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Sullivan, Benjamin David

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Nanoscale Optoentropic Transduction Mechanisms

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Bioengineering

by

Benjamin David Sullivan

Committee in charge:

Professor Michael J. Heller, Chair
Professor Sadik Esener, Co-Chair
Professor Dennis Carson
Professor Xiaohua Huang
Professor Geert Schmid-Schönbein

2007
The Dissertation of Benjamin David Sullivan is approved, and it is acceptable in quality and form for publication on microfilm:

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Co-Chair

Chair

University of California, San Diego

2007
To Mom, Dad & Amy,

Thanks for putting up with me for the past ten years.

I promise to smile more
“Controls, controls, controls.”

- David A. Sullivan
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ABBREVIATIONS

FRET.................................................................fluorescence resonant energy transfer

PCR.................................................................polymerase chain reaction
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VITA

1993  Diploma, Acton-Boxborough Regional High School

1997  Bachelor of Science Biomedical Engineering, Summa Cum Laude
       Boston University

1997-2001  Research Engineer, Schepens Eye Research Institute
           Harvard Medical School

2001-2003  Teaching Assistant, Department of Bioengineering
           University of California, San Diego

2003  Master of Science, University of California, San Diego

2004-Present  Chief Scientific Officer, OcuSense, Inc.

2007  Doctor of Philosophy, University of California, San Diego

PUBLICATIONS

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Dehlinger DA, Sullivan BD, Esener S, Heller MJ. Electric-field-directed assembly
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Bioderivatized Nanoparticles”, J. Assoc. Lab Automation, accepted for
publication, October 2007.

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7,051,569 Systems and methods for calibrating osmolarity measuring devices
7,111,502 Systems and methods for reducing the effect of corruptive signals during nanoliter osmometry
7,204,122 Systems and methods for calibrating osmolarity measuring devices

BOOK CHAPTERS


ABSTRACTS


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FIELDS OF STUDY

Major Field: Engineering (Bioengineering)

   Studies in Biomedical Engineering  
   Professor Charles DeLisi, Boston University

   Studies in Neural Networks  
   Professor Robert Hecht-Nielsen, University of California, San Diego

   Studies in Ophthalmology  
   Professor David A. Sullivan, Harvard Medical School
ABSTRACT OF THE DISSERTATION

Nanoscale Optoentropic Transduction Mechanisms

by

Benjamin David Sullivan

Doctor of Philosophy in Bioengineering

University of California, San Diego, 2007

Professor Michael J. Heller, Chair

Professor Sadik Esener, Co-Chair

Detection of rare, single base pair mutations of genomic DNA within complex biological samples requires exceedingly specific detection methods. All known molecular amplification protocols have been shown to be compromised by the thermodynamics of hybridization. The current dissertation explores bioelectromechanical analogues of information theory in an attempt to create a noise-tolerant detector. Specifically, modulation of intermolecular DNA fluorescent
resonant energy transfer (FRET) systems are used to transmit repetitive codes across a noisy channel. By exploring the optical transduction of the free energy of hybridization into a time-varying signal, we gather direct evidence for complementary, mismatched, and nonspecific binding of singly detectable nanoparticle constructs. Electrophoretic actuation was used to drive molecular transduction systems. Responses were observed using a custom built epifluorescent microscope through continuous and co-modulated detectors. FRET dynamics of two subsequences of the p53 gene revealed sequence and mismatch-dependent behavior. Theoretical models of modulation suggested that substantial improvements in specificity are achieved through eigenvector decompositions of modulated FRET signals.
Chapter 1: Introduction

1.1 Introduction

Cancer is genetic in origin. The onset, aggressiveness, and prognosis of the disease are governed by specific mutations within a given cancer [Kirk BW 2002, Kochethu G 2006, Nordgard SH 2007, Zheng HT 2005]. The efficacy and toxicity of applied chemotherapies are also correlated to genetic changes [Smith NF 2007].

The results of a 325 patient longitudinal study demonstrating such a correlation between prognosis and mutation in chronic lymphocytic leukemia (CLL) was reported in the New England Journal of Medicine in December 2000 [Dohner H 2000]. Patients were diagnosed as having B-cell CLL by a persistent lymphocytosis with greater than 5,000 lymphocytes per milliliter of blood, with over 95% of the leukemia classified as CD19+, CD5+ and CD23+. Using a fluorescent in situ hybridization panel of DNA probes, chromosomal abnormalities were identified in 268 of the 325 patients, while 52 patients showed no evidence of
aberration. The type of mutation was strongly correlated with the clinical severity of the disease and the time of survival. 17p deletions were the most severe, with a median survival time of 32 months, followed by the 11q deletion, 12q trisomy, undetected abnormalities and the 13q deletion, with survival times of 79, 114, 111 and 133 months, respectively. The severity of the 17p deletions also led to the fastest time to treatment (9 months), while those with milder karyotypes received treatment up to 92 months after diagnosis.

Could the physicians have improved treatment if they had been given this genetic information? There are a number of therapeutic options for CLL patients; for instance, the purine nucleoside analogs fludarabine, cladribine, and pentostatin are thought to induce apoptosis through either the nuclear p53/Bax, mitochondrial cytochrome c, or the extrinsic Fas/caspase pathway in rapidly dividing cells [Robak T 2006]. Supplementation with cyclophosphamide treatment inhibits DNA repair mechanisms, and has been shown to improve clinical outcomes of fludarabine alone [Eichhorst BF 2006, Wierda WG 2006]. There are also several emerging CLL therapies including the monoclonal antibodies alemtuzumab (anti-CD52) and rituximab (anti-CD20), as well as stem cell transplants and standard treatments such as chlorambucil and steroids [Brugiatelli M 2006, Fraser G 2007, Wierda WG 2006]. Further, since immunosuppressive CLL treatments often result in long term depletion of proper B cells, secondary side effects such as increased infections and the emergence of secondary tumors are common, and the decision to forgo or delay
chemotherapy in elderly or otherwise counter indicated patients is a valid treatment path to consider [Brugiatelli M 2006].

The mechanism of chemotherapy could be chosen according to each individual genotype. Patients with a 17p deletion lack p53 functionality; precluding the initiation of apoptosis as a viable treatment mechanism. A trial evaluating the efficacy of combination fludarabine and cyclophosphamide found that the majority of nonresponsive patients carried the 17p deletion prior to therapy [Eichhorst BF 2006]. By contrast, recent small scale trials evaluating the targeted antibody alemtuzumab showed that a percentage of patients responded favorably to this type of treatment, although remission only lasted between 3-17 months [Lozanski G 2004]. Similarly, favorable responses to flavopiridol, a cell-cycle inhibitor, were shown in 5 of 12 patients with 17p deletions [Byrd JC 2004]. These data led an to independent conclusion by Grever, et. al., that: “selection of therapy based on high-risk genomic features represents an appropriate treatment approach supported by currently available published data,” especially for allogenic immune therapy or other investigational agents.

Unlike many disease states where only a few therapeutic modalities exist, CLL has an abundance of options to choose from. An unambiguous genotyping assay would seem to have clear utility within this disease. Yet a recent panel on the management of the disease was quite clear in its ambivalence for late-stage genotyping:
The identification of recurrent chromosomal abnormalities, as demonstrated by fluorescence in situ hybridization (FISH), has been shown to be an important marker providing relevant information on the progression of the disease and response to therapy…The determination of the aforementioned prognostic markers is desirable in all patients but cannot be a mandatory requirement of clinical practice for a variety of reasons, including the lack of harmonization and standardization of the tests; furthermore, as yet, there is no evidence that altering therapy on the basis of these factors confers an advantage to the patient. The decision to treat a patient should not be taken on the basis of these biological and molecular markers. However these factors are useful in predicting the outcomes for individual patients and may be useful in selected patients prior to commencement of therapy. [Brugiatelli M, Haematologica 2006; Dec, 91:1662-1673]

A survey of genotyping diagnostics across other forms of cancer shows a similar pattern; clinical signs, phenotypic and gene expression biomarkers are more common than genotypes, despite the underlying causality associated with specific mutations and epigenetic factors. Epidermal growth factor receptor, E-cadherin, ERCC1, are used for lung cancer; tyrosinase expression for melanoma; mammaglobin and prolactin inducible peptide expression are common markers for breast cancer, and cytokeratins are common protein markers for a variety of tumors [Ghazani A 2006, Niedernhofer LJ 2007, Ranieri JM 2005, Reinholz MM 2005, Ring AE 2005].

So why, if genotyping carries such a wealth of mechanistic information, is it not the standard of care? If we revisit the Dohner study, several implications about late-stage genotyping seem to support the panel’s conclusion. First, existing clinical markers such as white cell count, lymph node diameter, and cell surface phenotype
were as effective as the genetic tests in determining prognosis. Given the complexity and expense of fluorescent in situ hybridization, it seems impractical to perform such a test based on this information. Second, survival rate was independent of chemotherapy.

The mutations described within the Dohner study were gross chromosomal defects; representing the gain or loss of hundreds of thousands of bases within the genome, ranging from 145 kilobase (17p) to 500 kb (11q) aberrations [Lozanski G 2004]. Once a cancer has acquired one of these genome-scale mutations, the progression of the disease is likely deterministic.

The broad consensus in the medical community is that early detection is needed in order to improve therapeutic outcomes. Increasing evidence points to the accumulation of genetic damage as the causative mechanism behind the transition to malignant cancer [Hainaut P 2001, Busuttil RA 2005, Kleihues P 1997]. Prevailing tumor progression models suggest that the cumulative effects of six to eight mutations result in the transition to metastases [Calabrese P 2005]. Analysis of morphologically benign tumors indicated that DNA instability, p53 mutations, and VEGF expression were common well before tumors could be classified as malignant [Iwasa M 2001, Jin Y 2007, Sun A 2003]. Therefore, there is a good chance that smaller changes within the genome precede deletions, loss of heterozygosity, or the chromosomal translocations that are strongly correlated with metastatic cancer.
Thus, the ability to detect single base pair mutations, or polymorphisms of rare genomic DNA is a fundamental challenge for early cancer detection. In order to detect these polymorphisms, a few molecules of DNA must be identified amidst a dense background of proteins, cellular debris, and similar DNA. The technical requirements to achieve this resolution are formidable, and as of yet have not been addressed.

While many papers claim to have achieved this milestone, the context in which single nucleotide discrimination is achieved follows substantial sample preparation and polymerase chain reaction (PCR) amplification. The premise of this thesis was to explore the following aphorism, as set out by my thesis advisor, Dr. Michael J. Heller: “It’s like going to the moon; you can build a spaceship to take you there, but if you can’t get the landing right, there’s no point in going at all. If people had actually done what they said they’ve done, we’d all be on our yachts by now. It’s still PCR.” To paraphrase, the need for a rare DNA genotyping platform is so great, that if current technology had solved the problem, we would already be using it. As a society, we are coming up on a quarter century of PCR based methods. More recently, singly detectable nanoparticles, such as quantum dots, promised to revolutionize genotyping. In the time span of this thesis we have seen that promise come and go as the real world limitations of nanoparticle detection became apparent. Dr. Heller assures me that the same enthusiasm and realization followed lanthanide chelates in the early 1980’s.
Another implication of Dr. Heller’s argument is that in order to “get to the moon,” a detection platform must exhibit superior receiver operator characteristics, require minimal sample preparation, be inexpensive, rapid, multiplexed, and have the ability to screen large volumes of clinical sample. For instance, no matter how sensitive the tip of an AFM probe may be, it is unlikely that such a technology could process 100 mL of a bronchial wash in a reasonable amount of time.

Our approach to rare molecule detection is predicated on communications theory. Specifically, we hope to create molecular analogs of noise-tolerant coding systems. These mathematical constructs allow information to be sent across a noisy channel with arbitrarily small probabilities of error [Shannon C 1948, MacKay D 2003]. In this treatment, the information is the presence or absence of a particular sequence of DNA within a complex biological sample. Noise comes from a variety of sources, most pertinent being mismatched hybridizations and nonspecific binding. The signals we use are photon fluxes rather than electronic currents or bit streams.

The lack of precedence for this approach means that many fundamental questions have yet to be answered. How do we communicate with molecular systems? How do we interpret output signals? How do you build robust molecular components when chemical syntheses are dominated by stochastic processes? What is a bit? How do you construct parity? It will likely require a special set of circumstances to translate these mathematical concepts into physical systems; the fields of molecular biology, chemistry and communications theory have
traditionally shared little. I hate to even say the word, but this is what “nanotechnology” should be about.

This dissertation is primarily focused on the basics of the information analogy. Chapter 1 establishes the problem statement in quantitative terms and provides a survey of existing genetic detection methods in relationship to these concepts. Chapter 2 examines electrophoretic actuation of intramolecular fluorescent resonant energy transfer constructs as a rudimentary input/output system. Chapter 3 reports on comodulated detection and other forms of high speed actuation. Chapter 4 summarizes a theoretical model of actuation and establishes boundary estimates for redundant codes. Finally, Chapter 5 details the challenges of precision nanoparticle synthesis.

1.2 Hybridization as a Thermodynamic Process

Figure 1.1 depicts the chemical makeup of DNA. At room temperature, two complementary single strands of DNA will spontaneously hybridize to form the double helix of double stranded DNA (dsDNA).

Classical Watson-Crick base pairing requires that adenine (A) associate with thymine (T), and cytosine (C) pair with guanine (G). There are two hydrogen bonds between AT pairs and three hydrogen bonds between GC pairs. A large percentage of GC content within a helix will make it more stable than sequence
with high AT content. The DNA helix is, on average, 2 nm wide with 0.34 nm rise per base. One full turn of the helix occurs every 10 bases, or 3.4 nm.

Single stranded DNA is considered to be floppy, with many degrees of freedom per base. The persistence length is estimated to be on the order of 1.4 nm for ssDNA [Kuznetsov SV 2001]. Double stranded DNA is generally considered to be a rigid rod with a significantly longer persistence length of around 50 nm or 150 bases [Okonogi TM 1999]. When in solution, DNA is constantly twisting, compressing and bending with correlation times between 1-100 ns (10 MHz-1 GHz) for 50mer strands [Harris SA 2007, Okonogi TM 1999, Hennig D 2004]. While still in close proximity, theoretical relaxation times for bases which have been forcefully separated are on the order of 1000 ns (100 kHz to MHz) [Hennig D 2004]. Because of the incessant motion of the DNA molecule, the instantaneous free energy is well described by a Gaussian random variable, or more specifically, an oscillator inside a heat bath [Schallhorn KA 2005, Harris SA 2007].

Hybridization is generally considered to be comprised of three stages: diffusion, nucleation and zippering [Bhanot G 2003, Wartell RM 1985]. In a well mixed solution of two complementary single strands of DNA, the diffusion time is generally limiting. Once in proximity, an initial nucleation event involving the alignment and interactions of around five complementary nucleotides is followed by a zippering of the remaining base pairs [Hagan MF 2004]. Nucleation can happen anywhere along the two strands, and once in a proper position, the relaxation of the remainder of the strand proceeds rapidly.
Double stranded DNA is in equilibrium with its single stranded counterpart [Plum GE 2002]. The non-covalent interactions within the two strands of DNA allows the reaction to proceed in the reverse direction. Typically, this is achieved by raising the temperature. The temperature where the rate of association of the strands is equal to the rate of dissociation is called the melting point, or \( T_m \). At any point in time, there is a 50% probability that DNA will be single stranded at its melting point. Initiation of melting is thought to occur in many places along a DNA strand, with a preference for AT rich segments [Kafri Y 2002, Kalosakas G 2004].

Like any spontaneous chemical reaction, hybridization corresponds to a reduction in free energy (\( \Delta G \)). The free energy change of hybridization has been estimated to be roughly -1 kcal/mol per base pair, which corresponds to an association constant \( K \) of \( 10^{11} \) for a 15mer double stranded oligonucleotide [Plum GE 2002]. At a constant temperature and pressure, we recall that:

\[
\Delta G = \Delta H - T\Delta S
\]

where \( \Delta H \) represents the change in enthalpy, \( T \) is the absolute temperature, and \( \Delta S \) represents the change in entropy. For simplicity, we can consider the enthalpic contributions to be largely due to hydrogen bonding and hydrophobic interactions between adjacent nucleotides. The entropy term relates to the degrees of freedom of the system [Sears FW 1975]:

\[
S = k_B \ln \Omega
\]
where $k_B$ is Boltzmann’s constant ($R/N_A$) and $\Omega$ is the number of microstates. Constraints on the system (i.e., structure) reduce the allowable microstates and reduce the entropy.

Spontaneously folding biomolecules like globular proteins tend to bury hydrophobic amino acids within the interior of their tertiary structure. DNA is similar; hydrophobic bases are held within the center of the helix while the negatively charged phosphate backbone is exposed to the polar solvent. DNA will remain hybridized as long as there are enough stabilizing hydrogen bonds and interior hydrophobic interactions to balance the electrostatic repulsion of the phosphate backbone and entropic penalty of helix formation.

This process is dependent upon a host of environmental variables including temperature and cationic distribution, as well as being sequence specific [Chen C 2004]. Longer probes may become trapped in local minima that exhibit significant secondary structure such as loops and bends, analogous to misfolded protein intermediates [Ansari A 2002]. Moreover, the local environment may impose mechanical stresses on the DNA that prevent the molecular system from finding its global minimum, as is the case in situ with cellular scaffolding, or in close proximity to rough or highly charged surfaces in heterogeneous assays.

Salts play a critical role in DNA stability. Sodium ions are shown in close proximity to nucleotides in Figure 1.1. A more accurate depiction would include a diffuse layer of ions and water molecules that extend out into solution. Within this layer, positive ions exist at a higher concentration than within the solvent as a
whole. Characteristic length scales (Debye length) of decay of the diffuse layer to bulk concentrations is $\approx 1$ nm at physiological conditions. The balance between electrostatic repulsion, mechanical tension, and osmotic pressure is central to the structure of the helix [Grodzinsky A 1995]. Increasing the amount of salt will collapse dsDNA towards the Z-form of the helix, while reducing salt will increase electrostatic repulsion between the bases and extend the strand beyond its the native B-form [Jares-Erijman EA 1996]. Local variations in the valence of cations can also over- or understabilize portions of the helix. For instance, magnesium ions stabilize DNA to a greater extent than sodium ions [Bourdélat-Parks B 2004]. Other molecules, such as formamide, can interrupt the hydrophobic interactions within the interior of DNA and destabilize the helix at room temperature.

Typical $\Delta H$ values for an oligonucleotide are around -80 kcal/mol for a 20mer. The structuring of the double helix leads to a decrease in entropy, such that $-T\Delta S \approx 60$ kcal/mol at 298 K [Kushon SA 2003]. Therefore, as long as the temperature remains low, the favorable reduction in free energy obtained by forming interactions between bases offsets the reduction of entropy caused by the structuring of the helix. As the temperature rises the entropic penalty of helix formation overcomes the bonding enthalpy, hybridization becomes unfavorable, and the strands dissociate. Too little salt in solution and hybridization also becomes unfavorable. $T_m$ for 15-20 bp strands are strongly dependent on GC content, but are generally found between 40-70°C. 9 bp oligonucleotides melt near room temperature (20-30°C).
1.3 Thermodynamic Barriers to Rare DNA Detection

Free energy minimization is a time dependent process. Complex biological samples offer an abundance of local minima that prevent single stranded DNA from finding its perfect complement. Thermodynamically favorable interactions (hydrogen bonds, hydrophobic, and ionic interactions) between proteins, inorganic surfaces, and other nucleic acids are the principal delay. The path to a free energy minimum is greatly extended in the presence of this complexity, where a single stranded DNA can partially hybridize to many loci within chromosome, interact with soluble proteins, and get trapped within cellular debris. Cooperative nonspecific interactions can be more stable than a complementary double helix. Scientists have several terms for nonspecific binding: background is one. The others are generally adjectives unbecoming of this dissertation.

Deviations from classical Watson-Crick base pairing are a consequence of local minima within nonideal environments. Alignment of a few noncomplementary bases is insufficient to inhibit oligonucleotide hybridization if the free energy change remains favorable. Although mismatches carry lower enthalpic benefit because of the lack of hydrogen bonds, they also have lower unfavorable entropic penalty, presumably because a mismatched helix is more disordered than its perfect complement. A example of a G/T mismatch is shown in Figure 1.2. Typical $\Delta H$ values for a single mismatch within a 20mer are around -62
kcal/mol with a correspondingly lower \(-T\Delta S \approx 50\) kcal/mol [Kushon SA 2003]. The overall complex is less stable than the perfect match, and depending upon where the mismatch is located and which base it replaces, the free energy difference \((\Delta \Delta G)\) can range between < 1 to 5 kcal/mol with a reduction of \(T_m\) of less than 2 °C [Bottema C 1993, Bhanot G 2003, Kushon SA 2003]. For a 25 base pair oligonucleotide, there are 975 possible single- and two base pair mismatches = 3 \cdot (25C_1 + 25C_2).

Typically, short genetic probes diffuse throughout a sample in order to locate and bind to a complementary DNA sequence. Passive hybridization allows oligos to repeatedly explore local free energy minima such as mismatched binding sites or nonspecific traps. To find its complement, a mismatched strand must dehybridize, diffuse, and renucleate to its proper place on the genome.

Numerous studies describe single nucleotide discrimination based on kinetics of hybridization [Tolley SE 2003, Peterson AW 2002]. Peterson reported that for a first order Langmuir isotherm describing 25 base pair oligos: 

\[
x_{eq}/(1-x_{eq}) = c_T K_A,
\]

where \(x_{eq}\) is the fraction of hybridized capture probes at a microarray surface, \(c_T\) is the soluble DNA concentration and \(K_A\) is the equilibrium constant, \(K_A\) for the perfect match, 1 base pair mismatch, and 2 base pair mismatch were 6\times10^{7} M^{-1}, 1\times10^{7} M^{-1}, 2\times10^{6} M^{-1} \) respectively [Halperin A 2006, Peterson AW 2002].

Because there are potentially many mismatched regions along a genome, moderate differences in \(K_A\) are neutralized by the sheer number of competing reactions. Bhanot et. al., demonstrated that for a randomized set of 25 nucleotide
long, complementary oligonucleotides against the *Saccharomyces cerevisiae* genome, probes could stably bind to over 20 sequences along the genome, with some binding to 100 different loci. Greater than 70% of the random probe set was improperly bound after $10^4$ seconds if either the probe or target concentrations were low ($10^{-15}$ M, or $10^5$ copies of DNA per mL). Thus, in the presence of genomic DNA, the abundance of mismatched minima present concentration dependent barriers to complementary hybridization.

In order to drive the likelihood of rare DNA recognition, concentrations of labeled oligonucleotide must be maximized, yet in practice, the lowest possible oligo concentrations are used to minimize nonspecific binding [Hloch P 2001]. Optimal passive hybridization may require several days for oligonucleotide probes to find their complement within complex biological samples [Bhanot G 2003].

Assay protocols in molecular biology attempt to remove less stable DNA hybrids by lowering the salt concentration, raising the temperature, and adding other destabilizing agents such as detergents and formamide to disrupt base pair interactions [Oldenburg SJ 2002]. Inspection of theoretical equilibria (Figure 1.3) show that by increasing stringency, both matched and mismatched sequences are shifted towards dissociation. A relatively significant single base pair mismatch ($5^\circ C$ lower melting point) provides a $\approx 2:1$ difference in hybridization probability when the temperature is raised from room temperature to $315^\circ K$ (48$^\circ$C). Washing away mismatched probes comes at a cost; to achieve the 2:1 signal to noise ratio, 26% of the matched hybridization is lost. Given that single base pair mutations
within early stage cancer may occur at a frequency of 1:10,000 cells, and that certain sequences may have 100 competing mismatched hybridization reactions of equivalent stability, ideal detectors must carry a signal-to-noise ratio upwards of 1:10⁹, and that’s without nonspecific binding.

For rare DNA, hybridization probability and free energy overlap between complementary and mismatched probes are the primary thermodynamic barriers to detection. Not only is the a priori probability of finding a rare sequence low, but the chance of hybridizing to a mismatch is orders of magnitude higher than complementary sequence. Therefore, it can be reasonably concluded that hybridization alone is insufficient to discriminate single nucleotide mutations within rare, early-stage cancer cells.

