded through the ectopic cos site uninhibited (Fig. 3.3b). While additional data is still needed, these results support the results by Feiss (ref. [23]) and Emmons (ref. [24]) indicating that termination does not solely occur due to recognition of the cos site. Thus, we should now be able to proceed with the tests of the proposed termination models.

3.3.3.1 cosφ21 Termination Control Experiment

It is here that we must now point out an obstacle that has significantly delayed the progress of this project. After this initial control experiment was perform we proceeded to test the models, beginning with the velocity model. However, while these tests were underway, we realized we needed to perform another control experiment. We need to demonstrate that we can observe termination in general at a cosφ21 site in our tweezer assay. This need, however, introduces new hurdles to overcome. In our tweezer force clamp assay, we employ a force feedback program that maintains 5pN in the tether during packaging. Termination can be identified when the force drops from 5pN to 0pN. In order to test that we can observe such DNA cleavage at a cosφ21 site, we need to create a biotin labelled DNA construct with the cosφ21 site positioned at a point greater than 36,377bp (75% of the lambda genome length) from the initiation cosλ site. Furthermore, our protocols are based on using un-expanded proheads, which rupture when 90% filling
is achieved. After this rupture, packaging resumes but the packaging rate, resistance force, and capsid pressure return to the levels they were during the initial packaging state (i.e. high speeds, zero capsid pressure, and zero resistance), which are not appropriate conditions for termination\textsuperscript{28}. Therefore, this long control termination DNA construct requires that the $cos^{\phi21}$ site be placed at a distance of 75\%-90\% the lambda genome length from the initial $cos^\lambda$ site. Lastly, we need to have a fairly high yield and high concentration of this DNA because lambda packaging in the tweezers is typically inefficient and our \textit{pre-stalled} and \textit{in situ} protocols exhibit greater efficiency with relatively high concentrated DNA. Furthermore, since efficiency also tends to decrease with increasingly longer DNA constructs, we need to do whatever possible to maximize the tweezer packaging efficiency in order to make these experiments feasible.

The design and construction of such a DNA construct was undertaken by our collaborators starting approximately a year ago, but proved difficult. Since this is still not finished, this study could not be pursued as a part of this dissertation; instead I turned my attention to finishing the studies described in Ch. 2. Nevertheless, in the remainder of this chapter I will present the findings of our preliminary studies and outline our present future plans for completing these studies, since these considerations will be essential for whoever continues this project in the future. The method by which this construct is being generated is similar to the plasmid transformation method used for the short termination construct, however, transformation grows increasingly more difficult as the plasmid size grows and, in this case, the final plasmid length is extremely long: \textasciitilde49.2kbp. The plasmid has been designed to have the $cos^\lambda$ and $cos^{\phi21}$ sites separated by \textasciitilde40kbp, which is \textasciitilde82.5\% of the lambda genome length. Since it was decided that this control was
absolutely necessary to determine the termination mechanism via our tweezer assay, we have put all tests of these models on hold until we verify that we can observe termination at the \( \text{cos}^{\phi 21} \) site. Nonetheless, we still have some preliminary results from testing the velocity model that are presented below. The manner by which the other models will be tested our outlined below and will be pursued once this long control DNA construct has been created and proven that we can observe termination at a \( \text{cos}^{\phi 21} \) site within our tweezers.

### 3.3.4 Testing the Velocity Model

To discern which mechanism triggers termination, we must first begin by testing the velocity model. To do so, we need to reduce the packaging rate to that which occurs during the terminal stage of packaging. As previously stated, it was demonstrated that lambda phage can terminate packaging as early as 75% filling but with a low probability\(^23\). Since it was also demonstrated that the probability of lambda phage termination increases with increasing filling\(^23,24\), to maximize the efficiency of our experiments, we endeavor to reduce the velocity of packaging near that which is expected near 100% filling. Fuller et al. (2008) reported that lambda packages at \(~400\text{bp/s}\) when 75% filled and \(~250\text{bp/s}\) when it is 90% filled. Extrapolating from their results, we estimate that lambda packages at \(~150\text{bp/s}\) near 100% filling. We can reduce the packaging velocity to such rates by: lowering the ATP concentration, lowering the temperature in the experimental environment, and/or using a slow packaging mutant terminase. Our lab has previously demonstrated that using \([\text{ATP}] = 2.5\mu\text{M}\) yields packaging rates \(< 100\text{bp/s}\) and using the lambda terminase with a T194M gpA subunit.
mutation yields packaging rates of < 100bp/s. In addition, packaging at 4°C is reported to yield packaging rates of ~120bp/s or less\textsuperscript{16,30}.

As mentioned before, termination can be identified in our tweezer assay by observing a drop in force from 5pN to 0pN while packaging with a force clamp program. So, to perform this experiment, we slowly package our termination construct using one or more of the packaging rate reduction methods described above while looking for the force to drop to 0pN at the position of the \textit{cos}\textsuperscript{921} site (7.7% filling). Since capsid pressure and the load force are near zero at 7.7% filling\textsuperscript{15}, if we observe termination under this slowed packaging condition, then the velocity model will be supported and both the capsid-filling and energy-monitor model will be ruled out\textsuperscript{21-24}. However, if termination is not observed at the ectopic \textit{cos} site while packaging slowly, we cannot immediately rule out the velocity model. There are a couple other conditions that may need to be met in order to observe termination here. Normally in the cell there is free terminase in solution. Our collaborators (Feiss & Catalano) have proposed that free terminase subunits in solution may participate in termination. In addition, \textit{in vivo} the lambda phage capsid expands after 30% filling and is then stabilized by the protein gpD\textsuperscript{15,31}. Thus, it is also possible that termination might only occur if a gpD stabilized expanded capsid is being used during packaging. Therefore, if termination is not observed by simply slowing packaging, we may need to also test with terminase subunits in solution and/or use pre-expanded, gpD coated capsids. If, after this, termination is still not observed, then the \textit{cos}\textsuperscript{921} termination control experiment described earlier becomes particularly important. A positive result from this control in conjunction with a null result from the velocity model tests would provide strong support against the velocity model.
3.3.4.1 Results

As stated earlier, we performed a couple tests of the velocity monitor model, but no termination was observed. We reduced the packaging velocity using the T194M mutant terminase and by using an ATP concentration of 2.5µM. In each case, all events showed the phage complex package over the position of the $cos^{\phi 21}$ site, as seen in figure 3.5a&b by the example blue packaging traces that cross over the $cos^{\phi 21}$ site, which is indicated by the red dotted line (see methods for brief description of how the position of the $cos^{\phi 21}$ site was identified in the tweezers assay). Note that, for the [ATP]=2.5µM case, the traces exhibit a lot of pausing and slipping. This is normal behavior of lambda in low ATP conditions and thus has nothing to do with termination or the $cos^{\phi 21}$ site. These results argue against the velocity model, but the other conditions as well as the control previously discussed still need to be checked before making a final conclusion.
Figure 3.5: Examples of slowed lambda packaging traces (tether length in kilo base pairs Vs time in seconds) resulting from the use of the lambda termination construct (Fig. 3.4b) and either a mutant terminase or low ATP. Note that the initial delay in initiation has been cropped out from each trace. The traces in plots A) and B) have been offset by 30 and 45 seconds respectively for display purposes. The position of the ectopic cos site in each plot is represented by the horizontal dotted line at ~9.3kb. A) T194M terminase used with our lambda termination construct (plasmid method – Fig. 3.4b) in saturating ATP conditions. The T194M terminase yields packaging rates much < 100 bp/s. B) Wild type terminase used with our lambda termination construct (Fig. 3.4b) in 2.5μM ATP. Under this [ATP], wild type terminase yields packaging rates much < 100 bp/s.

3.3.5 Testing the Energy-Monitor Model

To test this model we will utilize the unique capability of optical tweezers by directly applying additional force during packaging as the motor approaches the cosφ21 site. Doing so will increase the load on the motor, as would occur at high procapsid filling when the force resisting DNA confinement rises. If termination is observed at the cosφ21 site, then this energy-monitor model will be supported. We must note that while it is known that applying external force causes the packaging velocity to decrease, thus convolving this model with a velocity model, results from the velocity model test will
help us distinguish it from the results of this energy-monitor model test. A test of this
model can be performed in two ways: 1) Applying a quick impulse force when the $cos^{\phi_{21}}$
site is reached, thereby not permitting the motor velocity to slow down before the
expected point of termination. 2) Applying a high force throughout the entire duration of
packaging, which would yield a combination of slow packaging as well as a high load
force. Observing a null result from the velocity model test and a positive result from (1)
would provide support for a strictly energy-monitor mechanism. If this result was
observed, we would expect a test of (2) to also be positive but the conclusion would
remain the same. On the other hand, observing a null result from the velocity-monitor
test, a negative result from (1), and a positive result from (2) would support the
possibility that termination works by a combination of the velocity-monitor and the
energy-monitor models.

Lastly, even if the velocity model were supported by the results of the velocity-
monitor experiment, these experiments would serve as a control against the energy-
monitor model. But, in the case that termination is not observed by slowing the motor or
applying additional force, then the capsid-filling model will be supported. Though, an
explicit test of the capsid-filling model would still be necessary, which is described next.
As stated earlier, the test of this energy-monitor model has been put on hold until the
control $cos^{\phi_{21}}$ site termination experiment has been performed.

3.3.6 Testing the Capsid-Filling Model

In previous work we have shown that the internal capsid pressure can be varied by
changing the ionic screening of the DNA, which can be done by using different ions (e.g.,
$Na^+$ vs. $Mg^{2+}$ vs. Cobalt Hexamine$^{3+}$) and differing ionic concentrations$^{29}$. We plan to
test the pressure sensor model by watching for termination while varying ionic concentrations and ectopic cos site positions. If we find termination efficiency to depend on ionic concentration, then we will have support that terminase arrests packaging and cleaves the DNA by sensing the internal capsid pressure. Also, if tests of one of the other models yield a positive result, this experiment should still be performed to test for the possibility that termination utilizes two mechanisms: a pressure sensor as well as one of the other sensors (velocity or force monitor).

3.4 Conclusion

While there appears to be a large consensus that the general mechanism for termination is based on a headful (pressure) mechanism with considerable structural evidence provided more recently for the P22 phage, it still isn’t completely certain. Previous studies have not fully separated the convoluted mixture of low velocities, high forces, and high filling, thus making it difficult to claim one model over another or even to claim the use of a combination of sensors. The fact that lambda requires both a cos site as well as another trigger for termination demonstrates it is reasonable to consider the possibility of multiple sensors triggering termination. Our study here provides a method by which the proposed mechanisms can be tested not only separately but also directly. Though we think this experiment can provide evidence for a general mechanism of termination for dsDNA bacteriophages, we must acknowledge that the results of this study could be unique to the lambda phage (especially if the results are different than the capsid filling model). On the other hand, the significant sequence homology between the endonuclease domains of different viral terminases suggests they may have a common mechanism.
Thus far we have developed a *pre-stalled* phage packaging protocol that yields significantly greater event frequency in the tweezer assay than the previously used *in situ* protocol. The use of this protocol will aid in testing these mechanisms since many of the conditions under which these models need to be tested typically reduce the event frequency relative to that observed under normal conditions with wild type reagents. With this *pre-stalled* protocol and the short DNA termination construct, we have performed experiments that support, for the case of lambda and other phages with termination DNA sequences, that termination is not triggered purely by recognition of the *cos* DNA sequence (as expected) and that termination does not occur when slowly packaging under low ATP conditions or with the mutant terminase T194M, which provides support against the velocity-monitor model. The remainder of the velocity-monitor experiments as well as the energy and pressure monitor model tests are pending until the *cos*φ21 termination control experiment is performed. Once the DNA construct needed for this control experiment is available, these experiments will be performed. Until then, we’ve clearly outlined how each of these termination models will be directly tested.

### 3.5 Acknowledgements

We thank James Tsay, Nicholas Keller, Alan Schweitzer, and Mariam Ordyan for thoughtful discussions and technical assistance. We also thank our collaborators Jean Sippy, Michael Feiss, and Carlos Catalano for helpful discussions and providing protein reagents. We would also like to thank Shelley Grimes for helpful discussions. This work was supported by the NIH and NSF.
3.6 Materials and Methods

3.6.1 Optical Tweezers Instrument

All phage packaging was performed using a dual optical trapping system under a 5pN force feedback mode as previously described in [15].

3.6.2 Production of lambda Terminase and Procapsids

Lambda terminase used in these studies was an *E. coli* cell extract prepared by our collaborators (Feiss Lab, U. Iowa) as previously described in [15]. Also, the procapsids used here were purified by our collaborators (Feiss Lab, U. Iowa) as described in [15].

3.6.3 DNA Constructs

For all cases of PCR described below we used the 5Prime PCR Extender System and the standard non-high fidelity protocol provided by this kit. Also, 20ng of plasmid DNA was used for each 50ul PCR reaction.

3.6.3.1 10kb lambda Packaging Construct

A 13,881bp plasmid, pJM1, containing the full lambda *cos* site was provided by the Feiss Lab (U. of Iowa). Using this plasmid as a template, PCR primers were chosen to produce a biotin and digoxigenin labeled 13.7kb (13,747bp) DNA construct with the *cos* site positioned 10kb from the biotinylated end. The forward and reverse primers used were Dig-5’-TCGATAATCGTGAAGAGTCGGCGAGCCTGGTTAG-3’ and Biot-5’-TACGTCGAAGTGACCAACTAGGCGGAATCGGTTAG-3’ respectively.

3.6.3.2 Lambda Termination Construct

*First Construct Design: Ligation Method*

This was constructed by producing two pieces of DNA by PCR, cutting them with a restriction enzyme, and then ligating them together. The first piece is a 5kb piece of
DNA containing a lambda \textit{cos} site and an NcoI restriction site that was PCRed from circularized lambda DNA. The forward and reverse primers used were 5'-

\textbf{TTACTACCGATTCCGCTAGTTGGTC-3'} and 5'-

\textbf{TCTT(CCATGG)TGTTGCCTGCTTTATGCTCTATAAAGTAG -3'} respectively. The second is a 10kb piece of DNA containing a \textit{cos}^{\phi 21} site and an NcoI restriction site that was PCRed from the plasmid pJB2 (provided by Feiss Lab, U. of Iowa). The forward and reverse primers used for this segment were 5'-

\textbf{TCTT(CCATGG)CATATCGATGGGCAACTCATGCAATTATTGTG -3'} and Biotin-

\textbf{5'-GCGATAAGTCGTGTCTTACCGGGTTGGGAC-3'} respectively. The 5kb piece was designed to have an NcoI restriction site on the right end while the 10kb piece was designed to have an NcoI site on the left end and a biotin label on the right end (the bold bracketed sequences in the primers are the NcoI sequences). These were digested with NcoI and then ligated together. Furthermore, the primers were designed such that the final construct length is 15kb, but the lambda \textit{cos} site is placed ~11kb from the biotinylated end while the \textit{cos}^{\phi 21} site is positioned ~8kb from the biotinylated end.

\textit{Second Construct Design: Plasmid Method}

A 13,749bp plasmid was created by our collaborators (Feiss Lab, U. Iowa) in which a \textit{cos}^{\phi 21} site was inserted into their \textit{cos}^{\lambda} containing plasmid, pJM1, such that the two \textit{cos} sites were 3,736bp apart (7.7\% the length of the lambda genome). Using this as a template, we used forward and reverse primers, Dig-

\textbf{5'-TTAGTCTCCGACGGCAGGCTTCAATGAC -3'} and Biotin-

\textbf{5'-TTCTTGACAGTGACAGACTGCGTGTTGGAC-3'} respectively, to PCR generate a 13,424bp biotin and digoxigenin labelled construct with 13,077bp between the \textit{cos}^{\lambda} site
and the biotinylated end of the construct as well as 9,341bp between the $cos^{\phi21}$ sites and the biotinylated end of the DNA.

### 3.6.4 Pre-Stalled Packaging Protocol

This method begins by initiation packaging as one would in bulk. In a 1xTM (25mM tris-HCl pH 7.5 & 5mM MgCl$_2$) we combine β-mercaptoethanol, the DNA construct of interest, wild type terminase, and ATP. We let this incubate for 5 minutes so terminase can bind and nick the DNA. We subsequently add lambda proheads, let incubate for ~1min, and then stall packaging by adding γS-ATP. We let this incubate for ~5minutes. Final volume is 5ul. Finally we add 1ul of these stalled complexes to 5ul of two times 1xTM washed streptavidin coated polystyrene beads and let them incubate on a rotator for 20 minutes.

The anti-procapsid beads are prepared by initially washing protein G coated polystyrene beads twice in 1xPBS, followed by two more washes with 1xTM. Lambda anti-sera is then added to these beads, which are then incubated for ~30 minutes on a rotator at room temperature. Afterward, the beads are washed another two times in 1xTM and finally suspended in 1xTM for use in the tweezers.

### 3.6.5 Tweezer Assay for Pre-Stalled Phage Complexes

In our dual-trap optical tweezer setup, we captured anti-procapsid beads in one trap and the pre-stalled complex coated beads in the other trap. These beads were bumped into one another and then separated until a tether was formed. We titrated the amount of pre-stalled complexes added to the streptavidin beads to the point that only single tethers formed during this fishing procedure. The beads were separated until a tether that surpassed the distance between the $cos^{\phi21}$ site and the biotinylated end was
observed and achieved 5pN. At this point we waited in ATP for packaging to reinitiate while our system maintained a 5pN force during packaging. After sufficient DNA was packaged for the experiment, the beads were slowly pulled apart until the tether broke. The observation of only a single tether breaking to zero force, after separating the beads by a large distance, was used to confirm that we had only a single tether during the trial.

3.6.6 Identification of the \( \text{cos}^{\varphi 21} \) Site Position in the Tweezers Assay

Our raw packaging trace data was converted to tether length distances in base pairs using the method described in chapter 5. However, the tether length includes not only the DNA length, but also the diameter of the prohead, which can vary slightly, and the portal/terminase complex length. While we know the diameter of lambda proheads to be \(~50\text{nm} (~150\text{bp})\) \(^4\), we cannot simply subtract this length from the trace tether length calculation mentioned above because we don’t necessarily know the orientation the prohead has bound to the anti-prohead bead. In spite of this, we would still expect that the tethers we manage to obtain in the tweezers are primarily those where the prohead’s portal is roughly facing away from the bead surface. Furthermore, the tether length conversion assumes the bead diameters are fixed, but they in fact vary (the manufacturers provide the bead diameter distribution for a given batch of beads). When performing the experiments, we can roughly identify by eye the typical bead size and thus only accept and use beads of similar size. However, the diameters of the beads accepted can still vary as much as \(~100\text{nm} \text{ (or } ~300\text{bp})\). Therefore, with all this as well as the actual base pair position of the \( \text{cos}^{\varphi 21} \) site within the DNA construct, we roughly estimate a window for the \( \text{cos}^{\varphi 21} \) site to reside within a given packaging trace.
To aid in evaluating this $\cos^{\phi 21}$ site window, we created a Biotin and Digoxigenin labelled DNA construct with a length equal to the base pair position of the $\cos^{\phi 21}$ site within our long lambda termination construct (plasmid method). This construct was PCR generated using the lambda termination plasmid and the forward and reverse primers Dig-5’-GGGCGGCGACCTCGCGGTTTTTCACTATTTATG-3’ and Biotin-5’-TTCCTGACAGTGACAGACTGCGTGTTGG-3’ respectively. The 5Prime PCR Extender System was used here along with the kit’s standard non-high fidelity protocol. Once the DNA was generated, we tethered it between streptavidin and anti-digoxigenin beads and then stretched it within the tweezers to measure the $\cos^{\phi 21}$ site tether length window that results from variations in bead diameter. Then a rough estimation of the additional prohead diameter/orientation distance in base pairs was incorporated with this window size/position to yield the final window in which the $\cos^{\phi 21}$ site is expected to reside.

Though there are factors that make the exact position of the $\cos^{\phi 21}$ site uncertain within our experiments, termination can still be confirmed if, when it occurs, it most often occurs within this window we’ve estimated for the termination site.

3.7 References for Chapter 3


Chapter 4
Building High Resolution Dual-Optical Trap Tweezers

4.1 Abstract

The development of single-molecule manipulation instruments and methods, such as optical tweezers and magnetic tweezers, has greatly enhanced the understanding of many biological systems. As the field of single-molecule biophysics has advanced, so too have the demands on the instrumentation. Several groups have develop improved instrumentation to extend the resolution of optical tweezers measurements to the sub-nanometer level. As one of my thesis research projects I built a new dual-trap optical tweezers setup employing several improvements described by Moffitt et al. as well as implementing some improvements of our own design. Here we provide a description of the techniques used for our new instrument and a comparison with the previous system used in our lab.

4.2 Introduction

Since the late 1980s, many tools and methods have been developed to investigate the intricacies of biological systems at the single-molecule level.1 In particular, the optical trap2 has played a major role in the development of single-molecule biophysics research.1 Advancements in this field has led to the development of the more commonly used dual-trap optical tweezers, which are capable of applying piconewton forces and resolving nanometer-level displacements.3,5 Such tools have facilitated research that has greatly enhanced our understanding of many biological systems. However, often with greater understanding comes questions of greater detail. And in this case, to answer such
questions, investigative tools with greater resolution are needed. In 2006 Moffitt et al. described an approach to increase the spatial resolution of the dual-trap optical tweezers design to resolve subnanometer distances (Note that other labs have also developed different approaches for obtaining similar resolution\textsuperscript{6,7}). Their approach employed (1) techniques to reduce instrumental noise and drift by minimizing the effect of environmental factors; (2) methods that reduce measurement noise; and (3) a "differential" measuring technique between two optical traps.\textsuperscript{8-13} However, we did not simply build an exact replica of the system developed by Moffitt et al, because the exact details on their system are not given in the literature and the estimated cost of their system was at least 10x higher than our available budget. As such, we have endeavored to create a dual-optical trapping system that would significantly improve our resolution by utilizing some similar approaches as described by Moffitt et al. Methods we used include improving our own optical alignment methodology; testing optical components and using those that contribute lower noise; implementing design features that minimize external environmental noise contributions; utilizing dual-trap detection; and using measurement techniques that reduce the measurement noise. We show here how the use of such methods have helped us develop a system with ~1 nm spatial resolution, which is significantly improved over the ~10 nanometer resolution of our previous system. In this chapter we describe the key measures taken in designing and building the new system as well as present a comparison between our older lower resolution system and this new improved system.

We would like to emphasize from the beginning of this chapter that the goal of this project was not to conduct a detailed scientific/engineering study of the factors
governing optical tweezers resolution and to build an optimum system without regard to construction time or cost. Rather, it was to build a new instrument with improved stability and resolution over our previous system, within the budget we had available, that could be used to attempt measurements of the incremental step size of the phage lambda motor as well as to assist with our tests of the lambda termination mechanism and properties of HARP (chapters 2 & 7). Since this was not intended to be an engineering project, we did not quantitatively document results from tests of noise reduction techniques and methods, as this would be very time consuming. In building this system, we took the following approach. If a particular method or technique we tested yielded any measurable improvement then it was implemented, and only a qualitative discussion of these considerations is given in this chapter. Tests of the fully assembled system, however, are presented to show the increased performance over the prior system. In addition, applications of the system to measurements of the phage lambda step size are presented in Ch. 6.

4.3 Experimental and System Design Background and Implementation of Design

4.3.1 Background: Dual-trap Optical Tweezers in a Nutshell

In brief, our dual-trap optical tweezers work as follows: A 1064nm continuous wave laser is split into two beams of equal power and of orthogonal polarization. One of these beams is either reflected from a mirror (which can be tilted by a piezoelectric-driven actuator) or passed through an acousto-optic deflector (AOD) so that the downstream trap resulting from this beam can be translated. After expanding the beams, they are passed through a microscope objective, which focuses the light and thus creates two optical traps. A clear glass flow cell chamber (Fig. 1.1 in chapter 1) is positioned
just after the objective so that the traps are formed within the chamber. The light subsequently passes through another objective, which recollects the outgoing beams. The light from the stationary beam exiting this objective is imaged onto a position sensing detector (PSD) in such a manner as to measure a signal proportional to the mean angular deflection of this beam. In our case, experiments are typically performed by flowing micron sized polystyrene beads that are coated with biological molecules into the flow cell where they can be captured by the optical traps. Deflections of the trapping beams, which result from the response of the traps to external forces of the surrounding fluid and/or the biological molecules on the beads, are measured by the PSD to measure nanometer displacements and piconewton forces of the molecules under study. The beads can then be manipulated with the AOD or piezo-electric steered mirror as desired for the experiment while monitoring forces acting on the beads. Additional details for our older design can be found in ref. 14.

4.3.2 Building Details of the Higher Resolution Dual-Trap Optical Tweezers

A thorough "user’s manual" was written describing how the new tweezers instrument we discuss in this chapter was built and has been included in the appendix of this dissertation. The manual was written in the fashion of a guide to building and aligning our high resolution tweezers for the inexperienced student. It includes all the details of the equipment used, rational for design and equipment choices, and detailed basic instructions for how the system was built. Additional information not given in this chapter can be found in the dissertation appendix.
4.3.3 Background: Environmental Noise Considerations

Since many biological translocation motors function with rates that range from nanometers/s to hundreds of nanometers/s\textsuperscript{15-21}, with step sizes on the order of 1 nm, measurements of such motors can be affected by any external noise that produces signals of similar frequencies (i.e. < 300Hz). Therefore, to improve the spatial resolution for measurements with such motors, methods must be employed that reduce external signals within this low frequency regime. Noise sources that can produce such low frequency signals primarily come from room vibrations, temperature changes, and air fluctuations. Vibrations from the building, nearby noisy equipment, noisy classrooms, elevators, and/or air fluctuations (among other sources) can couple to the tweezers through its optical table and/or individual optical components to produce resonant modes of oscillation in the table and optical components at frequencies of 30-300Hz. Moreover, air fluctuations also contribute to spatial variations by causing air density fluctuations, which vary the refractive index of the air and consequently affect the pointing stability of the trapping beam. In fact, it’s been found that gentle air flow can cause an optical trap to move by tens of nanometers at low frequencies.\textsuperscript{9}

Fluctuations at very low frequencies (<1Hz), i.e. long term drift, become relevant for the occasional experiment that must be conducted on long time scales while maintaining high spatial resolution. Temperature changes, in particular, can yield instrument fluctuations on these large time scales (minutes to tens of minutes or more).\textsuperscript{9,10} The dimensions of the instrument’s optical components are susceptible to change with a change in temperature. Moreover, the rate of dimensional change depends on the material of which the components are made. As such, it becomes important to minimize
temperature fluctuations to the system and to use optical components of the same material where possible and with low thermal expansion coefficients. There are multiple ways in which heat can be transferred to the instrument that must be considered: air temperature (convection), human contact with any of the optical components (conduction), and laser heating (radiation). And temperature changes due to any of these transfer methods can yield long term instrument drift.

4.3.4 Accounting for Environmental Noise/Drift

Since Moffitt et al. do not present any explicit breakdown of the contribution of various factors to the overall noise/drift, we sought to test and thereby confirm the effectiveness of some of these environmental noise reduction techniques before using them within the system. To help us decide whether to apply the technique, we also considered cost (in time and money) to decide whether various approaches would be feasible and worthwhile for improving our spatial resolution.

To assess the effect of air currents we measured the effect of a beam’s pointing stability as a result of: blowing air across a beam path, using various beam path lengths, confining the optical setup to a box, confining the beam paths to a tube, and confining the beam paths to a tube while also covering the optical setup to a box. Consistent with the recommendations by Moffitt et al. (and Block et al. – ref. 7), our measurements showed that air flow significantly affects the beam’s pointing stability. In practice, we found that a setup with the beam confined to tubes yielded the greatest improvement in the beam’s stability and that the addition of an exterior box only had a relatively minor effect on improving the stability. Therefore we designed our system to have: the shortest beam paths possible (within our chosen design constraints); beam paths confined to black
anodized aluminum tubing; a plastic (high density polyethylene – HDPE) box, which had
½” walls lined with black-anodized aluminum foil and internally-baffled chambers for
separate sections of the optical system, to cover the entire tweezer setup. Additional
advantages of this box is that it reduces the buildup of dust on the optical components and
also reduces that chance that a user inadvertently bumps or spills fluid on any of the
components.

To test for noise contributions from room vibrations we tested how a beam’s
pointing stability was affected by: having the lights on or off; having electronic
equipment (PSD amplifier, laser and laser power supply, etc.) on or off the optical table;
floating and not floating the optical table on pneumatic isolators; manually oscillating the
table; talking in the room (i.e., acoustic noise); different optical mounting components
(such as adjustable mirror mounts vs. fixed mirror mounts and 1” vs. ½” mounting posts)
and components made of aluminum vs. stainless steel. Note that our lab is already
located in the basement of the building, away from elevators, so no effort was made to
build the system in any other room since this room already has among the lowest room
vibrations in the building. Under some of these conditions we were unable to observe
any noise contributions or improvements in the beam stability in which case we chose the
option that was expected to be a good compromise between cost, effort, and performance
for our system. In general, conditions that did yield noticeable reductions in
measurement noise (lights off, equipment off the table, no talking, stainless steel
mounting posts, 1” mounting posts) were consistent with those suggested by Moffitt.
Since the use of these measures did not add too much to the cost or effort to building the
instrument, we ultimately built the system with: 1” diameter stainless steel mounts
throughout the setup and stainless steel optical components wherever possible; as mentioned above, black anodized aluminum foil to minimize ambient light effects; all electronic equipment off the optical table; and the table floating on pneumatic isolators. We also put the laser cooling water pump, which makes a great amount of noise, in a separate room and built the tweezers in an enclosed, isolated room.

Lastly, to test temperature affects, we measured the beam stability while heating the room and consequently found temperature changes in the air to cause significant changes in the beam stability. However, while it was not feasible for us to create a temperature controlled room, the use of stainless steel optical components, which have lower thermal expansion coefficients than aluminum, and use of a box over the system that helps insulate the optical components (as well as reduce air fluctuations and to keep out ambient light) helped to reduce the effect of these temperature changes. In addition, as mentioned, the system was built in an isolated separate, dedicated room so that a temperature controlled environment could be created in the future if desired.

4.3.5 Background: Measurement Noise Considerations

Thus far we have considered and discussed how the resolution of an optical tweezers system can be improved by reducing the noise contributions from external environmental sources and instrument components. However, another form of noise results from the forces that cause the beads in the optical traps to undergo Brownian motion. Despite any efforts to minimize the external noise contributions, an optical tweezers system’s resolution will ultimately be limited by these Brownian motion fluctuations and can be quantified by the system’s signal to noise ratio (SNR) where higher SNR corresponds to greater spatial resolution.\textsuperscript{11} Moffitt et al. (2009) use this
measure to compare the resolution of three different measurement configurations: a
single-trap setup, a dual-trap setup where displacements of only one trapped microsphere
are measured, and a dual-trap setup where displacements of both trapped microspheres
are measured. We will denote the signal to noise ratios of these setups as $SNR_1$,
$SNR_{2,single}$, and $SNR_{2,double}$ respectively. The arrangement of the single-trap setup is a
single trap of stiffness $\kappa$ that contains a bead with drag $\gamma$ that is tethered to a flat surface
via a strand of DNA with contour length $L$ and stiffness $\kappa_{DNA}$. On the other hand, the
arrangement of the dual-trap setup is two beads with drag coefficients $\gamma_1$ and $\gamma_2$ that are
captured in the two traps with stiffnesses $\kappa_1$ and $\kappa_2$ and are tethered to one another by a
strand of DNA with stiffness $\kappa_{DNA}$ and contour length $L$. With these arrangements, they
ultimately demonstrate that $SNR_1 < SNR_{2,single} < SNR_{2,double}$, which means the dual-trap
setup with dual detection yields the greatest resolution, where the maximum achievable
$SNR_{2,double}$ is defined as (assuming negligible hydrodynamic coupling)

$$SNR_{2,\text{double}} = \frac{\kappa_{DNA}\Delta L}{\sqrt{4k_BT B \gamma_{\text{eff}}}}$$

and $k_B$ is Boltzmann’s constant, $T$ is the temperature, $B$ is the measurement bandwidth,
and $\gamma_{\text{eff}} = \gamma_1\gamma_2/(\gamma_1 + \gamma_2)$.

The power of the dual detection method arises from the
ability to subtract away the measured noise, arising from either environmental noise
sources or Brownian motion, that is common to both beads with the coordinate $x. = x_1 - x_2$, where $x_{1,2}$ are the deflections of the beads in trap 1 and 2 respectively. The noise that
is common to both beads results from the fact that they are tethered, and due to the fact
that certain sources of environmental noise, such as laser pointing stability, produce
common-mode noise. Movement of one bead away from the other bead, due to a
Brownian fluctuation, will pull the other bead with it, which is referred to as the symmetric motion. By subtracting the separate signals $x_1$ and $x_2$ from one another, the resulting coordinate $x_-$ exhibits less noise, and consequently a greater SNR, than that of either $x_1$ or $x_2$ alone. Thus yielding a system with greater resolution. As an aside, the coordinate $x_-$, as it is defined above, assumes the traps have equal stiffnesses and the beads have equal diameters/drag. This is not a requirement to achieve the maximum SNR but it is easier to describe and design a system that uses beads of equal size and traps of equal stiffnesses.$^{8,11}$ A discussion of the general case can be found in refs. 6 & 9.

