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High-throughput image cytometer for detection of circulating tumor cells and contrast-enhancement filtering for automated 3D image segmentation of cartilage tissue explants

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HIGH-THROUGHPUT IMAGE CYTOMETER FOR DETECTION OF CIRCULATING TUMOR CELLS AND CONTRAST-ENHANCEMENT.Filtering for Automated 3D IMAGE SEGMENTATION OF CARTILAGE TISSUE EXPLANTS

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

Bioengineering

by

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2007
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University of California, San Diego

2007
To My Parents

—for the continuing support
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<tr>
<td>CCD</td>
<td>charge-coupled device</td>
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<tr>
<td>CTCs</td>
<td>circulating tumor cells</td>
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<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole</td>
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<tr>
<td>DAQ</td>
<td>data acquisition</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>FIFO</td>
<td>first in first out</td>
</tr>
<tr>
<td>FOV</td>
<td>field of view</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>mWBCs</td>
<td>mononucleated white blood cells</td>
</tr>
<tr>
<td>NA</td>
<td>numerical aperture</td>
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<tr>
<td>PCR</td>
<td>polymerize chain reaction</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerize chain reaction</td>
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<tr>
<td>SNR</td>
<td>signal to noise ratio</td>
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<td>TDI</td>
<td>time delay integration</td>
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Chapter 4, in part, contains material being prepared for publication. Nguyen LK, Sah RL, Price JH, “Least squares designed contrast-enhancing FIR filters improve segmentation of 3D confocal images”. The dissertation author is the primary researcher and author of this publication.
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PUBLICATIONS


ABSTRACT OF THE DISSERTATION

High-Throughput Image Cytometer for Detection of Circulating Tumor Cells and Contrast-Enhancement Filtering for Automated 3D Image Segmentation of Cartilage Tissue Explants

by

Lam K. Nguyen
Doctor of Philosophy in Bioengineering
University of California San Diego, 2007

Jeffrey H. Price, Chair
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The first report of the presence of tumor cells circulating in blood was published over a century ago. The ability to routinely detect and characterize these circulating tumor cells can have a profound impact on diagnostic staging, therapy decisions, monitoring therapy efficacy, and long-term patient management. Existing methods of detection which are based on expression of proteins, are either plagued by high false positive rates or have not yet demonstrated consistent results. An alternative method of detection that exploits the morphologic differences between tumor and normal cells has great potential; however, there is not yet an instrument with sufficient speed and resolution to make this technique practical. A high-throughput, high-resolution image cytometer was developed to address this problem. A spiked cell model was used to measure its performance at finding circulating tumor cells and a performance comparison was made to an existing commercial image cytometer.

Better understanding of cartilage growth and repair is necessary for developing more effective therapy for articular cartilage repair. The confocal microscope permits experimental conditions that preserve the natural state of chondrocytes within the surrounding matrix. However, in the absence of any quantitative techniques for processing the data, the confocal microscope has been limited to a few
qualitative roles. A contrast-enhancing technique was developed to enable automatic segmentation of confocal data sets for routine quantitative three-dimensional studies of thick cartilage tissue.
Chapter 1

Introduction

1.1 Circulating Tumor Cells

The presence of tumor cells circulating in blood was first reported over a century ago [10]. Since then, various types of cancers have been shown to shed tumor cells into the circulation [6, 27, 76]. The ability to routinely detect and characterize these circulating tumor cells (CTCs) can have a profound impact on diagnostic staging, therapy decisions, therapy monitoring, and long-term patient management. For example, instead of the currently performed bone marrow aspiration which is complicated, time-consuming and uncomfortable for the patient, simple blood tests would allow repeated and frequent testing to detect the presence of as well as the change in number of CTCs as an indicator of the progression of the disease. In addition, adjuvant treatment can be better administered; patients who are more likely to relapse receive more aggressive or different forms of adjuvant treatments, whereas those at lowest risks of disease recurrence could forgo the morbidity of adjuvant treatment. Furthermore, analysis of the genotype and phenotype of CTCs also might give insights into the mechanisms of resistance to systemic therapy and the characteristics necessary for invasion and growth at metastatic sites.

Currently, the presence of cancer in the regional lymph nodes and bone marrow are the most valuable prognostic indicators of metastatic cancer. However, many patients considered node negative eventually develop recurrent disease. And since tumor cells can lay dormant for a long time in bone marrow and are not destroyed
by the body’s defense mechanisms, their presence does not give an accurate measure of the progression of the disease or the efficacy of treatment [16]. On the other hand, the 24-hour survival time of CTCs is relatively short [87]. Accidental detection of these cells in the circulation is unlikely unless there is a constant influx of tumor cells. Therefore, CTCs are most likely present during disease progression. The detection of CTCs provides a valuable prognostic indicator of disease progression and a measure of treatment efficacy which have been confirmed by numerous clinical studies in recent years.

1.1.1 Clinical Significance of Circulating Tumor Cells

Numerous studies have looked at the presence of CTCs as a prognostic indicator of disease progression in various types of cancer. Hardingham et al. analyzed blood samples of 94 colorectal cancer patients and showed a clear cut prognostic value for the estimation of the survival time [49]. Shariat et al. looked at the presence of prostate specific antigen (PSA) mRNA in the blood of prostate cancer patients after surgery and demonstrated its correlation with more aggressive disease progression and an inferior prognosis [101]. Halabi et al. found a significant increase of the risk for a relapse in prostate cancer patients, if CTCs could be detected after surgery [45]. Stathopoulou et al. reported a significant negative prognosis after detecting CTCs in 128 breast cancer patients. Patients with CTCs in blood after removal of the primary tumor and before receiving adjuvant therapy had a significantly lower survival time and a reduced disease free interval [110]. Xenidis et al. [123], Gaforio et al. [36], and Giatromanolaki et al. [37] also reported the detection of CTCs having significant prognostic value for the estimation of the survival time and disease free interval. Patients with CTCs had a shorter survival time as well as a shortened disease free interval. Jotsuka et al. investigated 100 node negative breast tumor patients and showed that patients with CTCs either before or after surgery had a significantly shorter disease free interval than those who had no CTCs at any time. Patients who were CTC positive at both times had the worst prognosis [58]. Most recently, Cristofanilli et al. measured the levels of CTCs in 177 metastatic breast cancer patients before initial treatment and at first follow-up visit and showed that the levels of CTCs were a significant predictor
of progression-free interval and overall survival time [27, 28]. These studies indicate that the detection of CTCs can be used to determine whether to initiate or continue adjuvant treatment or avoid unnecessary therapy altogether.

Other investigators have also investigated the idea of monitoring CTCs as a measure of treatment efficacy. Smith et al. monitored the presence of CTCs during systemic therapy in 22 breast cancer patients and found a 83% correlation between clinical response towards therapy and the detection of CTCs [108]. Giatromanolaki et al. correlated the efficacy of a systemic adjuvant chemotherapy with the presence of CTCs in 32 patients (early stages I/II and IIIA) and concluded that the presence of CTCs before surgery was predictive for an inferior therapeutic outcome, irrespective of which therapeutic regimen was used [37]. Xenidis et al. reported differences in the response of 161 breast cancer patients towards different systemic therapeutic treatment between groups of patients with and without CTCs immediately after cessation of the therapy. A sub-group of patients with a lymph node status less than n3 but detectable CTCs after therapy had a 4-fold risk of relapse. Furthermore, there were significant differences depending on the therapeutic regimen being used (Figure 1.1) [123]. Hayes et al. studied the level of CTCs during treatment in breast cancer patients. They detected CTCs in six of eight patients with progressive disease and in three of four patients with stable disease, but they only found CTCs in one of seven patients who had complete or partial remission. Furthermore, in four of twenty patients, whose primary tumor was HER-2 negative, HER-2 positive CTCs were found [51]. Overexpression of this gene occurs in some breast cancer and is associated with increased disease recurrence and worse prognosis. Although the detection of newly gained HER-2 expression during therapy implies an inferior prognosis, it also offers a possibility for Herceptin therapy which is only effective in breast cancer where HER-2 is overexpressed. This shows the importance of CTC analysis in the framework of individually tailored therapeutic regimens.
Figure 1.1: Presence of CTCs and Response to Adjuvant Therapy. Cumulative disease-free interval rates according to the type of adjuvant chemotherapy regimen used. (A) No CTCs detected after adjuvant therapy; (B) CTCs detected after adjuvant therapy. (CMF: cyclophosphamide, methotrexate and 5-fluorouracil; FEC: epirubicin, cyclophosphamide and 5-fluorouracil; TEC: taxotere, epirubicin and cyclophosphamide) [123].
1.1.2 Existing Methods of Detecting Circulating Tumor Cells

Current techniques of detecting CTCs can be classified into two main categories: molecular and cellular approaches. Cellular techniques use immunocytochemical methods to identify and characterize individual tumor cells. Molecular techniques are based on the detection of nucleic acids (DNA or mRNA) that are differentially expressed in tumor cells and normal blood components.

Molecular Techniques

Circulating DNA is detected in the plasma of normal individuals, but higher quantities are found in the blood of cancer patients. Furthermore, genetic alterations in plasma DNA corresponding to those in the primary tumors have been identified in patients with breast cancer [24, 75, 102, 105], lung cancer [25, 38], colorectal cancer [52] and head-and-neck cancer [79] suggesting that some of the DNA is tumor-derived. As a result, many research groups have used polymerize chain reaction (PCR) amplification to detect the presence of tumor-derived DNA in blood as a means of detecting a very small number of CTCs.

However, detection of tumor-related DNA in the plasma only indicates the presence of circulating DNA, not necessarily circulating tumor cells. Furthermore, there is uncertainty about the half-life of DNA in the blood stream. They can last a long time in the circulation [120] such that positive results may persist. Such persistence would be problematic in using this technique to monitor disease progression or treatment response. Furthermore, to use PCR efficiently, molecular analysis of the primary tumor of each patient is necessary to identify the unique changes that distinguish the tumor DNA from the DNA shed by the surrounding hemopoietic cells.

Messenger RNA (mRNA), on the other hand, has limited viability outside of the cell as it is highly susceptible to degradation by blood RNAses [61]. Detection of tumor-associated mRNA (specific expression or significantly upregulated expression of genes in tumor cells) in blood indicates the presence of circulating tumor cells. Reverse transcription polymerize chain reaction (RT-PCR) is used to detect tumor-associated mRNA. The first step in the process involves the isolation of
mononuclear cells from the blood sample. The usual approach is density-gradient centrifugation, and recently, immunomagnetic separation to enrich samples for epithelial cells [6, 27, 28, 121]. The RNA is then extracted from the cells and reverse transcriptase is used to transcribe target transcripts into complementary DNA (cDNA). The cDNA is then amplified with PCR with specific primers for the transcript of interest. The target sequences are either tissue-specific differentiation markers or oncofetal antigens (tumor-associated antigens present in fetal tissue but not in normal adult tissue). Reactions are run with both positive controls (RNA from a cancer cell line) and negative controls. The quality and efficiency of the reaction is controlled by coamplification of house-keeping genes.

Various studies of the techniques from model systems estimate the sensitivity of detection is up to one tumor cell in $10^7$ peripheral-blood mononucleated cells [43, 78]. On the other hand, published studies have also reported widely varied results of detection sensitivity based on the same marker or combination of markers. For example, two studies analyzing the relative sensitivity of markers in the blood of breast cancer patients found that cytokeratin-19 mRNA is more likely to be detected than EGFR, cytokeratin-20, or mammaglobin mRNA [39, 44]. However, other studies found that cytokeratin-19 is present at higher rates in healthy individuals suggesting that mammaglobin may be a more useful marker [104]. One explanation is the heterogeneity of expression. Although the primary tumor expresses the marker, heterogeneous expression could mean that the target marker might not be present in the tumor cells circulating in the blood [15, 53, 60]. Some investigators try to overcome this issue with the use of a multimarker assay. A study used RT-PCR to detect four mRNA markers ($\beta$-HCG, c-Met, GalNAc-T, and MAGE-3) in the blood of breast cancer patients. The individual target markers were detected respectively in 34%, 11%, 24%, and 14% of the patients with 69% of the patients being positive for at least one marker [112]. The use of multiple markers in the screening increases the sensitivity of the test; however, it may result in a decrease in specificity. In addition, other studies also have reported a high number of false positives with RT-PCR techniques. Various markers used to identify tumor cells have been found to be expressed in normal individuals [13, 15, 44, 53, 127]. This is caused by illegitimate expression [82] (the expression in normal cells of small amounts of RNA of genes that have no real physiological role in those cells).
and pseudogenes [97, 99] (DNA segments with sequence homology to target RNA sequences). Although these events are uncommon, the extremely high sensitivity of RT-PCR leads to false positive results.

Although RT-PCR is extremely sensitive in detecting the presence of mRNA, it suffers from the lack of tumor-specific markers, the highly-varied sensitivity levels between studies, and the false positive results. Furthermore, the mRNA extraction process destroys any CTCs that are present, eliminating their visual confirmation of tumor origin.

**Cellular Techniques**

Cellular techniques isolate the individual tumor cells from the blood with immunocytochemical methods using either flow cytometry or image cytometry. The advantage of these techniques is the ability to further characterize the CTCs at a molecular level. Furthermore, unlike RT-PCR in which the tumor cells are destroyed to extract the mRNAs, the tumor cells are intact allowing visual confirmation and elimination of false positives. The main disadvantages of cellular techniques are slow analysis rates and lower detection sensitivity compared to molecular techniques [63, 107, 108].

Since only a small number of CTCs are found in blood (typically, less than 10 cells/ml), most researchers use an enrichment step to discard most of the blood cells before differentiating the CTCs from other blood components. Most research groups have used immunomagnetic techniques for enrichment [95] using magnetic beads that are linked to antibodies with affinity to specific cells. Cells are then separated with powerful magnets. Separation is performed with either positive selection using antiepithelial antibodies, or negative selection using leukocyte-specific antibodies as illustrated in Figure 1.2. Enrichment is also accomplished with density centrifugation, in which the tumor cells together with white blood cells are separated from the large number of red blood cells under high g-forces with Ficoll columns. Another enrichment technique, although less common, utilizes filters with small pore sizes such that most leukocytes and erythrocytes pass through leaving only larger tumor cells behind.

Even with enrichment, the CTCs are still greatly outnumbered by the remaining
Figure 1.2: **Principles of Immunomagnetic Separation.** Positive selection with antiepithelial antibodies conjugated to magnetic beads. Negative selection with magnetic beads conjugated to anti-CD45 antibody (an antigen expressed by all leukocytes). Adapted from Ring et al. [95].

white blood cells. Most researchers employ immunocytochemical methods based on monoclonal antibodies against epithelial-specific antigens to identify the CTCs. The most common target antigens are the cytokeratins — a family of 20 related polypeptides that are part of the cytoskeleton of epithelial cells. Without tumor-specific markers, and working on the assumption that tumor cells shedding from solid tumors tend to retain the intermediate filaments of their progenitor cell type, detection of cytokeratins in an environment where no cytokeratin expression is expected (such as in peripheral blood) is used as a substitute marker for CTCs originating from solid tumors. There is evidence that the majority of circulating epithelial cells are malignant rather than normal cells; however, they do not prove that all circulating cytokeratin-positive cells are tumor cells. The screening of large numbers of cells for specific marker(s) to identify the CTCs is time-consuming and often performed with automated or semi-automated flow and image cytometry systems.

In a flow cytometer, a single-cell wide stream is flowed at high speed through laser beam(s) and a set of detectors. Measurements of the interaction between the light beam and the cells (such as scattering of light and/or emission of any attached
fluorescence probes) are used to characterize the cells. Tens of thousand of cells can be analyzed per second in a flow cytometer. Many investigators have reported sensitive techniques for detecting CTCs with flow cytometry [73, 92]. While flow cytometry has the capability to analyze large numbers of cells quickly, similar to RT-PCR, it also does not permit visual confirmation of the tumor cells making rejection of false positives difficult.

On the other hand, as the name implies, image cytometry systems acquire an image of the cells from which cell measurements are derived. Image cytometers generally consist of a microscopy platform, a light source (halogen lamp, arc-lamp, laser, etc.), a detector (photomultiplier tube or camera), automation hardware and control software that drives the system, and image processing software that extracts the measurements from the images. With these systems, the cells are spun down on to glass slides into a thin layer with cytocentrifuges. Similar to flow cytometry, these systems employ cell-type-specific fluorescence markers to differentiate the tumor cells from the blood cells. The main advantage of image cytometry over other techniques is the availability of image records of the cells and the ability to visually inspect the cells. This capability allows trained personnel the ability to confirm suspected CTCs and to reject false positives. However, the main disadvantage of image cytometers is speed. They are typically several orders of magnitude slower than flow cytometry systems.

Limitations of Current Techniques

All current techniques of detecting CTCs suffer from the presence of false positives. The extremely high sensitivity of RT-PCR that allows it to detect minute amounts of RNA of interest also makes it vulnerable to amplifying the rare but normal occurrences of illegitimate expression and pseudogenes [13, 15, 44, 53, 97, 99, 127]. Similarly, cellular techniques using flow or image cytometry rely on the specificity of the markers used to delineate the tumor cells from the blood cells. However, markers thought to be specific to CTCs have been shown to be expressed by blood cells in some cases. Furthermore, various steps in the sample preparation process can also affect the inclusion of false positives. Generally, any detection system will include a small number of false positives. Given the same sensitivity,
the best system is the one with the ability to eliminate the most false positives to achieve the highest specificity. For RT-PCR, there is not yet a proven method for eliminating illegitimate expression and pseudogenes. With cellular techniques, using multiple markers is the typical strategy to improve specificity. Using multiple markers, only cells positive for epithelial-specific marker(s) and negative for leukocyte-specific marker(s) are classified as CTCs. With image cytometry systems, a trained professional verifies the result and makes the ultimate classification decision based on visual inspection of the morphology features of the cells.

A related problem is the specificity of markers used to detect CTCs in current techniques. Ideally, one would want to use a marker that is specific to the tumor in the patient. Molecular analysis of cells from the primary tumor of individual patients is the only way to obtain tumor-specific markers. For the majority of cases, this is not possible. With the exception of a few cases, the markers used to detect CTCs are only epithelial-specific, or at most organ-specific, but not tumor-specific. This implies cells that are positive for these markers, although likely to be, are not necessarily originated from tumors. Various studies have reported positive results for these markers in healthy individuals. There are hypotheses of different scenarios such as injury or surgery during which healthy epithelial cells can enter the circulation. For example, many research groups discard the first few milliliters of the sample during a blood draw to prevent contamination by skin cells. Furthermore, even if molecular analysis of the tumor of individual patients and tumor-specific markers were found, heterogeneity in expression does not guarantee that these markers would also be expressed in the CTCs [15, 53, 60].

Expression heterogeneity also affects the sensitivity of current techniques. Since all current techniques rely on the presence of a certain marker, the sensitivity of the technique is directly proportional to the level of expression of that particular marker. As a result, most researchers are forced to compromise, having to choose either sensitivity or specificity. Setting the detection threshold too high may miss some of the CTCs. While setting it too low results in a large number of false positives. Alternatively, some groups try to increase the sensitivity by employing multiple markers based on the premise that at least one marker is expressed by the cell [112]. While this can increase the number of positive cells, it might do so at the expense of specificity.
The limitations of current techniques are primarily due to the use of markers to identify CTCs. To overcome these limitations, we propose a method for detecting CTCs using a custom high-throughput image cytometer that exploits the morphological differences between tumor cells and normal cells for their identification.

1.1.3 Proposed Method of CTC Detection based on Morphological Features

The current gold standard for identification and classification of tumor cells from normal ones is that performed by a pathologist. That is why most of the existing rare-event detection systems present a small subset of suspected CTCs to a trained person for the final classification decision. Pathologists identify tumor cells based primarily on morphological cues. Tumor cells have different appearance from normal ones. Compared to white blood cells, CTCs are generally larger, have larger nuclei with small cytoplasm (higher nuclear/cytoplasmic ratio), and have different nuclear staining patterns. The pathologists use these morphological differences to distinguish the tumor cells. Such analysis requires viewing the cells at high-resolution to obtain the necessary morphological information. While it is feasible to image at high resolution a very small subset of cells (tens to hundreds of cells) as a final verification step, it is impractical do so for the millions of cells in a typical sample with the existing image cytometry systems. Most of the existing systems do not have the spatial resolution required for obtaining accurate morphological measurements. On the other hand, those systems with the requisite resolution have very slow scan rates making analysis of the high number of cells in a typical sample impractical. This is one of the reasons why existing systems utilize immunochemistry markers to identify CTCs even though heterogeneous expression of these targets is well known [15, 53, 60]. It is much faster and easier to scan the sample at low resolution and process the marker’s ON/OFF signal.

To overcome the inconsistency of immunochemistry markers, we propose a method of CTCs detection based on the cells’ morphological features. To make this possible, we have developed an image cytometer that has both high-resolution and high-throughput. High-resolution is necessary to obtain precise morphological measurements to achieve accurate classification. On the other hand, high-
throughput is necessary to process the sample in a reasonable amount of time to make the method practical. High-resolution and high-throughput are often contradictory requirements. Existing cytometry systems usually compromise or sacrifice one to achieve the other. Our design employs methods that are fundamentally different from most of the existing cytometers. The next section gives an overview of existing image cytometry systems and introduces the key design principles of our cytometer that allows it to achieve both high-resolution and high-throughput.

1.1.4 Overview of Image Cytometers

Cytometers are instruments for counting, examining, and measuring cell properties for quantitative analysis. There are two main types of cytometers: flow cytometers and image cytometers. In flow cytometers, a single row of cells, suspended in a fluid stream, and flowing at high speed is interrogated by a light beam to produce the measurements. In the case of image cytometers, the cells are attached to a substrate such as microscope slides or microtiter plates and imaged with a camera or photomultiplier tube to produce two-dimensional images. Measurements of the cells are then extracted from these images. Flow cytometers are capable of analyzing thousands of cells per second while image cytometers are much slower with speeds ranging from tens to hundreds of cells per second. Both types of cytometers have been used for rare-event applications including CTC detection. However, for detecting CTCs, image cytometers offer the advantages of providing much more information about morphology, and more importantly, the capability of visual reexamination of any cell of interest in the sample either with image(s) of that cell or by relocating and viewing it directly in the sample. This is extremely useful for visual confirmation of CTCs and rejection of false positives. On the other hand, the current speeds of image cytometers are still too slow to make routine CTC detection practical. Much of the research in this dissertation is to develop an image cytometer with high throughput for practical detection of CTCs.

Automation determines the speed of image cytometers. Most image cytometers are microscopes with automated subsystems which include illumination source(s) (intensity and spectral), lateral motion of the specimen, axial motion of the objec-
tive lens or the specimen for focusing, image acquisition and image analysis. Some large-scale systems also have automated specimen loading capability. Among these, latency associated with the mechanical motion of moving the specimen and the efficiency of light collection are the two fundamental factors limiting the speed of the cytometer. Because of the microscope’s limited field of view, the area of a typical microscope slide is made up of thousands of fields of view. With a traditional 2D camera, thousands of stage movements are required to image the entire slide. In addition, for applications with dim fluorescence, extra integration time necessary to accumulate sufficient signal also decreases the system’s speed. To overcome these limitations, image cytometry systems employ different designs to improve the performance. Based on the design, existing cytometers can be divided into two classes: incremental- and continuous-scanning. The following sections describe the design principles of each class of machine.

**Incremental Scanning Image Cytometers**

In an incremental-scanning image cytometer, the microscope slide is stopped at each field of view while the image is being acquired. Figure 1.3 illustrates the incremental-scanning procedure. The cell measurements are obtained from the fluorescence image while the phase images are used for determining focus. Focus is determined by examining a series of images captured at different axial positions. A measure of focus is calculated for each image in the series to create a list of focus measures vs. axial positions. This list is used to estimate the best focus position, to which the objective lens is positioned. The fluorescence image is then captured. The system moves laterally to the next field-of-view and repeats the process until the entire microscope slide is imaged. There are many research and commercial cytometer systems based on this design. Some of the well known commercial systems include GE Amersham’s IN Cell Analyzer 3000, Beckman Coulter’s IC100, and ChromaVision’s ACIS.

All incremental-scanning cytometers share the same drawback. The thousands of lateral movements of the specimen and the sequential axial motion of the objective lens are the fundamental rate limiting steps of incremental scanning systems. Only a small fraction of the total time is utilized to capture the fluorescence im-
Figure 1.3: **Incremental Scanning Sequence.** At each field of view, test images are acquired at different axial position to determine the best focus position. The objective is moved to this new location for acquisition of the fluorescence image. This process is repeated for each field of view until the entire area is scanned. Adapted from Bravo et al. [18].
age, while the remaining majority of the time is spent moving the specimen and finding focus. The maximum scanning rate reported is 3.45 fields of view per second [17] with most systems being much slower. To increase scanning speed, some of these systems employ a two-pass strategy in which the slide is first scanned at low resolution to identify regions of interest. These areas are then re-scanned at high resolution. For some applications, this approach is acceptable. However, for detecting CTCs with morphological measurements or in applications with dim fluorescence, this strategy does not work since a high resolution image is required for each and every cell.

**Continuous Scanning Image Cytometers**

Continuous-scanning cytometers employ a different method to increase scanning speed. Instead of stopping at every field of view, the specimen moves across the objective lens in a continuous motion from one edge of the scan area to the other. In addition, the conventional 2D camera is replaced with a linear array of detectors arranged perpendicular to the direction of the specimen motion. The combination of continuous stage motion and linear array sensor produces 2D images in the same way a fax machine scans a document. With this design, the thousands of small stage movements of the incremental system are replaced with smooth, long stage motions that dramatically reduce the scanning time. Furthermore, with image acquisition occurring during specimen motion, virtually 100% of the time is efficiently utilized to capture the signal from the specimen. To further improve scanning speed, some continuous-scanning cytometers also employ time-delay-integration (TDI) sensors (linear array sensors with multiple integration stages) to increase the sensitivity of the system.

Early continuous-scanning cytometers have no autofocus. With the specimen constantly in motion, maintaining focus is extremely challenging. There are two methods of autofocus that have been reported for continuous-scanning cytometers: dynamic autofocus during specimen motion, and following a pre-recorded focus profile. With the latter method, before the actual scan, focus is determined at a series of points along the scan path to which a profile is fitted. The objective lens is directed to follow this profile during the actual scan. Focus accuracy is de-
terminated by the number of data points used for the fit. More data points result in better focus performance but they increase scanning time. With dynamic focusing, autofocus is performed on-the-fly while the specimen is in motion resulting in the most efficient use of time. Because of the level of difficulty involved, only two systems are known to employ this method of autofocus. Table 1.1 compares existing continuous-scanning cytometers that published data on autofocus method. At the time of this writing, there are also two commercial continuous-scanning cytometers: Hamamatsu’s NanoZoomer Digital Pathology (Bridgewater, NJ) and Aperio’s ScanScope (Vista, CA); however, they were not included in the comparison since information about their autofocus method and focus performance were not available. Of the four systems in the comparison, only three reported experimental data on focus performance. Two of these systems had focus errors that were larger than the depth of field of the system [80, 117]. While these results were acceptable for their intended applications, this level of focus performance is not adequate to obtain accurate morphological measurements necessary for CTC detection. The remaining third system was developed in our laboratory and demonstrated promising on-the-fly focus performance suitable for CTC detection [19]; however, it is an early prototype and it does not have the capability to routinely scan the large number of cells required for detecting CTCs. On the other hand, because of its potential performance, this on-the-fly autofocus concept was adopted as part of the design of the instrument developed in this thesis for CTCs detection. The next section describes this on-the-fly autofocus concept in detail.
Table 1.1: Previously Reported Continuous-Scanning Cytometers. A comparison of previously reported continuous-scanning systems. Adapted from Bravo [19]

<table>
<thead>
<tr>
<th>Image Sensor:</th>
<th>Linear CCD 1024 pixels × 1 row</th>
<th>Linear CCD 1024 pixels × 1 row</th>
<th>2-D array in TDI mode</th>
<th>2-D array in TDI mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pixel size:</td>
<td>13 µm</td>
<td>13 µm</td>
<td>6.8 µm</td>
<td>6.7 µm</td>
</tr>
<tr>
<td>Test line rate:</td>
<td>4 KHz</td>
<td>1.5 KHz</td>
<td>345 Hz</td>
<td>3370 - 6750 Hz</td>
</tr>
<tr>
<td>Autofocus method:</td>
<td>Servo loop with two dedicated linear CCDs</td>
<td>During the scan, at spaced points on slide</td>
<td>Predefined focus before scan (3 AF position in a 3.2 mm strip)</td>
<td>Parallel analog hardware with 9 CCD line arrays</td>
</tr>
<tr>
<td>Focus measure:</td>
<td>I.O.D.</td>
<td>NR</td>
<td>Energy of mid-frequencies</td>
<td>Energy of high frequencies</td>
</tr>
<tr>
<td>Ranges:</td>
<td>3 µm</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Error:</td>
<td>0.2 - 1.0 µm</td>
<td>NR</td>
<td>Maximum: 1.1 µm, average: 0.4 µm</td>
<td>Best: 0.014 µm; average: 0.07 µm^a</td>
</tr>
<tr>
<td>Optics</td>
<td>13×, NA 0.5</td>
<td>13×</td>
<td>25×, NA 0.5</td>
<td>20× NA 0.75, 40× NA 0.75</td>
</tr>
<tr>
<td>Specimen sampling</td>
<td>1 µm</td>
<td>1 µm</td>
<td>0.272 µm</td>
<td>0.167 – 0.337 µm</td>
</tr>
<tr>
<td>Microscopy mode:</td>
<td>Brightfield</td>
<td>Brightfield</td>
<td>Fluorescence</td>
<td>Brightfield, Phase-contrast, Darkfield, Fluorescence</td>
</tr>
<tr>
<td>Stage speed</td>
<td>4 mm/s</td>
<td>1.5 mm/s</td>
<td>93.8 µm/s or 0.0335 mm²/s (not including focus time)</td>
<td>2.34 mm/s</td>
</tr>
<tr>
<td>Focus step</td>
<td>NR</td>
<td>NR</td>
<td>25 nm</td>
<td>10 nm (piezoelectric drive accuracy)</td>
</tr>
<tr>
<td>Application</td>
<td>Prescreening of cervical smears DNA content (absorbance)</td>
<td>Metaphase spreads detection</td>
<td>Fluorescence microscopy FISH (Spot counting)</td>
<td>Fluorescence microscopy DNA content</td>
</tr>
<tr>
<td>Results</td>
<td>C.V. of I.O.D.: 9 - 6.5%</td>
<td>N/R</td>
<td>10% MTF degradation (at 1 cycle/µm)</td>
<td>C.V. of G1: 8%^b</td>
</tr>
</tbody>
</table>

AF – Autofocus; NR – not reported in listed reference; I.O.D. – Integrated optical density; C.V. – Coefficient of variance
a. Test conditions: Closed-loop autofocus with no stage motion. Specimen: Ronchi rulings (2000 lines/inch), Focus update rate – 27 Hz. Microscope in brightfield mode, tests were carried with 20× and 40× optics, and focus feedback gain of 0.2 and 0.4.
b. Specimen: NIH 3T3 cells with DAPI stain. There were 769 cells detected, mostly in G0 phase
On-the-fly Autofocus for Continuous-Scanning Cytometers

The principle of on-the-fly autofocus that enables high-resolution continuous scanning was demonstrated by our laboratory [19]. This approach is based on the concept of volume imaging as illustrated in Figure 1.4. Axially displaced focal planes in the specimen are focused on to corresponding different imaging planes in the image space. This allows for parallel testing of multiple focus planes eliminating the lengthy sequential focusing testing suffered by incremental-scanning image cytometers. Parallel testing was accomplished in our prototype with an array of fiber imaging bundles (see Figure 1.5). By arranging the ends of the bundles in a staircase pattern, different imaging planes are transmitted to the sensors at the other ends of the bundles. However, the system was only able to achieve a peak pixel rate of 4 MHz. Furthermore, the system has other severe limitations. The custom made fiberoptics are fragile, require complicated optomechanical supports and make adjustment of the system time consuming and difficult. In fact, the fibers were so fragile that one of them broke even before development was completed. In addition, even though the system optically acquires focus information in parallel, the electronics outputs the focus measures in a sequential and random order. This results in a complicated data acquisition scheme that involves excessively oversampling the focus measure outputs followed by a search through the acquired data to extract only a small number of useful data points. Compounded with the spatial/temporal delays caused by the side-by-side arrangement of the fiber bundles, focus data processing and calculation require complicated software algorithms that are highly CPU dependent. Therefore, the focus response of the system is not deterministic but depends on the loading state of the CPU. This resulted in misses in focus updates at rates up to 4.5%. The system is also unstable; it requires frequent and time-consuming calibrations. Although the system demonstrated for the first time that the parallel autofocus scheme works, the system itself is slow, not optimized, and too unreliable for routine use with large samples such as screening for CTCs. A major part of the research in this dissertation is to incorporate this on-the-fly autofocus concept to develop a robust and reliable image cytometer with the necessary speed and resolution for routine and practical use in detection of CTCs.
Figure 1.4: **Volume Imaging Concept.** Axially displaced focal planes in the specimen are focused on to corresponding different imaging planes in the image space.
Figure 1.5: Imaging Fiber Bundles for Simultaneous Acquisition of Focal Test Planes. Imaging fiber bundles were used for simultaneous acquisition of multiple focal planes as part of the initial on-the-fly autofocus demonstration system. By arranging the ends of the fiber bundles in a staircase pattern, different focal planes in the specimen are transmitted to sensors at the other ends of the bundles. The system demonstrated promising autofocus performance; however, it required enhancements and further development before it can be used routinely scan large samples. The design required frequent calibration and the fragile fibers made this process time-consuming and difficult. Furthermore, spatial delays associated with the side-by-side arrangement of the bundles led slows down the response of the autofocus system and resulted in misses in focus updates of up to 4.5% of the time. Adapted from Bravo [19].
Specifications of a Continuous-Scanning Image Cytometer for CTC Detection

At this point, it is useful to discuss the performance requirements of the instrument necessary for practical and routine CTC detection. The typical volume of blood sample used in CTC detection is about 10 ml. With the estimated ratio of one CTC for each million nucleated white blood cells, this volume is small enough to make the test manageable and also reasonably large to ensure detection of CTCs if they are present. After isolation to eliminate the red blood cells, approximately 10 million cells remain in the sample. To be practical, the instrument must be capable of analyzing this number of cells in a relatively short time period. The exact required efficiency remains to be determined, but it is reasonable to estimate that the instrument must be able to process samples from multiple patients each day. We estimate that the instrument must process a sample every two hours. White blood cells are about 10 µm in diameter. Assuming spacing of about 20 µm between cells in a monolayer, the cell density on the slide would be 2500 cells/mm² with a total area of 4000 mm² per sample (approximately 4 microscope slides). In order to achieve resolution sufficient for morphological measurements, the instrument must have high numerical aperture (NA ≥ 0.5). Taking into account Nyquist sampling, Table 1.2 lists the camera pixel rates necessary to complete the sample in the two-hour time period. The required pixel rates of 6.17–17.15 MHz are within the capability of existing scientific-grade cameras. The main challenge in the development of the instrument is to accomplish autofocus at this scanning speed in order to maintain image quality.

