Intrinsic Cytometry
based on Computational Microscopy

by

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ABSTRACT

With the goal of understanding cells, we propose to study the intrinsic properties of individual cells by combining visual observation from large field-of-view computational microscopy with separation of cells via an integrated label-free microfluidic platform. This intrinsic cytometry will benefit from the parallel and gentle separation of label-free cells via a microfluidic platform and parallel tracking of multiple cells via a large field-of-view microscopy in contrast to the gold standard, flow cytometry, which is able to rapidly and singly identify multiple cell properties via scattering of external fluorescent cell markers.

In this thesis, a prototype of this integrated platform was designed and fabricated. The prototype consisted of a large field-of-view digital in-line holographic microscopy system and a microfluidic deterministic lateral displacement array which separated particles based on size. Each system was first characterized separately and later integrated such that individual cells inside the deterministic lateral displacement array could be recorded and tracked with the large field-of-view digital in-line holographic microscopy system, showing the promise of our proposed intrinsic cytometry.

In future studies, if a microfluidic platform can be designed to investigate multiple intrinsic properties of individual cells on the same platform, the intrinsic cytometer will enable a large pool of quantitative measurement data of cell intrinsic properties that can potentially be used for cell characterization and diagnostics.
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Chapter 1 : Introduction

Visual observation has played an important role in our understanding of cells. Specifically, optical microscopy has been at the forefront of the technological tools that have helped shape important fields in biology and medicine such as cell biology and pathology. With the long history of visual observation, we are starting to appreciate the complex dynamics of multicellular systems and to recognize that investigation of this high level of complexity is beyond what can be obtained via visual inspection through a traditional microscope. As N. Scherf and J. Huisken have pointed out, further studies of complex multicellular systems require 1) a large pool of quantitative measurement data to study variability and obtain statistical significance, and 2) maintaining of the health and integrity of the specimens under study [1]. Cell separation and sorting are one way to manipulate and study cells in quantity and/or for specific cell properties that are not possible with visual observation alone.

1.1 Extrinsic Properties

Extrinsic properties are properties of cells that require extrinsic labels (e.g. antibody-conjugated fluorophores or magnetic beads) to become apparent. Conventional flow cytometry, which often relies on external labels to distinguish between cell types, has proven to be a powerful cell characterization and separation method in cell biology, biomedical diagnostics, and therapeutic applications. By extrinsically labelling cells with fluorophores conjugated to antibodies complementary to the biochemical markers of interest, conventional flow cytometry can run the cell sample in a single stream through an interrogation point and collect fluorescence scattering data of each cell in order to precisely detect cell type and measure its quantity with throughput up to 50,000 cells/second.

Nonetheless, the key operation concept, which relies on extrinsic labels, is also its limitation. First, biochemical markers might not be evident for cells in primitive states or not available for some specific populations of cells such as the rare circulating tumor cells (CTCs) found in blood. There is a pioneering work on capturing of CTCs by using epithelial cell-specific markers such as antibodies complementary to epithelial cell adhesion molecules (EpCAM) [2]. However, non-epithelial CTCs as well as epithelial CTCs that lose EpCAM expression will not be detectable. Second, the binding of cells with extrinsic labels can interfere with normal cell functions and induce cytotoxicity, making it more difficult to detect the original cell state [3]. Third,
sometimes native unaltered post-analysis samples are needed for further investigations or for therapeutic purposes, e.g. purified platelets are needed for transfusion or bacteria-cleansed blood are returned to its donor [4]. Last, flow cytometry with extrinsic labelling typically increases cost and complexity. Due to complicated preparation steps, the operating personnel needs to be trained and due to long preparation time, point-of-care diagnostic applications are more challenging to implement.

1.2 Intrinsic Properties

Intrinsic properties, on the other hand, are physical biomarkers that do not rely on extrinsic labels for the properties to become apparent and therefore can overcome the limitations of the extrinsically labelled cell separation techniques. Examples of the most commonly studied intrinsic properties include but are not limited to cell size, shape, deformability, polarizability, refractive index, and density [4].

Different macro- and micro-scale techniques have been devised to measure these intrinsic properties. One example of macroscale techniques is the principle of resistive pulse sizing (e.g., Coulter counter) for measuring size. A Coulter counter draws cells suspended in electrolyte through an interrogation orifice one-by-one and detects any change to the electrical impedance that is proportional to the volume of electrolyte that has been displaced by the particle. Another example of successful macroscale techniques is density gradient centrifugation (DGC), which separates cells based on their density. In DGC, cells are suspended in the media whose density increases in the direction of gravitational field, and after centrifugation, the cells will migrate to the point where the density of the cells and the density of the surrounding media match. The most common application of DGC is blood separation where blood plasma, platelets, leukocytes and erythrocytes are separated in layers. Subtypes of leukocytes can also be separated by changing the suspension medium density gradient to match their density and re-centrifuge. DGC, however, can suffer from incomplete separation between layers, activation of force-sensitive cells, and discontinuity of a batch processing technique.

Several label-free cell separation techniques in microfluidic systems have been developed to measure and study the intrinsic properties of cells. The benefits of microfluidic label-free separation techniques include the microscale interface and accessible forces for gently manipulating single cells and the possibility of continuous flow processing mode. Because of
these attractive features, microfluidic label-free cell separation techniques based on cells’ intrinsic properties might be the tool that we need to study cells in large scale without altering their properties, and will be the focus of this thesis.

1.3 Existing Methods
Microfluidic label-free methods that measure or separate cells based on these intrinsic properties are generally classified in two main categories: active techniques that rely on external force fields and passive techniques that rely on channel geometry and hydrodynamic forces for functionality [5]. We will give an overview of a few methods categorized by the intrinsic property being studied.

1.3.1 Mechanical Properties
Altered mechanical properties of cells have been linked to abnormality and diseases [6]. *Ex vivo* and *in vivo* biochemical factors as well as foreign organisms or pathogens can induce structural changes in cell membrane, cytoskeleton and cytosol, leading to changes in mechanical biomarkers, e.g. cell size, shape, or deformability. For examples, cancer cells are known to change their deformability in order to improve their motility and metastatic potential. Malaria-infected erythrocytes become stiffer than their normal counterparts. These biomechanical insights have been exploited to develop label-free cell separation techniques in order to separate and identify these cells based on deformability marker. For example, a simple microfluidic design for passive malaria-infected erythrocyte separation relies on naturally observed behavior of stiffer leukocytes and infected erythrocytes which are more likely to marginate to vessel walls than the normal more deformable erythrocytes in small blood vessels [7]. Another clever design that has been demonstrated to efficiently capture >90% of circulating tumor cells (CTCs) from unprocessed whole blood [8]. This technique uses varying gap-size microfluidic ratchets to aptly allow deformable particles to pass through while obstructing the flow of stiff particles. The ratchets with varying gap size enable simultaneous sorting and separation of both size and deformability markers.

Discussions on the cell size marker are reserved a future dedicated section.

1.3.2 Electrical Properties
Electrical biomarkers (e.g. electrophoretic mobility, polarizability) have been used to separate cells in hope that they are reflective of cells’ complex biological structures and characteristics.
Label-free techniques that use active electric force field to manipulate cells according to their electrical properties include electrophoresis and dielectrophoresis. Electrophoresis relies on different magnitude or direction of the Coulomb force the electric field exerts on a particle with a net charge. In contrast, dielectrophoresis relies on the interaction between particles’ dipole and the gradient of electric field [9]. As an example, continuous separation of *Escherichia coli* based on their intracellular polymers has been demonstrated in our lab, using dielectrophoresis and media with spatially varying conductivity gradient [10]. The same technique has been applied to separate viable and nonviable cells of budding yeast *Saccharomyces cerevisiae* and murine pro B cells [11], [12]. Due to high sensitivity and throughput, an adaptation of the same technique has been implemented to characterize neutrophils in different activation states [13].