1.4 Existing Genetic Detection Methodologies

1.4.1 Direct Hybridization of Labeled DNA

The simplest approach to detection is to attach a radioactive or fluorescent label to a strand of DNA. A labeled oligonucleotide is referred to as a “probe.” Depending on the intensity of illumination, organic fluorophores can produce 10⁴-10⁵ photons before they bleach [Braun D 2003]. These photons are emitted in every direction, making detection of single fluorophores difficult under even the most
ideal conditions, and virtually impossible in a complex, autofluorescent biological environment.

A large variety of labels have been developed to improve the sensitivity of DNA probes. For example, chemiluminescent enzyme conjugated DNA catalyzes the spontaneous emission of thousands of fluorescent 1,2-dioxetane substrates per oligonucleotide [Nagayama K 1996]. Chemiluminescent constructs virtually eliminate autofluorescence from the surrounding cell matrices because they don’t require an excitation light. This trick provides almost a 100-fold improvement in sensitivity as compared to single fluorophores [Jablonski E 1986]. However, enzyme labels often lead to strong nonspecific binding between the protein and the environment, and given the high gain of chemiluminescence, the chance of false positives is high. Other labels, such as far infrared fluorophores, try to minimize mostly green autofluorescent background by moving excitation spectra towards the 700-800 nm region with emissions up to 1000 nm. A similar approach to autofluorescent background reduction is the use of time resolved imaging of Eu$^{3+}$ chelates, whose fluorescent lifetimes far exceed that of a traditional organic fluorophore [Scorilas A 2000, Heinonen P 1997]. Other labels take a more brute force approach by increasing the number of organic fluorophores per oligonucleotide, such as long strands of fluorescent protein-conjugated polymers [Gaylord BS 2005]. Preference for a particular type of label is more often defined by its relative practicality, expense, and equipment requirements rather than its performance.
More recently, highly fluorescent nanoparticles known as “quantum dots” have become popular labels for direct hybridization assays because of their large Stokes shift, which allow them to be illuminated hundreds of nm away from their emission [Parak WJ 2003]. Moreover, quantum dots have a far narrower spectrum than organic fluorophores, enabling efficient multiplexing. Under the proper conditions, quantum dots are individually detectable. The downside to large surface area nanoparticle labels is their propensity to form nonspecific interactions with biological molecules, surfaces, and other nanoparticles.

Despite the advent of individually detectable nanoparticles, single molecule resolution has yet to translate into a viable platform for mapping the genetic changes associated with early stages of cancer progression. Sensitive assays are generally compromised when converted to complex biological samples such as tissue sections, smears, or whole blood. In particular, nanoparticle probes meant to detect DNA by hybridization regularly bind to cell matrices, proteins and other sections of the genome [Bentzen EL 2005]. Once again, this background represents the lower limit of detection for rare targets.

Direct hybridization assays can take on two broad classes defined by their sample preparation: in situ (in the cell) and in vitro, where nucleic acids are extracted from cellular confines.

The preparation of cellular samples for in situ hybridization requires an extensive process of paraformaldehyde fixing, enzyme digestion of matrix proteins, and denaturation of genomic DNA to expose hybridization targets. Probes are
incubated at high stringency, followed by substantial washing to minimize background signals.

Originally, in situ hybridization made use of radioactively labeled DNA as probes. The advantage of this approach was that even single grains of radioactivity were visible after exposure to film. Exposure times could last upwards of a week to achieve this sensitivity, but because the emitted spectrum required no excitation light, there were few background photons. One of the many disadvantages of isotopic detection was that the long integration times of radioactive probes resulted in low spatial resolution, making internal controls difficult [Levsky JM 2003].

Modern fluorescent in situ hybridization (FISH) protocols use labeled probes against kilobase segments of genomic DNA. An example of an interphase FISH from our lab is shown in Figure 1.4. Immediately apparent is the dominant, anisotropic background fluorescence of the DAPI nuclear counterstain. 5.4 kilobase alpha satellite probes are shown in blue, while 190 kilobase probes for HER-2 are shown in green. The constituent fluorescent channel of the HER-2 gene shows a 2:1 signal-to-background ratio for individually detectable genetic loci.

Absent from traditional fluorescent in situ hybridization methods are high resolution assays, and specifically, the ability to perform single nucleotide mutation profiles of biopsied tissue. As a result of the autofluorescent background and propensity for nonspecific binding within cellular matrices, the shortest individually detectable FISH probes are on the order of a few hundred base pairs [Tanke HJ 2005]. This also makes multiplexing within FISH difficult, as
computational multi-fluorophore deconvolutions are required to segregate the low resolution spots [Levsky JM 2003].

Clinically, FISH genotyping has found utility as a confirmatory, secondary test for subtyping patient populations, as well as within panels of diagnostic tests for acute leukemias [Navolanic PM 2005, Nachman J 2005]. The most popular commercial FISH test is tied to the targeted breast cancer antibody Herceptin, although its intended use statement indicates that it is not to be used for diagnosis. FISH tests have traditionally focused on the assessment of chromosome scale insertions/deletions, loss of heterozygosity, translocations, or the semi-quantitative classification of copy number [Hirsch FR 2005, Dei Tos AP 2005, Munné S 2005, Yeon CH 2005, Ronski K 2005, Hartmann A 2004, Huang S 2005].

The limited information associated with kilobase FISH probes has, perhaps, limited their clinical utility. For example, in situ hybridization also plays a role in the quantitative estimation of minimal residual disease following leukemia remission, where the test carries significant specificity advantages over both RT-PCR and traditional metaphase cytogenetics [Wang YL 2001]. However, while RT-PCR is able to distinguish the subtype of the t(9;22) derived protein product (i.e., p190 vs. the less active p210 tyrosine kinase) FISH lacks the resolution to do much more than verify the existence of a translocation [Wang YL 2001]. As such, FISH is not recommended as a cost-effective first line diagnostic for chronic myeloid leukemia, despite evidence of its superior sensitivity.
In-situ hybridization is also very time consuming and cumbersome. Tissue and cell preparations must be carried out with great care. Adding to the complexity, reagent binding conditions must be controlled with regard to reaction time, probe concentration, temperature, pH, ionic strength of electrolytes, and the presence of additional denaturing or chaotropic agents. In conjunction with these parameters, the subsequent washing steps determine the ultimate sensitivity and specificity of the FISH assay. Small changes in procedure or the nature of the cell preparation introduce intraobserver variation over time and between clinical laboratories [Thomson T 2001, Vera-Román JM 2004, Levsky JM 2003].

Labeled probes also found utility within *in vitro* assays. Examples of these techniques include Northern blots and microarrays. *In vitro* sample preparation require nucleic acids to be isolated from a population of cells or tissue through centrifugation, homogenization, sonication, precipitation and often, PCR amplification. Purified products are immobilized on a substrate or run on a gel and exposed to labeled probes. These protocols greatly reduce the background noise from nonspecific binding to the cell matrix, but the gross inefficiencies of traditional *in vitro* sample preparation result in significant losses of genetic material [Heller MJ 1992, Coombs NJ 1999, Kuske CR 1998]. Method variations in sample preparation can result in a 20-fold difference in DNA recovery [Kuske CR 1998]. As a result, *in vitro* protocols typically require far higher concentrations of starting material than FISH based methods. For rare targets, this is a critical problem.
An example of *in vitro* direct hybridization of labeled DNA to a nylon microarray is shown in Figure 1.5 (courtesy of Dr. David Sullivan and Steve Richards, during my tenure at The Schepens Eye Research Institute affiliate of Harvard Medical School, Boston MA, 6/1/1999).

Lacrimal glands were obtained from adult BALB/c mice, which were either untreated, or orchiectomized and administered vehicle or testosterone for 2 weeks, as well as from mice (Tfm) with dysfunctional androgen receptors and their Tabby controls. Tissues were pooled according to group, processed for the isolation of DNA-free RNA and analyzed for differentially expressed mRNAs. Radiolabeled cDNA libraries were synthesized from isolated RNA, hybridized to the array, washed, and exposed to autoradiography film for 65 hours.

Regions of localized and diffuse nonspecific binding of the cDNA probes were immediately apparent atop the microarray. Background emission severely compromised the quantification of underexpressed sequences. We found that while the sensitivity of the arrays could be greatly enhanced through software, inter-array inconsistencies could not be isolated. Negative controls with lacrimal tissue samples were always positive. Negative controls were not detectable if the RNA samples originated from the spleen, lymph nodes or meibomian glands. Throughout the studies we could identify only one gene that showed consistent differential expression in the same direction in the lacrimal gland. Overall, these results are indicative of mismatched cross-hybridization, as well as other nonlinearities within the capture probes such as self-hybridization. It should be emphasized that while
modern microarray protocols have reduced readout noise through thermal preconditioning and capture probe optimization, the limitations of starting material, sample preparation, and mismatch detection remain.

Direct hybridization assays remain subject to the thermodynamic limitations of hybridization. The inability of hybridization to discriminate matched from mismatched sequences means that improvements in the sensitivity of labeled DNA does not improve confidence of detection.

1.4.2 Amplified Detection of DNA

Figure 1.6 shows a comparison of the traditional noisy channel coding theorem, where a signal is subject to corruption during transmission, and the analogous molecular communications system where the signal integrity prior to transmission can be compromised. As an analogy, take the communications system created between two tin cans connected by a string, passed across neighboring, slightly cracked windows at night. If the string is taught and the cans are clean, you can whisper to your friend on the other end. If your parents are snoring loudly in the next room, some of the words may get jumbled across the string. This is additive noise following transmission. In response to the snoring, you can always speak more loudly, provided that your parents don’t wake up. However, if your big sister comes in the room and insists on talking into your tin can, the chances of you
getting any word in edgewise is very, very slim. In this case, amplifying the received signal does not preserve the integrity of the information source.

The same is true of molecular systems. Mismatched and nonspecific binding corrupt the information source prior to transmission, and compromise the integrity of amplification. High gain is only useful if you can be absolutely sure that the information is intact. Like direct hybridization assays, mechanisms of amplification such as PCR, rolling circle amplification (RCA), enzymatic invasive cleavage, gold nanoparticle accumulation assays, and so forth, transmit information about the presence or absence of the probe molecules, not the genomic DNA. Any time a high gain amplifier relies upon that bit of information, the thermodynamic limitations to detection still exist.

The two most popular amplifiers, PCR and isothermal ligated RCA, in particular, incorporate mechanisms for improving the specificity of detection beyond hybridization alone. PCR provides a kinetic noise tolerance. Because the equilibrium association constant of matched primers is higher than that of mismatched primers, the same number of thermal cycles will produce far fewer mismatched amplicons. Noise from isolated single primer extensions will increase linearly with cycle number while those sequences between primer pairs will exhibit geometric amplification. It can be argued that the noise tolerance of PCR is the single reason that it dominates molecular biology. However, because PCR is dependent upon primer binding to drive recognition, it is bound by the thermodynamic limitations of hybridization. Biases in PCR efficiency resulting

Clinical data support this conclusion. At low target levels, PCR amplification becomes inconsistent within complex biological samples [de Vries TJ 1999, Ghossein R 2000, Ranieri JM 2005]. Data indicate that mRNA from circulating metastases are expressed at levels over ten times below the threshold for reliable nested RT-PCR detection [Szenajch J 2002, Champelovier P 1999]. To understand the magnitude of this problem, nested RT-PCR techniques used in the studies above are amongst our most sensitive and noise-tolerant methods; PCR products from primary amplifications are reprimed and amplified with a second primer set in order to further reduce noise from mismatched primer pairs. As expressed levels of mRNA generally range from 100-10,000 copies per cell [Zhang L 1997, Kanno J 2006], PCR is three to five orders of magnitude away from providing a reliable genotype of rare, early stage cancer cells.

For ligated RCA amplification, specificity is increased by the use of a ligase enzyme to lock a hybridized probe to a genomic target. Upon ligation, the circularized probe cannot diffuse away from the target, providing a template for primer extension. Even though DNA polymerase may introduce random errors during extension, the large number of repeats of the circle average out errors to a negligible degree after a few hundred copies are produced. Fluorescent probing of
the extension products provide unambiguous evidence as to the sequence of the circular product. Note that the reporting does not verify the sequence of the genomic target, only the extended probe. Limitations come about from mismatched ligation, which occurs at a rate of roughly 1 in 500 [Lizardi PM 1998]. While possibly the highest known signal-to-noise ratio for singly detectable events, this level of specificity is also insufficient for early cancer detection.

Intrinsically, PCR and RCA are very sensitive given their high gain. Single molecules can been amplified and detected under ideal conditions [Lizardi PM 1998, Nakano M 2003]. Yet inefficiencies in sample preparation contribute to the poor performance of the real-world trials listed above, as it is impossible to decouple sample preparation from detection. Building a detector on top of lossy separation and purification procedures is likely to be bound by similar limits as the aforementioned RT-PCR studies. High gain amplification also has other practical drawbacks, including the very real possibility of contamination within a testing laboratory [Kirk BW 2002].

1.4.3 New Approaches to Single Base Pair Discrimination

In response to these barriers, considerable effort has been made in the last decade to develop methodologies that enable detection of single nucleotide polymorphisms without PCR amplification [Ref List B] (Note: the term single
nucleotide polymorphism is used here in the general sense, rather than the acronym SNP which has been co-opted by geneticists to mean a common variant in the genome sequence that occurs in at low frequency throughout the population [Guttmacher AE 2002]). Some of the more well-known techniques available in the literature include:

- nanoparticle assays based on quantum dots, plasmon resonant nanoparticles (nanoshells), fluorescent polymer beads, gold nanoparticles, and immunomagnetic precipitations;
- device based methodologies such as quartz crystal microbalances, electrochemical detection, fiber optic microbead arrays, surface plasmon resonance, nanochannels, nanoelectrodes;
- spectroscopic techniques including SERS, TCSPC, functionalized AFM, NSOM, MHz-TIRF, and near field approaches, microsphere whispering mode galleries;
- biochemical techniques centered around enzyme assisted detection, modified nucleic acids, PNA, LNA, fluorescent conjugated polymers;
- mechanistic approaches that employ fluorescence resonance energy transfer, nanoparticle aggregation or molecular beacons.

A survey of a few of the approaches that have reported signal-to-noise ratios in mismatch discrimination are included below.

As reported by Taton et. al., gold nanoparticle accumulation assays begin by functionalizing 13 nm gold particles with upwards of 200 strands of capture
DNA. The active particles hybridize target strands in solution, and are then rehybridized to a second capture probe on a microarray surface. Aggregates of nanoparticle assemblies exhibit a much sharper melting transition than a population of dsDNA [Storhoff JJ 2000]. Cooperative interactions between aggregates confer this transition. If we look at the probability of melting for each strand as a random variable, then the melting of the cluster will be convolution of each of the independent equilibria. Essentially, thousands of aggregated nanoparticles dissociate in near unison as the gain of the melting transition is increased. Following this thermal stringency, a hydroquinone reduction of silver atop the gold nanoparticle provides a large optical amplification of the remaining gold nanoparticles to facilitate detection [Taton TA 2000]. The expected discrimination between matched and mismatched nanoparticle aggregates is reported to be 4.3:1, vs. 1.6:1 for fluorescent dsDNA stringency. Experimental values showed a discrimination of 10:1 for the nanoparticle assay with a loss of 80% of initial signal, and a 2.6:1 ratio for Cy3 labeled fluorescent probes [Taton TA 2000].

Hybridization to concentrations as low as 50 fM were reported, although no data were given on the specificity at those low levels. Gold nanoparticle probes were used at 5 nM for capture [Taton TA 2000]. No data were given for complex biological solutions. Taton claims that the lack of observed nonspecific silver formation or nonspecific binding of nanoparticles to the bare glass surface ensures the integrity of direct, high gain detection. Conditions for this claim were not given. The case where mismatched nanoparticles integrate within a cluster of matched
oligonucleotides is not discussed. Mismatch discrimination in mixed samples was not reported. More recent publications showed similar detection limits of 150,000 copies of sonicated genomic DNA within ideal aqueous buffers, which corresponded to 500 ng of genomic DNA in 5 µL, or 50 fM [Bao YP 2005].

The most sensitive, long-lived, individually detectable label available, the plasmon resonant nanoparticle (PRP), was shown to have a detection limit near 1x10^6 molecules before the nonspecific background overcame the specific hybridization [Oldenburg SJ 2002]. At 25 pM the ability to count individual PRPs gave a signal-to-noise ratio of 158.2, whereas the same sample’s total intensity gave a signal-to-noise ratio of 10.5. A concentration of 0.83 pM produced a 9.8 and 0.1 signal-to-noise ratio for individually counted and total intensity. This study gives a good example of the promise of individually detectable nanoparticles, which demonstrated a two order of magnitude improvement over total intensity in the same assay [Oldenburg SJ 2002]. It should be noted that this study used a two base pair deletion as the polymorphism, rather than single base mismatched probes.

A surface immobilized enzyme cleavase assay followed by RCA amplification was shown to have a 30:1 match to single mismatch ratio at 10 pM (100 attomol synthetic DNA / 10 µL) [Nie B 2005]. In this assay, an immobilized, 5’ dabcy1 modified probe partially hybridizes a double stranded target. The excess single stranded overlap region of the immobilized probe is cleaved by a 5’ exonuclease that exposes a free phosphate on the immobilized probe. An RCA primer is ligated to those strands which have been freed of the dabcy1 blocking
group. Nie claimed that the rate of exonuclease cleavage was 300-fold higher for matched targets as compared mismatched targets, although this cited from a study in which mismatches were located specifically at the overlap intersection [Lyamichev V 1999]. No data was given for mismatches within body of the double stranded target.

Lanthanide chelates were shown to have an average match to mismatch signal-to-noise ratio between 10-40 at limiting concentrations of 1.5 pM (5x10⁷ molecules / 50 µL well) in an ELISA-type well assay [Heinonen P 1997]. Immobilized, surface quenched molecular beacon hairpin DNA molecules which fluoresce upon hybridization exhibited an 8:1 signal-to-noise ratio at 2.6 µM, dropping to 1.42:1 ratio at 150 nM [Du H 1997]. Solution phase excimer-monomer molecular beacons demonstrated a 10:1 signal-to-noise ratio at 5 nM [Fujimoto K 2001]. Magnetic microparticle assisted capture and subsequent conjugated fluorescent polymer detection of purified targets provided an 8:1 signal to noise ratio for 5 nM single mismatches, falling to 2:1 at 1 nM.

Electrophoretic dehybridization showed an absolute single mismatch signal-to-noise ratio of 5:1 with a reduction of initial fluorescence to 75% after 45 seconds of applied stringency [Sosnowski RG 1997]. This report is somewhat misleading because the authors reported endpoint signal-to-noise ratios from normalized starting points. Moreover, the authors did not hybridize at stringency, which would have greatly reduced the starting concentration of mismatched fluorescence. The authors did not take advantage of information within the dynamic signatures of
dehybridization. It is likely that these factors would significantly improve observed signal-to-noise ratios.

### 1.5 Gaussian Random Variable Description of Hybridization

As mentioned above, the instantaneous free energy of a DNA probe is well described by a Gaussian random variable [Schallhorn KA 2005, Harris SA 2007]. Not only is DNA subject to thermal fluctuations within an aqueous solvent, but the anisotropic environment within a complex biological sample. Therefore, probes of the same sequence may be more or less stably bound at any given instant, or point in space.

The free energy distributions of two Gaussian distributed probe sets are shown in Figure 1.7A. The application of a free energy threshold, whether by heat, chaotropic buffers, or electric field, creates an inverse relationship between how confident we are in eliminating the mismatched probe with how much signal we retain from the matched probe. The more the threshold is moved to the left (to ensure that only the most stable, lowest free energy probes remain), the more we are confident that the signal is specifically bound. Increased confidence comes at the expense of the amplitude of detectable signal. The problem becomes more pronounced when detecting single base pair differences between probes, or when the genetic material of interest is at very low signal-to-noise ratios, as shown in
Figure 1.7B. The integral of the Gaussian distributions up to the threshold in Figure 1.7 are closely related to the contour shown in Figure 1.3.

Figure 1.7C demonstrates the impact of nonspecific binding. The free energy mean and variance of nonspecific interactions within an assay are poorly defined, but are largely predicated on material interactions within the samples. With enough parallel cooperative interactions, nonspecific attachments may become more stable than properly bound reporter probes. At some point in a passive assay, nonspecific background will prevent stringency from improving detectability.

1.6 Detection Theory

Bayesian detection theory is constructed to minimize the expected cost of choosing a hypothesis $H_1$ (i.e. a signal exists within the noise) over $H_0$ (there is no specific signal) [Karl WC 1998]. For a multidimensional vector of measured information $y$, there exists an optimal partition of space $\mathbb{R}^n = \{y : m(y) = 0\} \cup \{y : m(y) = 1\}$, such that the probability of false positives $P_F$ is minimized, and the probability of detection $P_D$ is maximized [Hecht-Nielsen R 2001, Karl WC 1998]:
\[ m(y) = \begin{cases} 1 & \frac{p(y|H_1)}{p(y|H_0)} \geq \frac{p_0(C_{10} - C_{00})}{p_1(C_{01} - C_{11})} \\ 0 & \frac{p(y|H_1)}{p(y|H_0)} < \frac{p_0(C_{10} - C_{00})}{p_1(C_{01} - C_{11})} \end{cases} \] (3)

\( C_{10} \) represents the cost of choosing hypothesis \( H_1 \) when the true state was \( H_0 \), etc.

The apriori probabilities \( p_0, p_1 \) are perhaps unavailable for molecular detection, but can be estimated for certain cases, i.e., \{0.5, 0.5\} for heterozygotic genotyping, and \{0.00001, 0.99999\} for early stage, circulating metastases.

For the case of deciding between two unidimensional, equivariate Gaussian sequences with zero cost for true statements and equivalent costs for false positive and false negative results,

\[ \frac{p(y|H_1)}{p(y|H_0)} = e^{-\frac{(y-\Delta m)^2}{2\sigma^2}} \] , \[ m(y) = \begin{cases} 1 & y \geq \sigma^2 \ln\left(\frac{p_0}{p_1}\right)/\Delta m + \Delta m/2 \\ 0 & \end{cases} \] (4)

where the \( m(y) \) is the optimal partition given a single scalar observation \( y \), with a differences in means of the Gaussians \( \Delta m \) [Karl WC, 1998]. For passive hybridization assays, \( y \) would be equivalent to the residual fluorescence after stringency. The free energy overlap will determine the relative performance of \( m(y) \), with the optimal stringency conditions placing the free energy threshold at the intersection of the two distributions for a \{0.5, 0.5\} cost.

The performance of this decision rule is described by the receiver operating characteristic (ROC) curve, which is a plot of \( P_F \) vs \( P_D \) (Figure 1.8). For a passive
hybridization assay, the ratio of sensitivity to specificity is bound to the receiver operating characteristic. Changing stringency parameters moves along the curve, but does not change its shape.

The Bayesian definition of false positives and negatives is as follows:

\[
P_F = \int_{y: m(y) = 1} p(y|H_0) dy, \quad P_D = \int_{y: m(y) = 1} p(y|H_1) dy
\]

(5)

where \( y \) is a vector of observations. Integrating the conditional probability to the partition \( m(y) \) for each dimension of observation corresponds to the shaded area under the curve in Figure 1.7A. Independent observations produce a multiplicative benefit for reducing false detection. The observations can be spread out in time (redundancy), over multiple dimensions (classification), or both.