Table 1.2: Estimated Performance Requirements. Assuming 20 µm spacing between cells, the instrument must achieve the pixel rates listed below in order to image 10 million cells in a 2-hr period.

<table>
<thead>
<tr>
<th>NA (µm)</th>
<th>Rayleigh Resolution</th>
<th>Nyquist Sampling</th>
<th>Required Pixel Rate (MHz) (for 2-hr scan)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>0.61</td>
<td>0.30 × 0.30</td>
<td>6.17</td>
</tr>
<tr>
<td>0.60</td>
<td>0.50</td>
<td>0.25 × 0.25</td>
<td>8.89</td>
</tr>
<tr>
<td>0.75</td>
<td>0.41</td>
<td>0.20 × 0.20</td>
<td>13.89</td>
</tr>
<tr>
<td>0.85</td>
<td>0.36</td>
<td>0.18 × 0.18</td>
<td>17.15</td>
</tr>
</tbody>
</table>

a Calculated for λ = 500 nm
1.2 Automated 3D Segmentation for Quantitative Analysis of Cartilage Tissue Explants

Better understanding of cartilage growth and repair is necessary for developing more effective therapy for articular cartilage repair. To ensure normal functioning of these complex cellular processes during experimental studies, the natural state of chondrocytes within the surrounding matrix must be preserved requiring special measurement techniques. The confocal microscope permits such experimental conditions with its ability to non-destructively image deep inside the tissue allowing repeated measurement of the same tissue region over periods of time. However, with the absence of any quantitative techniques for processing the data, the confocal microscope has been limited to a few qualitative roles. The goal of this research is to develop an automated segmentation technique for processing the images that will enable routine quantitative three-dimensional studies of thick cartilage tissue.

Damage to articular cartilage is common in young active people. It is well known that articular cartilage has limited ability to heal once damaged. Current total joint replacement techniques have limited lifespan and do not restore full activity making them unsuitable for young or active patients. Major efforts have been looking at using tissue and cellular engineering techniques in the development of repair systems for cartilage. However, current tissue engineering therapies have yet to show consistent results (formation of normal articular cartilage and integration with host tissue). A better understanding of the repair mechanisms would likely lead to more efficient techniques. While in vivo experimental studies are desirable, they are difficult to conduct and their results are extremely difficult to assess since it is difficult to measure or to control the experimental conditions. As a result, most studies are performed on explant cultures of cartilage tissue fragments. These systems provide a well-controlled environment for studying repair mechanisms while mostly preserving the normal structure of the cells within their extracellular matrix. Studying chondrocytes in this three dimensional (3D) environment requires special imaging capability. The confocal microscope is potentially a great instrument for this task because of its ability to non-destructively image cells relatively deep inside the tissue which allows for repeated measure-
ment of the same tissue region over periods of time. Despite these potentials, the confocal microscope is currently limited to a few qualitative visualization roles for two reasons: 1) its slow acquisition speed and 2) labor-intensive manual delineation of object boundary or segmentation for quantitative analysis. To enable routine quantitative analysis of tissue in 3D, these two limitations must be overcome. Several investigators are addressing the limitation of slow acquisition with the developments of new high-speed confocal microscopes [9, 34, 48, 68]. Part of the research in this dissertation focuses on the latter challenge. The objective of this research is to develop and to establish the accuracy of an automated 3D segmentation technique for confocal fluorescence images of chondrocyte nuclei. This work will lay the foundation for future quantitative analysis methods of cells in 3D.

Accurate automatic segmentation is important and difficult. Initial data reduction by retaining only the regions of interest (ROI) obtained by segmentation is crucial when dealing with the massive raw 3D data. Furthermore, as all subsequent quantitative analyses depend on the accuracy of segmentation, this initial step is fundamentally important. Unfortunately, segmentation of fluorescence confocal data is difficult. All confocal systems rely on a small aperture to block out-of-focus light and allow only the in-focus light to reach the detector. While this provides excellent depth sectioning, it also allows only a very amount of the signal to reach the detector. As a result, confocal data often have very low signal-to-noise ratios (SNR). In addition, the variability of objects’ fluorescence intensities in biological specimens is so large that it typically covers almost the entire dynamic range of the imaging system. Therefore, most segmentation techniques of fluorescence data are iterative and/or require operator’s assistance [68].

The contrast enhancement technique presented here is designed to allow fast and accurate segmentation of 3D confocal data. The technique was previously developed to process 2D fluorescence images [91]. The contrast enhancement is achieved through convolution of the raw image data with a FIR filter. The purpose of this FIR filter is to boost the intensity of the foreground object pixels while suppressing the values of the background pixels. In effect, the filter stretches the contrast so that a single-point threshold operation is possible.

The goals of this research are: a) to develop an automated 3D segmentation technique for quantitative analysis of confocal fluorescence images, and b) to mea-
sure the accuracy of the technique at segmenting chondrocytes in cartilage tissue explants.

1.3 Dissertation Overview

The specific aims of this dissertation research are:

- To develop an instrument with high-throughput and high-resolution to enable practical use in detecting CTCs in blood based on morphology
- To measure the instrument’s performance at finding CTCs in blood
- To develop an automated 3D segmentation technique for quantitative analysis of confocal fluorescence images, and measure the accuracy of the technique at segmenting chondrocytes in cartilage tissue explants.

Chapter II focuses on the development of the image cytometer. An overview of autofocus and TDI imaging is presented. An image-splitting optical assembly allows simultaneous sampling of multiple focal planes that enables on-the-fly autofocus. In addition, utilization of a TDI camera increases the system’s sensitivity. The result is high scanning speed at high resolution with maintained image quality. Detailed descriptions of the optical, mechanical, and electrical subsystems are given with discussions of design considerations, advantages and limitations. A discussion on the importance of the control software and a description of its design and implementation conclude the chapter.

Chapter III focuses on the evaluation of the instrument. First, each subsystem was evaluated separately to establish its characteristics. Second, a Ronchi ruling was used to conduct a series of experiments to establish the instrument’s performance under optimal condition. Finally, to determine the system’s performance at finding CTCs, a comparison with an incremental-scanning cytometer was carried out on a spiked cell model. The same samples were examined by both instruments to compare sensitivity and specificity.

Chapter IV focuses on the development and testing of the contrast enhancement technique for automated segmentation of 3D confocal data sets. Contrast enhancement is achieved with a non-linear optimal filter that boosts the intensity
of foreground voxels and suppresses the intensity of background voxels. The theory behind the filter design is presented along with a description of its implementation on a distributed network of computers. Evaluation of the filter’s performance was carried out on different types of data and compared with common contrast enhancement filters. Qualitative visual comparisons and quantitative comparison with manual segmentation of cartilage tissue explants are presented. A discussion of the results completes the chapter.

Chapter V discusses the performance of the cytometer, the meaning of the results and a comparison of the system to existing instrumentation for detecting CTCs.
Chapter 2

High-Throughput Continuous-Motion Scanning Image Cytometer

2.1 System Overview

The system is designed to achieve both high-throughput and high-resolution. Increasing speed is accomplished in parallel with image acquisition and in utilizing the higher sensitivity of time-delay-integration (TDI) cameras to shorten scanning time. Figure 2.1 illustrates the optical light paths of the system. Wavelengths longer than 600 nm are reflected through the side-port to an image-splitting module that simultaneously measures focus at several focal planes. This parallel focus testing enables on-the-fly autofocus that allows collection of fluorescence signals to occur at the same time. As a result, 100% of the time is spent collecting the fluorescence signal (in incremental-scanning systems, the fluorescence signal is collected only during a fraction of the total time). The fluorescence signal (emission wavelengths, \( \lambda < 600 \text{ nm} \)) is transmitted to the top-port at which it is captured by a high sensitivity TDI camera. With the TDI camera, the specimen is imaged with a long continuous stage motion that eliminates the latency associated with thousands of stop-and-go movements of an incremental-scanning system (see Figure 2.2).

The next section gives an overview of autofocus in image cytometry and the
Figure 2.1: Continuous-Motion-Scanning Cytometer Diagram. The diagram shows the light paths in the Continuous-Scanning Image Cytometer. Wavelengths longer than 600 nm are reflected to the image-splitting module for parallel testing of focus data. Fluorescence signal (emission wavelengths, $\lambda < 600$ nm) is transmitted to the TDI camera for image acquisition. This design enables on-the-fly autofocus that allows 100% time-efficient collection of fluorescence signal. Coupled with the increased sensitivity of the TDI camera and the elimination of stop-and-go stage movements, the scanning time is reduced significantly. Adapted from Bravo [19] and Thieleking [115].
advantages of TDI imaging over a conventional 2D camera. The remaining sections describe the design and the implementation of the optical, the electrical, the mechanical subsystems and the control software.

Figure 2.2: Slide TDI Raster Scanning. With a TDI camera, image formation occurs with continuous stage motion. The latency associated with the thousands of stop-and-go movements is eliminated that results in much shorter scanning time. Furthermore, the thousands of non-contiguous images that conventionally produced by incremental-scanning cytometers are replaced by tens of contiguous image strips.

2.2 Autofocus and TDI Imaging

2.2.1 Autofocus in Image Cytometry

The Need for Autofocus

Autofocus is essential in automated cytometry. Focusing by manual means is too inconsistent and subjective to ensure repeatability. This is likely to affect the accuracy of subsequent image processing and measurements extracted from these images. For applications requiring scanning large areas at high resolution where depth of field is limited, it has been shown that refocusing in each field of view is necessary to maintain image quality [18, 40]. Furthermore, autofocus is necessary to compensate for the effects of mechanical instability (drift and thermal expansion), irregularity in glass slides and coverslips, and variability in specimen thickness. Mechanical instability is well documented: 0.058 µm/h [65] and 0.2 µm
in 10 minutes in specially controlled conditions [11], and maximum drift of 0.1 \( \mu m \) per 30 seconds [74]. Also, variations in focus position between fields of view on a microscope slide was demonstrated to be up to 6 \( \mu m \) [18]. Autofocus is critical in time-lapse microscopy over long periods of time (hours to days) and especially in cases where time-lapse is performed over large areas consisting of multiple fields of view.

Autofocus performance in microscopy is much more critical compared to other imaging systems. Unlike macroscopic systems in which slight changes in object distance are often not noticed in the resulting image, sub-micron changes in the distance between the specimen and the objective lens can cause drastic degradation in image quality. Generally, the need for autofocus is a function of the numerical aperture (NA) of the objective and the thickness of the specimen. With low NA objectives (NA<0.5), the depth of field is often larger than the thickness of the specimen. In these cases, it is possible to find focus only at a few locations on the slide, identify the average plane of focus, and scan along this plane without additional focusing [80]. For intermediate resolution (0.5<NA<1.0), the depth of field decreases to around 1 \( \mu m \) which is about the thickness of a cell on a microscope slide. Without focusing at each field of view, the specimen would almost always be out of focus. For high resolution objectives (NA>1.0), where the depth of field is only a fraction of the thickness of the cell, an in-focus image often means collecting a series of optical sections and using projection or other 3D processing techniques to reconstruct the image.

**Autofocus Implementations**

Most autofocus methods can be classified in one of two categories: position sensing and image content analysis. Position sensing methods typically involve independent calibration of the best focus position. They also require a single well-defined surface from which to reflect light or sound. In biological microscopy, there are typically two reflecting surfaces, the coverslip and the slide. Furthermore, biological specimens have variable thickness and best focus is not necessarily obtained at the surface of the glass or a certain offset from it. For these reasons, position sensing methods are generally not used to perform autofocus in biological
microscopy, but rather to maintain a constant distance between the specimen and the objective lens once focus has already been achieved in experiments lasting over long periods of time [65]. Image content analysis methods, on the other hand, measure the focus directly from the characteristics of the image. The best focus is determined by comparing these characteristics in a series of images obtained at different axial positions. Since they rely solely on the information in the image, focus is determined directly and these methods do not require an independent reference. However, their main drawback is speed, which is limited by the frame rate, the axial positioning time, the evaluation of the image characteristics, and the search range. A major part of this dissertation research is to optimize the speed of autofocus by collecting and analyzing the test images in parallel.

Image content autofocus systems are based on the idea that well-focused images contain more information and detail than unfocused ones. A criterion function is used to evaluate images at different specimen distances. These criterion functions return a value that indicates the relative sharpness of focus. The specimen distance corresponding to the largest value is taken to be the distance at which the image is best focused.

Many criterion functions have been reported in the literature [11, 33, 40, 50, 90, 125]. Generally, they can be classified as follows:

**Frequency Domain Functions:** These functions are based on the Fourier transform. High frequency components of the Fourier spectrum are associated with the edge information. Well-focused images have sharp edges and therefore contain more high frequency content.

**Information Content Functions** These functions work on the principle that well-focused images have higher entropy than unfocused ones. A measure of the entropy of the image is used to evaluate the focus of the image.

**Gradient Functions** Gradient functions are used to extract edges. Since well-focused images have sharper edges, they are expected to have higher gradient values.

**Gray Level Variance** These functions are based on the observation that well-focused images have higher intensity variation than poorly-focused ones. As
Figure 2.3: **Ideal Focus Criterion Function.** An ideal focus function should satisfy the following criteria: **unimodality:** the function has only one peak (no local maxima); **accuracy:** difference (in position) between the in-focus image and the peak of the function should be zero ($\epsilon = 0$); **reproducibility:** the width of the function near the peak is narrow ($\nu$ is small); **range:** the width of the function away from the peak is wide ($\eta$ is large); **efficiency:** evaluation of the function is accomplished quickly. Adapted from Groen et al. [40].
a result, gray value variance of the image can be used as a criterion function to evaluate focus.

A good criterion function should satisfy the following criteria [40]:

- **unimodality**: The criterion function should only have one peak corresponding to the optimal focus position, and monotonically decreases as the object is moved away from this optimal point.

- **accuracy**: The peak of the function should coincide with the position of best focus.

- **reproducibility**: The peak should be sharp ensuring good reproducibility.

- **range**: The function should be broad allowing evaluation of focus over a large range.

- **speed**: The function should be efficient so that evaluation of focus can be achieved quickly.

Figure 2.3 shows an ideal focus criterion function [40].

Performance of these criterion functions in biological microscopy applications has been investigated in several comparative studies in the last two decades. Criterion functions based on the gray level variance in the images were found to be the best criterion for coarse focusing as they are insensitive to noise over a broad range. However, in fine focusing, they may lead to incorrect focus position especially when the image contains inhomogeneous features [32, 50, 125]. Criterion functions based on the information content of the image (entropy) were reported not to perform well [33, 50]. On the other hand, some of the gradient and frequency domain functions were reported to be the best [33, 40, 90, 125]. Although frequency domain functions perform well, they are rarely implemented in autofocus systems because computationally they are too expensive for many applications. In this instrument, we use dedicated hardware with analog filters (focus measurement circuits, Section 2.4.2) to perform the focus analysis in real time.

The criterion function used in this instrument is based on the high frequency content of the image. Image resolution or sharpness occurs at best focus. Details
blur as the image is defocused and the resolution is lost. Focus can be measured by analyzing the Fourier frequency spectrum with highpass or bandpass filters to isolate the high frequencies. Typical filters have the form of the first derivative or the Laplacian (a measure of the second derivative) of the image intensity. For the focus measurement circuits in this instrument, active analog filters of higher order are implemented for better high-frequency discrimination. The focus measurement circuit implements the criterion function as the integral of the square of the high frequency components normalized by the square of the image brightness [19]

\[
F_z = \frac{\iint \left( \frac{dI_{xy}}{dx} \right)^2 \, dx \, dy}{\left( \iint I_{xy} \, dx \, dy \right)^2},
\]

(2.1)

where \( I_{xy} \) is the intensity of pixel location \((x,y)\). A high-pass filter represented by the derivative selects the range of high frequency. The result is then squared and integrated. Normalization by the square of the integrated intensity corrects for fluctuation in illumination. The focus measure is calculated as a function of axial \((z)\) position.

### 2.2.2 Time Delay Integration (TDI) Imaging

Conventional cameras which have a two-dimensional array of pixels are good for capturing still or slow-moving objects. To image fast-moving objects, these cameras require high-speed shuttering or strobed illumination. As the speed of the object increases, these techniques are increasingly difficult and expensive to implement to obtain high quality images.

Linescan cameras, on the other hand, are designed to image fast-moving objects. With a single row of pixels, linescan cameras use the motion of the object to create the vertical dimension of the image as illustrated in Figure 2.4. Unlike conventional area array cameras whose image sizes are limited by the dimensions of the array (e.g., 640×480, 1K×1K, etc.), linescan cameras build continuous images not limited in the vertical dimension. The length keeps growing as long as the object is moving. Well known examples of linescan imaging are the fax machine and the flatbed scanner.

Image information is transferred out of the sensor one pixel at a time through the readout register. Each type of sensor typically has a readout register located
Figure 2.4: **Linescan Imaging.** Linescan imaging uses a single line of sensor pixels to build up a two-dimensional image. Adapted from Dalsa application note [5].

Figure 2.5: **Comparison of Area Array vs. TDI sensors.** Area array, line scan, and TDI sensors all typically have one readout register located below the photoactive region. Because image information is read out one pixel at a time, the high pixel number of the area array requires a finite amount of time to transfer out. During this time, the sensor is unable to capture another image and therefore cannot reliably image fast-moving objects. On the other hand, linescan sensors have only one row of photoactive elements. With each clock signal, the charge is transferred to the readout register and the sensor is immediately ready to capture the next line of image as the previously line is being read out — allowing them to reliably image fast-moving objects. With TDI sensors, the transfer of charge between integration stages is synchronized to match the motion of the object. This allows the sensor to integrate the signal over a longer period of time resulting in higher sensitivity.
below the photoactive region as shown in Figure 2.5. Area arrays, with many pixels, take a finite amount of time to transfer the image out of the sensor to the acquisition system. During this readout period, the camera is essentially blind and therefore cannot reliably capture fast moving objects. On the other hand, linescan sensors, with a single row of pixels, require much less time to transfer the image information to the readout register. This process is initiated by a lineclock trigger. The trigger signals the end of exposure and the current image line is transferred out of the photoactive pixels into the readout register which is not responsive to light. The image information is then transferred out of the readout register to the acquisition system one pixel at a time. While readout is occurring, exposure of the next line happens in parallel. As a result, linescan cameras have the advantage of exposing a new image while the old image is still being read out — making them able to reliably image fast-moving objects.

As mentioned above, linescan cameras require a lineclock trigger. The motion of the object needs to be properly synchronized with the start of new image lines. Typically, an encoder is attached to the moving web or conveyor belt on which the objects are sitting. The encoder monitors how fast the objects are moving and provides the appropriate lineclock signal to the camera. Linescan cameras also require some method of exposure regulation. Exposure is dependent on the time between lineclock pulses. This means that exposure is dependent on the speed of the object. Some linescan cameras have built-in exposure control; however, most do not. As a result, most linescan camera systems control the illumination intensity as a function of the objects’ speed.

A Time-Delay-Integration (TDI) camera is an extension of a standard linescan camera; however, it provides much higher sensitivity. In a linescan camera, each line of the image is exposed once and sent to the acquisition system. A TDI camera has more than one row of photoactive pixels (see Figure 2.5) and each image line is integrated multiple times.

In a linescan camera, when the camera receives the line clock trigger, the line of image is transferred to the readout buffer and is read out pixel by pixel. At the same time, the next line of image is being exposed. In a TDI camera, when the camera receives the line clock trigger, the lines of image are shifted down by one row. The data in the lowest row is transferred to the readout buffer for pixel-by-
pixel readout while integration of the rows above occurs. This technique of multiple cycles of shifting and integration is how the TDI achieves longer integration time and hence increased sensitivity as illustrated in Figure 2.6.

The amount of increased sensitivity depends on the number of rows over which the line is integrated. Typical TDI sensors have 24, 48, 96 or even up to 256 integration stages. Higher sensitivity permits greater scanning speed at normal lighting conditions or normal scanning speed at low light conditions.

Figure 2.6: How TDI Works. Time Delay and Integration is the concept of accumulating multiple exposures of the same (moving) object, effectively increasing the integration time available to collect incident light. Adapted from Dalsa application note [5].

As with any system, there are trade-offs with the TDI system in order for it to achieve higher sensitivity and the ability to capture fast-moving objects. Compared to the standard linescan camera, the TDI camera requires much more careful synchronization and alignment. The transfer of the line of image from row to row must match the motion of the object or blurring will occur. In addition, it is important that the direction of motion is perpendicular to the sensor. The portion of the object imaged in a pixel in the first row must also be integrated in the same column at the last row or blurring will occur. The direction of the motion of the object also must match the transfer direction of the integration stages. Careful
attention must be paid to flipping and mirroring effects created by lenses and other optical components in the system.

Overall, with careful synchronization and alignment, TDI cameras are excellent at imaging fast-moving objects in low-light conditions with high sensitivity. Because of these advantages, this instrument was designed to utilize a TDI camera for sampling focus and for imaging while the specimen is in motion resulting in fast scanning time.

2.3 Optical Image Splitting Assembly

In this instrument, focal planes sampling for autofocus is performed in parallel, overcoming the speed limitation of sequential testing that hampered many other systems. Parallel testing of multiple focal planes is accomplished with an image splitting assembly. The image information from the microscope is divided into multiple channels which are simultaneously measured. The details are described in the following sections.

2.3.1 Image Splitting Module

Multiplanar imaging utilizes an array of CCD sensors, each of which is focused on a different plane in the specimen. This is achieved with beamsplitters as shown in Figure 2.7. In this design, a series of 50%–50% beamsplitters and mirrors divides the image from the microscope into eight channels. To simplify the arrangement and to eliminate the need for equal optical path lengths, the space containing the beamsplitters and mirrors assembly is collimated with an appropriate lens at the input. The images from each channel are then reformed by additional optics that also correct for differences in magnification. With this design, the even number of channels and careful layout of beamsplitters and mirrors for an even number of reflections create eight non-mirror-imaged outputs that can be easily registered. This is especially important for the TDI cameras because image formation depends on the direction of stage motion. Since different image planes correspond to different specimen planes, placing cameras at different distances from the magnification correction optics allows for acquisition of different planes in the
specimen. For large magnification and small axial displacement, the relationship between the specimen space and image space is approximated as

\[ \delta z = M^2 \Delta z \]  

(2.2)

where \( M \) is the lateral magnification, \( \delta z \) is the axial displacement in the image space, and \( \Delta z \) is the corresponding axial displacement in the specimen space. For example, with a 20× objective lens, a 0.5 µm displacement in the specimen space would have a corresponding 200 µm shift in the image space. This axial magnification effect facilitates the positioning of sensors in the image space to accurately sample the specimen space.

Figure 2.7: **Optical Image Splitting Module.** To allow simultaneous sampling of multiple image planes, an optical assembly of beamsplitters and mirrors delivers the image information to eight focus-measuring channels.

Because of the high number of optical components in the assembly, careful considerations were put into the selection of the components to maximize light transmission efficiency. Different beamsplitters and mirrors from several manufacturers were evaluated. Low polarizing beamsplitters from Opto Sigma (model
00602TT01, Santa Ana, CA) were selected to minimize channel-to-channel differences (Figure 2.8). The beamsplitters use a special combination of metallic and dielectric coatings on the internal splitting face to produce an even split in intensity with minimal polarization and minimal absorption. The reflected and transmitted beams each contain about 45% of the incoming intensity with only 10% absorbed. Also the s- and p- polarization components are within 10%. Similar right angle prisms (model 055-0150, Opto Sigma) were selected for the mirrors. These 25 mm×25 mm×25 mm beamsplitters and prisms were chosen to keep the assembly relatively compact and still accommodate a sizable diameter light beam. Larger components were not chosen since the 25 mm cubes were sufficiently large to transmit the small field of view imaged by the TDI sensor. Furthermore, the smaller cubes minimize the length of glass that the light travels through, which reduces scattering of light. These beamsplitters and mirrors are made of BK7 glass. BK7 glass was chosen for its low cost and because the image splitting module is designed for wavelengths only between 600 nm and 900 nm. The components were cemented together with refractive index matching adhesive (model 65, Norland Products, Cranbury, NJ) to eliminate reflections at air gaps between components. All remaining glass-air surfaces were coated with anti-reflection coating to reduce reflection and improve transmission. For stability and support, the entire assembly was mounted on a 1/2-inch thick plate of glass.

2.3.2 Magnification Correction

The unequal path lengths between channels and changes in focus in a simple lens system alter the magnification which changes the spatial frequency content of the image. With typical magnification errors less than 10%, relatively simple and cost effective optics can be employed that introduce little light loss and eliminate the additional computational burden. The lens system for magnification correction is shown in Figure 2.9. Since the light coming out of each channel of the beamsplitter system is collimated, lens 1 focuses the light to an image at its focal point serving as the object for lens 2. Magnification correction is achieved with lenses 2 and 3, which have a combined magnification that is given by the thin lens doublet
Low Polarizing Beamsplitters. Low-polarizing beamsplitters from Opto Sigma were selected to minimize the differences in intensity between imaging channels.

\[ M = \frac{f_2 f_3}{(s_{2o} - f_2) (d - f_3) - s_{2o} f_2} \]  \hspace{1cm} (2.3)

where \( f_1, f_2 \) and \( f_3 \) are the focal lengths of the respective lenses as labeled in Figure 2.9, \( d \) is the distance between lenses 2 and 3, and \( s_{2o} \) is the difference between \( f_1 \) and \( e \) which is the distance between lenses 1 and 2. The magnification is changed by moving lens 2. The procedure for calibrating the system is as follows.

One channel is chosen to be the reference channel. The camera of this channel is positioned exactly at the focal point of lens 3. A reference image is captured using a known thin test sample (e.g., a micrometer slide) from this channel for comparison with the other channels. For each remaining channel, the 2D test target is displaced axially to the desired plane of the volume space. The position of the camera with respect to lens 3 and the location of lens 2 are adjusted until the image is in focus and the magnification matches the reference image. Compared to typical magnification correction lens systems, this lens system is simple. Because it is required to work in only a small range of wavelengths (between 600 nm and 900 nm), the chromatic aberration is not a factor and a complex multi-element lens system is not necessary.

In addition to correcting magnification differences between channels, the lens
Figure 2.9: Magnification Correction Optics. A simple zoom-lens system at the output of each optical channel is used for matching the magnification between imaging channels. Desired magnification is achieved through adjustment of lens 2. System also provides additional magnification to ensure Nyquist sampling at the TDI sensors. Table 2.1 lists the objective lenses commonly used in our lab with their corresponding Nyquist sampling period and the additional magnification required to achieve these conditions. The lenses were selected with appropriate focal lengths to accommodate these conditions. Plano convex lenses were chosen for lenses 1 and 3 with 80 mm and 60 mm focal lengths, respectively (model 011-2460-A55 and 011-2450-A55, Opto Sigma, Santa Ana, CA). A biconcave lens with −50 mm focal length was selected for lens 2 (model 017-0425-A55, Opto Sigma). These lenses were chosen with 50 mm diameter (the largest possible to still fit in the space between the channels) to minimize aberrations. All lenses are made of BK7 glass and anti-reflection coated to reduce light loss.

2.3.3 Optical Coupling to Microscope Side Port

The light coming from the side-port of the microscope must be collimated before entering the image splitting module. The selection of this collimating lens is important. Although all rays emanating from the focal plane of the lens will be collimated, only rays going through the focal point of the lens will be parallel to the optical axis. Off-axis rays are transformed into a collimated beam diverging
from the optical axis as shown in Figure 2.10. The collimating lens needs to be selected with a certain focal length such that the divergence of the off-axis points is less than 25 mm (the size of the beamsplitters) at the exits of the image splitting module. To keep divergence low, the focal length of the lens should be long. On the other hand, a short focal length of the lens is also desired to keep the assembly as compact as possible. A compromise was made and the selection of the collimating lens was based on the requirements of the most commonly used objective lens, 20× 0.75 NA.

Figure 2.10: **Collimation of Off-Axis Rays.** Off-axis points in the focal plane of a lens emerge as collimated light but cause a diverging light cone rather than a parallel beam [55].

Since the longest light path in the module is 270 mm and the beam is not to be more than 25 mm at the end of the path, based on Figure 2.11 the required focal length of the lens should be calculated.

<table>
<thead>
<tr>
<th>Objective Lens</th>
<th>Nyquist Sampling (µm/pixel)$^a$</th>
<th>Image Width at Side-Port (mm)$^b$</th>
<th>Additional Magnification Required$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20× 0.5 NA</td>
<td>0.449</td>
<td>9.19</td>
<td>1.45</td>
</tr>
<tr>
<td>20× 0.75 NA</td>
<td>0.360</td>
<td>7.38</td>
<td>1.8</td>
</tr>
<tr>
<td>40× 0.95 NA</td>
<td>0.311</td>
<td>12.75</td>
<td>1.05</td>
</tr>
</tbody>
</table>

$^a$ Rayleigh criterion, $d = (1.22 \cdot \lambda) / (\text{NA}_{\text{objective}} + \text{NA}_{\text{condenser}})$ where $\lambda = 750$ nm, $\text{NA}_{\text{condenser}} = 0.52$

$^b$ Image width of a 1024-pixel wide image (width of the TDI sensor)

$^c$ The additional magnification required to achieve Nyquist sampling
length can be calculated as
\[
\tan \alpha = \frac{1/2 h_i}{s_i} = \frac{1/2 h_o}{f}
\]  
and since the image height is 7.38 mm (see table 2.1), the required focal length is
\[
f = \frac{s_i \cdot h_o}{h_i} = \frac{270 \text{mm} \cdot 7.38 \text{mm}}{25 \text{mm}} = 79.7 \text{mm}
\]  
A 80 mm focal length plano convex lens (model 011-2880-A55, Opto Sigma, Santa Ana, CA) was chosen. The lens was antireflection coated to reduce light loss.

Figure 2.11: **Collimating Lens Calculation.** Diagram illustrating the constraints for calculating the focal length of the collimating lens. Adapted from Thieleking [115].

### 2.3.4 Mechanical Integration with Microscope

Integration of the image splitting assembly with the microscope includes: the mechanical coupling of the assembly to the side-port of the microscope, the mechanical support of the optical components, the means for adjustment of the magnification optics, the support and means for alignment of the camera at the end of each imaging channel, the thermal management, and the light shielding.

**Mechanical Support and Coupling to Microscope:** A 1/4-inch thick aluminum plate provides the mechanical support for the entire assembly as illustrated in Figure 2.12. The image splitting assembly is elevated by a platform to the level of the microscope’s side-port. The lenses of the magnification correction optics are supported by lens holders bolted the aluminum plate. The cameras are supported by XYZ-translation stages attached to the aluminum
plate at the end of each imaging channel. Coupling to the microscope is
achieved with a side-port connector which also holds the collimating lens.
Mechanical drawings of the support components are listed in Section A.1.

Adjustment of Magnification Optics: For each imaging channel, adjustment
of lenses 2 and 3 of the magnification optics are necessary to minimize mag-
nification differences between channels. Lens holders for lenses 2 and 3 are
translation stages that can be adjusted with lead screws through a range of
approximately 3 cm. Mechanical drawings of the magnification optics com-
ponents are listed in Section A.1.3.

Camera Alignment: Each camera must be carefully aligned so that they image
the same region in the field of view. The camera is supported by a manual
linear XY-stage (model MT-XY, Newport Corporation, Irvine, CA) which
provides 9.5 mm travel in each direction. Axial positioning is achieved with
a custom linear stage actuated by a 25-mm micrometer (model 150-801,
Thorlabs, Newton, NJ).

Thermal Management and Light Shielding: The entire assembly is housed
inside an enclosure. The rigid structural aluminum frame protects the com-
ponents inside. Black opaque plastic panels shield the system from stray
light. The enclosure also partially insulates the assembly from outside tem-
perature fluctuation to maintain a relatively constant temperature inside.
Figure 2.12: **Focus Measurement Assembly.** Three-dimensional model of the focus measurement assembly. The optical image splitting assembly is in the middle with four imaging channels on each side. The inset shows the assembly next to the microscope with the top of the enclosure partially removed.
2.4 Focus Measurement Electronics

Once the image information is divided into eight channels by the optical system, the information is then converted into an electronic signal from which the focus information is extracted. A camera is positioned at the output of each optical channel. The analog video signals from these cameras go to an array of focus measurement boards. These boards process the high volume of image data in real time in hardware and produce a single focus value for each channel. These focus values are then processed by an appropriate algorithm to determine the best-focus positions. The details of these processes are described in this section.

2.4.1 Time Delay Integration (TDI) Camera

There is no off-the-shelf TDI camera that would fit into the 50-mm spacing between channels defined by the optical system. Custom TDI cameras were developed for this project. A two-board design was selected to meet the physical constraints. Only the CCD sensor, the clock drivers and the necessary signal conditioning components were placed on a board small enough to fit in the space between the channels. The video processor chip and the remaining electronics were placed on a larger board position at a right angle to the sensor-board in the space between channels.