1.3.3 Combination of >1 Intrinsic Properties

One can argue that any microfluidic label-free techniques that can be observed visually with a microscope can be used to extract cell size information. Therefore, size measurement has already been incorporated with several intrinsic markers, e.g. deformability [14] and polarizability (in our lab). Separation techniques based on deformability marker, particularly the ones involving size exclusion methods (e.g. the previously mentioned work that used varying gap-size ratchets [8]) can also claim to have sort particles based on size since size and deformability are dependent on each other. Two works that have impressed the author on integrating more than one intrinsic properties are the works by Beech *et al.* which has demonstrated the sorting of cells with three intrinsic properties, namely size, shape, and deformability. Beech uses a deterministic lateral displacement array which is inherent to size manipulation, and adds control of particle orientation via channel height to study morphology as well as adding control of shear stresses to study the deformability [15].

1.4 Intrinsic Cytometry based on Computational Imaging

To better understand the complexity of cells, we would like to study and understand the connections between the intrinsic properties of cells. We propose to study the intrinsic properties of individual cells by combining visual observation via large field-of-view computational microscopy with separation of cells via an integrated label-free microfluidic platform. The microfluidic platform gently manipulates cells such that cells with different intrinsic properties are laterally separated. Individual cells will be tracked as they transverse the platform (Figure
Large field-of-view microscopes are favored because tracking over large field of view would not only allow us to locate the positions of cells with respect to time in order to identify their encoded intrinsic properties, but also, in the future studies, allows us to link multiple intrinsic properties for individual cells. We visualize the integrated microfluidic platform in future studies to have a square-centimeter footprint, which cannot be observed by a conventional scientific-grade microscope with a single scan. Computational imaging, especially the digital in-line holographic microscope, helps us overcome this limit. The intrinsic cytometry will benefit from the parallel and gentle separation of label-free cells via a microfluidic platform and parallel tracking of multiple cells via a large field-of-view microscopy in contrast to the gold standard, flow cytometry, which is able to rapidly and singly identify multiple cell properties via scattering of fluorescent cell markers. The intrinsic cytometer will enable a large pool of quantitative measurement data of cell intrinsic properties that can potentially be used for cell characterization and diagnostics.

![Image: Figure 1-1 Schematics of the proposed intrinsic cytometry system, adapted from Bose Research Fellow proposal.](image)

As a proof-of-concept, we have designed and fabricated an integrated platform consisted of a large field-of-view digital in-line holographic microscopy system and a microfluidic deterministic lateral displacement array which separated particles based on size. Further
discussions and motivations for choosing the field-of-view digital in-line holographic microscopy system and the deterministic lateral displacement array will be discussed along with the overviews of microscopy and size separation techniques in the next two sections.

1.5 Microscopy
1.5.1 Optical Microscopes

Overview

Over three centuries, the optical microscope has proven to be a powerful instrument in the fields of biology and medicine, extending the visual capability of human naked eyes. In 1665, Robert Hooke employed a simple compound microscope to observe a thin slice of cork and was the first to accurately describe what is now known as the cell wall of plant cells in his historically well-known science book, Micrographia [16]. Hooke’s work inspired Antonie van Leeuwenhoek and the others, leading to the development of microscopes and the discovery of cells and microorganisms [17]. Early microscopes suffered from image distortion due to the low quality of glass and imperfect lens shape [18]. It was not until the middle of the 19th century that rigorous optical principles were formulated and the configurations and quality of modern-day microscopes were achieved [18], [19],[20]. New techniques have been continuously developed to improve the state-of-the-art microscope and enhance its functions for specific applications. For example, phase-contrast and differential interference contrast techniques improved image contrast for unstained, transparent cells [21], [22]. Fluorescence microscopes allowed imaging of sensitively and specifically stained specimens such as cell subcomponents, tissue components and pathogens to study their structures and functions [23]. Confocal microscopes optically scanned specimens at different depths, which enabled the reconstruction of three-dimensional images of specimens [24]. Attempts have been made to obtain higher spatial resolution and overcome the diffraction limit of optical microscope, such as super resolution techniques [25]. Because of their imaging quality and wide range of functionalities, microscopes are ubiquitous tools in research labs and hospitals.

Advantages and Limitations

Despite the diffraction limit, optical microscopes are sufficient for studying cells and microorganisms as well as for imaging the outputs of microfluidic devices because of their micron-scale resolution. However, the standard optical path in a research-grade microscope
includes properly aligned lenses and other optical components to form an image, rendering the research-grade microscope bulky and expensive. Most importantly, standard optical microscopes suffer from the decreasing field of view (FOV) as the magnifications of the objective lens increases. Studies of large-scale biological populations or of large-scale microfluidic devices are not efficient as they generally require manual or automated mechanical scanning of the microscope stage.

1.5.2 Digital In-Line Holographic Microscope

Overview

An innovative two-step lensless imaging system, which is now known as holography, was introduced by Dennis Gabor in 1948 [26]. The original Gabor holography was an in-line system intended to circumvent a magnetic lens aberration problem in electron microscopy as it avoided the usage of the magnetic lens altogether. Since its conception, the process involves 1) recording of object diffraction pattern and 2) wavefront reconstruction from the recording. Gabor recognized that when an object is illuminated by a single coherent point source, the scattered wave from the object interferes with the unscattered reference wave from the point source, allowing the amplitude and phase information of the object wavefront to be encoded as intensity on the recording material. Ultimately, an image of the original object can be computationally reconstructed from the recorded interference pattern, which is also known as a hologram, meaning a total recording [27].

Gabor holography only received mild interest in its early days because images reconstructed by the system were distorted by twin-image effects [27]. Because of the system’s inability to distinguish between positive and the negative phase shifts, the image of the original object as well as its second image at the symmetrical position would be reconstructed from the recorded interference intensity. When the image of the original object was reconstructed, its twin image, which was out of focus, was also present. It was not until laser technology was improved in terms of power and coherency and the twin-image problem was minimized that holography gained interest [28]. In the early 1960s E.N. Leith and J. Upatnieks suggested an off-axis geometry to avoid the twin-image problem [29]. The off-axis geometry described a system where there is an offset angle between the reference and object waves, in contrast to in-line geometry where the reference wave is in general alignment with the object wave.
Holography can be classified into two main categories based on recording materials and reconstruction methods: analogue holography and digital holography. In analogue holography, a hologram containing the object information is recorded as an alteration in the transmission profile of a photochemical medium [27]. After recording and processing, the photochemical medium is then illuminated by the coherent reconstruction wave in order to reconstruct the image of the original object at various depths. Digital holography, on the other hand, replaces the photochemical media with an electronic imaging sensor, and replaces optical reconstruction with digital computation. We will focus on digital holography because while analogue holography is time-consuming due to the photochemical processing before image reconstruction, digital holography, with improving image sensor and image processing technology, can digitally reconstruct the image of the original object at different reconstruction planes rapidly after the hologram is recorded with the image sensor and stored in the computer [28]. The reconstruction algorithm was first demonstrated in the electron microscope and was later applied to the optical microscope [30].

Gabor’s initial work recorded the hologram digitally and reconstructed the image optically. In the first digital holographic experiments in 1967, J.W. Goodman and R.W. Lawrence recorded the hologram by a vidicon camera as a 256x256 3-bit pixel array and digitally reconstructed the image with the computation time of five minutes [31]. In 1994, Schnars and Jüptner were the first to use a charged coupled device (CCD) camera directly connected to a computer to record the hologram and reconstruct the image digitally by numerical methods [32]. Since the hologram contained the whole-field information about the object, one could digitally reconstruct the image of the object at different reconstruction planes as permitted by the depth of field of the system, all of which could be used to form a three-dimensional rendering of the object. A basic setup of the digital holographic microscopy (DHM) generally consists of an illumination source to provide the reference wave, an interferometer to create beam paths for the reference wave and object wave, a digitizing camera to record the interference patterns and a computer with the necessary programs to reconstruct the image of the original object [28]. Digital holographic microscopy has been successfully demonstrated for biological applications, particularly for counting and tracking cells in three-dimensional space. For example, it has been used to track sedimenting unlabeled red blood cells and HT-1080 fibrosarcoma cells in a three-dimensional collagen tissue model without mechanical focus adjustment [33]. Digital holography has also
been utilized to non-invasively count unlabeled adherent cells directly in cell culture vessels with better accuracy as compared to the conventional manual cell counting [34]. More recently, digital holography has been combined with optical manipulation to simultaneously observe, track, and manipulate flowing particles in microfluidic channels [35], [36].