Analysis of redundancy within Gaussian distributed radar pulses serves as a direct analogue for this hypothesis, as shown below [Karl WC 1998]. \( N \) independent samples of a Gaussian process are collected, the measurement covariance is reduced by \( N^{1/2} \):

\[
\frac{p(y|H_1)}{p(y|H_0)} = \prod_{i=1}^{N} e^{\frac{2\Delta m y_i - \Delta m^2}{2\sigma^2}} = e^{\frac{2\Delta m (\sum_{i=1}^{N} y_i) - N \Delta m^2}{2\sigma^2}}
\]

(6)
\[ m(y) = \begin{cases} 1 & \frac{1}{N} \sum_{i=1}^{N} y_i \geq \frac{\sigma^2 \ln \frac{p_0}{p_1}}{N\Delta m} + \Delta m/2 \\ 0 & \text{otherwise} \end{cases} \]

Reduction of variance is a direct result of the linearity of the detection integrals. As each successive measurement of the reporter probe process is made, the decision rule increases performance. Ultimately, \( \lim_{N \to \infty} P_D = 1 \), \( \lim_{N \to \infty} P_F = 0 \) which can be seen in the explicit definitions for false positives and true positives after repeated samplings from a Gaussian distribution [Karl WC 1998]:

\[
P_D = \frac{1}{\sqrt{2\pi \sigma^2/N}} \int_{-\infty}^{\infty} e^{-\frac{N(x-\Delta m)^2}{2\sigma^2}} \, dx = \text{erf} \left( \frac{N^{1/2}(t - \Delta m)}{\sigma} \right)
\]

\[
P_F = \frac{1}{\sqrt{2\pi \sigma^2/N}} \int_{-\infty}^{\infty} e^{-\frac{N\sigma^2}{2\sigma^2}} \, dx = \text{erf} \left( \frac{N^{1/2}t}{\sigma} \right)
\]

(7)

\[
t = \frac{\sigma^2 \ln \left( \frac{p_0}{p_1} \right)}{N\Delta m} + \Delta m/2
\]

The effects of redundancy are shown in Figure 1.9. This suggests that modulation of the free energy of hybridized probes should homogenize the average of the population. Independently modulating the free energy is not straightforward within an assay. One way to accomplish this would be to repeat the assay many times and
average the results. This technique has been shown to improve detection, although this is expensive and could require excessive patient samples [Szenajch J 2002].

Another way to modulate free energy is to repeatedly disrupt hybridization and observe the rate of relaxation. In other words, create an oscillatory system that reports on the free energy of binding through its frequency response; essentially a hybridization spectroscopy.

It is unlikely that individual frequencies provide an orthogonal basis for DNA oscillators. In fact, our research shows that driven DNA oscillators produce signals with a wide range of correlated frequencies. For unknown signals \( x(t) \) in white noise \( w(t) \), observations can be transformed into the now familiar multidimensional Gaussian basis [Karl WC 1998]:

\[
y_i = \begin{cases} \ x_i + w_i \sim N(0, 1 + \lambda_i) & \text{if } H_1 \text{ is true} \\
\ w_i \sim N(0, 1) & \text{if } H_0 \text{ is true}
\end{cases}
\]  

(8)

such that the resulting threshold takes the form of

\[
\frac{p(y|H_1)}{p(y|H_0)} = \prod_{i=1}^{N} \frac{e^{-y_i^2/2(1+\lambda_i)}}{\sqrt{1+\lambda_i} e^{-y_i^2/2}}
\]

(9)
where $\lambda_i$ are the eigenvalues of the series expansion of the measured process. Identification of the proper eigenvectors within a frequency space shall be a critical factor in detection of signals.

1.7 Optoentropic Transduction Mechanisms

Given the aforementioned limitations of hybridization probability, nonspecific binding and free energy overlap between mismatched and matched probes, it can be reasonably argued that an ideal detection system must 1) be built upon a lossless sample preparation, such as sonication or lysing without further purification, 2) inundate the sample with a high concentration of probe, 3) correlate $H_1$ with the free energy of binding of individual probes.

Analog detection theory gives us a clear impetus to increase the number of dimensions of analysis (spectroscopy) and to increase the number of observations (modulation) of a system in order to determine the presence or absence of matched target. In order to do so, we propose that intramolecular fluorescent resonant energy transfer systems (FRET) will serve to convert the motion of a oscillatory DNA system into an observable signal indicative of the free energy of binding. FRET is a distance dependent dipole coupling between organic fluorophores or nanoparticles, where close proximity between a donor and acceptor results in a non-radiative transfer between the pair of molecules when the donor is in an excited state. The
efficiency of energy transfer is dependent upon the spectral overlap of the donor emission and acceptor excitation, as well as the orientation of the constituents [Jares-Erijman EA 1996, Wang L 2003]. Typically, green donors couple to red acceptors. Resonant energy transfer is also seen between quantum dots and organic fluorophores, as well as gold nanoparticles and quantum dots, although the mechanisms are less well understood [Gueroui Z 2004].

For the two-state oscillator shown in Figure 1.10, the closed state represents a fully hybridized FRET system, free of influence beyond thermal background. Upon application of an external driving force, the fluorophores shift equilibrium toward the open state, represented by a partially dehybridized system. The stable half of the acceptor strand remains bound to the target sequence, as ideal actuation perturbs the FRET construct without fully dehybridizing the probe.

FRET efficiency between fluorophores tethered to adjacent oligonucleotides falls off at the sixth power of distance [Wang L 2003, Jares-Erijman EA 1996]. Close together, the fluorophores couple to produce a predominantly red signal, and when far apart, the system emits in the green, as the coupling between fluorophores is interrupted. Induced changes in the structure of the oscillator will project onto the average FRET efficiency between donor and acceptor. Equation 1 suggests that at constant temperature, an increase of free energy in the system will result in a change in configuration of the molecule, both in structure and the number of interactions. We have coined the term “optoentropic” transduction to mean that changes in the structure of the DNA in response to an increase of free energy in the
system will be converted to a time-varying optical signal. It is difficult to see how changes in the enthalpic interactions between bases will be directly mapped onto the FRET pair, as bubble nucleation sites can occur within the interior of the helix, and not just at the end [Kalosakas G 2004]. Therefore, we chose to emphasize that these constructs may bias reporting of the entropic contribution to free energy, rather than the full \( \Delta G \), although the kinetics of rehybridization should include enthalpic components.

An immobilized optoentropic transducer should partially dehybridize upon application of an external driving force (Figure 1.10A). Position and sequence dependent opening energetics have been observed with imino proton exchange NMR, and rehybridization of DNA hairpin molecules have been shown to be slowed by entropic traps during the post-nucleation hybridization process [Ansari A 2002, Chen C 2004]. Therefore the kinetics of both opening and rehybridization are likely to depend upon the complementarity of the target DNA involved in assembling the transducer.

In order to modulate hybridization, the externally applied force must provide enough leverage to open the transducer. Estimates of the force required for the initiation of DNA unzipping are widely varying in the literature (between 15-200 pN), and depend strongly on how fast the forces are applied [Cocco S 2002]. Previous work by Heller et. al., have shown conclusively that electrophoretic actuation of FRET systems carry enough force to perturb binding, and temperature jump studies from pulsed lasers have also shown the ability to melt DNA
oligonucleotide structures in rapid fashion [Heller MJ 1997, Ansari A 2001, Braun D 2003]. The proper combination of actuation speed, force and the secondary implications in terms of photo- and thermodynamic stability have yet to be determined. It is also unclear whether it is sufficient to modulate the equilibrium of the construct, beneath the full dehybridization threshold.

For soluble transducers, application of an external force will likely lack sufficient gradients to dehybridize a construct, but rather compress or stretch the system up application (Figure 1.10B). Soluble constructs are meant to explore the mechanical modes of the DNA system, converting oscillations within the molecule into optical signals. It is well known that dsDNA is more rigid than single stranded DNA, with persistence lengths in excess of 54 nm as compared to just 1.4 nm for ssDNA [Abels JA 2005, Kuznetsov SV 2001]. Because of the increased degrees of freedom of a mismatched helix, mismatched systems should display distinct dynamic modes. Our hypothesis is that mismatches will preferentially absorb lower frequency oscillations than the corresponding matched system. It is unknown to what extent the differences in mechanical eigenmodes will correlate with sequence specific changes within DNA systems. Nor is it understood what effect the introduction of relatively massive nanoparticles will have on the frequency response, or whether this will enhance or suppress differentiation between matched and mismatched signals. However the potential for frequency multiplexing seems self-evident if different sized nanoparticles do, in fact, mediate resonant behavior.
Figure 1.1 Chemical structure of DNA exhibiting classical Watson-Crick base pairing. Modified under the GNU Free Documentation License, Version 1.2: http://en.wikipedia.org/wiki/Image:DNA_chemical_structure.svg
Figure 1.2 Chemical structure of DNA with a GT mismatch. Single base pair mismatches destabilize the strand by interrupting the local structure of the helix, changing the equilibrium free energy.
Figure 1.3. Sigmoidal equilibria for dsDNA formation versus temperature. The 1 base pair mismatch is set to have a 5 °C lower melting point than the match. Raising the temperature improves the signal-to-noise ratio, but lowers the total amount of signal. Specificity and sensitivity are inversely related using passive stringency because of this effect. Detection methodologies which amplify secondary labels are dependent upon hybridization for specificity.
Figure 1.4. Her2/Neu Fluorescent in situ hybridization. Image courtesy of Alexander Hsiao, UCSD Bioengineering, Heller Lab. A: Composite image, of B: Rhodamine labeled HER-2, C: FITC labeled chromosome 17 alpha-satellite probes, and DAPI as a nuclear counterstain.
Figure 1.5: Example of a directly hybridized radioactive cDNA microarray. Image courtesy of Dr. David Sullivan and Steve Richards, The Schepens Eye Research Institute affiliate of Harvard Medical School, Boston MA. After developing software to eliminate post-hybridization artifacts, it became clear to us that the problem with direct hybridization microarrays extended beyond readout noise. Readily apparent are regions of localized and diffuse nonspecific binding of the radioactive probes. Unknown is the degree of cross-reactivity between capture probes. The information content of passive hybridization is low.
A) Traditional Noisy Channel Communications

![Diagram of traditional noisy channel communications]

B) Molecular Communications

![Diagram of molecular communications]

Figure 1.6. (A) Traditional noisy channel coding assumptions [Shannon CE, 1948]. Information can be corrupted during transmission over the channel by a variety of additive noise sources. (B) Molecular communications must also consider that mismatched hybridization and nonspecific binding add additional components into the transmitted signal. Amplification of the Signal is therefore insufficient to recover the information when the noise sources are larger than the information source.
Figure 1.7. A) Gaussian free energies of equal concentrations of matched and mismatched probe. Application of stringency improves confidence of detection at the expense of signal, which is represented by the shaded area. B) For single nucleotide polymorphisms or rare DNA, here shown at a 1:3 ratio, false positive and false negative detection become prominent. Rare DNA can be found below 1:10,000 to 1:10^6 concentrations of target to mismatch. C) Nonspecific binding can be more stably bound than either the match or mismatch, posing a significant limitation to rare detection.
Figure 1.8. Receiver operating characteristic (left) shown for a Gaussian system (right). The ROC curve is an invariant feature of the system, defined by the marginal probabilities of binding. Applying thermal stringency to differentiate will only move along the ROC curve. Threshold A provides a measure of stringency, lowering the probability of false positives to 8%, reducing the probability of detection to 58%. At Threshold B, there is a higher chance of detection (83%) and a higher chance of false detection (17%).
Figure 1.9. A) Redundancy reduces free energy overlap and improves the probability of detection. B) Left: The receiver operating characteristic improves with independent observations. Right: As N increases, $P_D$ and $P_F$ exhibit asymptotic behavior.
Figure 1.10. A) Nanoscale Optoentropic Transduction Mechanism. When closed, the donor fluorophore (gray) comes into proximity of the acceptor fluorophore (white), eliciting a fluorescent response from the acceptor. Application of external driving force destabilizes a portion of the assembled system, interrupting the energy transfer between the fluorophores and blue-shifting the emission. Removal of the driving force allows the system to rehybridize. Repeated cycles of actuation and relaxation produce a dynamic response which can be analyzed to extract information about the complementarity of the probes. B) A soluble version using plasmon coupled gold nanoparticles. Mismatched DNA should exhibit distinct mechanical modes upon actuation.
Chapter 2: Information from Modulated FRET Systems

2.1 Molecular FRET System Components

To test the hypothesis that fluorescent systems can convey information about the complementarity of binding, we examined the ensemble behavior of steady state, association and dissociation kinetics of various fluorescent constructs. FRET within quantum dot, fluorescent protein, and DNA templates exhibited very different efficiencies. It was also shown that fluorescent systems convey a wealth of information about their immediate environment, exhibiting different amplitudes and dynamics depending upon the DNA sequence, linker chemistry, and buffer.

The fluorophores and nanoparticles used for these experiments are shown in Figure 2.1, and their absorption and emission spectra in Figure 2.2. The experimental DNA sequences along with their hybridization position in relation to a small sequence of the human p53 are shown in Figure 2.3. Cy3 is a common green donor fluorophore with an emission maximum at 562 nm and an extinction
coefficient of 150 mM\(^{-1}\) cm\(^{-1}\). Quantum dots have a very sharp emission peak in comparison to fluorophores, and continuously absorb blue shifted light of their emission spectra, which is useful for eliminating background. UV light below 300 nm tends to heat samples, cause autofluorescence, and sometimes photochemistry. We found that a good balance between emission and background for the quantum dot was between 350-400 nm excitation for a 565 nm emitting quantum dot. I like to think that the QSY-7 quencher was originally developed as a fluorophore, but failed miserably and was sold off as a quencher. We found that QSY-7 was superior to the more expensive Black Hole Quencher at extinguishing Cy3 and the 565 quantum dot emission. Finally, we chose an unusual FRET acceptor in Texas Red X (TRX), since the emission maximum is only slightly shifted from the Cy3 peak. However, there is a strong coupling between the two fluorophores due to the overlap in Cy3 emission and TRX excitation.

DNA was purchased and PAGE purified from TriLink Biotechnologies, San Diego, CA. Stock concentrations of DNA were diluted into 100 \(\mu\)L deionized water, diluted 100 fold (stock 1: 10 \(\mu\)L to 1 mL), and then diluted 10-fold again (stock 2: 50 \(\mu\)L – 500 \(\mu\)L) before absorbance at 260 nm was measured using a Perkin Elmer Lambda 800 UV/Vis spectrophotometer. DNA purity was ascertained using a 2 nm resolution scan between 220-320 nm. The ratio of \(A_{260}\) to the manufacturer supplied extinction coefficient (\(\varepsilon\)) was used to convert absorbance to molarity. To keep concentrations within the linear regime of absorbance spectroscopy, values were diluted below 1 OD in a 1 cm quartz cuvette, prior to
calculating the higher stock concentrations. Stock 1 concentrations were generally in the $\mu$M range.

Fluorescent spectra were obtained using a Perkin Elmer Lambda LS-55 fluorometer within a high-walled microscale quartz cuvette. Typically, excitation wavelengths were at least 25 nm off of the minimum recorded wavelength to ensure that bleeding from the optical filters was minimized. For Cy3, a 535 nm excitation was used for spectral recordings between 550 to 800 nm. A 10 nm excitation slit, 20 nm emission slit, and 1500 nm/s scan speed were standard.

It should be noted that the quantum dots used in this study were a custom synthesis from Quantum Dot Inc., prior to acquisition by Invitrogen, Inc. The early generation Quantum Dot product featured a proprietary polymer passivation of the shell, with streptavidin immediately conjugated to this polymer. The short distance between streptavidin and core was amenable to resonant energy transfer. Second generation quantum dots were modified to include a medium-chain polyethylene oxide coating with the streptavidin conjugated to the exterior of the PEG layer. PEG was reportedly added to reduce the nonspecific binding of quantum dots during in situ assays. Characterization of these second generation quantum dots showed erratic behavior during stability tests. We attributed this to higher nonspecific binding between the quantum dots themselves. The PEG insulation also eliminated our ability to quench the particles. Recently, Invitrogen re-released the “original” quantum dots [Qdot® 565 ITK™ Streptavidin Conjugate Kit, Invitrogen]
Inc., Carlsbad CA], although the stability of these new nanoparticles did not compare to the custom synthesis.

2.2 Steady State Binding of Mismatched DNA

To demonstrate the ability of DNA to bind a variety of mismatched sequences, we constructed a quantum dot based fluorescent resonant energy transfer (FRET) sensor (Figure 2.4). Sensors were constructed by loading streptavidin functionalized quantum dots, with a ten fold excess of 51mer biotinylated DNA (p53B51WT: 5’-Biotin-GAA-CAG-CTT-TGA-GGT-GCG-TGT-TTG-TGC-CTG-TCC-TGG-GAG-AGA-CCG-GCG-CAC-3’) in a 250 mM sodium phosphate buffer, and mixed with a 100-fold excess of complementary, 1 base pair mismatched, 2 base pair mismatched, and noncomplementary DNA labeled with QSY-7 quencher probes (p53cQSY7, p53m1bpQSY7, p53m2bpQSY7, RCA5QSY7). When in close proximity to the quantum dot, the QSY-7 molecule provided a nonradiative pathway, lowering the probability that a quantum dot would release a photon following illumination. We found that saturated quenching of a quantum dot required upwards of 20 QSY-7 molecules to be bound to the surface of the nanoparticle.

FRET is selective enough that a non-complementary strand of DNA, i.e., a strand with an unfavorable free energy of hybridization to the p53B51WT,
effectively does not change the fluorescent output of quantum dots in solution despite being in gross, 100-fold excess. The small reduction in total fluorescence is thought to be due to absorption of the excitation light. It may also signify that a small number of non-complementary strands have bound to the quantum dots through nonspecific binding, or that there is a transient interaction between non-complementary strands and the quantum dots that reduces the average fluorescence very little. In comparison, two base pair mismatched, singly mismatched, and complementary strands (spectra #3, #4, #5, respectively) achieve nearly identical reduction in fluorescence. It is difficult to suggest that quenching of the quantum dots was achieved through any other process than hybridization, given the lack of effect of the noncomplementary strands. The excess of quencher probe was added to ensure the equilibrium favored the hybridized state and to minimize time related differences in hybridization kinetics between polymorphisms. An 80% reduction in quantum dot fluorescence was seen with an excess of matched, 1bp and 2bp mismatched quencher.

Fluorescent proteins were shown to have a far more pronounced FRET response than quantum dots (Figure 2.5). Cy3 conjugated streptavidin [Sigma, S6402] was functionalized with a 1:1 concentration of biotinylated capture DNA from (p53B51WT) and mixed with equimolar amounts of complementary, 1 bp mismatched, 2 bp mismatched, and noncomplementary QSY-7 quencher probes (p53cQSY7, p53m1bpQSY7, p53m2bpQSY7, RCA5QSY7) in a 0.5 M NaCl, 0.05 M sodium phosphate buffer. 56% quenching was achieved with equal
concentrations of quencher to Cy3 streptavidin. Again, the matched and mismatched probes hybridized with equal efficiency.

It is not known how many Cy3 molecules are conjugated to Sigma S6402. Typically, N-hydroxysuccinimidyl esters are used to couple organic fluorophores to the solvent-exposed primary amines of lysine residues. Sigma reports lot-to-lot variance of 3-9 moles of fluorophore per mole of streptavidin. To get an estimate of the sensitivity and distance dependence of FRET, a 5’ biotinylated, three base pair oligonucleotide control was synthesized with a 3’ QSY-7 quencher [5’-Biotin-TTA-QSY7-3’]. At 0.34 nm per base, with short-chain carbon linkers between both functional groups, the QSY-7 was displaced by an estimated 1-2 nm. The quenching was reduced with the Cy3 emitting 70% of its initial value (Figure 2.6). If we examine the FRET efficiency curve, \( E = \frac{R_0^6}{(R_0^6 + r^6)} \), an estimated Förster radius \( R_0 \) of 5.1 nm suggests that the response should be close to linear between the extrema of the hybridized probe and displaced quencher [Wahlroos R 2006, Wang L 2003]. As there may be multiple Cy3 molecules per streptavidin, the FRET efficiency is likely underestimated for some and overestimated for others. Regardless of the exact blend of efficiencies, the fact that a displaced QSY-7 quenches less than one and two base pair mismatched probes provides further evidence that mismatched oligonucleotides readily hybridize with roughly the same stability as a complementary strand.
2.3 Bulk FRET Kinetics

A central hypothesis of exploiting oscillatory information is that differences in complementarity correlate to the dynamics of hybridization. Ensemble measurements of association kinetics give an indication of average behavior. The long time scales reported in bulk hybridization kinetics are so much longer than post-nucleation hybridization, that DNA interactions can be treated as binary on/off events. In other words, a bulk dynamic relaxation requiring fifteen minutes does not mean that hybridization requires 15 minutes. Bulk curves report on a the multiplicative probability of binding - essentially the equilibrium constant ($K = k_a/k_d$), and the diffusion limited probability of interaction. The difference between hybridization kinetics and bulk dynamics is similar to the difference between whole cell and patch clamp recordings of single ion channel recordings in excitable tissues.

Diffusion dominates bulk DNA kinetics, so it is essential that the solutions are well mixed prior to interpretation. Technically, this presents a challenge since the rectangular wells of microscale cuvettes are incompatible with even the smallest teflon stir bars. The strongest dynamics are seen within initial slopes, and slight variations in trigger time create large variations in the subsequently aligned recordings. Data showed that pipette-based mixing was insufficient within the rectangular cuvette. Pipette-based mixing was slow, ran the risk of introducing bubbles, and the mechanics of the rectangular cuvette created large pockets of
inaccessible fluid, producing anisotropic gradients and overlapping diffusive dynamics within a single kinetic trace.

To ensure adequate mixing, a timed protocol was implemented where all of the components needed for hybridization, except for one last reagent, were premixed and allowed to equilibrate in a 1.5 mL skirted microcentrifuge tube. The fluorometer was triggered to start recording full spectra (550-800 nm, 0.5 nm resolution) every 30 seconds. Immediately after the first spectra was complete, the last reagent was added to the mixture, the tube was capped, vortexed, briefly spun, and pipetted into a cuvette, checked for bubbles, and inserted into the fluorometer within the next 30s. Spectra were recorded for 15 to 30 minutes to allow the FRET response to steady state. With some practice, the protocol became repeatable, although it required a skilled hand. The fluorometer was kept at 22.5 °C with a Peltier water jacket surrounding the cuvette. The room temperature fluctuated upwards of four degrees day-to-day. We suspect that some of the variability was incurred via ambient temperature exposure during the vortexing step. Some variability may have also been attributed to the rapid pipetting step, since it was assumed that the mixing was incomplete upon withdrawal of the pipette, and an unequal amount of reagent could have followed the tip, despite our best efforts to the contrary.

FRET kinetics were studied for three systems: the left hand side (LHS) of the p53 template, the right hand side (RHS) of the p53 template, and a so-called Qatar strand (Fig 2.3). The p53 systems carried mismatches on the probes, while
the Qatar carried a mismatch on the target. Three types of triggers were used to examine dynamics: salt, p5351WT target, and the Texas Red labeled probe. For the TRX probe trigger, the Cy3 and target strands were prehybridized in 0.5M NaCl, 0.05 M sodium phosphate (hybridization solution). In general, equal volumes of 200 nM target strand and 200 nM probes, and hybridization solution were mixed to give 50 nM final concentration of triggered FRET system.

Figure 2.7 shows a typical family of spectra for the left hand side of the p53 sequence. Before triggering, the Cy3 peak is far higher than the Texas Red peak, which is superimposed on top of the Cy3 tail. As time progresses, the Cy3 peak falls as an increasing percentage of probes hybridize and become donors for the Texas Red acceptor. The absolute fluorescent amplitude for each peak is shown in the inset of Figure 2.7. Although it appears that the Texas Red fluorescence is decreasing, it is difficult to gauge visually, given the simultaneous decrease of the superimposed Cy3 spectrum.

Figure 2.8 shows a comparison between the left hand side and right hand side of the p53 strand, as well as a control with deionized water added instead of hybridization solution. The control shows essentially no FRET after 30 minutes. Because the DNA was purchased as a lyophilized sodium salt, trace amounts of hybridization are visible. In comparison, the LHS and RHS show considerable shifts after triggering. Of particular interest, the complementary FRET on the left hand side showed a very different response than the right hand side. The LHS response (decreased Cy3 with slight changes in TRX) was consistent over
complementary, 1 and 2 bp Texas Red mismatches, as well as between the p53RCy3 probe and the p53cCy3 probe. The p53RCy3 probe had an internally modified cytosine and three extra base pairs that bridged a gap between donor and acceptor probes, whereas the p53cCy3 was end modified and had a gap between the donor and acceptor. Both the RHS and LHS fluorophores were separated by four bases. This is strong evidence that FRET is sequence dependent, and that local variations in the helix structure influence both the dynamic and steady state behavior of adjacent FRET probes.