The camera is based on the TDI096AAU TDI CCD sensor (EG&G Reticon, Sunnyvale, CA). The sensor has 1024 13-µm² pixels and 96 integration stages. The camera design is based on the one-chip 10-bit video digitizer/processor XRD9853 (Exar Corporation, Fremont, CA) with timing signals generated by a programmable logic device (PLD) (model EPM7064STC44-10, Altera Corporation, San Jose, CA). A 10-bit DAC (model ADV7127KRU50, Analog Devices, Norwood, MA) provides the analog video output for the focus measurement circuit (section 2.4.2). A digital video output is also available for future expansion. When operated as part of the array, the camera is driven with an external line-clock provided by the Array Timing Controller (see Section 2.4.3). The camera has a pixel rate output of 10 MHz with a maximum line rate of 9.225 kHz. Eight of these cameras were built and seven were used in the array. The detailed schematic of the camera is
Custom-built TDI cameras are an important part of the auto-focus system allowing the specimen to be examined while moving at high speed with high sensitivity. A two-piece form factor was selected to fit the spacing between channels of the Parallel Imaging Module. See A.2 on page 209 for details.

Later in the development process, it was discovered that the DAC creates an offset in the analog video output which causes a decrease in the dynamic range of the system especially in low-light conditions. To restore the appropriate black level of the video signal and to add an additional gain stage, a video conditioning circuit was added in each channel between the camera and the focus measurement board (see Figure 2.14). The circuit is built around a DC-restore video amplifier (model ZXFV4089, Zetex Inc., Hauppauge, NY). With an adjustable DC reference level, the circuit can put the black-level lower than its original value, further amplifying the AC portion of the video signal.

2.4.2 Focus Measurement Circuit

In this instrument, the focus criterion is based on the contrast of the image. Contrast is at the highest when the image is in focus. The image is blurred and the details are lost as the image goes out of focus. The high frequency content
Figure 2.14: Video DC Restoration. A) Oscilloscope screen capture showing the analog video signal of the TDI camera at the camera’s output (top trace) and after the pre-amp stage of the focus measurement circuit (bottom trace). The signal is of a Ronchi ruling (parallel black lines) showing 50 mV peak-to-peak with a 150-mV offset. B) after removing offset (restoring DC level) and 2× amplification; the signal is now 100 mV peak-to-peak. C) an image of the DC restoration circuit. D) the schematic of the circuit.
of the image can be used to evaluate contrast. The instrument employs an array of special purpose mixed analog/digital circuits (one circuit for each TDI camera) for the evaluation of the focus criterion function (Equation 2.1). These focus measurement circuits are a modified version of the hardware previously developed in our lab for incremental scanning cytometry [18] and later commercialized by Q3DM (San Diego, CA). Q3DM was acquired by Beckman Coulter in 2003 and these focus measurement circuits are no longer commercially available.

Each circuit measures focus directly from the video signal of the TDI camera. A block diagram of the circuit is shown in Figure 2.15 together with the waveform reference points (a)–(f) that were used to obtain the waveforms in Figure 2.16. These waveforms demonstrate the process performed by the different sections of the circuit in order to produce the focus measure, or focus index. The input video signal is fed into the focus index section (top row of Figure 2.15) and the digital timing control section (center of Figure 2.15). The focus measure is calculated by the filter, transient removal, squaring, and integrator sections of the circuit. The result is sampled and amplified by the autogain section. A second integrate and sample-and-hold sequence calculates the average illumination (bottom of Figure 2.15). When the focus index and average illumination are ready to be digitized by the computer, the digital section generates the trigger signal to initiate the conversion process. Following the conversion, the computer applies the gain bits to the focus index and normalizes it by the average illumination completing the operations of Equation 2.1. A more detailed description of the circuit can be found elsewhere [18].

Seven focus measurement boards (model Afx-3000, Q3DM) were modified to perform the focus evaluation tasks of the instrument (Figure 2.18). The modifications included: 1) adapting the circuit to work with TDI video signal; 2) modifying the circuit to work with higher 10-MHz video signal; and most importantly 3) synchronizing the circuits to improve the system’s efficiency and reliability.

The focus measurement circuit was originally designed for conventional RS-170 area array cameras. The circuit filters and integrates the high frequency content for every field of the video signal. The circuit depends on the horizontal and vertical sync signals to generate the appropriate internal timing as well as the external interfacing signals. The concept of a field or frame does not exist in TDI imaging.
and the vertical sync is absent from the TDI video signal. To adapt these circuits to work with TDI video signals, synchronization signals are generated separately and delivered to the circuits. The Array Timing Controller (described in Section 2.4.3) performs this function through: a) a line clock that synchronizes the cameras and the focus measurement circuits, b) a “window” signal that removes the transients between video lines from the calculation, and c) a “frame” signal that defines a virtual video frame over which the focus measure is integrated.

The focus measurement circuit designed for 4-MHz video signal of RS-170 cameras was modified to work with 10-MHz video signal of the TDI cameras. The modification consisted of replacing the RC networks that specify the end point frequencies of the filter section of the circuit to correspond to the higher video rate.

In the previous system of parallel focus [19], the focus measurement boards were not synchronized which required a complicated and inefficient processing scheme resulting in unreliable operation and missing of focus updates up to 4.5% of the time.\footnote{The focus data from different channels were generated independently and with random timing. Focus data had to be digitized continuously at very high rate by the computer. A software} To ensure reliable and deterministic performance at high line rate, the fo-
Figure 2.16: **Focus Measurement Circuit Waveforms.** Analog waveforms after different stages of the circuit: a) a video line input of a stage micrometer (10-\(\mu m\) spacing); b) amplified signal without sync pulse; c) filter output passing only high frequencies; d) window applied removing end-of-line transients; e) squaring; f) integration of signal as a measure of high frequency content. The locations for obtaining these waveforms are labeled in Figure 2.15. Adapted from Bravo et al. [18].
cus measurement circuits of this instrument were modified to work synchronously for efficient data acquisition and processing. The modification involved removing the crystal oscillator from individual focus measurement circuits and running them from a single master clock. At startup, power is first applied to the circuits, and after a few seconds delay, to the master clock. This ensures that all circuits initialize at the same time resulting in synchronized operation as seen in Figure 2.17. With synchronized focus measurement circuits, the system operates efficiently. All the channels in the array present data at the same time. A trigger signal prompts the computer to acquire data from all channels when necessary. The volume of data processed by the computer is very small ensuring fast focus update rate without losing any data.

Figure 2.17: Synchronized Focus Measurement Array. Scope screen capture showing the trigger signals of the eight focus measurement circuits (lower) and two focus index outputs of channels 3 and 5 (upper). All channels are synchronized simplifying data acquisition of focus information. (Screen captured with an Agilent 54645D Oscilloscope with 54659B Data Module.)

algorithm then searched through the large volume of data for valid data points. With this method focus update was missed when the computer could not keep up with the data rate during periods of high activity.
Figure 2.18: **Focus Measurement Array.** Seven focus measurement circuits (Afx-3000, Q3DM, San Diego, CA) were modified for TDI cameras and synchronous operation (one circuit shown is not used). The Array Timing Controller is at upper-left corner. The master clock circuit is on the right side of the enclosure.
2.4.3 Array Timing Controller

The Array Timing Controller provides control and synchronization of the TDI cameras and focus measurement circuits. To synchronize the TDI cameras, the controller provides a line clock signal to all cameras which initiates the start of integration of a line. The integration time is adjustable in 800-ns increments between 433.6 µs and 1.338 ms through a bank of DIP switches on the controller. For the focus measurement circuits, the controller provides a “frame” signal which specifies how many lines to be included in each focus measurement, and a “window” signal which tells the focus measurement circuit to ignore the transients at the beginning and end of each video line. The frame size is adjustable between 4 to 511 lines and is set by a bank of DIP switches. These functions were implemented in two programmable logic devices (EPM7064STC100-10, Altera, San Jose, CA). A block diagram of the controller is shown in Figure 2.19 and the schematic of the controller is presented in Section A.2.1.

Figure 2.19: Array Timing Controller Block Diagram. Block diagram of the Array Timing Controller. The controller sends a line clock signal to the TDI cameras which initiates the start of a new line. “Frame” and “window” signals are sent to focus measurement circuits to define the interval over which focus information is extracted. Integration time of the TDI camera and focus measurement interval (or frame size) are set by DIP switches.
Figure 2.20: Block Diagram of the Electrical Subsystems.
2.4.4 Focus Data Acquisition Hardware

Each focus measurement circuit outputs two analog signals (focus index and average illumination) and two digital signals (two gain bits) totaling 14 analog signals and 14 digital signals for the seven focus measurement boards in the array. One of the two NI PCI-6031E Multifunction Digital Acquisition (DAQ) boards in the system (National Instruments, Austin, TX) is used to read the signals from the array into the computer. Because of the lack of digital lines on the boards and availability of extra analog channels, all the digital lines are connected to analog input channels. The digitized voltages are then thresholded in software to determine the logic states. A block diagram illustrating the connections is shown in Figure 2.21 and the detailed connection listing is presented in Section A.3.

2.5 Microscope Automation/Modifications

The instrument is engineered around a Nikon Eclipse TE300 microscope (Nikon Instruments, Melville, NY). Various components of the microscope were motorized or modified for automation allowing operator-independent operation. This section describes the details of these modifications.

2.5.1 XY-Stage

XY stage motion is accomplished with a NEAT OFS-6060 motorized microscope stage (Danaher Motion, Salem, NH), powered by GeckoDrive G210 microstepping stepper motor drivers (GeckoDrive, Santa Ana, CA) and controlled by a ValueMotion PCI-Step-4OX PCI motion controller board (National Instruments, Austin, TX). The motorized stage has an approximated 5 in \times 5 in travel, large enough to accommodate microscope slides or microtiter plates. Custom stage inserts hold microscope slides and grating test samples (see Section A.1.1). For accurate position feedback during scanning, the Y axis is fitted with a linear encoder (see Sections 2.5.7 and 2.7.2).
Figure 2.21: DAQ Connection Diagram. Diagram showing the wiring connections between the components of the system and the computer through the two multifunction data acquisition cards.
2.5.2 Z-Axis

High-resolution Z-axis motion is actuated by a P722.17 200-µm piezoelectric positioner (PIFOC) powered by a E-660.L0 amplifier (Physik Instrumente, Auburn, MA) and controlled through a NI PCI-6031E Multifunction data acquisition (DAQ) board (National Instruments, Austin, TX). For large Z-axis motion, the fine-focus knob is driven with a Vexta PH265M stepper motor (Oriental Motor Corporation, Tokyo, Japan), powered by a GeckoDrive G210 microstepping stepper motor driver (GeckoDrive, Santa Ana, CA) and controlled by a NI ValueMotion PCI-Step-4OX PCI motion controller board (National Instruments). The PIFOC and stepper motor work in complementary fashion providing both high-resolution and large travel-range capability to the Z-axis.

Figure 2.22: Fine-Focus Knob and Optical-Path-Changeover Automation. Working in complement to the PIFOC, the motorized Fine-Focus knob enlarges focusing range to accommodate large uneven specimens. The motorized Optical-Path-Changeover facilitates fast light-path switching that allows both optimal light collection of fluorescent channels and maximal transmission in bright-field mode used for focusing during scanning.
2.5.3 Filter Cube Selection

The filter-cube cassette is motorized for automated and software-controlled filter selection during scanning. The cassette is actuated by a HS-805BB Mega-Torque Servo (Hitec RCD, Poway, CA), driven by a mini-SSC-III servo controller (Scott Edwards Electronics, Sierra Vista, AZ) and controlled through the computer serial port by software written in LabVIEW (National Instruments, Austin, TX). The cassette has four positions. One of the four positions is kept unoccupied for transillumination imaging modes. The remaining slots can accommodate up to three excitation-dichroic-emission filter cubes.

Figure 2.23: Filter Cube Cassette Automation. Automation of the Filter Cube Cassette allows automated, operator-independent scanning of multiple fluorescent channels. For each scanning strip, all channels are collected before moving on to the next strip to minimize the effects of thermal drift.
2.5.4 Wavelength Splitting

The focusing is done in phase-contrast mode since it provides much better contrast signal. During scanning, the focusing light is directed toward the focus measurement array through the side port while fluorescence emission is directed toward the trailing camera at the top port. Typically, a 20%–80% beamsplitter in the Optical-Path-Changeover would be used in this case to split the light to both the side port and the top port. However, to maximize both trans-illumination transmission and collection of fluorescent emission from the sample, the 20%–80% beamsplitter was replaced with a custom dichroic mirror (model 580DCSP, Chroma Technology Corporation, Rockingham, VT). Light with wavelength longer than 600 nm is reflected to the focus measurement array through the side port of the microscope. The remaining light which consists mostly of the fluorescent light from the specimen is transmitted through to the scientific grade camera at the top-port of the tri-ocular head. Band-pass barrier filters specific to each fluorescent dye is placed in the camera rotational mount to select the desired wavelength (see Section 2.5.8 on page 63).

2.5.5 Optical-Path Switching

Motorized control of the Optical-Path-Changeover is necessary for efficient collection of fluorescence data. Only during the first pass of each scan must the light be transmitted to both the focus measurement array and the trailing camera. In subsequent passes, since the focus positions have been already determined, 100% of the light should be directed to the trailing camera for maximum efficiency. The Optical-Path-Changeover is motorized with a Vexta PH265M stepper motor (Oriental Motor Corporation, Tokyo, Japan), powered by a GeckoDrive G210 microstepping stepper motor driver (GeckoDrive, Santa Ana, CA) and controlled by a NI ValueMotion PCI-Step-4OX PCI motion controller board (National Instruments, Austin, TX).
2.5.6 Arc-Lamp Shutter and Neutral-Density Filters

To protect the specimen from unnecessary exposure to excitation light, the arc-lamp shutter and two neutral density filters are motorized with HS-422 servos (Hitec RCD, Poway, CA), driven by a mini-SSC-III servo controller (Scott Edwards Electronics, Sierra Vista, AZ) and controlled through the computer serial port by software written in LabVIEW (National Instruments, Austin, TX). This allows operator-independent control of the fluorescent excitation source shutter and four levels of intensity.

2.5.7 Linear Encoder

The motion of the XY stage is achieved with leadscrews driven by stepper motors. Since imaging is done with TDI cameras, in which the transfer of the charge between rows of sensors must be carefully synchronized with the motion of the specimen, the motion velocity is just as important as positioning accuracy. Fluctuations in velocity which cause mismatches between specimen motion and charge transfer in the sensor would result in blurring of the image in the direction of motion. In a positioning system of this type, velocity fluctuation is primarily caused by the leadscrew with a smaller component contributed by the stepper motor.

Stepper motors’ inherently discrete mode of operation causes velocity ripple at the motor step rate, especially at low step rates, although microstepping can minimize low speed velocity ripple. Above the motor’s primary resonance (~1 revolution/s) the ripple amplitude driven by step rate falls off rapidly, until, at intermediate speeds (~5 revolutions/s), the effects of individual steps disappear and are replaced with a component dominated by the manufacturing tolerances of the stepping motor’s magnetic poles. Microstepping cannot reduce this effect. At still higher speeds (~25 revolutions/s), as pole modulation exceeds 1 kHz, it is swamped by the system’s mechanical inertia, and no longer produces a signature [1]. The instrument is designed to operate from 0.167 to 3.25 revolutions/s (0.17–0.33 μm/pixel; 1–10 kHz line rate; 3.94 steps/μm; 4000 steps/rev) which is well within the range affected by the stepper motor.

The leadscrew is the principal accuracy determining element. Leadscrews ex-
hibit two types of error: 1) a cumulative lead error, which is usually monotonic in nature, and 2) a periodic component, which is cyclic and varies over each revolution of the screw. The manufacturer specifies a $4 \mu\text{m/cm}$ and $25 \mu\text{m}$ for the cumulative and periodic errors, respectively.

To minimize blurring caused by the velocity fluctuations, the Y axis of the stage is fitted with a linear encoder (model MPE-BF-S, Opti-Cal, Templeton, CA) of which the output is used to drive the line clock of the trailing camera. The encoder’s resolution is $10 \text{ nm}$. To achieve line clock corresponding to the desired pixel size (typically in the range of $0.17$-$0.33 \mu\text{m/pixel}$), the encoder output pulses are divided down through a divide-by-$N$ ring counter. The output of the ring counter is then put through a programmable pulse stretcher to eliminate multiple transitions (as seen in Figure 2.24) caused by jitter in the stage. The details of the circuit are presented in Section A.2.3.

Figure 2.24: Linear Encoder Signal Conditioning. Stage motion jitter occurs near the transition of the divide-by-N ring counter would cause the counter to shift back and forth quickly between full-count and new-count multiple times resulting in multiple output pulses as seen in the middle section of trace 1. If these extra pulses are allowed to reach the TDI camera, they will erroneously initiate integration of new lines. By feeding pulse train through a pulse stretcher circuit before delivering it to the TDI camera, the extra erroneous pulse are prevented from reaching the TDI camera. The details of the conditioning circuit are presented in Section A.2.3. Adapted from Thieleking [115].
Although this solution solves the issue of blurring, it creates a side effect. Fluctuations in the stage speed change the duration between line-clock pulses. Since the trailing camera has no independent exposure control, this in turn affects the integration time of the sensor resulting in an output image with non-uniform exposure. This effect is more prominent when the stage motion is slow to accommodate dim specimens.

Figure 2.25: Opti-Cal Linear Encoder on Y-axis of XY stage. The stage has a linear encoder on the Y-axis (model MPE-BF-S, Opti-Cal, Templeton, CA). The encoder has a native resolution of 10 nm. The output is fed through a divider circuit to provide the desired resolution for driving the TDI camera.

2.5.8 Rotational Mount for Trailing Camera

TDI acquisition requires accurate alignment of the camera to the motion of the specimen. A total misalignment (either translational or rotational) of one pixel or less across the length of the TDI sensor does not affect image quality [5]. The 1024 pixels on the Hamamatsu ORCA-100 camera dictates a maximum accept-
Able misalignment of 3.4 arc minutes. To ensure this level of accuracy, a custom zoom-rotational camera mount is constructed from a Nikon CCTV zoom-lens and a rotational stage (M-481-A, Newport Corporation, Irvine, CA). In combination with software calibration and alignment tools (section 2.8.4), this mount allows adjustment of the pixel-size up to an accuracy of 0.001 μm and a rotational accuracy of 0.3 arc minutes. The mount also contains a slot for filters to block out unwanted light from reaching the trailing camera. See Section A.1.4 on page 206 for details.

![Zoom-Rotational Camera Mount](image.jpg)

**Figure 2.26: Zoom-Rotational Camera Mount.** In combination with software tools, the zoom-rotational camera mount allows accurate alignment of the TDI camera to the specimen. See Section A.1.4 on page 206 for details.

### 2.5.9 2D Camera in Focusing Array

At the start of scanning each strip, the specimen must already be in focus in order for the system to keep the specimen in focus during the scan. In addition,
the TDI cameras in the focusing array only produce valid data when the specimen is in motion. While it is possible to switch the trailing camera into 2D mode and move the objective through focus to find the best focus position, the ORCA’s 9 frames/s maximum frame rate and high-pass filtering the images in software would take too long to be practical. The TDI camera of a dim channel in the focusing array was replaced with a conventional 2D RS-170 camera. Together with a focus measurement circuit, this setup can quickly move through focus and put the starting position in focus in matters of seconds.

Figure 2.27: 2D Camera in Array. The TDI camera of a dim channel in the array is replaced with a conventional area-scan camera. Together with a focus measurement circuit and moving the objective lens through focus, this camera is used to place the specimen in focus at the beginning of the scan of each strip.

2.6 Illumination

Since the focusing light is split into eight paths, assuming equal distribution, each path is at best only one eighth as bright as the original beam. A bright light source is necessary to provide sufficient light to each channel. Since the CCD sensor has highest sensitivity between 700 nm and 800 nm as shown in Figure 2.28, the
desired light source should have high output power at these wavelengths. Other factors including ease of intensity adjustment, heat generation, lifetime, cost of ownership and operation are also considered in the selection of the light source. Four types of light sources were evaluated: mercury vapor arc-lamp, metal-halide lamp, diode laser and high-power halogen lamp.

2.6.1 Evaluation and Selection of Light Source

![Graph: EG&G Sensor Quantum Efficiency]

Figure 2.28: **EG&G Sensor Quantum Efficiency.** The TDI096AAU TDI CCD sensors used in the focusing cameras are most efficient between 700 nm and 800 nm [3].

**Mercury Vapor Arc-Lamp**

Mercury vapor arc-lamps are conventionally used as excitation light source for fluorescence microscopy. They have a broad spectrum with several intense peaks. When used as a transillumination source, appropriate measures must be taken to block out ultra-violet and infrared radiation from getting to the eye-piece and excessive photobleaching and heat damage to the sample.

A 100W mercury arc-lamp (HBO 103W/2, Osram, Munich, Germany) in HMX-4 lamp housing was attached to the transillumination tower with an adapter (Nikon Instruments, Melville, NY). Nikon transillumination heat filter and a 610-nm long-pass color glass filter (model NT46-430, Edmund Optics, Barrington, NJ) were
used to filter out the infrared and light with wavelengths shorter than 600 nm. While being very bright, the small arc discharge needed to be spread out to provide uniform illumination. Various diffusers and diffuser combinations were used to improve uniformity (model SPL-LSDKIT-50mm, Physical Optics Corporation, Torrance, CA).

**Metal-Halide Lamp**

Metal halide lamps are similar to mercury vapor lamps, but instead of just mercury, they also contain sodium/scandium iodide and sometimes rare earth metals combined with halogens in the halogen group of the periodic table. They have a purer white light than mercury vapor, which tends towards the blue end of the electromagnetic spectrum.

A 150W metal halide lamp module (model NMH-1, Nikon Instruments, Melville, NY) designed for differential interference contrast (DIC) microscopy was evaluated as the focusing illumination source. The lamp housing contained the appropriate ultra-violet and infrared filters. The light was delivered to the microscope through a liquid light-guide which scrambled the light to provide uniform illumination. Wavelength selection was done with a 610 nm longpass color glass filter (model NT46-430, Edmund Optics, Barrington, NJ).

**Diode Laser**

High intensity and monochromaticity make lasers excellent illumination sources for microscopy. Recent advances in solid-state laser technology have made available diode lasers in a wide range of wavelengths ranging from ultraviolet to infrared. For this application, diode lasers offer an advantage of long lifetime over conventional illumination sources (halogen lamp, arc-lamp). The laser is typically coupled to the microscope through an optical fiber because it offers great flexibility in the setup and arrangement of optical components. On the other hand, data collected with laser illumination are degraded by speckle noise. Speckle noise is caused by high spatial coherence of the laser source and the different propagation modes of the coupling optical fiber. As a result, lasers have been used primarily in spot-scanning applications. Several techniques have been reported for reducing the effects of the
Figure 2.29: **Light Source Spectral Comparison.** Light of wavelengths longer than 600 nm is reflected by the dichroic filter to the side port for focusing. The spectra of light sources evaluated shown in comparison to the transmission curve of the dichroic filter. Types of light source evaluated: 1) 100W mercury arc-lamp (HBO 103W/2, Osram, Munich, Germany) in HMX-4 lamp housing attached to the transillumination tower with an adapter (Nikon Instruments, Melville, NY), 2) 300W halogen lamp (model DRA, Osram, Munich, Germany), 3) 150W metal halide lamp (model NMH-1, Nikon Instruments, Melville, NY) and 4) 635 nm 5 mW diode laser (model HL6335G, Thorlabs, Newton, NJ). The spectra were measured with an USB2000 spectrometer through a 1000-µm diameter fiber (Ocean Optics, Dunedin, FL). (See Figure A.8 for transmission curve of the fiber).
speckle noise including digital post processing, multi-length fiber coupling [31], and rotation or vibration of the optical fiber [7, 81]. The simplest method is to vibrate the optical fiber coupling the laser to the microscope. The vibrations cause continuous changes in the propagation modes and rapid movement of the speckle pattern at the exit face of the fiber. As long as the motion of the speckle pattern is sufficiently fast compared to the integration time of the camera, the speckle noise can be averaged out.

Figure 2.30: **Laser Speckle Reduction.** Effects of fiber vibration on speckle: **top)** images of a micrometer slide (10 µm spacing) without (upper) and with (lower) vibration of the optical fiber, **bottom)** histograms of intensity distributions without (left) and with (right) vibration of the optical fiber. Without vibration, the speckle pattern grossly degraded the image and dominated the histogram with a broad distribution and large number of saturated pixels. With vibration, the background is smoothed out resulting in a bimodal distribution in the histogram of the foreground (black lines) and gray background. Data was collected with an EG&G 1024 x 96 lines TDI array used in full-frame mode digitized with an IC-PCI framegrabber (DALSA Coreco, St. Laurent, Quebec, Canada) on a Nikon Diaphot 300 inverted microscope using a Plan Apo 20× 0.50 NA objective with strobed illumination of 1.7 ms pulse duration from a 670 nm diode laser.

To investigate the feasibility of using the lasers as a light source, a low power,
low cost 670 nm 5 mW diode laser (model HL6722G, Thorlabs, Newton, NJ) controlled by a diode laser driver (model LD2000, Thorlabs, Newton, NJ) was evaluated. The laser was coupled to the microscope through a 2 m long, 600 nm core, 0.37 NA, multimode fiber (model M29L02, Thorlabs, Newton, NJ). The microscope end of the fiber was attached to an XYZ stage that allows optimization of light delivery into the collector lens of the transillumination light path. To reduce the speckle, the middle section of the fiber was coiled into several turns and attached to an electromagnet. The magnet was driven with a 60-Hz AC current to produce a 5-mm amplitude oscillation at the attachment point between the magnet and fiber. The reduction in speckle is apparent as illustrated in Figure 2.30.

**Halogen Lamp**

Early experiments during the development of the TDI cameras performed with the microscope’s built-in halogen lamp indicated that the halogen lamp might be a good light source. These experiments were done with just one TDI camera attached directly to the side port of the microscope. Preliminary calculation indicated that a higher power lamp would be required once the optical image splitting module is installed. Various lamp models of different filament sizes and power rating were evaluated. The industry standard DRA (120V, 300W) lamp was determined to be the best option.

The original 12V, 100W halogen lamp provided with the microscope was replaced with a DRA halogen lamp (Osram, Munich, Germany). The lamp was powered through a variable autotransformer (model 3PN1010B, Staco Energy Products Company, Dayton, OH). The lamp intensity was controlled by varying the output of the transformer (0–140 V). Because of the high amount of heat generated, the lamp housing was left uncovered during the tests and the lamp was switched on for very short periods of time.

**Selection of Light Source**

Although mercury vapor arc-lamps and metal-halide lamps are bright, their spectra are spiky. In addition, most of the energy is in the blue region leaving little energy in the desirable part of the spectrum (greater than 600 nm). Furthermore,
typical arc-lamps have very short lifetime (approximately 200 hours) requiring frequent replacement and alignment.

With its monochromaticity, laser diodes seem ideal. Speckle noise can be minimized with the demonstrated technique. However, a much more powerful laser than the one evaluated would be required to provide adequate brightness once the image splitting module is in place. Such powerful lasers are expensive. Furthermore, intensity control is generally limited with lasers. Generally, intensity is adjusted either with polarizers or neutral density filters.

Of the options evaluated, the high-wattage DRA halogen lamp is the best option. Its broad spectrum in the red region matches well with the sensitivity of the TDI CCD sensors. As an incandescent lamp, the intensity is continuously variable through controlling the supplied voltage. With the same pin dimensions as the Nikon original lamp, the DRA high-wattage lamp is a drop-in replacement utilizing the same lamp housing base together with the illumination optics. However, since the DRA lamp is three times the power of the original lamp, a new intensity controller and active cooling are necessary to dissipate the heat generated. These modifications are described in Section 2.6.2.

<table>
<thead>
<tr>
<th>Light Source</th>
<th>Hg Arc-Lamp</th>
<th>Metal Halide</th>
<th>Diode Laser</th>
<th>Halogen</th>
</tr>
</thead>
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<td>Excellent</td>
<td>Good</td>
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<td>1,500 hours</td>
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<tr>
<td>Ownership Cost**</td>
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<td>$0.40 – $0.46</td>
<td>$0.02 – $0.10</td>
<td>$0.008 – $0.01</td>
</tr>
</tbody>
</table>

* Although the diode laser evaluated is a low-power low-cost model, high-power model required for this application costs in the thousands of dollars.

** Calculated for each hour of operation.
2.6.2 High-Intensity Light Source

The bright-field illumination is provided by a 120V, 300W halogen lamp replacing a 12V, 100W lamp supplied by the microscope manufacturer. Active cooling is necessary to remove the heat generated by the lamp to protect the nearby light collection optics. In addition, a new controller is necessary to control the intensity of the lamp.

Water Cooling

Incandescent lamps are less than 10% efficient. Most of the energy consumed is output as heat. For this 300W lamp, about 270 W of heat are generated. The collector lens is approximately one inch away from the lamp. Without proper cooling, damage to this collector lens such as cracking or pitting would result due to exposure to high heat.

To control the temperature of the lamp housing, water is circulated through a copper plate attached to the metal base containing the lamp socket. Heat is then removed from the water as it goes through a radiator that is actively cooled with forced air. The cooled water returns to a reservoir from which it is pumped to the lamp housing again (see Section A.5.2 on page 218). The closed-loop water cooling system is effective in maintaining the operating temperature of the lamp as illustrated in Figure 2.31. The system also includes four temperature probes to monitor temperatures of the lamp housing, the inlet, the outlet and the ambient air.

Lamp Controller

The lamp controller is based on a DC-controlled dimmer module (model K8003, Velleman, Gavere, Belgium) (see Section A.5 for the schematics). The heart of the module is a phase controller chip TEA1007. The magnitude of the DC input voltage changes the duration of the output trigger pulse going to the gate of the triac which in turn controls how long the AC current is applied to the lamp during each AC cycle. This allows intensity control of the lamp from the computer through an analog output channel of the DAQ board (refer to Figures 2.20, 2.21 and Section 2.8.2 for details). A DC power supply and a selection switch are also
Figure 2.31: **Water Cooling of High-Intensity Halogen Lamp Housing.** Lamp housing with water-block cooler (upper-left). Enclosure with pump, fan and radiator (upper-right). Plot showing the effectiveness of water cooling during a 30-minute ON period. Without cooling, the lamp had to be switched off prematurely to prevent damage. The temperature probe is inside the lamp housing approximately 3 cm from the bulb. (Lamp Control Voltage = 8 V.)
included in the controller to allow manual control of the lamp intensity.

### 2.7 Image Acquisition

#### 2.7.1 Scientific-Grade TDI Camera

The fluorescence data is acquired with an ORCA-100 cooled scientific camera that is modified to also work in TDI mode (model C4742-95, Hamamatsu Corporation, Bridgewater, NJ). The pickup device is a progressive scan interline CCD with 1280(H) × 1024(V) effective pixels. In TDI mode, only the center 1024 horizontal pixels are active creating a 1024-pixel wide and 1024 integration stages TDI camera. The pixels are square at 6.7 µm × 6.7 µm. The sensor is Peltier cooled with passive air radiation system. The camera has a maximum data rate of 20 MHz and a maximum line rate of 13.289 kHz in TDI mode. Camera control and mode switching (full-frame / TDI) is accomplished through a RS-232 interface. Synchronization in TDI mode is achieved through a line-clock input signal. The parallel digital 12-bit camera output is transferred to the computer through a framegrabber with 32 MB onboard memory (model NI-PCI 1422, National Instruments, Austin, TX).

![Hamamatsu ORCA-100 Quantum Efficiency](image)

Figure 2.32: Hamamatsu ORCA-100 Quantum Efficiency.

The camera is attached to the top port of the microscope through a custom zoom and rotational mount (see Section 2.5.8). This camera mount allows precise adjustment of magnification and angular alignment that are especially important
in TDI imaging. The mount also provide a means for filters to be inserted in front of the camera to block out unwanted signals.

2.7.2 Linear Encoder Driven Line-Clock

With TDI cameras, velocity mismatch of 2–4% is generally acceptable [5]. Preliminary testing discovered noticeable periodic blurring in the acquired images caused by fluctuations in the X-Y stage velocity. Further testing found that the stage fluctuation is about 12.6% (section 3.8 on page 117). To maintain image quality, a linear encoder is installed on the Y-axis of the stage. The encoder provides a sync signal to synchronize the charge transfer between stages of the camera with the motion of the specimen. The encoder has a native resolution of 10 nm. This output is put through a programmable divide-by-N counter to achieve the desired resolution appropriate for the experimental setup (i.e., with a 20× 0.75 NA objective lens, Nyquist sampling requires a pixel-size of 0.22 μm; the encoder output is divided down by 22 to give a pulse for each 0.22 μm that the stage travels).
Figure 2.33: **High-Throughput High-Resolution Image Cytometer.** A picture of the instrument that shows the components of the systems. The optical-splitting-assembly and TDI focus measuring cameras are housed in the black enclosure to the left of the microscope. The array of focus measurement circuits are in the aluminum enclosure on the left side of the table. All the wirings that interface the components of the system to the computer resides behind the array of focus measurement circuit. The trailing camera is at the top-port of the microscope. The PIFOC controller can be seen in the background to the right of the microscope. Servo controller, 2D focus measurement circuit, stepper motor drivers, halogen lamp controller, trailing camera controller, and temperature logger are on the shelf below the table.
2.8 Instrument Control Software

Serving a complementary role to the hardware, the control software is just as equally important. Unlike previous field-by-field cytometer systems whose hardware events are essentially sequential – one event finishes before the next event will start – hence the timing of events is not critical, hardware events in this instrument occur in parallel and therefore need to be carefully synchronized. In addition to controlling the basic automation and integration of the hardware components of the instrument, the software also provides the necessary and crucial timing and synchronization between the subsystems in order for the instrument to work.