A number of methods and factors have been identified, some of which derive from the above SNR relationship, that yield the greatest resolution. It has been shown that it is best, from a noise perspective, to use the following: (1) the smallest possible bead diameter (but no smaller than the laser wavelength); (2) the shortest tether length possible (but limited by the point at which trap cross-talk begins and/or transverse and axial (line connecting focal points of each trap) Brownian fluctuations begin to couple); (3) high forces (limited by the forces that can be maintained by the biological system being studied), which must at least be greater than 5pN; (4) the lowest possible measurement bandwidth allowed by the system being studied (However, the data should be collected with a high sampling rate to avoid aliasing and then filtered down to the desired measurement frequency/bandwidth.).$^{11,12}$

We would also like to note that while SNR is not directly dependent on trap stiffness, it is still possible to reduce SNR within a given measurement bandwidth by using higher trap stiffnesses. The variance of the displacement of a bead in a trap per unit frequency can be defined as
\[ \langle \Delta x^2(\omega) \rangle_{eq} = \frac{2k_B T}{\gamma (\omega_c^2 + \omega^2)} \]

Where \( \omega_c = \kappa / \gamma \) is the corner frequency, which is the point at which the bead displacement response \( \langle \Delta x^2(\omega) \rangle_{eq} \) becomes significantly reduced with increasing \( \omega \).\textsuperscript{22}

The total noise in a measurement is proportional to the area under this variance-frequency curve, which is constant when integrated over all frequencies. However, if we increase \( \omega_c \), the noise is spread over a larger range of frequencies, thus the noise (area) within a given finite measurement bandwidth is reduced. Consequently, SNR increases for any signal within this bandwidth. Also, since \( \omega_c \) is a function of \( \kappa \) and \( \gamma \), SNR can be increased by increasing trap stiffness (decreasing trap compliance) and/or decreasing drag (for example by decreasing the size of the beads).

Lastly, high trap stiffness is also recommended because it allows for higher achievable forces while still operating within the linear force-displacement response regime of the trap. Furthermore, it prevents the bead deflections from being too great. If the deflections become too large the scattered light can get clipped by the aperture of the downstream objective, which can lead to a nonlinear relationship between force and PSD detector signal, which is very difficult to calibrate.\textsuperscript{11} This same clipping effect can result from a misalignment between the two objectives in the system.\textsuperscript{11} \textit{Thus, well aligned objectives and high trap stiffness become particularly important in constructing a system with high spatial resolution.}

4.3.6 Accounting for Measurement Noise Considerations

In creating our tweezer system we made an effort to follow the recommendations by Moffitt et al. for reducing the effects that Brownian noise, the system design, and the
experimental methods have on the measurable resolution. Since our laser wavelength is 1064nm, we used beads of 2 micrometer diameter. We attempted to use beads with diameter closer to one micrometer as suggested, but the experiments became difficult and inefficient. In particular, we observed that the trapping force on smaller beads was reduced. This made it difficult to not only capture a bead but also to keep it in a trap. Too much drag while moving through the buffer or molecular forces that were too great could easily lead to ejection of the bead from the trap. Additionally, if we had beads captured in the two traps, tether formation between the traps occurred much less frequently than with 2μm diameter beads.

To ensure DNA stiffness was sufficiently high for improved resolution and so that the DNA force response was linear with bead displacement, we always operated at forces greater than 5pN. Tether lengths were never greater than 10 kilobase pairs (~3400 nm). Furthermore, data collected at tether lengths less than ~1 kilobase pairs (~300-400 nm) was ignored because, in our system effects, cross-talk between the two traps began around this distance. In collecting the data, we sampled at rates of 50kHz or more and then digitally filtered down to much lower measurement bandwidth frequencies (typically hundreds of Hertz or less) to yield higher SNR. For simplicity, we designed the system to have traps of equal stiffness while using beads of equal size in each trap (2μm). We also designed the system to have higher trap stiffnesses (or lower compliances; compliance=1/stiffness) than our original tweezers system by developing a new, better objective alignment procedures to help yield the strongest traps possible while also reducing the clipping effects of the deflected laser light.
Initially we built this new system with only one detector but designed it so that a second detector could later be included. While the single detector system yielded greatly improved resolution over our previous system, slightly greater resolution was still desired. Therefore, we ultimately included the second detector so that we could exploit the dual detection method.

4.3.7 Other Measures Taken to Improve the System Design

As mentioned earlier, we retested some of Moffitt’s recommended design features in simplified optical set-ups before implementing them, however, we also took a handful of other measures not necessarily mentioned by Moffitt et al. in effort to yield the best possible resolution. We briefly mention those efforts here. Our laser can either be run in constant current mode or constant power mode. We tested the stability of the beam in both cases and found the pointing stability to be significantly better in constant current mode and thus chose this mode for all experiments. To further reduce noise and increase our trapping strength, great efforts were made to improve our optical alignment techniques. Most significant would be the quantitative method developed to align the two objectives with one another and with the beam. Also of note is that we made a greater effort (than in previous tweezer builds in our lab) to ensure the flow cell (or experimental plane) was positioned perpendicular with the beam path (also suggested by Moffitt in ref. 14). Beyond this, rather than positioning the PSD at the expected position of the second objective’s back focal plane image, we developed a simple empirical method for aligning the PSD surface with this image. This helps account for the possibility that the imaging lens is not placed exactly at the position intended for imaging the back focal plane as well as uncertainty in the exact position of the detecting surface
within the PSD encasing. These, and other, alignment techniques are thoroughly discussed in the appendix of this dissertation. Lastly, before the build, we performed experiments to identify whether steering the beam that forms the movable trap with an AOD or a piezo-electric mirror would yield better resolution. Ultimately we found both the AOD and piezo electric mirror actuator we had available generated unacceptable drift, therefore we chose to purchase a new piezo-electric mirror actuator with high stability and very low drift. A secondary consideration in choosing to use the mirror was that because the power loss in the beam after reflection off the mirror is essentially zero, whereas, after the beam passes through an AOD, the beam’s power is reduced by ~67-75% (over the range of angular deflections typically used in experiments on our tweezer setups). Since trap compliance increases with decreasing power, it would be more favorable to have higher power passing through the objectives so as to generate a stronger trap (i.e. lower trap compliance) and thus increase the signal to noise ratio within a given bandwidth.

4.4 Results

A photo of our improved optical tweezers system prior to the installation of the second detector can be seen below (Fig. 4.1). In the section where the beam is split, reflected off a movable and stationary mirror, and then recombined, the distances between optical elements were too small to conveniently enclose the beam in tubing. Therefore, a small plastic box lined with anodized aluminum foil was used to cover these elements, which is seen in the upper right hand corner. See the dissertation Appendix for a cartoon diagram of the system.
Figure 4.4.1: The new higher resolution dual-trap optical tweezers setup with single trap detection only. After this photo was taken, the uncovered walls and lids seen in the image were covered with the black anodized aluminum foil as seen in the top chamber. Prior to an experiment, hinged lids were folded down over each chamber and securely clamped down to the table.

4.4.1 Reduced Noise for New Single Detection System

In their optimal configurations, and in conjunction with an improved calibration method (see chapter 5), the old and new tweezer setups yielded trap compliances of 19.7nm/pN and 11nm/pN respectively, which demonstrates that we achieved a much lower trap compliance as desired. Secondly, while implementing the optimal measurement methods and low noise configurations, we measured the displacement of a 2µm diameter bead in the stationary trap of the old system and the new system. The data
was collected at a high sampling rate was later digitally filtered down to 1kHz prior to processing.\textsuperscript{11,23} At all frequencies, the new system exhibited significantly reduced noise (blue trace, Fig. 4.2b). Example displacement measurements over a 60 second period with the two systems can be seen in Fig. 4.2a. The old system clearly exhibited greater drift and high frequency noise than the new system. Since these measurements were done using only one detector measuring and only one trapped bead, these improvements can be attributed to reducing the effect of external environmental noise/drift sources on the measurement.

Additional comparisons could not be performed because the original system was decommissioned as a result of a “dead” laser. The limited data presented here from that system was collected before it was decommissioned.

![Figure 4.2: Displacement measurement of a 2µm diameter bead in the stationary trap of our old and new single detection dual-trap optical tweezers systems. The data was digitally filtered to 1kHz. Red traces correspond to measurements on the old system while blue traces correspond to measurements with our improved system. a) Example traces of the displacement of a bead in a trap over time. b) Power spectral density of the example traces in (a).](image-url)
4.4.2 Greater Resolution with Dual Detection

Since one interest in our lab is to use this system to measure the DNA translocation step size of the lambda bacteriophage, which translocates DNA at rates of hundreds of base pairs per second\(^{14}\), we needed to test the system’s ability to resolve expected lambda step size displacements at such rates. The Bustamante Lab previously showed that the φ29 virus, which is similar to the lambda virus, makes 10bp steps that are composed of 4 rapid, successive 2.5bp steps\(^{24}\). We tested the system’s resolution by stretching a strand of dsDNA by various distances near ~2.5bp (~0.8nm) and at various rates of 100s bp/s and <100 bp/s. We found that we could successfully observe steps on the order of nanometers at rates below 100bp/s, but only while employing the dual-detection method. This is as expected since this method allows noise that is common to both trapped beads to be eliminated, which subsequently yields a higher signal-to-noise ratio. Figure 4.3 is an example of how the dual detection method allows us to resolve nanometer displacements at stretching rates of ~500bp/s while the single detection method fails. Fig. 4.3b is a magnified section of the DNA stretching traces in Fig. 4.3a where the DNA was stretched at 50steps/s and ~10bp/step (~3nm/step). One can clearly observe distinguished steps by eye in the blue trace of Fig. 4.3b, which corresponds to the dual detection DNA stretching displacement, but not in the single-detection DNA stretching trace (red).

Pairwise distribution analysis is a method that is often used to more quantitatively assess the occurrence of steps within a signal. Simply stated, this method takes the difference between all points within a given data set and plots the distribution of these
differences. A stepping function with steps of equal size (in time and length) that are separated by equal time intervals would yield a pairwise distribution that exhibits spikes at integer multiples of the step length. If noise were then added to the original data set, the resulting pairwise distribution would exhibit a sinusoidal character. By applying this method to the data in Fig. 4.3b, we are able to better assess whether the signals are exhibiting steps. As a result, we find the pairwise distribution analysis of the dual detection method (blue distribution) exhibits a well-defined, sinusoidal-like distribution with peaks separated by the ~10bp step displacements performed in this test. On the other hand, the single detection method (red distribution) exhibits an irregular distribution with shallow valleys and peaks that do not correspond to the ~10bp displacements.
**Figure 4.3:** Resolution comparison of our new laser tweezers using single detection (red traces) vs dual detection (blue traces) methodology. A 10kbp strand of DNA tethered between two 2µm microspheres was stretched with ~10bp/step (~3nm/step) at rates of 50 steps/s, which corresponds to ~500bp/s, while sampling at 50kHz. The force on the DNA increases during stretching, starting with ~15pN and ending with ~50pN. Note that traces in (a) and (b) have been offset for display purposes. a) Stretching displacement (in base pairs) versus time (in seconds) after filtering down to 1 kHz sampling rate. b) Magnified section of the traces in (a). c) Pairwise distribution of the stretching traces from (a). Distributions have been smoothed so the peaks of the dual-detection method can be better defined.

While the steps in this example are fairly clear, efforts to resolve subnanometer level steps has proven to be difficult and inconsistent where successful observations typically occur under conditions of either slow packaging rates, high forces, or a combination of slow packaging and high applied external forces. Beyond this, virus complexes do not always package as processively at high forces and low velocities (obtained by using low ATP concentrations) as in the DNA stretching example above, which can make it more difficult to resolve the step size of the motor. Additionally, for
reasons not fully understood, some viral DNA packaging events exhibit more and larger fluctuations than others; similar effects were reported by Moffitt et al. We believe this is a result of a change in environmental conditions from one trial to another. See chapter 5 for more discussion on this topic. We have found that changes in the data processing (resampling and smoothing) can affect the ability to resolve steps. As such, we believe that by optimizing the data processing, we can more consistently reach subnanometer resolution under conditions of low packaging rates, high forces, and the lowest obtainable environmental noise. For these reasons we mention in the conclusion what additional measures might have yielded a system that more consistently resolves subnanometer level steps during viral packaging

4.5 Conclusion

We’ve shown here that by employing many, but not all, of the noise reduction techniques and methods suggested by others we have been able to build a fairly “low” cost (less than ~$100,000) dual-optical trapping system with greater resolution than any system previously built in our lab, i.e. nanometer to subnanometer spatial resolution. Comparisons of an old system in our lab to our new system have shown a notable reduction in measurement noise over the entire spectrum of frequencies relevant to the experiments typically performed in our lab, 0.1 – 1000Hz. We’ve also shown that the use of a dual-detection method in a dual-optical trapping system does in fact yield greater spatial resolution, as previously demonstrated by others. This system will now permit us to ask and explore questions we were previously unable to investigate. Our initial efforts that take advantage of the improved resolution of this new system can be seen in chapter 5, which investigates the step size of the phage lambda DNA packaging motor.
Though we succeeded in achieving greater resolution, our ability to resolve subnanometer distances is inconsistent. It is possible that consistent subnanometer resolution might have been obtained had we also implemented some of the following techniques used by other groups: create a temperature controlled room, place all electronic equipment in a separate room, use a very thick optical table with low resonant mode frequencies, seal the system enclosing box and fill it with helium (a gas whose refractive index is insensitive to temperature and thus less susceptible to causing laser beam deflections). While the use of smaller beads can reduce the measurement noise, we use 2µm beads because experiments have proven to be especially difficult with smaller beads (i.e. efficiency of obtaining single DNA molecule packaging events is substantially reduced). Lastly, the use of remotely controlled motorized actuators to control the stage that maneuvers the flow cell could make a big difference because it eliminates user effects (heat transfer and mechanical vibrations imposed on the stage by the user as well as room heating resulting from body heat).

4.6 Acknowledgements

We thank Nicholas Keller for his collaboration and equal contribution in building this system. We also thank Alan Schweitzer, UCSD Physics Department Electronics Shop and Machine Shop, and Jeffrey Moffitt for advice, thoughtful discussions, and technical assistance.

4.7 Materials and Methods

Materials and methods for building this system are described in the appendix of this dissertation. Otherwise materials and methods used in testing this system are described below.
4.7.1 Measuring system noise

With the old system and the new system, we measured the displacements of a 2µm diameter polystyrene bead (Spherotech, Inc.) that was held in the stationary trap. Tests were performed in 1xPBS for a duration of one minute while sampling at high rates of 25kHz with the old system and 200kHz with the new system to avoid aliasing. The data was then decimated with digital filtering algorithms down to a sampling rate of 1kHz. The data was converted to distances in nanometers using calibration parameters, which were determined using the techniques described in refs. 23 & 24. The power spectral density distributions were generated using the Matlab pwelch function.

4.7.2 Dual detection tests

A biotin and digoxigenin labelled 10,051bp segment of lambda DNA was generated by PCR using the primers Biotin-5´-CTGATGAGTTGTCCGTACACAACGCGTAATC-3´ and Digoxigenin-5´-ATACGCTGTATTCCGCAACCCGTAGGAAACG-3´. This DNA was incubated with 2µm diameter streptavidin coated polystyrene microspheres (Spherotech, Inc.) in 1xPBS. The amount of DNA used was titrated such that single tethers formed after bringing one such trapped DNA coated bead into contact for ~1-2 seconds with a second, anti-digoxigenin polystyrene bead that is trapped in the other optical trap. Once a single tether is obtained, the beads are separated from one another by ~10bp/step at a rate of 50steps/s. Measurement forces ranged from ~5pN to ~60pN. Data was sampled at 50kHz and later digitally filtered down to 1kHz. Pairwise distributions were generated by taking the difference between all measured points in the 1kHz filtered data set. The
absolute value of these results were then binned in 0.1bp bins and smoothed with the Matlab smooth function, which uses a sliding window averaging algorithm.

4.8 References for Chapter 4


Chapter 5

Accurate measurement of force and displacement with optical tweezers using DNA molecules as metrology standards

5.1 Abstract

Optical tweezers facilitate measurement of piconewton-level forces and nanometer-level displacements and have broad applications in biophysics and soft matter physics research. We have shown previously that DNA molecules can be used as metrology standards to define such measurements. Force-extension measurements on two DNA molecules of different lengths can be used to determine four necessary measurement parameters. Here, we show that the accuracy of determining these parameters can be improved by more than 7-fold by incorporating measurements of the DNA overstretching transition and using a multi-step data analysis procedure. This method results in very robust and precise fitting of DNA force-extension measurements to the worm-like chain model. We verify the accuracy through independent measurements of DNA stretching, DNA unzipping, and microsphere contact forces.

5.2 Motivation in the Context of Dissertation Research

Since our newly built dual-trap optical tweezers (as described in Chapter 4) was designed to have significantly improved resolution over previous systems built in our lab, the calibration method became particularly important in evaluating the true resolution of this system. Unfortunately, as will be described below, we discovered that our previous calibration method did not always determine the calibration parameters as accurately as desired. Consequently, I developed a new calibration method that yields more accurately
determined calibration parameters. Results in Chapter 6 demonstrate the utility and benefits of this new, well-calibrated, high resolution optical tweezer setup.

5.3 Introduction

In the method referred to as optical tweezers, micron-sized dielectric particles are trapped in aqueous solution by focused laser beams and can be used as transducers for measuring small forces and displacements acting on the microscopic scale.\(^1\)\(^-\)\(^3\) This method has found wide applications in both biophysics and soft-matter physics research.\(^4\)\(^-\)\(^11\)

Most optical tweezers configurations require calibration to measure forces and displacements in physical units.\(^2\)\(^,\)\(^3\)\(^,\)\(^12\)\(^-\)\(^14\) We previously introduced a method by which double-stranded DNA molecules could be used as standards to determine all of the necessary measurement parameters.\(^13\) We have used this approach in a number of biophysical studies involving DNA manipulation.\(^15\)\(^-\)\(^18\) More broadly, the use of standards to ensure accurate measurements is a concern of the field known as metrology.\(^19\) With growing interest in the study of nanoscale structures in the physical and biological sciences and engineering comes an increasing need for readily accessible standards to ensure the accuracy of measurements across different laboratories. The advantage of DNA as a nanoscale standard is that a given molecule has well defined physical properties. For example, the length of DNA can be controlled in increments of a single basepair (~0.34 nm) and exact copies of the molecule can be produced using standard techniques in molecular biology.

The DNA-based metrology method is based on the fact that DNA has a well-defined force-extension relationship that can be measured by stretching DNA molecules
with optical tweezers (Fig. 5.1). We consider here DNA stretched between two trapped microspheres, but the approach is adaptable to other common configurations, such as when a single optical trap or atomic force microscope cantilever is used to stretch a DNA molecule tethered to a surface. The method allows four measurement parameters to be determined: trap compliance, force scale factor, displacement scale factor, and displacement offset. A trapped microsphere subject to an external force $F$ is displaced from its equilibrium position by a distance $\Delta x = \gamma F$, where $\gamma$ is the trap compliance. This induces deflection of the trapping laser beam, which is measured by a position-sensing photodetector (PSD), such that $F = \alpha V_{PSD}$, where $V_{PSD}$ is the measured PSD signal and $\alpha$ is the force scale factor. The separation between the two traps is controlled by optics that steers one of the trapping beams; specifically, by a control voltage $V$ that tilts a piezo-actuated mirror. The separation $d$ between the two traps is given by $d = \beta (V - V_0) + r_1 + r_2$, where $\beta$ is the length scale factor, $V_0$ the displacement offset factor, and $r_1$ and $r_2$ and the radii of the microspheres. $V_0$ is the control voltage when the microspheres first come into contact, where the distance between the traps is $d = r_1 + r_2$. 
Figure 5.1: Experimental geometry for DNA force-extension measurement in a dual optical trap system. The distance between the traps is $d$, the end-to-end extension of the DNA is $x$, the radii of the microspheres are $r_1$ and $r_2$, the force on the microspheres is $F$, and their displacements from the trap centers are $\Delta x_1$ and $\Delta x_2$.

To determine these measurement parameters we take advantage of the fact that the elasticity of DNA is well described by the worm-like chain (WLC) model.\textsuperscript{20, 21} For sufficiently large molecules ($\gtrsim 3 \text{ kbp}$) and stretching forces (>1 pN) the force-extension relationship is accurately described by the model function

$$\frac{x}{L} = 1 - \frac{kT}{4FP} + \frac{F}{S}$$

where $x$ is the end-to-end extension of the molecule, $L$ is the molecular contour length, $P$ the persistence length, $S$ the stretch modulus, and $kT$ the thermal energy (~4.14 pN-nm at room temperature).\textsuperscript{15, 20-22} As described previously, measurements of force-extension curves for two DNA molecules of different lengths provide sufficient information to determine all four parameters by fitting the model to the data.\textsuperscript{13}

5.4 Investigating Original DNA Metrology Calibration Method

After testing this procedure with several different optical tweezers systems, however, we found that certain measurement parameters were not always as accurately determined as desired. As an example, a set of measurements of DNA molecules of lengths 10,051 bp and 13,747 bp, prepared as described previously,\textsuperscript{15} were recorded with
a newly built dual-trap optical tweezers system. The DNA was tethered between 2.2 μm diameter polystyrene microspheres. This system is similar to that described previously\textsuperscript{13} except a piezo-actuated mirror is used for beam steering instead of an acousto-optic deflector. The system was calibrated using the procedure described previously, but an independent check revealed a discrepancy. Specifically, it led to a 33% underestimate of the force at which the well-known overstretch transition of DNA occurs.\textsuperscript{21, 23-27}

To investigate the source of this discrepancy we systematically examined how the quality of the fits of the WLC model to the data varied as a function of the measurement parameters. We found that nearly equally good fits could be obtained for a fairly wide range of $\alpha$ and $\gamma$ values, in which higher $\alpha$ values could be compensated for by lower $\gamma$ values, or vice-versa. This is illustrated in Fig. 5.2a, in which we calculate the average coefficient of determination,$^{28} \langle R^2 \rangle$, to characterize the quality of fits to the 10,051 bp and 13,747 bp data sets. Values of $\alpha$ and $\gamma$ varying over a wide range from 40 to 58 pN/volt and 8.5 to 15.5 nm/pN, respectively, yielded reasonable fits ($\langle R^2 \rangle$=0.95 to 0.975 out of a maximum value of 1). This analysis indicates that the previous fitting procedure\textsuperscript{13} is limited in its ability to accurately constrain both $\alpha$ and $\gamma$. 
Figure 5.2: Dependence on measurement parameters of the quality of fits of the WLC model to averaged force-extension measurements with 10.1 and 13.7 kbp DNA molecules. Greyscale shades indicate regions where the average coefficient of determination, $\langle R^2 \rangle$, is $<$0.95 (white), 0.95-0.975 (light gray), 0.975-0.99 (medium gray), 0.99-0.995 (dark gray), and $>$0.995 (black), out of a maximum of 1. (a) Dependence on trap compliance, $\gamma$, and force scale factor, $\alpha$, using the prior method. (b) Dependence on trap compliance, $\gamma$, and displacement offset factor, $V_0$, using the new method (in which $\alpha$ is determined independently).

The above analysis suggested that the accuracy could be improved if $\alpha$ or $\gamma$ could be constrained with additional experimental information. In this letter, we present an improved approach in which we determine $\alpha$ independently through measurements of the DNA overstretch transition and subsequently determine the other three measurement parameters through a multi-step analysis. We describe the method in detail and present independent measurements confirming that it results in greatly improved accuracy.
5.5 **Methods and Results of New DNA Metrology Calibration Technique**

Systematic studies have shown that the overstretch transition occurs at a characteristic force that depends on solution conditions (ionic strength and pH) and this feature has been used in some studies to confirm force measurements.\textsuperscript{29,30} The overstretch plateau is clearly seen in our measurements (Fig. 5.3a) and can be analyzed to determine the characteristic overstretch force $F_{os}$, defined as the half-way point through the plateau.\textsuperscript{27} To determine $F_{os}$ we fit second-order polynomials to the two portions of the raw PSD voltage data before and after the overstretch plateau and a fit a line to the plateau region (Fig. 5.3a). We determine the PSD voltage $V_{os}$ corresponding to $F_{os}$ as the point on this line half-way between the two points where the line intercepts the polynomials. We then calculate the force scale factor as $\alpha=F_{os}/V_{os}$, where we use $F_{os}=63.6$ pN based on measurements reported in the literature\textsuperscript{27} for conditions similar to ours (10 mM Tris-HCl, pH 7.5, 150 mM NaCl). We obtain $\alpha=54.2 \pm 0.2$ pN/volt (average over measurements on 34 molecules).
Figure 5.3: (a) Averaged force-extension measurements in instrument units for the 10.1 kbp DNA (grey points) and fits (dashed lines) to identify the mid-point of the overstretching transition (black point). (b) Fits of the WLC model (black lines) to a typical pair of force vs. extension measurements on the 10.1 kb and 13.7 kb DNA molecules (grey points).

Next, to determine the length scale factor, $\beta$, we use the fact that, according to the WLC model (Eq. (1)), the extension ($x$) of DNA is equal to its contour length ($L$) at a particular force, which is 33.4 pN for values of the persistence length ($P=45$ nm) and stretch modulus ($S=1275$ pN) appropriate to our solution conditions. After converting PSD voltage to force using $\alpha$ determined above, we determine the displacement control voltages $V_1$ and $V_2$ that yield $F=33.4$ pN for each of the two DNA molecules. We then calculate $\beta=(L_2-L_1)/(V_2-V_1)$, where $L_1$ and $L_2$ are the contour lengths of the two molecules. This method has the advantage of determining $\beta$ in a manner does not involve
curve fitting and does not depend on the values of $\gamma$ and $V_0$, which we determine in a subsequent analysis step. We obtain $\beta = 979 \pm 7$ nm/volt (average over 34 measurements of 10.1 kbp molecules and 33 measurements of 13.7 kbp molecules).

Lastly, to determine the series compliance of the traps, $\gamma$, and displacement offset factor, $V_0$, we perform two-parameter fits for $\gamma$ and $V_0$ of the WLC model to each force-extension data set for both DNA lengths (Fig. 5.3b) where $\alpha$ and $\beta$ are fixed to the values already determined above. To quantify the quality of the fit between the predicted WLC model $(x, F)$ values (Eq. (1)) and observed $(x, F)$ values we calculate the coefficient of determination $R^2$, where the observed values are evaluated by the equations $x = \beta (V - V_0) - \gamma F$ and $F = \alpha V_{PSD}$ and a particular set of $\alpha$, $\beta$, $\gamma$, and $V_0$. Then, with $\alpha$ and $\beta$ fixed to the values determined above we iterate over $\gamma$ and $V_0$ values to find the set that yield the greatest $R^2$ (i.e., the global best fit) and thus the best determination of $\gamma$ and $V_0$ for our system. Fitting was done over the range from 4-40 pN to minimize effects of instrumental noise at low force and the onset of the overstretch transition at high force, yielding excellent fits with $\langle R^2 \rangle = 0.994$, and the values $V_0 = -8.330 \pm 0.003$ and $\gamma = 11.0 \pm 0.1$ nm/pN (average over 34 measurements of 10.1 kbp molecules and 33 measurements of 13.7 kbp molecules).

This approach results in a dramatic improvement in the robustness of the determination of the measurement parameters. Whereas the prior method determined all four parameters through fits to the WLC model, the present method reduces the number of fit parameters to only two ($V_0$ and $\gamma$). The quality-of-fit analysis of the prior method (Fig. 5.2a) showed that $\alpha$ and $\gamma$ were not tightly constrained. In contrast, the improved
method determines $\alpha$ independently, and a similar quality-of-fit analysis (Fig. 5.2b) shows that these remaining two parameters are tightly constrained (0.02 volts range in $V_0$, and 0.1 nm/pN range in $\gamma$).

To independently check the accuracy of the determined force scale factor, $\alpha$, we conducted measurements of another characteristic DNA property different than the overstretch transition: the force required to unzip double-stranded DNA, which has been previously reported in the literature.$^{31-33}$ Here, we unzip a section of $\lambda$ phage DNA ranging from base 8015 to 4392 (Fig. 5.4a) and measure an average PSD voltage of 0.286 volts during unzipping (average over measurements on 44 molecules). The known unzipping force from the literature, accounting for our solution conditions and DNA sequence, is 16 pN which implies $\alpha=16/0.286=55.9$ pN/volts, which agrees to within 3% with the value of 54.2 we determined above. We note that the agreement of both the overstretch transition and unzipping transition with literature values also validates that force is a linear function of PSD voltage ($F=\alpha V_{PSD}$) for our system.
Figure 5.4: (a) Measurement of DNA unzipping with the λ DNA construct (grey points). The dashed line indicates the average unzipping force. (b) Measurement in instrument units of contact force between two microspheres vs. separation between the two traps (average of 64 measurements), used to check the displacement offset factor, $V_0$. Dashed lines mark the $V_{PSD}=0$ crossing point. The small increase to $V_{PSD}>0$ is due to cross-interaction when the two optical traps are very close (i.e., trap #1 exerts force on the microsphere in trap #2). (c) Measurements of contact force vs. trap separation in the linear portion of the $F<0$ regime (grey points; three individual measurements shown) and linear fits to each dataset (solid lines). (d) Typical force-extension measurement with the 13.4 kbp molecule (grey points) and comparison with the WLC model prediction (solid line).

To independently check the accuracy of the displacement offset factor, $V_0$, we conducted measurements of the contact forces between the two trapped microspheres in the absence of DNA. When the traps are moved together and the microspheres come into contact, $V_0$ is the control voltage at which the force transitions from near-zero to negative values (Fig. 5.4b). This measurement yields $V_0=-8.341 \pm 0.006$ volts, in excellent
agreement with the value of -8.330 determined above. The difference between these values corresponds to a deviation of $\beta \Delta V_0 = 11$ nm.

Microsphere contact measurements also provide a way to confirm the compliance, $\gamma$. After the microspheres touch, when the traps are moved closer by a distance $d$ the microspheres are pushed a net distance $d = \gamma F_c$ from their trap centers, where $F_c$ is the contact force (Fig. 5.4c). We only include contact events in which there is no slipping of the microspheres in a direction perpendicular to $d$. By measuring the slope of the linear portions of the $F_c$ vs. $d$ plot we obtain $\gamma = 10.46 \pm 0.03$ nm/pN (standard deviation in the mean, average for 92 pairs of microspheres), which agrees to within 5% with the value of 11.0 nm/pN determined above.

To confirm the accuracy of the displacement scale factor, $\beta$, we conducted force-extension measurements with a third DNA molecule having a different length ($L = 13,424$ bp) than the other two used for metrology (Fig. 5.4d). This data is used to derive a check as follows. Recall that DNA extension is given by $x = \beta (V - V_0) - \gamma F$ (Fig. 5.1) and $x = L$ at $F = 33.4$ pN. Thus $\beta = (L + \gamma F)/(V^* - V_0)$, where $V^*$ is the observed displacement control voltage for which $F = 33.4$ pN. Fixing $\alpha$, $\gamma$, and $V_0$ to the values determined by our procedure above, we find $\beta = 980 \pm 1$ nm/volt (average from measurements on 59 molecules), which agrees to within 0.1% with the value of 979 determined above. We note that these measurements also agree well with the prediction of the WLC model, which further supports this method as a way of determining accurate measurement parameters.
5.6 Conclusion

In summary, multiple confirmations via independent measurements show that the proposed method is much more accurate than the prior method. The greatest absolute improvements are in the determination of $\alpha$ and $\gamma$. The relative improvements in accuracy over the prior method\textsuperscript{13} can be characterized by the difference in present vs. prior values divided by the difference in present vs. confirmed values, indicating improvements of 10-fold in $\alpha$, 19-fold in $\beta$, 8-fold in $\gamma$, and 7-fold in $V_0$.

5.7 Acknowledgements

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5.8 References for Chapter 5


Chapter 6

Bacteriophage Lambda DNA Packaging Step Size

6.1 Abstract

Members of the ASCE (Additional Strand Catalytic E) superfamily of NTPase proteins exhibit a broad range of functions while sharing a number of properties including sequential motifs, structural domains, and in some cases the formation of oligomeric ring structures. To gain insight into the structure-function relationship of proteins within this family, we use high resolution dual-trap optical tweezers to examine the packaging dynamics of such an ASCE member, the bacteriophage λ terminase motor protein gpA. In this chapter we present preliminary results from the initial stage of our investigation, which is to directly measure the DNA translocation step size for λ. Our initial results unexpectedly indicate that λ makes ~5bp steps, which differs greatly from predictions for other viruses and from direct observations made for phage φ29. Besides providing an example of two ASCE members exhibiting a possible mechanistic difference in their common function of DNA translocation, such results could also potentially explain part of the difference in packaging rate between λ and φ29. However, this result must be considered preliminary; much more data needs to be collected to affirm this finding.