In order to better understand the energy transfer kinetics between matched and mismatched strands, the individual Cy3 and TRX fluorescent responses were extracted from the FRET response through a vector decomposition. First, the background light from a 535 excitation was subtracted from the FRET spectrum. The background decayed sharply and was negligible after 570 nm, but its initial value was about 70% that of the Cy3 peak, thus required accounting. Independent emission spectra for Cy3 and TRX were recorded in response to a 535 excitation and normalized by the area under each curve (Figure 2.9). Matrix division by Gaussian elimination ($x=A_{(502,2)}B_{(502,1)}$, in MATLAB 5.0, The MathWorks, Natick MA) allowed us to extract the relative contribution of each fluorophore at each time.

Resulting vectors for 51mer target triggered LHS probe set are shown in Figure 2.10. In comparison to the inset of Figure 2.7, the Cy3 and Texas Red vectors are symmetric, confirming energy transfer, rather than collisional
quenching. Per the protocol, the initial conditions were unavailable. However, the endpoints show the matched strand demonstrating the fastest, strongest response, followed by the 1 base pair mismatch. The 2 base pair mismatch showed the least deflection over time. There are very small differences between complementary and 1 base pair mismatch kinetics.

A similar interpretation results from prehybridized kinetics (Figure 2.11), except that no difference can be seen between the match and 1 bp mismatch. The 2bp mismatch clearly lags behind. Because the Cy3 probe is prehybridized, these kinetics are perhaps more indicative of differences between matched and mismatched kinetics.

Figure 2.12 shows salt triggered data for the Qatar sequence. Again, faster average kinetics were observed for the matched as compared to the 1 bp mismatched sequence. Fitting of the kinetic data showed agreement with a sigmoidal solution \(1/(1+e^{-kt})\) rather than a \(1-e^{-kt}\) curve (Figure 2.13). Sigmoidal solutions are typically solutions to the second order rate equations, where \(d[P]/dt = [P](1-[P])\), where \([P]\) represents the concentration of the products of the reaction.

During the course of the bulk kinetics experiments, it was noticed that internally modified fluorophores had a much higher quantum efficiency than their end-labeled counterparts. An example of this difference is shown for equimolar amounts of internal and end-labeled Cy3 and Texas Red in identical buffers (Figure 2.14). This suggests that information from fluorescent systems can also report on the immediate environment.
It is well known that certain dyes like ethidium bromide produce an enhanced fluorescent response when intercalated within the bases of double stranded DNA molecules. It is also known that ethidium bromide has more affinity for GC base pairs than AT pairs [Heller MJ, personal communication]. To further explore the sequence specific effects of a fluorescent dye molecule associating with different bases in a DNA sequence, polynucleotide tails were added to the 3’ end of a portion of the p53 target (Figure 2.15). By keeping the sequence of the probe constant, but varying the bases at which the fluorophore resides, we were able to examine the relative affinities of the fluorophore for each type of nucleotide. Melting curves were generated by observing the change in absorbance at 260 nm, baseline subtracted and normalized to their maximum value to facilitate comparison.

The resulting differences in melting curves and $T_m$ between the sequences indicate that the DNA/fluorophore interactions were different depending on which type of base (A, T, G or C) the Texas Red X fluorophore was allowed to interact with. These results provide evidence that the type of adjacent nucleotide may produce bias in the dynamics characteristics of that fluorophore. Further, this also suggests that methylation may have an impact on fluorophore affinities, opening the way for epigenetic analysis within oscillatory dynamics.

Overall, these data support the conclusions in Chapter 1 that not only do mismatched probes readily hybridize, but that depending on the sequence, the kinetics of binding are very similar. Also, it can be seen that FRET responses are
sequence dependent, and that fluorescent behavior is critically dependent on the immediate environment.

2.4 Preliminary Electrophoretic Devices

Early work by Heller et. al., demonstrated that the ends of double stranded oligonucleotide probes spent more time in solution than the center of the probe [Heller MJ 1974]. Our hope was that low levels of electrophoretic force below the dehybridization threshold could initiate oscillatory behavior from the frayed ends of intermolecular fluorescent resonant energy transfer (FRET) constructs.

It is expected that externally driven FRET systems will provide detailed information about the instantaneous free energy of detected species. Position and sequence dependent opening energetics have been observed with imino proton exchange NMR, and rehybridization of DNA hairpin molecules have been shown to be slowed by entropic traps during the post-nucleation hybridization process [Chen C 2004, Ansari A 2001, Ansari A 2002]. Therefore the kinetics of both opening and rehybridization are likely to depend upon the complementarity of the target DNA involved in assembling the FRET system. Similar mechanisms have been used to quantify intramolecular hairpin dynamics using thermal denaturation, but these methods have not been applied to mechanisms appropriate for intermolecular modulation [Ansari A 2002, Braun D 2003]. It was the purpose of
the following experiments to explore whether low frequency actuation of these constructs can provide a measure of differentiation from nonspecific binding. Along the way, we found that the devices used to actuate oscillations are as important as the molecular constructs in generating useful data. We also found that electric fields alone are generally insufficient to modulate hybridization of solution phase constructs, which migrate rather than dissociate.

Early implementations of electrophoretic actuation systems were built to interface to the optical system of the LS-55 fluorometer. Figure 2.16 shows a three chamber setup with the upper and lower chambers separated by a 2% high strength agarose gel. The upper and middle chambers were fashioned from fired borosilicate glass pipettes, and the lower buffer chamber consisted of a 50 mL Falcon tube. The quenched quantum dot sample (as shown in Fig 2.4) was added to the middle chamber in 0.1 M sodium phosphate, pH 7.0. The upper and lower electrode chambers were separated by approximately 5 cm and connected to the positive and negative terminals of a BioRad PowerPac 1000 power supply. The device could withstand voltages of upwards of 50 V, or currents of 25 mA, giving a field amplitude of roughly 1,000 V/m. We were never able to definitively show that the quencher probes were removed from the quantum dots. Shown in Figure 2.16 are the results from quantum dots accumulating within the lower gel from the middle buffer, the effect of which outweighed any removal of quencher. The setup was also prone to impressive failures when heat and pressure built up within the gel plugs during actuation. Of note, we did see oscillations of ethidium bromide / DNA
solutions, although hindsight suggests that these could have been quantum yield fluctuations of the ethidium dye upon application of the field.

The second generation device was motivated by the theory that the QSY-7 probes did not dehybridize upon electric field application because the field gradient between quencher probe and quantum dot was too small. The QSY-7 probe was chosen for its formal positive charge in hopes that a differential force between the quantum dot and the QSY-7 would be sufficient to separate the strands. Since quantum dots carry an intrinsic negative charge, a more likely scenario was that the charge on the QSY-7 probe overstabilized its interaction with the quantum dot. Therefore, we decided to immobilize FRET constructs in hopes that by anchoring the donor, enough force could be applied to remove the quencher probes. Further, the large gel constructs were replaced with a thin cellulose acetate disc [Alltech #2129, 25 mm diameter, 0.45 μm pores] in order to minimize resistance and Joule heating.

A suspension of 4% low-melt agarose [Bio-Rad, 162-0017] in 0.1 M sodium phosphate and an equivalent volume of micron-sized, biotinylated agarose beads [Sigma Aldrich, B0519] was heated to melting in a microwave and allowed to cool to 60°C. The cellulose acetate disc was spin coated at several thousand rpm. In order to prevent premature cooling, 500 μL of the hot suspension was aspirated, and 4-5 drops were manually applied to the spinning disc. The gel carrier was deemed necessary to prevent alternate current paths from circumventing the agarose beads.
The coated filter paper was placed in a small petri dish, followed by successive incubations of 10 µL of 0.2 µM streptavidin functionalized quantum dot, biotinylated capture probe (p53B51WT), and quencher (i.e., p53m2bpQSY7). Figure 2.17 shows the device used to apply fields across the filter paper, which was sandwiched between an upper and lower buffer chamber. The entire device was placed in a bath with buffer up to the sidewalls of the upper chamber. The upper chamber was also filled with buffer. The outer rim of the cellulose disc was sealed with polydimethylsiloxane adhesive, which also served to gasket the upper and lower chambers. Platinum electrodes were clipped into each chamber and fed into the power supply.

Figure 2.18 shows a series of fluorescent images taken in a UV transilluminator of the cellulose acetate discs. In 2.18A, the levels of bead supported quantum dot fluorescence before quenching are shown. 2.18B shows the same membranes following incubation with capture probe and quencher. 2.18C shows the effects of thermal stringency and electric field in removing the quenchers. For the successful removal of quencher, 0.25X Tris borate EDTA (TBE) was heated to 40-45 ºC. Discs were run for five minutes at 200 V with resultant currents of ≈10 mA. Disc #1 was a control which only saw heated buffer, but no quencher. It remained bright. Discs #2 and #3 were quenched with two base pair mismatched probes. #2 was exposed to 45 ºC buffer for 5 minutes, while #3 was exposed to electric field and buffer for 5 minutes. The other two membranes saw only quencher for comparison. Subsequent experiments showed that there was
considerable variability in the resultant currents, and differences between stringency and field were minimal at best. The heating and low salt buffer seemed to carry a more profound influence than the field in this configuration. We suspected that the sealing was poor and that the gel coating of the membrane provided little resistance to the current, allowing alternate current paths to largely circumvent the beads.

Electric force on a formal point charge is directly related to the potential gradient at a specific point:

\[
\vec{E} = -\nabla \phi \quad \text{and} \quad \vec{F} = q(\vec{E} + \vec{v} \times \vec{B})
\]

per the well known electrostatic relationships. The field gradient in Figure 2.17 and 2.18 was compromised by the continuous decrease in potential through large volumes of buffer and gel. Both buffer and gel present series impedances which reduce the effective gradient by the time the field reaches the molecular system. To minimize this effect, reduce heating, and provide real-time imaging of the fluorescence removal, the spin coated membrane was placed in direct contact with a gold coated slide [Erie Microarray, Portsmouth NH, C09-5076-M20] and sealed with electrical tape. 5 µL of 11 µM fluorescent p53RCy3 probe was incubated atop the membrane for an hour within the petri dish to facilitate diffusion into the agarose. 50 µL room temperature 0.1X TBE was placed atop the membrane, and a platinum counter-electrode was positioned above the construct and positively biased. Figure 2.19 shows a series of frames following the 5V, ≈1.2 mA removal of p53cCy3 under the direct influence of the electric field. The lower total current
from the low conductivity 0.1X TBE allowed for more of the flux to be assumed by the DNA. The Nernst-Planck constitutive equation details this balance:

$$\vec{I}_i = \frac{z}{|z_i|} \mu_i c_i \vec{E} - D_i \nabla c_i + c_i \vec{v}$$ \hspace{1cm} (11)

where $z$ is the valence, $\mu$ is the mobility, $c$ is the concentration, $D$ is the diffusion constant, and $\vec{v}$ is the velocity vector. Each individual ionic flux $\vec{I}_i$ (mol/s·cm²) has contributions from electrical mobility, diffusion, and convection. The total flux is the sum of each of the individual fluxes, which is related to the measured current per area by the average valence times Faraday’s constant. Ionic species within solution (including DNA) act as a parallel resistances to current. Low conductivity buffers like TBE, take up less of the total current as compared to more dissociable buffers such as sodium phosphate, allowing DNA to assume a larger flux. By minimizing the volume of buffer between counter-electrode and construct, DNA mobility is maximized. Eliminating the cellulose membrane and agarose beads in favor of a thin layer of functionalized polyacrylamide atop the gold slide further improved repeatability.

2.5 Low Frequency Electrophoretic Actuation

In order to examine the information content of electrophoretically actuated FRET transducers, a polyacrylamide model of \textit{in situ} hybridization was developed (Figure 2.20). The polyacrylamide gel serves as a physical barrier between the
electrolysis products at the surface of the electrode and the biological molecules bound to its surface. This is known as a permeation layer. To maximize surface functionality, biotin dextran [Sigma-Aldrich B9264] was incorporated into the gel layer. 14 µM biotin dextran was prepared in a 19:1 acrylamide:bis, TBE buffer solution. All buffers were prepared with 18.2 MΩ deionized water [Millipore]. 100 µL of the acrylamide solution was combined with 0.35 µL of TEMED [Sigma-Aldrich] and 0.35 µL of ammonium persulfate. The activators were stirred into the acrylamide rather than using aspiration to avoid excess oxygenation during mixing. 15 µL aliquots of biotin dextran acrylamide were immediately placed within a circular, 5 mm diameter wells fashioned from a hole punched, residual stress-free insulating tape adhered to gold coated slides. A partially wrapped borosilicate glass slide [Parafilm-M] was rapidly tilted on top of the acrylamide and a 200 g weight placed on top of the assembly to provide a uniform hydrophobic surface that was reasonably sealed from the oxygen atmosphere. The assembly was cured for 1.25 hours at room temperature, upon which time a hybridization solution (0.5M NaCl, 0.05 M sodium phosphate, pH 7.0) was poured over the slides. Capillary action lifted the top glass enough to allow the slides to be sheared apart with minimal effort. Normal forces were avoided during separation. Excess polymerized gel around the circumference of the well was carefully removed using a lint-free cloth. The polymerized dextran acrylamide was washed twice with hybridization solution.

Independent of, equimolar amounts of p53B51WT functionalized Cy3 streptavidin were mixed with complementary or 1bp mismatched QSY-7 modified
DNA (p53cQSY7, p53m1bpQSY7), vortexed, briefly spun, and then allowed to hybridize for 3 hours. Stock solutions of DNA were reconstituted in pH 7.0, 0.1 M sodium phosphate and concentrations verified by measuring absorbance at 260 nm. Functionalized Cy3 streptavidin samples were stored at 4°C in o-ring sealed plastic tubes prior to use. After washing the acrylamide constructs with hybridization solution, excess fluid on top of the gel layer was removed via suction at the top right corner provided by a lint-free cloth. 5 µL of the prehybridized, quenched Cy3 system was added to the center of the gel and incubated for five minutes before washing and immersing in hybridization solution at room temperature until analysis. We found that higher concentrations and short incubations reduced diffusion into the subsurface of the acrylamide.

Shortly following the gel surface modification, the DNA constructs were analyzed under a custom built epifluorescent microscope mounted on an air-stabilized optical table. Gold slides were attached to an electrically insulated, current controlled Peltier stage [LDT 5910B, ILX Lightwave, Bozeman MT] located under a rotary Leica head fitted with long-range air objectives. Light from a 100W halogen lamp was passed through a dichroic mirror and focused onto the sample, with the return light passing vertically through the dichroic and a green (Cy3) emission filter before being recorded by a Orca-ER CCD camera [Hamamatsu C4742, Hamamatsu City, Japan] driven by custom written software. A 64x64 pixel subarray of the 12-bit camera field was set to 8x8 binning mode to
provide maximal contrast at ≈100 Hz A/D conversion through a National Instruments PCI-1422 board [National Instruments, Austin TX].

A 32-gauge platinum counter electrode [13-766-10B, Fisher Scientific] was positioned adjacent to the gel construct, guided by micromanipulator probes [P110/360MT, Probing Solutions, Dayton NV] and connected in series with a programmable 1 kV, 100 mA power supply [237 SMU, Keithley Instruments, Cleveland OH] as well as in series with an external 100 Ω resistor. Parallel connections across the Keithley and resistor were fed into the analog inputs of a USB DAQ [6009 National Instruments] in order to simultaneously record applied power and estimate aliasing over time. The platinum electrode from the high Keithley terminal was connected to an exposed half of the gold slide with insulating electrical tape. Immediately before analysis, a second, circular, 3 mm diameter hole made within residual stress-free insulating tape was placed over the center of acrylamide construct to mitigate low-resistance current paths originating from the acrylamide-well interface. 400 µL of 0.01X TBE buffer was pipetted on top of the 3 mm well, taking care to create a prolate spheroid of buffer with an off-center minor axis such that any bubbles that may release during electrophoresis were guided away from the objective’s focal point. Finally, the upper electrode was positioned flat against the surface of the second layer of insulating tape, roughly 3 mm removed from the outer edge of the gel construct with the long axis of the wire spanning the diameter of the acrylamide. It was found that displacing the counter-electrode provided more repeatable quantification.
 Constructs were first exposed to an electronic stringency protocol in order to precondition the system (Figure 2.21). The gold slide surface was biased negative compared to the upper electrode from -1V to -6V in -0.1 V/s increments. Following stringency, any bubbles that were observed to accumulate along the edge of the tape were removed by gentle agitation from a pipette tip.

Polyacrylamide bound streptavidin DNA constructs were subjected to a sequence of three hundred monophasic square wave oscillations at -3V and -6V at 10 Hz, with the lower gold electrode biased as the cathode. Earlier qualitative findings had demonstrated that certain fully hybridized constructs did not oscillate when -3V, 10 Hz was applied across the electrodes, but began to respond when given a higher power. The early results suggested that the thickness of the gel was the rate-limiting factor in coupling electrophoretic fields to the constructs.

Along with the complementary and mismatched DNA, controls included: unmodified acrylamide constructs, fluorescent streptavidin bound to the polyacrylamide, and fluorescent streptavidin bound to the acrylamide containing only a capture probe. Unmodified constructs with free Cy3 labeled DNA added to the 0.01X TBE buffer were also observed in order to control for fluorescent molecules migrating in and out of the focal plane. A set of five experiments were repeated for each type of DNA under each oscillatory influence.

Time series data were detrended by subtracting a 256th order zero-phase forward and reverse lowpass filter, normalized by 2 standard deviations, and multiplied by a full-length, centered Hanning window prior to Fourier analysis.
Normalized peak amplitudes were calculated as the mean and standard error of the maximum amplitude between 9 and 11 Hz, normalized by the sum of the data within each window.

We observed a sequence and power dependent oscillatory response across the FRET constructs. Figure 2.22 depicts a typical small signal output of a hinged transducer in response to -3V, 5Hz actuation. Harmonics of the square wave response were clearly visible in the Fourier spectrum.

As shown in Figure 2.23, 10 Hz, -3V had little effect on the constructs at room temperature, but clear differences in amplitude were evident upon raising the voltage to -6V. The data showed an increase in oscillatory response with complementarity. The matched system (Group 6) was significantly enhanced as compared to its level at lower power (p < 0.034, as calculated by a student’s t test). The groups did not show a significant inter-group difference using a Tukey’s test (mean difference/√(mean squared error/harmonic mean)).

Cy3 streptavidin (Group 7) exhibited relatively constant fluctuations in response to electrophoretic actuation. This positive control remained consistently high at each actuation mode, likely as a result of the environmental modulation of the quantum efficiency of the fluorophore [Braun D 2003]. The steady response of the fluorescent streptavidin clearly contrasted the power-dependent oscillations of the fully assembled DNA system.

We found no correlation between normalized peak amplitude and power measured across a 470 Ω resistor in series with the gel constructs during the
electronic stringency ramp, indicating that differences in oscillation amplitude were not attributed to inherent construct variations, such as alternate low-resistance current paths in parallel with the FRET systems. Other random effects such as bubbles or electromechanically induced displacements within the constructs were uniformly distributed throughout the data and are unlikely to produce bias. Thus, the change in oscillatory responses are likely due to electrophoretic effects within the FRET system.

In order to test sensitivity gains resulting from the oscillatory behavior, serial dilutions of single base pair mismatch transduction systems were processed for electrophoretic actuation as described above. A -3V to +1.4V square wave at 5 or 10 Hz was applied to the gel constructs using an Agilent 33120A function generator for 30 minutes in order to accumulate the $2^{17}$ samples needed to facilitate a quality fast Fourier transformation of the data. The slight positive voltage was applied in order to help mitigate long-term pH shifts as a result of the electrolysis. Estimates of surface concentration assume that the FRET systems were bound uniformly across the gel construct, with areas calibrated against full frame images of a well characterized 80 µm diameter electrode, resulting in roughly 9 µm$^2$ per 8x8 binned pixel image. Spectra were calculated by taking the absolute value of the Fourier transform of the autocorrelation of the detrended, normalized time series. The center frequency of the data shown in Figure 2.24 was verified against spectral densities of the applied voltage.
Aliasing within the system prevented quantitative comparisons of the long-term recordings between subsets. We were unable to eliminate memory caching within the software, which broadened peaks and made direct comparisons impossible at such low signal-to-noise ratios. It was evident that a concentration dependent response of the actuated FRET system remained. Clear peaks in the frequency domain were visible down to near single molecule levels, despite being roughly 5,000 times lower than the autofluorescent background. The amplitude of the oscillations likely contain significant amounts of superposition between actual FRET responses and quantum yield fluctuations.

While it is premature to ascribe molecular mechanisms to explain the oscillatory differences between mismatched and matched behavior, power dependency indicates that a certain level of force is required before the differences in probe hybridization appear in response to electrophoretic actuation. It can also be concluded that on average, random fluctuations due to Brownian motion did not dominate the applied electrophoretic force within the FRET system.

Ideally, applied forces should provide enough leverage to unhinge the FRET pair without completely removing reporter probes from the target, as shown in Figure 2.20. Estimates of the force required for the initiation of DNA unzipping are widely varying in the literature (between 15-200 pN), and depend strongly on how fast the forces are applied [Cocco S 2002]. The electronic stringency results shown in Figure 2.21 demonstrated that steady state fields carried sufficient force to remove complementary oligonucleotide probes at higher temperatures. At lower
temperatures the constructs were more stable, remaining bound despite the application of the electric field.

Voltages were kept below the full dehybridization threshold in an attempt to prevent large-scale diffusional dynamics from blurring the results. The monophasic square wave actuation used to discriminate matched from mismatched responses (between negative voltages and ground) was used in hopes that any dehybridized probes were transported away from the bound constructs and could not diffuse back towards the field of view. Electrophoretic modulation of oligonucleotide FRET systems did seem to produce sequence dependent dynamics. It is our current interpretation that the equilibrium of the frayed ends of the molecules was altered by the application of the low frequency fields, rather than actual in-phase motion as a result of the actuation.

In order to correlate dynamic FRET output with actual mechanical motion of the molecules, it is likely that the frequency of modulation will have to be significantly increased. It is known that phonon modes of DNA begin in the GHz range, while transverse modes of long DNA segments have been observed using microwave absorption techniques [Woolard D 2002, Prohofsky EW 2004, Edwards GS 1985, Van Zandt LL 1986]. Neither of these modes are amenable to FRET reporting, as fluorescent lifetimes are generally observed on the order of 1-10 ns [Krishnan R 2003]. However, hybridization kinetics of short hairpin loops have been measured in the 1-100 kHz range, well within the sampling rates available to a modulated fluorescent transduction system [Ansari A 2001, Braun D 2003].
It is not clear whether electrophoretic actuation will be able to attain sufficient field strengths to effect modulation of hybridization at these frequencies. At 25°C the mobility of a sodium ion is $5.19 \times 10^{-4} \text{cm}^2\text{V}^{-1}\text{s}^{-1}$ [Grodzinksy A 1995]. A 10 V signal applied to electrodes spaced at 100 µm would enable a sodium ion to move roughly 50 nm at 100 kHz. While potentially sufficient to affect constructs located within the double layer, bulk modulation of nanoscale mechanisms seems unlikely at high frequencies using electrophoresis.

Since the ability to modulate FRET constructs located at micron-scale distances away from an electrode surface seem to be directly tied to the effects of electrophoresis, it is likely that the capacitance of the device will be the rate-limiting factor in modulation frequency. The power dependence of actuation supports this conclusion. It is well known that bubbling falls off sharply with electrophoretic frequency, even at high voltages. At 10 kHz or higher, electrolysis products may be formed within a short distance away from a non-polarizing electrode surface, but likely recombine almost as quickly as they are formed. In order to use electrical modulation to affect the state of hybridization at high frequencies, DNA constructs would have to reside within this short distance away from the electrode surface. In fact, Heaton et. al., demonstrated 300 mV DC electronic stringency within surface layers of an SPR sensor.