Digital holography with in-line geometry for microscopic imaging, also known as digital in-line holographic microscopy (DIHM), is a subtype of a digital holographic microscopy [37]. In DIHM, one does not need an interferometer, but relies on a constraint that the object is weakly scattering such that a significant portion of the illumination wave is unscattered by the object and acts as the reference wave. One prominent benefit of DIHM is its potential to be more portable than DHM due to requiring fewer components. Similar to DHM, DIHM has been successfully used to image biological specimens such as mammalian carcinoma cells [38], unicellular marine plant cells (the diatom *D. brightwellii*) [39], the sectioned head of a fruit fly (*D. melanogaster*) [39], as well as track flow through a microfluidic channel or track motion of bubbles, bacteria, and algae in water [40], [41]. Due to its portability, DIHM has been designed for underwater observation, e.g. lake and ocean, in order to observe algae, bacteria, and motion of various plankton species [30]. To further improve portability and reduce the complexity of the system, DIHM has been demonstrated with a light emitting diode (LED) as the primary source instead of a laser [42] and has been applied to classify viable and non-viable MCF-7 cells [43], and quantitatively measure cell locations and estimate percentage confluence [44]. J.P. Ryle *et al.* studied effects of varying coherence length by using two types of sources: laser and LED, and reported that higher visibility interference fringes were formed and a higher degree of spatial resolution was achieved when the coherent laser light was used as the primary source [38]. Nevertheless, coherent sources such as lasers were associated with speckle effects, a source of noise which occurred from backscattered laser light that formed interference patterns on the image sensor [44]. Using partially coherent sources such as an LED coupled with a pinhole reduced the speckle effects.

**Advantages and Limitations of DIHM**

We will summarize several attractive characteristics of DIHM as best described by W. Xu *et al.* [45]. First, DIHM has a simple setup without objective lenses and the need to accurately align then. DIHM only requires three hardware components: a laser or a LED, a pinhole, and an image
sensor. Second, a single recorded two-dimensional hologram contains all the information about the three-dimensional structure of the object. While the standard optical microscope requires scanning of the object at different depths to create a 3-D image, one could reconstruct the image of the object at different depths by computations from a single recorded hologram to generate 3-D rendering. Third, the maximum lateral resolution as predicted by the diffraction theory, which was on the order of the laser’s wavelength, could be achieved. Moreover, the depth resolution or depth of focus of 1 mm (more than 100 times the depth resolution of a conventional microscope) has been demonstrated with DIHM [37]. Fourth, staining and sectioning of biological samples could be avoided as DIHM could record the encoded phase information along with the amplitude information, and reconstruct the image of the object at different depths within the depth of focus. Finally, while conventional microscope required mechanical or optical focusing of the object before recording, DIHM does not. DIHM would be more suitable to track and observe the motion of target objects in a 3-D space. Overall, with DIHM, more information about the object can be conveniently extracted from one recording. If the interface of DIHM has been made user friendly, it could be a more powerful equipment than the conventional microscope for certain applications. One main limitation of the DIHM is the constraint on the object to be weakly scattering, which could limit the types and concentrations of specimens being observed.

1.5.3 A New Type of DIHM with Wide Field of View

Overview

In 2008, A. Ozcan and U. Demirci reported a lensfree technique for wide-field monitoring of blood cells, NIH/3T3 fibroblasts, murine embryonic stem cells, and AML-12 hepatocytes flowing through microfluidic channels [46]. The setup included a collimated optical beam, e.g. an incoherent white light source or a coherent laser, and an image sensor, e.g. a complementary metal-oxide semiconductor (CMOS) sensor or a charged coupled device (CCD) camera. This technique relied on recording of shadow images (classical diffraction patterns) of cells in the Fresnel regime with the goal of tracking the cells and not of observing the object fine features. In their early work with classical diffraction patterns, a depth of focus of up to 4 mm was reported [47] and the effects of classical diffraction signatures as a function of illumination wavelength, depth position of the object, substrate thickness and refractive index were studied [48]. It was not until 2009 that the Ozcan group implemented a similar setup with a CMOS sensor with smaller pixel size and an insertion of a pinhole butt-coupled to the LED in order to increase the spatial
coherence of the system, and recorded the holographic diffraction patterns of cells instead of their classical shadows [49]. The holographic diffraction patterns were shown to have more finely detailed and stronger fringes as compared to the weaker fringes of the classical shadows, improving uniformity of the holographic signatures for the same cell types and enhancing the differences in the holographic signatures among different cell types. The main differences between this setup and conventional DIHM were 1) the geometry of the newer setup provided a unit fringe magnification of objects at the image sensor while the geometry of the conventional DIHM yielded >1 magnification, which meant that fewer pixels of the image sensor were needed to represent the type and the location of the objects and higher throughput (higher field of view) could be obtained with the newer setup; 2) the newer setup did not rely on the nearly perfect spatial coherence of the light source, rendering the coherent laser and small pinhole unnecessary; 3) Early implementation of the newer setup did not require high spatial resolution and reconstruction algorithms as the only purposes were to identify the types and locations of the cells. This technique has more recently been demonstrated for wide field-of-view fluorescence imaging [50], [51] as well as for color imaging [52] of biological specimens. Two key examples of applications of this technique are a high-throughput semen analysis platform where count, speed, and dynamic trajectories of motile sperms were automatically quantified [53], and a high-throughput blood analysis platform where red blood cells and white blood cells were automatically counted and the concentration of hemoglobin in whole blood was measured [54]. The spatial resolution (~1.4–1.5 μm) achieved by the blood analysis platform has been demonstrated to have the potential to perform three-part differential white blood cell analysis cells. Granulocytes were visually easy to distinguish from monocytes and lymphocytes because of the granules inside cytoplasm, which could be observed in the reconstructed image and was shown to be comparable to the microscope images. The visual difference between the lymphocytes and monocytes, however, was so small that it seemed less convincing that one could discern lymphocytes from monocytes with the reconstructed images. Due to its compactness, the goal of this latter system has been to create a portable microscope and it has been applied as a portable lensfree imaging platform which could be mechanically attached to the camera unit of a cell phone, especially for diagnostics in resource-limited settings [55]. Super resolution techniques have also been applied to the system to obtain spatial resolution below the pixel size of the camera [56]–[58]. We would like to emphasize here that except for [59] where
antibody microarrays were applied in the microfluidic channel to capture leukocytes and detect secreted cytokines in order to quantify the ratio of CD4 and CD8 T-lymphocytes, microfluidics has been applied to this novel imaging technique mostly as microchannels for the sample to be conveniently loaded and unloaded, and not for what microfluidics could fully achieve.

**Advantages and Limitations**

This wide field-of-view imaging technique inherited the advantages and limitations from conventional DIHM systems. The main limitation of this technique, which is inherited from conventional DIHM, is that the sample needs to be weakly scattering, meaning only a certain type and concentration of object can be observed with this system. The main advantage of the newer technique is its capability for imaging a wide field of view (> 10 times improvement compared to the conventional 10X objective lens [60]) while maintaining the simplicity of the DIHM setup.

1.6 **Size Separation**

For the prototype of the proposed intrinsic cytometer, we chose to develop a method to measure the size of cells because cell size is a well-accepted commonly-used coarse parameter to distinguish between cell types, even though there is cell-to-cell variability of cell size among the same cell type. Label-free size separation techniques include but are not limited to mechanical filters, pinched flow fractionation, inertial, and deterministic lateral displacement (DLD), which we will discuss next. The criteria for a desirable size-separation technique for our cytometer are 1) separation able to resolve ~1 μm differences in diameter across an approximate size range of 5-20 μm, 2) easy integration with other microfluidic modules, 3) clog-free, and 4) sufficient throughput (>10 cells per second).