6.2 Introduction

P-loop NTPases have two main structural groups: the Kinase-GTPase (KG) group and the Additional Strand Catalytic E (ASCE) group\(^1\). The ASCE superfamily are a diverse group of proteins that share a structurally conserved core with a number of
sequence conserved motifs that are involved in ATP binding and hydrolysis\(^1\-^6\). This superfamily contains a number of subfamilies, such as RecA-like ATPases, Ftsk/HerA dsDNA translocases, AAA+ enzymes, and Superfamily 2 (SF2) helicases, that participate in a wide range of tasks including protein unfolding and degradation; protein transport and translocation; ATP synthesis; and DNA recombination, transport, and translocation\(^1\-^7\). The mechanisms by which these tasks are performed are not fully understood. Nonetheless, because these proteins all share a structurally conserved core, a detailed mechanistic understanding of some such proteins can provide great insight for the entire ASCE superfamily.

Crystal structures and sequence homologies have placed a number of dsDNA bacteriophage motor proteins within the ASCE superfamily\(^8\-^{15}\). Within this family, two major viral subfamilies have formed: the Ftsk/HerA family and the terminase large subunit (TLS) family\(^{16,17}\). Of these phages, single-molecule methods have been used to investigate the motor dynamics and mechanisms of the phage φ29’s motor, which is a ringed Ftsk/HerA NTPase that translocates dsDNA and is composed of five gp16 proteins subunits. Bustamante and coworkers have provided evidence that φ29’s subunits work together in a highly (but not strictly) coordinated fashion to translocate DNA in 10 base-pairs (bp) steps increments or “bursts”, which are composed of four rapid and successive 2.5bp steps\(^18\). Acquiring such detailed understanding of NTPases, or any protein for that matter, can be quite difficult and often limited to systems that can be studied using single-molecule techniques. However, studying additional systems would be particularly useful for understanding the mechanisms of a superfamily (ASCE) of proteins with such a breadth of functions. Another good candidate would be the phage λ, whose packaging
proteins have been extensively studied at a genetic and biochemical level and whose basic packaging dynamics have been studied (at low resolution) at the single-molecule level. The phage λ’s gpA terminase protein (the large terminase subunit protein) has been shown to be homologous with a number of SF2 helicases. Furthermore, lambda has many of the other TLS ATPase classifying characteristics, which include: having a large terminase subunit (gpA); forming subunits called protomers (one gpA plus two small gpNu1 proteins) that form a homomeric ring (supposedly tetrameric) around dsDNA and subsequently bind, cleave, and translocate dsDNA. The DNA translocation does not occur until the cleaved DNA terminase complex binds to a preformed viral capsid.

Prior to the φ29 single-molecule experiments, biochemical studies of phages φ29 and T3 estimated that these motors translocate DNA by 2bp per ATP hydrolyzed. However, this is an estimate based on measured ATP consumption during packaging in bulk assays. Factors that can affect the accuracy of this estimate include futile ATP hydrolysis by complexes that do not package, inefficient packaging by only a small fraction of the complexes in the bulk reaction, and failure to account for potential slipping events in which sections of DNA come out and need to be re-packaged. In addition, a proposed packaging mechanism of the phage T4 suggests that DNA is also translocated by 2bp per ATP hydrolyzed. Such reports may lead one to believe that other viruses, such as lambda, may also make 2bp DNA translocation steps. However, the discrepancy observed in the φ29 step size between the biochemical estimates and the single-molecule determination demonstrates the value and importance of direct observations through single-molecule techniques in understanding the mechanisms of
these viruses. Here we use single-molecule techniques to investigate λ terminase’s translocation mechanism and present preliminary results for λ’s step size, which are surprisingly different from that of φ29.

6.3 Results and Discussion

To gain knowledge about a general structure-function relationship among the ASCE NTPases and/or differences/similarities between the FtsK/HerA and TLS virus NTPase subfamilies, we’ve begun studying λ packaging dynamics with a newly built high resolution dual-optical trap tweezers instrument with nanometer to sub-nanometer resolution (depending on measurement bandwidth). This instrument is described in detail in chapter 4. In brief, the experiments consist of holding two 2 micron diameter polystyrene beads, which are tethered by a γ-S-ATP stalled λ DNA/terminase/prohead complex, in two optical traps at fixed positions. By introducing ATP into the buffer, DNA packaging is reinitiated and subsequently measured using a partial position clamp measurement mode in which the position of the traps are incrementally moved every time the force reaches a predefined threshold force. In other words, the traps are held at a fixed position while packaging proceeds, which results in an increase in bead displacement and measured force. Once the beads are displaced from the center of the traps such that the measured force is $F_{\text{max}}$, the traps are moved toward one another until the measured force is $F_{\text{min}}$, after which the traps are again fixed until $F_{\text{max}}$ is achieved. This measurement mode allows us to acquire numerous fixed position packaging traces over a given force regime from a single packaging complex. Henceforth we will refer to these fixed position packaging traces, in which the force rises from $F_{\text{min}}$ to $F_{\text{max}}$ while the trap positions are held fixed, as “segments”. The bead displacement measurements are
later converted into DNA contour length using the calibration procedures described in Chapter 5.

As previously discussed in chapter 4, our ability to resolve nanometer to sub-nanometer sized steps can be very difficult if the packaging rate is too fast, especially when measuring viral packaging, which exhibits imperfect processivity. Therefore, since lambda packages DNA fairly rapidly with an average rate of ~600bp/s in saturating ATP (1mM)\textsuperscript{20}, we reduced the packaging rate down to ~50bp/s by using a much lower ATP concentration of 5μM. In our preliminary experiments, we performed 12 of these partial-position clamp trials, during which we measured 1 to 8 position clamp segments. In total we collected 63 position clamp segments of data. By visual inspection alone, instances of stepping (abrupt changes in length packaged) could be observed in many of these segments of data. However, whether stepping could be observed in individual segments depended on the level of noise in individual records. Figure 6.1a provides two instances demonstrating recorded segments that appeared to either show (black trace) or not show (blue trace) resolved steps. In the black trace, somewhat equal-sized discrete steps that are monotonically increasing can be resolved because the size of the noise fluctuations between steps is relatively small. On the other hand, the size of the fluctuations the blue trace exhibits are often as big or bigger than the size of the steps observed in the black trace; so, even though the blue trace increases, indicating translocation and thus stepping, the level of noise measured in this instance obscures the stepping behavior.

Interestingly we noticed that the position clamp segments that displayed the most visually resolvable steps were collected together in a given trial (i.e. with the same virus complex). We found that approximately 3 of the 12 trials yielded position clamp
segments with frequent apparently resolved steps while stepping was much more sparse and short lived among the other 9 trials. Factors such as thermal convection of the fluid within the sample chamber (induced by laser-heating); and/or added noised caused by Brownian fluctuations and hydrodynamic drag on extra DNA molecules attached to the beads; and/or larger environmental noise during some measurements (e.g., floor vibrations) could cause increased noise in the measured signal during some events to make steps unresolved. Alternatively, although it seems unlikely, it is possible that different individual complexes could exhibit inherently differing stepping behaviors.

**Figure 6.1:** Comparison of lambda DNA packaging sections that do and do not exhibit stepping behavior. a) Lambda packaging traces collected with our high resolution dual-trap optical tweezers. Black trace is an example that shows step like behavior. Blue trace is an example packaging trace that portrays large fluctuations but no evidence of stepping. b) Pairwise distributions that correspond to the data shown in Fig. 6.1a.
From these position clamp segments of data, we selected sections of data over distances of approximately 50bp. After decimating and box-car filtering the data, we found that steps predominantly quantized in ~5bp increments could sometimes be observed. Figure 6.2a shows a few example packaging sections that have been decimated down to 1kHz and clearly exhibit step-like behavior with ~5bp increments. To investigate statistically for the presence of steps of a constant size, we calculated the pairwise distribution (PWD) from these translocation traces, which can be seen in figure 6.1b and 6.2b. The PWD is a distribution of the differences between all points in a trace (see sections 4.4.2 & 4.7.2 of chapter 4 for more on this). As is the case for a PWD of a step function, the black trace in figure 6.1b (same trace can be seen in Fig. 6.2b) exhibits a sinusoidal-like distribution with peaks that occur at approximately integer multiples from the first peak’s displacement difference, where the position of the first peak represents the step size distance, which occurs at ~5bp in these distributions. In contrast, the PWD corresponding to the blue packaging trace in figure 6.1a, which is seen in Fig. 6.1b (blue trace), shows a slowly decreasing curve with mild fluctuations but no recognizable oscillatory behavior that would be indicative of stepping.

Now, of the oscillatory PWDs we have obtained, the distributions do not always overlap perfectly and show slight offsets and variations in peak amplitudes. Figure 6.2 show a few packaging traces (Fig. 6.2a) that exhibit steps of ~5bp (see the PWDs in Fig. 6.2b). In these examples the amplitudes of the PWDs vary because the time between steps vary in these traces while the slight offsets are likely due to variations in the level of noise from one measurement to another. For PWDs that overlap well, an average PWD would be expected to provide us with a better determination of the measured step size.
However, when averaging the PWDs we find that offsets observed in the PWDs and PWDs that do not oscillate all contribute to a background from which peaks are difficult to resolve. Specifically we find that the average PWD does yield a peak at ~5bp, however, peaks positioned at multiples of 5bp are barely (or not at all) resolvable above the background distribution. This demonstrates the need to develop techniques that will more consistently generate traces with clear resolvable stepping.

**Figure 6.2:** Examples of λ phage DNA translocation that occurs in ~5bp steps. 

a) Sections of an example λ packaging traces under 5uM ATP conditions and measured with a dual-optical trapping system. After removing some environmental noise by utilizing differential detection, the 100kHz sampled data was decimated to 1kHz and box-car filtered prior to plotting here. During these events, the complexes underwent loads of ~18 – 25pN. The three traces here have been displaced vertically (by arbitrary distances) for display purposes. b) The pairwise distributions for the traces in (a) after box-car filtering. Note that the black trace is the same as that shown in Fig. 6.1. The color of the trace in plot (a) corresponds to the color of the traces in plot (b).
A step size of ~5bp was unexpected, but interesting, because it differs from φ29’s reported 10bp quantized packaging increments consisting of rapid bursts of four 2.5bp steps. We found no evidence that these ~5bp steps were comprised of “bursts” of smaller substeps. Because these results come from a very limited number of measurements on a new optical tweezers system, with a new calibration protocol, and with new data analysis routines we must consider other possible explanations for the discrepancy between this step size and that reported for φ29; it may be that our calibration is incorrect or the step size may actually be smaller than 5 bp and we are only able to resolve multiples of the smaller step size. However, if, in fact, the steps are equal to the 2.5 bp observed for φ29, then our trap compliance (γ) and force scaling (α) calibration parameters would have to be significantly off to yield such a discrepancy. In fact, the product αγ would have to be 2 times greater than it should be, which seems unlikely. However, it is possible that we are unable to resolve steps less than 5bp, as we currently have no independent test of the limits of our resolution. Furthermore, it is possible that this apparent ~5bp step could, for example, be a 6bp step measured with an ~1 bp systematic measurement error. The φ29 stepping results of Bustamante and coworkers show that the mean PWDs obtained under different ATP concentrations can be slightly offset from one another by 1-2 bp. It is possible that lambda actually only uses three of its putative four terminase subunits to make 6bp bursts that are composed of three (potentially unresolvable) 2bp steps, which is analogous to φ29 using 4 of its 5 subunits to make a burst of four 2.5bp steps. Alternatively, it is possible that lambda still makes 2.5bp steps but only utilizes two subunits per cycle and that we are unable to resolve the smaller 2.5bp steps here.
More interestingly would be the possibility that λ actually makes 5bp steps per ATP hydrolyzed. Such a result could shed light on how it is that different viruses package at different rates. Some factors that can affect a virus’s rate of packaging are the delays between steps, the number of subunits that make steps per cycle, and the step size. However, in this case, since lambda packages ~4-5 times faster than φ29, a step size of ~5bp would only be able to account for ~40-50% of lambda’s faster packaging rate (assuming lambda also makes “bursts” of four steps like φ29). Nonetheless, this is still a possibility. Though, as interesting as our result might be, recent studies of phage P74-26, which is more like λ than φ29, provide evidence from the crystal structure and other biochemical experiments that each terminase subunit translocates DNA by ~2.4bp. The results of φ29 as well as the predicted step sizes of P74-26, T4, and T3 suggest that dsDNA viruses in general have similar step sizes (~2-2.5bp per ATP hydrolyzed), which puts our observation into question. Additionally, given that the ATPase domain is believed to be closely connected with the DNA translocation mechanism and that all these viruses (including λ) are a part of the ASCE NTPase superfamily, it seems like these different viral motors would make steps of similar or the same size.

Of course, our results are only preliminary. To confirm the lambda step size, we need to collect much more data at this ATP concentration (5uM) as well as over a range of other ATP concentrations and then generate an average pairwise distribution from all the data. Additionally, a useful independent test would be to reproduce the φ29 stepping experiments conducted by Bustamante and coworkers to see if we indeed measure the same 2.5 bp reported step size in this case. If we are indeed able to resolve the discrete motor steps corresponding to single hydrolysis events, further and more detailed analysis
of the data, such as analysis of the distribution of dwell times between steps or bursts of steps, can be used to elucidate additional information about the gpA translocation mechanism as well as the extent of subunit coordination, which is also likely to shed light on the structure-function relationship of members of the ASCE superfamily of NTPases.

6.4 Conclusion

We present here initial high resolution measurements of λ bacteriophage’s DNA translocation steps. These preliminary results indicate that λ makes ~5bp steps, but whether these steps represent a “burst” of multiple single ATP hydrolyzed substeps or simply a single substep from one hydrolyzed ATP is unclear. In either case, the implications to our understanding of viral motor mechanisms and the mechanisms of ASCE superfamily proteins would be significant. However, significantly more data needs to be collected and control experiments performed to affirm these findings, especially since they differ so greatly from stepping predictions and observations made for other viruses. Once the λ step size can be confirmed, further experiments and analysis can be done to reveal information about this virus’s packaging mechanism.

6.5 Acknowledgements

We thank Nicholas Keller for his collaboration on this project. We also thank Michael Feiss and Jean Sippy for providing the λ reagents and Jeffrey Moffitt for providing additional, useful information about the single-molecule φ29 stepping analysis.

6.6 Materials and Methods

The prestalled λ DNA/terminase/procapsid complexes were prepared and attached to beads exactly as described in chapters 2 & 3. The tweezers measurements were conducted using our high resolution dual-trap and dual-detection tweezers instrument
which is described in chapter 4 and the dissertation appendix. Experiments were conducted using a partial position clamp mode in which the complexes reinitiated packaging by subjecting them to a buffer with 5uM ATP and allowed to package with the traps held at a fixed position until the force achieved a predefined threshold force $F_{\text{max}}$. Once this threshold force was reached, the traps were moved toward one another until the measured force dropped down to a predefined lower force $F_{\text{min}}$. These two steps were then repeated until all but ~1000bp of our ~10kbp packable λ construct was packaged. The data was collected with a 100kHz sampling rate. After evaluating the differential displacement coordinate (see chapter 4) and converting the results to contour length using our calibration, the results were decimated to 1kHz and box-car filtered. PWDs were generated by taking the absolute value of the difference between all points in the packaging trace just previously generated. The resulting PWD was then box-car filtered.

6.7 References for Chapter 6


origins of chromosome segregation, cell division and viral capsid packaging. *Nucleic acids research*, 32(17), 5260-5279.


Chapter 7

Dynamics of DNA Reannealing Protein HARP

7.1 Abstract

The exposure of damage susceptible single-stranded DNA (ssDNA) is a common occurrence during normal cellular activity. Replication protein A (RPA) is a protein that rapidly and stably binds ssDNA but is also capable of unwinding double-stranded DNA (dsDNA). On the other hand, the forked-DNA binding protein HARP, which is recruited to bind DNA in the presence of RPA, has been found to not only prevent further DNA unwinding but also to reanneal complementary ssDNA in an ATP-driven manner. Together these proteins act to protect and/or reduce the quantity of exposed ssDNA.

Using a DNA unzipping construct prepared by a novel construction method, which we present here, and the real-time manipulating and measurement abilities of dual-trap optical tweezers, we conducted preliminary work in an effort to develop a new approach to investigate the properties and mechanisms of RPA and HARP. Here we present the preliminary findings of these investigations. Many of the results reveal properties that agree with previously identified properties for these proteins, and thus provide independent verification of these properties, but the methods we introduce hold promise to reveal new features as well. Thus far, we have obtained results indicating that RPA rapidly binds ssDNA, prevents spontaneous reannealing of complementary ssDNA (after sufficient time has elapsed for RPA binding), and that RPA will unwind dsDNA whose strands are experiencing a load. Additionally, we have obtained evidence that HARP binds only to forked DNA (and that it can do so in the absence of RPA), is not a helicase,
and prevents DNA unzipping. Beyond this, a small amount of evidence has been obtained potentially implicating HARP in forcible DNA reannealing and supporting that HARP is recruited to bind forked DNA in the presence of RPA; however, the infrequency of these observations and particular uncertainties in the experimental conditions raise questions about the certainty of these findings. Nonetheless, we have also obtained results that propose HARP also acts to prevent reannealing of complementary DNA strands, resist high forces, and possibly slow reannealing in an ATP-dependent way. These preliminary findings demonstrate how the optical tweezers assays developed here can be used to determine qualitative features and properties of RPA and HARP.

Continued work with these assays and a recently built high resolution, low drift tweezers system (see chapter 4) should allow us to settle on our current findings and to quantify other properties of these proteins, such as binding rates and DNA unwinding/reannealing rates. However, for this dissertation, extending this project, which was from the beginning a "side project", was given lower priority than finishing the work on viral DNA packaging described in Ch. 2.

7.2 Introduction

DNA replication, repair, and transcription are regularly occurring multistep cellular processes that are accomplished by using a tremendous amount of molecular machinery to perform numerous subfunctions\(^1,2\). One such function common to these processes is that of DNA unwinding (also referred to as ‘unzipping’), which can occur actively by proteins such as helicases or passively by DNA breathing, which is promoted by A/T rich sequences in high temperature and/or salt conditions\(^1,5\). The unzipping of DNA by these means produces forked and single-stranded DNA. In the case of helicase
unzipping in eukaryotes, the damage susceptible ssDNA is coated by the single-stranded binding protein (SSB) RPA, which protects the chemically reactive nitrogenous bases from other reactive molecules within the cell. Whereas, in the case of unzipping by DNA breathing, RPA simultaneously protects exposed ssDNA and progressively unzips the DNA further by "wedging" its way between the two annealed strands at a DNA fork junction. Extensive studies of RPA have revealed that this protein initially binds to an ~8 base pair (bp) segment of ssDNA and subsequently undergoes a conformational change (independent of ATP) that causes it to extend and bind over an ~30bp region. If such RPA binding occurs just before a DNA fork, this conformational change leads the protein to push its way between two annealed strands, thereby unzipping the DNA.

Uncoated complementary strands of ssDNA near a fork will automatically and rapidly reanneal in the appropriate conditions whereas RPA-coated complementary single DNA strands will remain stably unzipped. However, besides reducing the benefits of maintaining a double-stranded structure (extreme stability, mutation resistance, code correcting capacity), long-lived stable RPA coated ssDNA strands can potentially interfere with transcription, replication, and/or repair. As such, how are such RPA coated complementary single strands of DNA reannealed? Moreover, what keeps dsDNA from being completely pealed apart by passive RPA unzipping?

The protein HARP, HepA-related protein, (previously known as SMARCAL1 and DNA-dependent ATPase A) was the first protein identified to have ATP-driven annealing helicase activity (i.e. DNA ‘rezipping’ activity). In 2009 evidence was provided that HARP preferentially binds to forked DNA and actively reanneals ssDNA (whether coated or uncoated by RPA) by hydrolyzing ATP and subsequently pulling the strands
together, which, in the case of RPA coated ssDNA, causes the RPA to dissociate. This same study also demonstrated that HARP ATPase activity is stimulated by forked DNA but that ATP is not necessary for HARP to bind to forked DNA. Additionally, because mutant HARP is known to be responsible for the human disorder Schimke Immunoo- osseous Dysplasia (SIOD), which impacts multiple physiological growth functions in human development and subsequently leads to an average lifespan of 9.2 years, it is suggested that DNA rezipping activity of HARP is particularly important for normal growth within a human. Since revealing the function of HARP, another protein with a similar annealing helicase function has been identified, AH2 (Annealing Helicase 2), which differs from HARP in that AH2: lacks a conserved RPA-binding domain; does not interact with RPA; and contains an HNH motif, which is commonly found in bacteria and fungi and is often associated with nuclease activity.

While there is strong evidence that HARP is an ATP-driven annealing helicase, it is not completely certain that HARP forcibly reanneals DNA. Furthermore, the dynamics and detailed mechanism of this protein have yet to be studied. Laser tweezers is a tool very well suited to investigate such unknowns because it has the capability to unzip dsDNA and hold this DNA partially unzipped while measuring forces on the DNA. Single molecule unzipping experiments have previously been performed to characterize DNA unzipping and to investigate properties of proteins that act on unzipped DNA, such as SSBs or helicases. Optical tweezers measurements of DNA unzipping have demonstrated that unzipping DNA produces a characteristic force-extension curve; while pulling on two annealed complementary DNA strands in opposing directions, the force applied rises to ~15 pN after which the force exhibits a sawtooth plateau as the strands
are pulled away from one another. In normal physiological salt conditions, the average force of this plateau is $\sim 15\text{pN}$\textsuperscript{12,15,16}. Greater cation concentrations yield lower average unzipping plateau forces\textsuperscript{16}. It is understood that unzipping DNA by such methods does not break the bonds between a single base pair at a time. Rather, each “tooth” within this plateau represents a segment of adjacent multiple bases of varying number (typically clusters of tens of base pairs) that simultaneously break from their complementary bases\textsuperscript{16}. The shape of this “tooth” (and force at which it occurs) is dependent on hydrogen bonding between bases, stacking energy (energy between adjacent base pairs within a sequence), overall electrostatic interactions, and entropy loss from the change in the DNA structure upon unzipping\textsuperscript{16}. Therefore, with an unzipping construct in hand, laser tweezers appear to be perfectly suited for affirming the function of HARP and RPA and for further characterizing their mechanisms.

Before we could attempt studies of HARP, however, we needed to develop a special DNA construct and develop techniques for manipulating it with optical tweezers. Although such measurements have been reported by a few other groups in the literature, such measurements are technically challenging, and full details are not given in the published literature. In this chapter we present a novel method for preparing a generalizable, high yielding unzipping construct designed for use in single molecule experiments. With this construct we present a new method for investigating the properties and mechanisms of RPA and HARP using dual-trap optical tweezers. We present preliminary findings for these proteins, which support the ssDNA coating and dsDNA unzipping activities of RPA as well as provide evidence of strong forked DNA binding, slow rezipping, and forcible rezipping activity of HARP.
7.3 Results and Discussion

7.3.1 Novel Single-Molecule DNA Unzipping Construct

7.3.1.1 Construction and Results

Previous single-molecule DNA unzipping studies have developed an array of DNA hairpin or unzipping construct designs\textsuperscript{12,13,15,16}. However, we have found that many of these designs and constructs have limitations that make single-molecule experiments inefficient and/or suboptimal for the study at hand. Some such limitations include low yielding production of the construct, short unzipping length, and short distances between strand labels. We overcome these limitations by avoiding the use of inefficient ligation procedures and instead utilize the power of PCR, nicking enzymes, and rolling circle replication.

In a nutshell, we prepare our unzipping construct by first amplifying a segment of lambda DNA by PCR using one unlabeled primer and one digoxigenin labeled primer where one of Lambda’s Nt.BbvCI (NEB Inc.) nickable sites is positioned somewhere in the middle of the PCR product. In rolling circle replication a polymerase will peel away a single strand of DNA from its DNA complement as it adds nucleotides to the replicated strand. With this knowledge, after nicking our PCR generated DNA, we label the DNA strand whose 3’-end is at the nicking position by using the polymerase Klenow and a limited selection of nucleotides (Thymine and Biotin-Cytosine). By limiting the nucleotides, we force Klenow to stop polymerizing after the first couple of nucleotides are added, which allows us to keep a large distance between the two labels. However, to ensure Klenow does not proceed past the first two nucleotides (by way of erroneous
polymerization), we subsequently quench the Mg\(^{2+}\) in the reaction with EDTA. See figure 7.1 for a cartoon of this construct design.

![Diagram](image)

**Figure 7.1:** Cartoon of the dual labeled general unzipping construct designed for single-molecule experiments. The construct contains a dsDNA arm and a dsDNA unzipping segment. The length of these segments are tunable.

Because PCR can generate a tremendous quantity of identical DNA segments and because restriction endonucleases and polymerases are highly efficient, this method produces very high yields and concentrations of this unzipping construct. Also, this method not only allows the arm and unzipping segment lengths to be tuned to the desired length but allows the distance between the label positions to be tuned, which is done by adjusting the position of the forward and reverse primers relative to the nicking site and one another. For our experiments we have designed our constructs to have a long dsDNA arm separating the two labels because we have interest in monitoring the forces applied to the unzipping construct just prior to stretching the construct. Because of this need, we cannot have the labels too close. Typically with our dual-trap tweezers, when the traps are less than ~1000 base pairs (~300-400 nm) from one another, trap-trap cross-talk
begins to affect the measured force, resulting in reduced measurement accuracy. In other words, when the traps are close enough, the trap from one bead begins to impose forces on the bead in the neighboring trap, causing it to inappropriately be displaced, thus resulting in a measured force even if the beads are not tethered. Ergo, we created four constructs using this method, each with an arm length of ~9.2kbp and unzipping segment lengths of ~0.9, ~1.6, ~3.6, and ~7.8 kbp (Table 7.1).

**Table 7.1:** Length details of four unzipping constructs prepared for single-molecule experiments by our new method.

<table>
<thead>
<tr>
<th>Construct Full Size</th>
<th>Arm Length (bases)</th>
<th>Unzipping segment Length (#bases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10kb</td>
<td>9166</td>
<td>885</td>
</tr>
<tr>
<td>10.7kb</td>
<td>9166</td>
<td>1550</td>
</tr>
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<td>12.8kb</td>
<td>9166</td>
<td>3622</td>
</tr>
<tr>
<td>17kb</td>
<td>9166</td>
<td>7784</td>
</tr>
</tbody>
</table>

Using dual optical tweezers we can partially unzip these constructs and hold them open, thus creating forked DNA and two strands of single stranded DNA, which can be used in our HARP and RPA experiments. The manner in which this is performed can be seen in figure 7.2 where we unzip the 10.7kb unzipping construct (see methods for additional details). Note, however, that we completely unzip the construct in this example. We begin by tethering the unzipping construct between an anti-digoxigenin
microsphere and a streptavidin microsphere, each of which are trapped in the two tweezer traps (Step 1). By moving the traps away from one another the dsDNA arm elongates and eventually becomes taught (Step 2). Then, as the traps continue to be separated, the force pulling on the two strands rises until it reaches the unzipping force (~15pN in normal physiological ionic conditions) and the DNA begins to unzip, exhibiting a sawtooth force vs. extension trace (Step 3). To avoid completely unzipping the DNA, the operator needs only to stop separating the traps during step 3 before the end of the unzipping construct is reached.
Figure 7.2: Procedure for unzipping DNA using dual-trap optical tweezers and the force vs. trap displacement trace resulting from completely unzipping a 10.7kb unzipping construct. Step 1: The unzipping construct is tethered between two trapped beads. Step 2: The traps are moved apart from one another, causing the beads to pull on the two DNA strands and a measured rise in force. Step 3: Sufficiently high force is achieve such that the DNA begins to unzip as the traps are moved apart.

Trials with these constructs have demonstrated the success of this construction method. In general, with other construction procedures, not all steps are expected to be 100% efficient, in which case only a fraction of the initial starting DNA ever manages to assemble into complete unzipping constructs. In addition it can be difficult to quantify the amount of complete, properly prepared construct. In some experimental setups, such as those that use surface tethering assays, high yields and concentrations may not be of great importance, and thus there is less need to attempt to quantify the final product$^{12-15}$. 
However, in experiments where DNA is tethered between beads, such as with dual-trap optical tweezers, high concentrations of an unzipping product become important. To make experiments with such tools feasible, we would need approximately one out of every 10 beads (or less) captured to have an unzipping construct attached. Since one microliter of 5% w/v concentrated 2μm diameter polystyrene beads contains on the order of 10 million beads, there is a tremendous surface area available for the unzipping construct to coat. If we start with a 100ng/ul (though we typically have higher concentrations) sample of purified 10.7kb PCR amplified DNA and assume the DNA nicking and polymerization is 100% efficient, then 1ul of this will contain ~9 billion unzipping constructs, nearly 1000-fold greater than the number of beads. Such concentrations would surely make it possible to coat each bead with numerous constructs. Furthermore, even if we assume that only 50% of the starting DNA is properly prepared into the designed construct, we would still have well over the number of beads. Lastly, high concentrations are important because they increase the rate at which the constructs bind to the beads. In other words, it decreases the diffusion distance between the beads and the construct. If we wish to mix an equal number of moles of beads and unzipping constructs but the molar concentration of the construct sample is much lower than that of the beads, a much greater volume of the construct sample will be require, which will dilute the beads when they are mixed together, thus increasing the diffusion distances and increasing the binding time. Nonetheless, in our trials with these constructs, we found that the final yield of complete, properly prepared constructs is so high that, in order to ensure the formation of single tethers between the beads, the samples need to be significantly diluted before attaching them to streptavidin beads. Thus demonstrating that
this method successfully produces high yields and concentrations of an unzipping construct.

The trials with these constructs have also shown that most constructs yield identical force-displacement traces, which demonstrates the consistency this method provides. Occasionally an unzipping trace is observed where unzipping occurs slightly later or earlier than the point it should (based on the design) or the force drops to zero during unzipping before the end of the construct is reached. Such occurrences can be observed when the DNA is either nonspecifically nicked somewhere other than the nicking site or if the DNA happened to have a nick prior to the digestion. Such events, however, are rare and thus inconsequential. Aside from this, we’ve also occasionally noticed the breaking of tethers when holding the construct partially unzipped. This is mostly likely due to the breaking of the digoxigenin – anti-digoxigenin bond, which is much weaker than a biotin-streptavidin bond and is dependent on force, rate of applied force, and ionic conditions\textsuperscript{17}.

At this point it is worth noting some details about the character of unzipping these constructs. First, as the traps are separated, the shape of the trace during the initial rise in force is the same as that of stretching dsDNA, which is expected because as the beads initially pull on the construct, they are only pulling on the dsDNA arm. In contrast, stretching ssDNA typically exhibits an early slow rise in force followed by a rapid steep rise in force – steeper than that seen while stretching dsDNA. Figure 7.3 affirms this by comparing an unzipping trace to a 10kb dsDNA stretching trace.
Figure 7.3: Comparison of a force-displacement 10.7 kb unzipping trace with a 10kb dsDNA stretching trace. The unzipping trace exhibits a similar initial rise in force as in the case of stretching dsDNA. Note that the tether in the dsDNA stretching trace has broken before achieving the overstretching plateau that is common of dsDNA stretching.

Second, the use of dual-trap optical tweezers for these experiments allows us to also monitor the rezipping of DNA in real time. We’ve found that the force-displacement trace measured during rezipping can retrace the unzipping trace but that it is dependent on the rate at which the beads are moved toward one another. The slower the beads approach one another, the closer the rezipping trace will match the unzipping trace.

Despite this, in general, uninhibited rezipping is an extremely rapid process. We’ve found that complementary single strands of DNA at a fork junction will typically reanneal at rates > 25,000 bp/s without the force dropping more than ~ 5σ (~3 pN in normal physiological ionic conditions where σ is the standard deviation of the unzipping force plateau) below the plateau force. The reason for imperfect reannealing under rapid bead retraction rates is likely a result of slight misalignments between the single strands that can occur by completely slackening the strands very quickly.
7.3.1.2 Limitations

Thus far we’ve discussed the advantages of this method and construct, but the particular design used here is not without limitations. First of all, this construct is not a hairpin. Once completely unzipped, the construct can no longer be used for experiments and another construct will need to be tethered between the beads to perform another experiment. However, if a hairpin was needed, it would be possible to ligate a hairpin on to the unzipping segment. This would require first identifying a restriction site that produces an overhang within the unzipping segment. Alternatively, a specific restriction enzyme site can be incorporated in the primer (to its 5’ end) that corresponds to the unzipping end of the construct. Second, designing an oligo that forms a hairpin as well as an overhang that is complementary to the restriction site overhang. Then, prior to the nicking step, these pieces can be ligated to one another. However, since ligation can be inefficient, the resulting product is likely to be low. Nonetheless, after completing the remaining steps (nicking and polymerizing) the product will be a mixture of complete hairpins and unzipping constructs.

Another potential limitation of this design is the need for a nicking site within the PCR amplified DNA, which implies the original template DNA must contain a nicking site. Also, if a particular DNA sequence is needed for an unzipping experiment, this method may not work if the template DNA contains multiple nicking sites and, in particular, if it contains nicking sites within the segment of DNA that is needed for unzipping. See section 7.3.1.3 for a discussion of how this limitation might be overcome.

In contrast to other methods, this method is also limited by the fact that it does not allow for more than one dsDNA arm. While unzipping this construct, of the two ends
being pulled, one will be ssDNA and the other will be a combination of ssDNA and the dsDNA arm. However, by designing a construct where in the PCR primer contains the nicking site, it is possible to make the dsDNA arm so short that, during unzipping, the short oligo from the dsDNA arm will peel away, thus making it so that the user is only pulling ssDNA during unzipping.