During the course of this dissertation, considerable effort was spent trying to functionalize the surface of gold slides with the FRET systems shown herein. Our work focused on direct binding of thiolated capture probes to the gold surface.
Unfortunately, we were never able to obtain sufficient signal from a monolayer of fluorescent energy transfer systems to facilitate reliable quantification with the optical systems available to us. Detailed research into this subject revealed that there is a strong inverse correlation between the length of DNA and the attainable surface coverage, and that thiolated 48mer probes bind at over an order of magnitude lower capacity than thiolated 24mer oligos [Steel AB 2000]. Our capture strand was 51 bases long, and given that a monolayer of fluorophores was difficult to reliably detect, it is now unsurprising that we were unsuccessful at detecting differences within a sparse monolayer of quenched probes.

The design of the FRET constructs may also play a role in discerning the differences between complementary and mismatched probes. The proximity of the mismatch to the frayed end as well as interactions with surrounding base pairs may significantly affect the behavior of oscillatory systems. The lack of information surrounding the rates of prenucleated rehybridization makes it difficult to claim that all sequences will behave identically under electrophoretic perturbation. In particular, local free energy minima along the rehybridization isotherm could significantly affect the dynamics of certain sequences [Ansari A 2002]. The probability of exploring secondary structures during rehybridization may also vary with the position and type of mismatch, in which case the relative magnitude of oscillatory response between complementary and mismatched sequences would be difficult to predict. Nevertheless, our interpretation that matched oscillations are
greater than mismatched oscillations are consistent with earlier electrophoretic microarray data [Heller MJ 1997].

In contrast to microarray formats where µm electrode spacing enables kV/m field densities to dehybridize oligonucleotides, the ability to modulate the hybridization of surface bound constructs with a macroscopic counterelectrode suggests possible application of this method to in situ hybridization, where paraffin embedded tissue sections preclude the use of microarrays for electrophoretic actuation.

### 2.6 Modulation of Quantum Dots

The idea of scanning through a field of surface bound, individually detectable quantum dots was one of the original concepts of this dissertation. At the time, the idea of a “virtual array” was popular; a large concentration of solution phase nanoparticles would collect sheared DNA to drive the probability of recognition. The particles would then be electrophoretically addressed to a surface in order to facilitate analysis. Immunomagnetic separations work in a similar manner to this, but suffer from low recovery efficiency. The virtual array approach offered a method of lossless sample preparation, since the nanoparticles would never be removed from the sample buffer. The original hypothesis included the provision that quantum dots with matched targets would oscillate at a different
frequency than nanoparticles with mismatched targets or nonspecifically bound particles lacking a FRET system. Since quantum dots were reminiscent of point sources of light, comparisons was drawn to the analysis of a pulsars within a dense field of stars.

The following factors were complicit in preventing implementation of such a detector: 1) Solution hybridized quantum dots became sterically hindered, inhibiting binding to a biotinylated surface, 2) quantum dots were susceptible to considerable nonspecific binding, as in other studies [Gill R 2005, Gerion D 2003], 3) low frequency electrophoretic modulation causes everything to oscillate at the same frequency, just different amplitudes, 4) quantum dots fluctuate in the presence of unbound quencher probe, 5) semiconductor quantum dot FRET is very insensitive, requiring an excess of acceptor to appreciably change the rate of emission.

The experiments on quantum dot modulation were performed atop platinum microelectrodes patterned onto a silicon substrate [Nanogen, Inc]. There was no permeation layer to this chips. These trials were not meant to explicitly model the in situ environment, so we chose a microelectrode format to incur less variability and higher electric fields than the manually positioned counter-electrode preparation described above. Figure 2.25 illustrates the quantum dot assembly.

Chips were immersed in piranha etch for 30 minutes (70% H₂SO₄, 30% H₂O₂). NOTE: piranha etch and organics produce a very strong exothermic reaction [Herne TM 1997]. Washes should be performed in a covered glass petri dish placed
atop a hot plate in the hood, in an area with no lab cloth surrounding the etch. The etch solution would boil depending upon the level of organic material present on the chips. Only about 10 mL of solution needs to be prepared at any time). Piranha etch was poured off into a waste container, and chips were thoroughly rinsed with Millipore deionized water. 1 mg/mL biotin dextran polymer [Sigma B264] was baked onto the chip surface at 80 °C for one hour. Halfway through the quantum dot trials, it was recognized that using ethanol as a solvent for the biotin dextran provided much better surface uniformity than aqueous solutions. Typically, 2 μL of second generation 2 μM streptavidin functionalized quantum dots [Qdot 565 ITK Streptavidin Conjugate, Invitrogen] were incubated on the chip surface at room temperature in a humidity chamber for one hour. The humidity chamber was formed using a covered petri dish with ≈1 mL deionized water pipetted around the walls of the dish, making sure that the surface tension at the walls kept the fluid segregated from the chip. A 200 g weight was added to the top of the dish to seal the chamber. Quantum dots were rinsed in a 1X TBE solution to preserve photostability (Figure 2.26). This resulted in a self-evident green spot in the center of the chip, which could easily saturate our CCD detector under high gain. Successive incubations of biotinylated p53B51WT capture probe, and the appropriate quencher probes were performed in between washes with hybridization solution. Chips were kept continually hydrated once the quantum dots had been bound to the surface. Independently, a 16-fold excess of prehybridized complementary quencher was shown to reduce 600 pM quantum dot fluorescence
by half (23.3 to 9.95, and 22 to 10, arb units) after 800 seconds of solution-phase incubation. This was done to confirm that the new generation of ITK quantum dots allowed FRET quenching.

Interestingly, it was found that adding capture probe to quantum dots in solution prevented their attachment to the chip surface. Equal 1 µL volumes of 1.6 µM prehybridized quencher (p53B51WT capture probe with p53cQSY7 in hybridization solution) and 0.4 µM streptavidin quantum dots were mixed to provide a 4:1 mixture of DNA to quantum dot in 0.25 M NaCl, 0.025 M sodium phosphate. A similar solution of capture probe and noncomplementary RCA5QSY7 quencher probe was prepared. No signal was evident on the chip surface from either of the preparations after rinsing with 1X TBE. Prior experience indicated that quantum dot quenching saturated after the addition of an almost 20-fold excess of biotinylated probe, so it seems unlikely that a fourfold excess would result in a large concentration of free biotinylated probe that could deactivate the streptavidin surface of the chip. That said, no further purification of the quantum dots was performed before addition to the surface. An extended 51mer capture probe is roughly equal in length to the diameter of the quantum dot. Single stranded DNA half the size of our capture probe have been shown to nonspecifically wrap around the outside of quantum dots to the point of affecting hybridization efficiency [Gerion D 2003]. This precedent suggests that in order to implement a virtual array, a multifunctional nanoparticle with two types of binding affinities would be required. The surface binding moiety would also likely need to be attached by a
long chain linker. However, multifunctional nanoparticles are generally non-trivial to synthesize [Derfus A].

In preparation for modulation, microchips were manually connected using micromanipulator probes. Probe tips were dragged across connector pads until a visible scratch was formed. During this time, the chips were kept under hybridization solution, since the connection required microscopy to validate both position and connection. Chips were washed in 1X TBE, and then washed again in a 1/100\textsuperscript{th} dilution of TBE to raise the resistance into the 2.3-2.8 MOhm range, as measured by an Agilent 4263B LCR meter at 1kHz. Addition of 2.5 \(\mu\)L of 1X TBE lowered the resistance to 330 kOhm. Similar resistances were observed upon addition of an equivalent amount of quencher probe suspended in 1X TBE during experiments. Of note, we were unable to quench surface bound quantum dots to the same degree as solution phase particles. Under microscopy, addition of an excess of prehybridized quencher reduced fluorescence was monitored for 229 seconds, resulting in a decrease from 779 to 730 twelve bit counts. Background was typically recorded between 200-210 counts.

Modulation of surface bound, complementary quenched quantum dots is shown in Figures 2.27 and 2.28. Figure 2.28 shows a series of dynamic range adjusted frames to better depict the extent of modulation.

The quantum dot labeled surface reveals a very heterogeneous pattern, indicative of clustered nanoparticles. This is not observed with organic
fluorophores. Nonspecific clusters of quantum dots could grow to be many layers thick and change the environment modulated DNA.

An intriguing property of quantum dots was the nearly undetectable baseline oscillation in the presence of an applied electric field. Organic fluorophores respond strongly to electrophoretic actuation even without a FRET couple. Serial incubations of capture probe and complementary quencher resulted in the strong modulation shown in Figure 2.28. However, the addition of free, non-hybridized RCA5QSY7 probe also resulted in strong modulation.

Additional experiments are required to understand the causality of this oscillation. The hybridized quencher systems were thoroughly washed following incubation and stored in a large volume of hybridization solution before preparation for modulation, which consisted of extensive buffer exchange into 0.01X TBE to raise the resistance. It seems unlikely that residual free probe remained in solution after this treatment. Over time, it is possible that the quencher dehybridized from the capture probe in the low salt buffer, although we did not observe a gross increase in fluorescence over time.

Quantum dots have a relatively large surface area and do not require a specific orientation for resonant energy coupling. Because of this, free quencher in close proximity to the quantum dot could theoretically affect emission. Quenching in the presence of 100-fold excess of free quencher was not seen in solution at steady state (Figure 2.4). During modulation, the depth of quenching was far
greater than that accessible with passive incubation of either prehybridized or noncomplementary QSY-7 probe.

The morphology of the biotin dextran layer is not known, yet it is permeable and supports electrophoresis. Immobilization of quantum dots on this layer seems to reduce access to the surrounding streptavidin, given the relatively low quenching of surface bound nanoparticles. Reduced access to streptavidin could also be the result of steric hindrance within the monolayer of nanoparticles.

Electrophoretic actuation could conceivably overcome the diffusive barrier to the underside of the layer and concentrate free quencher probes to be in close proximity to the quantum dots, which is otherwise unfavorable. This theory does not explain the discrepancy in amplitude between complementary hybridized quencher probes and the decrease in emission during modulation, as there should be the similar concentrations of quencher in close proximity before and during modulation. One possibility is that hybridized quencher probes exist in a partially hybridized state and are pulled in closer to the quantum dots upon actuation.

It has been suggested that changes in pH are solely responsible for the oscillation of the quantum dots. Figure 2.29 displays the intensity of quantum dots under modulation with and without the addition of quencher. Quantum dots on biotin dextran were prepared as described above, and rinsed with 0.01X TBE. Quencher probes and biotinylated capture probes were prehybridized in 1X TBE. To control for potential salt effects, quantum dots were exposed to 2.5 μL 1X TBE for several minutes, which dropped the impedance to 330 kOhm. The chip was
thoroughly flushed with 0.01X TBE until the impedance reached 2.7 MOhm. Application of 3 V, 2 Hz actuation showed no evidence of oscillation. Following modulation, the 0.01X TBE buffer was exchanged for the prehybridized quencher probe in 1X TBE, which dropped the impedance to 225 kOhm. After incubation, the chip was again flushed with 0.01X TBE (to 2.4 MOhm) and exposed to 3 V, 2 Hz actuation. The results shown in the bottom of Figure 2.29 clearly demonstrate a 2 Hz oscillation in response. This is strong evidence that pH alone is not responsible for the observed oscillations, although under high voltages greater swings in pH may result in a more pronounced change of fluorescence intensity.

Chapter 2 in part, is a reprint of the material as it appears in Nano Letters: Sullivan BD, Dehlinger DA, Zlatanovic S, Esener SA, Heller MJ. Low-frequency electrophoretic actuation of nanoscale optoentropic transduction mechanisms. Nano Lett. 2007 Apr;7(4):950-5. The dissertation author was the primary investigator and author of this paper.
Figure 2.1 Fluorescent components used in various FRET experiments. The quantum dot and oligos are not shown to scale. Quantum dots are roughly 15-20 nm in diameter, whereas the diameter of streptavidin is on the order of 2 nm, and the organic fluorophores are about 1.5 nm wide [Jares-Erijman 2003].
Figure 2.2 Absorption (dotted) and emission (solid) spectra of optical components. QSY-7 shows only an absorption spectra because it is non-fluorescent.
Figure 2.3. Subset of the DNA library used in FRET experiments.

\[
\begin{align*}
p53\text{B51WT} & \quad 5'-\text{Biotin-GAA-CAG-CTT-TGA-GGT-GCG-TGT-TTG-TGC-CTC-TGG-GAG-AGA-CCG-GCG-CAC-3}' \\
p53\text{cQSY7} & \quad 3'-\text{QSY7-CTT-CTC-GAA-ACT-CCA-CCC-AC-5}' \\
p53\text{mbpQSY7} & \quad 3'-\text{QSY7-CTC-GTC-GAA-ACT-CCA-CCC-AC-5}' \\
p53\text{mbpQSY7a} & \quad 3'-\text{QSY7-CTG-GTC-GAA-ACT-CCA-CCC-AC-5}' \\
p53\text{mbpQSY7b} & \quad 3'-\text{QSY7-CTC-GTT-GAA-ACT-CCA-CCC-AC-5}' \\
s\text{RCA5QSY7} & \quad 3'-\text{QSY7-CTA-CTC-GTC-AAG-ATG-CAC-C-5}' \\
\text{BiotinQuencher} & \quad 5'-\text{Biotin-TTA-QSY7-3} \\
p53\text{RCY3} & \quad 3'-\text{A-AAC-ACG-GAC-AAG-ACC-CTC-5}' \\
p53\text{Cy3F} & \quad 3'-\text{Cy3-C-ACG-GAC-AAG-ACC-CTC-5}' \\
p53\text{cTRX} & \quad 3'-\text{CTT-GTC-GAA-ACT-CCA-CCG-A-Texas Red X-5}' \\
p53\text{mbpTRX} & \quad 3'-\text{CTT-GTC-GAA-ACT-CCA-TGC-A-Texas Red X-5} \\
p53\text{mbpTRX} & \quad 3'-\text{CTT-GTC-GAA-ACT-CCA-TGC-A-Texas Red X-5}' \\
p53\text{Cy3C} & \quad 3'-\text{BiotinTEG-CGC-ACA-AAC-ACG-GAC-AG-5} \\
p53\text{Cy3End} & \quad 3'-\text{BiotinTEG-CGC-ACA-AAC-ACG-GAC-Cy3-5}' \\
p53\text{BTRX} & \quad 3'-\text{G-ACC-CTC-TCT-GGC-CGC-GTG-Biotin-5}' \\
p53\text{mbpBTRX} & \quad 3'-\text{G-ACA-CTC-TCT-GGC-CGC-GTG-Biotin-5}' \\
p53\text{BTRXEnd} & \quad 3'-\text{TRX-CC-CTC-TCT-GGC-CGC-GTG-Biotin-5}' \\
\text{QatarC} & \quad 5'-\text{Biotin-CAC-GAG-AGA-CTC-AGG-GGC-GTG-CCG-ATC-GGC-TCC-TCA-GGT-CAA-GTC} \\
\text{Qatarlp} & \quad 5'-\text{Biotin-CAC-GAG-AGA-CTC-AGG-GGC-GTG-CCG-ATC-GGC-TCC-TCA-GGT-CAA-GTC} \\
\text{QatarCy3} & \quad 3'-\text{Cy3-GC-TAG-CCC-AGG-ATG-CCG-GTC-5}' \\
\text{QatarTRX} & \quad 3'-\text{TC-TCT-GAG-TAC-TGC-TCC-CCG-A-Texas Red X-5} \\
\end{align*}
\]
Steady State Quantum Dot Quenching

Figure 2.4. Quenching of a 565 nm quantum dot with an excess of QSY-7 probe. One and two base pair mismatched oligonucleotides readily bind to the capture oligonucleotide, as evidenced by the equivalent reduction in fluorescence to the matched strand.

<table>
<thead>
<tr>
<th>#</th>
<th>Construct</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Quantum Dot Alone</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>Noncomplementary</td>
<td>3’-QSY7-CT\text{a}-cTC-G\text{tc}-A\text{ag}-atg-\text{CaC}-c-5’</td>
</tr>
<tr>
<td>3</td>
<td>2 base pair mismatch</td>
<td>3’-QSY7-CT\text{c}-G\text{Tc}-G\text{Aa}-ACT-CCA-C\text{GC}-AC-5’</td>
</tr>
<tr>
<td>4</td>
<td>1 base pair mismatch</td>
<td>3’-QSY7-CT\text{c}-GTC-G\text{AA}-ACT-CCA-C\text{GC}-AC-5’</td>
</tr>
<tr>
<td>5</td>
<td>Complementary</td>
<td>3’-QSY7-CT\text{T}-G\text{TC}-G\text{AA}-ACT-CCA-C\text{GC}-AC-5’</td>
</tr>
</tbody>
</table>
Figure 2.5 Steady state quenching of fluorescent protein constructs. Neither addition of noncomplementary quencher nor biotinylated capture probe quenched the fluorescent protein. A reduction of roughly half of the fluorescence resulted from a 1:1 addition of biotinylated capture probe and complementary, 1- and 2 base pair mismatched QSY-7 quencher probes.
Figure 2.6. Differential FRET response between a hybridized p53cQSY7 probe and a biotinylated QSY-7 quencher linked to the 5’ end of a three base pair oligo [5’-Biotin-TTA-QSY7-3’]. Inset: estimated FRET efficiency with 56% and 30% quenching corresponding to the hybridized probe and biotinylated quencher, respectively.
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Figure 2.13 Sigmoidal fit is a near perfect estimate of the eigenvalue kinetics. Also shown is the single exponential fit returned by MATLAB after 2,000 cycles of optimization.
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Figure 2.19. Fluorescence removal via direct platinum counter electrode.
Fluorescent protein transducer model of *in situ* hybridization. To explore actuation parameters, 1:1 concentrations of quencher were hybridized to the capture probe, and then bound to a streptavidin-activated acrylamide surface. Fluorescence from the donor (streptavidin) to QSY-7 quencher (light circle) is modulated by an applied driving force that converts mechanical motion into a time varying optical signal.
Figure 2.21. (Top) Electronic Stringency at 45°C for a complementary 19mer Cy3.5 labeled fluorescent probe. A majority of the bound probe is removed at -3V at 45°C. (Middle) Normalized fluorescent response of surface bound constructs during application of electronic stringency preconditioning. Note that at room temperature, 25°C, the voltages are below the dehybridization threshold, as no significant change in fluorescence is observed. (Bottom) Voltage used for preconditioning, -1V to -6V, 25°C. Background autofluorescence was measured at a value of 280 units.
Figure 2.22. (Top) Driven transducer response. Raw fluorescence data was detrended by subtracting a low pass filtered version of itself, and then normalized by 2 standard deviations to recover small signal fluctuations. (Middle) The applied 5 Hz, -3V voltage oscillation at room temperature. (Bottom) Absolute value of the discrete Fourier transform of the fluorescent signal.
Figure 2.23. (Left) 10 Hz, -3V oscillation. (Right) 10 Hz, -6V oscillation. Group Definitions: 1. Free Cy3 DNA; 2. Acrylamide Constructs Alone, negative control; 3. Control; wild type p53 capture only; 4. One Base Pair Mismatch; 5. Two Base Pair Mismatch; 6. Complement; 7. Cy3 Strep Alone, positive control. Complementary oscillations (Group 6) were significantly different at 10 Hz, -6V than at 10 Hz, -3V with a Student’s unpaired t-test, \( p < 0.034 \).
Figure 2.24. Power spectral densities for different concentrations of electrophoretically actuated transduction mechanisms.
Figure 2.25. Quantum dot on biotin dextran layer atop platinum electrode microarray.
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Figure 2.27. Modulation of quantum dots on platinum microarrays, raw data. The two lower left electrodes are modulated in the middle and bottom frames.
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Figure 2.29. Addition of quencher initiates quantum dot blinking. At ≈16 frames per second, the 2 Hz signal features extrema every 8 frames. The grouped bars represent the two inversely biased electrodes involved in modulation. (Top) Qdot intensities following addition of 1X TBE, followed by flushing and modulation in 0.01X TBE. (Bottom) Qdot intensities following addition of prehybridized quencher in 1X TBE, followed by flushing and modulation in 0.01X TBE.
Chapter 3 : Beyond Low Frequency Electrophoretic Actuation

3.1 Plasticity within Electrophoretic Modulation

Assuming, for a moment, that our system is a linear, time invariant system (which it is not), it should obey superposition and scaling laws: \( f(ax+by)=af(x)+bf(y) \). What we observe is somewhat different. Conversion of an electrical voltage pulse into an electrophoretic flux is conditioned by the impedance of the redox reactions and geometry of the permeation layer.

**Oxidation (Anode):** \[ 2 \text{H}_2\text{O} \rightarrow \text{O}_2 + 4\text{H}^+ + 4e^- \]

**Reduction (Cathode):** \[ 2 \text{H}_2\text{O} + 2e^- \rightarrow \text{H}_2 + 2\text{OH}^- \]

At steady state below the redox potential of water (-1.23 V at the cathode), charges at an electrode surface are quickly shielded by soluble counter-ions. Outside of a few Debye lengths, there is essentially no more electric field [Grodzinsky A 1995]. When in the presence of dissolved salts, anions can compete with hydroxide to
donate electrons. In multivalent ionic solutions the redox reactions become complex, providing parallel redox reactions that lowers the barrier to electrolysis. As a result, bubbling occurs at a much lower voltage with high conductivity solutions. Macroscopic bubbling is catastrophic for our experiments since the bubbles can get lodged between the electrode and permeation layer, altering the electrical and optical properties of interest. Lowering the conductivity of a solution allows greater flux with less bubbling, but at the expense of greater swings of pH. The reaction of electrolysis products with buffer salts determines the rate at which the pH changes spill out into the bulk and potentially affect linearity.

Zwitterionic buffers such as L-histidine were found to stabilize DNA hybridization at a permeation layer-protected platinum anode with relatively low conductivity. The tertiary amine on the L-histidine imidazole group (pK_a=5.97) becomes protonated at the anode and acts as a counter-ion [Edman CF 1997]. In response to a 200 nA applied current, 50 mM L-histidine attained an equilibrium of pH 5.9 in 10 seconds (down from a baseline of pH 7.5), measured 7 µm above the electrode surface. At 500 nA (or roughly 3.1 V), the L-histidine reached a steady state of pH 4.5. It is likely that the pH was lower closer to the anode [Edman CF 1997]. L-histidine does not support hybridization unless protonated, which means that electrically addressed DNA in L-histidine is only temporarily stabilized as the histidine decays to its non-protonated form. Therefore, we chose not to use the standard zwitterionic buffers during our actuation in order to better control the initial hybridization conditions.
The TBE (Tris-Borate-EDTA) buffer used in the experiments of Chapter 2 contains an aqueous solution of 89 mM Tris Borate, and 2 mM ethylenediaminetetraacetic acid disodium dihydrate [Sigma Aldrich T6400]. At a 1/100\textsuperscript{th} dilution, there could be as much as 40 \( \mu \)M sodium to help stabilize DNA, although it is not clear how much of the sodium remains complexed to the EDTA. It is also likely that the buffer exchange from hybridization solution to TBE leaves residual sodium to transiently stabilize hybridization. All of the modulation experiments were done as quickly as possible after buffer exchange.

The conversion of an electrical pulse into soluble ions is in many ways the equivalent of injecting charge across a p-n junction in a diode; except that soluble ions are heterogeneous charge carriers with a greatly reduced mobility. Once the redox potential of the primary solvent has been exceeded, current can readily flow into the circuit. As mentioned, there is a space and time component to the flux of ions. The linear cable equations describe the injection of current into a lossy parallel capacitor/conductor network [Weiss TF 1996]:

\[
\lambda_C^2 \frac{\partial^2 v(x,t)}{\partial x^2} = v(x,t) + \tau_M \frac{\partial v(x,t)}{\partial t} - \lambda_C r_o I_e(x,t) \tag{12}
\]

where \( v(x,t) \) is the voltage throughout the permeation layer, \( I_e(x,t) \) is the applied current pulse. Integration of \( I_e \) over all \( \{x,t\} \) gives the total injected charge (\( Q_e \)). \( r_o \) is the resistance per unit length of the bulk solution, \( \lambda_C \) is a space constant and \( \tau_M \) is a time constant defined as:

\[
\lambda_C = \sqrt{\left( \frac{a}{2 \rho G} \right)} , \quad \tau_M = \frac{c_m}{g_m} \tag{13}
\]
where \( a \) is the radius of the gel through which current is flowing, which for the Nanogen arrays is 80 \( \mu \)m. \( G \) is the conductance per unit area of hydrated permeation layer, \( \rho \) is the resistivity (\( \Omega \cdot \text{m} \)), \( c_m \) is capacitance per unit length and \( g_m \) is conductance per unit length. As with other linear equations, the time and space dependence of the resulting voltage can be acquired by convolving the forcing function with the impulse response of the nondimensionalized Equation 12:

\[
v(x, t) = \frac{r_o \lambda C Q e}{\tau M \sqrt{4\pi (t/\tau M)}} e^{-(x/\lambda C)^2/(4t/\tau M)} e^{-t/\tau M} u(t) \tag{14}\]

Equation 14 indicates that a larger permeation layer will result in a much smaller potential amplitude at the surface, given the same time to develop. Based on this equation, we would expect the amplitude to drop off with a characteristic rate proportional to the square of the normalized space dimension [Weiss TF 1996].