Due to the high level of complexity required in the software, careful considerations were put into its development. The following criteria were carefully followed throughout the process:

**Organization:** The overall design of the software as well as the subsystems are well laid out before being implemented. Such careful planning ensures cleanly written software and prevents quick fixes and patch works.

**Understandable:** Effort was put in to make the code understandable, which saves myself and others, in the future, significant debugging and maintenance time.

**Editable:** The combination of understanding the code and well-designed/well-organized architecture makes editing the code easy and quick without disrupting the rest of the program.

**Minimize Modular Independence:** Software code was made modular without strong dependence between unrelated modules (i.e., changing one module does not bring down the entire application).

**Strong Cohesion:** Each module is part of a closely related set of functions knowing exactly which parts need to be changed if edits are required.

The software was written with LabVIEW 7.1, a graphical development environment by National Instruments (Austin, TX). Besides providing native support for most of the interfacing hardware in the instrument, LabVIEW also has an efficient user interface creation tool which facilitates the development of the software. The
following sections describe in detail the different sub-components of the instrument control software.

### 2.8.1 Software Architecture

The main program of the control software consists of two while loops running together to create a state machine as illustrated in Figure 2.34. One loop handles the user interaction, translating the user’s actions into system events (hardware and/or software) which are put into the queue and sent to the other loop, which carries out the events in the queue.

**Figure 2.34: Software Architecture.** The main program consists of two while loops running together to create a state machine. One loop handles the user interaction, translating the user’s actions into system events (hardware and/or software) which are put into the queue and sent to the other loop, which carries out the events in the queue. With this architecture, the control software is extremely modular, flexible and scalable. Additional features are easily accomplished either by defining a new execution order of existing events or adding new event(s) to the Event Queue Execution loop without affecting existing features of the software.

**Event Queue Building** loop consists of a User Interface Event structure which handles user interaction (i.e., changing front panel controls, mouse clicks, keyboard keystrokes) and associates them to appropriate system events (e.g., increasing the value of a slider changes the intensity of the lamp). The User Interface
Event structure effectively generates a software interrupt for each defined user action. The advantages of using a User Interface Event structure over a traditional polling method are less CPU usage and guaranteed no lost events (all events are queued by the operating system when the program is busy and subsequently delivered).

**Event Queue Execution** loop is a queued message handler which executes events from the event queue generated by the first loop. In some cases, the execution of some events can generate other events which are added to the queue. Events can be either hardware events (i.e., moving the objective, capturing an image) or software events (i.e., estimating the best-focus position, calculating the average brightness of an image). Typically, events are executed in the order they were received; however, the structure of the queue permits access to individual elements allowing reordering of the queue — putting high-priority tasks at the front of the queue and pushing low-priority tasks to the back.

The main component of the Event Queue Execution loop is a case structure with many cases. Each contains the instruction for a specific hardware or software event. The execution of a system task can invoke just one of these cases or a combination of cases. Data and variables of the system (state of the system) at the completion of one case are passed to the beginning of the next case.

With this architecture, the control software is extremely modular, flexible and scalable. Additional features are easily accomplished either by defining a new execution order of existing events or adding new event(s) to the Event Queue Execution loop without affecting existing features of the software.

The control software is organized into modules, each responsible for a different aspect of the operation of the system. The following sections describe these modules in detail.

### 2.8.2 Hardware Control

Control and automation of the hardware components is one of the main tasks of the control software. The Hardware Control module is responsible for initialization, control and monitoring of the status of the following hardware sub-systems:

**Halogen Lamp:** The software controls the transillumination source through a
16-bit analog output working in conjunction with the lamp controller. The lamp intensity is proportional to the output voltage of the analog control signal (section 2.6.2).

**Hg Arc-lamp and ND Filters:** The software controls the motorized arc-lamp shutter and neutral density filters (section 2.5.6) to provide the correct level of excitation during fluorescence acquisition.

**XY Stage:** the position (relative or absolute), speed and acceleration of the specimen (section 2.5.1).

**Z Motion:** the position of the PIFOC (fine-resolution, short-travel) and position of the Fine-Focus Knob motor (coarse-resolution, long-travel) (section 2.5.2)

**Light Path Changeover:** selection of the appropriate light path during multiple-pass scanning (section 2.5.5).

**Filter Block:** selection of the appropriate fluorescence excitation/emission filter (section 2.5.3).

**Trailing Camera:** adjustment camera parameters (gain and offset)

**Focus Array:** measurement of array line rate

### 2.8.3 Auto-Focus

Autofocus is the most important task performed by the software. As the specimen is moving across the objective lens, the software takes in and analyzes the focus information provided by the focus measurement array and calculate the new position of the objective to keep the specimen in focus. There are two main processes involved with this task: acquiring and processing the focus information quickly and efficiently, and the more challenging process of normalizing focus data coming from different channels in which the responses are different.
Figure 2.35: **Hardware Controls.** Screen capture showing controls for hardware sub-systems. These controls provide an easy and intuitive method for setting up the parameters of the system for a scan.
Performance Requirement

The performance requirement of the autofocus module is governed by the response time of the PIFOC. When commanded to change position, the PIFOC requires a finite amount of time to make the transition and to settle into the new position. For the 200-µm PIFOC of this instrument, the manufacturer specifies a 20-ms settling time. Therefore, a complete focus update cycle (i.e., focus data acquisition, processing, and calculation new focus position) must be completed in less than 20 ms.

Focus Data Acquisition

Most of the focus processing and data reduction has been done in hardware by the focus measurement circuits, very little data remains to be acquired and processed by the computer. Each of the seven focus measurement circuits produces a set of focus measurements (focus index, average illumination, and two gain bits) for every virtual frame. The size of the virtual frame is set by the Array Timing Controller (section 2.4.3). For most of the experiments, the size of the virtual frame was 128 lines (or about 44 frames every second). The four signals from each channel are connected to four analog inputs for a total of 28 analog input channels for the entire focus measurement array (section 2.4.4). Because the array is synchronized (section 2.4.2), it provides a trigger signal that initiates the acquisition of data from all circuits of the array at the end of the virtual frame when the focus data becomes valid. Ten samples per signal are acquired at 2000 Hz and averaged to reduce noise. For each trigger, a total of 280 samples (4 signals/circuit × 10 samples/trigger × 7 circuits) are acquired which are easily buffered by the 512-sample onboard FIFO. For continuous acquisition at 44 Hz, a requisite constant data rate of 24.640 kbytes/s was tested and verified on the system (16 bits/signal × 4 signals/circuit × 10 samples/trigger × 7 circuits × 44 Hz).

Focus Data Processing and Focus Calculation

The responses of the focus measurement channels are different and these differences must be accounted for and normalized out. The light transmission through each channel is different. In addition, the cameras and focus measurement circuits
in different channels have different responses. Since all these factors affect the magnitude of the output signal which is the metric for determining focus, the responses from the channels need to be carefully normalized prior to focus calculation in order to get the correct result.

As a calibration step in setting up the system, the objective lens is moved through focus in small increments with a Ronchi ruling\textsuperscript{2} as the specimen to produce through-focus curves as shown in Figure 2.36. These curves characterize the responses of the different channels and they give the following calibration parameters for subsequent focus calculation:

---

\textsuperscript{2}Ronchi rulings are equal bar-and-space square-wave gratings, having very high contrast ratio and fine edge definition. Aligning the lines of the grating parallel to the direction of charge transfer in the TDI sensors creates very high contrast output video signals. Furthermore, since the features of the Ronchi ruling are constant and one dimensional across the field of view, it does not cause blurring along the direction of charge transfer even when it is not in motion. Therefore, the Ronchi ruling is the ideal test specimen for calibrating the autofocus system. The Ronchi ruling used with the system has 2000 line-pairs per inch (model NT56-614, Edmund Optics, Barrington, NJ).
Figure 2.36: **Focus Data Acquisition.** The software assists the user with the calibration of the system. Focus information of all channels is quickly captured and displayed for visualization along with various related parameters. This makes the calibration process efficient and allows easy diagnosis and troubleshooting of any problem that may arise during the operation of the system.
Camera Positions: the peak location of each curve gives the axial position of the corresponding camera in the focus sampling range.

Offsets: the average value of the tails of each curve away from the central peak gives the offset for that channel. This is especially important if the channel has a large offset or small peak value.

Scaling Multipliers: the ratios of the value of the highest peak over the value of the other peaks give the scaling multipliers (or normalization factors).

During scanning, the above parameters are used to calibrate the incoming focus measurements prior to using them for calculating the best focus position. For every focus measurement coming in from each channel, the gain bits are first applied to the raw focus index and the result is divided by the average illumination (section 2.4.2). The offset is then subtracted and the scaling multiplier is applied to normalize out the response differences between the channels.

The Power Weighted Average algorithm is used to calculate the best focus position. This algorithm had been previously demonstrated to provide robust performance [90]. The best focus position is estimated as

\[ w_a = \frac{\sum z F^n_z}{\sum F^n_z} \] (2.6)

where \( w_a \) is the weighted average, \( z \) are the axial test positions, \( F_z \) are the corresponding focus measures at those locations, and \( n \) is the weight.

Equation 2.6 was modified to accommodate multiple focus measure inputs of this instrument. For each cycle of focus correction, the focus update \( z_{update} \) is calculated as

\[ z_{update} = \frac{\sum z (z - z_{trailing\_cam}) F^n_z}{\sum z F^n_z} \] (2.7)

where \( z \) is the axial position of the focus measurement cameras, \( z_{trailing\_cam} \) is the axial position of the trailing camera, and \( F_z \) is the normalized focus index value.

The new position of the objective lens is then calculated as

\[ z_{new} = z_{previous} + K * z_{update} + z_{offset} \] (2.8)

where \( z_{new} \) and \( z_{previous} \) are the new and previous positions of the objective lens, respectively, \( K \) is the feedback gain, and \( z_{offset} \) is a correction offset. Adjustment
or tuning of the feedback gain is necessary to ensure stability. Oscillation in the response of the system is corrected by decreasing this feedback gain. The non-uniform response or non-symmetric spacing of the channels sometimes can create a small offset in the output. The $z_{offset}$ term can be used in these cases to cancel out the offset.

During scanning, the system sometimes encounters conditions which cause it to take large correction steps such as the presence of a large piece of debris or a large clump of cells. Often as a consequence of these large steps, the system cannot recover and loses track of the specimen. A maximum update parameter is used to limit the size of the correction step in these situations. Correction updates larger than the maximum update parameter are reduced down to the size of the maximum update.

![Auto-Focus Parameters Control](image)

Figure 2.37: **Auto-Focus Parameters Control.** The control displays the calibration parameters (channels’ locations, offsets, and normalization factors) and allows selection of which channels to include in the focus calculation. The power weight, the feedback gain, the additional offset, and the maximum update parameters can be easily input by the user to tune the response of the autofocus system.

**Focus Profile Recording and Playback**

For multi-color scans, several passes are required to acquire all the colors. Autofocus is performed only during the first pass and the focus positions (objective lens axial positions) are recorded as a function of the specimen’s lateral location. The recording is then played back during subsequent passes. For recording, the D/A output to the PIFOC controller is looped back to an analog input channel on
the data acquisition board (see Figure 2.21). Triggering and synchronization with
the specimen’s lateral position are accomplished by using the Y-axis step signal
and two counters on the data acquisition board (see Figure 2.21 and Section 2.8.7).
The system is set up to record the position of the objective lens at intervals of 1/10
of the camera’s field of view in the Y-direction (e.g., 22 µm or 89 motor steps for
the 20× 0.75 NA objective lens).

2.8.4 Calibration and Alignment

TDI Calibration

With TDI imaging, because the transfer of the lines of image through the
integration stages must coincide with the motion of the object, the projection of
the specimen on the sensor must have the correct magnification, otherwise blurring
will occur in the direction of motion. The software provides a calibration tool to
be used with the zoom lens (section 2.5.8) in the mount of the trailing camera to
achieve the required magnification. The calibration procedure involves continuous
imaging of a microscope micrometer slide while the stage is stationary. The black
bars are thresholded and their centroids calculated. Since the spacing between bars
and the pixel-size are known, the magnification can be calculated and displayed
on the screen in real time. With the aid of this tool, the correct magnification can
be achieved in matters of seconds.

Angular alignment is another important criterion with TDI imaging. The por-
tion of the specimen imaged in a pixel of the first row of the TDI sensor must also
be integrated in that same column as the object passes over the last row; other-
wise smearing will occur. The software also provides a Camera Alignment aid to
facilitate this process. The procedure is as follows:

- Capture an image.

- Prompt the user to select a small region-of-interest (ROI) in the image. This
  ROI should contain features that are different from the rest of the image.
  (Specimens with repeating features such as Ronchi ruling should not be used
  here.)
• The specimen is moved so that the ROI is at TOP-CENTER of the field-of-view (FOV).

• The specimen is then moved so that the ROI is at the bottom of the FOV.

• The lateral displacement of the ROI between the TOP and BOTTOM positions is used to calculate the angular displacement and the result is displayed on the screen.

• The user is prompted to adjust micrometer on the camera mount (section 2.5.8) until the displacement is acceptable (less than 1 pixel) while the displacement is continuously calculated and displayed.

With the aid of this utility, the required angular alignment can be achieved in matters of minutes.

**Slide Leveling**

Under most circumstances, the PIFOC’s 200-µm range is large enough to accommodate any tilt of the microscope slide; however, to maximize autofocus accuracy and optimize image quality, the plane of the specimen should be made as perpendicular to the optical axis as possible. The autofocus performance is a compromise between accuracy and range. With a limited number of testing planes, narrower spacing between the testing planes gives better accuracy but is only capable of tracking small changes in focus. Wide spacing, on the other hand, can track large variations but with diminished accuracy. As a result, it is desirable to maximize autofocus accuracy by eliminating as much as possible any tilt in the slide and to have autofocus correcting only the inherent variations present in the specimen. Furthermore, due to the large number of integration stages on the trailing camera, it is desirable to minimize any unnecessary axial variations in the specimen as it travels across the field of view to avoid blurring.

Taking advantage of the adjustability of the slide insert (section A.1.1), the Slide Leveling utility helps the user flatten out the specimen in preparation for the scan. Under most cases, the total tilt of the scan area is brought to within 10 µm in just one iteration of the utility. The utility starts by finding the best focus positions at the center-top and the center-bottom points of the scan area (the
average tilt in the Y-direction). By knowing the relative distances between these points and the leveling screws, it estimates the axial positions of the objective lens at these points at which the leveled specimen would be in focus. The objective lens is then placed at one of these new positions while the user is prompted to adjust the leveling screw until the image is in focus. A similar process is carried out to level the specimen in the X-direction.

**XY Stage Speed**

Since the magnification to the focus measurement cameras is fixed and the line rate is set by the Array Timing Controller, the only way to match the motion of the specimen with the transfer of charges in the sensors is by varying the stage speed. In addition, the stage speed may also need to be varied to control the exposure. In all of these cases, the stage speed must match the transfer of the charge from row to row to avoid blurring. The stage speed is calculated as

\[
\text{stage speed}[\text{steps/s}] = \frac{3.940[\text{steps/µm}]}{\text{pixel size}[\text{µm/pixel}]} \times \text{line rate}[\text{Hz}] \tag{2.9}
\]

### 2.8.5 Image Acquisition

Care must be taken in transferring data from the TDI camera in order to prevent data loss. Unlike conventional 2D cameras which output data one frame at a time with a pause between frames, TDI cameras output a continuous stream of pixel data. The ORCA-100’s maximum data rate is 20.48 MB/s (10 kHz line-rate × 1024-pixel line width × 2 bytes/pixel). Although the Peripheral Component Interconnect (PCI) bus can transfer up to 132 MB/s, this maximum transfer rate is shared among all the devices on the bus. Depending on the number of devices and requests on the bus, the actual sustained transfer rate is much lower. Furthermore, the operating system’s interrupts also introduce large delays on the bus, longer than the acquisition time of a line of image data which may result in data loss. To prevent this, a ring-buffered acquisition is setup in the onboard buffer of the framegrabber to capture the stream of image data coming from the TDI camera.

A ring-buffered acquisition is a multiple-buffer continuous acquisition that uses multiple image buffers in a buffer list which is filled up as images are acquired.
When all the buffers in the list are full, the acquisition begins writing data at the beginning of the buffer list, similar to a circular buffer or ring. As a result, during delays associated with the PCI bus or operating system, the onboard buffers will continue to be filled until the system resources are freed to catch up with the image acquisition.

Figure 2.38: Ring Buffer for Image Acquisition. Diagram illustrating the buffer configuration for acquisition of image data from the TDI trailing camera. Two levels of buffering ensure no data loss. The ring buffer residing in the framegrabber ensures that image data from the camera is safely captured during periods of high activity on the PCI bus. A second linear buffer in the system memory buffers the data before writing it to the hard drive.

Similarly, the data rate written to the hard disk of the system is highly dependent on the PCI bus activity and the system load. A second buffer is set up in the system’s random-access-memory (RAM) to buffer the data before it is written to the hard disk.

Data is constantly transferred from the TDI camera to the onboard memory at a relatively slow rate without delays. When the PCI bus is freed up, data is
then transferred to system RAM in high-speed bursts. Finally, the image data is written at low priority to the hard disk when the operating system permits.

2.8.6 Scan Configuration

An important and useful function of the software is the Scan Configuration utility. This tool provides an easy and efficient way to configure experiments (see Figure 2.39). The parameters for all hardware settings for each (color) pass during the scan are individually configured. These settings are written to a configuration file as shown in Figure 2.40 creating an automatic record of the experimental setup. Furthermore, each new set of configurations is also stored as a method available for use at a later time under the Method menu. This is useful in cases where many slides need to be scanned with the same settings or if the same experiment needs to be performed multiple times.

The user can create a new method from scratch or from an existing one. In each method, the user can create as many channels as required. Once created, channels can be reordered or deleted. For each channel, the following parameters are available:

**Channel Label:** a short descriptive describing the channel

**Focus Mode:** if the channel is the first on the list, the focus mode will be **Record** (auto-focus is performed during the scan of this channel and the objective lens positions are recorded); for subsequent channels, the focus mode would typically be **Playback** (positions recorded during the scan of the first channel are played back). Under special cases, the user can select to perform auto-focus for every channel; however, this is inefficient and unnecessary.

**Focus Offset:** because of chromatic abberation, different colors come into focus at slightly different positions in z. Playing back the focus profile recorded while scanning one color would cause the specimen to be out of focus for signal of a different color. This setting allows the user to specify an offset to be used for each channel during playback. The offset for each channel can be set manually or it can be determined automatically using the **Calculate Channel Offsets** function (lower-right corner of the panel).
Figure 2.39: **Scan Channel Configuration Control.** Setting up a scan is fast and easy with the Scan Channel Configuration tool. The tool allows easy configuration of hardware parameters for each channel (dye color). The settings for each set of experiments are in a method file. These files can be easily recalled later if similar experiments need to be conducted. The hardware settings of the experiments are also written to an output file that go together with the image data.
### General Information
- **ScanID:** Scan20050809
- **Output Directory:** /E/TEMP
- **Collection Mode:** 0

### Scan Area
- **Top:** 164000
- **Bottom:** 206000
- **Left:** 170000
- **Right:** 200000

### Channel 0 Config
- **Focus Mode:** 0
- **Focus Offset:** 0.000000
- **Arc-Lamp:** FALSE
- **ND Filter 1:** 1
- **ND Filter 2:** 0
- **Dichroic Filter Position:** 0
- **Optical-Path Selector:** 0
- **Stage Speed:** 750
- **Camera Gain:** 0
- **Camera Offset:** 0
- **Save Images:** FALSE

### Channel 1 Config
- **Focus Mode:** 1
- **Focus Offset:** -0.300000
- **Arc-Lamp:** FALSE
- **ND Filter 1:** 1
- **ND Filter 2:** 0
- **Dichroic Filter Position:** 0
- **Optical-Path Selector:** 3
- **Stage Speed:** 5000
- **Camera Gain:** 0
- **Camera Offset:** 0
- **Save Images:** TRUE

### Microscope
- **Objective:** 2
- **PIFOC Model:** 1

### Array Cameras
- **Array Line Rate:** 5580
- **Average Pixel Size:** 0.330000
- **Array Positions:** 0.2549897|0.5103008|0.4828271|0.5243169|0.3369883|0.3746048|0.0000000|0.3950707|0.0000000|0.3950707|0.0000000
- **Array Normalization:** 0.123411|0.066440|1.000000|0.3950707|0.0000000|1.584888|0.0000000|0.0000000|0.0000000
- **Array Offsets:** 0.2549897|0.5103008|0.4828271|2.5243169|0.3369883|0.3746048|0.0000000|1.1584888|0.0000004|0.0000000

### Focus Parameters
- **Best Focus Algorithm:** 5
- **Gain:** 2.000000
- **Offset (um):** 0.000000
- **Camera Pair 0:** 4|5|0.5627426|4.4846350|99.7900000|2.4094223|0.0529559|0.0000000|0.0000000|0.0000000
- **Camera Pair 1:** 4|6|1.3163324|0.8062477|99.7896423|0.9433504|-0.4539861|0.3162103|-0.1597425|0.0453412|-0.0053109
- **Camera Pair 2:** 4|8|0.7035798|1.7280193|99.7941110|0.1725943|-0.0049654|0.0001149|0.0000308|0.0000016|0.0000004
- **Camera Pair 3:** 4|8|2.0514023|0.6611445|98.8899999|0.5124843|0.0179665|0.0012272|0.0009421|0.0000142|0.0000002
- **Camera Pair 4:** 5|8|0.8385247|1.2384984|98.8899999|0.6579040|0.0306294|0.0028582|0.0003354|0.0000403|0.0000030

### Trailing Camera
- **Trailing Cam Line Rate (Hz):** 8370
- **Pixel Size (um/pixel):** 0.220000
- **No. Strips:** 34
- **Frames/Strip:** 48

Figure 2.40: Example Scan Configuration file
performs through-focus curves for each channel and it uses the peaks of the focus curves in each channel to determine the offsets for the different colors.

**Lamp Voltage:** this typically controls the intensity of the halogen lamp during scanning of the first channel; however, the halogen is also available if required for subsequent passes.

**Arc-Lamp:** this setting controls the shutter of the arc-lamp.

**ND1 & ND2:** these settings control the positions of the neutral density filters giving four different fluorescence excitation intensity levels.

**Dichroic Filter Position:** this setting selects which filter cube to be used with each channel. The microscope has four filter positions and the first position is typically left empty for bright-field modes.

**Optical-Path Selector:** this selects the optical path for each channel. For the first channel (focus recording), the selector is typically set to position D which splits the focusing light to the focusing camera array at the side port and the fluorescence emission to the trailing camera at the top port. For subsequent channels (focus playback), the selector is typically set to position A which passes all of the light to the trailing camera at the top port.

**Stage Speed:** for the first channel (focus recording), the stage speed is determined by the line rate of the focusing camera array (section 2.8.4); for focus playback channels, the stage speed together with the illumination setting determines the exposure of the trailing camera. If the specimen is bright, the speed is set high to limit exposure. On the other hand, if the specimen is dim, the speed is set low to allow longer exposure. The corresponding line rate for the trailing camera is displayed in **Line Rate**.

**Clock Source:** the line clock of trailing camera is typically driven with the linear encoder; however, the linear encoder is noisy at very slow speed (very dim specimens). In those cases, the camera is set to be driven by a clock generator since the effects of the speed fluctuation are not apparent.
**Save Images:** if checked, the software saves the images acquired for the channel. Typically, during test scans, this setting is not checked since the scan can produce a large amount of image data.

**Camera Gain:** this setting controls the gain of the trailing camera. Range=[0:255].

**Camera Offset:** this setting controls the offset of the trailing camera. Range=[0:255].

### 2.8.7 Timing and Synchronization

The synchronization of hardware subsystems during scanning of a strip is the most critical and difficult part of the software. There are several processes occurring in parallel and they must be properly synchronized. Due to the nature of TDI imaging, the main challenge involves starting these processes while the specimen is in motion, at the exact moment a specific point in the specimen (the starting point of the strip) passes over the objective lens. These tasks include:

- the specimen must already be moving at the required constant speed as the starting point crosses the objective lens,

- start capturing image data from the trailing camera at the moment the starting point passes over the objective lens,

- begin moving the objective lens to maintain focus at the moment the starting point passes over it and not before, and

- start recording (or playback) of the focus profile at the moment the starting point passes over the objective lens.

Implementation of these tasks are detailed in the following sections.

**Trailing Camera and XY-Stage**

The framegrabber is to begin acquiring image data from the trailing camera at the starting position of each strip. In addition, to avoid blurring and to maintain uniform exposure, the specimen must be moving at a constant speed at the starting point of the scan. To achieve both of these conditions, the the specimen is moved
back in the Y-direction N steps from the starting point. N is chosen to be large enough to allow the stage to accelerate up to the constant speed required during scanning before reaching the starting point.

\[
N \text{[steps]} = (v \text{[steps/s]} \cdot 1 \text{[s]}) + (a \text{[steps/s}^2\text{]} \cdot (1 \text{[s]}^2)) \tag{2.10}
\]

where \(v\) is the stage speed during the scan, and \(a\) is the acceleration. To create a start-of-acquisition trigger for the framegrabber, the step signal of the Y axis is fed into the input of counter 0 on the DAQ device 1 (see Figure 2.21). The counter is set up to work in divide-by-N mode (the counter's output issues a pulse for every N input pulses). As the stage passes the starting point, the output of the counter would fire triggering the framegrabber to start acquiring image data. The number of image lines to be captured is calculated from the length of the strip and the appropriate buffers are allocated. The camera would stop once the desired number of lines have been captured. The stage is programmed to maintain constant speed for at least 1024 lines (the number of integration stages of the trailing camera) beyond the stopping point of the strip before slowing down and stop ensuring the constant specimen speed through the end of the strip. The trailing camera requires a line clock signal or it would time out. During the length of the strip, the trailing camera is driven by a line clock derived from the output of the linear encoder (see Section 2.7.2); however, when the stage is stationary, it is driven by counter 0 of the DAQ device 3 configured as a frequency generator. Selection of which line clock to be fed to the camera is achieved with a flip-flop circuit and a multiplexer (see Figure 2.21). The start-of-acquisition trigger described above also provides the “SET” signal to the flip-flop which switches the line clock source from the frequency generator over to the linear encoder at the start of the strip.

**PIFOC and XY-Stage**

At the starting point of the strip, with the stage stationary, the software performs a two-stage focus search using the 2D camera in the array (section 2.27): 1) a coarse search using the fine-focus-knob motor, and 2) a fine search using the PIFOC. The result of the search is used to place the PIFOC at its midrange position (for maximum range during the scan) and the objective lens at the best-focus
position found during the search. Since the focus data is not valid until the specimen is moving at the correct speed, the PIFOC must be held constant during the XY-stage acceleration period and must not start moving until the starting point of the strip has been reached. While the focus calculation is updated continuously in the background, a software routine monitors the direction and position of the stage and it would only activate the PIFOC once the stage is moving in the scanning direction and having passed the starting point.

**Focus Profile Recording and Playback**

The trailing camera in this instrument is a monochrome camera. Scanning specimens with multiple dyes requires multiple passes with filter sets to acquire the different colors. For these types of scan, autofocus is performed only during the first pass and the focus positions (or profile) is recorded as a function of the stage position. The focus profile is played back in subsequent passes during the acquisition of the additional colors. Recording and playback begins at the starting point of the strip. The start-of-acquisition trigger described in Section 2.8.7 also triggers software modules to begin record or playback the focus profile as the starting point passes over the objective lens. The step signal of the Y axis is fed to counter 1 of DAQ device 1 which is set to divide-by-N mode to provide the sampling clock during recording and playback (see Figure 2.21). The counter is set to provide a sample clock that would record or playback the focus positions ten times for every field of view. For example, the 1024×1024-pixel field of view of the camera with a 20× 0.75 NA objective is about 225 µm×225 µm at Nyquist sampling. The sample clock is set to give out a pulse for every 22.5 µm traveled in the Y direction, or one pulse for every 89 steps (stage resolution = 3.94 steps/µm).

### 2.8.8 Slide-Scanning Routine

The procedure for scanning a slide is illustrated by the flow chart in Figure 2.41. A scan of a slide consists of the acquisition of image data from multiple strips that make up the area of the scan. Each strip, in turn, consists of one or more data channels (colors). The operation of slide scanning is controlled by two main software modules: Scan Sequencer — which handles the high-level strip-to-strip events,
and Strip Scan — which controls the low-level synchronization tasks between the different subsystems during the image acquisition of a channel in a strip. Detailed description of each module is presented below.

**Scan Sequencer Module**

After the scan is initiated, Scan Sequencer moves the slide to the top-left corner of the scan area to start the acquisition of the first strip. For each channel in the strip, Scan Sequencer moves the appropriate dichroic filter, neutral density filter(s) into the light path and selects the illumination source according to the settings specified by the user (see Figure 2.41). It then calls Strip Scan to perform the image collection. Once Strip Scan finishes, Scan Sequencer switches filter(s), selects light source, and repeats the process until all channels of the strip have been scanned. The slide is then moved to the start of the next strip and the sequence repeats until all strips of the scan has been processed.

**Strip Scan Module**

Strip Scan is responsible for synchronizing the stage motion, the autofocus, and the trailing TDI camera to produce well-focused images during the scan (see Figure 2.42). When invoked by Scan Sequencer, Strip Scan first determines the order of the current channel in the strip. If it is the first channel, Strip Scan uses the 2D camera in the array to determine the best focus position at the start of the strip to which it positions the objective lens using the Z-motor. This position is stored and reused for subsequent channels in the strip. The stage is then moved back a certain distance in the opposite direction to the scan direction. This gives the stage time and distance to accelerate up to constant speed as it passes the start of the strip. Next, Strip Scan determines which focus module(s) to invoke. Typically, the autofocus is performed and the focus profile is recorded during data collection of the first channel. For subsequent channels, this focus profile is then played back. After initiating the focus module(s), Strip Scan initiates the image acquisition module which controls the camera. At this point, the focus and the image acquisition modules are waiting for a trigger signal which activates them. This trigger signal is provided by an external circuit (see Section 2.8.7). The stage
Figure 2.41: **Slide Scanning Flowchart.** The procedure for scanning a slide. The scan of a slide may consist of one or more strips. And for each strip, it may consist of one or more channels (dye colors). The data acquisition of each color in the strip is performed by the Strip Scan module (see Figure 2.42).
Figure 2.42: **Strip Scan Flowchart.** The procedure for scanning a channel (color) of a strip on the slide.
is commanded to accelerate to the speed specified by the user. As it passes the start position of the strip, the external circuit triggers the focus and image acquisition modules. During the stage motion, image data is acquired into buffer(s) (see Section 2.8.5) while the focus module(s) maintain image quality. When the stage arrives at the end of the strip, Strip Scan stops the focus module(s) and waits for the image data to be written to the hard drive. With the images safely stored on the hard drive, Strip Scan stops and returns control to Scan Sequencer.

2.8.9 Metadata

Metadata is composed of the Greek (meta) and Latin (data) words for information. Together, they literally mean “data about data” or information about another set of data. The control software has a Metadata module that embeds information into the image files. Typically, this additional information contains the hardware settings of the systems; however, the user can add any additional information one would like to be included. With this type of metadata, the images themselves contain the record of the experimental setup and conditions.

The metadata is based on the well known Extensible Markup Language (XML) standard. The user can define one’s own data to be included and there is no size limits on how large the metadata can be. The metadata is appended to the end of the image file so that it does not interfere with other software programs that do not support it. The end of the metadata is terminated with a 16-bit string “LKNMTDT####” where # is the size of the metadata in bytes. This allows the metadata reader to determine where from the end of the file to start reading the data. An example of the metadata is shown in Figure 2.43.
Figure 2.43: Metadata Viewer. An example of the metadata that is embedded in the image files.
2.8.10 Miscellaneous

The software also has several features that assist the user in the operation of the system:

**Email Notification:** For very large scans that take a long time to complete, the user can tell the system to notify oneself through email when the operation is completed or more importantly if any errors had occurred.

**Temperature Monitor:** The system monitors the temperature of the water cooling system and the camera array enclosure. If any of these temperatures falls out of their normal operating range, an alarm will sound and the user is notified through email.

**Hard Disk Checking:** Before starting the scan, the system calculates how much disk space will be needed to store the data. If there is not enough disk space remaining, the system notifies the user and does not allow the scan to proceed.
Chapter 3

Cytometer Performance Evaluation

3.1 Introduction

The cytometer developed in this dissertation is a complex instrument that depends on the interaction between many subsystems for proper operation. Degradations in performance in one part or at one stage of the system often get transmitted and propagated to other parts or stages. Therefore, to properly evaluate the performance of the system, individual components must be carefully and systematically characterized. This chapter describes this process. First, the individual subsystems (optical, electrical, and automation software) were evaluated separately. Once the characteristics of these subsystems have been established, the performance of the whole system was evaluated.

The performance of the system depends on both the instrumentation and the specimen. Evaluation of the results must be done in the context of the quality of the specimen. First, to separate out the effects of specimen quality, a Ronchi ruling (an equal spacing line grating) was used in a series of experiments to establish the performance of the system under optimal conditions. The performance of the system was then measured on cell specimens. The effects of specimen quality on overall performance were examined. Finally, using a spiked cell model of CTCs, a comparison with an incremental-scanning cytometer was carried out to determine the relative performance on CTC detection.
3.2 Image Splitting Optical Assembly

3.2.1 Simultaneous Multiplanar Imaging

Figure 3.1 qualitatively demonstrates simultaneous imaging of multiple focal planes achieved with the image splitting module. A microscope micrometer slide was tilted such that only a portion of the field of view was in focus at any focal plane. The camera was first placed in channel 1 and focus was adjusted so that the top part of the field of view was put into focus. With the focus knob locked, the camera was then moved to channel 2. The lenses of the magnification system and the camera position were adjusted until the image had the same magnification as the one previously obtained in channel 1, and the in-focus portion of the image were translated down. Similar steps were done with channels 3 and 4 to produce the images shown in Figure 3.1. The trailing camera (ORCA 100) was used in 2D mode to capture these images instead of the focus measuring TDI cameras because the slide was stationary.