Another limitation, as mentioned before, is that the anti-digoxigenin/digoxigenin bond is dependent on the applied force and rate of applied force on the construct, which may limit the forces that can be tested in experiments where proteins inhibit unzipping. Fortunately it is possible to label both strands with biotin. However, a slightly different tethering method would need to be developed to prevent both biotin labels of a single construct from binding to the surface of the same streptavidin bead. One way in which this can be accomplished is first capturing two streptavidin beads in the two traps and then flowing a sample of the constructs by these beads while bumping the beads together. The flow will help keep the DNA elongated, thus making it more likely that only one biotin will bind to the beads prior to bumping them together.

Lastly, the length of unzippable DNA is limited by the maximum length that can be amplified by PCR. Commercially available long range PCR kits typically do not advertise the ability to produce DNA segments greater than ~40kb. And even such 40kb constructs are fairly low yielding. Moreover, the PCR DNA must be either purified or dialyzed in this method, both of which tend to reduce the yield further for long DNA products and/or dilute the construct sample. A possible way to create constructs of greater length would be to generate large plasmids that can be digested to have one blunt
end, one 5’ overhang, and one nicking site. Although, creating large plasmids and with substantial yield can be difficult.

7.3.1.3 Generalizability of Method

While we have discussed many of the limitations of the particular design used here, we’ve presented options that can be used to overcome most of its limitations. Consequently, the adaptability of this method for generating a variety of unzipping constructs makes it nearly generalizable. A key feature in making this method generalizable is having a nicking site present in the template DNA within the segment to be amplified by PCR. Two potential issues can arise from this. Either the template DNA has no nicking site or it has more than desired. In the case of no nicking sites, a nicking site can be inserted into the digoxigenin labeled primer. Although, because of the previously mentioned cross talk, force measurements of this construct within the first ~300-400nm of trap displacement would not be reliable. Alternatively, DNA vectors containing a nicking site can be transformed into a plasmid. The resulting plasmid can be grown and extracted from E.coli and then used as a template for the PCR in our method. Otherwise, when the DNA of interest has multiple nicking sites, this method fails. One possible solution is to search for sequences of other nicking enzymes within the DNA. Though, if none exist, and the DNA is a plasmid, it may be possible to insert a vector containing the sequence of a nicking enzyme that the DNA does not already contain. It is this possible abundance or lack of available nicking sites that prevent this method from being applicable to any DNA and thus from being a completely general method. Notwithstanding, this method can still be applied to many DNA templates, is tunable,
simple, and can produce both high yields and high concentrations of an unzipping construct.

7.3.2 HARP Acting on Bare Forked DNA

7.3.2.1 Evidence that HARP Impedes Unzipping

Now, with an unzipping construct and a tool for manipulating it in real time, we are equipped to probe how the protein HARP interacts with DNA. Our initial experimental mode consisted of unzipping DNA in the presence of various concentrations of HARP. Occasionally in concentrations ≥ 80nm HARP (and in the presence of 1.5mM ATP), a rise in force above the unzipping plateau was observed during unzipping at rates of roughly 450nm/s (Fig. 7.4). Often the forces rose by tens of piconewtons above the unzipping plateau before the force abruptly dropped back down to the unzipping plateau (Occasionally the force did not drop, which we address later.). Such results can only occur if something initially impedes further unzipping at the fork and then dissociates or partially dissociates to permit further unzipping. This result suggest that HARP tightly binds to either dsDNA or forked DNA to prevent the DNA from unzipping further. Additionally, out of several tens of trials performed, a rise in force above the unzipping plateau was never observed at the onset point of unzipping. Rather, such instances only occurred after some DNA had been unzipped and at random positions within the unzipping plateau. This supports the proposal that HARP does not bind until forked DNA is present⁸. However, these results alone still do not discern whether HARP binds to forked DNA or simply the dsDNA immediately upstream from the two unzipped single strands of DNA at a fork.
Notably, while HARP also appears to impede unzipping in the presence of the non-hydrolyzable UTP (as will be discussed later) – suggesting that hydrolysis is not required for HARP to bind, it would be worth conducting this same experiment in the absence of ATP or any ATP analog. While studies have previously shown that HARP can bind forked DNA in the presences of UTP\(^8\), such experiments might reveal whether HARP requires a nucleotide for stable binding and/or whether the presence of a nucleotide affects the strength of HARP-DNA binding. While these experiments have been performed in lower concentrations of HARP and have occasionally shown similar results, albeit less often, further testing is still needed.

![Figure 7.4: Force vs. trap displacement trace of unzipping DNA in the presence of HARP. In the presence of ≥ 80nm HARP (and in the presence of 1.5mM ATP), unzipping is occasionally impeded as exhibited by the rise in force above the unzipping plateau as the traps are displaced. Typically the impeded fork ruptures, causing the force to return to the unzipping plateau force, thus allowing for further unzipping.](image)

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\(^8\) Reference to be included.
7.3.2.2 Evidence that HARP Resists and Temporarily Sustains High Forces

In performing the experiments described above as well as in subsequent experiments described below, we also observed high rises in force above the unzipping plateau that did not rupture, suggesting HARP may be capable of resisting very high unzipping forces. To probe further, we tested whether these high forces could be sustained by holding the trap positions fixed once a fairly high force was achieved ($\geq 30\text{pN}$). In doing so we observed slow reductions in force with intermittent “pauses” (a fixed force that was sustained for a given period of time) of varying duration (Fig. 7.5). Note that while the impeded unzipping construct example in figure 7.5 was only stretched up to approximately 30pN, forces greater than 30pN were also attained in other trials of this sort. It has been shown previously that other DNA binding proteins (particularly the restriction enzymes BsoVI, XhoI, and EcoRI) are also capable of resisting similarly high forces when unzipping enzyme-bound dsDNA; these studies showed that such enzymes typically resist tens of piconewtons and even resist as much as ~45pN$^{18}$. Thus, our observations could be due to a fairly strongly bound HARP protein at forked DNA that slowly slips back along the dsDNA portion, which would result in further unzipping and thus a reduction in measured force, while occasionally catching the DNA. Alternatively, these results could be due to dissociation and rapid association of HARP at the fork. Moreover, similar reductions in force were observed when conducting these experiments in the presence of UTP but such events occurred less often than under ATP conditions. This suggests that ATP may yield greater HARP-fork stability than UTP.

Notwithstanding, to uncover the actual mechanism that describes this behavior will
require additional experimentation. Finally, since RPA and helicases are capable of generating high enough forces to overcome the unzipping plateau forces\textsuperscript{5,19}, which can range from ~ 10-20pN depending on the conditions, it seems reasonable to propose that one of HARP’s functions may be to resist high unzipping forces, given the evidence presented here.

\textbf{Figure 7.5:} Force vs. Time trace of an unzipping construct exhibiting impeded unzipping in the presence of HARP where the tweezer trap positions are held fixed once a high force ($\geq 30\text{pN}$) has been achieved. Note that this example comes from an experimental procedure used in a later section where, after impeded unzipping was observed, the beads are moved toward one another and then separated once again. Nonetheless, its resulting character during the high force and fixed trap extension period is the same as observed with the experimental mode described in this section. Here the beads are being separated until ~ 250s have passed, showing a rise in force due to impeded forked DNA. The traps are held at a fixed position from this point on and a slow reduction in force is observed that ultimately ends with a tether break.
7.3.2.3 Evidence of Forcible Rezipping by HARP

To explore whether HARP can forcibly rezip DNA as suggested indirectly by the studies of Yusufzai and Kadonaga (2008), we performed experiments in which we first partially unzipped a naked DNA unzipping construct in the presence of HARP, fixed the tweezer trap position, and then waited. If HARP does in fact forcibly reanneal DNA, then a rise in force above the unzipping plateau should be observed during this waiting stage. Out of ~30 trials in $\geq 80\text{nM}$ HARP and 1.5mM ATP we observed a few instances in which the measured force rose slightly above the unzipping plateau by $\sim 2\text{-}3\text{pN}$ (Fig. 7.6).

![Example force vs trap displacement and force vs time traces for a test trial of forcible rezing by HARP. Initially $\sim 700\text{bp}$ of the DNA construct is unzipped and then the traps are held at fixed positions while in the presence of HARP (80nM) and ATP (1.5mM). These traces show a slight rise in force above the unzipping plateau and conclude with a break in the tether.](image)

The low yield and small force rise put these few events into question. And while previous work has shown that *in vitro* tweezers experiments don’t exhibit the same level of activity as *in vivo* experiments, we still must consider the possibility that these events are false readings. Besides possible forcible rezing by HARP, slight increases in force...
could have also resulted from instrument drift or an aggregated particle or "dirt" within the experimental chamber that interferes with the DNA or microspheres, or falls into the trap, during the experiment. As a control, numerous trials were also performed with 1.5mM UTP instead of 1.5mM ATP; no rise in force above the unzipping plateau was observed in any of the trials, providing additional support that these few events may in fact have resulted from HARP forcibly rezipping DNA. Although we have evidence that HARP forcibly rezips DNA, in order to more conclusively assert this claim, many more trials with ATP and UTP are needed as well as control experiments for drift. Also, this project should benefit from the lower-noise instrument described in Ch. 4.

In the event that these observations are displays of authentic forcible rezipping by HARP, it is worth noting a couple features of the results. First, knowing that it takes ~15pN to unzip DNA, we note that, because RPA is capable of passively unwinding two complementary strands of DNA, it must at least be capable of resisting the ~15pN of force. The finding that HARP forcibly reanneals RPA coated complementary ssDNA strands then suggests that HARP is capable of generating as much as ~15pN of force in order to displace the RPA that has overcome this ~15pN unzipping force. Taking this one step further, the slight rise in force above the unzipping plateau seen in fig. 7.6 suggests that HARP may be capable of generating as much as ~17-18pN of force. Initially it may seem that the results in figure 7.6 suggest HARP only applies a few additional piconewtons in order to force a section of ssDNA together. However, if HARP is in fact forcibly reannealing the strands, then any force the traps apply to a HARP-bound fork DNA complex would be applied directly to HARP and no longer to the DNA. Furthermore, our evidence that HARP resists unzipping and slows reannealing
demonstrates that HARP likely binds to forked DNA and that any tension in the ssDNA strands pulls directly on the HARP molecule. Therefore, the rise in force seen in figure 7.6 suggests that HARP may be capable of generating up to ~17-18pN. Additionally, it may be that HARP only generates a moderate force on the DNA as a way of regulating reannealing. If its force generation was too great, it might force important replication/repair proteins to dissociate before they have completed their tasks. However, in order to understand these force generating features, as well as other features such as the initial delay or the rate of rezipping, much more data needs to be collected.

7.3.2.4 Evidence that HARP Slowly Rezips DNA

In exploring the behavior of this protein further, we developed another mode of experiments that revealed other interesting features. This measurement mode was guided by the behavior we observed while unzipping and rezipping the construct in the presence of HARP. This mode consists of: (1) pulling on the two strands of an unzipping construct until ~700bp are unzipped, (2) holding the trap positions fixed for ~1 minute, (3) moving the traps toward one another for ~1-3 seconds by a distance corresponding to the reannealing of approximately a few hundred base pairs, (4) holding the traps at a fixed position for ~1-2 minutes, (5) separating the traps once again until the DNA fully unzips or the tether breaks. Here we present results for experiments conducted in the presence of ≥ 80nm HARP and 1.5mM ATP. Figure 7.7 displays the results of a typical trial performed using this experimental mode.
**Figure 7.7:** Force vs trap displacement plots of unzipping and rezipping DNA in the presence of 80nM HARP and 1.5mM ATP using the measurement mode described in the text. Step 1 & 2: The DNA construct is unzipped ~700bp and held open for ~1min. Step 3: The traps are moved toward one another for a few seconds by a distance corresponding to approximately a few hundred base pairs. Step 4: The traps are held fixed for ~1-2 minutes. Step 5: The traps are separated until the DNA fully unzips.

During step 3, after having incubated in HARP, the construct does not spontaneously reanneal as it normally would when moving the traps together. Rather a drop in force occurs that is atypical of reannealing DNA in the absence of any proteins (Recall, as mention earlier, protein-free complementary ssDNA strands typically reanneals DNA at rates of > 25,000bp/s without displaying drops in force greater than a few piconewtons.) This suggests that HARP has bound itself to the DNA fork and is impeding rezipping. During this DNA relaxation there is also an observed decrease in elasticity because the tether is now composed of both dsDNA and ssDNA. Previous studies have shown that ssDNA elasticity depends on pH and ionic conditions, where, at
a sufficiently high monovalent and/or multivalent ion concentration and within the low force regime (<20pN), the stretching of ssDNA exhibits a slowly rising force “plateau”, which is believed to be a consequence of formed secondary structures (e.g. hairpins). Because such a plateau was observed in a study using similar ionic conditions as used here, it is reasonable to expect that some hairpins may be forming from the exposed ssDNA as the tension is reduced during this step. Consequently, HARP proteins may bind to these newly formed forks, which could possibly shorten the tether length (and thus increase the force) but, if so, would not be expected to shorten the tether length substantially. As an aside, the possibility of multiple hairpin-forks bound by HARP could provide another possible explanation for the slow decrease in force observed in the high force measurements discussed earlier (see section 7.3.2.2). It could be that, at these high forces, HARP dissociates from the hairpin-forks, which lead to a short increase in extension and thus a slight drop in measured force.

During step 4, where the trap positions are held fixed just after relaxing the tension in the DNA, we observed a slow rise in force toward the unzipping plateau. This suggests that the single strands near the fork are slowly reannealing. Subsequently the observed rise in force above the unzipping plateau during step 5 indicates that something is bound to the fork and inhibiting unzipping, presumably HARP, as observed in the experiments discussed earlier. This experiment was performed numerous times, each time displaying nearly the same behavior. Our earlier finding that HARP impedes DNA unzipping suggested that HARP binds to forked DNA. However those results could not distinguish whether HARP binds to forked DNA or simply dsDNA, both of which can impede unzipping if bound tightly enough. The results from this method demonstrate
that both unzipping and rezipping are impeded in the presence of HARP, thus providing much stronger evidence that HARP binds to forked DNA. If it only bound to dsDNA, then step 3 should have exhibited spontaneous rapid rezipping character. Also, after holding still for ~1 minute, upon separating the traps the force would not necessarily immediately increase above the unzipping plateau if HARP nonspecifically binds to dsDNA, which was always the case for the experiments performed here.

With our support that HARP binds to forked DNA, we now note that, consistent with previous bulk biochemical assays, we find no evidence that HARP exhibits any helicase activity. As in the case of RPA, helicases are capable of unzipping DNA in normal in vivo conditions, which implies they are capable of generating greater than ~15pN. In these experiments, as we hold the DNA construct partially unzipped and open, an unzipping force of ~15pN has already been applied and thus any unzipping protein need only generate a small amount of force (e.g. ~1-2pN) to overcome the next local force barrier (tooth within the sawtooth force plateau) to further unzip. With this force assisted unzipping, any helicase or unzipping protein (such as RPA) would be expected to easily unzip the DNA, which would exhibit a drop in measured force below the unzipping plateau during step 2. However, in all our measurements using this measurement mode or any other previously described measurement mode, a drop in force was never observed in the presence of HARP (all concentrations tested, 10nM – 300nM) with ATP or UTP (1.5mM), providing strong support for the previously reported finding that HARP is not an unzipping helicase \(^8\). As a control, section 7.3.3 presents results in which we directly observe such a drop in force due to RPA induced DNA unzipping.
7.3.2.5 Slow Rezipping Character in ATP

We conducted the above experiments under both ATP and UTP conditions and found the overall results to be very similar except during step 4 when slowed reannealing was observed. In the presence of ATP, the force rose slowly, fairly processively (i.e. increasing without frequent interruptions), and typically returned to the unzipping plateau force (Fig. 7.8). Whereas, in the presence of UTP, three phenotypes were observed where the force rise would stall before achieving the unzipping plateau in each case. See the next section for discussion on experiments conducted with UTP.

Figure 7.8: Force vs time example trace of slow rezipping in the presence of HARP (80nM) and ATP (1.5nM) during stage 4 of the measurement mode described in the text (section 7.3.2.4).
In the trace displayed in figure 7.8, the measured tension slowly rises, while occasionally exhibiting pauses, until it reaches ~15pN. After this step, further unzipping is impeded upon separating the traps (data not shown in Fig. 7.8). While most trials in ATP exhibited similar results, additional experimentation and controls are still needed to ascertain the exact cause of this behavior. However, until then, we propose a few possibilities, the first being that a single HARP molecule (or complex) is bound to the fork, translocating, and actively rezipping the DNA, but at a slower rate than naked DNA would spontaneously rezip. Specifically, for this example, approximately 500bp are rezipped in approximately 20 seconds, which corresponds to a rezipping rate of ~25bp/s – much slower than that of reziping in the absence of proteins (>25,000bp/s).

Alternatively, since the force is less than 15pN, it could be that a fork bound HARP is passively moving along with the fork as the DNA spontaneously rezip and is slowing the reziping by friction or by occasionally gripping and releasing grip on the fork. Akin to this, it could be that multiple HARP molecules are binding and dissociating from the fork during reziping where each HARP, while bound, impedes reziping. And very closely related to this is the possibility that hairpin structures form in the ssDNA while relaxing the DNA, consequently creating many forks to which HARP can bind. We propose a few possibilities for this scenario: (1) The binding of HARP to each fork shortens the tether between the beads by a small amount, thus yielding an increase in measured force, (2) HARP binds to the hairpin forks and then each HARP molecule translocates until it runs into a translocating HARP on an adjacent fork, or (3) Some combination of possibilities (1) & (2). While possibilities (1) and (2) seem plausible, it is not clear whether they can completely explain the observed behavior. Option (1) depends on the number of possible
hairpins present and/or the tether length reduction that occurs if HARP-fork binding condenses DNA. The variability of these dependencies would not necessarily be expected to fairly consistently yield a return to the unzipping plateau force. More importantly though, the difference observed in the slow force rise behavior when exposing HARP to ATP vs UTP (see UTP discussion below) suggests ATP may play a role in the shortening of the tether (possibly by ATP powered HARP translocation). As such, this observation disfavors the explanation of DNA condensing by HARP. Option (2) seems more plausible but, with enough hairpin structures, the force rise might be expected to stop before reaching the plateau force. Lastly, for the slow observed rise in force, we propose a least-interesting scenario in which non-physiological HARP aggregates are obstructing the fork, thus causing impeded reannealing.

In addition to the results described above, we also observed one event (out of ~25 trials) that showed a force rise above the unzipping plateau, thus providing another example to support the possibility that HARP forcibly reanneals DNA (Fig. 7.9). In the case that HARP is in fact acting as an active or passive reannealing protein as described among the first two possibilities above, such measurements can be used to reveal information about its active or passive translocating dynamics. More generally though, these experiments can help shed light on how and why HARP may impede rezipping. Lastly, given that this event was rare, we still must consider the possibility (as we did earlier) that this could be a result of instrument drift or an artifact resulting from some aggregated particle interfering with the microspheres or DNA.
Figure 7.9: Force vs time trace of one event collected in which the slow force rise, measured during step 4, rose above the unzipping plateau during an experiment in the presence of 80nM HARP and 1.5mM ATP. The horizontal solid line just above 14pN is the average force of the unzipping plateau that occurs just prior to the DNA relaxation (step 3). This suggests HARP is forcibly rizipping DNA.

7.3.2.6 Slow Rezipping Character in UTP

Using UTP as a control against these ATP experiments, we found, as stated earlier, that the measurements yielded similar results but with a difference primarily occurring during step 4 of the measurement mode (i.e. the slow rezipping stage). Just as in the case of ATP, the UTP conditions yield a force decrease below and increase above the unzipping force plateau during steps 3 and 5 respectively. These results support previous findings, once again, that HARP binds forked DNA with either ATP or UTP and they support our earlier observations that HARP impedes DNA unzipping and rezipping. Otherwise, the three phenotypes we observed during step 4 include: (1) a relatively fast rise in force that stops and stalls at a force below the unzipping plateau, (2) a very slow
rise in force that eventually stalls after only a small increase in force, or (3) no rise in force (Fig. 7.10).

Figure 7.10: Force vs time example traces of all 5 steps of the measurement mode used in this section. Experiments performed in the presence of HARP (80nM) and UTP (1.5mM). The bracketed sections correspond to step 4, the second fixed extension step. The left plot provides an example of phenotype (1) (quick force rise to a “stall”), whereas the right plot provides an example of phenotype (3) (no rise in force).

As in the case of the ATP results, at this point, we can only propose some possible explanations for these observations. The quick rise in force followed by a stall in phenotype (1) could result from fork-bound HARP that is either actively (by UTP hydrolysis) or passively rezipping initially, but then stalls. Although, this explanation seems unlikely since Yusufzai and Kadonaga report that HARP does not exhibit reannealing activity in the presence of UTP. Similarly, phenotype (2) could also be HARP bound to forked DNA while actively or passively rezipping, albeit very slowly. A possible but less likely scenario for this phenotype could be that
HARP is slowly accumulating on the DNA because of high [HARP] thus causing the DNA to condense/shorten, which would yield a slow increase in force. However, based on our evidence that HARP primarily binds to forked DNA, this option seems unlikely relative to others. Tests for DNA condensation by HARP could be performed by measuring tether length changes of a piece of ssDNA or dsDNA in the presence of high [HARP]. As for phenotype (3), the stall without any observable rise in force could be a consequence of HARP locking strongly on the fork and remaining unmoved because of the absence of hydrolyzable nucleotides. Despite these possible explanations, each of these phenotypes observed with UTP could still also result from some of the reasons proposed earlier for the ATP results, such as rapid dissociation/association of HARP or the least-interesting scenario of non-physiological HARP aggregates obstructing the fork.

Though the exact reason for the impeded rezipping in the presence of ATP or UTP is unclear, one distinction is worth noting: During this step of the procedure, the measured force typically stops increasing before reaching the unzipping plateau force in the presence of UTP while, on the other hand, it typically rises to or above the unzipping plateau force in the presence of ATP. This provides anecdotal evidence that HARP is an ATP-dependent, active reannealing protein.

7.3.3 RPA Acting on Forked DNA

Previous studies have presented findings that HARP has an RPA binding site but that this binding site is not absolutely necessary for annealing helicase activity. While these results support the possibility that HARP is not simply an RPA remover, other findings have shown that HARP is promoted to bind to forked DNA where RPA coats the ssDNA, suggesting RPA still plays a role in HARP’s activity. To further
investigate the activity of HARP in the presence of RPA, we first performed some basic single-molecule characterization of the interaction between RPA and an unzipping construct.

7.3.3.1 RPA Coats ssDNA Rapidly

We began by first incubating an unzipping construct (never yet unzipped) in 3nM RPA for approximately 1 minute and then proceeded to unzip and rezip the DNA at trap separation rates of ~ 450 nm/s and with a range of time (< 1s to minutes) between the unzipping phase and reziping phase. Of all trials performed (several tens of trials), the initial bead separation always yielded unzipping just as it appears in the absence of RPA. However, upon relaxing the tension (i.e. moving the beads toward one another), the ssDNA did not spontaneously and rapidly rezip. Rather, the measured force dropped below the unzipping plateau, indicating that RPA impedes reziping (Fig. 7.11). This supports the well-known fact that RPA is a single-stranded binding protein and it demonstrates that RPA can bind to ssDNA within seconds of its exposure. After unzipping the DNA, RPA is able to bind to the exposed ssDNA strands. And since RPA coated strands cannot reanneal, moving the traps together immediately relieves the tension in these coated ssDNA segments. If RPA was a dsDNA binding protein, we would have observed a rise in force above the unzipping plateau at the onset of unzipping or during unzipping.
Figure 7.11: Unzipping DNA in the presence of RPA (3nM). Top plot is a force vs trap displacement plot of an example of unzipping and rezipping DNA in the presence of RPA. Bottom plot is a time vs trap displacement plot corresponding to the data displayed in the top plot. Initially the unzipping construct is incubated in RPA for ~1 minute before unzipping here (not shown). The construct is unzipped, exposing ssDNA for < 3 seconds. During the relaxing step, the top plot shows a drop in force below the unzipping plateau, indicating that RPA rapidly binds to ssDNA.

In performing these measurements we also observed that if the construct is quickly relaxed after exposing ssDNA to RPA, we still observe the force to drop below the plateau force. However, after relaxing to zero force, upon unzipping again the behavior is very similar to unzipping in the absence of RPA. This suggests that initially RPA bound to the ssDNA during the unzipping step but that it may not have had sufficient time to completely and/or stably bind the ssDNA strands. Thus, in relaxing the tension in the strands, various sections of unbound ssDNA likely attempt to reanneal and cause weakly or incorrectly bound RPA to dissociate. Given the multistep process and sequence by which RPA is understood to bind ssDNA, rapid exposure of ssDNA to RPA...
is likely to result in uncoordinated binding and consequently numerous RPA molecules that are incorrectly or weakly bound.

7.3.3.2 Force Assisted Unzipping by RPA

On the other hand, if an unzipping construct was held open for a longer duration, tens of seconds to minutes, then the measured force slowly drops while holding the trap positions fixed. With enough time, the force reached zero piconewtons. However, for the results discussed here, the experiments were performed by waiting until the force dropped to ~3-7pN, after which the traps were brought back together (separated by less than 10kb – i.e. the dsDNA arm length) to release the remaining tension in the strands. After waiting for ~1-2 minutes, the traps were pulled apart. During this final trap separation, DNA unzipping was typically observed to initiate at a much larger trap displacement than an unzipping construct unladen by proteins and the resulting stretching trace overlapped with the previous relaxing trace. In this case, holding the fork open for a long period of time, provides RPA sufficient time to bind the ssDNA stably. However, again, given its binding mechanism, it still likely binds in an uncoordinated manner, leading to a combination of partially and fully bound RPA molecules. Despite this, holding the fork open also provides RPA an opportunity to bind properly and sequentially. The RPA that binds to ssDNA near the fork will peel the fork open, after its conformational change, and then create a short site for another RPA to bind. As this process continues, the result is a series of properly and sequentially ssDNA-bound RPA molecules. This slow RPA binding and DNA peeling is likely what leads to the slow drop in force observed when the trap positions are held fixed. Moreover, this process of RPA peeling the fork open occurs more readily in this instance because the fork is
already under tension and thus only requires a slight amount of additional force to unzip the DNA more. Lastly, because there was sufficient time for RPA to bind stably (and correctly near the fork), the RPA coated ssDNA strands are unable to reanneal when the DNA is relaxed. And as a result, when separating the traps again, the force doesn’t rise until later because the tether length between the beads and the fork is increased.

Also of note is the shape of the relaxation curve after exposing ssDNA to RPA molecules during these measurements. Recalling our earlier discussion on HARP impeded rezipping, RPA impeded rezipping also yields a force vs trap displacement relaxation curve that rises more gradually than that of stretching/relaxing dsDNA because the tether incurring a force is composed of both dsDNA and ssDNA. As we pointed out earlier, the formation of hairpin structures within the ssDNA segments are possible in our ionic conditions. However, given RPA’s unzipping capabilities, if any hairpin structures happen to exist during these measurements, RPA will most likely have unzipped them (especially under the applied load used here). Therefore, the force response curve measured during the relaxation step in these experiments is not expected to include any contribution from the presence of hairpins but rather only result from dsDNA and linear protein laden ssDNA. If we find that hairpin formation is occurring and interfering with the earlier experiments that only use HARP, the use of RPA may become a necessary tool for overcoming such an issue.

Lastly, while performing these measurements an interesting and unexpected observation was occasionally seen during the second trap separation (after the ssDNA was coated by RPA). Occasionally, rather than unzipping upon reaching a stretching force of ~15pN, the force was observed to rise above the unzipping plateau by ~3-10pN.
This behavior was subsequently followed by a rupture in the impeded unzipping, a rapid drop in force to ~15pN, and then continued unzipping as the traps were further separated (Fig. 7.12).

![Figure 7.12](image)

**Figure 7.12:** Force vs trap displacement of unexpected unzipping impedance observed during unzipping/rezipping/unzipping DNA measurement mode in the presence of RPA. Step 1: The initial quick partial unzipping and rezipping performed in the presence of RPA. Note that the rezipping force plateau does not perfectly overlap the unzipping plateau. Step 2 (data not shown): Unzip a few hundred bases, wait until the force drops to ~7pN, move the traps together to relax all tension, then wait ~1-2 minutes at zero force. Step 3: Separate the traps to unzip the DNA. In this case, during this step, unzipping was unexpectedly impeded, as seen by the rise above the unzipping plateau. Here, after the locked fork ruptures, the DNA is further unzipped and then relaxed.

Because this behavior is a rare occurrence and because no studies have previously implicated RPA in dsDNA binding or fork binding, we suspect this may be a secondary effect of the unique experimental circumstances occurring during these experiments that are atypical of *in vivo* conditions (e.g. holding DNA partially unzipped in such a way that it forms a “T” shape conformation). One proposal is that two RPA molecules bound to the ssDNA strands nearest the fork form a weak bond between one another, which could
temporarily prevent unzipping until this bond ruptures. However, another interesting and reasonable proposal is, given RPA’s multistep binding mechanism and its multiple DNA binding sites, a single RPA molecule binds its first two DNA binding domains to one strand at the fork and then, after a conformational change, reaches across the fork where the second two DNA binding domains bind to the opposing DNA strand (Fig 7.13).

Figure 7.13: Cartoon of a proposed RPA-Forked DNA structure that might explain the occasional unexpected impeded unzipping observations. Here a single RPA is shown to partially bind to both complementary ssDNA segments.

7.3.4 HARP and RPA Acting Together on Forked DNA

As mentioned earlier, while studies have previously shown that RPA is not absolutely necessary for HARP to perform annealing helicase activity, other studies have shown that HARP is promoted to bind in the presence of RPA, suggesting that RPA may still play a useful role in HARP’s overall mechanistic process. Additionally, since force assisted RPA can unzip dsDNA, RPA can be used to minimize the number of forks present in a single unzipping construct prior to performing measurements in the presence of HARP. Here we perform experiments in which we first incubate partially unzipped DNA in RPA long enough to ensure the ssDNA strands are completely and stably bound by RPA and then move this RPA coated construct (in a relaxed state) into the presence of
HARP and ATP. More specifically, we follow the measurement mode used in the previous section (see Fig. 7.12), but add one additional step. After having demonstrated that the ssDNA is well coated by RPA in step 3, after relaxing the tension to zero, we move the construct into the presence of HARP and ATP and wait tens of seconds to minutes before separating the traps one last time (Step 4) – see figure 7.14. The trace corresponding to the final trap separation in step 4 (yellow – trace exhibiting the overstretching force plateau at ~60pN) overlaps with the trap separation trace shown from step 3 (red). This indicates that the ssDNA is likely still coated by RPA. However, as the DNA is stretched further, the force rises well above the unzipping plateau and even exhibits dsDNA overstretching, which is a consequence of the dsDNA arm. This experiment has proven to be difficult to perform and so, thus far only a few such measurements have been conducted. However, two of the three trials performed exhibited the behavior shown in figure 7.14.
Figure 7.14: Force vs trap displacement traces from a combined RPA & HARP experimental procedure. Steps 1-3 as well as the resulting data sets shown here are the same as in figure 7.12. After relaxing the DNA tension in step (3), the partially open unzipping construct, with RPA coated ssDNA, was moved into the presence of 10nM HARP and 1.5mM ATP. After waiting for tens of seconds to minutes, the traps were separated once again (step 4). Here we see the force rise above the unzipping plateau and even exhibit a dsDNA overstretch force response.

At this point it is worth noting that the HARP experiments discussed earlier in section 7.3.2 exhibited much less impeded unzipping and rezipping behavior in HARP concentrations tested below 80nM (e.g. 27nM and 10nM HARP). However, these results suggest that HARP, even at this low concentration, may have bound tightly to the fork to impede further unzipping. It also appears to be consistent with the findings that RPA promotes HARP binding to forked DNA\textsuperscript{21-24} since RPA and/or HARP at low concentrations are not expected to impede unzipping, especially not impeded unzipping that permits such high tensions as observed in these results.

These results do not show what would have been expected of a rezipping protein. Were HARP functioning here as it has been proposed to function, then the RPA coated
ssDNA strands should have been reannealed by HARP, causing RPA to dissociate. This would have resulted in a final stretching trace that overlaps with the initial stretching trace observed in step (1), followed by unzipping or a rise in force above the unzipping plateau due to HARP still being tightly bound to the fork. However, despite the lack of any observed rezipping, only a few trials were performed and thus many more trials as well as controls need to be performed before discussing whether HARP in fact reanneals RPA-coated complementary ssDNA.

7.4 Further Discussion

Using our newly created unzipping construct we obtained many results that shed light on the mechanisms and properties of HARP and RPA. Some of the observations are consistent with earlier findings, while others are new or unexpected. However, we would like to emphasize that all the HARP and RPA results presented here are preliminary and require additional data collection and controls before making assertions about the mechanistic features of these proteins. Nonetheless, these findings are still notable and important for guiding our ongoing and future HARP and RPA investigations.