**Mechanical filters**

Mechanical filtration based on size exclusion is a simple and intuitive approach to separate particles based on size. Typically, microfluidic channels are designed with obstacles and gaps that allow small objects to pass through while obstructing the passage of large objects. Four types of mechanical filters have been demonstrated in microfluidics: weir-type, pillar-type, cross-flow, and membrane [4]. Weir-type filtration provides gaps between the weirs and the top cover. Pillar-type filtration uses array of microposts to block larger cells in direction of the flow. Cross-flow filtration also uses weir structures or arrays of microposts to block large cells but in
the direction perpendicular to the flow. Membrane filtration utilizes a membrane with a well-defined pore size to block larger cells. Mechanical filters have been applied to blood cell separation [61], [62] and separation of the circulating cancer cells from blood [63],[64]. It is simple and is particularly sufficient for separation of small cells for downstream analysis [5]. Because this technique relies on the blockage of larger cells with obstacles, the main limitation of this technique is clogging because the obstructed large cells could block the flow and prevent the smaller cells from passing through the gap, reducing the recovery of smaller cells. Moreover, the performance of this technique could deteriorate if larger cells can deform and pass through the obstacles. Because of these limitations, mechanical filters are not the perfect candidate for measuring cell size in our intrinsic cytometer.

Pinched Flow Fractionation

Pinched flow fractionation is a passive separation technique, operating with a simple hydrodynamic principle in laminar flow. Cells from the sample inlet are forced to line up against the wall of the microfluidic channel by the buffer inlet. Based on the location of their center of inertia with respect to the wall (i.e. size), cells will follow the streamlines in which their center of inertia reside into different radial outlet branches [65]. This technique has been demonstrated with leukocyte enrichment [66] and combined with functionalized 3.09 and 5.6 μm polystyrene microspheres to distinguish between two DNA sequences in the HBB gene [67]. In contrast to the mechanical filtration approach, this technique is not limited by clogging issues and the critical size range for this technique is easily tunable by adjusting the flow rates of the two inlet channels. However, with the increasing flow rates, the inertial forces on particles could increase and affect the motion and trajectory of cells, reducing the separation efficiency. Furthermore, the outlet branch structure is not favorable for downstream integration with another microfluidic device.

Inertial

Inertial size-separation relies on the balance of two inertial forces: the shear-induced lift force and the wall-induced lift force [68]. These forces, which are inherent to the motion of cells inside a microchannel, determine the equilibrium lateral positions of cells based on their size relative to the microchannel dimension, enabling size separation of cells. Spiral microchannels are the most common structure of the inertial size-separation device. The curved structure can provide
additional fluid dynamic forces for increasing the precision of particle positioning inside the channel [68]. For example, spiral channels have been used to separate neuroblastoma cells and glioma cells with a reported 80% separation efficiency, >90% cell viability, and a throughput of ~10^6 cells/min [69]. While clog-free inertial sorting can achieve a high efficiency rate and a high throughput, the separation resolution of this system can significantly suffer from particle-particle interactions of samples with high particle concentration, and sample dilution is generally required before applying the technique.

Deterministic Lateral Displacement (DLD)

Deterministic lateral displacement utilizes a slanted array of microposts to bifurcate the fluid flow and deterministically separate cells based on their center of mass in bifurcated flow streams. After travelling through the array, cells with diameter above and below the designed critical size will have designated nonzero lateral displacement (bump mode of motion) and zero lateral displacement (zigzag mode), respectively. Deterministic lateral displacement has been demonstrated to separate microspheres based on size with the resolution of ~ 10 nm [70]. This technique has also been used to fractionate whole blood components (with 100% separation of lymphocytes and monocytes from red blood cells and the plasma) and isolate blood plasma (with no cellular contamination) from whole blood with no dilution and with a flow rate up to 1 μL/min (1000 μm/sec) [71]. Due to the high separation resolution that this technique can achieve, DLD has been used to measure platelet activation [72]. After exposure to thrombin and 4°C refrigeration, activated platelets were observed to experience an increase in size. D.W. Inglis et al. demonstrated a parallel DLD device for the enrichment of leukocytes from undiluted whole blood with the flow rate up to 115 μL/min [73]. More recently, the DLD array has been integrated with inertial focusing and magnetophoresis to isolate circulating tumor cells (CTC) from whole blood with 97% yield of CTCs at up to ~10^7 cells/s [3]. We chose to integrate DLD in our platform because it has been shown to achieve high separation resolution and throughput. Fractionation of particles based on a range of critical sizes simply requires stacking up of micropost arrays with slightly different parameters. Most importantly, the separation of particles using this technique is deterministic as long as the flow inside the device is consistently laminar, which mean that undesired stochastic events are less likely to deteriorate the separation efficiency. In addition, the flow rate inside the device is highly flexible, which increases the
potential of DLD to be successfully integrated with other techniques that may have constraints on flow rates. DLD does have some limitations, including the potential of clogging, the effects of particle-particle interaction, and the effects of particle deformability on the effective hydrodynamic size. Weighing in all the advantages and limitations of the DLD compared to other size separation techniques, we believe that the DLD is the best candidate for our intrinsic cytometer.

1.7 Thesis Overview

We will begin Chapter 2 by giving an overview of the DLD principles, followed by modelling of the design parameters and design considerations to achieve the desired critical diameters for particle separation. Two mask designs for our DLD devices are then illustrated along with the fabrication protocols. The experimental setup protocol as well as the experimental results are presented with the discussions.

In Chapter 3, we will discuss the theory behind the working of the traditional and the wide-field DIHM system. Test sample, imaging setup, imaging reconstruction methods, and tracking algorithms used are elaborated in the Materials and Methods section. Next, the results from small-scale and large-scale reconstructed images of microspheres and cells are described and discussed. Lastly, the results from tracking experiment of unstained suspension cells inside a DLD platform are included.

We conclude in Chapter 4 by discussing our contributions, suggested improvements, and future directions of the proposed intrinsic cytometry.
Chapter 2 : Size-based Separation by Deterministic Lateral Displacement

2.1 Introduction

Deterministic lateral displacement (DLD) arrays passively separate particles based on their sizes with unrivaled resolution [70]. While passing through a designed array of obstacles, particles are laterally separated. Results can then be read optically from particle exit positions. Due to the flexibility of its design parameters, such as device dimensions and required flow velocity, DLD is attractive to be included as a size-measurement module on an integrated cell analysis platform. In this chapter, we will review the mechanism by which DLD operates as well as compute a flow profile in a simple two-dimensional device model to estimate its “size threshold”, which is regarded as the “critical diameter”. As a first-order approximation, critical diameter is calculated from two design parameters, which yields a fairly good estimate. We will explore how other device geometric parameters can contribute to the accuracy of this estimate. Furthermore, to test the theory, single-array and chirped-array DLD devices will be designed and fabricated. Fabricated devices will be characterized with polystyrene microspheres of known, narrow size distributions. Devices are then used to perform size measurement of cells. Unlike most other DLD papers, we are primarily interested in the accuracy of each individual particle measurement; therefore, we will investigate in-depth the sources of measurement errors as well as the sources of stochasticity that could be introduced to a deterministic separation. Furthermore, we offer insights into some common experimental issues and how to avoid them.