Figure 3.1: **Tilted Slide Demonstrating Simultaneous Imaging.** Images of a tilted slide collected simultaneously from four channels of the parallel imaging module. Translation of the in-focus region (indicated by the arrows) in the images demonstrates simultaneous sampling of multiple image planes.
3.2.2 Transmission Efficiency

The optical image splitting assembly contains a substantial amount of glass and high number of glass-air surfaces. Light loss due to scattering and reflections at glass-air interfaces affects the contrast of the image information being transmitted through it. The transmission efficiency of each channel was measured and compared to theoretical values.

Referring to Figure 3.2, the light paths in the channels have between 75 mm and 150 mm of glass, and either 10 or 12 glass-air surfaces (see table 3.1). Ignoring scattering loss and using the manufacturer’s specified values of 0.5% reflection loss at each glass-air interface and 45%–45% split (with 10% absorption) in each beamsplitter, the maximum theoretical transmission in the channels are 8.58% and 8.67% for 12 and 10 glass-air surfaces, respectively (\(0.45^3 \cdot 0.995^n\), where \(n\) is the number of glass-air surfaces).

Figure 3.2: Image Splitting Module Light-Path Diagram. Diagram showing the light paths inside the image splitting module. Channels have different path lengths in glass and different numbers of glass-air interfaces. See table 3.1.

To measure the transmission efficiency, the system was set up for Köhler illumination, and the light levels at several locations in the system were measured with a power meter (model 840, Newport Corporation, Irvine, CA). A 100W mercury
Table 3.1: **Image Splitting Module Transmission Efficiency**

<table>
<thead>
<tr>
<th>Glass-Air Interfaces* in Glass (mm)</th>
<th>Path Length in Glass (mm)</th>
<th>Measured Transmission (%)</th>
<th>Theoretical Transmission (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH1</td>
<td>12</td>
<td>100</td>
<td>4.73</td>
</tr>
<tr>
<td>CH2</td>
<td>10</td>
<td>75</td>
<td>4.06</td>
</tr>
<tr>
<td>CH3</td>
<td>10</td>
<td>100</td>
<td>5.48</td>
</tr>
<tr>
<td>CH4</td>
<td>12</td>
<td>125</td>
<td>5.77</td>
</tr>
<tr>
<td>CH5</td>
<td>12</td>
<td>150</td>
<td>6.28</td>
</tr>
<tr>
<td>CH6</td>
<td>10</td>
<td>125</td>
<td>4.91</td>
</tr>
<tr>
<td>CH7</td>
<td>10</td>
<td>100</td>
<td>3.80</td>
</tr>
<tr>
<td>CH8</td>
<td>12</td>
<td>125</td>
<td>3.38</td>
</tr>
</tbody>
</table>

*Eight of the glass-air interfaces are from the lenses: two from the collimating lens and six from the three magnification correction lenses.

vapor arc-lamp filtered with a 600 nm long-pass filter and attached to the transillumination tower provided the light source. The Nikon 20× 0.75 NA Ph2DL objective lens was used in bright-field mode. The light power was measured at the following locations:

1. before the objective lens with the field diaphragm fully open
2. before the objective lens with the field diaphragm partially constricted to be slightly larger than the field of view of the microscope viewed through the eyepieces
3. after the objective lens (the power meter was placed in the space of the fluorescence filter cassette)
4. at the image plane of the side-port of the microscope
5. after the collimating lens (before the beamsplitter assembly)
6. at the output of each channel (including magnification correction lenses)

The measured values converted to percentage are plotted in Figure 3.3. Light illuminating the area of the field of view comprised only about 19% of the available illumination. The transmission losses in the objective, the microscope, and the collimating lens were 89.5%, 58.4%, and 79.5%, respectively. The transmission
efficiencies of the channels range from 3.38% to 6.28%, all much less than their theoretical values.

Figure 3.3: **Optical Transmission Efficiency.** Fluor 20× 0.75 NA Ph2DL objective in brightfield mode. Arc-Lamp with 600-nm long-pass filter. Measured with Newport Optical Power meter at 626 nm. Only about 19% of available illumination is received by the objective. The transmission loss in the objective is about 90%. The transmission loss in the microscope is 58.4%. The transmission loss at the collimating lens is 79.5%. The transmission efficiencies of the imaging channels range from a low value of 3.38% to high value of 6.28% all less than the maximum theoretical value of 8.67% (the total transmission of all channels is 38.39%).
3.2.3 Resolution

The point spread function (PSF) is a measure of the optical quality of an optical system. Using the method described by Boreman [12], the PSF was measured for each channel to determine the optical quality of the image splitting assembly. The PSF is the image of a point source. The degree of spreading (blurring) is a measure of the quality of the optical system. A Nikon Plan Apo 60× 1.40 NA Oil objective lens was used to image sub-resolution fluorescent microbeads with a Hamamatsu OR100 camera. For reference, the PSF of the microscope at the side-port was measured without the optical image splitting assembly. Also for comparison, the PSF of the two outputs of a Nikon Dual Imaging Module (a commercial two-channel image splitting product that also contains a beamsplitter and relay optics) were also measured. For each condition, twelve PSFs were collected from different regions of the field of view (that included regions both in the center and near the edges). These values were then averaged. The results are plotted in the top part of Figure 3.4. The full-width-half-max (FWHM) values are listed in the legend. The Fourier transforms of the PSFs were taken to determine the effects of the image splitting assembly on the modulation transfer function (MTF).

The comparison of the MTFs is plotted in the bottom part of Figure 3.4. The microscope’s FWHM was found to be 0.240 µm and used as a reference value. With the addition of the image splitting assembly, all channels degraded the resolution to some degree ranging from 2.08% at best (0.245 µm, channel 4), to 39.6% at worst (0.335 µm, channel 7), with an average value of 16.8%. For comparison, the degradation caused by the Dual Imaging Module were 6.67% and 13.3% for an average of 10%. Similar results are observed with the MTFs. The cutoff frequencies (typically defined at 10% of maximum) with the image splitting assembly are lower in comparison to reference value. In addition, there are unusual undulations in the frequency spectrum of channels 7 and 8. The results show that the transfer functions of the channels are not substantially degraded compared to the microscope’s.
Figure 3.4: **Image Splitting Module PSF/MTF Comparison.** Experimental Conditions: Nikon Plan Apo 60× 1.40 NA Oil. Hamamatsu ORCA100 camera, subresolution fluorescent microbeads 0.17 μm. SP = side-port; DIT-L = Dual Imaging Module Left-Port Output; DIT-R = Dual Imaging Module Right-Port Output.
3.3 TDI Camera Performance

Following the optical image splitting module, the TDI cameras of the focus measurement array are the next component in the optical system. To measure the response of the system including the TDI cameras, the edge spread function (ESF) was measured for each channel of the array according to the method described by Boreman [12]. A razor blade aligned parallel to the charge transfer of the cameras served as the edge. The video signal from each camera was captured with a framegrabber (model IC-PCI, Coreco Imaging, St. Laurent, Quebec, Canada). To improve signal to noise, 96 video lines were averaged to obtain the ESF. The derivative (first difference) of the ESF was taken to get the point spread function (PSF). The modulation transfer function (MTF) was then obtained through a Fourier transform of the PSF. The results are plotted in Figure 3.5. These MTFs, which include the response of the focus measurement cameras, are worse than the ones obtained without them (Figure 3.4). The cutoff point is lowered to about 2.5 cycles/µm for most channels with channel 2 being the lowest. Furthermore, the MTFs of channels 5 and 8 show some unusual characteristics between 0.5 and 1 cycles/µm which indicates some sort of distortion.

3.4 System Mechanical Stability

The axial locations of the focus measurement channels are some of the determining inputs in the calculation of best focus. These values are measured at the beginning of the scan as part of the calibration. If the cameras were to move during the scan, the accuracy of the focus calculation and performance of the system would be degraded. Because of thermal expansion, there is a possibility of this happening as the system heats up. To investigate this scenario and to establish the effects of temperature on the system, a series of experiments was performed to determine its thermal stability of the system.

To investigate the effects of temperature on the focus measurement channels, the location of maximum focus index of each channel was recorded as a function of time at 2-minute intervals over a 10 hour period. Figure 3.6 shows the movements of the channels as a function of time due to temperature changes during different
Figure 3.5: Modulation Transfer Function of Focus Measurement Channels. A comparison of the MTFs of the focus measurement channels. MTFs were obtained from measuring the edge spread function of a razor blade. Experimental Conditions: Nikon Plan Apo 60× 1.40 NA Oil objective lens, bright-field, TDI focus measurement cameras, Coreco IC-PCI framegrabber.
parts of the day. Over a 10 hour period, the channels drifted as much as 2 µm; however, they moved together. This means that although the channels drift, the relative spacings remain the same. Because of this, the autofocus calculation was modified to use the relative spacing values instead of the absolute location values as its inputs.

It was also necessary to investigate the mechanical stability of the specimen holder. For scans with multiple colors, since the trailing camera is a monochrome camera, each color is acquired separately and sequentially. There are two options for doing this: 1) scan the entire slide with one color, then switch filters and proceed with the next color or 2) scan all colors for each strip before moving to the next strip. With our existing hardware, it would be more efficient with method 1; however, concern about thermal effects would necessitate implementation of method 2. To determine the appropriate approach and also to investigate the possibility of any other mechanical instability not related to thermal effects, the following experiment was conducted. A microscope slide with NIH 3T3 fibroblasts was used to investigate the mechanical stability of the system. The focus positions at the four corners of a 7.620×21.844-mm² scan area of a microscope slide containing NIH 3T3 fibroblasts were repeatedly determined at 5-minute intervals at 0.1 µm resolution over a 24 hour period. The results are presented in Figure 3.7. During the experiment, the four corners drifted axially approximately 5 µm; however, the entire slide moved together such that the corners’ relative positions remained constant. The results established that method 2 was necessary and there was no other mechanical instability. As a result, automation to the filter cube slider, the arc-lamp shutter, and the neutral-density filters were added to the system.

3.5 Stage Speed Fluctuation

Using the linear encoder, the speed fluctuation in the Y-axis of the stage was measured. The linear encoder was set to 100:1 down sampling such that it would output a pulse for every 1 µm traveled. The output pulse was then lengthened to 40 µs with the line clock conditioning circuit (see A.2.3). The output pulse train was digitized with an A/D channel of the PCI-6031E DAQ card at 100 kSamples/s. The stage was moved through 280,000 steps (71.12 mm) near the center of the
Figure 3.6: **Thermal Stability of Focus Measurement Array. top)** The positions of the focus measurement channels as a function of time, **bottom)** The relative displacement of the focus measurement channels compared to the 2D camera as a function of time. The positions of the focus measurement channels (position of maximum focus measure) drifted over time; however, they drifted together so that the relative displacements between channels remained constant. Channel 2 was the most unstable (highest SD), hence was excluded from best focus calculations in subsequent experiments.
Figure 3.7: **System Mechanical Stability.** The positions of the four corners of a $7.620 \times 21.844 \text{ mm}^2$ area of a slide were recorded at 0.1-μm resolution over a 24 hour period at 5-minute intervals. The system drifted approximately 5 μm over this time period. However, the entire system moved together so that the relative positions of the slide remained constant. TL = top-left; TR = top-right; BL = bottom-left; BR = bottom-right.
stage at 10,000 steps/s (2.54 mm/s). A small portion of the output pulse train is shown in the top panel of Figure 3.8 in which the non-uniform spacing between the pulses can be seen. The bottom panel of Figure 3.8 shows the distribution of time periods between pulses. With a stage resolution of 3.937 steps/µm, the mean of the distribution is expected to be at 397.3 µs. However, the distribution is centered around 417.15 µs (5% error from the expected value). Furthermore, the fluctuation in speed is relatively large ranging from a minimum of 210 µs to a maximum of 620 µs with a standard deviation of 12.6%.

3.6 Autofocus Performance

There are no standards for evaluating the autofocus performance of scanning cytometers. Autofocus performance depends on both instrument parameters and specimen conditions. On the instrument side, autofocus depends on parameters such as the numerical aperture, the axial focus sampling intervals, the virtual frame size, the number of focus measuring channels, the focus search range, and the focus update frequency. On the specimen side, the cellular density (image content), the specimen thickness and the slide flatness affect the performance of autofocus. Therefore, any evaluation of the autofocus performance must take into consideration not only the instrument’s performance but also the effects contributed by the specimen. In addition, it is sometimes impossible to separate out components to evaluate their individual performance. Rather, the entire system must be evaluated as a whole.

The primary measure of the autofocus performance is focus accuracy. Since the true focus position is not known, we used the best focus position determined with incremental scanning as an estimate of true focus (see 3.6.1). The focus accuracy was then defined as the difference in focus positions obtained between the incremental scanning and the continuous-motion scanning.

To evaluate the instrument, the numerical value of focus accuracy was compared to the system’s depth of field. The depth of field is defined as the axial range through which an objective can be focused without any appreciable change in image sharpness. Therefore, we define acceptable focus accuracy to be one half of the depth of field (i.e., a difference in focus positions between the incremental scan and
Figure 3.8: **Stage Speed Fluctuation.** Stage was moved at 10000 steps/s while setting the linear encoder to output a pulse every 1 μm (external divider set at 100:1 with pulse stretcher set at 40 μs. The data was digitized at 100,000 samples/s. The non-uniform spacing between pulses in the top graph shows the fluctuation in stage velocity. The bottom graph shows the temporal distribution of the output pulses. With the stage resolution of 3.937 steps/μm, the period between output pulse should have been 397.3 μs. The minimum and maximum periods measured were 210 μs and 620 μs, respectively. The speed fluctuation (1 SD) is about 12.6%.
the continuous-motion scanning of less than half the depth of field is acceptable). At high NAs, the depth of field is primarily determined by wave optics, while at lower NA the geometrical optics dominates. Using different criteria for when the image becomes unsharp, many authors have proposed different formulas to describe the depth of field. In the evaluation of the performance of this instrument, we used the definition by Inoué and Oldenburg [54] which combines the wave and geometrical depths of field as

\[ d_{\text{tot}} = \frac{\lambda n}{NA^2} + \frac{n}{M \cdot NA} e \]  

where \( d_{\text{tot}} \) is the total depth of field, \( \lambda \) is the illumination wavelength, \( n \) is the refractive index of the imaging medium, \( NA \) is the numerical aperture of the objective lens, \( M \) is the lateral magnification, and \( e \) is the resolution of the detector.

For the experimental setup that was used to evaluate the performance of the system which included a 20× 0.75 NA Ph2DL objective, 700 nm illumination source, 30.45× magnification at the image plane, and 6.7 µm camera pixel, the total depth of field was 1.538 µm. Thus, a focus difference of 0.769 µm or less was deemed acceptable.

A series of experiments was conducted to evaluate the autofocus performance of the system. First, the software module for the autofocus was tested to verify that it satisfied the timing requirement. The static and the dynamic behaviors of the autofocus loop were characterized. Then, the focus accuracy was measured first using a Ronchi ruling and then specimens containing cells. In these experiments, the incremental autofocus was used as the reference to determine the performance of the system’s parallel autofocus. Consequently, one of the first tasks was to establish the variance of incremental autofocus prior to using it as the reference. This process is described in the next section.

### 3.6.1 Incremental AutocuFOCUS Variance

To evaluate how well the instrument performs in keeping the specimen in focus, the focus positions calculated by the instrument need to be compared to the true focus positions in the specimen. However, the true focus positions are not known. The best we can achieve is estimating the true focus positions with an independent
method. For all the autofocus performance experiments, the best focus found with the more proven incremental focus testing was used as an estimate of the true focus position. Being estimated, these measures have errors associated with them. Before using them as a basis to evaluate the performance of the system, it is necessary to establish their goodness as an estimate of the true focus positions. In other words, we need to determine how large the typical error of the estimate is or the variance of the measure.

A microscope slide containing NIH 3T3 fibroblasts was used to measure incremental autofocus variance. The process involved finding the best focus position twice, one immediately after the other, for each field of view in the 34×97 scan area. The distribution of the difference between the two best focus positions over the entire scan area gave the value for the measure’s variance. The best focus position was determined with a 10-µm search at 0.1-µm steps with 30-ms delay between steps using the 2D camera in the array. The search was performed in phase-contrast with a 20× 0.75 NA Ph2DL objective lens. The result is plotted in Figure 3.9. The standard deviation (SD) of the difference was 0.364 µm.

3.6.2 Focus Loop Timing Performance

Table 3.2 lists the execution time for the software subroutines in the autofocus loop. After a 15.6 ms initialization delay at the start of the loop, the software portion of each focus update cycle took a total of 10.9 ms.

For each focus update, the objective lens actuator (PIFOC) takes about 20 ms to move and settle at the new position. Combining this hardware settling time with the software execution time, the total time for each focus update cycle is 31.9 ms (or maximum update rate of about 31 Hz). However, this is a very conservative timing scheme. Since the PIFOC travels most of the distance (80–90%) during the first half of its response time, the focus update cycle can be shortened by executing the software part of the cycle during the second half of the PIFOC’s response time which shortens the update period to 20 ms. With this approach, a large increase in update rate is achieved for a slight decrease in accuracy. The idea behind the compromise is to achieve the high update rate necessary to track fast-moving specimens.
Figure 3.9: **Incremental Focus Variance.** A slide containing NIH 3T3 fibroblasts was used to determine the variance of incremental autofocus. For each field of view in the 34×97=3298 FOV area, the best focus position was found twice, one immediately after another. The distribution of the difference is plotted above. The search was performed with the 2D camera in the array using 10-µm range, 0.1-µm steps, and 30-ms delay between steps. A 20×0.75 NA Ph2DL was used in phase-contrast mode. Mean = −0.0014 µm, SD = 0.364 µm, skewness = 0.230, kurtosis = 143.532
Table 3.2: Focus Loop Timing Performance

<table>
<thead>
<tr>
<th>Software Task</th>
<th>Hardware Event</th>
<th>Execution Timea (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(LabVIEW Subroutine)</strong> Focus Data Acquisition Offset Subtraction &amp; Scaling <strong>(ARRAY_Get_Measurements_DAQmx.vi)</strong></td>
<td>Read from 28 analog input channels</td>
<td>10.3</td>
</tr>
<tr>
<td>Best Focus Calculation <strong>(Best_Focus_Algorithm.vi)</strong></td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td><strong>(PIFOC_Move_DAQmx.vi)</strong> Move Objective Lens</td>
<td>Write to analog output channel</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>10.9</strong></td>
</tr>
</tbody>
</table>

The execution times were measured with LabVIEW’s Performance Profiling tool for a scan with over 15,000 focus updates. There was an initialization delay of 15.6 ms.

3.6.3 Parallel Autofocus: Feedback Loop Stability

The autofocus system is a closed loop system with the image content being the feedback signal. The system constantly works to correct for the error between the current position of the objective lens and the calculated best focus position. The system is configured for negative feedback. The feedback signal is inverted on its way round the control loop to increase the stability and the accuracy of the system. However, this scheme can fail if the input changes faster than the system can respond to it. When this happens, the negative feedback signal begins to act as positive feedback, causing the output to oscillate. This phenomenon leads to overcompensation and unstable behavior.

To explore the system’s boundary of stability, the autofocus feedback loop was commanded to maintain focus at different gains and update rates. Higher gain settings increase the size of the corrections which correspond to the distance that the PIFOC has to move the objective lens. Decreasing the update rate slows down the response of the system. To examine these effects, the position of the PIFOC was recorded to observe any oscillatory behavior. Figure 3.10 shows the results. While holding the update rate at 44 Hz, increasing the gain to 1 caused the system to take larger correction steps which resulted in the larger standard deviation values. With further increase in the feedback gain, the system started
to oscillate. Similarly, decreasing the update rate quickly set the system into oscillation.

Figure 3.10: **Stability of Focus Feedback Loop.** To test the stability of the autofocus feedback loop, the system was initiated to keep focus and the position of the objective lens was recorded over a period of time. The plots show the stability of the system as a function of the feedback gain. (The stage was stationary with Ronchi ruling as the specimen. Autofocus was performed under phase contrast with a Nikon Plan Fluor 20× 0.75 NA Ph2DL objective lens in phase-contrast. Power-Weighted-Average algorithm, w = 8.)
Figure 3.11: **Focus Feedback Loop - Unstable Conditions.** Feedback gain and update-rate were altered to explore conditions that would cause instabilities. 

A) Gain = 1; Update rate = 44 Hz. Compared to conditions in Figure 3.10, the higher feedback gain increased SD to 0.3177 µm and a very brief initial oscillation (∼1 s),  

B) Gain = 2; Update rate = 44 Hz. The high gain resulted in larger correction steps. The system could not keep up and resulted in oscillation.  

C) Gain = 1; Update rate = 35 Hz. Slowing the update rate to 35 Hz caused the system to oscillate for a few seconds before settling to a stable position.  

D) Gain = 1; Update rate = 25 Hz. The system could not keep up with further decrease in update rate and put the system into oscillation. (The stage was stationary with Ronchi ruling as the specimen. Autofocus was performed under phase contrast with a Nikon Plan Fluor 20× 0.75 NA Ph2DL objective lens in phase-contrast. Power-Weighted-Average algorithm, w = 8.)
3.6.4 Parallel Autofocus: Keep Focus Tracking

Having determined the static feedback loop stability, simulations of focus tracking were performed to characterize the dynamic behavior of the autofocus system. The Ronchi ruling was again used as the specimen to provide a consistent image content input. The stage was stationary. To simulate changes in focus, the fine focus knob was programmed to move the objective nose piece up and down in a trapezoidal wave pattern. In order to compensate for the changing distance between the objective and the specimen, the autofocus system commanded the PIFOC to contract and to expand to keep the lines of the Ronchi ruling well focused. The root mean square error of the difference between the fine-focus-knob and PIFOC positions was used as the measure of performance.

Figure 3.12 shows the results of the simulations. Initially, with a small focus-change slope of 0.5 µm/s simulating a relatively flat specimen, the system was able to track the focus changes well (RMS error = 0.286 µm) with a modest feedback gain of 0.1. The focus profile was smooth with very little oscillation at the extrema. When the focus slope was increased to 5 µm/s to simulate the upper end of the conditions encountered with cell specimens, the small 0.1 gain could not keep up with the changes in focus. The PIFOC lagged behind the fine-focus-knob almost all of the time. When the feedback gain was increased to an adequate level, the system was able to track the focus changes. However, the error was larger (0.439 µm) and there were noticeable oscillations at the extrema.

In addition, in the second scenario the system also exhibited non-symmetric behavior showing slightly better tracking ability with the down slope than the up slope. This is caused by the different responses (shape/width of the focus function curves) of the focus measuring channels. The channels do not have identical response — some have wider focus function curves than others. Typically, this is not apparent when the system is working near the best focus position at which each channel’s output is close to the peak of its focus function curve. However, when the system is far away from the best focus position, the channels with wider focus function curves have higher signals compared to those with narrower ones as illustrated in Figure 3.13. Thus, the channels with wider focus function curves affect the result of best focus calculation more in these conditions.
Figure 3.12: **Focus Tracking Dynamics.** Focus tracking was simulated by driving the fine-focus knob in a trapezoidal-wave pattern and let autofocus try to compensate. Different slope values were used to explore the dynamics of the system. With a small slope (0.5 \( \mu \text{m/s} \)) simulating slowly-changing focus, a gain value of 0.1 was able to compensate for the change. However, with the steeper slope value (5 \( \mu \text{m/s} \)), the gain needed to be increased to 0.6 in order compensate for the focus change. The larger gain also caused more oscillatory behavior which is most noticeable at the flat portions of the wave patterns. The stage was stationary with Ronchi ruling as the specimen. Autofocus was performed under phase contrast with a Nikon Plan Fluor 20\( \times \) 0.75 NA Ph2DL objective lens. Focus update was at 44 Hz with six focus measurement channels (1, 3–6 and 8).
Figure 3.13: **Effect of Non-Uniform FFC on Best Focus.** Illustration showing the effects of non-uniform focus function widths on the calculation of the best focus. **A) uniform focus function curves:** the ratio of focus measures is the same when the system is displaced by the same amount in both directions; therefore, the autofocus performance is identical regardless of the direction of displacement; **B) non-uniform focus function curves:** when the system is near the best focus position, contributions from the two channels are approximately equal. However, when displaced far away from the best focus position, the ratio of focus measures is dependent on the direction of displacement and thus also is the autofocus performance. For example, in this particular case, the system would be more sensitive to a focus shift to the right (+Z) than it would be to a shift to the left (−Z).
3.7 Scanning Performance on Ronchi ruling

The initial performance evaluation of the system was performed with a Ronchi ruling. The uniform parallel lines of the Ronchi ruling provided consistent image content for these initial tests. The lines of the Ronchi ruling were aligned parallel with the direction of the charge transfer in the TDI sensors. The Ronchi ruling was tilted to a slope of 0.638 μm/mm and a 21.82-mm strip was repeatedly scanned with incremental and continuous scanning to determine the focus accuracy. A Nikon 20× 0.75 NA Ph2DL objective lens in phase-contrast mode was used for all the experiments in this series. For comparison, an incremental scan was first run to determine the focus positions along the strip. It was then immediately followed by a continuous scan. With the incremental scan being used as a reference, the focus positions along the strip obtained with the two methods of scanning were compared to determine the continuous scanning focus accuracy. The incremental scan portion of the test was slow taking approximately six minutes to complete. This means that there was a six minute time difference between the starts of the incremental and the continuous scans. It was possible that the specimen could have drifted during this time which would contribute to the difference in the focus positions obtained by the two scanning methods. To investigate this possibility and to get a measurement of the drift if there was any, the comparison was run consecutively for 200 times. The difference in focus positions between two consecutive incremental scans gave a measure for the drift, to which the error between incremental and continuous focus positions was compared.

Over a period of approximately two months, many sets of these experiments were performed to measure the performance, to debug the hardware and software, and to fine tune the system. The results are summarized in Figure 3.15 and table 3.3 lists the parameters of these experiments.
Figure 3.14: **Focus Accuracy on Ronchi-Ruling.** A) Scan with error approximately equals to the average error, B) Example of scan with minimum error, C) Focus profiles of 200 trials showing drift, D) Comparison of RMS errors. Tilt = 0.638 μm/mm; Scan Length = 21.82 mm; 200 Trials; Incremental-Scanning RMS error: min = 0.0844 μm; max = 0.7108 μm; mean = 0.1879 μm; SD = 0.1148 μm. Continuous-Scanning RMS error: min = 0.2268 μm; max = 1.1254 μm; mean = 0.3997 μm; SD = 0.1428 μm.
Figure 3.15: **Summary of Focus Accuracy on Ronchi ruling.** Results of a series of experiments during which the system was being debugged and fine-tuned. For each data set, the Ronchi ruling was scanned 200 times each in both incremental and continuous mode. **Top)** a plot of all data sets, **Bottom)** a close-up view of the latter and better data sets.
Table 3.3: Parameters of Continuous Scans on Ronchi ruling.

<table>
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<tr>
<th>Date</th>
<th>Date Set Name</th>
<th>Gain</th>
<th>Weight</th>
<th>Max Update (um)</th>
<th>Lamp Voltage (V)</th>
<th>Continuous-Scan RMS Error (um)</th>
<th>Mean</th>
<th>SD</th>
<th>Incremental-Scan RMS Error (um)</th>
<th>Mean</th>
<th>SD</th>
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</table>

Six focus measurement channels (1, 3–6 and 8) were used for all scans.
3.8 Scanning Speed

The scanning speed is limited by how fast the system can reliably maintain focus. A series of experiments were carried out to determine the system’s maximum scanning speed. Table 3.4 lists the results for the different experimental conditions. For each condition, the system was set to scan a 22 mm strip of the specimen. Starting at a relatively slow stage speed, the illumination was adjusted to obtain good signal levels from the focus measurement channels. Once optimized, the system was set to scan the strip three times. The stage speed was slowly increased and the same process repeated at each speed increment until the system could no longer maintain focus. Judging the system’s ability to maintain focus was done visually through inspection of the acquired images. The highest speed at which the system could maintain focus during all three passes was recorded as the maximum scanning speed. The high contrast of the Ronchi ruling allowed the system to work in both bright-field and phase-contrast mode, while sufficient contrast could only be obtained with the cell specimen in phase-contrast mode.

Table 3.4: Maximum Scanning Speeds. The scans were performed with a Nikon 20 × 0.75 NA Ph2DL objective lens. The average sampling density of the focus measurement cameras was 0.33 µm/pixel and the trailing camera’s sampling density was 0.22 µm/pixel.

<table>
<thead>
<tr>
<th>Mode</th>
<th>Specimen</th>
<th>Lamp Voltage (volts)</th>
<th>Array Cameras Line Rate (Hz)</th>
<th>Trailing Camera’s Line Rate (Hz)</th>
<th>Scan Speed (mm²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brightfield</td>
<td>Ronchi ruling</td>
<td>6.8–7.2</td>
<td>8224</td>
<td>12336</td>
<td>0.611</td>
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<tr>
<td>Phase Contrast</td>
<td>Ronchi ruling</td>
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<td>7184</td>
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<td>Phase Contrast</td>
<td>Cells</td>
<td>7.0–8.5</td>
<td>5580</td>
<td>8370</td>
<td>0.415</td>
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</table>

The system’s overall scan time was also measured. Besides the actual scan during which autofocus, stage motion and image acquisition occur, there are other software and hardware events taking place before and after the actual scan that contribute to the total scanning time. These events consist of setting up hardware parameters such as illumination, filters, framegrabber, etc. Table 3.5 lists the average execution times of the events that take place during scanning of a strip of the specimen. For example, the overall time of a 22 mm strip was measured to be 24.7 seconds during which the actual scan portion only took 11.95 seconds. This greater than 50% overhead is mainly due to the inefficient implementation of
hardware components unrelated to autofocus (control of light source and filters). Table 3.5 also lists alternative implementations that would eliminate most of this extra overhead. The research effort and resource were concentrated on optimizing autofocus performance and not on these aspects of the instruments since they are readily available and have been demonstrated.

Table 3.5: System Performance - Overall Scanning Speed. The execution times for the different software / hardware events during scanning of a strip of the specimen. For a 22 mm strip, the actual scan and overall times were 11.95 s and 24.7 s, respectively. Experimental conditions: Nikon 20× 0.75 NA Ph2DL objective lens, phase-contrast, 8370-Hz trailing camera line rate, Ronchi ruling specimen

<table>
<thead>
<tr>
<th>Order of Execution</th>
<th>System Event</th>
<th>Execution Time</th>
<th>Current Implementation</th>
<th>Optimal Implementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1′</td>
<td>Position specimen at beginning of strip / back trace</td>
<td>~ 5 s (^a)</td>
<td>(depends on length of strip)</td>
<td>~0.5 s with bidirectional TDI</td>
</tr>
<tr>
<td>2</td>
<td>Find best-focus at beginning of strip</td>
<td>3 s</td>
<td></td>
<td>&lt; 1 s with secondary displacement sensor(^b)</td>
</tr>
<tr>
<td></td>
<td>Adjust Halogen Lamp Brightness</td>
<td>5.3 s</td>
<td></td>
<td>10 ms with shutter</td>
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<tr>
<td></td>
<td>Open Arc-Lamp Shutter</td>
<td>2 s</td>
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<td>10 ms with shutter</td>
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<tr>
<td></td>
<td>Switch Dichroic Filter / ND Filters</td>
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<td></td>
<td>100 ms with filter wheels</td>
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<tr>
<td></td>
<td>Allocate Framegrabber buffers</td>
<td>0.4 ms/frame</td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>Move specimen back N steps</td>
<td>~ 1 s (^a)</td>
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<tr>
<td>4</td>
<td>Set external timing circuit</td>
<td>0.1 s</td>
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</tr>
<tr>
<td>5′</td>
<td>Scan (move stage, keep focus, acquire images)</td>
<td>strip length / line-rate</td>
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<td></td>
</tr>
<tr>
<td>6′</td>
<td>Write image data to disk</td>
<td>~20 MB/s</td>
<td>Faster with future disk drive technology</td>
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</tr>
</tbody>
</table>

\(^a\) Writing image data to disk starts during the scan (after 1/3 of the strip has been scanned) and continues during the back-trace of the next strip

\(^b\) Stage settings: speed = 50000 steps/s; acceleration = 10000 steps/s\(^2\); resolution = 3.937 steps/µm

\(^b\) In the current implementation, the system searches a 50-µm range to find the best-focus position. With a secondary sensor that tracks the surface of the cover slip, the system would need to search only a small range ~5 µm
3.9 System Performance on Detecting CTCs

3.9.1 Focus Accuracy on Cell Specimens

Prior to determining the system’s performance on finding CTCs, a series of experiments similar to the ones with the Ronchi ruling were carried out with specimens containing NIH 3T3 fibroblasts. The objectives of the experiments were to determine the system’s performance on scanning cells and the effects of specimen quality on focus accuracy. The specimen preparation is described elsewhere [91]. For each data set, alternating incremental and continuous scans of the same strip were repeated 10 times each. Two types of specimens were used to study the effect of specimen quality: one set of slides was scanned immediately after preparation in which the cells were relatively flat, the other set was allowed to dry for several weeks in which the cells had started to wrinkle and no longer lay in a single flat layer. Figure 3.16 shows a comparison of the scans on a fairly good quality slide. There is relatively good matching in focus positions between the incremental and continuous scans with an overall RMS error of 0.6821 µm which is less than half of the depth of field (1.538 µm). With the dried-out slides, there were large fluctuations in focus between field-of-views which is indicated by the jagged profile of the incremental focus positions (blue trace). The system was not fast enough to track the fast-changing focus profile and the best it could do was to effectively average out the fluctuations as shown in Figure 3.17 which led to the overall RMS error of 1.2863 µm.