Since the function of RPA is reasonably well understood, our basic characterizations of RPA in the tweezers has served as a tool for understanding various observations made while only HARP was exposed to forked DNA. Being that RPA is capable of unzipping DNA and in fact did so in our measurements, we were able to ascertain, in conjunction with our observation that HARP binds to forked DNA, that HARP is not a helicase. Both of these observations are consistent with previous findings\textsuperscript{3-8}. Further supporting this are our observations that HARP impedes unzipping, which also independently corroborates previous findings\textsuperscript{21-24}. Now, in characterizing
RPA, we deduced from the RPA-fork construct relaxation experiments that the observed drop in force below the unzipping plateau indicated that the strand were not reannealing; the strands were not reannealing because they were blocked by RPA (since it is known that RPA binds to ssDNA only). Therefore, when we observed a similar drop in force while relaxing forked DNA that had been incubated in HARP, we were able to deduce that either the ssDNA strands or the fork was occluded by HARP. And since our earlier findings suggested it may bind to forked DNA, this finding provided additional support that HARP binds to forked DNA rather than ssDNA or dsDNA, which is a finding that was previously reported by Yusufzai and Kadonaga.

As for the slow force rise observed after relaxing the tension in a HARP-forked DNA complex, while we cannot say for sure at this point the reason for this behavior, we favor the interpretation that HARP forcibly rezips the DNA and translocates with the fork in an ATP driven manner. The fact that this slow force rise behaves differently in ATP vs UTP, where the ATP situation typically results in the force at least returning to the force plateau (~15pN), supports the possibility of some kind of ATP driven reannealing while also disfavoring the possibility that HARP is condensing the DNA in this particular circumstance (see earlier discussion). Then, the few observations of a rise in force above in the unzipping plateau (when a construct is in the presence of HARP and ATP) provide some evidence that HARP may be capable of generating force. We reiterate, however, that more trials and controls need to be collected to better determine the reason for this slow force change behavior. In addition, we must also keep in mind the few tests for forcible rezipping when using both HARP and RPA. Here we observed strong binding of HARP to the DNA fork with RPA coated ssDNA but no evidence of rezipping. Finally,
while these results provide evidence that HARP is promoted to bind to forked DNA where the ssDNA is coated with RPA, they also suggest that HARP may not require RPA to perform its functions. This has also been suggested by a collection of findings from previous studies. In particular, previous in vitro studies have demonstrated that HARP can bind forked DNA in the absence of RPA and that HARP with a disrupted RPA binding site still performs annealing helicase activity. Furthermore, results from damaged DNA in vivo studies have suggested that RPA recruits HARP to bind to regions of ssDNA that form during DNA damage and repair.

To continue this work, considerable effort has been made in building a new dual-trap optical tweezers system with significantly improved resolution and reduced drift (see chapter 4). Not only will this system be useful for eliminating the possibility of drift from time dependent measurements in which drift might be a concern, but it should also help us more accurately determine velocities and/or forces that HARP and/or RPA might generate. Besides using this new system to perform additional trials and controls of all experiments discussed in this chapter, it will be used for other future investigations of HARP and RPA, some of which include: Identifying the energy barrier for HARP binding by performing the impeded unzipping experiments at different stretching rates; continuing investigations of the slowed rezipping behavior exhibited by HARP and its dependence on ATP, UTP, and [HARP]; quantifying the effect of RPA on limiting rezipping by varying [RPA] and RPA incubation time as well as by studying the kinetics of RPA unbinding by washing it away; quantifying RPA induced DNA unzipping by varying [RPA], applied force, and salt concentrations; and exploring conditions that might permit RPA to wedge into dsDNA. Lastly, should we conclude in our future
investigations that HARP translocates or passively rezips DNA, then a fixed force ("force clamp") measurement mode can be used to evaluate the rate at which HARP moves along the DNA.

7.5 Conclusion

In this chapter we have presented a novel, simple, and nearly generalizable method for creating long and high concentrated unzipping constructs for use in single-molecule experiments. Using this method we have prepared unzipping constructs for investigating properties and mechanisms of HARP and RPA in dual-trap optical tweezers. In our preliminary measurements we have made observations that support previously reported character of HARP and RPA. Specifically, we have obtained evidence that HARP only binds to forked DNA, does not exhibit helicase activity, and that it might have ATP-driven forcible DNA rezipping activity. In addition, we have obtained results indicating that RPA only binds to ssDNA, binds ssDNA rapidly, is capable of unzipping DNA in the appropriate conditions, prevents DNA rezipping, and that RPA might recruit HARP to bind to forked DNA. However, as a result of the capabilities of our assay, we have obtained results indicating that HARP may (1) also impede and slow rezipping, where UTP much more frequently disrupts this processive slow reannealing than does ATP, and (2) be capable of resisting high forces. Overall, the results of these experiments demonstrate that this unzipping, dual-trap tweezers assay is capable of extracting a wealth of information about the properties and mechanisms of HARP, RPA, and potentially many other DNA binding proteins. In fact, some of the experimental methods used in this study can potentially be refined to develop standard,
general protocols for identifying particular characteristics or functions of DNA binding proteins.

7.6 Acknowledgements

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7.7 Materials and Methods

7.7.1 Unzipping Construct

Four segments of DNA were PCR amplified from lambda DNA with lengths: 10kb, 10.7kb, 12.8kb, and 17kb. Each of these used the forward primers: 5´-CTGATGAGTTCTCGTGCCTGACAACTGCGTAATC-3´; 5´-CATCATCATGCAGAACATGCGTGACGAAGAGCTG-3´; 5´-GCGACAATCAACAGAGGAGGAGAAGAGTGACAGC-3´; and 5´-GCGAGTATCCGTACCATTCAACTGGCACGAAGA-3´ respectively while they all used the same digoxigenin labeled reverse primer: Dig-5´-ATACGCTGTATTCCAGCAACACCGTCAGGAACACG-3´. The resulting samples were PCR purified using the QIAquick PCR Purification Kit (Qiagen) and their concentrations were quantified. 1μg of each of these were then digested by Nt.BbvCI (NEB Inc.) for about one hour at 37 °C. Subsequently, the nicking sites are biotinylated by combining digested DNA with Klenow (exo-) and biotin-dCTPs and dTTPs such that the final concentrations are ~17ng/μl, ~80units/μl, ~0.01mM, and ~0.07mM respectively.
This is then incubated for 15 minutes at room temperature. The Mg\textsuperscript{2+} in the reaction is then quenched by added EDTA. The final unzipping DNA constructs are stored at 4 °C.

### 7.7.2 Buffer and Microsphere Preparation

We refer to our buffer solution as the “annealing helicase” buffer (AH buffer), which is based on the buffer conditions used in [8]. 1xAH contains 10mM Tris-HCl pH 7.9, 20mM KCl, 2mM MgCl\textsubscript{2}, 0.5mM DTT, 0.1mM EDTA, 1.5mM ATP. HARP or RPA experiments were performed in solutions where the protein was suspended in a 1xAH buffer. HARP concentrations tested were: 10, 27, 80, 150, 300 nM HARP. Only a concentration of 3nM RPA was tested in this study. Anti-digoxigenin beads were prepared by first washing 100μl of 0.5% (w/v) 2μm diameter Protein G coated polystyrene microspheres (Spherotech Inc.) with 50μl 1xPBS buffer. We then added 2ul of 200μg/ml of anti-DIG and incubated for ~20-30 minutes. This is then washed twice more in 50ul 1xAH buffer and lastly resuspended in 20μl of 1xAH. Similarly, 20μl of 0.5 (w/v) 2μm diameter streptavidin beads were washed 2x in 20μl 1xAH and resuspended in 20μl 1xAH with 0.3μl of 100mg/ml BSA added. The DNA beads were prepared by combining 2.5μl of the washed anti-DIG beads and 5μl 1xAH buffer with enough unzipping construct such that the final concentration of the unzipping construct is ~100pM. This entire volume was then incubated for ~20-30 minutes prior to diluting in 500μl of 1xAH, which was then injected into the experimental flow cell chamber. ~3-5ul of the washed streptavidin beads were also diluted in 500μl of 1xAH prior to injecting into the experimental chamber.
7.7.3 HARP and RPA Preparation

All experiments performed here use human HARP and RPA. These proteins are prepared according to the procedures used in [8].

7.7.4 Optical Tweezers Instrument, Data Collection, and Data Processing

All experiments in this chapter were performed using a dual optical trapping system as previously described in [25]. Typically DNA coated beads and streptavidin beads were injected from a top and bottom channel of our experimental flow cell chamber (see chapter 1) into the central channel where the experiments were conducted. In cases where RPA or HARP were studied alone, the central channel contained the protein. Otherwise, the case where both proteins were used in series, we filled the main channel with RPA while injecting HARP into the main channel from the top channel. Measurements were performed by first capturing a DNA coated anti-digoxigenin microsphere in the system’s stationary trap and a streptavidin microsphere in the system’s movable trap. The movable trap was then moved toward and away from the stationary trap, causing the beads to move toward one another, bump into another, and then move apart (“fishing”). Fishing was continued until a single unzipping construct is tethered between the two beads. The microspheres were then manipulated according to the measurement modes described in the result/discussion sections above. While performing these manipulations, the PSD (position sensing detector) signal and the acousto-optic deflector (AOD) input frequency are recorded throughout the duration of the trial to track the force on the unzipping construct and the position of the traps respectively. The PSD and AOD recorded voltages and frequencies were converted to piconewton forces and
nanometer displacements using the calibrations parameters acquired using the method described in [26].

7.8 References for Chapter 7


Chapter 8

Appendix

8.1 Section 1: Overview and Design, Requirements, Rational for Chosen Equipment and Optics, and Construction Notes

8.1.1 Overview and Design

This document describes in detail our recent effort to build an affordable dual-trap optical tweezers setup with greater resolution than previously obtained in our lab\(^1\). The Bustamante lab previously built a dual-trap optical tweezers system with subnanometer resolution, albeit at a hefty price\(^2-7\). We designed a system that employed many of their more affordable suggestions and that would satisfy the needs of our planned experiments with Lambda, T4, and phi29 phages as well as other biological systems/molecules. The following describes as many of the details as we could recount in building this particular system. It includes our detailed design, chosen instrumentation and components, detailed description of how the system was built, and the rational for all just said. As I had never built an optical system before and since no optical tweezers building guides existed for the completely inexperienced, I wrote this article not only to document our system for future reference but also as an aid for the inexperienced. At the least we hope that this write-up will provide a better starting point for future students in our lab to learn and build upon should greater improvements be desired.

8.1.1.1 Basic Design

In a nutshell the system consists of a 1064nm continuous wave laser whose beam is first passed through an opto-isolator and then through a two lens telescope for beam expansion. The expanded beam is split into two orthogonally polarized beams. One beam is reflected off a stationary mirror while the other is reflected off a piezo-electric controlled movable mirror. After reflection, the two beams are “recombined” and sent through a second two lens telescope for expansion again. Subsequently the beams pass through a microscope objective, which focuses the beams, thus creating two optical traps within a specimen chamber (flow cell) that directly follows this objective. The outgoing light passes through another objective, consequently recollimating the beams. The beams are split once again where the non-movable (or stationary) beam passes through a lens that images the deflections of any particle trapped in the stationary trap onto a position sensing detector (PSD). In the case of a dual detection system, after the final beam splitting, the other beam (movable beam) is similarly passed through an imaging lens and onto a second PSD. See figure 8.6 for a simple cartoon image of this layout. Another version of this layout containing all the important numbers and distances can be found in figure 8.7. Note that the system figures 8.6, 8.7, 8.11-8.48 are not to scale.
8.1.1.2 Basic Experiment

A typical experiment is performed by injecting into the flow cell polystyrene microspheres that are coated with the biological molecules to be studied. Using the video feed of the imaged flow cell, the operator maneuvers the flow cell to capture the beads in the traps. Using a program that controls the position of the movable mirror, the operator moves the bead in the movable trap toward and away from the bead in the stationary trap so the biological interaction of interest can occur. Deflections of the bead in the stationary trap are detected and measured by the downstream PSD. And in the case of a dual detection system, deflections of the bead in the movable trap are measured by the second PSD.

8.1.2 Important Requirements

8.1.2.1 Beam Transmission Criteria

The laser beam profile is Gaussian shaped. In order to transmit >99% of the incoming laser light, all lenses and mirrors need to have an aperture diameter at least 1.5x the beam waist.

8.1.2.2 Lens Orientation Criteria

According to the optical guide provided by CVI Melles Griot, a lens will exhibit much less spherical aberration if it is oriented such that parallel rays are in contact with the side that has the smallest radius of curvature (i.e. the most curved). So, for a plano-convex lens, it is best for the parallel rays to enter or exit from the curved surface.

8.1.2.3 Beamsplitter Criteria

For a beamsplitter to work as designed the beam must enter the appropriate face. The face which the beam must enter is typically designed by most manufacturers with a pencil dot on one of the top corners of the cube. Two surfaces should correspond to the side in which this dot is located, thus the beam can enter either surface.

8.1.3 Optical Height

Newport’s 562 XYZ stages have an optical height range from 3.375 inches to 3.875 inches. We chose the optical height to be 3.625 inches, which is half way between these two extremes, to ensure there would be plenty of distance to adjust the objective vertically in the case that we made a small mistake elsewhere in our design or in the case that the beam height changed slightly as a consequence of the upstream optics.
8.1.4  Mounting Pedestals

To minimize the measured noise, we decided to use (whenever possible) stainless steel pedestals for all optical components that can potentially affect the amount of noise in the beam.

8.1.4.1 Custom Pedestals

I designed pedestals to be made out of stainless steel for mounting the laser head, opto-isolator, and the Mad City Labs stage. The laser mounting pedestal was designed such that after the head was clamped into the wedge cut, the beam height would be 3.625 inches as mentioned above. The other mounts were also designed so that the beam would pass directly through the center of the opto-isolator and hit the center of the mirror on the Mad City Labs stage. We had the campus research machine shop manufacturer these mounts for us. Note that two sides on each mount are unfinished because they didn’t need to be and it made them cheaper to make. The sketches I submitted to the machine shop are provided at the end of this document (Figures 8.8-8.11).

I also designed and machined myself an ~1” thick aluminum baseplate to raise the flow cell stage such that the center of the flow cell was at the appropriate working height. This baseplate was also designed so that the flow cell stage could be mounted directly to the table rather than being clamped down with the standard Thorlabs CL5 clamps that we use elsewhere in the system.

8.1.4.2 Other Pedestals

All the lenses, the first polarizing beam splitters, and the first two mirrors that reflect or pass the laser light as well as the On-Trak PSD have been mounted on new 1” diameter, 2” tall Newport stainless steel circular pedestals (with a foot). To position each optical element such that the beam passed/hit as closely as possible to the center, 1” diameter Newport stainless steel circular spacers were used. Note that the mounting threads on the PSD are ¼-20 while the threading on the stainless steel pedestals is 8-32. Because of this I had to rethread the pedestal to have ¼-20 threading and the spacers to have holes slightly bigger than the ¼-20 thread diameter.

Newport stainless steel pedestal model # PS-2
http://search.newport.com/?q=*&x2=sku&q2=PS-2

Clamping forks used:  Clamping Fork, Captive 1/4-20 Screw, Standard Slot, For 1.0-in. Pedestals, Model# 9916
http://search.newport.com/?x2=sku&q2=9916

All other optical elements that shouldn’t affect the level of noise in the laser beam (the black anodized tubing, imaging lenses, imaging mirrors, imaging LED, Camera, imaging IR filter, the attenuator and PBSC (polarizing beam splitting cube) that just
precede the PSD) were mounted upon the standard Thorlabs 0.5” aluminum posts and post holders that we have in the lab.

8.1.4.3 Mount Spacers

As will be discussed later, the PBSCs deflect the beam upward, thus leading to a slight increase in the beam height. Because of this we’ve had to use spacers for all optics mounted on the circular 1” diameter, 2” tall pedestals downstream of the second PBSC so the beam would pass or hit as closely as possible to the center of the optical component. Specifically, in addition to these pedestals, the 1” diameter lenses required the use of a ½” & ¼” spacer, mirrors required the use of a ½” & 1/8” spacer, and the PSD required the use of a ¼”, a 1/16”, and two 1/32” spacers (note: The small differences in the thickness of the 1/16” and the two 1/32” spacers positioned the center of the PSD closer to the beam height than 1/8” spacer did.).

Newport 1” diameter stainless steel spacer model numbers: ½” – PS-0.5E; 1/4” – PS-0.25; 1/8” – PS-0.125; 1/16” – PS-0.063; 1/32” – PS-0.031.

https://www.newport.com/1.0-in.-Pedestal-Spacers-and-Extensions/856261/1033/info.aspx#tab_orderinfo

8.1.5 Optical Mounts/Stages

8.1.5.1 Lens Mounts

We used the standard 1” diameter Thorlabs lenses mounts for all but one lens, model # LMR1. We used one large 2” diameter lens for the imaging, which was mounted using a Thorlabs 2” diameter lens mount with model # LMR2


8.1.5.2 Mirror Mounts

We used two new Suprema Clear Edge Mirror Mounts (stainless steel), each with 2 Locking Actuators, 8-32 (M4). One was 1” diameter for the stationary beam after the first polarizing beam splitter and one was 2” diameter for the first dichroic mirror. Note: Originally we purchased the mirror mounts with 2 non-hex key locking actuators and later discovered it would be useful to have actuators with hex key holes. So, for the 1” mount, we replaced the pivot ball with a Newport hex key actuator (model # AJS100-0.5) we purchased for the objective stages. This allowed us to translate the mirror by small amounts. Then, for the 2” mount, we replaced both actuators and the pivot ball with hex key actuators. We then used these two non-hex key actuators for adjusting the vertical position of the objective stages.

http://search.newport.com/?q=*&x2=sku&q2=SN200-F3K
http://search.newport.com/?q=*&x2=sku&q2=AJS100-0.5
8.1.5.3 Polarizing Beam Splitting Cube Mounts

For the first 2 beam splitters in the system we used new Newport Pint-Sized Prism Mounts, 0.25 to 1.00 in., ±3.5 °, 8-32, model# 9481. These are designed to be mounted on the 1” diameter stainless steel pedestals and supposedly are more stable than the standard Newport prism mounts. For the last beam splitter (just before the PSD) we used an old Thorlabs prism mount we had in the lab. We assumed that the light passing through the beam splitter is not affected by any noise resulting from the 0.5” aluminum post and Thorlabs prism mount used. However, we plan to swap this out with the Newport stainless steel pedestal and prism mount if we put in another PSD because the reflected light should be affected by noise resulting from the PBSC mounting.

http://search.newport.com/?q=*&x2=sku&q2=9481

8.1.5.4 PSD Mounts

As mentioned above, we used new 1” diameter, 2” tall Newport stainless steel circular pedestals and spacers to mount the PSD where these were redrilled and rethreaded to accommodate the ¼-20 threading on the PSD.

8.1.5.5 Objective Lens Stages

We used new stainless steel Newport stages for this system. Specifically one left handed and one right handed 562 ULTRAAlign™ Precision Linear Stages, 13mm Travel. (model # 562-XYZ and 562-XYZ-LH)

http://search.newport.com/?q=*&x2=sku&q2=562-XYZ

As mentioned earlier, for each of these we used one of the non-hex key actuators from the Suprema mirror mounts for controlling the height of the stage while the x and y positions are controlled by hex key actuators (model # AJS100-0.5).

http://search.newport.com/?q=*&x2=sku&q2=AJS100-0.5

A Newport dovetail rail (model # 562-RAIL-3.7) was then screwed to the top of these stages. And on either end of the dovetail rail, an objective lens mount (model # 561-OBJ) was clamped. Note that the objective lens mounts used here were previously used in other tweezer systems in the past. We cleaned, lightly sanded, and oiled them before use. Only one is used for an objective whereas both are used in the alignment of the stage, which will be described later. Also note that the edges of the stage surface are beveled and so the objective lens mounts were clamped to the beginning of the bevel (i.e. the mounts do not hang over the bevel. The entire bottom surface of the mount is in contact with surface area of the stage.)

http://search.newport.com/?q=562-RAIL-3.7
http://search.newport.com/?q=*&x2=sku&q2=561-OBJ
8.1.6 Magnification, Objectives, & Operating Range

8.1.6.1 Magnification

As will be discussed in the section on objectives, the diameter of the back aperture of our Olympus objectives is 7.2mm. We decided we wanted to fill the back aperture exactly. It is debated among various optical tweezers labs whether over filling, under filling, or exact filling of the back aperture will produce the best trap. I believe the Bustamante lab slightly over fills\(^4\) whereas Mohammed Mahamdeh showed that slight under-filling is best, primarily for small beads (i.e. 1um or less in diameter)\(^9\). Because it isn’t completely clear which option is best, we chose the intermediate option of exact filling the back aperture. We also chose this option because it made it a bit easier to select lenses for the magnification required to just fill the back aperture.

The initial beam diameter of the IPG (additional details listed later) laser is 1.6mm (where the Gaussian profile falls off by e\(^{-2}\)). In order to completely fill the back aperture of the objective, the beam must be magnified by 4.5x (i.e. \(\frac{7.2\text{mm}}{1.6\text{mm}} = 4.5\)). Our system has been designed to have a beam expander before and after the Mad City Labs stage mirror. So the net magnification must satisfy \(M_{\text{tot}} = M_1 \times M_2\), where \(M_1\) is the magnification of the first telescope and \(M_2\) is the magnification of the second telescope. We chose \(M_2\) to be 1.5, and so, in order to satisfy this equation, \(M_1\) must be 3. Since the Mad City Labs stage only has a 0.5 inch diameter mirror, it is important to verify that beam diameter just after the first beam expander still satisfies the beam transmission criteria stated above. However, since the mirror on the Mad City Labs stage is at a 45° angle to the incident laser beam, we must determine the effective incident diameter of the mirror, which we call \(D_{\text{mirror}}\), while we call the diameter of the of the laser beam after being magnified \(M_1\) times by the first beam expander, \(D_{\text{laser}}\).

![Figure 8.1: Laser beam incident on an angled mirror.](image)

\[
D_{\text{mirror}} = \frac{0.5''}{\sqrt{2}} = 0.35'' = 8.98 \text{ mm}
\]

\[
D_{\text{laser}}^{after M_1} = 5\text{ mm}
\]
Check that we have satisfied the beam transmission criteria:

\[ D_{laser}^{afterM_1} \times 1.5 = 7.5 mm < D_{mirror}^{eff} \]

Thus, since the criteria is satisfied, the mirror will reflect >99\% of the incoming laser light.

8.1.6.2 Objectives

We use two 60x water immersion infinity corrected Olympus microscope objectives. The specifics of the objectives used are:

<table>
<thead>
<tr>
<th>OLYMPUS</th>
<th>OLYMPUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>UP PlanApo/IR</td>
<td>UP PlanApo</td>
</tr>
<tr>
<td>60x/1.20 W</td>
<td>60x/1.20 W</td>
</tr>
<tr>
<td>∞/0.13-0.21</td>
<td>∞/0.13-0.21</td>
</tr>
</tbody>
</table>

A couple notes about these specifications. 1.20 W means the objective is a water immersion lens with a numerical aperture of 1.2 while the 0.13-0.21 refers to the range of coverslip thicknesses in millimeters that can be placed between the front lens of the objective and the plane of the focal point.

These objectives have an adjustable knurled collar (called a correction collar) that allows the user to correct for the distorted focused light that results from placing a glass coverslip between the front lens of the objective and the plane of the focal point. Our flow cells are made using Fisherbrand microscope cover glass slides (1oz. No. 1 slides, product# 12-545-M). Fisher advertises these slides to have thicknesses of 0.13 to 0.17mm. Fortunately this is within the range of values for which these objectives are capable of being adjusted.

I called Olympus some time during 2012 to find out the diameter of the back aperture in the objectives. I spoke with Olympus technician Ernie Marquez who sent me the information provided below that they use in determining the diameter of the back aperture in their objectives. Contact info for the technician I spoke to:

Ernie Marquez
Ernie.Marquez@Olympus.com
Olympus SEG Technical Support
Phone: 800-446-5967 press 3
(Tuesday thru Friday 9am-4:30pm PSD)
**Table 8.2:** How to calculate the diameter of the back aperture of an Olympus objective.

<table>
<thead>
<tr>
<th>Calculating the Back Aperture Diameter of any Olympus UIS objective</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D</strong> = 2 x N.A. x focal length divided by magnification</td>
</tr>
<tr>
<td>Where</td>
</tr>
<tr>
<td><strong>D</strong> = diameter of back aperture</td>
</tr>
<tr>
<td>N.A. = obj. numerical aperture</td>
</tr>
<tr>
<td><strong>Focal Length</strong> = focal length of tube lens divided by obj. magnification</td>
</tr>
</tbody>
</table>

In the case that Olympus UIS tube lens = 180mm focal length (Olympus UIS infinity corrected obj’s)

Then **D** = 2xN.Ax180/mag

Some important guides for the objectives:


I later called an Olympus technician (Ernie again) on 2/19/2013 to find out the location of the back focal plane for these objectives. I was told that back focal plane position is unique to each objective and one must contact them to find out the position. I was informed that the **back focal plane** for both of these objectives is **19mm from the object end (i.e. the end of the objective threads) toward the specimen end.**

I called Olympus a second time on 06/25/2013 to verify the location of the back focal plane. The technical support member with whom I spoke sent me a diagram. Below (Fig. 8.2) is a simple diagram I created that emphasizes the important features of that diagram. I was told that for about 90% of their objectives (UIS and UIS2) the back focal plane is 19mm from the shoulder of the objective (where the threads terminate) toward the specimen (as seen in the diagram below). The diagram they provided indicates the position of another back focal plane that is 6 additional millimeters toward the specimen, but this corresponds to objectives with a DIC prism slider, which we do not have. What matters is that **the back focal plane for all our objectives is 19mm from the shoulder toward the specimen.**
Infinity corrected microscope objectives are objectives that focus the emerging light at infinity. In order to observe the image with our own eyes, the emerging collimated light must be focused using a lens that is called the tube lens. The magnification of a particular infinity corrected objective is calculated by the following equation:

\[ M = \frac{f_{\text{tube lens}}}{f_{\text{objective}}} \]

Where \( f_{\text{tube lens}} \) is the focal length of the tube lens and \( f_{\text{objective}} \) is the focal length of the objective. \( f_{\text{tube lens}} \) varies from one objective manufacturer to another, but all infinity corrected objectives from a particular manufacturer are designed to have the same \( f_{\text{tube lens}} \) as specified by this manufacturer (though occasionally a manufacturer designs specialty objectives with a different value of \( f_{\text{tube lens}} \)). For Olympus, all of their UIS and UIS2 series objectives are designed such that \( f_{\text{tube lens}} = 180 \text{mm} \). I believe all the objectives in our lab are of the UIS Olympus series. Some of the newer ones may be of the UIS2 Olympus series. Also, we use M=60x objectives in our lab, which, based on the fixed \( f_{\text{tube lens}} \) and the equation above, implies that the objective focal length for all these objectives is the same:

\[ f_{\text{objective}} = \frac{f_{\text{tube lens}}}{M} = \frac{180 \text{ mm}}{60} = 3 \text{ mm} \]
Note however that the objective focal length is measured relative to the position of some unknown plane within the objective casing. And so, objective manufacturers have defined a distance from the objective front lens surface to the plane of the focal point, which they call working distance (WD). For many (if not all) of our Olympus objectives, the working distance is 0.28 mm.

In addition, as given in the table above, the back aperture diameter of an Olympus UIS objective is determined by the following equation:

\[ D = \frac{2 \times NA \times f_{\text{tube lens}}}{M} \]

Where NA is the numerical aperture, M is the objective magnification, and \( f_{\text{tube lens}} \) is the tube lens focal length. See the letter on this equation obtained from the Olympus technician Ernie Marquez at the end of this document. For our Olympus objectives NA=1.2, M= 60, and \( f_{\text{tube lens}} = 180 \) mm, which gives a back aperture diameter of \( D = 7.2 \) mm.

8.1.6.3 Back Focal Plane

Microscope objectives were originally designed to have the light of an illuminated specimen enter the objective at the small end and emerge from the “back” end, which is threaded. All parallel rays coming from a specimen that enter such a compound lens objective will cross at a plane within the objective known as the Back Focal Plane (BFP). Though in the context of laser tweezers, while we use the objectives in this same manner when we image the flow cell, we also use these objectives in the opposite way where we send a collimated beam of IR laser light into the back end. In this case, rotations of a collimated beam at this plane lead to translations of the focused light in the specimen plane, which, for us, is in the flow cell. This is useful for the purposes of moving one of our traps laterally and only laterally in the trapping (specimen) plane. This can be seen in the figure 8.3 below.
Figure 8.3: Diagram showing that rotations of a beam at the back focal plane result in linear translations of the focal point of the objective focused beam.
8.1.6.4 Approximate Lateral Working Range within Flow Cell

Recall that if we rotate the mirror by $\Delta \theta_m$, the beam rotates by an angle $\Delta \theta_{\text{beam}}$ from the first reflection to the second reflection, which is $2\Delta \theta_m$, as derived below.

![Diagram showing mirror rotations and reflected light rotation](image)

**Figure 8.4:** Diagram showing that mirror rotations cause reflected light to rotate by twice that of the mirror rotation angle.

Note from the diagram that:

$$\theta_{r_2} = \theta_i + \Delta \theta_m$$

We can derive $\Delta \theta_{\text{beam}}$ as follows:

$$\theta_{r_1} = \theta_i$$

$$\Delta \theta_{\text{beam}} = \theta_{r_2} + \Delta \theta_m - \theta_{r_1}$$

$$\Delta \theta_{\text{beam}} = (\theta_i + \Delta \theta_m) + \Delta \theta_m - \theta_i$$

$$\Delta \theta_{\text{beam}} = 2\Delta \theta_m$$

If a beam expander with magnification $M_2$ is placed after the beam steering device (AOD or tip-tilt piezo-electric stage), then the range between the traps within the flow cell can be approximated by:

$$\Delta x = \frac{\Delta \theta_{\text{beam}} * f_{\text{obj}}}{M_2}$$
Whereas, if NO beam expander is placed after the beam steering device, then the range can simply be approximated by:

$$\Delta x = \Delta \theta_{beam} \times f_{obj}$$

In our case, we have a beam expander with a magnification of $M_2 = 1.5$ after our Mad City Labs tip-tilt piezo-electric stage, which can rotate by $\Delta \theta_m = 5.185 \text{mrad}$. Thus, by the boxed equations above, we determine that we can rotate the beam by $\Delta \theta_{beam} = 10.37 \text{mrad}$ and subsequently determine that the range between the beads would be approximately:

$$\Delta x = \frac{10.37 \text{mrad} \times 3 \text{mm}}{1.5} = 20.74 \text{um}$$

However, the beads we use have ~2.2um diameters, which means we must subtract 2.2um from this estimated range to give us an effective range of:

$$\Delta x_{eff} = 20.74 \text{um} - 2.2 \text{um} = 18.54 \text{um}$$

Then using the dsDNA conversion of $0.34 \text{nm/base}$, which is true at $F \approx 33 \text{pN}$ (see details of my new calibration method\textsuperscript{10}), we can estimate the longest piece of DNA we can stretch between the beads. Note that at $F= 5 \text{pN}$ dsDNA length/base conversion is $0.318 \text{nm/base}$.

$$L_{Max}^{Stretching \ DNA} = \frac{\Delta x_{eff}}{0.34 \text{nm/base}} = \frac{18.54 \text{um}}{0.34 \text{nm/base}} \approx 54,529 \text{ bases}$$

Or, if we approximate a phage capsid to have a diameter of 100nm (the diameter of Lambda phage procapsid is actually ~60nm), then the longest piece of dsDNA we can package would be

$$L_{Max}^{packaging \ DNA} = \frac{18.44 \text{um}}{0.34 \text{nm/base}} \approx 54,235 \text{ bases}$$

\subsection*{8.1.7 Lenses}

Because we currently have many 1 inch diameter lenses mounts, we decided to use 1 inch (25.4mm) diameter lenses for all lenses the laser passes through. Furthermore, 1 inch diameter lenses still allow us to satisfy the transmission criteria previously described since the beam waist is never greater than the back aperture of the objectives (7.2mm). Also, we attempted to use achromatic lenses wherever possible since they presumably have less spherical aberration than regular plano-convex lenses.
The first imaging lens was chosen to be larger than 1 inch so as to capture as much of the imaging light as possible so that we can ensure good contrast on the TV and that the size of the imaging light as it enters the objective is sufficiently large to completely illuminate the entire field of interest within the flow cell.

8.1.7.1 Telescope Lenses

The first telescope downstream from the laser consists of a 1” diameter Thorlabs f=5cm achromatic 1050-1620nm doublet lens (model # AC254-050-C) followed by a 1” diameter Thorlabs f=15cm achromatic 1050-1620nm doublet lens (model # AC254-150-C), which, when spaced apart appropriately, yield an image magnification of \( M_1 = 3 \).

http://thorlabs.us/thorproduct.cfm?partnumber=AC254-050-C
http://thorlabs.us/thorproduct.cfm?partnumber=AC254-150-C

The second telescope downstream from the laser consists of a 1” diameter Thorlabs f=15cm achromatic 1050-1620nm doublet lens (model # AC254-150-C) followed by a 1” diameter Newport f=22.5cm, N-BK7, 1064nm anti-reflection coated, plano-convex lens (model # KPX108AR.33). When these lenses are spaced apart appropriately, they yield an image magnification of \( M_2 = 1.5 \).

http://thorlabs.us/thorproduct.cfm?partnumber=AC254-150-C
http://search.newport.com/?q=*&x2=sku&q2=KPX108AR.33

As recommended by various optics guidelines, the lenses in these telescopes were oriented such that the flat sides face each other and the curved sides face outward. See the tweezers diagram for clarity (Figure 8.7).