2.2 Design and Modeling

Deterministic lateral displacement (DLD) relies on a simple hydrodynamic principle: a particle in laminar flow follows a streamline at which its center of mass is located regardless of obstacle presence [74]. DLD consists of rectangular arrays of microposts with slanted rows, generating repetitive asymmetrical bifurcation of flow between adjacent posts in one row into two branches of fixed fluid flux at the subsequent row. As a result, particles can be separated from row to row according to positions of their center of mass in the gap between two posts (i.e. their effective radii if particles are adjacent to one of the posts). It is important to note here that, in this thesis, we will always refer to the collection of microposts in a line perpendicular to the flow direction as row and those along the flow direction as column, even if the figure is rotated by 90°. The use of rows and columns are intuitive in Figure 2-1.
In the simplest form, a deterministic lateral displacement array consists of one or more arrays of circular microposts with each row being laterally offset from its predecessor by a row shift distance (\(\sigma\)) as shown in Figure 2-1. For ease of streamline visualization, the row shift fraction (\(\varepsilon\)) can be set such that

\[
\varepsilon = \frac{1}{N},
\]

where \(N\) is periodicity and is a positive integer. Note that

\[
\varepsilon = \begin{cases} 
\frac{\sigma}{\lambda} = \tan(\theta), & \text{if vertical pitch = horizontal pitch = } \lambda \\
\frac{H}{\lambda} \tan(\theta), & \text{if vertical pitch}(H) \neq \text{horizontal pitch}(\lambda)
\end{cases}
\]

where angle (\(\theta\)) represents the alignment of each column relative to flow direction. \(\lambda\) refers to horizontal pitch or the distance between adjacent posts in each row, and in some cases vertical pitch or the distance between each row. Fluid streamlines are bifurcated at every post with a fraction \(\varepsilon\) of the total fluid flux flowing to the left of the post and the rest flowing to the right. Therefore, when negotiating posts (note position of particles in the first row of Figure 2-1), particles whose effective radii are larger than the width of the left-bifurcated streamline will keep passing around the obstacles to the right, as if they are being bumped in the same direction. They will be laterally displaced by one pitch after \(N\) rows. This mode of motion is called “bump mode or displacement mode”. Smaller particles will remain in the original streamline which cyclically
comes to the original lateral position after N rows. Since the original streamline and small particle trajectory alternately pass around the obstacles in either direction, this mode of motion is called “zigzag mode”. Particles with different hydrodynamic sizes can then be directed to different lateral exit positions or different outflows given enough rows and appropriate outlet geometry. The critical diameter, which is twice the width of the left streamline, determines the mode of motion each particle is subjected to. The critical diameter is an important design parameter and is theoretically derived in the next subsection. Note that in this thesis, only the case where the vertical pitch of the array is equivalent to the horizontal pitch is considered.

2.2.1 First-Order Approximation
In this subsection, we summarize the analysis by [75], in which the first-order approximation of the critical diameter was theoretically derived. The Navier-Stokes equation governing incompressible fluid dynamics states:

$$\rho \left[\frac{\partial u}{\partial t} + (u \cdot \nabla) u\right] = -\nabla P + \mu \nabla^2 u$$  \hspace{1cm} (2-2)

where $u$ is the fluid velocity, $\rho$ is the fluid viscosity, $\mu$ is fluid viscosity, and $P$ is pressure. In microchannels at low Reynolds number, viscous forces dominate inertial forces and fluid flow becomes laminar, meaning smooth and predictable. The inertial terms on the left-hand side of the Navier-Stokes equation can then be ignored and the equation is simplified to the Stokes equation:

$$\mu \nabla^2 u = \nabla P$$  \hspace{1cm} (2-3)

For simplicity, consider a two-dimensional pressure-driven flow through the gap of a certain size ($G$) between two posts in a DLD array with zero velocity at the post sidewalls and a fixed pressure gradient along the length direction (z-direction) as shown in Figure 2-2B:

$$\frac{\partial P}{\partial z} = -k$$  \hspace{1cm} (2-4)

with $k > 0$. Given the geometry of this problem, the velocity field must only be z-directed:

$$u_x = 0.$$  \hspace{1cm} (2-5)

The simplified Navier-Stokes equation becomes:

$$\mu \frac{\partial^2 u_z}{\partial x^2} = \frac{\partial P}{\partial z} = -k.$$

(2-6)

With the no-slip condition on the post boundary:
\[ u_z(x = 0) = u_z(x = G) = 0, \] (2.7)

the solution is a parabolic flow profile across the gap,

\[ u_z(x) = \frac{k}{2\mu} (Gx - x^2). \] (2.8)

As the DLD is designed so that the left-bifurcated flux is equal to a fraction \(\frac{1}{N}\) of the total flux, the width of the left-bifurcated streamline (\(\beta\)) can be predicted by solving the following equation:

\[ \int_0^\beta u(x)dx = \frac{1}{N} \int_0^G u(x)dx, \] (2.9)

which has solution

\[ \beta = \frac{G}{2} \left[ 1 + 2w + \frac{1}{2w} \right] \] (2.10)

where

\[ w^3 = \frac{1}{8} - \frac{N^{-1}}{4} \pm \sqrt{\frac{N^{-1}(N^{-1} - 1)}{16}}. \] (2.11)

The correct root of \(w^3\) is

\[ w = \left[ \frac{1}{8} - \frac{1}{4N} + \sqrt{\frac{1}{16N} \left( \frac{1}{N} - 1 \right)} \right]^{\frac{1}{3}} \left( -\frac{1}{2} - i \frac{\sqrt{3}}{2} \right). \] (2.12)

By assuming that the particles do not alter the streamlines and do not interact with each other, an analytical expression for \(D_c\) is then

\[ D_c = 2\beta = G \left[ 1 + 2w + \frac{1}{2w} \right]. \] (2.13)

Figure 2-3 is reproduced from [74] to show the comparison between the theoretical and the experimental results of \(D_c\), as \(G\) and \(\frac{1}{N}\) varied. The experimental results were from the studies of DLD with 22 combinations of \(G\) and \(\frac{1}{N}\) and with particles ranging from 2.3 \(\mu m\) ot 22 \(\mu m\).

Particles experiencing bump mode and zigzag mode were plotted as open points and solid points, respectively. The particle diameters were normalized by \(G\) before being plotted as a function of \(\frac{1}{N}\). The theoretically derived critical diameter from the plug flow model and the parabolic flow
model were plotted with a dashed line and solid line, respectively. Note that for the plug flow model (Figure 2-2A), the flow velocity is constant across the lateral direction (x-direction), and \( D_c = 2 \frac{G}{N} \). It was recognized that the theoretically derived expression for \( D_c \) was roughly accurate, but did not truly separate all the experimental points of the bump mode from those of the zigzag mode. There were some solid points representing zigzag mode on the side of the parabolic-flow-model \( D_c \) curve, where only bump-mode points were expected. Therefore, an empirical formula for \( D_c \) which separated all the bump-mode points from all the zigzag-mode points was needed and was derived in [76]. We used this formula as a design parameter.

\[
D_c = 1.4G \left( \frac{1}{N} \right)^{0.48}.
\]  

\[ (2-14) \]

*Figure 2-2 Theoretical 2-D flow profiles between two microposts: (A) plug flow and (B) parabolic flow.*
2.2.2 Assumptions and Validations

In order to derive the first-order approximation of the critical diameter, a few assumptions are made explicitly and implicitly in the previous subsection. They will be examined in this subsection.

First, low-Reynolds-number laminar flow through the gap is assumed. The Reynolds number (Re) is the ratio of inertial to viscous forces and can be written as:

\[ Re = \frac{\rho u l}{\mu}, \]  \hspace{1cm} (2-15)

where \( u \) and \( l \) are characteristic fluid velocity and characteristic length scale. In our system, the characteristic length scale is approximately 20 \( \mu \)m and phosphate buffered saline is used as buffer (\( \rho = 1000 \text{ kg/m}^3 \) and \( \mu = 1.05 \text{ mPa} \cdot \text{sec} \)). Microscale fluid flow is considered to be creeping flow (have negligible inertia) when \( Re < \sim 1 \), then fluid velocity has to be

\[ u < < \frac{\mu}{\rho l} \sim \frac{(1.05 \times 10^{-3} \text{ kg/m}^3 \cdot \text{s})}{(1000 \text{ kg/m}^3)(20 \times 10^{-6} \text{m})} \sim 0.0525 \text{ m/s}. \]  \hspace{1cm} (2-16)

However, fluid flow is still considered laminar when \( Re < \sim 2000 \), which implied that...
\[ u < \frac{\mu \times 2000}{\rho l} \sim \frac{(1.05 \times 10^{-3} \frac{kg}{m \cdot s}) \times 2000}{(1000 \frac{kg}{m^3})(20 \times 10^{-6} m)} \sim 105 \frac{m}{s}. \]

Strictly speaking, the Re \( \ll 1 \) restriction was applied when calculating the theoretical critical diameter. However, practically the maximum flow velocity inside the microchannel can be between 0.0525 m/s and 105 m/s, as it can be considered as a laminar flow with small inertia.