3.9.2 System Performance on CTC Detection

To test its performance at finding CTCs, the system scanned blood samples from a healthy individual that were spiked with tumor cells. Identification and classification of the CTCs from normal blood cells were done based on morphological measurements extracted from the scanned images. For comparison, the same samples were also analyzed with a commercial incremental-scanning cytometer. Except for the image acquisition process, the rest of the experimental conditions were identical between the two systems. The performance comparisons were made based on the number of CTCs detected, and the sensitivities and specificities of
Figure 3.16: **Focus Accuracy on a Good Test Slide.** Average of 10 trials. Strip length = 10.56 mm. Nikon 20× Ph2DL objective lens.
Figure 3.17: Focus Accuracy on a Low-Quality Test Slide NIH 3T3. Average of 10 trials. Strip length = 9.21 mm. Nikon 20× Ph2DL objective lens.
the systems.

Specimen Preparation

For *in vitro* experimentation, we developed a model of circulating cancer cells in peripheral blood using cultured cells mixed with mononucleated white blood cells. Tissue culture media and supplements were purchased primarily from Gibco Invitrogen (Carlsbad, CA), Mediatech, Inc (Herndon, VA), and Sigma-Aldrich (St. Louis, MO). The MCF-7 breast cancer cells were grown in media consisting of phenol-free RPMI, 10% FBS, 2 mM L-glutamine, 1% Pen/strep/fungizone, and 1% HEPES buffer (Omega Scientific, Inc. Tarzana, CA).

Starting with a sample of whole blood, we did an enrichment step to isolate the mononucleated white blood cells (mWBCs) and fix them. Breast cancer cells were stained with CellTracker Orange (Molecular Probes, Eugene, OR) while still in culture, for 45 minutes at 37 °C. CellTracker stain establishes “ground truth” to grade performance of classifiers. The cells were washed twice with PBS, removed from culture dishes with trypsin/EDTA, and resuspended in media. The cells were then centrifuged at 400g for 5 minutes, the supernatant removed, and fixed in suspension with 4% paraformaldehyde solution. The breast cancer cells were then mixed with mWBCs at a ratio of 1: 10000, after enumeration with a hematocytometer.

The cells were prepared for imaging by cytocentrifugation onto slides. In particular, we centrifuged the cells onto Silane-Prep™ slides (Sigma). Aminoalkylsilane, like Poly-L-Lysine coating, improves adhesion of tissues and cells by imparting a negative charge to the surface of the glass slide. Prior to mounting coverslips, we performed counterstaining for 1 hr at 4 °C with the stoichiometric nucleic acid stain DAPI (Molecular Probes, Eugene, OR) adapted from Hamada [46]. The DAPI solution consisted of 75 ng/ml DAPI, 10nM Tris, 10nM EDTA, 100nM NaCl, and 2% 2-mercaptoethanol. This preparation yields a bright nuclear signal with excellent resistance to photobleaching.

A total of four slides were prepared, each with two 22×22-mm² coverslips. Several cell densities were prepared to determine the performance of the system as a function of specimen condition.
**Image Acquisition**

**Continuous-Scanning System:** The samples were first imaged on the continuous-scanning system. For each strip in the scan area, two passes were made to acquire the DAPI and CellTracker Orange signal. Autofocus was performed during the collection of the DAPI signal in the first pass. The focus profile was then played back during the collection of the CellTracker Orange signal in the second pass. The emission filter of the DAPI filter cube (model 31000v2, Brattleboro, VT) was removed. During the first pass, the Optical-Path-Changeover was set to position D which had been modified with a custom dichroic mirror to split the focus light and the fluorescence light to the different ports of the microscope (see section 2.5.4). The combination of no emission filter in the DAPI filter cube and the custom dichroic mirror allowed simultaneous transmission of focus signal to the focus measurement array and DAPI signal to the trailing camera. During the second pass, a Spectrum Orange epifluorescent filter cube (model C7868, Chroma Technology) was used to select the wavelengths and the Optical-Path-Changeover was set to A which transmitted 100% of the light to the trailing camera. The samples were imaged with a Nikon Plan Apo 20× 0.75 NA Ph2DL objective lens at 0.22×0.22 μm² sampling density. The DAPI signal was scanned at 8370 Hz line rate. The CellTracker signal, being much dimmer, were scanned at 4604 Hz line rate. To measure focus accuracy, the best focus position of each field-of-view in the strip were determined prior to the scan of each strip and was compared to the focus profile generated during the scan. Table 3.6 summarizes the results of the scans. Some samples had large differences between incremental and continuous autofocus positions. The majority of the cases were caused by a dense layer of debris on those samples. The layer of debris caused the incremental autofocus to falsely lock on to it instead of the cells (Figure 3.18). The continuous autofocus, with lower resolution, did not pick up the debris and was able to lock on to the cells. Furthermore, visual inspection of the images acquired with continuous-scanning revealed that most of them was in focus.

**Incremental-Scanning System:** The samples were then scanned by a Beckman Coulter’s IC100 Image Cytometer (Fullerton, CA). Because of hardware limitations, the specimens were digitized at 0.3225×0.3225 μm² sampling density with
Figure 3.18: **Debris Layer on some CTC samples.** A dense layer of debris on some samples caused incremental autofocus to lock on to the layer of debris (**bottom**) instead of the cells (**top**).
a Hamamatsu ORCA C4742-80-12AG scientific-grade camera (Hamamatsu Corporation, Bridgewater, NJ) through a Nikon Plan Fluor 20× 0.5 NA Ph1 DLL objective lens. A four-color (DAPI/FITC/Texas Red/Cy5) epifluorescent filter set (model 86012v2, Chroma Technology) provided the wavelength selection for acquisition of the DAPI and CellTracker Orange signals. The DAPI signal was acquired with 10 ms integration time and camera gain set at 199. The CellTracker Orange signal was acquired with 60 ms integration time and camera gain set at 199.

**Image Processing and Features Extraction**

The image processing and the extraction of morphological measurements were performed with the image analysis software Cytoshop versions 1.6 and 2.1 (Beckman Coulter). Section A.4 lists the measurements extracted for each cell. Image segmentation was done on the cell nucleus (DAPI signal). The presence of CellTracker signal was used to identify tumor cells for grading the performance of subsequent classification methods.

**Cell Classification**

The classification of tumor cells and normal white blood cells was performed by manual setting of gates or thresholds on the morphological measurements to define the boundary between normal and tumor cell populations. Tumor cells are generally larger than normal mononucleated white blood cells (mWBCs). The manual gating method used two nuclear parameters that exploited this difference to separate the two populations. First, the tumor cells were separated based on size. A threshold value was defined for the area of the nucleus. Objects with area larger than the threshold were classified as tumor cells. A second gate based on the wiggle (the ratio of nuclear perimeter to area) was used to eliminate clumps of mWBCs that were falsely selected as tumor cells. Clumps of mWBCs might be segmented as a single cell and since having a large nuclear area, they might be falsely defined as a tumor cell. However, when comparing a tumor cell and a clump of mWBCs of the same nuclear area, a clump of mWBCs would have a higher wiggle value. As a result, the manual gating method defined cells with large nuclear area and small wiggle value to be tumor cells. The exact threshold values for area and
wiggle parameters were defined using an independent population of all tumor cells. The result of the classification was verified with the ground truth provided by the CellTracker staining. The method was applied to each of the incremental-scanning and continuous-scanning data sets. The sensitivity and specificity of each system were calculated and tabulated in Table 3.7. An example of tumor cells is shown in Figure 3.19.

Results

Figure 3.19: An example of tumor cells found in the spiked samples. An image in the continuous-scanning data set of sample A1 (strip0-frame13). **top-left** DAPI image; **top-right** CellTracker Image; **bottom** Merged Image (DAPI in blue, CellTracker in red)
Table 3.6: Summary of Continuous/Incremental Scanning Comparison on Eight Spiked Cell Samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Scan Area (mm$^2$) (Strips × FOV)</th>
<th>Cell Density (objects/mm$^2$)</th>
<th>Comments</th>
<th>Average RMS error (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>$13.967 \times 10.813$ (62 × 48)</td>
<td>2285</td>
<td>-Most strips lose focus toward the end of the strip.</td>
<td>2.6805</td>
</tr>
<tr>
<td>A2</td>
<td>-DRIED OUT – NOT SCANNED</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>$13.967 \times 13.066$ (62 × 58)</td>
<td>670</td>
<td>-The cell density is too sparse.</td>
<td>8.7310</td>
</tr>
<tr>
<td>B2</td>
<td>$13.967 \times 10.813$ (62 × 48)</td>
<td>1576</td>
<td>-Strips 5, 35, 40, 45 look good. - Most strips start at the wrong axial plane. - Specimen contains a layer of debris.</td>
<td>6.0981</td>
</tr>
<tr>
<td>C1</td>
<td>$14.193 \times 10.813$ (63 × 48)</td>
<td>4472</td>
<td>-Strips 12 – 18 contain regions of very few cells. - Strips 53 and 62 start at the wrong axial position. - Strip 4 contains two layers of cells.</td>
<td>2.1445</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.9211 excluding 11 bad strips)</td>
</tr>
<tr>
<td>C2</td>
<td>$13.967 \times 11.038$ (62 × 49)</td>
<td>1655</td>
<td>-Strips 6 contains sparse regions, loses focus toward the end. - Strips 38, 46-61 contain sparse regions with two cell layers. Continuous scans look good while incremental scans lock on to the other plane. - Strip 44 contains regions with two layers and start at wrong axial position.</td>
<td>1.9786</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.5936 excluding 20 bad strips)</td>
</tr>
<tr>
<td>D1</td>
<td>$12.814 \times 9.912$ (57 × 44)</td>
<td>2798</td>
<td>-In strips 19 and 39, debris on coverslip causes incremental scan to lose focus and could not recover. Continuous scan glides over debris without drifting off.</td>
<td>2.8809</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.7969 excluding 2 bad strips)</td>
</tr>
<tr>
<td>D2</td>
<td>$13.967 \times 11.715$ (62 × 52)</td>
<td>2108</td>
<td>-Slide contains a debris layer. - Most strips start at the wrong axial position, but become better toward the end of the strips</td>
<td>3.0429</td>
</tr>
</tbody>
</table>

Average RMS error is the error between focus positions obtained with incremental and continuous autofocus over the entire sample.
Table 3.7: **CTCs Classification Performance with Manual Gating.** A summary of the results comparing the performance of the systems at detecting CTCs using manual gating. The values obtained with the incremental-scanning cytometer are in parentheses.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Objects</th>
<th>True Tumor$^a$</th>
<th>Gated$^b$</th>
<th>True Positive</th>
<th>False Positive</th>
<th>False Negative</th>
<th>% Sensitivity$^c$</th>
<th>% Specificity$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>145078 (77569)</td>
<td>9 (10)</td>
<td>185 (44)</td>
<td>8 (7)</td>
<td>177 (37)</td>
<td>1 (3)</td>
<td>88.8889 (70.0000)</td>
<td>99.8780 (99.9523)</td>
</tr>
<tr>
<td>B1</td>
<td>12965 (75914)</td>
<td>6 (10)</td>
<td>971 (1682)</td>
<td>4 (10)</td>
<td>967 (1672)</td>
<td>2 (0)</td>
<td>66.6667 (100.0000)</td>
<td>92.5380 (97.7972)</td>
</tr>
<tr>
<td>B2</td>
<td>69637 (146385)</td>
<td>15 (15)</td>
<td>1262 (9379)</td>
<td>11 (12)</td>
<td>1251 (9367)</td>
<td>4 (4)</td>
<td>73.3333 (75.0000)</td>
<td>98.2032 (93.6004)</td>
</tr>
<tr>
<td>C1</td>
<td>285868 (104397)</td>
<td>6 (7)</td>
<td>626 (239)</td>
<td>5 (7)</td>
<td>621 (232)</td>
<td>1 (0)</td>
<td>83.3333 (100.0000)</td>
<td>99.7828 (99.7778)</td>
</tr>
<tr>
<td>C2</td>
<td>25506 (96790)</td>
<td>4 (7)</td>
<td>1216 (2473)</td>
<td>4 (7)</td>
<td>1212 (2466)</td>
<td>0 (0)</td>
<td>100.0000 (100.0000)</td>
<td>95.2474 (97.4520)</td>
</tr>
<tr>
<td>D1</td>
<td>78340 (59350)</td>
<td>12 (15)</td>
<td>594 (399)</td>
<td>10 (13)</td>
<td>584 (386)</td>
<td>2 (2)</td>
<td>83.3333 (86.6667)</td>
<td>99.2544 (99.3495)</td>
</tr>
<tr>
<td>D2</td>
<td>32133 (124984)</td>
<td>4 (7)</td>
<td>2284 (1200)</td>
<td>4 (6)</td>
<td>2280 (1194)</td>
<td>0 (1)</td>
<td>100.0000 (85.7143)</td>
<td>92.9036 (99.0446)</td>
</tr>
</tbody>
</table>

**MEAN**  
85.0794 (88.1973)  
96.8296 (98.1391)

**SD**  
12.4615 (12.5357)  
3.2175 (2.2153)

---

$^a$ True cancer cells identified with positive CellTracker signal and visually inspected

$^b$ Cells classified as tumor cells based on AREA and WIGGLE parameters

$^c$ % Sensitivity = \([\text{true positive}/(\text{true positive} + \text{false negative})] \times 100\)

$^d$ % Specificity = \([\text{true negative}/(\text{true negative} + \text{false positive})] \times 100\)
Chapter 4

Contrast-Enhancement Filtering for Automatic 3D Segmentation

4.1 Introduction

Three-dimensional microscopy imaging provides unique perspectives of the relationships of cells and glands in fixed thick sections, in vivo preparations and living explants. Using instruments capable of 3D cellular resolution such as confocal, multi-photon, deconvolution and tomographic microscopes, enables direct observations of the 3D patterns of molecules and organelles. On the other hand, slow acquisition speeds coupled with the challenges of developing 3D image processing and analyses have traditionally limited 3D microscopy to primarily qualitative observations of small numbers of cells relative to flow cytometers and advanced 2D image cytometers. Here we describe and report on exploration of a 3D segmentation method for chondrocyte nuclei in cartilage, where identifying the changes in spatial distribution of nuclei (or cells) with tissue growth is critical for understanding and stimulating repair this difficult-to-heal tissue.

Technological advances in high-speed confocal image acquisition, data storage, and computational power continue to reduce these limitations are paving the way for practical 3D cytometry. The single-spot laser scanning technique most commonly employed in confocal [30] and multiphoton [77] systems has fundamentally hampered signal-to-noise ratios (SNRs) at high acquisition speeds (e.g., optical sectioning \( \geq 30 \) Hz). Parallel systems creating tens to tens of thousands of spots
arrayed simultaneously on a specimen offer orders of magnitude higher combinations of sensitivity and speed than single-spot scanning. Parallel 3D microscopy techniques are based on variations of the Nipkow disk [88, 111, 113, 124], spatial light modulators such as the Texas Instruments Digital Light Processor (DLP; [14, 22, 48, 64, 68, 109]) and multifocal multiphoton microscopy [9, 21, 34]. These parallel techniques are capable of providing high-resolution optical section rates of 10–1000 Hz with orders of magnitude greater SNRs than single-spot scanning laser confocal microscopes. Computational power fundamental to automated 3D image analysis is projected to continue to increase by Moore’s law for at least another decade. The cost of hard-drive based storage has dropped faster than computing power for the last several years, with the cost of raw hard-drive based storage (not including enclosures, computers, etc.) to less than $1,000 per terabyte. These advances in 3D image acquisition, computational power and mass storage warrant development of the key components for fully automated 3D cytometry.

For 3D cytometry to achieve the ubiquitous use of a flow cytometer or to become practical from the point of view of pathologists who analyze tens to hundreds of tissue sections per day [106], high-speed acquisition must be combined with walk-away automation. Automated 3D cytometry requires unattended scanning and reassembly of many 3D fields-of-view, mechanized tracking of laterally undulating tissue sections, and accurate segmentation of 3D cell images. Achieving satisfactory 3D segmentation is a key step in achieving walk-away automation. Image segmentation creates a data structure of binary masks that locate the image points (pixels or voxels) describing each object. Segmenting the image: 1) is the first step in making cell measurements; 2) enables the data reduction option of storing only the regions of interest (ROIs) containing the cell images, which has proven useful in 2D cytometry [91] and may be even more critical for handling massive 3D images; and 3) provides the basis for rapid image-to-data and data-to-image relational database operations critical for intelligent and fast gating, sorting and visualization of cell types. Segmentation of biological microscope images is difficult because cells are amorphous in size and shape, and various subcellular regions often differ dramatically in intensity, creating images that occupy essentially the entire dynamic range. The task of analyzing even a small tissue section may involve processing and measuring millions of cells and demands real-
time computation. In addition, regardless of the ongoing imaging sensitivity and dynamic range improvements, the motivation to image faster will always give an advantage to the segmentation that performs most robustly and accurately even on low signal-to-noise (SNR) images.

There has been an increasing interest in automated image segmentation of 3D cell images and here we summarize those that have been developed for cell nuclei in fluorescence images. Initially, 3D image cytometry relied on interactive methods that are labor-intensive and require manual steps for delineation of nuclei in each of the 2D optical sections [29, 72, 94]. Less laborious interactive techniques involved the operator manually identifying individual nuclei by defining a point or a region in each nucleus [83, 96]. These techniques often performed well but were slow, typically taking minutes per nucleus. Fully automated techniques have also been reported. For example, a 3D nuclear segmentation method by Rigaut et al., which included hypomedian filtering (size 13, rank 113), local average filtering (size 3), histogram normalization, and mathematical morphology transformations with, successively, gray level opening with a planar structuring element (size 5), top hat transformation, geodesic reconstruction, particle deagglomeration by watershed method, and final discrimination by adaptive thresholding, was reported to work on 60 adult rat liver tissue sections but not on in situ cancer of the human esophagus [94]. The results included a DNA content histogram of 160 hepatic and 30 nonhepatic nuclei from five 3D images. Irinopoulou et al. [56, 57] segmented the nuclei in 2D by applying a median filter, global thresholding, an opening operation and a watershed distance transform for de-clustering, followed by 3D de-clustering using shape criteria. Results reported using two 3D images acquired from each of four different human prostate tissue samples of hyperplasia, PIN, well- and poorly-differentiated carcinoma included nuclear volume and DNA content on 248, 121, 209 and 135 cells, respectively [57]. No performance measurements were reported in these publications.

Ancin et al. [8] used an edge-enhancement-and-smoothing method based on anisotropic diffusion and the symmetric nearest neighbor principle, gray level opening, 4-kernel 3D anisotropic gradient filtering, voxel-by-voxel adaptive thresholding within each optical section, region splitting, watershed, detection and removal of objects not conforming to features of user-identified objects, re-segmentation that
maximized the entropy computed from co-occurrence matrices of the objects found in the first segmentation and the background, hole filling, and refinement of the object edges using local intensity variations. Accuracy, defined as the number of correctly recognized 3D nuclei compared with human observation, was 92%, with 4.6% of the nuclei too overlapping/clustered to be separated (under-segmented) and 2.8% of the nuclei incorrectly split into two or more objects (over-segmented). The algorithm was designed to automate counting the number of objects (cells, nuclei, etc.) per unit volume, and make related measurements of intensity, size, shape features and surface area. In a later study of rat hippocampal and testis tissues, computerized counting resulted in 14% less nuclei in a total of six 3D images containing 100-200 cells each [100]. Adiga and Chaudhuri [119] described watershed and rule-based merging of over-segmented cell nuclei and reported 2% error in nuclear counts vs. manual identification in 2D images containing 327 nuclear optical sections (note the difference in 2D vs. 3D object counting, negating comparison). Lin et al. [2] used intensity restoration, noise reduction, thresholding, region correction, and morphological filtering in a pre-processing step followed by a gradient-weighted distance transform, 3D watershed segmentation and post-processing over-segmentation reduction via object model construction and a watershed surface breaker. 3D nuclear counts yielded 97% accuracy vs. human observers on five confocal image stacks that comprised a total of 1,026 fluorescently stained cell nuclei from the CA1 layer of the hippocampus in rat brain. Chawla et al. [23] used these techniques in a software package called 3D-catFISH and achieved 3D nuclear counting accuracies of 96% on CA1 hippocampal cells and 94% on parietal cortical cells from rat brain, in an application that included classification of nuclei via automated RNA FISH co-localization. Lin et al. [71] improved on these methods by introducing a hierarchical recursive tree-based merging algorithm for over-segmented nuclei, which improved 3D nuclear counting accuracy from 90% to 96% in 10 image stacks comprising a total of 1,327 computer-identified DAPI-stained nuclei from the CA1 layer of the rat hippocampus.

These examples indicate that the bulk of the literature reporting 3D segmentation of cells addresses the challenges of over- and under-segmentation created from region-based (largely watershed) methods. Segmentation accuracy per se (defined as the accuracy of classifying voxels as object or background) was not reported in
any of these examples. Performance was instead reported as the object recognition accuracy (defined as correctly identified whole objects, e.g., nuclei). Proposed segmentation accuracy measurements [86] include: a 2D distance measure of the difference between manually and automatically segmented images by Levine and Nazif [66], who later added performance parameters such as region uniformity, region contrast and line contrast [67]; the proportion of correctly classified pixels in 2D nuclear segmentation by Price et al. [91] a measure of the probability of the error between the manually- and computer-segmented results by Lim and Lee [70]; the second order local entropy by Pal and Bhandari [85]; and a correlation measure [20] between the original and segmented images [85]. Methods for comparing different segmentation algorithms have also been proposed [126], but such comparisons have not yet been reported for cell segmentation. Voxel-by-voxel boundary/segment accuracy measurements for cell nuclei have likely not yet been reported because of the laborious nature of manually segmenting the images.

Here, we report an exploration of the segmentation accuracy of a previously reported 2D nuclear segmentation algorithm that we extended to 3D. This segmentation approach consists of contrast enhancement followed by automatic global thresholding for segmenting nuclei in cartilage tissue sections, where sparse cellularity reduces or eliminates touching/overlapping cell nuclei. The choice of a tissue exhibiting sparse cellularity enabled focusing on the voxel classification accuracy. This technique wasn’t prone to over-segmentation in 2D, and with the sparse cellularity we assumed no nuclear clusters would be present. The segmentation approach is based on our previously developed real-time segmentation for cell nuclei in cultured monolayers that achieved the highest known reported 2D segmentation accuracy of 93% [91]. The key to this technique is contrast enhancement achieved through convolution of the raw image data with a finite impulse response (FIR) filter designed by supervised nonlinear least squares. This FIR filter boosts the intensities of the foreground object pixels while suppressing the intensities of the background pixels. In 2D operation, the filter stretches the contrast to enable automatic global thresholding. For image $G$, FIR filter $K$, and image segment $S$, 
the segmentation operations are

\[ F = K \ast G \]

\[ S = \begin{cases} C_0, & F < T \\ C_1, & F \geq T \end{cases} \]  

(4.1)

where \( \ast \) is the convolution operator, \( F \) is the filtered image, \( T \) is the threshold, and \( C_0 \) and \( C_1 \) are the two classes of the segmented image. The operations above represent a two-class linear classifier with class separation enhanced by an FIR filter. Given an image \( G \) and the corresponding ideal (or standard) segmented image \( I_S \), the FIR filter is designed so that it best maps \( G \) into \( I_S \). The objective of the filter design is to minimize the pixel-to-pixel error between the ideal image \( I_S \) and the filtered image \( F \). The perceptron criterion, commonly used in neural network and other classification techniques, is the error measure for filter design.

\[ E = \begin{cases} \sum (I_s - F)^2, & A < F < B \\ 0, & \text{otherwise} \end{cases} \]  

(4.2)

where the error \( E \) is non-zero only when the filtered image \( F \) has not achieved the contrast enhancement defined by \([A, B]\). Minimization of \( E \) produces the filter \( K \) that creates the best contrast for subsequent threshold within \([A, B]\). Relaxing the requirement that background and object pixels be transformed to achieve an exact value (i.e., 0 and 255 respectively for 8-bit fluorescence images) by defining zero error outside \([A, B]\) was found to dramatically improve the resulting contrast in the previous 2D segmentation work [91]. With the contrast enhancement provided by this filter, the subsequent thresholding step was automatic and non-iterative in 2D [91] and may achieve similarly simple operation in 3D.

The supervised portion of this filter design technique was labor-intensive in 2D [91] and similar manual delineation of the surfaces of nuclei in 3D images appeared daunting. The laborious nature of manually segmenting nuclei in 3D meant that we were not likely to generate a large measurement test set. To simplify this challenge, we also imaged spherical fluorescence beads of known size to provide a \textit{a priori} knowledge of the ideal segment and utilized a computer-assisted 2D segmentation technique to speed creation of a segmentation standard test set for images of cell nuclei. The use of standard objects in 2D was not thought practical largely
because known specimens thin enough to fit within the depth of field of high-resolution optics are not widely available; beads of sufficient area to mimic cell objects also extend axially outside the depth of field and blur the images. With these modifications, we explored 3D least squares filter design for segmentation of fluorescent confocal images of cell nuclei. Designing the initial filters took days to weeks of computation on a single computer, motivating programming and analysis of parallel computation to further speed implementation.

The overall aim of this study was to implement fully automated 3D image segmentation and to evaluate its performance. The specific objectives were: 1) to extend the contrast-enhancement technique to 3D by utilizing spherical fluorescent beads to simplify the definition of ideal segments, 2) to implement the filter design algorithm with parallel processing on a cluster of computers to speed up processing time, 3) to qualitatively evaluate the contrast enhancement through visual comparison on cell nuclei, and 4) to quantitatively measure the effects of contrast enhancement on segmentation accuracy on samples of chondrocyte explants.

4.2 Materials and Methods

Fluorescent Beads in Tissue Preparation

Four microscope slides containing fluorescent micro-beads embedded in pulverized mouse liver tissue were used to simulate cells in biological tissue. One liver was collected from an unrelated Institutional Animal Care and Use Committee approved experiment. A tissue sample weighing approximately 0.5 g was pulverized with a mortar and pestle into a paste. One hundred fifty micro-liters of unfixed liver paste were deposited onto a well-slide (Part No. 12-560A, Fisher Research, Pittsburgh, PA). Five micro-liters of 4-µm and 10-µm diameter fluorescent micro-beads (Part No. F-8834, excitation peak 580 nm and emission peak 605 nm, Molecular Probes Inc., Eugene, OR) were then injected and mixed into the liver solution with the pipette tip. The fluorescent bead solution was sonicated prior to use to create a uniform slurry. The preparation was then fixed with 90% ethanol, covered with a coverslip (No. 1.5), and sealed with nail polish.
Image Acquisition and Ideal Image Segment Definition

The 3D images were acquired through Fluor 20× Ph3DL 0.75 NA, Fluor 40× Ph3DL 0.85 NA and Plan Apo 60× 1.4 NA oil objectives (Nikon Instruments Inc., Melville, NY) with a Bio-Rad MRC-1024UV laser scanning confocal system (Bio-Rad Laboratories, Hercules, CA) attached to a Nikon Diaphot 300 inverted microscope. For each data set, a Z-series was collected that contained the entire bead and several empty slices at the top and bottom of the stack. At each axial position in the Z-series, 100 images were collected. One of each of the 100 images was extracted to create the input Z-series \((G\) in Equation 4.1). Each set of 100 optical sections was then averaged to create a low SNR Z-series. A new Z-series \(I_s\) was then artificially constructed from the centroids of the averaged experimental Z-series, the known radii of the beads and the magnification of the system. The magnification was measured with a stage micrometer (Nikon Instruments Inc., Melville, NY). The stack dimensions were 64×64×36, 152×152×48, and 256×256×64 at sampling frequencies of 0.24×0.24×0.9 µm³, 0.19×0.19×0.5 µm³, and 0.13×0.13×0.2 µm³ for 20×, 40×, and 60× magnifications, respectively. Regions in the sample were selected so that each stack contained only one bead.

FIR filter solver algorithm

The Levenberg-Marquardt nonlinear least-squares routine [89] was used to design 3×3×3 to 13×13×13 FIR filters by minimizing the error of Equation 4.2. The filter coefficients were initially set to zero. Error minimization was stopped when the improvement was less than one percent and it had decreased in the previous 7 iterations. Empirically, seven successive improvements ensured convergence. Under these conditions, 10 to 20 iterations were required to design the filters. For comparison, linear least squares solutions were also found by forcing foreground pixels to 255 and background pixels to 0; Equation 4.2 was converted to \(E = \sum (I_s - F)^2\).

Computer Implementation and Parallel Processing

Nonlinear least squares design of 3D FIR filters is computationally intensive. Initial solutions for 2197 (13×13×13) coefficient filters using a 256×256×64 data
set on a desktop PC took weeks to complete. Filter design was sped up by parallel implementation of the algorithm on a virtual parallel computer made up of 14 Intel-based workstations, ranging from a Pentium Pro 200 MHz to Pentium III 733 MHz. The processors in these workstations were configured into nodes linked together by the network interface cards (NIC) via the campus network at UCSD. The Message Passing Interface (MPICH; Argonne National Laboratory, Argonne, IL) provided communication and synchronization between the processing nodes [4, 41, 42]. The virtual parallel computer was set up in a master-slave configuration. The master node was responsible for checking the status of the slave nodes, distributing the work, collecting and compiling the results, and optimizing work distribution to processors of different speeds. During each iteration, the master divided the data into segments and assigned each node one data segment at a time. As each node completed a data segment, it reported the results to the master node and began work on a new segment of the data. This task distribution scheme allowed the workload to be distributed proportionally to processing speed; faster nodes accomplished more. To minimize data transfer, each node stored the entire data set. During filter design, the master sent the filter coefficients and the two endpoints of each work segment; and each slave node sent back the curvature matrix and the beta vector at completion of a data segment.

Sample Preparation

The image quality in confocal microscopy depends on factors that include the index of refraction of the specimen and depth into the tissue. Aberrations decrease resolution as a function of depth more rapidly when the index of refraction of the tissue does not match the immersion medium. Two relative extremes, thin cell monolayers and thick tissue sections were imaged to provide comparative insight into how the method would degrade with depth.

**DAPI-stained NIH 3T3 cells in monolayers:** NIH 3T3 mouse fibroblasts (ATCC CRL 1658) were cultured at 37°C on 22×60-mm² washed and autoclaved coverslips (No. 1.5) in 5% CO₂ and Minimal Essential Medium with Earle’s salts, 10% fetal bovine serum, 100 g/ml gentamicin, and 0.26 mg/ml L-glutamine for several days prior to fixation and staining to create an evenly distributed monolayer.
They were then fixed for 2.0 hrs in 95% ethanol and air-dried. The slides were stained for 2.0 hrs with a 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) nuclear stain solution consisting of 100 ng/mL DAPI, 10 mM Tris, 10 mM EDTA, 100 mM NaCl, and 2% 2-mercaptoethanol [46]. The coverslips were then laid down on cleaned microscope slides with excess DAPI solution, and sealed with nail polish.

**DAPI-stained Bovine Cartilage Tissue:** Osteochondral blocks $2 \times 5 \times 0.2$-mm$^3$ were harvested from the femoral condyle region of the knee joint of a 3 week old bovine calf. The samples were maintained in a humidified 5% CO$_2$–95% air incubator at a temperature of 37°C in DMEM supplemented with 20% fetal bovine serum, 100 µg/ml ascorbate, 0.1M nonessential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin. The media was changed every two days for eight days, and then replaced with phosphate buffered saline for one day. The samples were then fixed with 4% paraformaldehyde for one day and stained with previously described DAPI solution for four days. These blocks were mounted on microscope slides with mounting medium containing DAPI (Vectashield H-1200, Vector Laboratories, Burlingame, CA), covered with cover glass (No. 1.5) and sealed with nail polish.

**Imaging, Processing, and Visualization**

Z-series of the nuclei were collected from the two specimens and processed to subjectively evaluate the contrast enhancement of filters designed on beads. Each contrast-enhanced Z-series was converted to a binary image by thresholding with the first minimum in the histogram after the background peak. For visualization, stereo pair images were created using Confocal Assistant version 4.02 (Todd Clark Brelje, University of Minnesota, MN) and 3D surface renderings of the segmented regions were created in MATLAB Release 12.1 (Mathworks, Natick, MA). The images contrast enhanced by the least squares designed 3D filters were compared to those derived from typical 2D sharpening and unsharp mask filters obtained from the literature (section B.1). The raw Z-series were thresholded and surface rendered the same way for visual comparison.
3D Segmentation Error Analysis

The filters were designed with the goal of expanding the separation between object and background classes in order to simplify thresholding. To evaluate the filters’ performance, the bovine cartilage data sets contrast enhanced by the designed filters were thresholded and compared to manually segmented versions of the same data sets. Segmentation accuracy was used as a measure of the filters’ performance. Manual segmentation was assisted by a software routine provided by Vala Sciences (La Jolla, CA) that used the watershed transform to do the segmentation. For each layer in the data set, the user marked the locations of the nuclei with a single point near the center and drew a boundary around them. These markers defined the locations of the local minima from which the catchment basins would form. The catchment basins enclosed by and not touching the user-defined boundary were classified as nuclei. For automatic thresholding, Otsu’s method [84] was used to segment the data sets after contrast enhancement with the least squares design filters. The method automatically selected a threshold value that would maximize the between-class variance. To describe the segmentation accuracy, a classification ratio was defined as \( 1 - \frac{\text{misclassified voxels}}{\text{object voxels}} \), where the number of object voxels is defined by the manually segmented data sets. This is a conservative measure that penalizes for both object and background classification errors, but it is scaled only by the number of object voxels.

For comparison, the segmentation accuracy after automatic thresholding was also calculated for four other versions of the data sets: original (no-filtering), and three filtered versions: sharpen, unsharp-mask, and a \( 7 \times 7 \) contrast enhancing filters [91] (see Section B.1 for the coefficients of the filters).