8.1.7.2 Specimen Imaging Lenses

The first lens downstream from the LED is a 2” diameter Thorlabs f=12.5cm, N-BK7, plano-convex lens (model # LA1384). The second lens is a 1” diameter f=12.5cm plano-convex lens (this lens is an old lens salvaged from a previous tweezers system and thus the remaining manufacturer details are unknown).

http://thorlabs.us/thorproduct.cfm?partnumber=LA1384

Since these lenses were simply used for the imaging, their quality was not critical. All that was necessary was that there were no significant smudges or scratches on the lenses. Also, the first lens was chosen, as mentioned previously, to have a 2” diameter so as to capture as much of the light from the LED as possible so as to produce bright imaging with high contrast. This was also done in effort to eliminate potential clipping of the image on the camera (i.e. if the image that illuminates the beads in the flow cell is not sufficiently large.).
8.1.7.3 Bead Deflection Interference Pattern Imaging Lens

When a bead in a trap is deflected, the outgoing refracted laser light produces a characteristic interference pattern at the back focal plane of the downstream objective. To image this interference pattern onto the downstream PSD, we used 1” diameter Thorlabs f=15cm achromatic 1050-1620nm doublet lens (model # AC254-150-C).

http://thorlabs.us/thorproduct.cfm?partnumbe

In effect, what we measure on the PSD are the angular deflections of the light due to a displaced bead. With calibration, we can then convert these measured deflections to linear translations of the bead.

8.1.8 Mirrors

8.1.8.1 Movable Mirror

It was our desire to choose the lightest mirror possible with the best reflectivity at an angle of incidence of ~45° because the weight of the mirror will dictate the maximum rate at which the stage would ultimately be able to move. We chose to use a gold mirror because of the available metallic plated mirrors, the gold plated mirrors were available in the smallest sized mirrors (0.5” diameter and ~3mm thick) while still maintaining very high reflectivity for both S&P-polarizations of 1064nm light while only minorly being affected by the angle of incidence. Also, even though dielectric mirrors tend to have higher reflectivity than metallic mirrors, the 0.5” diameter mirrors tend to be sold in thicknesses of 6mm (twice as heavy as the option found with gold plating). Note: Looking back at the specifications of available mirror options, it looks like a silver plated mirror, which I can find in 0.5” diameter and 3mm thick, could have been just as good if not a little better than the gold mirror option we chose with regards to reflectivity. Nonetheless, gold is still much better than aluminum and the amount of reflectivity is probably good enough. If a dielectric mirror was available in a thinner dimension, it would probably have been the best choice.

http://search.newport.com/?q=05D20ER.4

8.1.8.2 Stationary Mirror Opposing Movable Mirror

Here we used one of the 1” diameter dielectric mirrors from the previous T1 setup. The manufacturer of this mirror is unknown, but it is likely from either Newport, CVI, or Thorlabs. Nonetheless, as mentioned above, dielectric mirrors have more reflectivity (>99%) than metallic mirrors for 1064nm light, even at 45° angle of incidence. So, if anything, the slightly greater reflectivity of this mirror over the gold plated mirror used on the piezo-electric stage could be a contributing factor to slightly stronger trapping power in the stationary trap.
8.1.8.3 Dichroic Mirrors

Before and after the objective lenses we used a dichroic mirror for imaging purposes. For the mirror before the objective we used a fairly new 2” diameter CVI Optics dichroic mirror that was previously used in the old T3 system. We chose this very large diameter mirror to ensure that we don’t clip the imaging light. At 45° angle of incidence this mirror reflects 1064nm light while allowing 532nm (green) light to pass (transmit) through. I believe the part number is: BSR-15-2025. It can be found here:

http://marketplace.idexop.com/store/IdexCustom/PartDetails?pvId=16763#

In contrast, the dichroic mirror that comes after the objective lenses passes the 1064nm laser light while reflecting the 532nm light. This particular mirror was newly purchased for this system. It is from CVI Optics with part number: BSR-51-2025 and can be found here:

http://marketplace.idexop.com/store/IdexCustom/PartDetails?pvId=16780#

A plot of % transmission vs wavelength of P and S polarized light that is incident to the mirror at 45° can be found on the manufacturer’s website. It shows that the 532nm wavelength light will be almost entirely transmitted (i.e. entirely reflected) while most (but not all) of the P and S 1064nm light will be transmitted.

8.1.8.4 Imaging Mirrors

The system contains two standard mirrors used for the imaging. We found these mirrors, which were clean and unused, in the lab and their source is unknown. They are fairly large, rectangular, and mounted on Thorlabs 1” diameter mirror mounts (with 2 adjustable knobs) via some simple homemade clamps. These mirror mounts were then attached to the standard Thorlabs aluminum 0.5” posts and baseplates.

8.1.9 Polarizing Beam Splitters

After the first beam expander the beam is split and then is recombined after one beam reflects off the gold mirror on the MCL stage and the other reflects off a stationary dielectric mirror. In both cases small 0.5” (smallest size available) polarizing beam splitter cubes (PBSC) were used. One was newly purchased from Thorlabs with product number: PBS123, which is good for the wavelength range of 900-1300nm and can be found here:


The other PBSC, which also works for our 1064nm light, was obtained by selecting from what we scavenged from the previous T1 and T3 tweezer setups. We inspected them under a microscope and selected the one that was cleanest and had the fewest scratches/defects on the surfaces that light reflected or passed. Note that we used small
PBSCs here with the thought that a smaller cube would be less subject to external forces and thus contribute less noise to the beam. Also, as mentioned earlier in the section on magnification, the use of 0.5” cubes are still sufficiently large to capture the entire beam after being expanded by the initial telescope while still satisfying the beam transmission criteria.

Near the end of the beam path, just after the second dichroic mirror, we placed a large 1” PBSC that is suitable for 1064nm light. This PBSC was also selected from the PBSCs that were scavenged from the old T1 and T3 tweezer setups. It was mounted on an old Thorlabs kinematic prism mount and this mounted is on a standard Thorlabs 0.5” aluminum post and base bracket. Though we reasoned that the use of smaller PBSCs might reduce the noise observed in our measurements, we reasoned that light transmitting though the PBSC might not be perturbed by the cube and thus not subject to external forces that the cube receives. Also, it was cheaper to use what we already have and easier to put in confinement tubing with this cube and mount.

Finally, as previously mentioned, in effort to reduce noise, we purchased and used the best PBSC mounts we could find, which were Newport Pint-Sized Prism Mounts.

8.1.10 Attenuator and Filters

Prior to hitting the PSD, an attenuator was put in the beam but at an angle (i.e. not perpendicular to the beam path) to deflect back reflections. For optimal performance, the On-Trak PSD must receive a particular amount of power from the laser when set at a gain of 1. This optimal power on the PSD corresponds to a $V_{sum}$ output of 5V, but it will still work for input powers that correspond to a $V_{sum}$ output of 1 to 6V (actual range is 0-6V). This is indicated by the “Hi” and “Lo” LED indicators on the amplifier. If the power is too high or too low for the set gain, then the LED will turn on.

We set the amplifier to a gain of 1 and chose an attenuator that reduced the power to a level that yielded and output $V_{sum}$ as close to 5V as possible. We chose a 1” diameter Thorlabs reflective ND filter with an optical density (OD) of 0.5; Model: ND05B. It can be found here:

http://www.thorlabs.com/thorproduct.cfm?partnumber=ND05B

The use of this attenuator led to $V_{sum}$ being just a bit greater than 5V. It was mounted in an old rectangular fixed position lens mount (manufacturer unknown), which was then mounted on a standard Thorlabs 0.5” aluminum post and base bracket.

To keep ambient light from affecting our measurements, we inserted a Thorlabs 1” diameter 1064nm band pass filter into the PSD mount. Note that a rubber ring was inserted into the PSD mount so that the filter would fit snugly without rattling after the outer threaded ring was tightened in place. Part #: FL1064-10.

http://www.thorlabs.us/newgrouppage9.cfm?objectid=1000&pn=FL1064-10#2901
For the imaging we placed a filter just before the CCD camera to block out IR light while passing the visible blue light (450-495 nm) from our LED. It is a 2”x2” filter from Rolyn Optics (now out of business). The details of this filter are unknown to me but I am guessing it is a cut-off filter, though it is possible that it is a bandpass filter or laser-line filter.

### 8.1.11 Optical Table

We used the same optical table that was used for the original T1 system, which is a 3’ x 5’ x 4.25” Newport table, Model: VH3660W-OPT; Manufactured Date: 08-07-2001. The table is suspended on air at ~ 37 psi while the table legs rest firmly on the floor by the adjustable feet (not by the wheels), which have been tightened in place by a nut on the feet. To verify that this was enough pressure to suspend the table, we pressed each corner of the table down and up to check that it was floating between the bottom (contact with the table legs) and top stops. If any one corner is in contact with one of these stops, then we adjusted the screw on a lever beneath the table near the corner that regulates how much air pressure is being applied to that particular corner until that corner is found to be floating between the two stops.

Lastly, before using the table, all signs of rust and corrosion were lightly sanded away. The entire table was then coated with a thin layer of oil to prevent corrosion.

### 8.1.12 Beam Confinement Tubing

We positioned 1.5” diameter black anodized aluminum tubing along the entire beam path when possible. The tubing was anodized black so as to absorb any stray laser light and/or prevent reflections of stray laser light. The tubes were cut to fit as closely as possible to all optical elements without touching. The tubes were threaded and mounted using the standard Thorlabs ½” aluminum posts and post holders. These tubes were put in place to reduce potential noise that can result from air currents that pass through the beam path. In effort to close off all gaps where the tubing meets an optical element we used black tape to cover the holes as much as possible without allowing the tubes to make contact with any optical elements. The only place where tubing was not used is the square of optical elements where the beam is split and the two polarized beams either reflect off a stationary mirror or the mirror attached to the Mad City Labs movable stage. The spaces between the optical elements here were too small to fit tubing, so a small 20cm x 20cm plastic box was built to cover it. Finally, there is one tube that came before the attenuator, which is the last optical element before the PSD, that has a hole cut in its side to allow the beam reflecting off the attenuator to exit the tube. This hole was covered with a piece of transparent tape to keep air currents out.

### 8.1.13 Custom Enclosures

First, as briefly described above, the square arrangement of optical elements where the beam is split was covered with a small 20cm x 20cm plastic box to prevent air currents from passing through the beam path since the spacing between the optical elements is too small to fit the black anodized aluminum tubing. In addition, the inside of
this box was lined with black anodized aluminum foil. Two holes were cut into the box to allow the tubing/beam to enter and exit.

Second, a large box made of ½” white HDPE (high density polyethylene) plastic was constructed to enclose the entire tweezers setup. This was done to protect the system from air current, temperature changes, user disruptions, ambient light, and possible acoustic vibrations. The inside of the entire box was lined with black anodized aluminum foil. To further reduce air currents and temperature changes as well as to provide structural support, the box was built with three compartments. Multiple stoppers have been screwed to the table along the walls of the box to prevent it from moving. In addition the box has been clamped down to the table. One final note about the box: The beam that is dumped by the optoisolator is reflected off a mirror and sent out of the box through a hole and terminated on a beam block just outside the box. This hole is covered with a thin sheet of Plexiglass via double sided sticky tape. This was done to keep the air in the box from heating up since the beam block absorbs the energy from the beam and consequently heats the surrounding air. As for the beam that reflects off the attenuator at the end of the system, we didn’t bother sending it out of the box because the power of this beam is so low, we don’t expect it to produce significant heating when it hits the beam block inside the box.

8.1.14 Other Equipment and Construction Notes

8.1.14.1 Equipment Table

All the electronic equipment is set on a very heavy, sturdy table that was placed next to the optical table. As noted below, each item is either plugged into a surge protector (SP) or a power conditioner (PC) – only used for the equipment through which our measurement signal passes. This table holds:

- NI DAQ board SCB-68 E-series (connected to computer)
- Alligator low pass filter (PC)
- On-Trak PSD amplifier OT-301 (PC)
- IPG Laser YLM-2-LP and driver LDA-10 (SP)
- Mad City Labs piezo-electric tip-tilt actuator Controller ND1-AR10-TS (SP)

Note that the laser drive and the Mad City Labs controller are plugged into the enhanced noise suppression outlets on a Tripp-Lite isobar premium surge protector.

8.1.14.2 Lab Counter Adjacent to Equipment table

Additional electronic equipment was placed on a very heavy sturdy counter next to the equipment table. The following were placed on this counter:

- New Computer (PC)
- GS Sola Power Conditioner PC 150
8.1.14.3 Laser and Laser Settings

We used a 1064nm CW fiber laser with a 1.6mm beam diameter manufactured by IPG; Model: YLM-2-LP and Serial #:PL0602179 (Manufacture date: 03/2006). It is accompanied by a laser driver; Model: LDA-10 and Serial #: D0602179 (Manufacture date: 03/2006). Note that the light emitted from the fiber is linearly polarized.

We set the laser to work in automatic current control (ACC), which is a constant current mode, because we showed experimentally with the PSD that the pointing stability is significantly better than in automatic power control (APC), which is a constant power control mode that utilizes a feedback loop to measure and maintain the output power. The laser current set point was set to 1.80 amps, which yields a power of ~960mW. This setting was used for most tests of the laser and during the entire building process of the tweezers.

8.1.14.4 Laser Water Cooler

We drilled holes in the wall and fed the water tubes from the aluminum/copper base of the laser to the water cooler on the other side of the wall (in the hallway). We did this because the water cooler is very loud, vibrates a lot, and produces much heat. The cooler is from Fisher Scientific with BOM#: 213103181900, S/N: 106212013, and 115V, 12 amps, 60Hz, 1 phase. And lastly it is set to hold the water temperature at 20.5 C.

8.1.14.5 Opto-Isolator

We used an optical isolator originally used in the T3 system. It was purchased from Electro-Optics Technology (EOT). It has a clear aperture of 4mm and works for 1030-1080nm high power lasers. It can be found here:

http://www.eotech.com/product/94/10451080nm_High_Power/

It utilizes two polarizing beam splitter cubes and a Faraday rotator to prevent any back reflections of the beam off downstream optics from entering the laser fiber, which could damage the laser. The internal cylinder was rotated so that the output beam would be polarized at 45° to the surface of the table. The output polarization needs to be 45° so that when split by the first PBSC downstream, the two beams will be of equal power, thus allowing the two traps to potentially be of equal strength. I don’t remember exactly if this is correct, but I have a faint recollection that to more accurately rotate the internal cylinder so that the output polarization was at 45° we put an attenuator followed by a PBSC just after the optoisolator and rotated the optoisolator’s cylinder until we measured equal power of the PBSC’s reflected and transmitted beams.

The following explains how we took care of the energy dumped by the PBSCs. Flat metal rings or sleeves surround each PBSC. These sleeves, which have two holes, were rotated until a hole on the sleeve matched with a hole in the cylinder, thus allowing the “rejected” polarization to exit the cylinder. The “rejected” beam from the initial
PBSC is dumped onto a beam block just outside the box to prevent heating within the box. More specifically, a mirror was positioned so that the “rejected” beam would be reflected in such a way that it passed through a hole (covered with clear plexiglass) at the back of the box where a beam block was placed. Potential back reflections being dumped by the second PBSC are expected to be minimal and very low power, so we decided it wouldn’t be necessary to use a beam block here to capture this energy.

Also, to prevent reflections of the laser off the first PBSC within the opto-isolator from reflecting back into the laser collimator, we positioned the input end of the opto-isolator a couple centimeters from the end of the laser collimator and oriented the opto-isolator at a slight angle. In fact, we rotated it as much as we could, which wasn’t much since the aperture is only 4mm in diameter, without clipping the incoming or outgoing laser light. After estimating some dimensions and the potential rotation of the opto-isolator, some basic calculations indicate that any back reflected beams off the incoming surface of the opto-isolator or off the initial PBSC within the opto-isolator are sufficiently deflected so that they do not reenter the collimator.

8.1.14.6 Low Pass Filter

A low pass filter allows one to filter out from a set of data all frequencies above a user defined frequency. First, as is usually recommended, we used a low pass filter to ensure that our data is not aliased. Other than this, we use it to filter out the high frequencies that make our data noisy and are well over the time scales relevant to us. For this we used an 8-pole Bessel filter from Alligator Technologies Model: USBPGF-S1/L, which can be found here:

http://www.alligatortech.com/

The cutoff frequency is currently set at 200Hz but originally it had been set at 500Hz. It uses a Bessel filter. Also note that if using the BNC connector, which is wired for differential use, for single ended input, it is recommended that the In Lo terminal be connected by a very short wire to the A Gnd terminal. This is because the In Lo terminal is connected to the BNC’s neg wire and unless the In Lo terminal is grounded, it will float. Consequently the signal coming from the pos BNC wire will be referenced to this floating value, thus yielding incorrect output voltages. See the Alligator filter manual for details. Note that this low pass filter is primarily intended to be used for force clamp experiments.

8.1.14.7 Power Conditioner

We plugged the Alligator filter, On-Trak amplifier (both of which are not grounded), and the computer into a GS Sola power conditioner (PC 150, CAT. No 25-00 563, with total maximum output of 1.25A, 120V AC) to provide these electronics with a clean AC signal so as to prevent/reduce noise in our measurement signals that could come from the building outlets. It is also meant to protect this equipment from any potential building power surges.
8.1.14.8 Beam Steering

For the beam steering, we chose to use a movable mirror rather than an acoustic optic deflector (AOD) because we observed that horizontal position measurements of a beam exiting the AOD (as measured on a PSD) occasionally exhibited large scale drift for unknown reasons, which was not observed with a fix stationary mirror. Also, since the Physik Instrumente (PI) movable mirror that we had also appeared to drift (as the AOD did) and was difficult to mount/align, we decided to buy a piezo-electric tip-tilt stage from Mad City Labs (MCL) to control the movable beam, which presumably (according to their specifications) shouldn’t have this drift and very good pointing stability compared to what we had observed with the AOD and PI mirror. Specifically, we purchased the MCL stage model: Nano-MTA2X, SN: MCLS02634 and controller model: ND1-AR10-TS, SN: MCLC02634 with analog input ranging from -10V to 10V (i.e. the extended range model), which allowed up to 5.185 mrad of rotation. However, this corresponds to a maximum angular rotation of the reflected beam up to 10.37 mrad as discussed earlier.

Using Scotch Permanent Double Sided Tape, we attached a 0.5” diameter, 3.1mm thick gold plated Pyrex (borosilicate glass) mirror to the small customized aluminum mount that is attached to the top of the stage. We used double sided tape rather than glue in case we ever need to remove the mirror in the future and also because it wasn’t clear whether it would be any better to use glue over tape.

Important note about turning on the MCL controller: Before using, you must first send an input voltage of -10V into the controller then turn the controller on. Then you need to check with a voltmeter that the HV/10 output lies between -0.5 to -1.0 Volts. If it doesn’t, you need to adjust the offset screw near this output until the value lies within this range. This procedure effectively sets -10V from the computer as 0V from the controller to the stage (i.e. it sets -10V input to be one angular extreme of the stage’s possible angular positions). If you don’t do this, you won’t be able to use the mirror’s entire range and there will be a systematic shift in your positions.

Now, with regards to alignment, we chose to align the movable beam with the stationary beam while the Mad City Labs stage was set at a voltage of -9.5V (i.e. 0.5 volts from one of its extremes in its range of rotation). We chose to do this in case our alignment between the two beams was off or if the stage drifts, we could still bump the beads together. Current calibration of the system yields $\beta \approx 979$ nm/V (ref. 10) and thus a simple calculation can show, for beads that are on average ~2.2um in diameter, a drift of the MCL stage of ~2.75V in the wrong direction or a misalignment of the two traps by ~2.7um in the wrong direction will lead to the inability to bump beads together.

A Couple Notes After the System was Complete: After the system was done, we found that the beams did not exactly overlap at the input voltage of -9.5, but we were still able to bump the beads. Ultimately we found that the stage drifts on a daily basis for reasons still yet unknown (potential reasons include temperature changes in the room among others) but fortunately the drift has yet to lead to a displacement of the beam alignment where we would be unable to bump the beads.
8.1.14.9 Imaging LED

We used the old blue LED used in the previous T1 setup. If it is the same as was used for the old T3 setup, then this LED is a blue 3900 mcd 5mm SuperBrite (there is a bag of them in the metal cabinet in the basement). While this LED still yields decent imaging, a green light should theoretically work better. The specifications of the second dichroic mirror used indicate that green light is reflected with the greatest intensity whereas the intensity of the blue light is significantly reduced after reflection. Note that the bulb went out sometime near the end of 2013 and we replaced it with a new one that we found in a small bag in the small drawer dresser for storing optical components.

8.1.14.10 Imaging Camera

For imaging the beads we used the old CCD camera originally used in the previous T1 setup. It is a Watec LCL-903HS CCD camera.

8.1.14.11 Television

We used 12” screen, black and white, ProVideo CCTV Monitor; Model: VM-1201B, manufactured: Apr 2000.

8.1.15 Position Sensing Detector (PSD)

After many experiments in comparing the On-Trak PSD to the Pacific Silicon PSD, we determined, to the best of our ability, that the pointing stability measured on the On-Trak detector is less than that of the Pacific Silicon. Furthermore, the On-Trak detector is much easier to install. Therefore we purchased a new 2D silicon On-Trak PSD (encased in aluminum housing) and amplifier for this system, PSD Model: PSM2-10 (housing and detector; Model # of detector only: 2L10SP); Amplifier Model: OT-301. The chosen PSD has a 10x10mm active area, which is just large enough to measure deflections of the beam (7.2mm diameter) resulting from displacements of the bead in the stationary trap. These items can be found here:

http://www.on-trak.com/2lseries.html (Detector Only)
http://www.on-trak.com/psm.html (Housing and Detector)
http://www.on-trak.com/ot301.html (Amplifier Only)

Note that the x and y outputs from the On-Trak amplifier are normalized. That is, \( V_x(\text{output}) = V_x(\text{detector})/V_{\text{sum}}(\text{detector}) \) and similarly for y, which makes \( V_x(\text{output}) \) and \( V_y(\text{output}) \) independent of fluctuations/changes in the power of the beam. This means that each value of \( V_x(\text{output}) \) and \( V_y(\text{output}) \) correspond to a specific position on the PSD irrespective of the power. This also means one less step we would need to perform during our data analysis.

Also, as discussed earlier in the section on attenuators and filters, we set the gain on the amplifier to 1. In addition, the Cal/Zero switch on the back of the amplifier is set to the OFF position.
For purposes of alignment, I measured the position of the silicon detecting surface relative to the incoming aluminum housing surface with calipers and found it to be 0.53 inches (13.46mm) from the entering surface of the detector housing. I made a small scratch on the top of the detector housing where I measured the detecting surface to be. I also measured the total width of the housing to be 1.127 inches (28.63mm). See the diagram below.

![Diagram of PSD housing](image)

**Figure 8.5**: View of PSD housing from above with indication of the position of the detector surface.

### 8.1.16 Fluidics

I constructed a custom fluidics assembly that sits in a Plexiglass box, which I also made, that is meant to “hover” over the white box very near the flow cell so as to minimize the tube length (or dead volume). This assembly includes a 4-way fluidic valve from Hamilton (HVDP4-5 Valve Rev J; product #86788) that was purchased in 2011 and has had little use since then. For this valve I also used:

- Two plastic nuts (one for feeding in the tubing and one for blocking an unused channel), IDEX Health and Science product # P-252. The one used for blocking a channel was wrapped in Parafilm to keep the channel clean.
- Two ferrules to align the tubing with the channel holes properly (one for the incoming fluid and one for the outgoing fluid), IDEX Health and Science produce # P-248x (Ferrule assembly)
- One Hamilton Female Luer Adapter with ¼-28 threading (product #35031) for connecting the large 10ml BD syringes.

In addition for this assembly we used:
• B-D 18G 1½ syringe needles (product # 305196) for connecting our syringes to the plastic tubing that feeds the liquid into the valve. We cut the ends off these syringes so they weren’t sharp and to reduce the dead volume.

• Tygon tubing as connector tubing for connecting either two tubes or for connecting the syringe needle to a tube.

• Originally we used Intramedic polyethylene tubing PE50 (product # 427410), which had I.D. 0.023”, O.D. 0.038”. But we ran out one day and bought Teflon tubing from the stock room with I.D. 0.0177”, O.D. 0.035” from the Chemistry stock room. I think at some point in the past we did use the Teflon tubing, which seemed to work fine, but I think we used the BD PE50 tubing for this setup.

### 8.1.17 Flow Cell

The method I developed for making strong, well-sealed, uniform thickness, and lean flow cells with blunt-end cut capillary tubing is not described here, but I used the following materials:

• Fisherbrand Microscope Cover Glass (product # 12-545-1), 24x60-1; 1oz. These coverslips had holes drilled in them by a laser cutter at Carlos Bustamante’s Lab.

• Capillary tubing from King Precision Glass, Inc. with I.D. 0.025± 0.010mm, O.D. 0.100±0.010mm. They sold us 250 pieces of length 150.0±6.0mm each. (Glass type 7740). These were cut for use in the flow cell using a diamond scribe.

• Heat triggered epoxy sheets (or AbleFilm Adhesive Film from Ablestick, which is a Division of Henkel Electronic Materials LLC). We purchased 14 12”x12” sheets of 4mil thickness (561-1-004; Article no. 1199273) on 04/18/2011. I think we may have sold some of this to some other tweezers group. We store it at -80°C according to manufacturer storage conditions. Note that the previous epoxy sheets (also from Ablestick) were ~10 years old and were yielding leaky flow cells because the epoxy wouldn’t melt properly when heated so that a good seal could be formed. This issue does not arise with the new epoxy.

### 8.2 Section 2: Construction Procedure, Methods, and Details

#### 8.2.1 Initial Comments

• Throughout the building process, any unused beams or temporarily unused beams were blocked with a beam block. Also, for our protection, with the exception of determining the correct rotation of the laser collimator (described later), stray beams were always blocked (using a beam block) from extending past the edges of the table or through any plane parallel to the table. Note that such beam blocks were not always shown in the figures. Beam blocks were only shown in the figures when they were specifically needed during that step.

• Throughout the building of the system, centering a beam with an iris was achieved by first imaging the beam as it hit the surface of a closed iris using a
CCD camera and TV in a very dark lit room. The iris eye was opened slowly until only the very edges of the beam could be seen. Depending on what was being aligned, the iris or the upstream (or downstream) optics were repositioned until a very faint and symmetric ring of light could be seen on the iris. In some cases, rather than observing a ring, only three very faint points, all equidistant from one another (as the points in an equilateral triangle), could be seen.

- During the building process we frequently used a simple tool for finding the beam and doing quick checks of the height and lateral position of the beam. The tool involved a small rectangular piece of white foam board (~1.5-2” x ~6”) that was taped to a heavy L-bracket so that the long axis of the board was vertical. Initially after we clamp the laser into its mount, as described below, we put it in front of the laser, so that the laser was roughly centered laterally, and let a small hole be burnt onto the board. We then drew a crosshair on the board that was centered on this hole where the crosshair lines extended to the edges of the board. As mentioned above, the reflection of the beam off this white board could be seen with a CCD camera and TV. See Figure 8.12.

- When a beam is being centered with an iris eye, it is important that the iris is as perpendicular with the beam path as possible. Note that we only ever oriented these irises perpendicular to the beam path by eye.

- Throughout the building process, we were careful to never place any objects (especially optical elements) at the focal points of a lens (through which the laser is already passing). Doing so can be very, very damaging to the object.

8.2.2 Building Procedure

8.2.2.1 Laser Mounting and Initial Alignment

Because the laser beam does not exit the collimator (metal cylinder attached to the end of the laser fiber) parallel to the axis of the collimator but rather at an angle, we had to determine the correct orientation that would ensure the beam travelled parallel to the table after exiting the mounted collimator. We did this in the following way:

- First we placed the collimator in the V-block mount with the cylinder extending a few centimeters out from the wall of the mount. A small piece of Tygon tubing (O.D. ~0.5”) was cut and placed on the collimator (to protect it from being crushed when clamped) and then a metal block was placed over top of this and clamped/screwed lightly down to the V-block. It was tightened to a degree that it would prevent it from rattling about while still permitting it to be rotated about the axis of the collimator.

- I don’t remember the exact details of this part of the procedure, but in a nutshell we temporarily mounted the V-block mount to the position on the table where we intended it to be based on our design. Then we clamped an adjustable dielectric mirror some ways down the beam path so that we could reflect the laser beam out of the room and onto the wall in the
hallway. While using a small CCD camera, we rotated the collimator and traced out the circle it produced on the wall with a pencil. Afterward, we rotated the collimator until the beam hit the right most point on the circle (i.e. $y = 0$ on $y$-axis, or $\theta = 0$). Once positioned, we tightened the clamp covering the laser collimator until it was snug. Note: The beam doesn’t come out of the collimator perfectly collimated, it diverges a bit. This actually made it easier to see on the wall when tracing out the circle.

- The previous step is enough to get the beam parallel to the table, but we may also have performed a step where we double checked that the beam was in fact parallel to the plane of the table, I can’t remember. If we did, we probably did it in some way similar to the following:

  o We would have first removed the mirror and positioned an iris at two positions away from the laser head. Using the small CCD camera the irises were likely adjusted until the beam passed right through the center of the iris. After doing so, the base clamp of each would have been securely clamped to the table. Then we would have tightened a collar onto the post of the first iris to fix its height. We would likely then have taken this iris/post/collar assembly out of its mount and placed it in the second mount downstream (after removing the iris in the mount of course). It would then have been rotated to be as perpendicular as possible to the beam path and we would check with the camera whether the beam was passing directly through the center of the iris. If not, we would have gone through an iterative process of rotating the collimator, repositioning the iris bases and heights, redetermining the iris height near the laser, clamping the iris height, and moving the iris to the second iris mount. In doing this repetitively, we would have been able to get the beam path as parallel as possible to the table.

- **Determining Table Position for the Laser Collimator:**
  
  This was primarily based on where we wanted the flow cell stage to be mounted for the user and dimensions of our design. We initially placed the flowcell stage, with the flowcell mount attached, near the edge of the table where we expected the user to work and positioned it such that the micrometer extended \(~ 2 - 2.5\)” from the edge of the table. This distance was enough that the user would be unencumbered while using the micrometer and so that a box with 0.5” thick walls could be constructed over the setup without interfering with this stage. We then made a pencil mark on the table where the approximate position of the flowcell would be. It ended up being just above one of the bolt holes that was \(~ 7 \frac{5}{8}\) inches (~19.4cm) from this edge of the table. Then based on the dimensions of our design, this meant the beam at other end of the table
needed to be ~57cm from this bolt hole, or ~15.56cm (or ~6\(\frac{1}{8}\) inches) from the opposite edge of the table, which is about half way between two bolt holes.

The position of the laser collimator along the table’s long axis had more freedom. This choice depended on the overall length of our tweezer design, allocating extra distance for the box and possible design changes, and the most desired position for the user. Ultimately we ended up positioning the very end of the collimator at ~ 28 inches (~ 71 cm) from PSD end of the table.

- We then drew a line on the laser-side edge of the table in pencil using a long metal straight edge where the line was ~6\(\frac{1}{8}\) inches from this edge of the table. We marked points along the line where we planned to put the laser collimator mount, lenses for the telescope, and the system’s first mirror. Again I don’t remember the exact details, but one iris was placed somewhere between the position for the two lenses (it had to have been a position where the beam diameter wasn’t too small to see on the iris), while the other iris was placed at some point between the position of the second lens and the mirror. And finally a third iris was placed as far as possible to the end of the table. Each was aligned to the line on the table by matching the midpoint on the base bracket of the iris stand to the pencil line. Immediately after this alignment/positioning, each iris was securely clamped to the table. Also, each iris was rotated by eye so it was as perpendicular as possible to the pencil line. See Figure 8.13.

- Once the irises were in place, the V-block mount was rotated and translated until the beam passed through the first and last irises. If the middle iris was off, we likely translated it perpendicular to the beam axis until the beam was centered with the iris center. Translations of the V-block were likely performed by first bumping two ball tip kinematic stops against the base and securing them to the table. The V-block can then be translated along these (i.e. perpendicular to the beam axis) without translating along the beam direction. I don’t recall exactly how we performed the rotations (a process that can be difficult with flat edged base brackets), but we probably did it by first clamping a ball tip kinematic stop against one edge of the V-block that is parallel to the beam and then holding this end of the V-block against the two perpendicular stoppers while very slightly pushing the other end of the V-block in the direction of the beam axis. After which the stopper that is no longer in contact could be bumped up against the block and clamped down thus allowing one to repeat this process of translating/rotating if needed. See Figure 8.14.