The force on a charged nanoparticle attached to a DNA, assuming a uniform electric field, can be expressed as the balance between Stokes drag and the divergence of surface current around the particle [Grodzinsky A 1995]:

\[
f_z = \pi R \eta \left[ -6U + 4 \left( \frac{3\varepsilon \zeta E_o}{2\eta(1 + \frac{\varepsilon \zeta \sigma_m}{\eta \sigma R})} \right) \right] \tag{15}\]

where \( R \) is the radius of the particle, \( \eta \) is the viscosity, \( U \) is the resultant velocity of the particle, \( \varepsilon \) is the permittivity, \( \zeta \) is the potential drop across the double layer extending radially from the particle surface, \(-\sigma_m\) is the sum of all mobile charges within the double layer, and \( \sigma \) is the bulk conductivity. Coupled with the potential expression of Equation 14, the force on a nanoparticle under steady electrophoretic
actuation can be calculated given the size and charge of the particle, conductivity of the buffer, capacitance and conductivity of the permeation layer.

For speeds above 10 kHz, dielectrophoretic phenomena begin to dominate electric responses at low voltages (< 10 V). The inability of counterions to migrate within a viscous fluid fast enough to compensate for the applied voltage allows high frequency fields to emanate out into solution with a high amplitude, inducing electrokinetic behavior within particles [Squires TM 2005]. Higher frequencies therefore broadcast more energy instead of injecting charge to drive electrophoresis. It is widely believed that electrolysis stops at these high frequencies, although it is probably more accurate to state that as soon as water is split into hydrogen and oxygen, the products are driven to recombine before diffusion or convection let them move away. It is not clear whether dielectrophoretic forces could impart sufficient force to initiate dehybridization, especially for smaller nanoparticles, as DEP scales with the cube of the radius of the particle [Huang Y 2001]:

$$\vec{F} = 2\pi r^3 Re \left( \frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*} \right) \nabla E_{rms}^2$$  \hspace{1cm} (16)

In the dielectrophoretic frequency regime, electrophoretic force contributions have necessarily dropped; as the bulk movement of ions around a sphere would compete against the dielectrophoretic mechanism. Given sufficient amplitude, it may be possible to elicit high frequency movement of ions to recover electrophoretic
forces, but the inevitable Joule heating from such a powerful pulse would be prohibitive.

Deviations from the above linear equations occur when there are changes in the way an electrochemical system converts voltage pulses into fluxes. For instance, history in the system is a common nonlinearity. If the pH, temperature, or cation distribution local to an electrode has changed between a prior voltage pulse and the current pulse, the impedance of solution will have also changed. The phase and amplitude of the resultant electrophoretic pulse will be dependent upon the extent of bulk deviation. Also, changes in the molecular system itself such as dehybridization or a change in nonspecific binding environment upon perturbation by an external field can also contribute to nonlinear modulation responses. To borrow a term from neurophysiology, we call the change in amplitude as a result of history “plasticity.”

Figures 3.1 and 3.2 demonstrate a characteristic plasticity within a fluorescent modulation of Cy3 and TRX, respectively, performed on a Nanogen H2 100-site microarray cartridge. The Nanogen chips contain the same platinum on silicon array as used in the quantum dot experiments, but the array is covered with a 10 µm thick acrylamide permeation layer seeded with streptavidin. Presumably, the Nanogen chips provide the most repeatable fields available in an analog microelectrophoretic environment. Chips were rinsed 10 times over half an hour with 200 µL aliquots of deionized water to remove a protective arabinose coating atop the permeation layer. Biotinylated oligonucleotide probes in hybridization
solution (p53cBCy3 or p53cBTRX) were incubated within a fresh chip overnight to facilitate diffusion throughout the gel and binding to the embedded streptavidin. The chip was then flushed with 0.01X TBE in preparation for modulation. A 1.5 V, 2 Hz, 20 second pulse train was followed by a 60 s linear frequency sweep, which was followed by a second 20 second pulse train. The fluorescence (CCD) was recorded in parallel with the applied voltage at 48 Hz. The results of the pre- and post-sweep pulse trains for two separate electrodes are shown in Figures 3.1 (Cy3) and Figure 3.2 (TRX). The applied voltage is shown superimposed upon mean subtracted normalized fluorescence amplitude, the maximum of which was adjusted to match the maximum of the applied voltage for ease of visualization. The frequency sweep data is shown in Figures 3.3 (Cy3) and 3.4 (TRX).

Immediately apparent is the trend between pulse trains. The trend is dependent on when the pulse trains were performed, with fluorescent responses changing shape and amplitude following the frequency sweep. Trends are similar between electrodes. Clear differences between Cy3 and Texas Red are also apparent. The Nanogen chips are prohibitively expensive to average over a large number of cartridges, so this data is inconclusive as to whether the effect is cartridge dependent or fluorophore dependent. In either case, the variability is nonlinear. The low frequency modulation experiments of Chapter 2 were performed after a preconditioning step in an attempt to reduce nonlinearities, although there was no data to support this effect at the time. Preconditioning in
mechanical systems is also commonly used to minimize time variant changes in strain hysteresis.

Because there is no FRET system in place, the data shown in Figures 3.1 to 3.4 likely reflect the effects of the interaction between pH and temperature shifts resulting from electrophoretic actuation alone. It is also unlikely that there is substantial bulk transport of the oligos within the permeation layer as the actuation is symmetric and relatively fast for bulk transport. Voltages were kept low to avoid bubbling and substantial Joule heating.

Nonlinear trends were also seen within the frequency sweeps, which steadily decrease from 20 Hz to 2 Hz over 60 seconds (Figures 3.3 & 3.4). Again, Cy3 and Texas Red display clear differences. There does seem to be a region, between 500 to 1000 samples where a plateau is reached within each of the frequency sweeps. Figures 3.5 (Cy3) and 3.6 (TRX) show close up behavior of the 2 Hz end of the frequency sweeps. The Cy3 response exhibits stable doublets between applied sine wave peaks. There is little evidence of doublets during the pre-sweep 2 Hz pulse train, demonstrating a repeatable environmental nonlinearity. At the conclusion of the frequency sweep, the function generator defaults back to 20 Hz, after which a drop in amplitude can be seen. The close-up of the Texas Red frequency sweep shows evidence of doublets, although their amplitude greatly reduced. The Texas Red also responds with a greater magnitude overall. Without additional data, it is difficult to speculate on the mechanism behind these shifts.
Figure 3.7 depicts the Fourier spectra of the frequency sweeps for the different electrodes. The greater amplitude response of the Texas Red fluorophore is clear. Inspection of the Texas Red response reveals a rolloff with increasing frequency.

3.2 High Speed Amplified PIN Diode Detection

In lieu of an orthogonal basis of oscillation (i.e., different frequencies between matched and mismatched constructs), the rate of information must be increased to allow for multiple frequencies to be probed prior to bleaching. Higher modulation frequencies may also lead to different modes of actuation and a richer spectrum of information to help differentiate states of hybridization. Plasticity due to frictional heating and large pH shifts may also be mediated by a higher speed of modulation. Given the substantial nonlinearities evident within the low frequency actuation, there is a clear incentive to raise the speed of actuation. However, increasing the frequency of analysis does lead to additional technical challenges dependent on the transfer function of the system, the amplitude of modulation, hardware sensitivity, readout noise, and timing jitter.

While there has been considerable progress in the sensitive detection of FRET kinetics, most of the high speed platforms (ALEX, TCSPC, FCS, FLIM, etc.) make use of a confocal volume and photon counting hardware to detect single
fluorophores [Doose S 2007, Li H 2004, Jung J 2006, Kettling U 1998, Lee NK 2007, Maiti S 1997, Schwille P 1996]. Photon counting is based on the MHz sampling of photomultiplier tubes set to very high gain (>10^6). In this mode, even single photons carry sufficient energy to generate an avalanche within the PMT.

With low levels of illumination and low concentrations within the confocal volume, the rate of fluorescence is kept well below the sampling rate of the hardware. Impulse responses, autocorrelation times, and other dynamic responses are then averaged over many trials in order to compensate for the increased shot noise that accompanies the high gain PMTs.

Because of this high gain, the amplitude of the PMT response is independent of how many photons arrived within the sampling interval. As a result, analog responses are inaccessible with photon counting setups. Modulated signals within a large background of autofluorescence require intensity measurements over time. Secondly, for what we believe to be a useful detector, the detection volume will likely be far larger than a femtoliter, as patient samples can often be in the mL range.

For high speed analog detection within our budget, we initially opted to use amplified silicon PIN diodes (not avalanche photodiodes, which are considerably more expensive). The Thorlabs PDA36A offered visible detection within the 350-1100 nm range, with a peak response of 0.65 A/W @ 970 nm, as well as a built in preamplifier with a gain-bandwidth response of 1.5kV/A at 17 MHz, and 4.75 MV/A at 12.5 kHz on a high impedance load.
Illumination was provided by an Osram HBO 103W/2 mercury arc lamp, the light of which passed through an infrared short pass filter to remove unnecessary wear on the optics. During quantum dot experiments, the excitation light was then conditioned using an epifluorescent filter set [Chroma 32009, Qdot® 565]. Fluorescent signals were routed through a custom Leica head. Two PDA36A units were mounted on custom screw chucks that made use of the eyepieces as focusing elements to underfill the PDA36A 13 mm² detector area. The chucks allowed 1” filters to be placed in line with the detectors. When used in this mode, the dichroic mirror and excitation filter were left inside the epifluorescent cube, while Cy3 and Texas Red emission filters could be placed in line with individual detectors. Long-distance objectives were used in experiments to allow the various micromanipulator connections and Nanogen cartridge headroom. The objectives featured numerical apertures between 0.15 @ 10X, and 0.75 @ 100X (as compared to ≈1.4 for a good oil objective), which limited the amount of light available to our detectors.

The signal from the silicon detector was passed through serial stages of 10X and then 100X, AC coupled voltage amplifiers [Linear Technologies LTC6910-1]. This two-stage conditioning placed the output of the detector within the central bit range of the fixed range 9215A National Instruments DAQ at our disposal, which provided 16-bit sampling spread over a range of –10 V to 10 V. At the highest gain settings of the PDA36A, the fluorescent output of our epifluorescent setup provided
a DC deflection on the order of a few mV per channel between background and full fluorescence.

Figure 3.8 presents a Fourier magnitude spectrum of a 1 kHz modulation of complementary quenched quantum dots (p53cQSY7). 16-bit data were collected over 60 seconds at a sampling rate of 10 kHz. The harmonics of the mercury lamp are the dominant feature below 300 Hz. In response to actuation, a small peak is visible towards the right of the spectrum (signal-to-noise ≈ 4). The inset of Figure 3.8 provides a detailed view of that peak. The timing jitter of ADC is suspected to have been responsible for the broadening of the peak.

While we have not established the causality of this oscillation [Chapter 2.6], it does confirm that electrophoretic actuation is possible at higher frequencies. One of the main reasons for moving to higher speeds was to enable broad frequency sweeps to examine differences between matched and mismatched constructs in greater detail. We concluded that the setup, as described, was not sensitive enough to allow frequency sweep analysis for the Cy3 or Texas Red FRET systems. The excessively long integration time to produce a single peak of sufficient signal-to-noise prohibited integration over the many kHz that we had hoped to span.

Encouraged by the evidence of high speed electrophoretic actuation, we sought to improve the sensitivity of the system in three ways. First, we replaced the mercury lamp with a 5 W cyan LED [LXHL-LE5C 505 nm Luxeon LED]. Since the 100 W mercury lamp lacked a peak over the excitation filter, the concentrated power of the LED poured more light into our Cy3 excitation band. Second, we
optimized our optical filter setup, as shown in Figure 3.9. It was found that our old excitation filter had sufficient overlap with the Texas Red emission filter that even after conditioning with the dichroic mirror, illumination light was passing through the Texas Red channel to become the primary signal. With the help of Chroma Technology Corp, were able to find a cyan excitation filter with a $10^8$ rolloff above the low end of the red emission spectrum of the 645x75 filter. Interestingly, the 645x75 manufacturer specifications did not indicate that there was a pass-band below 500 nm. The spectra shown in Figure 3.9 were measured on our Lambda 800 by disassembling the dichroic cubes and mounting the individual components flush against the sample window of the spectrometer. Finally, we explored replacing the silicon detectors with high speed analog photomultiplier tubes and silicon APDs. Extensive research led us to invest in two avalanche photodiodes [C4777, Hamamatsu] with a flat amplification band of 300 kV/W between 10 kHz and 100 MHz, which is 100-fold more sensitive than the PDA36A in the MHz band. Unfortunately, a compromise was made in that the 0.1-10kHz band was sacrificed, as the rolloff of the APD made it less sensitive to the PDA36A in this region.

Perhaps the most obvious way to increase signal would be to increase the concentration of fluorophore. However, this is constrained by the available surface density. In a permeation layer system such as the one used in Chapter 2, or within the Nanogen H2 chips, long incubations of high concentration of fluorophore promotes diffusion into the interior of the gel without necessarily contributing to the specific signal of interest. Since many probes can get stuck within pockets of
the gel, it does get very bright. As mentioned, the preferred approach is to functionalize the surface of a hydrogel with a polymerized biotin layer, cover that layer with streptavidin, and then incubate with a high concentration of probe for a short time (or electronically address the area) to avoid stable gradients into the bulk of the gel. Of note, when biotin-dextran is baked onto the platinum chips, a gross excess of quantum dot is placed on top of the layer, yet a majority of fluorescence remains in the fluid above the chip following incubation. This indicates that we are saturating the available surface density of the platinum chip, and that further increases in signal would not be likely to occur with a higher concentration of quantum dot.

3.3 Comodulation

Recognizing that acquiring a high speed detector in the visible range for the 0-10 kHz band was becoming unlikely, we turned to a technique developed by Dieter Braun and Albert Libchaber at Rockefeller University that allowed us to drop the requirement of a fast detector [Braun D 2003]. By modulating not only the environment \(E\) of the DNA but the illumination, phase shifted intensities could be recombined to provide a good estimate of the amplitude \(A\) and phase \(\varphi\) of the molecular response, assuming a linear relationship between the environmental modulation and quantum efficiency \(q(E)\) of the fluorophores. Braun expressed
that for a given sinusoidal oscillation $E$, $q = q_o[A\sin(\omega t + \varphi) + 1]$, meaning that the small signal fluctuation of the quantum efficiency of the fluorophore would follow the modulation, offset by a delay and the baseline quantum efficiency of the molecular system $q_o$. Like Braun, we implemented an LED for illumination, which supplied a rectified excitation sinusoid:

$$I_{ex} = \Delta I_{ex} \Theta[\sin(\omega t - \alpha)]\sin(\omega t - \alpha) + I_{ex}^o,$$

where $\Theta$ represents the Heaviside step function. At any given point in time, the intensity $I$ results from the multiplication of the instantaneous quantum efficiency with the illumination intensity. A slow detector integrates that intensity over many periods of modulation:

$$I = \int qI_{ex} dt$$

(17)

Which, explicitly looks like:

$$I = \int q_o[A\sin(\omega t + \varphi) + 1]\left(\Delta I_{ex} \Theta[\sin(\omega t - \alpha)]\sin(\omega t - \alpha) + I_{ex}^o\right) dt$$

$$= Aq_o \Delta I_{ex} \int \sin(\omega t + \varphi) \Theta[\sin(\omega t - \alpha)]\sin(\omega t - \alpha) + I_{ex}^o Aq_o \sin(\omega t + \varphi)$$

$$+ q_o \Delta I_{ex} \Theta[\sin(\omega t - \alpha)]\sin(\omega t - \alpha) + q_o \Delta I_{ex}^o dt$$

$$= Aq_o \Delta I_{ex} \int \sin(\omega t + \varphi) \Theta[\sin(\omega t - \alpha)]\sin(\omega t - \alpha)dt + 0$$

$$- q_o \Delta I_{ex} \left(\cos(\pi)\right)_{0} + 2\pi q_o I_{ex}^o + I_{const}$$
Braun solved a detailed recombination of four phases \{0°, 90°, 180°, 270°\} and the background intensity \(I_{back} = 2\pi q_o I^0_{ex} + I_{const}\), to provide the following expression of amplitude and phase:

\[
A e^{i\phi} = \frac{4}{\pi} \left[ \frac{I_0 - I_{180}}{I_0 + I_{180} - 2I_{back}} + i \frac{I_{270} - I_{90}}{I_{270} + I_{90} - 2I_{back}} \right]
\]

This allowed us to use the sensitive Orca ER CCD to integrate each image at the corresponding phase rather than trying to observe photon-limited high speed modulation, which, as the oscillation gets faster, fewer photons are emitted per sinusoid. Braun also suggested that in order to correct for linear bleaching of the fluorophores that the intensities used in Equation 19 be averaged over a sequence of recordings \{\(I_{back}, I_0, I_{180}, I_{270}, I_{90}, I_{270}, I_{180}, I_0, I_{back}\)\}.

To implement this system, the internal clock of an Agilent 33120A function generator was bypassed and externally locked to the clock of an Agilent 33250A function generator. The 33120A signal was passed through an AC coupled audio-range power amplifier and then through two 5 \(\Omega\) power resistors in series with a power diode to prevent large reverse-biased currents from destroying the Luxeon LED. Current was kept below 700 mA as specified by the manufacturer. The LED was retrofitted into a mercury arc lamp housing using a micromanipulator assembly to allow focal length optimization. The housing was attached to the epi-fluorescent
head in place of the standard arc lamp. Software triggered, phase shifted excitation of the FRET system was driven directly off of the 33250A. Integration intervals were not aligned to a specific phase of the excitation, as there was a random delay between triggering and locking the two function generators. Integration times were limited to 10 seconds per intensity image by the CCD readout buffer.

To examine the comodulation of a FRET system, we prepared Nanogen 100-site H1 cartridges [#605033 lot B03844] for surface functionalization. After being flushed with 200 µL deionized water five times for four minutes each, 1 mg/mL biotin dextran was incubated for fifteen minutes, followed by a second 5x200 µL dH2O wash, and a 12.5 mg/mL avidin incubation. To maximize surface functionality, a second course of biotin dextran / avidin incubations were performed. Prehybridized, biotinylated 1.44 µM 1 base pair mismatch LHS FRET (p53B51WT, p53Cy3F, p53m1bpTRX) in 0.25 M NaCl and 0.025 M sodium phosphate was then incubated for one hour at room temperature. As a departure from earlier experiments, 20 mM sodium phosphate (0.0346 g dibasic sodium phosphate, 0.0215 g monobasic sodium phosphate, 100 mL deionized H2O) was used to flush the cartridge. Sodium phosphate was also used as a modulation buffer to ensure long term hybridization stability, given the expense of the Nanogen cartridges.

A representative series of comodulated intensities for 100 Hz, 4.0 V and 6.0 V actuation are shown in Figures 3.11 through 3.14. The series of phase shifted intensities (Figure 3.11) and the resultant amplitude and phase corresponding to the
real and imaginary part of Equation 19 (Figure 3.12), show no evidence of modulation at 4.0 V. At 6.0 V, a strong signal is evident (Figures 3.13 & 3.14). The average amplitude of oscillation is below 5%, and in general was found to be very small at these high frequencies which was consistent with the rolloff seen in Figure 3.7. Inspection of the phase image of Figure 3.14 shows that the two electrodes biased relative to one another also exhibit the proper phase dependence, roughly 180° apart. Concurrent with the results of Chapter 2, this is evidence for a power dependence on initiation of fluorescent oscillation.

However, given the non-orthogonality of quantum efficiency fluctuations, it is impossible to tell the relative contributions of FRET modulation as compared to changes in emission rate as a result of pH or temperature changes. Unlike the thermal actuation used by Braun the nonlinearities of the electric field would seem to preclude compensating for the thermal response with a pre-recorded fluorophore-only spectrum. Braun indicated that his thermal actuation carried bandwidth of 10 kHz and that the reaction cascade for quantum efficiency fluctuations required < 10 ns. Ideally, the system would respond to actuation far faster than the dynamics of interest. The doublets seen in Figure 3.5 seem to suggest that there is a level of refractoriness within our system.

Also evident in Figure 3.14 is the amplitude imbalance between the two electrodes used for modulation. It is not clear why these differences arose, as no DC bias was applied. This was periodically observed throughout our comodulation experiments, perhaps indicating variations in the permeation layer, electrodes, or
electrical connections. Examination of Figure 3.13 reveals what seems to be an illumination hot spot towards the lower left of the array, but this offset caused no bias in the 4.0 V image, so this is an unlikely cause of the imbalance. The phase image of Figure 3.14 also reveals a halo of influence between the electrodes, as well as a series of ripples extending radially past the periphery of the lower electrode.

During modulation, contrary to the suggestion by Braun, offsets caused by bulk fluorescence changes were not linear and were not eliminated by simply averaging frames. Figure 3.15 shows the steady state fluorescence following modulation in both the Cy3 and Texas Red channel. The outer rim of electrodes were floating, and are representative of the magnitude of the actuated electrodes prior to modulation. The inner four electrodes had been activated during sequential modulations. It is clear from the images that the Cy3 channel got brighter while the Texas Red channel became substantially dimmer. Since the Texas Red probe carried a single base pair mismatch, it is conceivable that it was being preferentially dehybridized during actuation. This is further evidence that the oscillation is not simply a quantum yield oscillation, but that the effects of pH are likely superimposed on FRET oscillations.

At 100 Hz, we did not expect to find gross shifts of pH over time. However, there was considerable electrode damage after prolonged or repeated modulations. This led to our hypothesis that despite having a symmetric voltage pulse, the buffer was asymmetric in its ability to compensate for acids and bases. Evidence of this
can be seen in Figure 3.16, where the amplitude and phase of a 100 Hz comodulated 1bp mismatch FRET is shown in 0.01X TBE buffer instead of sodium phosphate. Not only was the threshold seemingly higher, the pattern of oscillation was different than the characteristic sodium phosphate response, being localized along the outer edge of the electrode.

If there were indeed shifts in pH superimposed on FRET oscillations, we would expect the largest magnitude to emanate out from the center of the electrode, because the gradient from bulk has the least effect towards the center of the pad. Conversely, the highest field region of the electrode is likely at the corner, and we would expect field mediated modulation to concentrate here. The results do not support such a simplistic interpretation, unfortunately, but the drastically different behavior in sodium phosphate as opposed to TBE does raise interesting questions about the interactions between buffer and alternating electric fields.