4.3 Results

The purpose of this set of experiments was to determine the potential for 3D image segmentation by least squares designed 3D FIR filters. The report includes measurements of the advantages of distributed processing, an evaluation of the appearance of the resulting contrast-enhanced images and segments, and a comparison of segmentation accuracy.
Parallel Processing Performance in 3D FIR Filter Design

The parallel processing performance can be task-dependent and usually scales only partially with the number of processors due to interprocessor communication latencies and the additional computation required to reassemble the distributed results. Figure 4.1 shows the processing time comparison between a single processor and 14 processors for least squares design of filters ranging in size from 27 to 2197 coefficients and a 152×152×33 image. The processing time is actually longer for less than 125 filter coefficients and the parallel processing advantage increases to about 10-fold at 729 coefficients. This 10-fold speed advantage is consistently maintained up through the 13×13×13 filter with 2197 coefficients. As is typical, the speed increases shown in Figure 4.1 are less than might be thought possible from simply multiplying by the number of processors due to additional overhead and communication latencies. The effects of additional overhead are especially apparent in the case of small FIR kernels for which the extra overhead dominates. Early results indicated that filters of at least 9×9×9 were needed to perform good contrast enhancement. On a single processor, filters from 9×9×9 to 13×13×13 required 4.6 days and 21.9 days, respectively. Thus, the 10-fold speed gain realized by combining 14 CPUs to create this virtual multi-processing computer was very helpful. An example 9×9×9 nonlinear least squares designed FIR filter designed using this parallel technique is shown in Figure 4.2 (see B.1 on page 220 for filter coefficients). It has some characteristics analogous to a sharpening filter.

Linear Fit vs. Nonlinear Fit (Perceptron Criterion)

The computational requirement for an exact fit to the ideal image segment is dramatically less than for the perceptron-defined error of Equation 4.2 because it is solved by single-step linear least squares. The difference between the contrast-enhancement provided by linearly and nonlinearly designed 7×7×7 filters was compared to evaluate the need for the extra computation. Three dimensional mesh plots of selected optical sections from the original and filtered versions of the confocal image of a 4 μm fluorescent bead are shown in Figure 4.3. The contrast is visibly improved in the mesh plots of optical sections from both the linearly- and nonlinearly-designed FIR filters (shown in the columns B and C, respectively), as
Figure 4.1: **Parallel Processing Time Comparison.** Processing time comparison of Levenberg-Marquardt nonlinear least squares method for the design of contrast-enhancing FIR filters of sizes $3 \times 3 \times 3$ to $13 \times 13 \times 13$ on a $1.1 \times 10^6$-voxel dataset ($152 \times 152 \times 48$-voxel image). Computational speed improved about 10-fold for greater 729 coefficients. However, additional communication and network latency outweighs the benefit of parallel processing on lower numbers of coefficients. The single-processor tests were performed on a Pentium III 533 MHz CPU. The parallel-processor tests were performed on 14 processors ranging from a Pentium Pro 200 MHz to Pentium III 733 MHz.
Figure 4.2: 3D mesh plot of an example $9 \times 9 \times 9$ least squares designed FIR filter. The 3D array is displayed as nine 2D arrays: 1–3 (top), 4–6 (middle), and 7–9 (bottom). See B.1 on page 220 for numerical values.
compared to the mesh plots of the original images in column A. The contrast enhancement of the filter designed by nonlinear least squares is also greater than that of the linear least squares filter and it is especially visible in the optical sections 17 and 41 near the top and bottom of the bead. The improvement in contrast by the nonlinearly designed filter appears similar to that observed in the 3D plots showing 2D image contrast enhancement in the earlier study [91]. The improved contrast increases the distance between object and background pixel classes and motivates use of nonlinearly designed filters in spite of the additional computation required.

Figure 4.3: Mesh plots of bead demonstrating the effects of FIR contrast-enhancing filter. Intensity mesh plots of optical sections 17, 30 and 41 from a 55×53×55-voxel image of a 4 µm diameter fluorescent bead. A: Original images. B: Results of a 7×7×7 linearly designed, exact fit filter. C: Results of a 7×7×7 nonlinearly designed, classifier fit filter. Plots B and C were clipped at [0, 255] to magnify the threshold region and the optical sections in A were acquired within the 8-bit dynamic range. The image stack was acquired on a Bio-Rad MRC-1024 Confocal Microscope with a 60× 1.4 NA Nikon objective at 0.2×0.2×0.2 µm³ sampling.
Contrast-Enhanced Images

Nonlinearly designed (according to Equations 4.1 and 4.2) FIR filters were used to enhance the contrast of confocal images collected from monolayers of NIH 3T3 cell nuclei stained with DAPI. For comparison of the contrast enhancement, regular 2D sharpening and unsharp-mask filters were also applied to each 2D optical section of the dataset (See B.1 on page 220 for the filter coefficients). Figure 4.4 shows projections/stereo pairs of the original and filtered Z-series of a metaphase nucleus. The image enhanced with the nonlinear least squares designed filter demonstrates the most contrast; the details of the chromosome arms much better delineated. Dim chromosome arms on the periphery of the nucleus are much brighter and easier to distinguish from background. Both the brighter and dimmer portions of the nucleus show increased detail and contrast, indicating successful achievement of the goal of improving contrast for the purpose of simpler single-threshold segmentation. The example stereo pair of a 163-µm thick bovine cartilage tissue section in Figure 4.5 shows similar results; the contrast was enhanced, saturating most cell nuclei (at a digital intensity of 255) without increasing the noise. Deep tissue can be very dim in confocal microscopy and the deepest cells in Figure 4.5 do not appear as well enhanced.

Thresholded and Surface Rendered Images

To more easily visualize the differences between the original and filtered images that might be expected in automated image segmentation, the surfaces were rendered after thresholding. The intensity histograms of the confocal volumes were analyzed and the threshold chosen as the first minimum after the largest (background intensity) peak for both filtered and unfiltered images. Figure 4.6 shows examples comparing the surface renderings of the resulting segmented original and filtered Z-series of three nuclei stained with DAPI. Portions of the original images contain dim regions that were underselected (dim object voxels that were segmented as part of the background), and bright regions that were overselected (bright background voxels that were segmented as part of the object). Improvement in both underselected chromosome arms and overselected central regions can be seen in the filtered versions. Figure 4.7 shows similar results with the Z-series of
Figure 4.4: **FIR contrast-enhancing filter on a metaphase nucleus.** Stereo-pairs of a DAPI stained metaphase nucleus. A: original, B: 3×3 sharpening filter, C: 3×3 unsharp-masking filter and D: contrast enhanced with a 9×9×9 nonlinearly designed filter (See B.1 on page 220 for filter descriptions). In the sharpening and unsharp-masking versions, contrast is slightly improved and noise is also amplified. With the nonlinearly designed filter, the edges are much better defined and both dim and bright regions exhibit more detail; some of the dim chromosome arms are much more clear after filtering while noise is not substantially increased. The image was collected on a Bio-Rad MRC-1024 Confocal Microscope with a 60× 1.4 NA Nikon objective at 0.1×0.1×0.2 μm³ sampling. The stereo pairs were created with 0° and 15° views.
Figure 4.5: **Stereo Pairs of Cartilage section demonstrating FIR contrast-enhancing filter.** Stereo-pairs of a DAPI stained bovine cartilage tissue: original (top), and contrast enhanced with a $9 \times 9 \times 9$ nonlinearly designed filter (bottom) (See B.1 on page 220 for filter descriptions). In the filtered version the contrast was noticeably enhanced saturating most cell nuclei. The image was collected on a Bio-Rad MRC-1024 Confocal Microscope with a $40 \times 0.85$ NA Nikon objective at $0.266 \times 0.266 \times 0.5 \mu m^3$ sampling. The stereo pairs were created from $\pm 6^\circ$ views.
Figure 4.6: **Shaded 3D rendering of a metaphase nucleus demonstrating FIR contrast-enhancing filter.** Surface renderings of three nuclei from the raw data (left) and contrast-enhancing filtered (right). Both sets were thresholded using the first minimum after the largest (background) peak in the intensity histogram. The nuclei on the right show substantially more surface detail than those on the right. By appearance, underselected regions (from dim portions of the image) on the left are more appropriately represented on the right and overselected regions (from bright portions) on the left exhibit more detail on the right.
Figure 4.7: **Shaded 3D rendering of a section of bovine cartilage tissue demonstrating FIR contrast-enhancing filter.** Surface renderings of nuclei from the raw image (left) and after contrast-enhancement (right) of a $136 \times 136 \times 163$ \(\mu m^3\) region of bovine cartilage tissue. Both sets were thresholded using the first minimum after the largest (background) peak in the intensity histogram. In the raw image, brighter nuclei at the top of the stack are large and appear to be overselected and dimmer ones deeper in the stack are smaller and appear to be underselected. In the enhanced image, bright overselected nuclei are smaller and show more surface details (nuclei 1–6, 9, 12, and 15) whereas dim underselected nuclei are larger (nuclei 7, 8, 11, 19–21, and 24). The remaining nuclei in the insets appear to have the same size but with much more surface details in the enhanced version. This size variation indicates the need for gain correction with depth in future work. Small processing artifacts present in the enhanced image (e.g., the object below nuclei 16 and 17) can be easily eliminated with an object-size threshold.
Figure 4.8: **Overlay of shaded 3D rendering of a section of bovine cartilage tissue demonstrating FIR contrast-enhancing filter.** The raw (yellow) and contrast-enhanced (red) surface renderings from Figure 4.7 are superimposed. Note that the outer surfaces of the upper halves of the nuclei are predominantly from the original image and the outer surfaces of the lower halves primarily are from the contrast-enhanced image. This may indicate that the upper portion of a nucleus is dense enough to routinely shade the lower portion in confocal microscopy.
bovine cartilage tissue. Brighter nuclei were overselected and dimmer nuclei were underselected in the original image. In the filtered version, all cells show much more surface details, nuclei that were overselected became smaller and ones that were underselected became larger. In Figure 4.8, the raw (yellow) and contrast enhanced (red) surface renderings of Figure 4.7 are superimposed. It is interesting that contrast enhancement appears to predominantly shrink the upper halves and expand the lower halves of the nuclei. This is consistent with the upper portion of the object shading the lower portion. This raises the question of whether or not bright objects are routinely distorted by shading in confocal microscopy.

**3D Image Segmentation Accuracy**

The comparison of segmentation accuracy between the different methods of processing is summarized in Table 4.1. Of all the filtering methods, the non-linearly designed 3D filter resulted in the highest average classification ratio. The second best method (no-filtering) was an order of magnitude lower. The other three contrast enhancing filters performed even worse than the case of no-filtering and resulted in negative average classification ratios which indicated high numbers of misclassified voxels. Although there were individual cases in which one of the other filters had higher numbers (sharpen filter on samples 6–8), the non-linearly designed 3D filter had the most consistent performance (low SD). On the other hand, the classification ratios even for the best case were low. These low values are due to the combination of very low percentage of object voxels in the data sets and the conservative definition of the classification ratio. With background voxels making up most of the data (96.55%–99.51%), misclassification of a small percentage of background voxels has a big effect in lowering the classification ratio.
Table 4.1: **3D Image Segmentation Accuracy.** A comparison of the effects different contrast-enhancing filters on the segmentation accuracy of 3D data sets.

<table>
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<tr>
<th>Sample</th>
<th>Sampling Resolution (μm$^3$)</th>
<th>Data Size (voxels)</th>
<th>Foreground Objects</th>
<th>Classification Ratio: 1 – (misclassified voxels/object voxels)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. Voxels</td>
<td>Nuclei</td>
</tr>
<tr>
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<td>678289</td>
<td>262</td>
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<tr>
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<td>128</td>
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<tr>
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</tr>
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</tr>
<tr>
<td><strong>SD</strong></td>
<td></td>
<td></td>
<td>8461</td>
<td>36</td>
</tr>
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</table>
4.4 Discussion and Conclusions

We have demonstrated an accurate fully automated and non-iterative technique for segmenting nuclei from 3D confocal images. The technique comprises of a contrast-enhancement filtering step followed by automatic global thresholding. Qualitative and quantitative evaluations on cartilage tissue samples were presented.

To the best of our knowledge, this is the first time a fully automated non-iterative technique has been described. Furthermore, this is also the first time that segmentation accuracy has been calculated directly from differences in object voxels between manual and automatic segmentation of real biological specimens. Previous studies have only evaluated segmentation error based on synthetic objects [72, 83], qualitative classification based on visual inspection [72, 83], or utilizing other indirect measures (e.g., coefficient of variation of DNA content) [57, 94].

As will likely be true of any 3D image segmentation method for confocal microscopy of tissue, the technique degraded with imaging depth as seen in the deep sections of the cartilage tissue shown in Figure 4.7. This depth attenuation might be minimized with gain correction methods [69, 93]. However, because scattering and aberrations increase with depth, there will always likely be some limitation to thickness at which these techniques provide only reasonable image segmentation. For applications where the underlying data includes measurements of fine detail and pattern, the practical depth limit may be reached even before image segmentation degrades noticeably. For any application, the contrast enhancement should improve class separation at any depth.

Techniques were employed to speed implementation. The use of fluorescent beads for constructing the ideal image segments for design of the FIR filters is important because it substantially reduces the preparation time and may offer the opportunity to completely automate the process and to eliminate supervision. The computational challenge in designing these filters was met by taking advantage of a 10-fold speed improvement from a 14-processor virtual parallel computer. Real time operation of these filters on 3D images produced by fast 3D microscopy systems (operating at, e.g., 100 Hz) will require about 80 GOPS and it can be achieved by implementation on a bank of DSP chips. With Moore’s law of doubling
computer speed every 18 months projected to continue for at least a decade, the
cost of real-time implementation will continue to drop rapidly.

A fast, accurate and fully automated 3D segmentation technique will enable
3D cytometry for many applications. For example, there has been tremendous
interest in using 3D cytometry for DNA ploidy analysis of tissue biopsies as an
eyear marker of neoplasia [26, 29, 35, 47, 56, 57, 59, 94, 98, 114, 116, 122]. Three-
dimensional cytometry combines the advantages of flow and 2D image cytometry,
where a large number of cells can be studied, and the advantages of 3D cytometry,

where locational histological information is retained. Compared to cartilage tissue,
other types of tissues have more closely organized cell structures; therefore, they
will be more difficult to segment. In future work, we will improve, adapt, and
evaluate the technique for other tissue types. The results obtained in this study
demonstrated substantial promise for real-time automated 3D image segmentation
and motivate further study.

4.5 Acknowledgement

The text of Chapter 4 contains material being prepared for publication. Nguyen
improve segmentation of 3D confocal images”. The dissertation author was the
primary investigator and author of this paper.
Chapter 5

Discussion and Conclusions

5.1 Introduction

The dissertation research brought together the disciplines of biology, optics, and engineering to develop an instrument for detecting circulating tumor cells in blood. The ability to routinely find these cells has great significance in the diagnosis, selection of therapy, assessment of treatment efficacy, and long term management of cancer patients. A review of the current literature indicates that present methods of detection are either plagued by high false positive rates (molecular techniques) or have yet to show consistent results (cellular techniques using immunocytochemical markers). An alternative method of detection that exploits the inherent morphologic differences between tumor and normal cells has great potential; however, there is not yet an instrument with sufficient speed and resolution to make this technique practical. A high-throughput, high-resolution image cytometer was developed to address this problem. A spiked cell model was used to measure its performance. A performance comparison was also made with an existing commercial image cytometer.
5.2 How Did the Cytometer Compare to Existing Systems?

5.2.1 Autofocus

The instrument’s performance is determined by its ability to maintain focus. Compared to incremental scanning, the focus accuracy during continuous scanning was worse. The degraded performance could be attributed to several factors in the autofocus measurement subsystem.

Low Light Transmission Efficiency

First, light transmission efficiency in the optical train was very low (Figure 3.3). The light loss occurs at every stage in the optical path. Only about one fifth of the available illumination enters the objective lens. Of this amount, further losses result in less than 1% reaching the optical splitting assembly. About 96% is lost in the objective and microscope, with another 3% at the collimating lens outside the side port of the microscope. Inefficiencies (39%–73% transmission) in the optical splitting assembly further compounded the problem. Because of these transmission efficiencies and to compensate for them, the standard 100-W halogen lamp was replaced with a 300-W version. The replacement lamp (model DRA) was chosen over other types of light sources (arc-lamp, lasers, diode-lasers) not only because of its favorable spectral characteristic and ease of control, but also because of its filament size and density. The filament of the DRA is only slightly larger than that of the one it replaces which results in a very intense source when coupled to the microscope. There are other lamp models with much higher power ratings; however, their filaments are larger which result in lower intensity at the specimen. Although the DRA bulb was the best option at the time and it allowed the system to operate, future optimization of the transmission efficiency is still crucial.

The low light transmission slowed down the response of the autofocus system. To give the focus measuring cameras more time to collect light, their line rate was kept low. When working under phase-contrast with cell specimens, the maximum line rate of the focus measurement cameras was 5580 Hz. This is only about 60% of their maximum line rate of 9225 Hz. Furthermore, because of the low signal, focus
was updated less frequently as measuring focus over larger areas was necessary to obtain enough information. For most of the experiments, focus was updated every 128 lines (44 Hz or about 5.3 focus updates for each field of view). The slower response limits the size of focus change that the system can track. The autofocus can only track and correct the focus changes if the specimen remains within its measuring range. If the focus changes too quickly, by the next focus update, the specimen would have traveled out of the range and the focus would be lost. To keep the system from losing focus, the measuring range was increased by increasing the spacing between the measuring channels to better track focus. However, in doing so, a tradeoff was made in increasing the focus range at the expense of focus accuracy. This contributed in part to the worse focus accuracy.

**Degraded Resolution in Image Splitting Module**

The lower focus accuracy was also caused in part by the degraded resolution in the focus measuring channels. Measurements of the modulation transfer function (MTF) at different points in the system indicated resolution losses. Almost all components in the signal path degraded the resolution to some degree. The scattering of light as it travels through the beamsplitters and mirrors caused some of the resolution loss. Although minimized with antireflection coatings, the reflections at the many glass-air surfaces also degraded the resolution. The magnification correction lens system was not corrected for flat-field and therefore the distortion degraded the resolution. Some resolution was also lost in the cameras as light was converted into electrical signal. The combined loss in resolution mostly attenuates the transmission of high-frequency signals. Since the high-frequency portion of the spectrum changes most rapidly with focus, its attenuation decreases the system’s sensitivity to changes in focus. Consequently, the system only detects and corrects relatively large focus changes, thus lowers the accuracy.

**Bad Specimen Conditions**

Like many other image based autofocus systems, the performance depends on the quality of the specimen. High-content and flat specimens lead to better performance. Scanning tests performed on the Ronchi ruling, which had consistent
and high information content, resulted in very low average focus error — typically less than 1/3 of the depth of field. The experiments done on cell specimens of different quality had varied results. For well prepared specimens, the error was less than half the depth of field which was deemed acceptable (Figure 3.16). For low-quality specimens, whose focus changed quickly between fields of view, the system was unable to follow the fast-changing focus and took an average path through them (Figure 3.17). Similarly, the focus accuracy on the CTC samples depended on the quality of the specimen. The overall focus error for all the specimens was larger than the depth of field. Closer examination of samples A1, B1, B2, and D2 revealed that most of the images acquired with the continuous scans were in fact in focus and the error measure was invalid for these samples. These four samples had a dense layer of small cellular debris which was at a different focal plane from the cells (Figure 3.18). The continuous autofocus system, with its degraded resolution, was unable to pick up on the fine details of the debris and only detected the cells. On the other hand, the incremental autofocus system falsely locked on the layer of debris because it was denser. For the remaining three samples, C1, C2, and D1, this debris layer did not exist and both autofocus methods tracked the same layer of cells. In these three samples, the system lost focus 18% of the time (33 out of 182 strips). Considering the remaining 149 strips, the average focus error was about half the depth of field.

From detailed examination of all 430 strips in the CTC samples, the following situations were identified as the causes for the system to lose focus:

**Sparse or empty field of view:** The system was likely to lose focus if it encountered empty fields of view or ones containing only a few cells (less than 5 for 20× objective). When these fields of view occurred in the middle of the strip, the system continued to track focus if it encountered only one or two, but would lose focus after five or six consecutive ones. If the strip started on one of these fields of view, it would almost always lose focus.

**Starting at the wrong axial position:** At the beginning of the scan of each strip, the system uses incremental autofocus to find best focus and centers the continuous autofocus around that position. In specimens that contained a debris layer, incremental autofocus always chose the debris layer and centered
the system there. In this situation, if the cell layer was within the range of
the continuous autofocus, the system was able to detect the cells and to track
them. However, if the cell layer was outside the range, the system lost focus.

**Specimen containing two layers of cells:** When the system encountered strips
with two cell layers, it did one of three things: a) it picked the layer with
more cells and tracked it, b) it switched back and forth between the two
layers, or c) it switched from one layer to the other, tracked focus for a short
segment and then lost focus.

All situations above are related to the quality of the specimen. As with other
cytometry systems, the overall performance depends on both the instrumentation
and the specimen. The system can be made more sensitive to work with low
content specimen, and to have a larger range to response to large changes in focus.
Improvements in specimen preparation such as monolayer with uniform cellular
density and without debris will also increase the overall performance.

Table 5.1 compares the autofocus performance of the system to other continuous-
scanning systems with published autofocus performance data. The system devel-
oped in this dissertation has the highest line rate (8370 Hz), largest overall focusing range (200 \(\mu m\)), is one of two systems with the highest numerical aperture (0.75 NA), and the second highest sampling density (0.22 \(\mu m/\)pixel). Compared
to the system by Bravo [19], this instrument updates focus at a slower rate (44 Hz
compared to 56 Hz). The stage speed is also slower (1.84 mm/s vs. 2.34 mm/s);
however, stage speed is a function of line rate and sampling density. The sampling
density of the system by Bravo at the stated speed was larger (0.337 \(\mu m/\)pixel vs.
0.22 \(\mu m/\)pixel).
Table 5.1: Comparison of Autofocus Performance with other Continuous-Scanning Systems. Adapted from Bravo [19].

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Image Sensor:</strong></td>
<td>Linear CCD</td>
<td>Linear CCD</td>
<td>2-D array in TDI mode</td>
<td>2-D array in TDI mode</td>
<td>2-D array in TDI mode</td>
</tr>
<tr>
<td>Pixel size:</td>
<td>1024 pixels × 1 row</td>
<td>1024 pixels × 1 row</td>
<td>1317 × 1035 pixels</td>
<td>1280 × 1024 pixels</td>
<td>1024 × 1024 pixels</td>
</tr>
<tr>
<td>Test line rate:</td>
<td>4 kHz</td>
<td>1.5 KHz</td>
<td>345 Hz</td>
<td>3370 - 6750 Hz</td>
<td>8370 Hz</td>
</tr>
<tr>
<td><strong>Autofocus method:</strong></td>
<td>Servo loop with two dedicated linear CCDs</td>
<td>During the scan, at spaced points on slide</td>
<td>Predefined focus before scan (3 AF positions in a 3.2 mm strip)</td>
<td>Parallel analog hardware with 9 CCD line arrays</td>
<td>Parallel analog hardware with 7 TDI CCD arrays</td>
</tr>
<tr>
<td>Focus measure:</td>
<td>I.O.D.</td>
<td>NR</td>
<td>Energy of mid-frequencies</td>
<td>Energy of high frequencies</td>
<td>Energy of high frequencies</td>
</tr>
<tr>
<td>Range:</td>
<td>3 μm</td>
<td>NR</td>
<td>5 μm @ 56 Hz, 100 μm/slide</td>
<td>Best: 0.014 μm/s</td>
<td>44 Hz, 200 μm/strip, 10 cm/slide</td>
</tr>
<tr>
<td>Error:</td>
<td>0.2 - 1.0 μm</td>
<td>NR</td>
<td>Maximum: 1.1 μm; Average: 0.4 μm</td>
<td>Average: 0.07 μm²</td>
<td>Best RMS error: 0.594 μm; Average RMS error: 0.771 μm²</td>
</tr>
<tr>
<td><strong>Optics:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specimen sampling:</td>
<td>13×, NA 0.5</td>
<td>13×</td>
<td>25×, NA 0.5</td>
<td>20× NA 0.75, 40× NA 0.75</td>
<td>20× NA 0.75</td>
</tr>
<tr>
<td>Microscopy mode:</td>
<td>Brightfield</td>
<td>Brightfield</td>
<td>Fluorescence</td>
<td>Brightfield, Phase-contrast, Darkfield, Fluorescence</td>
<td>Brightfield, Phase-contrast, Fluorescence</td>
</tr>
<tr>
<td>Stage speed:</td>
<td>4 mm/s</td>
<td>1.5 mm²/s</td>
<td>93.8 μm/s or 0.0335 mm²/s</td>
<td>2.34 mm/s</td>
<td>1.84 mm/s</td>
</tr>
<tr>
<td>Focus step:</td>
<td>NR</td>
<td>NR</td>
<td>25 μm</td>
<td>16 μm (piezoelectric drive accuracy)</td>
<td>20 μm (piezoelectric drive accuracy)</td>
</tr>
<tr>
<td>Application</td>
<td>Prescreening of cervical smears DNA content (absorbance)</td>
<td>Metaphase spreads detection</td>
<td>Fluorescence microscopy FISH (Spot counting)</td>
<td>Fluorescence microscopy DNA content</td>
<td>Fluorescence Microscopy Detection of circulating tumor cells in blood</td>
</tr>
<tr>
<td>Results:</td>
<td>CV, of I.O.D.: 9 - 6.5%</td>
<td>N/R</td>
<td>10% MTF degradation (at 1 cycle/μm)</td>
<td>C.V. of O1: 8%³</td>
<td>Sensitivity: 85.07954%</td>
</tr>
<tr>
<td></td>
<td>10x 10 dB better than static</td>
<td></td>
<td></td>
<td>Specificity: 96.8296%⁴</td>
<td></td>
</tr>
</tbody>
</table>

AF - autofocus; NR - not reported in listed reference; I.O.D. - integrated optical density; CV - coefficient of variance

a. Test conditions: Closed-loop autofocus with no stage motion. Specimen: Ronchi ruling (2000 lines/inch). Focus update rate = 27 Hz. Microscope in brightfield mode, tests were carried with 20× and 40× optics, and focus feedback gain of 0.2 and 0.4.
b. Specimen: NIH 3T3 cells with DAPI stain. There were 769 cells detected, mostly in G0 phase.
c. RMS error compared focus obtained with incremental autofocus on samples of mWBCs spiked with MCF7 tumor cells. Best: sample with 2,058 fields of view; Average: from three samples with 6,974 total fields of view.
d. Average values from seven samples with over 500,000 cells.
It is difficult to compare autofocus accuracy between the systems since the testing conditions and specimens were different on each system. Of the systems that reported error measures, the two systems a) Shippey [103] and Tucker et al. [117, 118], and b) Bravo [19] reported the standard deviation of focus positions between repeated scans and not the actual focus difference between continuous autofocus and an independent focusing method (manual or incremental focusing). From our experience, repeated scan may overlap each other very well to produce small standard deviation values; however, they do not necessarily correspond to the real locations of the cells. The error comparison must be made with an independent and accepted method such as manual focusing by an experienced human operator or incremental autofocus. Furthermore, the values reported by Bravo were measured with a Ronchi ruling under static conditions. Only Netten et al. [80] reported the actual error difference between focus positions obtained during continuous scan and ones obtained with incremental method; however, the reported values came from a single 3.2 mm strip with a total of 10 data points. None of the published works performed the same thorough focus accuracy analysis that was done in this dissertation. The focus accuracy of our instrument was measured against the objective incremental autofocus method, on different specimens (Ronchi ruling, low quality and high-quality cell samples). The focus accuracy is also derived from the data collected from thousands of fields of view. We obtained the smallest error measures with the Ronchi ruling; however, such an optimal and ideal specimen does not reflect the typical cell sample and they were not used for the comparison. Instead, the error values listed in the table were the ones obtained with real cell samples. Although the autofocus system was less than optimal, the instrument was able to achieve focus errors that were about half the depth of field.

5.2.2 Throughput

Besides the high-resolution, the high-throughput was the other performance criterion of the instrument. Table 5.2 compares the throughput of the system with two commercial continuous-scanning instruments: ScanScope CS (Aperio Technologies, Inc., Vista, CA) and NanoZoomer Digital Pathology (NDP) (Hamamatsu Photonic Systems, Bridgewater, NJ). Both of these systems are marketed for dig-
ital pathology applications in which microscope slides of pathology samples are
digitized and stored at a central storage location. This allows convenient local and
remote access through the internet for archival, collaboration, consultation, and
teaching purposes. These two systems were not included in the above autofocus
comparison because data about their autofocus system and performance was not
available.

Table 5.2: Throughput Comparison with two Commercial Continuous-
Scanning Systems.

<table>
<thead>
<tr>
<th></th>
<th>Aperio’s ScanScope CS</th>
<th>Hamamatsu’s NDP</th>
<th>[Nguyen, 2006]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Image Sensor</strong></td>
<td>3 linear CCDs, 2048 pixels × 1 row each</td>
<td>3 TDI CCDs, 4096 pixels × 64 rows each</td>
<td>2-D array in TDI mode, 1024 pixels × 1024 rows</td>
</tr>
<tr>
<td><strong>Objective Lens</strong></td>
<td>20× 0.75 NA</td>
<td>20× 0.75 NA</td>
<td>20× 0.75 NA</td>
</tr>
<tr>
<td>Specimen sampling</td>
<td>0.5 µm</td>
<td>0.46 µm</td>
<td>0.22 µm</td>
</tr>
<tr>
<td>Microscopy mode</td>
<td>Brightfield</td>
<td>Brightfield</td>
<td>Brightfield, Phase-contrast, Fluorescence</td>
</tr>
<tr>
<td><strong>Data Bit Depth</strong></td>
<td>24-bit color</td>
<td>24-bit color</td>
<td>12-bit grayscale</td>
</tr>
<tr>
<td><strong>Throughput</strong> (megapixels/s)</td>
<td>5a</td>
<td>10.5b</td>
<td>8.37</td>
</tr>
</tbody>
</table>

a. 15 × 15 mm scan area in 3 minutes
b. 20 × 20 mm scan area in 3 minutes

Both commercial systems are equipped with 3-chip cameras to acquire color
images while our instrument only has a monochrome camera. This is currently
a disadvantage of our system; however, replacing the monochrome camera with
a 3-chip color camera is essentially a simple bolt-on replacement. The objective
lenses in the three systems are equivalent — all Plan Apo 20× high NA objectives.
The commercial systems have coarser sampling (0.5 µm/pixel and 0.46 µm/pixel)
since they work in brightfield mode and probably with relatively low NA condenser.
The sampling in our instrument is more conservative since we work primarily in
fluorescence mode and we want to achieve Nyquist sampling at DAPI’s emission
wavelength (λ = 460 nm). Comparing the throughput in terms of number of pixels
per unit time, our instrument came out second (8.37 Mpixels/s), behind NDP’s
10.5 Mpixels/s but faster than ScanScope’s 5 Mpixels/s. On the other hand, our
camera is only 1024-pixel wide which is half the size of ScanScope’s and only one
fourth of NDP’s. With the Plan Apo objective lens which is well corrected for flat-
field, our system’s throughput can be easily doubled by replacing the camera with a 2048-pixel wide version with little or no distortions at the edges. In addition, one should note that pathology samples are always stained with dyes to enhance the contrast, which as a side effect allow autofocus to work better, while our specimens are not. Also, the commercial systems only work in brightfield mode while our instrument works in brightfield, phase-contrast, and fluorescence modes. Although our system was able to achieve 12.3-kHz line-rate (or 12.3 Mpixels/s) in brightfield mode with the Ronchi ruling, we chose the 8.37 Mpixels/s value for the comparison because it better represents the typical performance of the system.

5.3 How Did the System Perform as a Tool to Find CTCs?

The performance of the system at finding CTCs is determined by the quality of the image data. Our method of CTC detection exploits the differences in morphological features between tumor cells and mWBCs. The performance of the method depends on the accuracy of the measurements. As the quality of the measurements degrades, so does the system’s ability to detect CTCs and distinguish the different cell types. Table 5.3 summarizes the comparison of CTC detection performance between the continuous-scanning instrument and Beckman Counter’s IC100 incremental-scanning cytometer.

Table 5.3: Summary of CTCs Detection Performance Comparison with the Incremental-Scanning Cytometer.

<table>
<thead>
<tr>
<th></th>
<th>Incremental System</th>
<th>Continuous System [Nguyen 2006]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Objects Found</td>
<td>685389</td>
<td>649527</td>
</tr>
<tr>
<td>Total CTCs(^a)</td>
<td>71</td>
<td>56</td>
</tr>
<tr>
<td>Identified CTCs</td>
<td>62</td>
<td>46</td>
</tr>
<tr>
<td>Average Sensitivity</td>
<td>88.1973%</td>
<td>85.0794%</td>
</tr>
<tr>
<td>Average Specificity</td>
<td>98.1391%</td>
<td>96.8296%</td>
</tr>
</tbody>
</table>

\(^a\) Number of CTCs in the image data sets identified with ground truth staining

The results indicate that the detection performance between the two systems
were comparable. The sensitivity of 85% means that the system would miss 15 out of every 100 CTCs. And the specificity of 96.8% means that there would be thousands of false positives for each typical 10-ml blood sample. In comparison with other microscopy based CTC detection methods, these sensitivities and specificities are relatively low. For example, Krivacic et al. [62] reported 95% sensitivity and 99.9997% specificity. Similarly, Allard et al. [6] reported greater or equal to 85% for sensitivity and 99.7% for specificity. However, the classification methods of both of these systems include a visual inspection step by a trained professional. After the initial automated classification by the machines, the subset of cells suspected to be CTCs were presented to the human operator for verification. With this visual inspection, the operator eliminated the majority of the false positives which resulted in the high specificity numbers. The addition of similar visual verification step to our classification method would likely to result in better performance as well.