8.2.2.2 Positioning the opto-isolator

- The opto-isolator was placed with the entering face of the opto-isolator at ~1.5-2cm from the end of the laser collimator. Then, as explained earlier,
the opto-isolator was rotated by hand so that the beam entered the opto-isolator at an angle so as to prevent back reflections from entering the collimator. The opto-isolator was rotated as much as possible without clipping the beam. Note: We later discovered after the beam was expanded that the beam was in fact being clipped as a result of over-rotating the opto-isolator. So, we later rotated the opto-isolator until no more clipping was observed. Fortunately this didn’t affect any of our downstream alignments. See Figure 8.15.

8.2.2.3 Positioning and Alignment of Telescope #1 Lenses

- First we must note that normally it would be best to align the mirrors first and then put all other optical elements. However, there were particular circumstances for which it was better to position and align the optical elements of this system in a different order. One such circumstance was that at this early stage in the setup the beam diameter is too small to accurately align downstream mirrors using irises, so we needed to expand the beam first. So, here we positioned and aligned telescope #1 first and then put in the stationary mirror downstream

  - Also, given what was just said, after positioning and aligning this telescope, you would think we would next position and align the two downstream mirrors that bend the beam around the corners of the table (i.e. the dielectric stationary mirror and dichroic mirror #1) but we didn’t. Instead, we first positioned and aligned the two PBSCs and the MCL stage mirror and then put in the stationary mirror, followed by the first dichroic mirror. The reason for this was because it was too difficult to rotate the MCL stage precisely while still ensuring that the beam, which filled most of the mirror at 45° AOI, remained centered on the mirror. Furthermore, the second PBSC was positioned and aligned before the stationary mirror because if we put the mirror in first, there wasn’t going to be enough room for the temporarily clamped base bracket along which the PBSC mount (with the custom pedestal alignment tool attached – see Figure 8.16) would be translated for alignment.

- With the irises in place, centered, and at the correct height, the lenses could be put in place. Based on our design, we marked with a pencil the positions on the table along the previously drawn pencil line where the lenses were to be placed. Again, I don’t remember the exact details about how we did this but the procedure was similar to the following:

  - Lens #1 was placed over its mark with the incoming beam hitting the more curved side while the beam exits from the flatter surface. It was first translated perpendicular to the beam axis by
eye until the beam hit the center of the lens and then rotated (also by eye) until it was perpendicular to the beam axis. Since we ordered fixed height posts (as planned), no adjustments to height needed to be made. I can’t remember if we tried to use the irises to align this lens. I have a faint recollection that we did but that we didn’t initially position the irises in a place that would be best for the alignment of this lens. I think the first iris was in a position where the beam diameter was still fairly small after emerging from the lens. If this is in fact how we aligned the lens, then we would have used the custom pedestal alignment tool I made for the 1” diameter pedestals to perform translations. More specifically, we would have translated the lens perpendicular to the beam axis until the beam was centered on two downstream irises (1st and 2nd irises). Such translations would have been performed by first clamping a flat edge up against the flat edge of the custom alignment tool and then down to the table. Then the lens could have been translated perpendicular to the beam axis. See Figure 8.17.

- Lens #2 was placed over its mark with the incoming beam hitting the flatter side while the beam exits from the more curved side. The lens was then rotated by eye until it was perpendicular to the beam axis. Just as for lens #1, the pedestal alignment tool was attached to lens #2 so that the lens could be translated perpendicular to the beam axis until the outgoing beam was centered on the two downstream irises (2nd and 3rd irises).
- Finally the beam had to be collimated. Collimation was achieved by translating lens #2 along the beam axis using the custom pedestal alignment tool while swapping one of the downstream irises back and forth between the two downstream iris mounts (2nd and 3rd iris mounts). More specifically this was done by adjusting the eye of the iris in the first mount until only the very edges of the beam were seen, then moving the iris to the second mount. If the thin ring of light observed on the iris was not as was seen while the iris was on the previous mount, then the beam was not collimated. With the iris still in place, the lens was translated until a ring of light was barely seen on the iris. The iris was then switched back to the first mount. If the same ring of light was not seen on the iris, then the beam is still not collimated and the iris eye was adjusted until a very thin ring was seen on the iris. This process was repeated until the ring of light observed on the iris looks the same when placed in both mounts. Note: The eye of the iris was only adjusted when it was in the first mount and the lens was only translated while the beam was hitting the iris in the second mount. It could be done the other way around but it is better to do it this way because converging or diverging of the beam due to very
small translations of the lens is more easily seen at the most distant iris. Once completely collimated this lens was clamped to the table. See Figure 8.18.

- **Note:** We later discovered we could use back reflections to align the lenses more precisely! I have a faint recollection that we later came back and used this method, which is described later for the alignment of telescope #2, to align the lenses and ensure that the beam passed through the center of the lenses but also trying to do so without affecting the collimation, which would result if either lens was unintentionally translated along the beam axis.

### 8.2.2.4 Positioning and Aligning the First Beam Splitter

- First we drew in pencil lines corresponding to the intended beam path of the first PBSC’s reflected beam using a metal L-square angle ruler and a long metal straight edge. Note that the position of this line was based on our design and did not necessarily follow along any line of bolt holes. See Figure 8.19.

- Next we centered two irises mounts (spaced far apart from one another) along the pencil line that the reflected beam travels.

- **Note on centering beam on mirrors and PBSCs:** You can’t see the beam on the mirrors or on the PBSCs, so it makes it difficult to center them with the beam. We found the best way to do this was by lightly holding small pieces of lens paper against the mirror or PBSC surfaces. This allowed us to still see, via the CCD camera, the entire surface of the mirrors and PBSCs as well as the beam profile without damaging/scratching the optical surfaces.

- Then, both irises upstream of the lenses in telescope #1, with their height collars, were taken and inserted into these two mounts so that the height of the PBSC could be adjusted as described in the next step.

- The beamsplitter was then positioned, at first by eye, such that the beam hit the center of the beamsplitter’s face, the reflection surface was at a 45° with the beam, and the reflected beam roughly followed the pencil line. Also, the beamsplitter was oriented with the beam entering the appropriate face as specified earlier in this document under the criteria section. After doing so, the pedestal was temporarily clamped to the table.

- Next the angle of the beamsplitter mount was adjusted (using the adjustment knobs on the mount) in order to get the beam height correct. This was done by first translating the irises to be laterally aligned with the beam, then by adjusting the tilt of the reflected beam until it passed through the center of both downstream irises. We continued alternating between lateral translations of the iris mounts and tilting of the PBSC mount until the beam passed through the center of both irises. Once the
correct tilt was attained, the iris mounts were translated back until they were centered along the pencil line.

- After adjusting for the height, the reflected beam was aligned to the pencil line by alternating between translations and rotations of the beamsplitter. Note that the beamsplitter mount does not have an adjustment knob to rotate the PBSC about a vertical axis (perpendicular to the table). So rotations were performed by slightly loosening the screw on the custom alignment tool followed by rotating the pedestal by hand. These rotations had to be done slowly and carefully so that no translations occurred while rotating the pedestal. **See Figure 8.19.**

- **Interesting observation that we had to adjust for:** After positioning and aligning the first PBSC we noticed that the transmitted (but not the reflected) beam was downwardly deflected by ~5 arcmins. We tried flipping the PBSC and we tried tilting it about the axis perpendicular to the axis of the transmitted beam but no matter what, it always deviated downward by ~5 arcmins. The manufacturers had no useful responses and simply stated that it is within their advertised specification. We later accounted for this deviation in height by adjusting the stationary mirror until the reflected beam was parallel to the table. The PBSCs were then adjusted until the movable beam was in line with the stationary beam.

### 8.2.2.5 Positioning and Aligning of the MCL Stage Mirror

- To begin, the MCL stage was set to -9.5V and remained at this value during the positioning and alignment of this mirror, the second PBSC, and the stationary mirror.

- As before, we used an L-square ruler, straight edge, and the dimensions of our design to draw a pencil line on the table for the intended beam path of the beam that reflects off the MCL mirror. We then took the iris mounts and the irises used in the previous step and positioned them at two distances away from the MCL stage and centered them along the pencil line.

- The alignment of the MCL stage was one of the most difficult to perform because its edges weren’t parallel to either of the beam paths and because the straight edges of its mount made rotations difficult. Also, as mentioned earlier, this was difficult to align because the expanded beam filled most of the mirror when it was rotated, thus making it difficult to translate or rotate while keeping the beam centered on the mirror. So, as discussed earlier, we did not attempt to precisely align this mirror, but only roughly aligned it and later adjusted the downstream PBSC to get this beam in line with beam reflected off the stationary mirror, which was not yet installed at this point. Ultimately we positioned the MCL stage mirror by eye such that the beam was centered on the mirror and that the reflected beam nearly passed through the center of the two downstream irises. The
reason for still positioning the irises was so that we could still roughly position the MCL stage where it should be according to our design, this way the remaining downstream optics could roughly be placed at the positioned originally planned. See Figure 8.20.

- Note: If any rotations of the MCL stage were needed, they were likely done very slowly and by hand, whereas if any somewhat precise translations were needed, we probably performed them as follows:
- This would have been done by putting a flat edge or ball tip kinematic stops against the back side of the MCL mount, translating it the desired amount, temporarily clamping the stage down to the table, putting another flat edge or ball tip kinematic stops against the appropriate side for the desired translation, and finally translating the MCL mount along this edge the desired distance. The net translation of these two such ~45° translations would have been a translation “up” along the incoming beam axis. See Figures 8.20&8.21.

### 8.2.2.6 Positioning and Aligning the Second Beam Splitter

- First, according to the dimensions of our design, a pencil line was drawn down the table along the intended path of the beam reflecting off the stationary mirror.
- Again, the two irises used in the previous step were taken and positioned at two distances away from the position at which the second PBSC was to be placed and centered with the pencil line.
- The mounted PBSC was positioned at the intersection of the pencil lines and oriented so that the incoming beam entered the correct surface of the cube (i.e. the incoming surface that is indicated by a dot on top of the cube). The position of the pedestal was adjusted until the beam entered the center of the cube and roughly passed down along the pencil line path.
- Finally, using the custom pedestal alignment tool, the second PBSC was rotated and translated until the reflected beam passed through the center of both irises. See Figure 8.22.

### 8.2.2.7 Positioning and Aligning the Mirror for the Stationary Trap

- We began by putting a beam block just after the first PBSC or the MCL Stage to block the movable beam.
- Next we roughly positioned the stationary mirror with the adjustable mount and roughly aligned it so that the beam was passing through the two downstream irises (still in place from the last step). Then we temporarily clamped the mirror down to the table.
• As mentioned earlier, since the PBSC transmitted beams were being deflected downward, we needed to readjust for the height. So, to find the new height, we first translated the two downstream irises perpendicular to the beam path until the beam passed through the center of the irises laterally. We then removed one of the irises and alternately adjusted the tilt of the mirror while adjusting the height of the iris and switching the iris back and forth between the two mounts to check whether the beam is parallel to the table. This procedure was repeated until the beam passed through the iris when positioned at either of the two mounts. At this point we had found a new beam height, which wasn’t much different than before, and had also maintained that the beam was parallel to the table. Next, we reinserted the other iris into the empty mount and adjusted its height until the beam was centered on the iris eye. See Figures 8.23.

• Once this was done, we needed to laterally adjust the beam, but first we needed to re-center the irises by translating them until they were centered on the pencil line again. Once in position, the stationary mirror was translated and rotated using the custom pedestal alignment tool until the beam passed through the center of both irises. Depending on how much the beam needed to be rotated, we either simply used the knobs on the adjustable mount for small rotations or carefully rotated the entire pedestal after slightly loosening the custom pedestal alignment tool for large rotations. See Figures 8.23 & 8.24.

• Then for more precise alignment, we temporarily placed the first dichroic mirror roughly in place so as to increase the laser length after the second PBSC. This extra laser length allows lateral or vertical deviations between the stationary and movable beams to more easily be seen. See Figure 8.25.

• A new mount was then placed at the end of the table and the second iris (with a collar to hold its height) from the previous step was inserted into this mount and then positioned such that the beam reflecting off the dichroic mirror was centered on the iris. This tilt of the dichroic mirror was also adjusted at this time in order to get the beam centered on the iris.

8.2.2.8 Readjusting the Height of the Movable Beam

• Since a new beam height was established in the previous step while aligning the stationary mirror, we needed to readjust the height of the movable mirror. We first unblocked the movable beam and blocked the beam reflecting off the stationary mirror.

• Next, we translated the second iris mount until the beam was laterally centered on the iris.

• We then adjusted the tilt of both PBSCs until the beam reflecting off the second PBSC passed through the center of both downstream irises. More specifically, this was done by alternating between tilt adjustments of the
first PBSC until the beam was centered on the first iris downstream of PBSC #2 and tilt adjustments of the second PBSC until the beam was centered on the second downstream iris. Note that in performing this vertical alignment, the second iris downstream probably required occasional lateral translations in order to acquire very precise vertical alignment of the beams. See Figure 8.26.

8.2.2.9 Double Checking the Lateral Alignment of the Stationary Mirror

- Now with the movable beam in vertical alignment with the stationary beam, we decided we should use this extended beam length to double check that the lateral alignment of the two beams is still good. To do so we first blocked the movable beam and allowed the stationary beam to pass. We then translated and rotated the stationary mirror using the custom pedestal alignment tool until the beam passed through the center of both downstream irises. See Figure 8.27.
- After the mirror was completely aligned, the mirror mount adjustment knobs were locked by tightening the Allen key screws locks.
- At this point the stationary and movable beams appeared (to the best of our ability) to overlap, to be parallel to the table, and to be passing along the desired path (designated by the pencil line on the table). But the overall beam height was now slightly different (a bit higher now) than it was after exiting the laser collimator, but not by too much. Fortunately for our design, the remaining downstream optical elements are either adjustable or were negligibly affected by this height difference.

8.2.2.10 Positioning and Alignment of Dichroic Mirror #1

- Using a L-square ruler and a long metal straight edge, we drew in pencil a line down the table’s long axis that was \(7\frac{5}{8}\) inches (~19.4cm) from the trap-side edge of the table and perpendicular to the pencil line that the beam followed after reflecting off the stationary mirror. See Figure 8.28.
- We then recentered the iris at the end of the table with the pencil line, positioned and centered a new mount to the pencil line a short distance away from the dichroic mirror, and inserted into the latter mount the iris used in the mount that immediately followed PBSC#2 during the previous step. We also blocked the movable mirror beam.
- Now, as was done previously while positioning/aligning the stationary mirror, the dichroic mirror was first positioned such that incoming beam hit the center of the mirror and then alternately translated, rotated, and tilted this mirror until the reflected beam passed through the center of both downstream irises. Note: The Suprema mirror mount we purchased to hold this dichroic mirror has a fairly thick clamping arm. We were careful
not to allow this large clamping arm to clip the reflected beam. We later observed while putting in the imaging optics that some of the blue light that passed through this dichroic mirror was clipped by this thick mirror mount arm. Fortunately it did not clip the area we were interested in imaging.

- After the mirror was aligned, the mirror mount adjustment knobs were locked by tightening the Allen key screws locks.

### 8.2.2.11 Positioning and Alignment of Telescope #2 Lenses

- To begin we marked the positions on the table where the lenses were to be placed according to our design. In order to implement one-to-one imaging of the MCL stage mirror onto the back focal plane (BFP) of the first objective that we had previously decided, it was very important that the first lens in telescope #2 be positioned as accurately as possible one focal length \( f_{\text{Lens 1, Telescope #2}} \) away from the mirror. We noted that the focal length of these lenses is not measured relative to the center of the lens. Fortunately the lens manufacturers also provide the focal distance relative to each surface of these fairly thick lenses, so we used these alternative focal lengths in positioning the lenses in this telescope.

- For this step we used the stationary beam only. So we kept the movable beam blocked.

- As mentioned earlier, at this point in the building process we discovered that we could better align the lenses by using back reflections on irises upstream of the lens. So, we put a new iris mount ~5 cm upstream from the position of the second lens. The irises downstream of the mirror were left in their mounts while new irises were put into the mounts that come before lens #1 and #2 in this telescope. The heights were adjusted until they were centered with the beam. See Figure 8.29.

- Lens #1 was now positioned at the first mark as accurately as possible. It was then alternately rotated and translated (using the custom pedestal alignment tool) until the reflected beam was centered on the upstream iris while the transmitted beam was centered on the downstream iris. More specifically, rotations were performed until the reflected beam centered on the upstream iris while translations were performed until the transmitted beam centered on the downstream iris. See Figure 8.29.

- Lens #2 was positioned over the pencil mark that designated its placement. This lens was then aligned in the same fashion as described for Lens #1.

- Lastly we needed to collimate the beam. But to yield more precise collimation, we added beam length by putting any type of IR reflective mirror at the end of the table (just in front of the iris that was at this end of the table) to reflect the beam around this corner. We then mounted an iris at the adjacent corner for which the beam was headed and adjusted the mirror until the beam was centered onto the iris (height of the iris was
unimportant). This extra beam length led to a larger diameter beam when the beam was diverging, which made it much easier when trying to collimate the beam very precisely. **See Figure 8.30.** Note: In retrospect, it would have probably been even better if we collimated telescope #1 by adding in another mirror or two to increase the arm length, which would have made the collimation of telescope #1 even more precise.

The actual collimation was performed using the same method as described for the collimation of telescope #1 except that here we used the first iris mount downstream of lens #2 as well as the iris and its mount placed further downstream as explained in the previous paragraph.

### 8.2.2.12 Positioning and Aligning Objective Stage #1

- **Finding the Image of the MCL Stage Mirror:** Originally our plan had been to position the BFP one focal length away from the second lens in telescope #2. However, after some investigation, we discovered that the image of the MCL stage mirror wasn’t at one focal length away from this lens. So, we ended up finding the image of the MCL stage mirror by centering the PSD on the pencil line downstream of dichroic mirror #1 near the point where we expected the image. Since the MCL stage mirror is a point of beam rotation, one should only observe beam rotations, not translations, at the image location. Thus, if the PSD was at the image and the mirror was rotated, no change in the position of the beam should be observed on the PSD. If the PSD was positioned anywhere other than the image point, than one would observe displacements on the PSD. Before implementing this idea we first blocked the stationary beam, placed an angled (so as to deflect back reflections) attenuator after the dichroic mirror to reduce the power to a level that resulted in PSD measured Vsum voltages within 1-6 volts, and then roughly positioned the PSD such that the Vx reading was near zero. We could then utilize the idea just described by translating the PSD along the beam axis while scanning the MCL stage mirror back and forth until the deviations measured by the PSD from one rotation angle to the next was zero or nearly zero over the entire angular range of the mirror. Once this position was identified, the PSD pedestal was clamped to the table and a mark was made on the table that was measured ~13.5mm (as discussed earlier) from the incoming surface of the PSD housing, which is the position of the actual PSD silicon detecting surface.

**Aligning the PSD to be Perpendicular to the Beam Axis:** Note: It was very important that we used the custom pedestal alignment tool here so as to hold the PSD perpendicular while allowing us to translate the PSD along the beam axis without permitting any rotations. Because the PSD has such great precision, we were able to determine this image point very accurately. However, in order to determine the position of the image
appropriately and accurately, it was necessary that the PSD be as perpendicular as possible to the beam path. This was done by using an L-square ruler to align and clamp a “tall” (~4-6”) L-bracket that was perpendicular to the beam path. The flat back of the PSD, while on its pedestal, was bumped up against this L-bracket until the two surfaces where flush. We then put in place a series of clamps that referenced the alignment of this L-bracket to ensure that the custom pedestal alignment tool was clamped to the PSD pedestal in such a way that the pedestal could be translated parallel to the beam axis. **This is far easier to show in pictures than to explain. See Figure 31 for the details.**

- **Positioning and Aligning the First Objective Stage:** To the top of the first objective stage we attached a rail. However, there is so much tolerance in the screw holes on the rail, it was likely that this rail was not aligned with the long axis of the stage. So, we developed a new method for aligning the axis of this rail, which was the axis along which the objective axis was to be aligned. To begin, on either end of the rail, we clamped a threaded Newport objective mount (both flush with the very edge of the stage). Then we positioned the stage by eye such that the beam path (i.e. the pencil line drawn beam path) was centered with the center of the Newport objective mounts and such that the downstream end of the stage was approximately 19mm from the position of the MCL stage mirror image, as found and marked in the previous step. Then two ball tip kinematic stops were bumped up against the center point of two adjacent sides of the stage’s base bracket and clamped to the table. **See Figure 8.32.** Note that we designated the center point as the position of the center screw hole on the top of the stage (not the center of the stage’s base bracket).

- **Next the beam block, just after the stationary mirror, was removed so that both the stationary and movable beams were passing through the objective mounts on the stage.** We checked that the MCL stage was still set to -9.5V. Then, a power meter was placed ~ 2 inches away from the end of the stage and clamped to the table. With this we measured the total power of both beams passing through the objective mounts on the stage.

- **We next inserted our stage alignment tool into the downstream objective mount (See Figure 8.33).** It is basically a cylindrical piece of metal with a hole drilled through the center and two outer diameters. It has a larger diameter for half its length, and then a smaller outer diameter for the second half that is equal to the diameter of the hole in the objective mount. On the shelf of this tool we applied double sticky tape so that the tool will stick (temporarily) to the objective mount once it was inserted. We marked with a permanent marker the midpoint on both objective mounts from above. In addition we made two markings on the outer surface (the larger diameter) of the alignment tool that were 90° apart from one another. Lastly, a thin flat, half-circle piece of metal was taped to cover half of the hole on this tool with the straight edge aligned with one of these markings.
• **Aligning the Center of the Objective Mounts with the Beam:** First, the alignment tool was rotated so that the bottom half of the objective mount was blocked. In doing so, the markings on the alignment tool and the objective mounts were aligned with one another. Once oriented correctly, the tool was pressed firmly against the objective mount so that it stuck well via the double sticky tape. First note that the thin plate of metal did not perfectly block half of the hole, thus we didn’t expect to measure 50% of the beam to pass through once the stage was aligned. All that mattered was that the power measured, while either half was blocked, was equal when the stage was well aligned. So, next we measured the power of the light that was permitted to exit. The alignment tool was then rotated so that the top half of the hole was blocked, after which we measured the power. If the measured powers were not equal, then we adjusted the stage’s height micrometer while rotating the alignment tool to block either the top or bottom half of the objective mount until the measured exiting power was the same for the top and bottom half of the objective mount. Once done, the height of the stage was in place and so we locked the height micrometer.

• **Alignment of the Objective axis with the beam axis:** This was a multistep iterative process in which we alternated between rotating and translating the stage. While it yields great alignment, it can be quite cumbersome. The rotation and translation steps are described separately below. First note that all lateral translations performed by turning the x and y stage micrometers were rotated using an Allen wrench, which allow for finer translations. This was possible because the x and y micrometers have Allen key holes just for this purpose.

• **Stage Rotations:** To begin, for ease of explanation, I will designate lateral positions by either ‘laser’ side or ‘objective’ side where ‘laser’ side is that which is closer to the side of the table with the laser, and ‘objective’ side is that which is closer to the side of the table with the objectives.

   We identified whether a rotation needed to be performed by inserting the stage alignment tool into the upstream objective mount with the laser side half of the beam blocked by the thin metal plate. We noted the power measured, removed the tool, inserted the tool into the downstream objective mount with the laser side blocked, and measured the power. If the powers measured were not equal, then we applied pressure to the corner of the stage opposing the ball tip kinematic stoppers and carefully rotated the stage by a slight amount. We repeated the power measurement at both objective mounts in the same way. This procedure was repeated until the power measured at both objective mounts (with the laser side blocked) was the same. **See Figures 8.34.** Note: We acknowledged that rotations performed in this manner are not perfect rotations about a point but rather also resulted in slight translations. But, by alternating between performing rotations in this manner and translations via the stage micrometers, we were still able to align the stage
well. We performed rotations in this way because it works sufficiently well without having to design a special tool for rotating the stage about a chosen point and furthermore this method was far more precise than the way in which the stages were previously aligned.

- **Stage Translations:** Now, we inserted the stage alignment tool into the downstream objective mount and measured the power transmitted when the laser side and the objective sides were blocked. If these were unequal, then we translated the stage perpendicular to the beam path using the stage’s micrometer that translates the beam in this direction until the laser side and objective side power measured was equal. Note that this could have alternatively been performed at the upstream objective. See Figure 8.35.

- **Completing the Alignment:** Since, as previously mentioned, the objective axis (and rail) was not parallel to the stage axis, translations of the stage here did not correspond to straight translations of the objective axis. Translations of the stage here, inevitably permitted small rotations, which would be verified if one measured the exiting power from both objective mounts with the laser side blocked and found the measurements to be slightly unequal. It could also be verified by measuring the laser side and objective side power at the upstream objective mount after the stage translation performed in the step above. Therefore, in order to best align the objective axis with the beam axis, we had to repeatedly alternate between performing these stage rotations and translations until the power measured at all four positions (i.e. laser side and objective side at both objective mounts) were equal.

- Lastly, once the stage was accurately and precisely aligned, it needed be clamped to the table. However, in the process of clamping, the stage may move. So, to minimize/prevent this, we slowly tightened clamps at each corner in a repeated X-like fashion. That is, we tighten one clamp a bit and follow it up by tightening the clamp at the opposing corner a bit, then move to an adjacent corner, tighten this clamp a bit and ended with slightly tightening the clamp at the corner opposing this. Then, we continued tightening all the clamps in the same fashion over and over until the stage was very securely clamped. See Figure 8.35.

**8.2.2.13 Checking the Trapping Ability and Strength**

- Before moving on with the rest of the setup, we needed to check that this objective stage was well aligned. To begin we screwed in one of the objectives into the downstream objective mount. See Figure 8.36.

- Next we positioned the flow cell stage. First a custom rectangular aluminum mount (~1” tall) that I milled was positioned so that the flow cell stage that sat upon it would hold the flow cell near the objective. The mount was then very securely screwed to the table. The flow cell stage
(with the flow cell mount and flow cell attached) was then placed atop of this and oriented such that the flow cell was ~1mm from the surface at the end of the objective and parallel to this surface as well. After achieving this, the stage was very securely screwed to the mount. Then a drop of water was squirted into this gap between the flow cell and objective.

- Next we positioned the LED on the opposite side of the flow cell and placed it as near as possible to the flow cell. Consequently, the light from this LED illuminated the flow cell, passed through the objective, and then passed through the dichroic mirror upstream.

- A standard mirror was then placed past the dichroic mirror so as to reflect the imaging light up the side of the table where we had room for our imaging lens and camera.

- The imaging lens was placed at an arbitrary distance away from the standard mirror and then the camera at one focal length away from this lens.

- The image was observed with a white piece of paper before and after the standard mirror. The standard mirror was rotated until the image was positioned on the camera. We used the white paper to help us align the image here.

- At this point, we observed concentric rings on the TV. The lens was now rotated, translated, and vertically adjusted by hand until the bright center of the rings was seen near the center of the TV. If the voltage on the MCL stage was changed, two bright centers were seen displaced by a small distance with concentric rings surrounding each center.

- Finally we placed the IR cut-off filter in front of the camera. The rings disappeared and the image of the flow cell and its contents were fairly clear.

- We separated the traps, then flowed beads into the flow cell and captured a bead in one or both traps. Then we translated and rotated the lens until the beads were well focused. If beads could not be seen, or only a faint circular shadow was observed, then it was possible that the flow cell was too far from or too close to the objective. If so, we translated the flow cell along the beam axis using the micrometer on the stage followed by adjustments of the imaging lens until the beads could be seen.

- Now the strength of the trap could be tested. This was simply done by vigorously moving the beads about in the flow cell. If the beads fell out of the traps easily, then the objective was clearly not very well aligned with the beam path. It was also important to check the strength of both traps because it was possible for one trap to be very strong while the other was weak. In the case of our system, a well aligned objective should yield traps that appear equally strong, which would be identified if beads in both traps flew out at the same time while moving them about vigorously. The actual maximum force that the traps could sustain before the beads flew out of the traps could only be determined after the rest of the setup was complete, however, one who is experienced with using another tweezer
setup can identify through this vigorous motion testing whether the traps were sustaining sufficient force to justify moving forward with the rest of the building process.

- When the strength of the traps was satisfactory, then we first locked the stage micrometers with an Allen wrench (just like we did for the upstream mirror mounts). Next we drew a line in pencil on the table around the perimeter of the base mount for the flow cell stage. Afterward the LED and flow cell stage was removed. If the trap strength was unsatisfactory, then the entire process of aligning the stage was repeated. In the case of an unsatisfactory alignment, it is likely the stage was moved while clamping or some power measurements during the alignment may have been falsely determined, which could have occurred if they stage alignment tool wasn’t securely attached to the objective mount via the double sticky tape during each measurement.

### 8.2.2.14 Positioning and Aligning Objective Stage #2

- We first reset the MCL stage voltage back to -9.5V.
- The rail, two objective mounts, and objective were attached to objective stage #2 just as they were for #1. The micrometer that translates the stage along its long axis was then turned until the stage had translated approximately half the micrometer’s maximum distance. The stage was then positioned and aligned by eye, which is to say the long axis of the stage was aligned with the pencil line and the entire stage was translated until the surface of the objective was ~1mm from the surface of the flow cell. The stage was then temporarily clamped to the table. A drop of water was squirted into the gap between the flow cell and this objective. The stage was then translated along the beam axis and perpendicular to it until a circular beam of IR light emerged, which could be seen on an IR card or off a white surface that was imaged with a CCD camera. See Figure 8.37.
- The stage was then translated along the beam axis until the outgoing beam appeared roughly collimated by eye. If the stage axis was not parallel to the beam axis, which it likely was not, then such translations were likely to yield clipping of the beam, so translations perpendicular to the beam axis were probably performed as well. If this rough collimation could not be achieved without crashing objective #2 into the flow cell, then it is likely that the flow cell was too far downstream of objective #1. If so, we would have slightly translated the flow cell upstream using the micrometer on the flow cell stage. This would have solved this issue. Keep in mind that this was only an initial rough alignment so as to roughly determine the position of the stage along the pencil line.
- When the outgoing beam roughly appeared to be collimated, an L-bracket was bumped up against the objective side of the stage and clamped to the
Two ball tip kinematic stoppers were then positioned against the stage and clamped to the table in the same way as was done with the first stage. The stage was then translated downstream via the stage’s micrometer until there was sufficient clearance for removing the objective. Once clear, objective #2, the flow cell stage, and objective #1 were removed. Stage #2 was then translated back upstream until it bumped up against the L-bracket again. Lastly, the power meter was positioned and clamped to the table a couple inches downstream of stage #2 and the clamps on the stage were removed.

- The objective axis of this stage was now aligned with the beam axis in the same fashion as was done for stage #1. That is, performing vertical and lateral translations and rotations of the stage while measuring the outgoing power of various halves of the objective mount holes. For more detail, see above step titled **Positioning and Aligning Objective Stage #1**.
- When the alignment was complete, the stage was clamped to the table in the same fashion as described for stage #1. An L-clamp was bumped up against the objective side of the stage again and clamped to the table. The stage was then translated back downstream so as to provide enough clearance to install both objectives. The objective lenses were screwed into the objective mounts and stage #2 was translated back upstream until it bumped up against the L-bracket. At this point the L-bracket and ball tip kinematic stoppers were removed.

### 8.2.2.15 Collimating the Beam after Stage #2

- First the flow cell mount was positioned within the pencil outline traced earlier. This mount was then be very securely screwed to the table. The flow cell stage was set on top of this and lightly screwed down. If all the micrometers were still set as they were when we used this stage earlier, then the flow cell should be displaced from objective #1 by approximately the same distance when it was slid into place. Next, the flow cell mounting bracket was very slowly lowered so that the flow cell slipped into the gap between the objectives without touching either. The mount was then screwed to the stage. Since the stage was only lightly screwed to its base mount, it was slightly rotated until the flow cell was parallel to the surface at the end of the objective. Once achieved, the stage was very securely tightened to the base mount.
- Drops of water were now squirted into the gaps between the flow cell and the objectives.
- We now placed a new iris and mount a few inches downstream of stage #2. The beam should still follow the pencil line, but if it wasn’t following it exactly, then we simply repositioned the mount and adjusted the iris height until the beam passed through the center of the iris.
Then, as we had done previously, we mounted an IR reflective mirror at the end of the table, just before the iris mount that was still there from an earlier alignment. Note: This iris mount was left in place so we knew the original beam path for the cases where we had to disassemble portions of the system and rebuild.

Also as before, an iris mount was positioned at the adjacent corner. The iris (with a collar to hold its height) just positioned two steps prior was placed in this mount and the mirror was adjusted until the beam passed through the center of the iris.

The beam was then collimated in same manner as described when we collimated telescope #2 but in this case stage #2 was translated along the beam axis by turning the micrometer a very small amount with an Allen wrench.

Lastly, the stage micrometers were locked with an Allen wrench just as we did for the upstream stage and mirror mounts.

8.2.2.16 Positioning and Aligning Dichroic Mirror #2 and the Imaging Optics

I don’t remember the exact details of how we performed this stage of the setup, but what follows is what I can recall. Note that the arrangement of imaging optics here was designed according to the method known as Koehler illumination.

First, we blocked the laser by placing a beam block between the two lenses of telescope #2.

Next, we roughly measured the new beam height, which was now slightly greater than the original planned height of 3.675”. Then we adjusted all the imaging optical elements used from the LED to stage #2 such that their centers were approximately at this beam height. For the LED, we adjusted it such that the long axis of the bulb was parallel to the table and that the bulb was positioned at this height from the table surface.