Second, particle diffusion in the device is assumed to be negligible. In laminar flow, particles mix only by diffusion. We have to ensure that the effects of diffusion on particles are minimal so that it does not significantly alter the lateral positions of the particles and obscure the result readout. The Péclet number (Pe) is generally used to show the ratio of rate of convection to rate of diffusion of particles which can be calculated by

\[ Pe = \frac{uw}{D}, \]

where \( w \) is the channel width and \( D \) is the diffusion coefficient, which can be calculated from the Stokes-Einstein relation,

\[ D = \frac{kT}{6\pi \mu a}. \]

where \( a \) is the hydrodynamic radius of the particle, and \( k \) and \( T \) are the Boltzmann constant and the absolute temperature, respectively. Microscale particles in general are minimally affected by diffusion. For diffusion effects, the worst case experimental condition is when particles are of the smallest size because smaller particles diffuse more easily and when fluid velocity is minimal because it provides longer time for particles to be in the channel and diffuse. In our system of 40 µm pitch, the smallest particle used here is roughly 4.5 µm in diameter and the minimum flow velocity is 0.75 mm/sec. The Péclet number for this condition is

\[ Pe \approx \left( \frac{0.75 \times 10^{-3} \frac{m}{s}}{0.046 \times 10^{-12} \frac{m^2}{s}} \right) (40 \times 10^{-6} m) \approx 6.5 \times 10^5 \]

meaning for a 4.5µm-diameter particle to laterally diffuse across the pitch and shadow the difference between the bump mode and zigzag mode of motion, the channel length of approximately 650000 times the pitch or 26 m is required. For the typical device length of 3.2 mm to 10.4 mm, diffusion is negligible. Note that this condition readily extends to particles of
roughly 0.6 pm in diameter before diffusion starts to take an effect in a 3.2 mm long channel and with a flow speed of 0.75 mm/sec.

2.2.3 Secondary Effects and Other Design Considerations

The first-order approximation for the critical diameter takes two device parameters into account: gap width and periodicity. Due to its simplicity, this relation is very useful for designing a DLD device. However, there are some other parameters in the device design that can affect the critical diameter. In this section, we will explore these secondary effects on our design qualitatively, as outlined by J.P. Beech [77].

Device height effects

The finite height dimension (y-direction) is ignored during the derivation of $D_c$. In Davis’ dissertation [76], the height dimension is readily negligible as the channel height is roughly five times the gap width. In our design, the channel height ($h$) is about the same size as the gap width.

To study the effects of finite channel height, we simulated a flow profile inside three-dimensional channels of varying heights (20 μm as in our devices, 50 μm as is the height of interest in [76], 100 μm as is the suggested 5 height/post diameter ratio in [76], and 1000 μm as this can represent infinite height) with the Microfluidics module of COMSOL Multiphysics® 4.2a modelling software. The cross section of the 3D channel can be observed from Figure 2-4A. The modelled channel represents a unit of a DLD array which consists of a 20 μm gap between two 20-μm-diameter circular microposts. Note that in the literature [78], the shapes of the DLD microposts are named by the geometries as seen from the top view. In our case, circular posts are actually cylindrical posts. No-slip conditions are applied along the post boundary. The equations governing the incompressible fluid flow inside this geometry are:

\[
\rho (u \cdot \nabla)u = \nabla \cdot [-P + \mu (\nabla u + (\nabla u)^T)] + F \tag{2-20}
\]

\[
\rho \nabla \cdot u = 0 \tag{2-21}
\]

Recall that $u$ is the fluid velocity, $\rho$ is the fluid viscosity, $P$ is the fluid pressure, and $\mu$ is fluid viscosity. $T$ is temperature and $F$ is external force density applied to the fluid element in addition to the pressure and viscous force.

The resulting 2D flow profiles from the middle cross sections of the selected channel heights are shown in Figure 2-4A. We choose to investigate the middle cross sections of the 3D flow profile
as a general example case study although one can argue that cells whose density usually almost matches the density of the suspension buffer (e.g. PBS and DI water) would float up to the channel ceiling.

The flow velocity data from these 2D flow profiles are also extracted as .xls files and inputted into MATLAB® 2014a software for analysis. Since the 1D flow profile in-between posts is symmetric, only the first half of the saved profile data of each channel height is plotted as shown in Figure 2-4B. Each flow-velocity plot is normalized by the area under the curve (i.e. the flux). The normalized 2D plug flow and 2D parabolic flow profiles where channel height is assumed infinite and negligible are also included in the same figure. The maximum difference between the 20 µm and the 50 µm high channel models is 0.24%, which occurs at the middle of the gap (distance of 10 µm from the post), and which can be neglected for our purposes. Similarly, the maximal difference for the 50 µm and higher modules is ~0.02%. Figure 2-4C plots the fluid flux over distance from the nearest post to the position of interest as a fraction of the total flux through the gap for varying channel heights. From this figure, one can determine the critical diameter (2×distance from post) for a given periodicity (1/fraction of fluid flow through gap).

Here, the maximum difference between the models accumulated from the difference in the flow profile. The maximum difference between the 20 µm and the 50 µm high channel models is 3.7%, which occurs at distance of 4.46 µm from the post, and which can still be neglected for our purposes. Similarly, the maximal difference for the 50 µm and higher modules is ~0.08%.

Figure 2-4D plots the critical diameter as a function of channel height for varying periodicity (N). The critical diameter and the channel height is normalized by the gap width in this figure. The maximum difference between all the models of different height is less than 1%.

According to the results from Figure 2-4(A-D), there are only slight differences between 20 µm height results and 50 µm height results with negligible difference between 50 µm and higher models. Our results in Figure 2-4(A-D) from the COMSOL® simulation data for 20 to 1000 µm channel height are consistent with Beech’s results for the models with 1 to 10 µm channel height. The results deviate more from theoretical parabolic flow model and approach plug flow model as the channel height decreases. This is reasonable because for a 3-dimensional model, the Stokes equation becomes:
\[ \mu \left( \frac{\partial^2 u_z}{\partial x^2} + \frac{\partial^2 u_z}{\partial y^2} \right) = \frac{\partial P}{\partial z}. \] (2-22)

For models with characteristic length in the y-direction (height) much larger than characteristic length in the x-direction (gap width), the second term on the left-hand side of Equation (2-22) can be ignored and Equation (2-22) becomes (2-6, as if the 3D model has transformed into a 2-D model, and the flow profile is more parabolic-like. On the other hand, for models with gap width much larger than channel height, the first term of Equation (2-22) is negligible and the flow profile on the x-z plane does not depend on x. As a result, the flow profile becomes more plug-like. In Figure 2-4E, \( D_c/G \) versus N of three different models are plotted. Interestingly enough, even though 3-D models allow us to theoretically compute critical diameters from more accurate flow profiles, 2-D model critical diameters (~12% maximal difference) are more accurate than the 3-D model critical diameter (~22% maximal difference) when they are compared to the more reliable empirical formula which was derived from experimental results of more than 22 combinations of G and N parameters (Figure 2-3).

**Post-size to gap-size ratio**

Post-size to gap-size ratio of the experimental points plotted in Figure 2-3 ranged from 0.32 to 1.36 for the work of Inglis et al. [74] and from 2.64 to 4.71 for that of Huang et al. [70]. Note that values from the work of Huang et al. are not given but are calculated from the gap width data and the assumption that the pitch remains the same for all the varying gap widths. The effect of post-size to gap-size ratio can be investigated in a similar manner as the previous device height study. \( D_c \) are calculated for various post sizes at fixed 20 \( \mu \)m gap width and constant 20 \( \mu \)m height. Figure 2-5 depicts COMSOL Multiphysics® simulation results for post-diameter to gap-width ratio of 0.1, 1, and 10. In Figure 2-5B, the maximum difference between all the models of post-size-to-gap-width ratio is less than 2.4% which occurs between the 0.1 and 1 ratio at distance of 1.15 \( \mu \)m from the post. Similar to the results from height study, in Figure 2-5C, the maximum difference between all the models accumulated from the difference in Figure 2-5B and became ~15% which now occurs between the 0.1 and 1 ratio at distance of 4.33 \( \mu \)m from the post. In Figure 2-5D, the maximum difference between all the models of post-size-to-gap-width ratio is less than ~11%. We have seen that the negligible maximal difference between flow profiles of different models (Figure 2-5B) could accumulate to a significant difference in Figure
At constant gap width, larger post diameters contain larger surface area for the no-slip boundary conditions and larger post diameters also make channel wider, which means that more liquid will be squeezed through the same gap width. In this case, the flow is more parabolic-like in models with larger post diameters.