The image quality is one of the main factors that affects the accuracy of our measurements. Out of focus images were the main cause of the performance differences between the two systems. In out-of-focus images, the edges of the cells were blurred and they appeared larger. When segmented, these blurry nuclei were about the same size as the tumor cells and were falsely identified as CTCs. This resulted in the high number of false positives for both systems. In the continuous-scanning instrument, the out-of-focus images were the result of the system losing focus. On the other hand, in the incremental-scanning instrument, out-of-focus images were the result of the system falsely tracking the layer of debris instead of cells (samples B2 and D2). Blurry images also caused closely-spaced cells, that otherwise would have been able to be separated, to be segmented as a single object. Since most of these clumps were gated out because they did not resemble a single tumor cell, CTCs that were part of clumps were not detected. This was one of the reasons why the continuous system found fewer CTCs. In addition, some CTCs were in regions in which the system completely lost focus; therefore, they were not detected. Clumping also caused the discrepancies in total number of objects detected in each sample between the two instruments. Because of its coarser sampling density (0.3225 µm/pixel), it was less able to separate closely-spaced cells which ended up with more clumps and resulted in a smaller total number of
objects. The continuous-scanning system with its finer sampling (0.22 \( \mu \text{m/pixel} \)), was better able at segmenting closely-spaced nuclei in samples that it could keep in focus (samples A1, C1, and D1).

The performance of the system can be increased by improving the specimen preparation. As seen from above, factors such as cell density and quality of the specimen preparation have a big impact on the overall performance of the system. The sample’s cell density, in part, determines the throughput of the system. Faster throughput can be achieved by increasing the cell density. On the other hand, too densely populated samples with closely packed cells create difficulties for the image analysis process that may result in data loss. Optimal cell density is a compromise between obtaining the highest speed and accurate segmentation. Furthermore, sample quality issues such as multiple cell layers and inclusion of debris also degrade image quality and the performance of the system. Although some effort has been already put into optimizing the specimen preparation process, a lot more work remains to perfect the technique.

Better overall system performance can also be achieved with improved classification techniques. The classification method employed was relatively simple. The purpose of the experiment was to compare the performance between the continuous and incremental systems, to see the effects of continuous-scanning on the performance, and not necessarily to optimize the overall method. The most obvious feature difference was used to distinguish the cell types. Much more sophisticated software algorithms and gating criteria can be developed to produce better sensitivities and specificities.

The main advantage of our method is its basis for detection. Because our method identifies cells based on morphologic differences between tumor and normal cells, it has advantages over techniques that are dependent on the detection of variably expressed proteins that are not uniformly expressed in all patients. As previously mentioned, existing marker-based studies have shown great variability in the results. Even when the same antigen is used, different research groups have reported different sensitivities and specificities. Similarly, RT-PCR studies have shown high variability in the results some of which was able to be attributed to illegitimate transcription and the presence of pseudogenes. Furthermore, another advantage of image-based technique over RT-PCR or flow-cytometry is the capa-
bility to reject false positives through visual verification. Because of these advantages, with future improvements in performance, our method has great potential in becoming the method of choice for detecting CTCs.

5.4 What Improvements Can Be Made to the System?

The throughput of the instrument was hampered by its ability to maintain focus. Autofocus performance was limited by the inefficiency in light transmission and the degradation of high-frequency signals through the focus measurement channels. One way to improve transmission efficiency is to decrease the number of channels. The current design splits light into eight channels. The large number of channels was intended to give autofocus a large range. However, from our experiments in brightfield in which light transmission was not the limiting factor, as little as four channels were sufficient enough to track focus. With fewer channels, more light will be transmitted to each focus measuring channel. Less integration time will be required and focus can be updated more frequently. With fewer channels, the focusing range will be smaller; however, the faster update rate will compensate and allow the system to track focus. Fewer channels also mean that light will travel through fewer beamsplitters and mirrors which reduces scattering of light and improves resolution. Other components in the optical path including the collimating lens and the magnification correction optics can also be improved to increase transmission efficiency and resolution.

The autofocus performance can also be improved with higher sensitivity TDI cameras for focus measurement. During the development of the instrument, the tight spacing between focusing channels did not allow the use of off-the-shelf cameras. Even though our custom cameras went through several development cycles, their performance was low compared to commercial products. In recent years, newer commercial solutions have become available in two-piece designs in which a small sensor head is separated from the rest of the camera’s electronics. These new cameras may be integrated into our instrument with few modifications. In addition, with fewer focusing channels, the integration of these cameras may be
simpler since there will be less mechanical restriction. The combination of efficient transmission, higher resolution optics and better performance cameras will increase the SNR of the focus measuring channels. The overall effect will be faster and more accurate autofocus which will increase the throughput of the system.

The autofocus performance can be further improved with a surface tracking method. Non-contact laser displacement sensors such as those available from Keyence (Woodcliff Lake, NJ) and Micro-Epsilon (Ortenburg, Germany) have 1–2 µm resolution, 5–10 mm range, 1–4 kHz bandwidth, and are capable of sensing transparent glass surfaces. Although these sensors are not accurate enough for keeping the specimen in focus, they can be incorporated into the system to work in a complementary fashion to the image-based autofocus and to provide a coarse estimate of the position of the specimen. By tracking either of the surfaces of the coverslip, the displacement sensor helps to confine the image-based autofocus system in a narrow zone. Because of this, the channels of the autofocus system can be spaced closer together to achieve a higher accuracy. In addition, by restricting the autofocus to a narrow range, the displacement sensor also prevents the autofocus system from losing focus when presented with empty or sparse fields of view.

Incorporating additional monochrome cameras or replacing the existing trailing camera with a 3-chip color camera will increase the throughput of the system. With the appropriate filters, multiple colors can be acquired at the same time which reduces the overall scanning time. We have worked with Hamamatsu to develop a 3-chip TDI camera with independent gain, offset, and integration controls for each chip, and we are in the process of incorporating it into the instrument. The camera also has a variable electronic shutter period at the beginning of each line, during which the camera is essentially blind. This feature will be used to eliminate the bleed-through of autofocus light to the trailing camera. In the current configuration of the system, the autofocus and fluorescent light are separated to different optical paths by a dichroic mirror. This only works for bright fluorescence signals such as DAPI. Dim fluorescence signals are overwhelmed by the bleed-through. Working in combination with the camera’s electronic shuttering, autofocus light will be pulsed ON during the shuttered period. This method of light delivery allows transmission of autofocus light to the focus measuring cameras without overwhelming the fluorescence signal at the trailing camera.
The overall scanning time can be further increased with the use of bidirectional TDI sensors. With these sensors, the direction of charge transfer between integration stages can be switched. This allows scanning of the specimen in a “switchback” pattern, in which adjacent strips are scanned in opposite directions. This method of scanning eliminates the time-consuming “fly back” periods associated with unidirectional TDI sensors.

Better and efficient automation of the microscope subsystems will also dramatically cut down the overall scanning time. In the current instrument, automation of the light sources and the filters were intended to make the scanning process independent from the operator. No attempts was made to optimize automation. In the next generation of the instruments, the shutters and the filter wheels will reduce the processes to the order of tens of milliseconds versus the current 5 to 10 seconds to accomplish in each strip.

Upgrading the X-Y stage with a better model with less speed fluctuation will help improve the quality of the images. Although the use of the linear encoder minimize blurring due to speed fluctuation in the stage, it does not correct for the differences in integration time. These fluctuations in intensity between lines of pixel in the images have big impact on fluorometric measurements. A more stable stage will minimize these fluctuations and will improve the quality of the image data.

Improvements in the sample preparation process will also be crucial to increase the performance of the system. Such improvements include changes to create more uniform density slides, with an even monolayer, and free of debris. The protocol will also need to be optimized to minimize clumping of cells. Such improvements will reduce the number of out-of-focus objects, eliminate cell loss due to clumping, and improve the system’s ability to focus — all of which will increase the sensitivity and specificity of the detection method.

The CTCs detection performance will benefit from more sophisticated classification techniques. The primitive method used in this dissertation utilized only two morphologic measurements in a simplistic manual gating process. Improvements in performance can be achieved by thoroughly exploiting all possible differences that may exist in the available measurements. Systematic identification and utilization of important, distinguishing, and independent features will result in more robust
classification techniques.

5.5 Conclusions

To meet the specific aims of the dissertation, a high throughput, high resolution image scanning cytometer was developed. The performance of the system at finding CTCs was demonstrated on a spiked cell model.

The first aim was to develop an instrument that can scan large areas at high resolution with high throughput. This aim was met with the design and construction of the continuous-scanning image cytometer with on-the-fly autofocus. The instrument incorporated an image-splitting system that allowed simultaneous sampling of focus at multiple focal planes. Together with TDI cameras and analog focus measurement electronics, the specimen was kept in focus as it was imaged by the trailing camera. The performance of the system was measured on Ronchi ruling along with cell specimens, and it showed to be less than the depth-of-field of the system.

The second aim was to evaluate the performance of the system at finding CTCs using a spiked cell model. Tumor cells were spiked into blood samples from healthy donors. The samples was cytocentrifuged on to slides and imaged by the instrument. Manual gating was selected to separate the tumor cells from normal mWBCs based on morphological measurements. An identical process was also performed on an incremental-scanning cytometer to compare their relative performance. The results showed the two systems to be comparable; however, sensitivity of 85% and specificity of 98.6% were low compared to other cell-based detection methods.

5.6 Future Directions

Along with the development of the high throughput, high resolution image cytometer, this dissertation also demonstrated the possibility of using this instrument to detect CTCs based on morphological measurements. The next steps to be done are the rigorous testing and verification of the method.

Once the optimization of the instrumentation has been completed, a series of tightly controlled spiked cell model experiments need to be performed to fully
characterize the overall performance of the system. In these experiments, exact numbers of CTCs are added to the samples, so that the true numbers of CTCs could be determined ahead of time. This will also allow the determination of the efficiency of the specimen preparation and the quantification of any cell loss during the process. To determine the threshold of sensitivity, a varying number of CTCs will be added to different sets of samples ranging from a few cells to hundreds of cells per sample. Also, slides will be scanned and analyzed multiple times to measure the system’s repeatability.

Following the spiked cell model, the method should be characterized on real/non-spiked samples. First, a mouse model should be used to further verify the technique. Tumor cells that have been in the circulation might have their morphology altered by mechanical forces of the circulatory system or attacks from the immune system. The mouse model should be created by transfecting the mouse with a tumor cell line that had been modified to express green fluorescent protein (GFP). The GFP is then used as the ground truth marker. Prior to cytocentrifuging, the samples should be run through a flow cytometer to enumerate the number of CTCs. Finally, the method then should be verified on large number of clinical samples. A performance comparison should be made with existing detection methods.

The development of a high throughput, high resolution image cytometer for CTCs detection also opens up many other possibilities in image cytometry. With its ability to image large areas at high resolution and high throughput, the instrument can be used for many other applications. For example, it can be used in drug discovery to look at the effects of chemical compounds on cell populations for libraries that contain hundreds of thousands of different compounds. It can also be used for digital/telepathology in which pathology slides are digitized for easy local or remote access, archiving, collaboration, consultation, or teaching purposes. It can also be used a general piece of lab equipment that is capable of imaging and measuring millions of cells in a short period of time. Because of the time saved, researchers can use their time more efficiently to do other tasks such as designing experiments, analyzing and interpreting the results.
Appendix A

Scanning Cytometer

A.1 Mechanical Drawings
NOTE: All the Units are in Inches.

UNLESS OTHERWISE SPECIFIED:
DIMENSIONS ARE IN INCHES
TOLERANCES: 0.001, 0.0005, 0.00002
FINISH 125 RMS
SIMPLIFY ALL BURRS AND
SHARP ALL BUMP EDGES
FILLET 1/16 IN DIAM AND TO 0.002
DO NOT SCALE THIS DRAWING.
NOTE: All the units are in Inches
A.1.2 Linear Encoder Mount

NOTE: All the Units are in Inches

QUALITY: AS SHOWN

188
190
A.1.3 Magnification Correction
PRESS FIT FOR OD=5/16" 2 PLCS
A.1.4 Trailing Camera Rotational Mount
A.2 Electrical Diagrams

A.2.1 Array Timing Controller

Figure A.1: Timing Controller Schematics

A.2.2 TDI Camera
Figure A.2: TDI Camera Schematics - Sensor Board
Figure A.3: TDI Camera Schematics - Main Board
A.2.3 TDI Line-Clock Conditioning

Figure A.4: TDI Line-Clock Conditioning
A.2.4 Lamp Controller

Figure A.5: Lamp Controller Schematics

Figure A.6: Velleman K8003 DC-Controlled Dimmer
## A.3 DAQ Connections

<table>
<thead>
<tr>
<th>Description</th>
<th>Software Variable</th>
<th>Port Label</th>
<th>Pin # (+) – (-)</th>
<th>Test Panel Channel</th>
<th>Device</th>
</tr>
</thead>
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<td>Focus Measurement Channel 1</td>
<td>F[1][0]</td>
<td>ACH19-ACH18</td>
<td>3 – 4</td>
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<td>A[1][0]</td>
<td>ACH11-ACH10</td>
<td>5 – 6</td>
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<td>1</td>
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<tr>
<td></td>
<td>G[1][0]</td>
<td>ACH13-ACH12</td>
<td>69 – 70</td>
<td>33</td>
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<tr>
<td></td>
<td>O[1][0]</td>
<td>ACH14-ACH13</td>
<td>71 – 72</td>
<td>34</td>
<td>1</td>
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<tr>
<td>Focus Measurement Channel 2</td>
<td>F[1][1]</td>
<td>ACH12-ACH11</td>
<td>7 – 8</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
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<td>A[1][1]</td>
<td>ACH13-ACH12</td>
<td>9 – 10</td>
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</tr>
<tr>
<td></td>
<td>G[1][1]</td>
<td>ACH15-ACH14</td>
<td>53 – 54</td>
<td>55</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>O[1][1]</td>
<td>ACH16-ACH15</td>
<td>77 – 78</td>
<td>36</td>
<td>1</td>
</tr>
<tr>
<td>Focus Measurement Channel 3</td>
<td>F[1][2]</td>
<td>ACH14-ACH13</td>
<td>11 – 12</td>
<td>4</td>
<td>1</td>
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<tr>
<td></td>
<td>A[1][2]</td>
<td>ACH15-ACH14</td>
<td>13 – 14</td>
<td>5</td>
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</tr>
<tr>
<td></td>
<td>G[1][2]</td>
<td>ACH17-ACH16</td>
<td>79 – 80</td>
<td>37</td>
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<tr>
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<td>O[1][2]</td>
<td>ACH18-ACH17</td>
<td>81 – 82</td>
<td>38</td>
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<td>Focus Measurement Channel 4</td>
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<td>ACH16-ACH15</td>
<td>15 – 16</td>
<td>6</td>
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<tr>
<td></td>
<td>A[1][3]</td>
<td>ACH17-ACH16</td>
<td>17 – 18</td>
<td>7</td>
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<td>G[1][3]</td>
<td>ACH19-ACH18</td>
<td>83 – 84</td>
<td>39</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>O[1][3]</td>
<td>ACH20-ACH19</td>
<td>85 – 86</td>
<td>48</td>
<td>1</td>
</tr>
<tr>
<td>Focus Measurement Channel 5</td>
<td>F[1][4]</td>
<td>ACH18-ACH17</td>
<td>59 – 60</td>
<td>55</td>
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</tr>
<tr>
<td></td>
<td>A[1][4]</td>
<td>ACH19-ACH18</td>
<td>53 – 54</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>G[1][4]</td>
<td>ACH21-ACH20</td>
<td>87 – 88</td>
<td>49</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>O[1][4]</td>
<td>ACH22-ACH21</td>
<td>89 – 90</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>Focus Measurement Channel 6</td>
<td>F[1][5]</td>
<td>ACH18-ACH17</td>
<td>55 – 56</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>A[1][5]</td>
<td>ACH19-ACH18</td>
<td>57 – 58</td>
<td>19</td>
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</tr>
<tr>
<td></td>
<td>G[1][5]</td>
<td>ACH21-ACH20</td>
<td>91 – 92</td>
<td>51</td>
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</tr>
<tr>
<td></td>
<td>O[1][5]</td>
<td>ACH22-ACH21</td>
<td>93 – 94</td>
<td>52</td>
<td>1</td>
</tr>
<tr>
<td>Focus Measurement Channel 7</td>
<td>F[1][6]</td>
<td>ACH22-ACH21</td>
<td>63 – 64</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>G[1][6]</td>
<td>ACH25-ACH24</td>
<td>95 – 96</td>
<td>53</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>O[1][6]</td>
<td>ACH26-ACH25</td>
<td>97 – 98</td>
<td>54</td>
<td>1</td>
</tr>
</tbody>
</table>

PIFOC Control Voltage  | PIFOC Out  | DAOC0UT-AOC0ND | 23 – 20 | 0 | 1 |
PIFOC Feedback Monitor | PIFOC Moniocr | ACH30-ACH29 | 67 – 68 | 32 | 1 |
PIFOC LVDT Monitor | PIFOC LVDT Monitor | ACH32-ACH31 | 59 – 60 | 20 | 1 |
PIFOC FZT Monitor (1.100) | PIFOC FZT Monitor | ACH31-ACH30 | 61 – 62 | 21 | 1 |
LAMP Intensity Control Voltage | LAMP Out | DAOC0UT-AOC0ND | 23 – 20 | 0 | 3 |
LAMP Intensity Feedback | LAMP Feedback | ACH2 – ACH15 | 17 – 18 | 7 | 3 |
2D Camera Focuss Index | ACH16 – ACH24 | 51 – 52 | 16 | 3 |
2D Camera Average Illumination | ACH17-ACH25 | 53 – 54 | 17 | 3 |
2D Camera Gain 0 | ACH18-ACH26 | 55 – 56 | 18 | 3 |
2D Camera Gain 1 | ACH19-ACH27 | 57 – 58 | 19 | 3 |

Figure A.7: Multifunction DAQ Board Pin Configuration
A.4 Cytoshop Cell-by-Cell Measurements

Reproduced from the Beckman Counter’s Cell Lab IC 100 Image Cytometer instruction manual (Part Number 627452B).

<table>
<thead>
<tr>
<th>Measurement Name</th>
<th>Common Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSDEV</td>
<td>Nuclear Pixel intensity absolute deviation</td>
<td>Absolute deviation of intensity of pixels in the nucleus</td>
</tr>
<tr>
<td>AREA</td>
<td>Nuclear area (um^2)</td>
<td>Area of the nucleus in microns squared</td>
</tr>
<tr>
<td>AREA**1/2</td>
<td>Nuclear area^1/2</td>
<td>Square root of the nuclear area</td>
</tr>
<tr>
<td>AVER_GS</td>
<td>Nuclear Mean Pixel Intensity</td>
<td>Average intensity (normalized to 1) for all the pixels in the nucleus</td>
</tr>
<tr>
<td>BATCH_ID_</td>
<td>BATCH_ID_</td>
<td>Batch ID, an integer assigned by the user</td>
</tr>
<tr>
<td>CHANNEL</td>
<td>Fluorescent Label</td>
<td>Channel index</td>
</tr>
<tr>
<td>CL_AVGCYT, i = 1, 2, 3, ...</td>
<td>Label 2 Cytoplasmic Mean Pixel Intensity</td>
<td>Average intensity in the cytoplasm (for channel i)</td>
</tr>
<tr>
<td>CL_AVGNUC, i = 1, 2, 3, ...</td>
<td>Label 2 Nuclear Mean Pixel Intensity</td>
<td>Average intensity in the nucleus (for channel i)</td>
</tr>
<tr>
<td>CIN_ABSDEV, i = 1, 2, 3, ...</td>
<td>Label 2 Nuclear Pixel Intensity ABSDEV</td>
<td>Absolute deviation of brightness of channel i pixels that overlap with the nuclear mask</td>
</tr>
<tr>
<td>CIN_AVG, i = 1, 2, 3, ...</td>
<td>Label 2 Nuclear Pixel Intensity AVG</td>
<td>Average brightness of channel i pixels that overlap with the nuclear mask</td>
</tr>
<tr>
<td>CIN_CM3, i = 1, 2, 3, ...</td>
<td>Label 2 Nuclear Pixel Intensity CM3</td>
<td>Third central moment of brightness of channel i pixels that overlap with the nuclear mask</td>
</tr>
<tr>
<td>CIN_CM4, i = 1, 2, 3, ...</td>
<td>Label 2 Nuclear Pixel Intensity CM4</td>
<td>Fourth central moment of brightness of channel i pixels that overlap with the nuclear mask</td>
</tr>
<tr>
<td>CIN_VAR, i = 1, 2, 3, ...</td>
<td>Label 2 Nuclear Pixel Intensity Variance</td>
<td>Brightness variance of channel i pixels that overlap with the nuclear mask</td>
</tr>
<tr>
<td>CINM_AREA, i = 1, 2, 3, ...</td>
<td>Label 2 Nuclear Area</td>
<td>Obsolete, same as AREA</td>
</tr>
<tr>
<td>CM3</td>
<td>Nuclear Pixel intensity CM3</td>
<td>Third central moment of intensity of pixels in the nucleus</td>
</tr>
<tr>
<td>CM3**1/3</td>
<td>Nuclear Pixel intensity CM3^1/3</td>
<td>Third root of the third central moment of intensity of pixels in the nucleus</td>
</tr>
<tr>
<td>CM4</td>
<td>Nuclear Pixel intensity CM4</td>
<td>Fourth central moment of intensity of pixels in the nucleus</td>
</tr>
<tr>
<td>CM4**1/4</td>
<td>Nuclear Pixel intensity CM4^1/4</td>
<td>Fourth root of the fourth central moment of intensity of pixels in the nucleus</td>
</tr>
<tr>
<td>COLUMN_</td>
<td>COLUMN_</td>
<td>Well column index</td>
</tr>
<tr>
<td>CORIM_AREA, i = 1, 2, 3, ...</td>
<td>Label 2 Area</td>
<td>Area of the cell in the correlated image</td>
</tr>
<tr>
<td>CORRI, i = 1, 2, 3, ...</td>
<td>Label 2 Total Fluorescence</td>
<td>Total integrated intensity of correlated channel i</td>
</tr>
<tr>
<td>Measurement Name</td>
<td>Common Name</td>
<td>Description</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>CORRI_CYT, i = 1, 2, 3, ...</td>
<td>Label 2 Cytoplasmic Intensity</td>
<td>Sum of intensities in the correlated channel i for all the pixels in the cytoplasm</td>
</tr>
<tr>
<td>CORRI_FLIC, i = 1, 2, 3, ...</td>
<td>Label 2% cytoplasmic</td>
<td>CORRI_FLIC = CORRI_CYT/CORRI</td>
</tr>
<tr>
<td>CORRI_FLIN, i = 1, 2, 3, ...</td>
<td>Label 2% nuclear</td>
<td>CORRI_FLIN = CORRI_NUC/CORRI</td>
</tr>
<tr>
<td>CORRI_IDX, i = 1, 2, 3, ...</td>
<td>Label 2</td>
<td>1 if the cell has data for correlated channel i, -1 otherwise</td>
</tr>
<tr>
<td>CORRI_NUC, i = 1, 2, 3, ...</td>
<td>Label 2 Nuclear Intensity</td>
<td>Sum of intensities in the correlated channel i for all the pixels in the nucleus</td>
</tr>
<tr>
<td>FRAMEID</td>
<td>Frame ID</td>
<td>Index of the image frame the cell belongs to (multiple frames can be imaged in one region)</td>
</tr>
<tr>
<td>HEIGHT</td>
<td>Nuclear height (um)</td>
<td>Height of the nucleus in microns</td>
</tr>
<tr>
<td>INTEG_GS</td>
<td>Nuclear Intensity</td>
<td>Integrated grayscale, sum of intensities (normalized to 1) for all the pixels in the nucleus</td>
</tr>
<tr>
<td>IQ_RANGE</td>
<td>Nuclear Pixel Intensity 25%-75%</td>
<td>Interquartile range is a measure of spread or dispersion; the difference between nuclear pixel intensities at 75% and 25%</td>
</tr>
<tr>
<td>ISBOUNDARY</td>
<td>ISBOUNDARY</td>
<td>1 if the cell touches the frame boundary; 0 if it does not touch</td>
</tr>
<tr>
<td>ISSTITCHED</td>
<td>ISSTITCHED</td>
<td>1 if the cell image was stitched across the frame boundary; 0 if it was not</td>
</tr>
<tr>
<td>PERCENT__0</td>
<td>Nuclear Pixel Intensity 0%</td>
<td>Brightness of the darkest pixel in the nucleus</td>
</tr>
<tr>
<td>PERCENT__5</td>
<td>Nuclear Pixel Intensity 5%</td>
<td>Brightness of the pixel that is brighter than 5% of the pixels in the nucleus</td>
</tr>
<tr>
<td>PERCENT_25</td>
<td>Nuclear Pixel Intensity 25%</td>
<td>Brightness of the pixel that is brighter than 25% of the pixels in the nucleus</td>
</tr>
<tr>
<td>PERCENT_50</td>
<td>Nuclear Pixel Intensity 50%</td>
<td>Brightness of the pixel that is brighter than 50% of the pixels in the nucleus</td>
</tr>
<tr>
<td>PERCENT_75</td>
<td>Nuclear Pixel Intensity 75%</td>
<td>Brightness of the pixel that is brighter than 75% of the pixels in the nucleus</td>
</tr>
<tr>
<td>PERCENT_95</td>
<td>Nuclear Pixel Intensity 95%</td>
<td>Brightness of the pixel that is brighter than 95% of the pixels in the nucleus</td>
</tr>
<tr>
<td>PERCENT100</td>
<td>Nuclear Pixel Intensity 100%</td>
<td>Brightness of the brightest pixel in the nucleus</td>
</tr>
<tr>
<td>PERIMETER</td>
<td>Nuclear perimeter</td>
<td>Perimeter of the nucleus</td>
</tr>
<tr>
<td>PLATE_ID_</td>
<td>PLATE_ID_</td>
<td>Plate ID, an integer assigned by the user</td>
</tr>
<tr>
<td>PLATE_IDX_</td>
<td>PLATE_IDX_</td>
<td>Plate index, from 0 to n-1 for a batch of n plates</td>
</tr>
<tr>
<td>REGION_INX</td>
<td>Well ID</td>
<td>Well index; numerical representation of absolute well position, i.e. well B5 has well index 16 on a 96 well plate</td>
</tr>
<tr>
<td>ROW_</td>
<td>ROW_</td>
<td>Well row index</td>
</tr>
<tr>
<td>Measurement Name</td>
<td>Common Name</td>
<td>Description</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>SCAN_DATE_</td>
<td>SCAN_DATE_</td>
<td>Obsolete, always zero in the database</td>
</tr>
<tr>
<td>SERIAL</td>
<td>Cell ID</td>
<td>Index number of the cell (identified by image segmentation based on nuclear stain, numbered consecutively)</td>
</tr>
<tr>
<td>STDDEV</td>
<td>Nuclear Pixel Intensity standard deviation</td>
<td>Standard deviation of intensity of pixels in the nucleus</td>
</tr>
<tr>
<td>TIME</td>
<td>TIME</td>
<td>Time (ms since the beginning of the run) when cell was imaged</td>
</tr>
<tr>
<td>TIME_SLICE</td>
<td>Time Slice</td>
<td>Time slice to which this row of measurements refers</td>
</tr>
<tr>
<td>TOTALCOUNr</td>
<td>Cell Count</td>
<td>Number of cells in the region (region is a well or a slide)</td>
</tr>
<tr>
<td>VARIANCE</td>
<td>Nuclear Pixel Intensity variance</td>
<td>Intensity variance of pixels in the nucleus</td>
</tr>
<tr>
<td>WIDTH</td>
<td>Nuclear width (um)</td>
<td>Width of the nucleus in microns</td>
</tr>
<tr>
<td>WIGGLE</td>
<td>Nuclear area/perimeter</td>
<td>Wiggle; perimeter divided by area of the nucleus. Measures how convoluted the boundary of the nucleus is; high for lobed nuclei</td>
</tr>
<tr>
<td>WIGGLE_NRM</td>
<td>Nuclear area/perimeter normalized</td>
<td>Normalized wiggle; perimeter divided by the square root of the area of the nucleus</td>
</tr>
<tr>
<td>X</td>
<td>Nuclear x coordinate</td>
<td>X position of the centroid of the cell within the region</td>
</tr>
<tr>
<td>X_SIZE</td>
<td>Nuclear x-size (pxl)</td>
<td>Width of the nucleus in pixels</td>
</tr>
<tr>
<td>Y</td>
<td>Nuclear y coordinate</td>
<td>Y position of the centroid of the cell within the region</td>
</tr>
<tr>
<td>Y_SIZE</td>
<td>Nuclear y-size (pxl)</td>
<td>Height of the nucleus in pixels</td>
</tr>
<tr>
<td>Z_FOCUS</td>
<td>Z position</td>
<td>Z position (in um) at which the cell was imaged</td>
</tr>
</tbody>
</table>
A.5 Miscellaneous

A.5.1 Ocean Optics Fiber Transmission Curve

Figure A.8: Ocean Optics Fiber Specification Sheet
A.5.2 Halogen Lamp Water Cooling System

![Halogen Lamp Water Cooling System Diagram]

**Figure A.9: Halogen Lamp Water Cooling System**

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
<th>Part No.</th>
<th>Qty.</th>
<th>Vendor</th>
</tr>
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<tr>
<td>1</td>
<td>Water Block</td>
<td>ast-2030</td>
<td>1</td>
<td>Sharka Computers</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><a href="http://www.sharkacomputers.com">www.sharkacomputers.com</a></td>
</tr>
<tr>
<td>2</td>
<td>Radiator</td>
<td>bip-120_2</td>
<td>1</td>
<td>Sharka Computers</td>
</tr>
<tr>
<td>3</td>
<td>Water Pump</td>
<td>4182K23</td>
<td>1</td>
<td>McMaster-Carr</td>
</tr>
<tr>
<td>4</td>
<td>Water Reservoir</td>
<td>9884T13</td>
<td>1</td>
<td>McMaster-Carr</td>
</tr>
<tr>
<td>5</td>
<td>Quick Disconnect</td>
<td>51545K28</td>
<td>2</td>
<td>McMaster-Carr</td>
</tr>
<tr>
<td></td>
<td>Tubing Couplings</td>
<td>51545K91</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Enclosure</td>
<td>S500T</td>
<td>1</td>
<td><a href="http://www.directron.com">www.directron.com</a></td>
</tr>
<tr>
<td>7</td>
<td>Temperature Sensors</td>
<td>DS18S20</td>
<td>4</td>
<td>Dallas Semiconductor</td>
</tr>
<tr>
<td>8</td>
<td>Temperature Transmitter</td>
<td>Kit 145</td>
<td>1</td>
<td><a href="http://www.kitrus.com">www.kitrus.com</a></td>
</tr>
<tr>
<td>9</td>
<td>Liquid Level Sensor</td>
<td>176268</td>
<td>1</td>
<td>Jameco Electronics</td>
</tr>
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</table>
Appendix B

3D Segmentation
### B.1 FIR Filters

#### B.1.1 3×3 Sharpening Filter

\[
\begin{bmatrix}
-1 & -1 & -1 \\
-1 & 9 & -1 \\
-1 & -1 & -1 \\
\end{bmatrix}
\]

#### B.1.2 3×3 Unsharp-Masking Filter

\[
\begin{bmatrix}
-0.1667 & -0.1667 & -0.1667 \\
-0.1667 & 4.3333 & -0.1667 \\
-0.1667 & -0.1667 & -0.1667 \\
\end{bmatrix}
\]

#### B.1.3 7×7 Classifier Filter

\[
\begin{bmatrix}
-0.0456 & -0.0233 & 0.0400 & -0.0985 & -0.0071 & 0.0012 & -0.0806 \\
0.0211 & -0.0510 & 0.0762 & -0.0806 & -0.0193 & 0.1190 & -0.0361 \\
0.0113 & 0.0905 & -0.1347 & 0.0430 & -0.1959 & 0.0524 & 0.0177 \\
-0.1997 & 0.2228 & -0.3395 & 2.0736 & 0.1581 & -0.3158 & 0.0027 \\
-0.0020 & 0.0402 & -0.0723 & 0.0577 & -0.0974 & 0.0426 & -0.0035 \\
0.0402 & 0.0342 & 0.0739 & -0.1737 & -0.0301 & 0.0452 & 0.0029 \\
-0.0794 & -0.0299 & 0.0120 & -0.1229 & -0.0004 & 0.0406 & -0.0795 \\
\end{bmatrix}
\]

#### B.1.4 9×9×9 Nonlinear Least Squares FIR Filter

\[
\begin{bmatrix}
-1.326176e-2 & 5.099670e-2 & 2.483405e-2 & -2.526362e-3 & -1.577056e-3 & -1.932957e-3 & 0.063786e-2 & 4.732018e-2 & -2.672643e-2 \\
\end{bmatrix}
\]
<table>
<thead>
<tr>
<th>x0</th>
<th>x1</th>
<th>x2</th>
<th>x3</th>
<th>x4</th>
<th>x5</th>
<th>x6</th>
<th>x7</th>
<th>x8</th>
<th>x9</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1.08636e+1</td>
<td>0.612330e+1</td>
<td>0.105499e+1</td>
<td>-3.40468e-2</td>
<td>-3.10063e-2</td>
<td>2.59644e+2</td>
<td>4.83078e+2</td>
<td>5.27584e+2</td>
<td>1.48876e+1</td>
<td></td>
</tr>
<tr>
<td>8.38325e-2</td>
<td>8.352596e+1</td>
<td>0.70426e+1</td>
<td>5.60447e+2</td>
<td>3.35842e+3</td>
<td>1.37353e+3</td>
<td>7.58358e+2</td>
<td>1.07858e+1</td>
<td>1.05385e+1</td>
<td></td>
</tr>
<tr>
<td>-5.18533e-2</td>
<td>0.745519e-1</td>
<td>-0.37423e-2</td>
<td>0.186493e+1</td>
<td>2.30815e+2</td>
<td>5.13957e+2</td>
<td>1.60229e+2</td>
<td>6.89793e+2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1.80850e+1</td>
<td>-4.73835e-2</td>
<td>-2.61533e-2</td>
<td>-2.63080e+2</td>
<td>-4.22696e+2</td>
<td>-7.70028e+2</td>
<td>-9.23582e+2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-2.36737e-1</td>
<td>0.30978e+1</td>
<td>9.68491e+3</td>
<td>-1.62187e-1</td>
<td>3.42020e+2</td>
<td>-1.40227e-1</td>
<td>2.79685e+2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1.12311e-1</td>
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