Since we chose to do one-to-one imaging where the lens was positioned $2f_{\text{lens}}$ from the BFP of objective #2, this implied that the LED also needed be placed $2f_{\text{lens}}$ from the lens. To begin, we roughly positioned the dichroic mirror downstream of stage #2 as close as possible while still providing enough room to insert the Allen wrench into the micrometer that translates stage #2 along the beam axis. For convenience, we chose to align the imaging optics along a line of bolt holes. So, the dichroic mirror base mount was aligned with and bolted down along a line of bolt holes. Also in positioning this mirror, we aligned it to the best of our ability by eye so that it was at a 45° angle with the IR beam and that the IR beam hit the center of the mirror. See Figure 8.38.

The distance the lens needed to be from the dichroic mirror was determined by first measuring the distance between the center of the dichroic mirror and the BFP of objective #2. This distance was then...
subtracted from \(2f_{\text{lens}}\) and lens was roughly placed this distance from the dichroic mirror. It was also oriented perpendicular and aligned (as best as possible by eye) with the line of bolt holes.

- Next the LED was temporarily placed at an arbitrary distance from the lens and oriented such that the bulb’s axis points down the line of bolt holes.
- Now, the LED makes an image that looks like the letter “H”. While holding a piece of lens paper over the surface of the lens, we adjusted the knobs on the LED mount until the “H” appeared laterally symmetric.
- Next we taped a piece of lens paper over the hole of the upstream objective mount on stage #2.
- Note that while the light from this LED is not a collimated beam like the laser, we get the best imaging if we align the imaging optics as if the LED did produce a collimated beam of light.
- We held a piece of lens paper over the hole of the downstream objective mount on stage #2 and translated dichroic mirror #2 perpendicular to the IR beam axis until the “H” image appeared to be centered over this hole. Once done, we clamped the mirror to the table and uncovered the downstream objective mount. This translation was performed by simply sliding the mount along the bolts (slightly loosened of course) that are used to secure the mount to the table. See Figure 8.38.
- Now we slowly rotated the mirror until the “H” image appeared to be laterally centered on the upstream objective mount. Note that the only way to rotate this mirror was to put a collar on the post to hold its height and slowly rotate the post within the mount. This was made a bit easier by having the post snuggly (Vs. tightly) tightened within the base mount, which allowed the mirror to be very slowly rotated.
- We continued alternating between translating and rotating this mirror until the “H” image was centered on both upstream and downstream objective mounts on stage #2.
- Now we remeasured the distance between the dichroic mirror and the BFP of objective #2. If the distance changed, then we carefully translated the lens along the line of bolts holes until it was at the appropriate distance from the BFP. This was done by sliding the base bracket of the lens along the straight edge of another bracket clamped to the table that was parallel to the line of bolt holes.
- Now the LED was unclamped from the table and a standard mirror was positioned at an arbitrarily chosen distance from the lens. It was positioned and oriented such that the incoming light coming from the center of the LED passed along a line of bolt holes and reflected off this mirror along the appropriate line of bolts holes. See Figure 8.39.
- The LED was positioned last with it centered along a line of bolt holes and placed from the mirror a distance = \((2f_{\text{lens}} - \text{distance between mirror and lens})\).
• The LED was adjusted laterally with the mounting knobs until it points straight down the line of bolt holes toward the mirror. At this point we held a piece of lens paper over the large downstream lens to view the “H” image. The tilt of the LED was adjusted until the “H” looked symmetric in the vertical direction.

• Now we translated the standard mirror vertically (doing our best not to rotate it) and perpendicular to the bolt hole line (the one parallel to the beams reflecting off this mirror) until the image of the “H” was centered on the large imaging lens. The lateral translations were done by sliding the base bracket along a flat edge on the table that is perpendicular to this same line of bolt holes or by sliding the base bracket along the bolts (on either side of the bracket) that are being used to secure the bracket to the table. See Figure 8.39.

• Then using the mirror mounting knobs, the mirror was tilted and rotated until the image of the “H” was centered on the lens paper covering the upstream objective mount.

• We continued alternating between performing these translations and rotations until the image of the “H” was centered on both the imaging lens and the upstream objective mount of stage #2. If at any point while alternating between these steps the “H” image became asymmetric, we adjusted the knobs on the LED until it appeared symmetric. Also, if the standard mirror was in fact translated, then the LED was also translated accordingly so that the distance between the LED and the imaging lens was 2f_lens.

• Realigning the downstream imaging optics: Now that the imaging optics after stage #2 had been aligned, the imaging optics previously mounted prior to stage #1 may have needed to be realigned. Note that this last stage didn’t require as much precision and accuracy as the rest of the tweezer setup and so the following steps did not have to be conducted as perfectly as described. We only had to position these optics until an image was produced on the screen that was satisfactory to the user. However, the following describes what we did to obtain the most desirable looking image of the beads and flow cell.

• Note that the imaging light that emerges from the objective is diverging. Ultimately we observed that this image was clipped a bit on the side by the large clamping arm of the mount for dichroic mirror #1. Fortunately this didn’t end up clipping the important portion of the image. As mentioned previously, the image, as it emerged from the objective, could be seen by holding a piece of white paper in the path of the light. While observing the flow cell image on this piece of paper, the flow cell was moved about until one of the capillary tubes was seen on the piece of paper.

• The standard mirror downstream of this was then rotated until the reflected image (viewed on this white paper) was reflected along a path that was parallel to the bolt hole lines on the table. The tilt of the mirror
was then adjusted until the reflected image was at approximately the same height at various distances from the mirror. See Figure 8.40.

- The lens that comes after this mirror was translated vertically and laterally until the image of the capillary tube was seen over the surface of the lens (seen with a piece of white paper or lens paper over the lens surface). This lens was also oriented perpendicular to the path of the reflected light. Note that the center of this lens should be at a height a bit greater than 3.675” from the surface of the table, which was the laser beam height after telescope #2.

- Now the camera was positioned so that its surface was \( f_{\text{lens#2}} \) away from the lens upstream from it. Its long axis was oriented as parallel as possible with the axis of the light that was reflecting off the standard mirror prior to this. Also, the height of the camera was adjusted so that the light emerging from this lens was producing an image on the surface of the camera (again, as can be seen with a white piece of paper).

- Now with the image of the flow cell projected onto the camera surface, adjustments needed to be made so that the image of the traps lied roughly at the center of the camera. As before, without the IR filter in place, concentric rings were observed on the TV. First we changed the MCL voltage so that the traps were displaced from one another and then translated the lens perpendicular to the path the light was following until the center of these concentric rings were seen on the TV. Such lens translations also required that we translate the camera because translations of the lens caused the image to fall off the detecting surface of the camera. We continued these translations until the trap centers were laterally centered on the camera surface (or TV screen). Then, we performed the same vertical translation of the lens and the camera to get the traps centered vertically.

- Finally, we injected some beads into the flow cell, put the IR filter in front of the camera and captured some beads in the trap. The trapped beads looked blurry if the lens was at the wrong distance from the camera. To get a clear image of the beads, we translated the lens along the light path until a clear image of the beads was seen. Slight rotations of the lens were also performed in order to achieve symmetric looking beads on the TV.

- One last comment about the imaging. The “H” image that the LED produced was the brightest portion of the image. If the image on the TV appeared a bit dull, it was because the light of the regions outside of the “H” were illuminating the region in which the traps were located. If so, we adjusted the tilt and angle of the LED until the brightest portions of the LED image (i.e. the “H”) were illuminating the region where the traps were located.
8.2.2.17  Positioning and Aligning the Last PBSC

- First we made a pencil mark on the table for the position of the lens that images the BFP of objective #2 onto the PSD (~30cm downstream the BFP of objective #2) and then removed the beam block that was blocking the beam earlier while positioning and aligning the imaging optics. Also we reset the MCL voltage to -9.5V.
- Then we placed the PBSC at a position between this line and the dichroic mirror while leaving enough space for the size of the lens mount and clamps.
- The height and lateral position of the PBSC were oriented (by eye) so that the incoming beam hit the center of the cube’s incoming face (i.e. the face that has a dot on it from above).
- The alignment of the beam that reflects off this cube wasn’t done as precisely as with the rest of the system (i.e. through the use of irises), but it was still reasonably well aligned. For the alignment of the reflected beam we used the vertically mounted white foam board with crosshairs drawn to indicate the original beam height.
- First we adjusted the tilt of the cube so that the reflected beam was parallel to the table. We did this by moving the foam board between two positions on the table while tilting the cube until the beam appeared to be at the same height on the foam board at either position on the table.
- Next was to orient the cube so that the reflected beam was 90° to the incoming beam. This was done by choosing a path that was perpendicular to the incoming beam path, which wasn’t necessarily a line of bolt holes, but it was parallel to a line of bolt holes. Also, we didn’t end up drawing a line on the table to follow (though it probably would have made things easier). Instead we just memorized the distance between the vertical crosshair line on the foam board and a nearby line of bolts holes. See Figure 8.41.
- Now with this unmarked path, we moved the foam board back and forth between two positions while rotating and translating the PBSC until the beam was laterally centered on the vertical crosshair line. As in all other alignment cases, translations were performed until the beam was centered on the board when it was at its near position and rotations were performed until the beam was centered on the board when it was at its far position.
- Once the PBSC was aligned, we placed a beam block some distance away on which the beam could terminate.

8.2.2.18  Positioning and Aligning the Last Lens (Imaging BFP of Objective #2)

- We began by positioning an iris between the PBSC and the position the lens was to be placed. We made sure to leave enough room for the lens to
be clamped to the table. We then positioned another iris \( \sim 2f_{\text{LastLens}} \) (where \( f_{\text{LastLens}} = 15 \text{cm} \)) from where the lens was to be clamped. We adjusted and moved these irises until the beam was centered on their eyes. See Figure 8.42.

- Now, just as we did for the lenses in telescope #2, the lens was put in place (the mark made on the table in an earlier step), after which we rotated and laterally translated it the passing beam was centered on the downstream iris while the reflected beam was centered on the upstream iris.
- The irises were removed once this lens was aligned.

### 8.2.2.19 Positioning and Aligning the PSD

- The procedure of putting in the PSD was almost exactly the same as when finding the image of the MCL stage mirror earlier except that here we were positioning the PSD at the image of the BFP of objective #2.
- First the expected position of objective #2’s BFP was marked on the table in pencil. Then an attenuator was positioned at an arbitrary position after the lens (except for at the lens’s focal point) and angled so as to deflect back reflections from reentering the laser. See Figure 8.43.
- At this point the PSD was placed over the pencil mark for its position (centering it by eye) and the same method used earlier to make the PSD perpendicular to the beam path was implemented.
- And lastly, as before, the PSD was translated back and forth along the beam path while we scanned the MCL stage mirror back and forth until the measured Vx deviation was nearly zero. The PSD pedestal was then temporarily clamped to the table while another straight edged bracket was put in place so that the PSD could be translated perpendicular to the beam path. Once the clamp was in place, any clamp that could interfere with these perpendicular translations was removed and the PSD was unclamped. The PSD was then translated until Vx measured as close to zero as we could manage. When this point was found, the PSD was very securely clamped to the table.

### 8.2.2.20 Final Comments

- After completing the build, we noticed on the TV that the traps were offset vertically by a small amount. This was expected because others’ experience has shown that this always happens. I believe Tom Perkins told us in an e-mail that we should expect this. We made an effort to reduce this displacement by adjusting the tilt of the dielectric mirror off which the stationary beam reflects. There was some concern that doing this might affect some aspect of the trap (such as its strength), but fortunately we were able to move the trap so that the beads were at the
same height without causing any noticeable differences to the stationary trap. Incidentally, the movable trap traversed horizontally across the TV screen as desired (Vs. diagonally).

- After we agreed not to make any additional changes, I built a box out of 0.5” thick, opaque white, HDPE (high density polyethylene) with three main chambers and hinged doors that opened from above. The box was designed to be screwed together with bolts so that it could be disassembled without having to disassemble any of the setup. The inside walls of the box were lined with black anodized aluminum foil, the box was assembled around the system, and then clamps were put in place around the box to prevent it from sliding about. A dielectric mirror was also put in the first chamber to reflect the opto-isolator’s dumped beam out of a hole near the corner of the box and onto a beam block. The hole was covered by a thin piece of clear Plexiglass. In addition, a couple small notches were cut out of the bottom of the wall near the PSD through which we feed in the laser cable and the wires for the LED, Camera, and PSD. See Figure 8.43.

- Another smaller box, which was taped together with foam tape and assembled out of thin clear Plexiglass, was created to cover the square of optical components where the beam was split and recombined. Such a box was built because the gaps between each element were too small to insert tubes that protect the beam from air currents. The inner walls of this box were also lined with the black anodized aluminum foil, and so, I made special rails, which clamped to the table, that allowed us to slide this box straight down over this square without disturbing any of the optical elements.

- We cut pieces of the anodized aluminum tubing to fit between all the optical elements. A small hole was drilled in each piece and then these holes were threaded. We then used normal mounts and 0.5” aluminum posts to hold these tubes in place. In any case when possible where gaps were still present, anodized aluminum foil was taped to the tubing to close off such gaps (such as the gap between tubes on either side of a lens). In addition to this aluminum tubing, a specially cut piece of card stock was attached to the large visible light imaging lens to reduce air current in the space between this lens and the adjacent dichroic mirror (#2). Also, the beam that reflected off the attenuator at the end of the system passed through a hole that we cut in the side of the aluminum tube upstream from this optical element. The hole was covered with transparent tape. This was similarly done for the beam dumped by the opto-isolator.

- Some makeshift clamps were attached to the table that kept the box doors closed tightly and that held the entire box tightly down onto the table.

- The custom fluidics assembly was installed last.
8.3 Addendum – Installation of Second PSD for Differential Detection

After completing the build as described in Sections 8.1 & 8.2, we tested this single detection system and found it to be more stable and to have greater resolution than our previous system, however, there was still some interest to achieve greater resolution. So we decided we would install a second PSD to detect deflections of the bead in the movable trap so that we could implement the differential detection technique introduced by Moffitt et al. in 2006. This decision meant backtracking slightly. Because we would now be measuring deflections in the movable trap, it became important that the movable beam pass through and reflect off very stable optical components downstream of the second object. This meant remounting the PBSC downstream of the second objective onto a 1” diameter pedestal and on a stable PBSC mount. Because this resulted in physical interference with nearby optical components, we also had to realign the optical components for the first PSD as well (i.e. the PSD for the stationary trap). A simple and detailed diagram of this new arrangement can be seen in figures 8.44 & 8.45.

8.3.1 Components and Instrumentation

8.3.1.1 Position Sensing Detector (PSD), Attenuator, and Filter

We purchased a second PSD identical to the one purchased for the stationary trap. That is an On-trak PSD with model # PSM2-10 (housing and detector; Model # of detector only: 2L10SP) and an amplifier with model # OT-301. Additional details about the can be found in the PSD listing in Section 8.1 of this document. Upon testing the new PSD with the amplifier gain set to 1, we discovered that the new PSD had greater sensitivity than the other PSD. As such, we were unable to use the same attenuator used prior to PSDstationary for the PSDmovable. When we tried, Vsum of PSDmovable was still greater than the maximum allowable Vsum (6V) for accurate Vx readings. Therefore we had to purchase a different attenuator. We chose a 1” diameter Thorlabs reflective ND filter with an optical density (OD) of either 0.5 or 0.6 (we can’t remember, though it was probably 0.6 since this PSD is more sensitive than the PSD used for the stationary trap); Model: ND06B while setting the PSD amplifier to 1. It can be found here: http://www.thorlabs.com/thorproduct.cfm?partnumber=ND06B

The use of this attenuator led to Vsum being ~5.2V. Similar to the attenuator for PSDstationary, this attenuator was mounted in a Thorlabs 1” diameter LMR1 lens mount, which was then mounted on a standard Thorlabs 0.5” aluminum post and base bracket. As was done with the PSDstationary, we inserted a Thorlabs 1” diameter 1064nm band pass filter into the PSD mount to keep ambient light from affecting our measurements. A rubber ring was inserted into the PSD mount so that the filter would fit snugly without rattling after the outer threaded ring was tightened in place. Part #: FL1064-10.

Also of note, the amplifier has an internal filter with a 15kHz bandwidth, thus rejecting all frequencies above this threshold. This is mainly important for the purpose of these high resolution dual-trap position clamp experiments. In contrast, an additional low-pass filter is connected to the amplifier for force clamp experiments (see Low Pass Filter in Section 8.1).
8.3.1.2 Mirrors, Mounts, and Pedestals

For remounting the PBSC, a new Newport Pint-Sized Prism Mount (model #9481) was mounted on a 1” diameter, 2” tall stainless steel pedestal (as used elsewhere in the system) with a 1/8” and a 1/16” in spacer. The attenuators were mounted in ThorLabs LMR1 mounts on standard Thorlabs 0.5” mounting posts. The PSDs were mounted on the 1” diameter, 2” tall stainless steel pedestals, which were rethreaded to allow ¼-20 screws, and slightly different combinations of spacers that led the center of the PSD to be as close as possible to the beam height (I don’t recall the exact choice of spacers used here, but it isn’t too important. The spacers used for the two PSDs were slightly different.). Lastly, a 1” metallic mirror was mounted in a left handed Suprema Clear Edge Mirror Mounts (stainless steel) with two 127-TPI locking actuators, model #SN100C-R2K-LH. Since all such components used here were used earlier in the system, additional information about these components can be found in Section 8.1 of this document.

8.3.1.3 PSD Wiring To DAQ Card

To maximize the resolution, we wired the PSDs differently to the DAQ card when operating in differential detection mode. As suggested by the “Field Wiring and Noise Considerations for Analog Signals” document on the National Instruments website\(^\text{11}\), we wired the PSD amplifiers to the NI DAQ card using differential wiring (check this) with bias resistors (37kΩ).

When acquiring data with a DAQ card at a high sampling rate, the slew rate, the card’s maximum rate of change of output voltages per unit time, becomes an important consideration. The smaller the difference in voltages input to the DAQ card, the faster the data can be accurately sampled. To account for the slew rate, we minimized the voltage changes between channels by only using two (adjacent) channels, one for each PSD, and by reversing the polarity of the wires from the second PSD. When the beads move toward one another due to some biological activity, the PSDs measure bead deflections in opposing directions. That is, if the bead deflection images are well centered on the PSDs, then such an event will lead one PSD to measure positive deflections while the other measures negative deflections. By changing the polarity of one of the PSD voltage inputs, the voltage differences between signals becomes significantly reduced.

8.3.2 Building Procedure

8.3.2.1 Positioning and Aligning PBSC

- The last PBSC was positioned and aligned is a similar manner as was previously done in section 8.2.2, step 17, except with a few extra steps for better alignment of the reflected beam.
- First, an iris needed to be positioned at the end of the beam path for the stationary beam. By the same method used earlier in the building procedure,
which is an iterative process of translating the iris perpendicular to the beam axis, rotating the iris within its bracket, and adjusting the height until the beam was centered with the beam iris eye. The iris translations were performed by first clamping a flat edged bracket to the table with the flat edge perpendicular to the beam axis. The flat edge of the iris bracket could then be slide against this clamped bracket to permit straight translations. Once centered laterally, the iris was securely clamped to the table, after which the iris could then be adjusted vertically and rotationally. The height was adjusted by eye until the beam was centered with the iris. The iris was rotated by eye until it appeared to be as perpendicular to the beam axis as possible. Height and rotation adjustments sometimes showed that the lateral position was off, in which was additional translations were performed.

- The PBSC, which is now mounted on the pint-sized prism mount, a 1” diameter, 2” tall stainless steel pedestal, and the appropriate spacers, was roughly positioned where it was previously placed and such that the beam is centered on the PBSC. Since we are now using different mounting components, we had to make sure it was distanced sufficiently far from the place where the downstream imaging lens so that enough table space is available putting an iris between the PBSC and this lens as well as for all the necessary clamps for these optical components. Consequently, the reflected beam will not necessarily pass down a line of bolt holes.

- Because the reflected beam didn’t pass down a line of bolt holes, the PBSC was rotated using the custom pedestal alignment tool (Figure 8.16) until the beam was parallel to a line of nearby bolt holes. This was done using the foam board tool (Figure 8.12) as described in section 8.2.2, step 17. See Figure 8.41.

- Along this beam path, two irises are positioned, one past the planned position of the mirror, which reflects the beam toward $\text{PSD}_{\text{movable}}$, and another midway between the PBSC and the planned position of the mirror. These irises are translated and rotated until the beam is centered with their iris eyes. Once centered, the mounts are securely clamped to the table and the irises are removed.

- The reflected beam was then made parallel to the table surface. This was done by first adjusting the height of an iris at the nearby iris mount until the beam was centered with the iris, after which a collar was clamped to the iris post to hold retain its height position. This iris was then moved to the distant iris mount, where the PBSC mount was then tilted until the beam was centered with this iris. The iris was then moved back to the nearby iris mount and this iris swapping procedure is repeated until the beam is centered with the iris at both positions without needing to adjust the iris height or rotate the PBSC mount. See Figure 8.46.
8.3.2.2 Positioning and Aligning the Imaging Lens and PSD for Stationary Trap

- The stationary trap imaging lens and the PSD were aligned just as described in section 8.2.2, steps 18 & 19.

8.3.2.3 Positioning and Aligning the Mirror for Movable Trap

- Since the plastic box that covered the entire setup had two septums/walls running along the long axis of the box that created three rectangular chambers of equal size, the mirror was positioned roughly centered in the middle chamber. Its exact position is somewhat arbitrary. The only criteria is that there is sufficient space for an attenuator between the mirror and planned position of the PSD. To make things easier, we chose to position the mirror such that the reflected beam would pass down a line of bolt holes. With a straight edge, we drew a line in pencil down a line of bolt holes and centered two iris mounts on the line, one at the end of the beam path on the table and the other roughly halfway in between. See Figure 8.47.

- At this point the mirror needed to be tilted so that the beam would be parallel to the table surface. We did so by placing the iris from the earlier step that has the collar in the second iris mount. We then positioned the mirror such that the beam was centered on the mirror and that the reflected beam would pass down the line of bolt holes. The mirror was then translated (with the custom alignment tool) in the direction of the incident beam until the reflected beam was horizontally centered on the iris. The mirror was then tilted (using the mirror actuators) until the beam was vertically centered on the iris. These two steps were repeated until the beam was centered on the iris.

- The mirror then needed to be adjusted so that the reflected beam followed the bolt hole line. The iris was first moved to the mount closer to the mirror. We then translated (with the custom alignment tool) the mirror along the axis of the incident beam until the reflected beam was centered with the iris. After this we moved the iris to the far mount and rotated (with the mirror actuators) the mirror until the beam was centered with the iris eye. This translation/rotation procedure was repeated until the beam was centered on the iris at both positions without needing to adjust the mirror any further. Once completed, the mirror was securely clamped to the table and the actuators were locked. See Figure 8.47.

8.3.2.4 Positioning and Aligning the Imaging Lens for Movable Trap

- First, along the reflected beam path, we marked the position for the imaging lens, which is 30cm from the BFP of objective #2. We then followed the procedure in section 8.2.2, step 18 except that, rather than inserting another iris downstream, we used the iris and distant iris mount that was used in step 3 of this section (8.3.2). When complete, the iris and mount between the PBSC
and the lens was removed. The iris mounts were removed afterward. See Figure 8.48.

8.3.2.5 Positioning and Aligning PSD for Movable Trap

- The PSD for the movable trap was positioned and aligned in the same way as the PSD for the stationary trap, which is described in section 8.2.2, step 19.

8.4 Additional Figures
Figure 8.6: Simple cartoon image of optical tweezers layout used here (without second detector).
Figure 8.7: Detailed cartoon image of optical tweezers layout used here (without second detector).
Figure 8.8: Custom mount design for MCL mirror stage.
Figure 8.9: Custom mount design for opto-isolator.
**Figure 8.10:** Custom V-block mount design for clamping the laser head.
**Figure 8.11:** Additional view of custom V-block mount design for mounting the laser head. The top plate for clamping is absent in this image.

**Figure 8.12:** Foam board tool. This tool is used for aligning vertical and lateral positions of the laser beam.
Figure 8.13: Diagram for building step 1 of section 8.2.2

1. Iris centers need to be centered with the pencil line.

2. Since beam does not pass through the center of the irises, the V-block needs to be translated and/or rotated until it passes through the center of the 1st and 3rd irises.

3. If beam is not centered with 2nd iris after step (2) above, this iris must be translated perpendicular to the beam path until it is centered.
Figure 8.14: Methods for rotating and translocating the laser head V-block mount for alignment.
Figure 8.15: Diagram for building step 2 of section 8.2.2

[Diagram of a series of objects, possibly representing a step-by-step process or a diagram of a system, with a note: "Opto-isolator positioned and oriented at an angle to prevent back reflections of the opto-isolators incoming surface from reentering the laser collimator but also so that the beam does not get clipped."]
Figure 8.16: Custom pedestal alignment tool. Used for aligning the cylindrical pedestal mounts.
Figure 8.17: Diagram for building step 3 of section 8.2.2

Lens translated until the outgoing beam follows original beam path, i.e. center outgoing beam as best as possible on the center of the downstream irises.
Figure 8.18: 2nd diagram for building step 3 of section 8.2.2

1. Lens translated perpendicular to beam path until outgoing beam is centered with the two downstream irises (2nd and 3rd irises). Note: A bracket clamped to the table and the custom pedestal alignment tool are used for this translation. Step (2) above shows how this tool is used to translate the lens along the beam path for beam collimation.

2. Lens translated until the outgoing beam is collimated.
Figure 8.19: Diagram for building step 4 of section 8.2.2

Rotate and translate the pedestal upon which this beam splitter and PBSC mount rest on until the reflected beam is centered on both irises. Centering on the irises will also require that the PBSC mount will need to be tilted to get the height correct.
Figure 8.20: Diagram for building step 5 of section 8.2.2

To translate the MCL stage "up", we would first slide the stage mount along the bracket in the direction of the arrow shown. Then, after temporarily clamping the stage mount to the table, another flat edge could be clamped to an adjacent side on the MCL mount and translated (see next figure).
Figure 8.21: 2\textsuperscript{nd} diagram for building step 5 of section 8.2.2

Now with this flat edge bracket against the MCL stage mount, the stage can be translated in the direction of the arrow shown. The resulting net translation after this two 45° translation is a translation “up” along the incoming beam.
To account for the downward beam deflection due to the PSSCs, the stationary mirror tilt is adjusted. But because the irises are laterally centered on the beam reflected off PSSC #2, the iris mounts need to temporarily be translated so that the irises are in alignment with the beam reflecting off the stationary mirror. Once done, one of the irises is temporarily removed and the other is switched back and forth while the mirror tilt is adjusted and the iris height is changed. The new beam height is acquired when the beam passes through the center of the iris at either mount.

After the new height is established, we reinsert an iris into the empty mount and adjust its height until centered with the beam. We then translated the irises back into place (i.e., when the iris mounts are centered on the pencil line on the table).

**Figure 8.23:** Diagram for building step 7 of section 8.2.2
Figure 8.24: 2nd diagram for building step 7 of section 8.2.2

With the irises now recentered along the pencil line, we rotated and translated the stationary mirror until the beam was centered with both irises. Rotations are performed by either using the knobs or by rotating the entire pedestal (depending on how much the mirror needs to be rotated).
Figure 8.25: 3\textsuperscript{rd} diagram for building step 7 of section 8.2.2
Figure 8.26: Diagram for building step 8 of section 8.2.2

1. Iris mount is translated until centered with the movable beam now shooting onto it.

2. Readjusting the beam height of the movable beam. Alternate between tilting the first and second PBSC mounts where the first is tilted until the beam reflecting off PBSC#3 is centered on the first iris, while the second is tilted until the beam is centered on the second iris.
Figure 8.27: Diagram for building step 9 of section 8.2.2
Figure 8.28: Diagram for building step 10 of section 8.2.2
Figure 8.29: Diagram for building step 11 of section 8.2.2
Figure 8.30: 2nd diagram for building step 11 of section 8.2.2
Figure 8.31: Diagram for building step 12 of section 8.2.2

L-clamp oriented to a line perpendicular to the beam axis (determined using a L-square ruler). This clamp is firmly clamped to the table. The long side of the L-bracket is coming out of the page and pushed flat against the back of the PSD.

1. L-clamp clamped to table first
2. Typical base bracket (with 90° angles) pushed against L-clamp and secured to table
3. PSD pushed up against L-clamp and translated until PSD reading is ~0V. Pedestal is then temporarily clamped to the table.
4. & 5. Custom pedestal alignment tool temporarily clamped to pedestal. Then base bracket (4) is pushed against base bracket (2) and the side of the pedestal alignment tool all while the screw on this tool is loosened and tightened until all the edges of the brackets/tools are flush with one another. Once done, base bracket (4) is secured to the table and the alignment tool is secured to the pedestal. Lastly the L-clamp and bracket (2) are removed and the clamp on the pedestal is removed so PSD can be translated along the beam axis.
Figure 8.32: 2\textsuperscript{nd} diagram for building step 12 of section 8.2.2
Figure 8.33: Custom stage alignment tool.

Stage Alignment tool. This tool is inserted (it is not threaded) into the objective mount and secured by double sticky tape. Depending on which quadrant is being checked, the tool is rotated until the yellow line on the tool is matched up with the yellow line on the objective mount. A half circle aluminum plate is taped to the surface to block light from exiting this half of the hole.

Objective mount. It attaches to the dovetail rail on the stage and the objective screws into it.

Apply double sticky tape to this surface so that it will hold this tool securely but temporarily to the objective mount.
Figure 8.34: 3rd diagram for building step 12 of section 8.2.2

1. Stage alignment tool swapped between these two objective mounts while the laser side half of the mount holes is blocked by the thin metal plate on the alignment tool.
2. Power is measured each time the alignment tool is swapped.
3. If the measured power at each partially blocked mount is not equal, then the stage must be rotated until it is equal. This is done by putting pressure on the corner shown and then carefully and slowly rotating the stage by hand as shown.
1. The partially blocked stage alignment tool is rotated so that the power passing through the laser-side half and objective-side half can be measured.

2. If the power is unequal, then the stage is translated until they are equal. This is performed by first applying slight pressure on the base of the stage toward the table so the stage doesn’t move while adjusting the micrometer for translation. The Allen wrench that has been inserted into the micrometer is rotated slightly and then the power on each half of the same objective mount is measured again. This process is repeated until the power measured on either half of the objective mount is equal.

Note: After repeating the procedures described in figures 16a&b to the point of completely aligning the stage, the stage will need to be clamped to the table. The clamps should be positioned near the corners of the base and repeatedly tighten in an X-like fashion where each clamp is tightened only a slight amount each iteration. See figure on right.

**Figure 8.35:** 4th diagram for building step 12 of section 8.2.2
**Figure 8.36:** Diagram for building step 13 of section 8.2.2
3. Stage temporarily clamped to table and translated downstream a sufficient distance for removing the objective.
4. Remove both objectives #2, outline base of flow cell stage on table in pencil and remove flow cell stage, then remove objective #1.
5. Translate stage #2 back upstream until it bumps up against the L-bracket. Put two ball tip kinematic stoppers in place as shown, then unclamp the stage and the L-bracket from the table.
6. Place a power meter after stage #2 and align and then clamped to the table in the same fashion as for stage #1.
7. L-bracket is bumped up against the objective end of the stage again and clamped to the table. The stage is then translated downstream after which the objectives are screwed in.
8. The stage is then moved back upstream until it bumps into the L-bracket. The L-bracket and ball tip kinematic stoppers can then be removed.

Figure 8.37: Diagram for building step 14 of section 8.2.2
Figure 8.38: Diagram for building step 16 of section 8.2.2
Figure 8.39: 2nd diagram for building step 16 of section 8.2.2

LED centered along bolt hole lines. It is tilted and rotated so that the “H” image seen downstream on the imaging lens is symmetric. Also, if the mirror ends up being translated, this is translated until the total distance between this LED and the imaging lens is still $2f_{\text{up}}$.

Standard mirror is translated vertically and laterally until “H” image is centered on the surface of the imaging lens. It is then rotated until the image is centered over the hold of the upstream objective mount on stage #2.
**Figure 8.40:** 3rd diagram for building step 16 of section 8.2.2
Figure 8.41: Diagram for building step 17 of section 8.2.2
Figure 8.42: Diagram for building step 18 of section 8.2.2
Figure 8.43: Diagram for building step 19 of section 8.2.2
**Figure 8.44:** Simple cartoon image of optical tweezers layout used here (with second detector).
Figure 8.45: Detailed cartoon image of optical tweezers layout used here (with second detector).
Figure 8.46: Diagram for building step 1 of section 8.3.2
Figure 8.47: Diagram for building step 3 of section 8.3.2

2. Mirror is tilted until beam is centered on this Iris, which is then used as a marker.

3. Mirror is rotated until reflected beam is centered on this Iris.

4. Iris from left mount, with collar, is put in this mount where mirror is then rotated with its actuators until reflected beam is centered on this Iris.

1. Iris mounted aligned with the beam to assist with alignment of the lens.
Figure 8.48: Diagram for building step 4 of section 8.3.2
8.5 References for Chapter 8