With regard to comodulation as an apparatus for detection, the reliance on a ratio to calculate amplitude and phase resulted in severe artifacts when the absolute value of the intensities were low. The amplitude images of Figure 3.16 show large, bright spots even though the phase shows no change. These were previously modulated (and burnt out) electrodes with very low fluorescence. This data does support that under normal conditions that the comodulated calculation of phase was due to oscillations, and not just offsets. The reliance on ratioed calculations would seem to preclude comodulation as being a candidate for effective detection, despite the advantages of its slow detector and inherent parallelizability.
Figure 3.1. Cy3 nonlinearity in response to sequential 1.5 V, 2 Hz frequency modulations. In between top and bottom traces, a 60 frequency sweep was performed.
Figure 3.2. Texas Red nonlinearity in response to sequential single frequency modulations. In between top and bottom traces, a 60 frequency sweep was performed.
Figure 3.3. Cy3 frequency sweep response from 20 Hz to 2 Hz, 1.5 V, 60 seconds. Fluorescence curves normalized as in Figure 3.1. The initial response varies, but the slower (right side) response shows similar average concavity.
Figure 3.4. Texas Red X frequency sweep response from 20 Hz to 2 Hz, 1.5 V, 60 seconds. Fluorescence curves normalized as in Figure 3.1.
Figure 3.5. Close up of low frequency portion of Cy3 sweep traces. Notice the very clear doublets between each sine wave period. Only slight evidence of doublets is evident in the pre-sweep traces.
Figure 3.6. Close up of low frequency portion of TRX sweep traces. Texas Red seems to respond more strongly than Cy3, and there is less evidence of doublets.
Figure 3.7. Cy3 and Texas Red frequency sweep Fourier spectra. The more strongly responding TRX gives a good indication of the rolloff of the transfer function of the Nanogen system.
Figure 3.8. 1 kHz actuation of quantum dots. 60 second recording of a 10 kHz sampled amplified PIN diode output waveform. The peaks to the left are noise from the mercury lamp. The quantum dot response is evident to the right, and shown in the inset.
Figure 3.9. Optical filter sets used for co-modulation. From left to right: 500x35x excitation filter (blue), 560LP long pass dichroic mirror (cyan), 580x40m2p Cy3 emission filter (dark green), 645x75 Texas Red X emission filter (red). Superimposed are the LXHL-LE5C 505 nm Luxeon LED emission (light green), as well as the overlapping, non-FRET spectra of Cy3 and TRX illuminated with a 535 nm excitation (black).
Figure 3.10. Comodulation Schematic. Software timed, phase locked function generators cycle through a series of offsets, which when integrated over a long time, produce an intensity image per phase.
Figure 3.11. Series of phase shifted images \(\{I_{\text{back}}, I_0, I_{180}, I_{270}, I_{90}, I_{270}, I_{180}, I_0, I_{\text{back}}\}\) for 4V, 100 Hz excitation.
Figure 3.12. Resulting amplitude and phase images from the sequence of images shown in Figure 3.11 for 4V, 100 Hz excitation.
Figure 3.13. Series of phase shifted images \{I_{\text{back}}, I_0, I_{180}, I_{270}, I_{90}, I_{180}, I_{270}, I_{90}, I_{\text{back}}\} for 6V, 100 Hz excitation.
Figure 3.14. Resulting amplitude and phase images from the sequence of intensities shown in Figure 3.13 for 6V, 100 Hz excitation.
Figure 3.15. Post-comodulation images of the FRET system. A) Cy3 Channel B) TRX Channel. The surrounding electrodes give the “before” view of fluorescent intensities for each channel. Note that the intensity of the Cy3 channel has increased, while the TRX fluorescence has decreased.
Figure 3.16. Comodulation in 0.01X TBE buffer. A) 100 Hz, 6.0 Volts, B) 100 Hz, 10.0 Volts. Very different pattern of modulation is observed with TBE as compared to sodium phosphate. The area demarcated by the white arrow is shown below in grayscale to improve contrast.
Chapter 4 : Theoretical Considerations

4.1 Thermodynamic and Stochastic Models of DNA Modulation

Given that a very limited amount of information is available describing the kinetics of externally actuated DNA systems, it is difficult to form an intuition about the expected behavior of such a system. Most direct measurements of actuated dynamics have been performed on hairpin constructs, which feature long loop regions connecting the fluorescent oligonucleotides. These loops provide access to local free energy minima (secondary structures, or misfolded intermediates) that may substantially lengthen the process of rehybridization as compared to short DNA segments [Ansari A 2001, Ansari A 2002, Jung J 2006]. The impact of single mutations on rehybridization kinetics has not been studied comprehensively, nor does there exist a suitable theoretical construct to accounts for the secondary structure that may influence kinetics. Further, FRET actuation has only been observed on ensemble systems; to our knowledge, the behavior of
single molecule fluorescent energy transfer systems have only been characterized without actuation [Schallhorn KA 2005].

Insight from explicit molecular dynamics simulations is limited by the timescales of interest, as well as the known errors in calculated melting forces [Piana S 2005]. Rehybridization times for actuated FRET systems are likely sequence dependent, and have been estimated to be on the order of tens to hundreds of microseconds [Ansari A 2002, Braun D 2003]. Molecular dynamics on small DNA segments are generally simulated over tens of nanoseconds [Piana S 2005]. Therefore, the goal of this study was to create a long time-scale model to examine whether the actuation of intermolecular FRET systems could be used to differentiate rare DNA from a large background of similar, but mismatched, DNA.

The following model attempts to characterize the dynamics of a thermally actuated FRET system as a stochastic process. For sufficiently large time steps, (i.e., 1 μs), the system was considered to be at equilibrium [Nina M 2005]. Differences between matched and mismatched constructs were contained within the transitions between open (melted) and closed (hybridized) states. A uni-directional, six base pair zipper model was used to represent the opening and closing of the acceptor strand with increased temperature [Kuznetsov SV 2001]:

\[
Z_j = \sigma_j \xi \left( \prod_{i} e^{- (\Delta H_i - T \Delta S_i + \frac{\delta G_{i-1,i} + \delta G_{i,i+1}}{2}) / RT} \right)
\]

(20)

\[
\sigma_j = 4.5 \times 10^{-5} e^{\frac{\delta G_{j,j+1}}{2RT}}
\]

(21)
where statistical weight of each microstate, $z_j$, was formed from the product of the cooperativity parameter $\sigma_j$, the entropy penalty $\xi$, and the Boltzmann weights of each intact base pair. Unbound Boltzmann weights were set to 1. $\xi = 0.1$ was used in place of a loop weighting function [Krueger A 2006]. The entropy weight was set to place the melting point of the strand near 30°C. Consistent with Kuznetsov, internal loops were not included due to the small size of the construct. For each microstate in the model, the free energy was calculated using $\Delta S_i = -25.2 \text{ cal/mol} \cdot \text{K}$, $T_{AT} = 337.3$ °K, $T_{GC} = 380.4$ °K, where $\Delta H_{AT} = T_{AT} \Delta S_i$ [Kuznetsov SV 2001].

Stacking interactions, $\partial G_{i,i+1}$, were also borrowed from Kuznetsov, represented by the average of the parameter sets measured by Wartell & Benight [Kuznetsov SV 2001, Wartell RM 1985]. Interactions between the donor and acceptor ends of the strands were assumed to be negligible, as FRET optimization suggests that fluorophores remain several base pairs away to avoid collisional quenching.

The probability of each base pair being intact, $p_i$, and the total fraction of intact base pairs, $\theta(T)$, are shown in Equations 22 and 23, respectively:

$$p_i = \sum_{k=\text{intact}} \frac{z_k}{Z} \quad (22)$$

$$\theta(T) = \frac{1}{N} \sum_i n_i \frac{z_i}{Z} \quad (23)$$

with $n_i$ corresponding to the number of intact base pairs in a given microstate, and $N$ representing the number of bases in the sequence. Only those microstates which have a particular base pair hybridized are included in the calculation of $p_i$. The partition function, $Z$, is the sum of all the statistical weights $z_j$. 
Two sequences, 5’-CAGACAT-3’, and 5’-CTCGTGT-3’, were used in the study. The lack of thermodynamic parameters describing the sequence specific impact of a true mismatch required us to postulate that a G->A or C->A mutation would carry similar free energy change as an actual mismatch, corresponding to a reduction in $T_m$ by < 5 °C. Therefore, to simulate single base pair polymorphisms, the third base pair from the 5’ side of each sequence was substituted with an adenine. The resulting sequence was considered a “mismatch.” Concurrently, two base pair mismatches carried adenine mutations on the first and third bases (Table 4.1).

DNA was assumed to be in equilibrium in this model in the sense that there was no history between time steps of the simulation. Melting and rehybridization was considered without hysteresis or rate dependence. Results from the zipper model are shown in Figures 4.1 and 4.2, for Sequences #1 and #2, respectively. The melting point of Sequence #1 was 302.9 K, 292.4 K, 287.5 K for the match, mismatch, and two base pair mismatch.

A concise description of the mechanics of DNA on microsecond timescales was unavailable. Presumably, the relative distance between the donor and acceptor would be governed by the hybridization equilibrium as well as the flexible polymer behavior of the DNA, which is commonly modeled as Gaussian [Cocco S 2002]. Indeed, experimental studies of single molecule DNA FRET systems with fluorophores located at opposing 5’ ends of the molecule indicate a Gaussian distribution of emission over 200 ms sample times. However, the zipper model
above suggests that the base to base probability of being dehybridized follows a sequence specific pattern, as evidenced by the nonuniform distance between successive curves in Figure 4.2A.

Therefore, in order to model the relative distance between the fluorophores on a microsecond time scale, two noise models were compared; the sequence-specific zipper model using the $p_i$ as an analogue of the distance distribution, and a Gaussian model using $\theta(T)$ as the mean distance between fluorophores with a normally distributed distance. Because the zipper model did not include internal loops, the probability of each base being dehybridized was directly related to the degrees of freedom of the remainder of the sequence.

The relative distance between fluorophores, $x$, was expressed on $\{0,1\}$ with 0 representing the closed state, and 1 representing the open state. As a result of this convention, the zipper model distance distribution was chosen to be a linear interpolation between points defined by $1-p_i$. For the Gaussian model, the distance distributions followed Equation 24, with $\sigma$ representing the standard deviation. A deviation of $\sigma = 0.25$ was chosen to fit the distribution of experimentally observed single molecule FRET efficiencies [Schallhorn KA 2005].

$$p_{\text{Gaussian}} = \frac{1}{\sqrt{2\pi}\sigma}e^{-\frac{(x-(1-\theta(T)))^2}{2\sigma^2}} \quad (24)$$

Open and closed boundary conditions for the Gaussian model are shown in Figure 4.3. Residence in a single state produced a noisy signal over time, which is shown in Figure 4.4. While it is known that FRET follows sixth-order relationship
to distance, the amplitudes of oscillation within this system were assumed to be small and a linear relationship was used to approximate the distance dependence [Braun D 2003]. At $x = 0$, the FRET system was at its brightest, fully hybridized state. At $x = 1$ the acceptor was at a maximum distance away from the donor with a minimum fluorescence.

Boundary conditions were chosen to be identical for matched and mismatched probes. When fully hybridized, small numbers of mismatches within oligonucleotide FRET systems have been shown to exhibit identical fluorescent amplitudes. This suggests that average distance between the donor and acceptor are similar within the closed state [Sullivan BD 2007]. Fully open single stranded DNA segments were assumed to have the same degrees of freedom for matched and mismatched strands.

Increases in temperature ($T$) shifted the center of the distance distribution toward the open state. Figure 4.5 shows the probability distributions of complementary and mismatched FRET systems along equally spaced samples of temperature for the Gaussian model. The matched system exhibits a higher density of states towards the closed boundary, although the difference is very subtle.

Dynamics were calculated in response to a series of temperature pulses. A nondimensionalized $T$, set between 0 and 1, represented temperatures between 270 and 340 °K. In order to model the effects of dampening on the driving force, square waves were smoothed out using rising and falling exponentials to create the pulse. Accordingly, the duty cycle of the temperature was split between a rising
exponential over $t_1$ seconds during actuation, and a falling exponential over $t_2$ seconds during dissipation as shown in Equation 25. It should be noted that for long $\tau$, the short rise times did not allow the temperature to asymptote to its maximum value. In these cases, the pulse amplitude was greatly reduced, oscillating between a much smaller range of temperatures than 270 K to 340 K.

$$T(t) = \begin{cases} 1 - e^{-t/\tau} & t < t_1 \\ e^{-t/\tau} & t_1 < t \leq t_2 \end{cases}$$

(25)

The lower limit for actuation times ($t_1$) was 20 $\mu$s. Dissipation times ($t_2$) were also limited to 20 $\mu$s in order to prohibit arbitrarily fast rehybridization dynamics. This constraint was within the measured closing times of a 5 base pair hairpin using temperature jump (10 $\mu$s) and fluorescence correlation spectroscopy (28 $\mu$s) [Ansari A 2002, Bonnet G 1998]. It is likely that the closing dynamics of a partially dehybridized strand of linear DNA would be faster than a hairpin DNA containing a loop, therefore these constraints should ensure a good approximation of equilibrium behavior.

In order to reduce simulation time, the probability space was discretized over 100 distributions between the maximum and minimum temperature. At each time step, the FRET distance was chosen from the probability distribution as determined by the instantaneous temperature and the corresponding noise model. Results were obtained for $t_1$, $t_2 = \{20, 40, \ldots 1000 \ \mu s\}$ and $\tau = \{5, 10, 100, 200, \ldots 1000 \ \mu s\}$ with a 1 $\mu$s sampling interval.
Matched, mismatched, and temperature signals were truncated prior to FFT analysis in Matlab (The Mathworks, Natick MA). A noise threshold was set to four standard deviations from the average matched and mismatched response within the 400-500 kHz band. Harmonics of the applied temperature signal above this noise threshold were used as a subset of peaks for comparison. The ratio magnitudes of matched and mismatched peaks within this subset were recorded for each parameter set in the simulation. No attempt was made to include fluctuations in quantum yield due to environmental modulation [Braun D 2003].

The frequency response of the system was found to depend strongly upon the time constant \( \tau \), and ratio of the actuation and dissipation periods (Figure 4.6). However, the differences between noise models was minor, with both the Gaussian and zipper model noise producing signal-to-noise ratios of near 2:1 for a single set of actuation parameters. This result suggests that the difference in amplitude at a given frequency has a greater dependency upon the average free energy of binding than the sequence specific changes within the unzipping or rehybridization. Similar signal-to-noise ratios were seen between the match and two base pair mismatch across both sequences of interest.

Three regions of behavior were found; a) the predominantly matched response with a long \( t_1 \), short \( t_2 \), b) the predominantly mismatched region with a short \( t_1 \), long \( t_2 \), and c) roughly equivalent oscillations throughout the majority of actuation parameters. Increasing \( \tau \) shifted Regions a and b towards the center of the actuation parameter set while broadening the area of variance between the
mutations. Increasing the number of temperature pulses did not change the average signal-to-noise ratio between matched and mismatched constructs for a given parameter set. Rather, it reduced the variance between trials. A detailed view of the predominantly matched response is shown in Figure 4.7, while a representative sample of the mismatch dominant region is depicted in Figure 4.8.

Inspection of Figures 4.7 and 4.8 suggest that as the actuation and dissipation times were shortened in relation to $\tau$, the temperature profile biased the FRET system towards either the closed or open states, respectively. The combination of biasing system above the melting point with a short dissipation time exploited the ability of the matched construct to rehybridize faster than the mismatch. Conversely, rapid, low-amplitude heating followed by prolonged cooling kept the FRET system near the closed state. In this region, the mismatched construct was able to dissociate before the matched system could respond.

At equal concentrations within Region $a$, the matched construct gives not only higher amplitudes of response, but higher frequency components above the noise threshold (Figure 4.9A). Doubling the concentration of mismatched constructs creates overlap in the upper harmonics (Figure 4.9B). Thus, the total amplitude of peaks above the noise threshold is a good indication of signal-to-noise ratio for a single set of actuation parameters. This result also indicates that the frequency components between matched and mismatched responses are non-orthogonal.
Without distinct frequency components to differentiate probes, we implemented a directed walk through parameter space in order to create a spectrum of responses for each sequence. Actuation parameters were swept through Regions $a$ to $c$ to $b$ (Figure 4.10). The resulting eigenvectors (Figure 4.11) were used to deconstruct responses from increasing ratios of matched and noncomplementary probes. At equal (1:1) concentrations, one output signal of the matched, mismatched and 2 base pair mismatched sequences were summed together, and peak amplitudes of the combined signal were recorded for each set of actuation parameters in the walk. Combined signals for a shortened walk are shown in Figure 4.12 for (1:1) and (1:5) concentrations. Note that for a (1:5) concentration, there was one matched response added to five responses of both the one- and two base pair mismatch. Combined signals for the case when no match was present were recorded at each ratio to provide negative controls.

Rounding amplitude normalized eigenvalues provided discrete estimates of concentration from the combined signal. The results of these predictions for the Gaussian noise model are shown in Table 4.2. Interestingly, the signal-to-noise ratio was dramatically improved over individual actuation parameters. Sequence #1 proved to be more difficult to predict than Sequence #2, which can be linked to the smaller area between matched and mismatched $\theta(T)$ in Figures 4.1 and 4.2. False positive detection was not seen for Sequence #2 until the ratio reached 1:1000. There were no false negatives predicted for either sequence, although the absolute concentrations became less reliable as the noise was increased.
The insensitivity of the ratio between matched and mismatched responses to noise models is a good indication that modulated FRET systems report the average free energy of binding of an oligonucleotide probe. It is likely that a much faster, non-equilibrium analysis would be necessary to differentiate the sequence specific dynamics during melting or rehybridization. However, it is not known whether such modes would improve signal-to-noise ratios at a single actuation frequency.

Significant increases in signal-to-noise ratio were achieved by examining the relative responses across different modes of actuation. Because predicted concentrations were calculated from amplitude normalized signals, the performance of a modulated detector could be quite robust across different lighting conditions. Furthermore, in spatially multiplexed formats where the number of DNA molecules per area is kept low, this technique could be very useful for improving the specificity of detection.

Chapter 4 in part, has been submitted for publication of the material to Physical Review E, entitled Stochastic Model of Externally Driven Fluorescent Resonant Energy Transfer Mechanisms. Sullivan BD, Dehlinger D, Heller MJ. The dissertation author was the primary investigator and author of this paper.
Table 4.1. Simulated sequences, listed 5’ to 3’, left to right.

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Figure 4.1. A) Probability of each base pair of the zipper being intact at a given temperature for Sequence #1. The leftmost curves represent the first base pair in the zipper. Matched curves are represented by the solid line. Mismatched curves are shown with a dashed line. B) The total fraction of intact base pairs at a given temperature. The intact fraction for each strand was used as the mean of the Gaussian probability distribution in Equation 5.
Figure 4.2. A) Probability of each base pair of the zipper being intact at a given temperature for Sequence #2. A nonuniform, sequence specific distance between base pairs is evident. In particular, the first two bases of the Match dehybridize relatively easily compared to bases three four and five, whose midpoints are clustered together above 300 °K. B) The total fraction of intact base pairs at a given temperature for Sequence #2.
Figure 4.3. Open and closed boundary conditions. The distributions represent the probability of the acceptor fluorophore being a distance $x$ away from the donor.
Figure 4.4. Acceptor FRET response of the system over time using the Gaussian noise model. The relative distance between the fluorophores and the intensity of the signal were inversely related.
Figure 4.5. Distance distributions over several equally spaced temperatures using the Gaussian noise model for matched (top) and mismatched (bottom) strands of Sequence #1. The matched distributions exhibit a subtle bias towards the closed state, while the mismatched distributions are more prominent towards the open state.
Figure 4.6. Ratio of matched to singly mismatched amplitudes for the zipper model (left column) and Gaussian model (right column) for Sequence #1. As the time constant, \( t \), lengthened, the regions of highest signal-to-noise shifted towards the center of the parameter set. The match responded most strongly along the left vertical edge, while the mismatch dominated along the lower horizontal edge.
Figure 4.7. Predominantly matched response. (Top) Amplitude vs. frequency of matched and mismatched responses. The noise threshold is shown as a horizontal line. (Middle) Applied temperature profile. The short dissipation time biases the temperature at a high level, keeping the construct near the open state. (Bottom) Time series of FRET responses to temperature actuation. Average of 40 traces. An extended $t_1$ and brief $t_2$ was found to produce a considerably stronger oscillatory response in complementary as compared to mismatched probes.
Figure 4.8. Predominantly mismatched response. (Top) Amplitude vs. frequency of matched and mismatched responses. (Middle) Applied temperature profile. The short actuation time biases the temperature at a low level, keeping the construct near the closed state. (Bottom) Time series of FRET responses to temperature actuation. Average of 40 traces. At these levels, the mismatched complex was able to dissociate before the matched system could respond.
Figure 4.9. A) Equal concentrations of match and mismatch give both higher amplitudes of matched response and higher matched frequency components above the noise threshold. It seems as if the match “responds faster.” B) As the concentration of mismatched constructs is increased, overlap becomes apparent in the upper harmonics, showing that the actual frequency spectra are non-orthogonal.
Figure 4.10. The directed walk through parameter space began at \( \{t_1=400, t_2=20\} \), increased \( t_2 \) by 10 until \( \{t_1=400, t_2=400\} \), then decreased \( t_1 \) by 10 until \( \{t_1=20, t_2=400\} \). All \( t \) were set to 100 in order to maximize the differences in frequency response.
Figure 4.11. Each point of the normalized eigenvector represents the relative amplitude of response at the given set of actuation parameters. Optimization of path through parameter space could reduce the length of the vectors.
Figure 4.12. Sum normalized, combined signals from equal (1:1) and low concentration (1:5) walks are shown in the solid lines. The dashed lines represent the negative control, where only the one- and two base pair mismatched signals were included for each set of actuation parameters. The presence of a match raises the combined signal at the beginning of the parameter set. As the concentrations of mismatched probes are increased, the differences between combined positive and negative controls are diminished. The reduced parameter set was used for illustrative purposes.
Table 4.2. Predicted concentrations of matched, mismatched, and 2 base pair mismatched sequences using the eigenvalues of a 20:10:400 walk through parameter space. The signal-to-noise ratio for Sequence #1 approached 1:100, where Sequence #2 approached 1:1000. There were no false negative calls for either sequence, although the concentration was overestimated at the lower concentrations.

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Chapter 5: Precision Nanoparticles

5.1 Nanoparticle Classification

A typical semiconductor quantum dot contains a ≈5 nm core of CdSe crystal passivated by a shell of ZnS. To make it water soluble, a hydrophilic polymer coating is bound to the core-shell structure. The outer polymer layer provides a scaffold for the covalent linkage of biomolecular recognition domains, whether streptavidin, antibody, or chemical. The total size of semiconductor quantum dots is on average 10-15 nm [Parak WJ 2003].

The emission wavelength quantum dots is directly related to the size of the core. When excited by light with a wavelength below its emission peak, a hole-electron pair is created within the confines of the core. The smaller the core, the higher the energy of the exciton, and the more blue the emitted light. At 5 nm, the CdSe core has an emission peak of 565 nm. The thousands of atoms within a semiconductor quantum dots make them significantly larger than organic
fluorophores, which are usually on the order of tens of atoms and around 1-2 nm [Jares-Erijman EA 2003].

Another nanoparticle of note is the metal nanoparticle, which is typically comprised of gold or silver. Gold and silver nanoparticles are individually detectable through scattered light in darkfield microscopy. Metal nanoparticles also exhibit unique optical properties which change depending upon their interaction with external electric fields [Rechberger W 2003, Bouhelier A 2005, Reinhard BM 2005, Sönnichsen C 2005]. Metal particles are also known to quench organic fluorophores when in close proximity (0-6 nm), but greatly enhance the organic fluorescent emission rate when they are displaced 7-20 nanometers away [Kulakovich O 2002, Malicka J 2003]. In other words, gold and silver nanoparticles can act as a distance dependent, nanoscale lens [Li K 2003].

Recently, metal quantum dots were synthesized by encapsulating eight to thirty atoms of gold within a fourth generation polyamidoamine, hydroxy-terminated dendrimer (G4 PAMAM-OH) [Zheng J 2004]. There is a rich literature on using the nucleophilic interior of dendrimers to sequester metal ions. Following a reduction step, the ions coalesce into a zerovalent metal nanoparticle which is simultaneously passivated by the dendrimer scaffolding.

Dendrimers have their own place in the nanoparticle hierarchy, and can take on a variety of characteristics depending on their composition and generation. The transition from being a wet sponge to being more like a rigid polystyrene ball can happen between generations four to eight [Crooks RM 2001]. When dendrimers are
terminated with primary amines, such as with PAMAM, the large positive charge density allows them to effectively bind to the negatively charged DNA backbone [Maiti P 2006].