Figure 2-4 Analysis of flow velocity profile and the corresponding critical diameters as channel height varies. (A) Middle-plane results of the simulated 3-D flow profiles between two posts of 20 µm post diameter and 20 µm gap at different device heights. (B) Normalized flow velocity versus distance from post at y=h/2 and x. Due to symmetry of the flow velocity between gap, only halves of the profiles are shown. (C) Fraction of fluid flow through gap as a function of distance from post. (D) Normalized theoretical critical diameter versus normalized height as periodicity varies. (E) Normalized critical diameter versus periodicity for N=10 estimated by three different methods.
Figure 2-5 Analysis of flow velocity profiles as post diameter to gap width ratio vary. (A) Middle-plane results of the simulated 3-D flow profiles between two posts of 2, 20, and 200 μm post diameter and constant 20 μm gap (not to scale). (B) Normalized flow velocity versus distance from post. (C) Fraction of fluid flow through gap as a function of distance from post. (D) Normalized theoretical critical diameter versus normalized height as periodicity varies.
Post shape effects

Circular post shape is simple, symmetrical, and is generally used in early DLD work. However, circular posts are prone to clogging particularly at the very top surface where fluid velocity is zero, trapping incoming particles. Later works explore other post shapes (Figure 2-6) in order to improve separation performance, reduce clogging, and develop favorable flow profile around the post [78]. Most shapes contain sharp corners against the flow direction aiming to minimize the zones surrounding circular posts with zero flow velocity, reduce clogging, and improve recovery rates. Examples of these shapes include triangular, streamlined, and diamond. Another advantage of triangular posts is their asymmetrical shapes that induce an asymmetric flow profile along the gap. \( D_c \) can then can be different for forward flow and reverse flow. This can be useful when fabrication footprint or fabrication cost is limited since one can have twice as many \( D_c \) for a device than the typical symmetric array. Meanwhile, I-shaped posts are known to induce a series of rotations in non-spherical particles in order to increase their hydrodynamic radius and facilitate separation [78]. Quadrilateral posts are used with inertial flow (Re > 1) to increase separation efficiency [78]. Airfoil and diamond posts have been shown by simulation to induce flow with low velocity gradient, meaning that there is less shear stress exerted on particles, and thus a reduction in deformability. As a prototype for the DLD, we chose to use the circular posts as they have been analyzed and used most often in the literature. However, based on the performance of the circular posts, different shape will be considered.

![Figure 2-6 Different post shapes that have been simulated and experimented with. (A) Circular (B) quadrilateral (C) triangular (D) airfoil (E) streamlined (F) diamond (G) I-shaped. Reproduced from [78]](image)
Sidewall effects

The performance of DLD strongly depends on a uniform flow profile along the gap particularly in regions that perform separation. In some designs, that includes regions near the sidewalls of the DLD arrays. Inglis reported degradation of separation efficiency near the boundary as the presence of the boundary disturbs the periodic flow patterns and reduces predictability and uniformity of $D_c$ in the array [79]. This can be corrected by modifying geometries near the boundaries. To ensure that the left-bifurcating stream from the (n-1)$^{th}$ gap second-closest to the left boundary has room to flow through in next row, the distances of the gap between the left boundary and nearest posts for the n$^{th}$ row have to be set to accept that stream and the stream right above it, which is:

$$G_L = G \frac{n}{\sqrt{N}}.$$ \hspace{1cm} (2-23)

n represents the position/order of row that is being considered, and recall that N is periodicity. The gap between the right boundary and the nearest post on the n$^{th}$ row has to be adjusted to push the stream in the gap to bifurcate to the left around the post and into another gap on the left of it in the next row.

$$G_R = G \sqrt{2 - \frac{n}{N}}.$$ \hspace{1cm} (2-24)

In most DLD designs, larger particles are to be displaced close to the right boundary; therefore, a factor of two is included to give more space for particles to flow through without clogging.

2.3 Materials and Methods

2.3.1 Fabrication

There are two main methods for DLD device prototyping. The first is to make an SU-8 mold, cast polydimethylsiloxane (PDMS), and plasma bond PDMS to glass slides. The use of PDMS has many advantages. For example, it is transparent and has low autofluorescence, enabling low-noise optical and fluorescent readings of DLD channels. Furthermore, it is biocompatible, ensuring minimal material impact on cell samples and maximal recovery of live cells. Another advantage of PDMS is gas permeability. During priming steps, this allows bubbles to be eliminated through PDMS walls besides through inlet and outlet reservoirs. Last but not least, PDMS is inexpensive and easy to mold. Combining this technique with plastic molding
technique would permit the making of batches of device replicas for experiment repetition in short amount of time. Nonetheless, two main limitations of PDMS devices are low aspect ratio and potential deformation under pressure. Deformation of PDMS structures is not desirable as accurate gap and post dimensions are important in device design and critical diameter prediction. Features with height-to-width aspect ratios of 2:1 are well accepted since high aspect-ratio features tend to adhere to the SU-8 mold during peeling-off process and tend to collapse during plasma bonding to glass slides. Low aspect ratio limitation, however, can be overcome by the use of another fabrication method, which is to etch silicon wafer, bond it to glass slides and sandblast holes for fluidic connection. With deep reactive ion etching of silicon wafer, aspect ratio of 5:1 has been shown in Huang et al. [70]. High aspect ratio is favorable because increasing channel height entails increasing throughput and decreasing clogging effects. In any case, a device height of 20 µm is selected to ensure compatibility with successful IDS models.

The PDMS-based method which is conceptually shown in and is described in details in section 2.3.1 and Appendix A is used to fabricate all devices reported in this thesis. That is because of ease in fabrication and because our device designs readily comply with the low-aspect-ratio restriction. The SU-8 molds were made in MIT’s Technology Research Laboratory, a class 100 cleanroom.

Figure 2-7 Schematic illustration of standard SU-8 photolithography and replica molding techniques.
The photomasks for photolithography step are chrome on quartz substrate made from Front Range Photomask Co. with critical dimension tolerance of ± 0.5 μm.

2.3.2 Mask Designs

Mask Design No.1

To facilitate the integration of DLD and IDS, the width of the DLD chamber in this mask design is chosen to be roughly equal to the width of the IDS inlet. This decision significantly impacts performance of our Design No.1 devices as will be discussed in the Results and Discussion section since the sidewall effects are ignored. It is important to note that sample inlet or the middle inlet is designed to be narrow and to be on the same order of magnitude as the gap width such that the distribution of the samples’ beginning positions is narrow. This is because the lateral displacement by DLD according to sample sizes is relative to the beginning position. Larger variation of samples’ beginning positions can cloud the true lateral displacements in devices that are not well designed or are limited in number of displacement redundancy because of small footprint. However, small feeding channels are easily clogged, but judicious sample preparation can minimize the chance of clogging.

Two devices from Mask Design No. 1 which were used most often in experiments are Device D3 and D4, whose dimensions are detailed in Table 2-1 and whose schematics are shown in
Figure 2-8.

<table>
<thead>
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<th>Device</th>
<th>Critical diameter ($D_c$)</th>
<th>Height ($h$)</th>
<th>Pitch ($\lambda$)</th>
<th>Gap width ($G$)</th>
<th>Periodicity ($N$)</th>
<th>Angle ($\theta$)</th>
<th>Length ($L$)</th>
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</tbody>
</table>

Table 2-1 Important DLD design parameters for Device D3 and D4 from Mask Design No. 1.