5.2 Poisson Statistics of Functionalized Nanoparticles

The unique optical properties of metal and semiconductor nanoparticles present an intriguing approach to improving the information rate within nanoscale FRET systems. For instance, Figure 1.10B depicts a “dumbbell” arrangement of oligonucleotide assembled nanoparticles. The interaction between metal nanoparticles in such a configuration could provide information about the long-range bending modes of DNA. Combinations of Figure 1.10A and 1.10B may also be possible, with an organic FRET system placed at the center of the dumbbell to provide access to modulated fluorescent enhancement as well as modulated FRET responses. It may also be possible to multiplex dumbbell assays in the frequency domain. If DNA acts as a linear spring, which for short oligonucleotides may be a good approximation, changing the size of the attached nanoparticle could alter the harmonic resonance of the construct. It would also stand to reason that mismatches within dumbbell constructs would preferentially favor low energy bending modes, since double stranded DNA is far more rigid than its single stranded counterpart.
Unfortunately, the ability to produce high yield, high purity nanoparticle constructs like those shown in Figure 1.10B is well beyond our current capabilities. Nanoparticles are intrinsically heterogeneous. Most semiconductor and metal nanoparticles are grown colloidally, which leads to high coefficients of variation within small particles [Xu X 2007]. The nano-sized crystals which grow in solution are not guaranteed to be spherical, and the optical properties of these particles are closely related to their geometry. A transmission electron micrograph (TEM) image of gold nanoparticles is shown in Figure 5.1. These particles were nominally listed as 20 nm is diameter [Sigma Aldrich G1652]. Further purification of nanoparticles based on size is possible, but like mismatched DNA stringency, broad, overlapping Gaussian distributions of nanoparticle morphology create an inverse relationship between purity and concentration.

Standard protocols for nanoparticle functionalization (i.e., adding the DNA to the nanoparticle) incorporate either a direct chemical linker such as thiol (DNA-SH) or secondary linkers such as biotin following streptavidin adsorption to the nanoparticle surface [Alivisatos AP 1996, Reinhard BM 2005]. Functionalization of nanoparticles after their colloidal synthesis follows the Poisson distribution [Karl WC 1998]:

\[ P(N = k) = \frac{\lambda^k}{k!} e^{-\lambda} \]  

(26)

where \( \lambda \) is the average number of DNA binding events in a time interval or region of space, and \( k \) is the number of DNA strands attached to a specific nanoparticle.
The Poisson distribution is a good approximation of large numbers of binary interactions. Functionalization can be reasonably assumed to be binary because the equilibrium constants strongly favor the bound state for either covalent or streptavidin/biotin binding. In other words, DNA is either bound or not bound. Nanoparticles are capable of hosting many DNA molecules, the upper limit of which would correspond to the maximum $k$. For high concentrations of nanoparticles in solution, $\lambda$ is equivalent to the stoichiometric ratio of DNA to nanoparticle.

Figure 5.2 shows the probability densities of DNA/nanoparticle binding at different starting ratios of DNA to nanoparticle ($\lambda$). The top graph shows the familiar Poisson distribution in response to a slight excess of DNA. As one would expect, some nanoparticles carry a single functionalization, while some have many, and some have none. A similar graph is shown for equal starting ratios of DNA to nanoparticle, resulting in a distribution where only 35% of the constructs are singly modified. Thus, not only are nanoparticles intrinsically heterogeneous, but post-synthesis functionalization creates a large percentage of multiply modified particles. In reality, the probability distributions of functionalizations should take into account both the intrinsic nanoparticle population variability as well as the Poisson statistics above, represented by the convolution of the probability densities of the two random variables ($i.e.$, a Poisson convolved with a Gaussian). Here we assume ideal nanoparticle populations for brevity.
Figure 5.3 depicts the ratio of singly modified nanoparticles to all other particles in solution as a function of the DNA to nanoparticle ratio. A distinct maximum is reached at $\lambda = 1.6$, corresponding to the top graph of Figure 5.2. At no time are more than half of the nanoparticles in solution singly modified.

Assuming you could easily separate the DNA modified nanoparticles from the unmodified particles, one possible way to get a high likelihood of singly modified constructs would be to titrate in DNA over time, basically adding very small amounts of DNA in relation to the gold particles, followed by purification after each step. Figure 5.4 shows that for a titration to be of high purity, at least $1/10^{th}$ of the concentration of the gold must be added at each step. Since real purification steps between titrations would be quite lossy, this approach would represent a very expensive, time consuming approach, and is rarely seen in practice.

A more common approach is to try to separate and purify singly modified nanoparticles from the soup of malformed constructs resulting from higher ratios of addition. However, it is very difficult to distinguish singly from multiply modified nanoparticles [Xu X 2007, Zanchet D 2001]. Unlike or small molecules or proteins, which are identical, the heterogeneity of nanoparticles limit the usefulness of traditional purifications.

A study by Zanchet et. al., in 2000, showed clear differences between singly and multiply DNA modified gold nanoparticles using 5 nm nanoparticles and 100 base pair DNA ($\approx 30$ nm) run on a 3% agarose gel. When the size of the DNA was
reduced even to 50 bp (≈ 15 nm), significant overlap between unmodified and modified gold was observed. The charge and volume changes resulting from DNA functionalization were insignificant compared to the intrinsic variance of even 5 nm gold particles. Useful DNA oligonucleotide probes are commonly 20 bp or lower, and individually detectable nanoparticles can be upwards of 40 nm, meaning that nanoparticle variances will dominate the contributions of the DNA. In this case, broad overlapping Gaussian nanoparticle populations should be separated by very small mean differences. Passive purification would result in either a low specificity, heterogeneous mix of probes, or very low yield of purified product. In many ways, this is a similar problem to differentiating between singly mismatched and perfectly matched DNA constructs.

5.3 Direct Nanoparticle Functionalization

Following work done by the Alivisatos group, we used thiol-modified 51mer DNA to directly functionalize the surface of gold nanoparticles [Loweth CJ 1999]. 5’ thiolated DNA (SH-DNA) was added to a 20 mM solution of Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) in Quantum Dot Incubation Buffer, which was comprised of a sodium borate, bovine serum albumin buffer (BSA). Bare gold nanoparticles were found to precipitate in the presence of TCEP alone. Dissolution of the TCEP in the BSA rich incubation buffer kept the nanoparticles
stable, as indicated by their deep red color. Prior to addition to the gold, the thiolated DNA was purified using an Amicon YM-10 spin column and reconstituted using 30 µL deionized water. SH-DNA was incubated with the gold for 4 days. For visualization purposes, a complementary fluorescent Cy3 probe was hybridized to the solution before gel purification on a 2% ethidium-bromide embedded agarose Egel [Invitrogen].

Figures 5.5 & 5.6 detail the results and associated controls for direct nanoparticle functionalization. In brief, modified gold particles exhibited gross heterogeneities (i.e., a smear) that prevented isolation of specifically functionalized particles. These results confirm the Zanchet study interpretation that large nanoparticles and shorter DNA are dominated by the intrinsic nanoparticle heterogeneity.

The nature of the interaction between the gold and the DNA was also unclear. Unmodified gold carried a negative charge, similar to the DNA. No interactions were seen between gold particles and unreduced SH-DNA, Cy3 probe, or bare DNA. DNA is known to nonspecifically bind to gold surfaces through hydrophobic bonds, although the lack of interaction with control sequences indicates that this was not a factor within this experiment [Herne TM 1997]. Introduction of the BSA incubation buffer had no electrophoretic effect on the gold, although some nonspecific binding was observed between the DNA and BSA (Fig 5.6, Lane 3). Upon addition of unpurified TCEP, all detectable DNA became complexed with the gold nanoparticles. These unpurified TCEP samples reversed
direction, suggesting that the acidic TCEP solution could have protonated the nanoparticle, resulting in strong electrostatic interactions between particles and DNA. Purification of the TCEP away from the SH-DNA restored the original gold particle trajectory. Gross heterogeneities became apparent after incubation of purified SH-DNA with gold (Fig 5.5 Lane 7, Fig 5.6 Lane 8-11).

5.4 Secondary Nanoparticle Functionalization

Secondary modification of gold particles was also explored, using streptavidin to both stabilize the gold sol and provide biotin binding sites. 50 nm gold colloid [Ted Pella Inc., 15708-20] was found to be stabilized by a minimum of 0.2 mg/mL streptavidin [Streptavidin SQ Roche; 1520679] in the presence of 0.16 M sodium chloride solution. Below the 0.2 mg/mL threshold, the gold particles turned blue after addition of salt. At the critically stabilized threshold, the gold particles were calculated to be at 37 pM concentration while the streptavidin was at 77 nM, a 2,061 fold concentration increase of protein over nanoparticle. The loading density of the nanoparticle is not known. However, the ratio of the surface area of a spherical 50 nm particle as compared to a 2 nm streptavidin molecule, \( \frac{s_{\text{part}}}{s_{\text{strept}}} = \frac{4\pi(25\times10^{-9})^2}{2\times10^{-9}\cdot2\times10^{-9}} = 1,964 \), which suggests that the streptavidin covers a large portion of the gold nanoparticle in order to passivate the particle from salt effects. If only half of the streptavidin is adsorbed to the particle surface
due to steric hindrance or less than perfect ordering, a thousand fold excess concentration of free streptavidin would remain in solution. Upon addition of prehybridized, biotinylated dumbbell dsDNA (with biotin labels located at each of the 5’ ends of a double stranded helix), no change in absorbance was observed with the streptavidin coated particles.

To reduce what we believed to be the influence of free streptavidin, a gentle, partial centrifugation protocol was developed to prevent flocculation. The protocol did not use membrane filters, again to prevent aggregation. Equal 400 µL volumes of streptavidin and gold nanoparticle were mixed to reach the critical stabilization concentration. The mixture was spun for 2 minutes at 6,000 rpm. 5 µL of the “pellet” (which consisted of a diffuse layer of gold solution near the bottom of the microcentrifuge tube) was resuspended in 1 mL 0.14 mg/mL BSA. The BSA allowed a second centrifugation step to proceed while maintaining the requisite density for diffuse pelleting. Gold nanoparticles would not spin down in deionized water after the first purification step. After the second 2 minute 6,000 rpm centrifugation, 995 µL of the supernatant was carefully drawn off the top of the solution. 50 µL of 0.25 M NaCl, 0.025 M sodium phosphate was added to the retentate. The gold particles remained a deep red color, indicating that they were still stabilized. Reconstituted samples were then split in two. To the test the functionality of the streptavidin coated particles, 15 µL of prehybridized, biotinylated 51mer dsDNA was added to a final concentration of 30 pM and incubated at room temperature. Absorbance spectra were recorded over three days,
as the concentrations of DNA and nanoparticle were low. Figure 5.7 shows that in each of the three test solutions, a significant decrease in absorbance was seen. Two of the control solutions showed little decrease, while a third showed similar decrease in absorbance relative to the tests.

The experiments did not conclusively demonstrate whether the change in absorbance was due to the DNA driven assembly of the nanoparticles as compared to simple aggregation, given that one of the controls showed a similar decrease over the time span in the absence of DNA. However, flocculated gold particles exhibit a significant blue shift in spectra that was not observed in this experiment. We are unaware of any other studies which have observed the spectroscopy of dumbbell assemblies of gold nanoparticles. The characteristic blue shift of flocculation is likely to be the result of much larger aggregates than was observed here.

TEM analysis of streptavidin coated nanoparticles joined by DNA also reveals gross heterogeneities within the population (Figure 5.8). TEM samples were spotted and dried on copper grids before imaging, which likely influenced the nanoparticle characteristics. TEM data does reveal interesting morphologies of the nanoparticles, such as the thin gray band surrounding the periphery of the gold, which could be the capping layer used to arrest nanoparticle synthesis, the adhered protein layer, or a combination of both.
5.5 Dendrimer Encapsulated Nanoparticle Growth

Collectively, the results in sections 5.3 and 5.4 indicate that stoichiometric functionalization following colloidal nanoparticle synthesis presents significant barriers to generating high yields of singly modified nanoparticles. In order to circumvent these barriers, we believe that precision nanoparticle syntheses must address both the Poisson statistics of functionalization and the Gaussian statistics of nanoparticle heterogeneity.

In collaboration with Dr. Xiaohua Huang, we formulated a strategy to grow nanoparticles directly off the 5’ end of an oligonucleotide. Direct synthesis circumvents Poisson statistics because the maximum number of allowable interactions \((k)\) at the end of a DNA is identically one. Our approach borrows from oligonucleotide phosphoramidite synthesis in that it allows a large excess of reactants to drive coupling reaction equilibria, resulting in > 95% theoretical completion for each step. Ideally, the nanoparticle growth would be incorporated into DNA synthesis prior to releasing the oligonucleotide from its substrate to facilitate the superior washing and recovery afforded by synthesizers.

Our approach, detailed in Figure 5.10, uses a hydrophilic, Tris(hydroxymethyl)amidomethane terminated, cystamine cored PAMAM dendron [Dendritich Nanotechnologies, Inc.] as a scaffold for nanoparticle nucleation and reduction. The dendrimer is cleaved using a reducing agent to expose a reactive thiol at the apex of the dendron. We explored two strategies to couple the dendron
to DNA: 1) the heterobifunctional linker, Sulfo-SMCC (sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate) [#22322, Sigma Aldrich] was reacted with a primary amine modified oligonucleotide, and following size exclusion purification to remove free SMCC [Amicon YM-10, 12 minutes], the maleimide-DNA was added to the reduced dendrimer, and 2) parallel one-pot reduction of thiolated oligonucleotides and cystamine dendrimers followed by size-exclusion purification [Amicon YM-3, 100 minutes] and passive oxidation of the reactants to reform disulfide bonds. The dendron (half of a dendrimer) could be grown off the end of the DNA, but PAMAM features a large concentration of primary amines during synthesis which would likely bind to the negatively charged DNA and interfere with successive reactions. Therefore, we opted for the fully formed dendron approach. Following dendrimer conjugation to DNA, the constructs were incubated with 0.75 mM aqueous AgNO₃, followed by a 10 minute exposure to focused, unfiltered mercury lamp light in a quartz cuvette.

Figure 5.11 shows the UV/Vis spectra during the growth of silver nanoparticles in the presence of G4 PAMAM-OH dendrimer. 1.5 µmoles aqueous silver nitrate [#209139, Sigma Aldrich] was added to 0.5 µmoles of G4 PAMAM-OH solution [#477850, Sigma Aldrich, in methanol]. The pH was adjusted with acetic acid to pH 8 in the manner of Dickson, in order to promote deprotonation of the tertiary amines on the interior of the dendrimer and sequestering of the silver ions [Dickson RM 2006]. The solution was then exposed for the times indicated in Figure 5.11. A clear increase in absorbance over time was observed, with less than
10% evaporation of the initial volume. While this data does not confirm that the nanoparticles are located within the dendrimer core, it can be said that the presence of dendrimer does not inhibit nanoparticle growth. The excitation and emission spectra of the silver/PAMAM-OH solution before and after photoactivation are shown in Figure 5.12. Clear differences in the amplitude and shape of the fluorescent spectra are evident following lamp excitation.

Preliminary, unrepeated results from our DNA/dendrimer coupling scheme are shown on a 4% agarose Egel [Invitrogen] in Figure 5.13. In our experience, dendrimers do not react with ethidium bromide, so that the bands in Figure 5.13 are representative of the position of DNA within the gel. DNA/dendrimer solutions were mixed with 0.75 mM AgNO₃ and exposed to lamp light before gel separation. Positive and negative controls for the first approach (+/- SMCC) and the second approach (+/- TCEP) suggest that both the SMCC and thiol binding had an effect on the DNA, while the negative controls showed essentially no change in the DNA mobility. Again, while this data is preliminary and does not confirm that the DNA is coupled to the dendrimer (as it could be nonspecifically bound), the results are promising. The thiol coupling was far more efficient than the SMCC approach, although there is a pH and time dependence on the SMCC reaction that we have not yet explored, so these results are unoptimized. Furthermore, TCEP is known to compete with maleimide coupling, so additional purification may improve the yields for this approach [Getz EB 1999, Schafer DE 2000].
Immediately apparent from the gel is that even if these results are due to DNA/dendrimer coupling, there still seems to be gross heterogeneities within the resultant nanoparticle population. No attempt was made to stir the solutions during photoactivation, and the setup was suboptimal in that there was a sharp intensity gradient over the reaction volume. Future experiments that address illumination may also improve monodispersity of the particles.
Figure 5.1. Typical distribution of gold nanoparticles. Image courtesy of Dr. Kenneth Vecchio.
Figure 5.2. Poisson distributions for a slight majority of DNA to nanoparticle $\lambda=1.6$, and a stoichiometrically equivalent mixture of nanoparticle to DNA, $\lambda=1$. 
Figure 5.3. The ratio of the probability of finding a singly modified nanoparticle vs. all other modifications \{0, 2, 3\ldots\} as a function of stoichiometry. The maximum concentration (at $\lambda=1.6$) will result in a solution of nanoparticles with less than half being singly modified.
Figure 5.4. Assuming it is possible to separate singly modified particles from unmodified particles but not multiply modified particles, Poisson statistics suggest a very slow titration to ensure singly modified particles. This approach is likely very lossy and expensive.
Figure 5.5. Gold nanoparticle functionalized with thiolated DNA. All samples were labeled with a Cy3 fluorescent probe for visualization prior to electrophoresis in a 2% E-gel.

Lane 3: Gold nanoparticles alone
Lane 4: Gold nanoparticles + bare DNA
Lane 5: Gold nanoparticles + SH-DNA
Lane 6: Gold nanoparticles + SH-DNA + TCEP Mix
Lane 7: Gold nanoparticles + purified SH-DNA

The Cy3 probe in Lane 3 alone is the most mobile and compact distribution. Addition of a bare 51mer (p53Target) resulted in a slight retardation. The thiolated probes showed more resistance to movement than the bare DNA, perhaps from cysteine dimers. Although not evident in the fluorescence image, distinct red bands were visible near the top of the wells in Lanes 3-5, indicating that the gold migrated slowly into the gel. Upon addition of TCEP in Lanes 6 & 7, very little DNA moved into the gel, indicating complexation with the gold nanoparticles. Lane 6 suggests that the acidic TCEP caused sufficient protonation of the particles that they moved in the opposite direction. Purification of TCEP from the thiolated DNA before addition to the gold recovered the negatively charged, DNA complexed nanoparticles.
Figure 5.6. Repeat of the experiment in Figure 5.5 with two sequences of thiolated DNA. All samples were labeled with a Cy3 fluorescent probe for visualization prior to electrophoresis.

Lane 1: Gold nanoparticles alone
Lane 2: Gold nanoparticles + SH-DNA
Lane 3: Gold nanoparticles + BSA + SH-DNA
Lane 4: Gold nanoparticles + bare DNA
Lane 5: Gold nanoparticles + SH-DNA + TCEP Mix
Lane 6: Gold nanoparticles + BSA + SH-DNA + TCEP Mix
Lane 7: Gold nanoparticles + TCEP Mix + Buffer
Lane 8: Gold nanoparticles + purified SH-DNA
Lane 9: Gold nanoparticles + purified Complementary SH-DNA
Lane 10: Gold nanoparticles + purified SH-DNA + BSA
Lane 11: Gold nanoparticles + purified Complementary SH-DNA + BSA

As in the previous experiment, the unreduced SH-DNA did not associate with the gold (Lanes 1-4), while reduced and purified SH-DNA complexed with the gold particles and bound the Cy3 probe (Lanes 8-11), which indicates that the association is most likely nonspecific, as Lanes 9 & 11 have a complementary DNA that should not hybridize to the Cy3 probe. The timing of BSA addition did not seem to have a protective effect from the TCEP (Lane 7).
Figure 5.7. Streptavidin adsorbed gold nanoparticle dumbbells. In triplicate, centrifugation purified gold nanoparticles were mixed with prehybridized, biotinylated dsDNA in 0.25 M NaCl, 0.025 M sodium phosphate and incubated for three days. Prior to DNA addition, samples were split in two, with controls receiving only salt solution. Day 1 was always the highest peak, followed by successive decreases in absorbance. The decreases over time were evident in the DNA assemblies. Two of the controls showed less decrease, although a third showed evidence of flocculation.
Figure 5.8. While isolated doublets were apparent within the sample (top left & top right), the majority of the sample revealed the gross heterogeneities of the nanoparticle population.
Figure 5.9. (Top) Example of first generation (G1) PAMAM dendrimer with a cleavable cystamine core. (Bottom) Fourth generation dendrimer with a hydrophilic Tris(hydroxymethyl) surface group.
Figure 5.10. DNA-dendrimer crosslinking approach. A) TCEP Reduction of disulfide bond cleaves the dendrimer into dendron segments. B) Purified dendrimer is added to an maleimide-terminated DNA (following SMCC coupling to amine-DNA). C) Alternatively, parallel reduction of HS-DNA and cystamine dendrimer is followed by purification to promote oxidation of DNA and dendrimer.
PAMAM/Silver nanodot spectra following photoactivation over time

Figure 5.11. UV/Vis absorbance spectra of AgNO₃ solution over timed exposures to mercury lamp light. The increase in absorbance indicates nanoparticle formation.
Figure 5.12. Emission (top) and excitation (bottom) spectra of PAMAM-OH / silver solution before and after photoactivation. A clear emission peak at 594 nm seems to indicate that the nanoparticles are weakly fluorescent. More indicative of nanoparticle growth is the increase in UV excitation below 250 nm.
Figure 5.13. Reaction of dendrimer with DNA, following nanoparticle growth.

Lane 4) NH$_2$-DNA + SMCC + TCEP reduced dendrimer (without purification)
Lane 5) NH$_2$-DNA + TCEP reduced dendrimer (without purification)
Lane 6) HS-DNA + TCEP + dendrimer, followed by purification
Lane 7) HS-DNA + dendrimer, followed by purification
Chapter 6: Conclusions

We have identified a nanoscale transduction mechanism that may provide greatly improved specificity within molecular diagnostics. We have also demonstrated that this mechanism permits modern information theory to be applied to the detection of DNA within complex biological samples. More specifically, we have shown that:

- electric field driven oscillatory systems constructed out of fluorescent resonant energy transfer assemblies exhibit a power and sequence dependence. Modulated signals were found to overcome the inverse relationship between sensitivity and specificity characteristic of Gaussian distributed processes. Our results also indicate that driven oscillations reduce the variance of free energy within single probe distributions. Looking forward, we hypothesize that high speed ultrasound shall facilitate analysis of the mechanical modes and entropic contributions to the free energy minima of bound oligonucleotides, which if true,
may lead to unique strategies to improve specificity and reduce variance within genetic assays;

- integration of oscillatory responses over time give near single molecule sensitivity within a high background. This finding is also very significant. While most single molecule detectors operate only under very favorable signal-to-noise environments, the ability to extract signals from a high background of autofluorescence and nonspecific binding has been elusive. We have demonstrated that driven oscillations of molecular systems several orders of magnitude below the fluorescent detection limit result in clear signals in the frequency domain. Eigenvector decomposition across a multiparameter walk through oscillation modes improved signal-to-noise ratios from 2:1 to upwards of 1000:1. As our research indicates that there should be a distinct difference between the frequency responses of matched and mismatched probes, we hypothesize that incorporation of additional dimensions of observation shall lead to greater specificity within responses of nanoscale optical transduction systems. Moreover, mechanical analogs of parity may be necessary to improve robustness within limited channel capacity. Future work should test this hypothesis, in order to increase our understanding of the interactions between nanoscale probes and biological targets within driven oscillatory systems;

- Construction of high tolerance, singly-labeled nanoparticle probes shall be of paramount importance for modulated detection. It remains unclear as to how nanoparticles of different mass or eccentricity affect the free energy of a probe
system. Dendrimer encapsulated nanoparticles grown atop a solid phase substrate may offer an ideal platform for this work. Finally, the Gaussian nature of defects within colloidal nanoparticle growth suggests that modulation of separation and purification protocols may help homogenize populations of nanoparticles. For instance, modulated gel chromatography, modulated HPLC, or modulated size exclusion separation are possibilities. Using size exclusion as a hypothetical case, modulated interactions would consist of continuously dilating and contracting the pore size within a sephadex-type gel.
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**Aptamers**


**Ion Channel Switch**


**Quartz Crystal Microbalance**


**Water Soluble Conjugated Polymers & PNA Probes**


**In Situ Hybridization**

**Biochannel Concentration**

**Colorimetric Reflection Substraction on Gold**

**Microcantilevers / AFM Tips**