The length of the DLD devices in this design are calculated such that particles in zigzag mode and in bump mode exit from different outlets. The expected lateral displacement of particles in bump mode is 100 $\mu$m and is used to calculate the device length ($L$):

$$L = \frac{x}{\tan \theta},$$

(2-25)
where $x$ is the desired lateral displacement of particles between bump mode and zigzag mode.

Table 2-2 shows dimensions along with estimated channel resistance of the inlet and outlet designs for these devices.

<table>
<thead>
<tr>
<th>Width</th>
<th>Length</th>
<th>Height</th>
<th>Estimated Channel Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu m$</td>
<td>mm</td>
<td>$\mu m$</td>
<td>Pa·sec/m$^3$</td>
</tr>
<tr>
<td>Inlet 1</td>
<td>112</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>Inlet 2</td>
<td>50</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Inlet 3</td>
<td>224</td>
<td>45</td>
<td>20</td>
</tr>
<tr>
<td>Total Inlet</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outlet 1</td>
<td>200</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>Outlet 2</td>
<td>200</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>Total Outlet</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2-2 Inlet and outlet design parameters for Device D3 and D4.

![Figure 2-8 Mask layouts of device D3 and D4 with corresponding expanded views of the DLD chambers.](image)

Mask Design No.2
The motivation for Mask Design No. 2 is to correct design mistakes in Design No. 1 and to incorporate an IDS channel next to the DLD devices. Two important changes in Design No. 2 are increasing the width of the DLD chamber and choosing the periodicity to be positive integers instead of a rational number. The widths of the DLD chambers are chosen such that there are up to 30 posts in one row. Since in this design the feeding channel is in the middle, enlargement of the DLD chamber width moves sidewalls further away from particle trajectories and thus reduces the sidewall effects mentioned in Section 2.2.3. A schematic of a device of interest from Mask Design No. 2 is depicted in Figure 2-9. Important device design parameters are given in Table 2-3. This device is a chirped array of four different critical diameters. Each critical diameter has a repeat of three arrays. The middle feeding channel is 112 μm wide and is slightly larger than twice the width of the first design.

Given the sample inlet channel width and the syringe pump flow rate applied to devices in our mask design no.2, the maximum velocity is calculated to be approximately 37 mm/s which does not satisfy the Re<<1 restriction. However, it is still considered to be laminar with small inertia.

<table>
<thead>
<tr>
<th>Critical Diameter ((D_c))</th>
<th>Height ((h))</th>
<th>Pitch ((\lambda))</th>
<th>Gap Width ((G))</th>
<th>Periodicity ((N))</th>
<th>Angle ((\theta))</th>
<th>Length ((L))</th>
</tr>
</thead>
<tbody>
<tr>
<td>μm</td>
<td>μm</td>
<td>μm</td>
<td>μm</td>
<td>-</td>
<td>degree</td>
<td>μm</td>
</tr>
<tr>
<td>6.65</td>
<td>20</td>
<td>40</td>
<td>20</td>
<td>20</td>
<td>2.86</td>
<td>2400</td>
</tr>
<tr>
<td>9.27</td>
<td>20</td>
<td>40</td>
<td>20</td>
<td>10</td>
<td>5.71</td>
<td>1200</td>
</tr>
<tr>
<td>11.85</td>
<td>20</td>
<td>40</td>
<td>20</td>
<td>6</td>
<td>9.46</td>
<td>720</td>
</tr>
<tr>
<td>14.39</td>
<td>20</td>
<td>40</td>
<td>20</td>
<td>4</td>
<td>14.04</td>
<td>480</td>
</tr>
</tbody>
</table>

*Table 2-3 Important DLD design parameters of the chirped array with four different critical diameters.*
2.3.3 Test Samples
To characterize separation performance of the devices, three types of carboxylate fluorescent polystyrene microspheres with diameter of 4.5 μm (Polysciences, Inc., carboxylate-modified, bright-blue, Catalog# 18340—diameter 4.44±0.109 μm), 6 μm (Polysciences, Inc., carboxylate-modified, yellow-orange, Catalog# 19395—diameter 6.1±0.289 μm, CV 5%), 10 μm (Polysciences, Inc., carboxylate-modified, yellow-green, Catalog# 18142—diameter 10.1±0.97 μm) and 15 μm (Life Technology, carboxylate-modified, red-orange, Catalog# F21012) are used as test particles independently. We are particularly interested in these size ranges because they represent size ranges of mammalian cells.
For single-sized microsphere experiments, the microspheres are washed and suspended in Dulbecco’s phosphate-buffered saline (DPBS) at the final concentration on the order of $10^5$ to $10^6$ particles per milliliter. For multiple-sized experiments, the microspheres of different sizes are mixed together at the same ratio by number of particles, also aiming for the total final concentration of on the order of $10^5$ to $10^6$ particles per milliliter. Fractions of the prepared microsphere solutions are run through Beckman Coulter Z2 Particle Counter to analyze distribution of particle sizes in the sample which is compared to the manufacturer’s datasheet and to DLD results.

In some cases, we were interested in testing the laminarity of the flow generated from three inlets and how different flow rates applied to different inlet channels interacted with each other to generate the flow that entered into the DLD chamber. Fluorescein sodium salt (CAS# 518-47-8) was dissolved in deionized water at the concentration of $\sim 1\mu$M and was used as flow tracer.

To show that the fabricated DLD devices are compatible and still functional with biological samples, the murine interleukin-3 dependent pro-B cell line BA/F3 is cultured and used as a test sample. The BA/F3 cells, which have been continually maintained in our laboratory, were cultured inside sterilized cell culture flasks according to the ATCC protocol and were passaged roughly every 4-5 days. The cell culture medium was prepared from RPMI-1640 with high glucose content, L-Glutamine, and phenol red indicator (ATCC® 30-2001™), supplemented with sodium pyruvate (1% v/v), MEM non-essential amino acids (1% v/v) and penicillin/streptomycin (1% v/v). Similar to microsphere sample preparation, BA/F3 cells are gently washed and suspended in DPBS at the final concentration on the order of $10^5$ particles per milliliter.

2.3.4 Test Setup

Sample preparation

- Microsphere sample
  1. Dispense 100 μL bead solution from bottle into a 1 mL Eppendorf with 900 μL of 0.1% tween® 20 DPBS solution. Keep track of bead concentration. The proportion of beads to buffer can be adjusted according to bottle concentration. Vortex bead container before dispensing.
  2. Centrifuge the Eppendorf according to bead size.
<table>
<thead>
<tr>
<th>Bead Diameter (μm)</th>
<th>G – Force (cm/s²)</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>594</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>334</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>120</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>53</td>
<td>2</td>
</tr>
</tbody>
</table>

3. Carefully drain the liquid until only the beads are left in the bottom.
4. Dispense 1 mL filtered DPBS-T into the Eppendorf. Vortex.
5. Apply bead sample to the filter of slightly larger pore size, e.g. 10 μm pore size for 4.5 μm beads, 35 μm pore size for 6, 10, and 15 μm beads. Vortex.
6. Dilute the washed solution to the final concentration of 10⁵ to 10⁶ particles/ml.
7. Take 100 µL out for coulter counter analysis.

- Cell sample
  1. Load cells suspended in cell culture media into a 15 mL Falcon tube.
  2. Centrifuge at 1500 rpm for five minutes. In the meantime, thaw fluorescent stain, Calcein AM, in water bath for 2 minutes.
  3. Gently drain the media out of the Falcon tube without discarding the cells.
  4. Dispense 1 mL of DPBS-T and 1 μL of thawed Calcein Am into the tube. Vortex.
  5. Wrap the tube with aluminum foil to prevent exposure to light. Wait for 30 minutes.
  6. Apply cell sample to the filter of slightly larger pore size, e.g. 35 μm. Vortex.
  7. Dispense a few drops of sample onto a glass slide for observation under microscope.
  8. Take 100 µL out for coulter counter analysis.

Device Pre-conditioning:

- Microsphere sample
  1. Load a 10 mL syringe (Becton, Dickinson and Company) with 0.1% Tween® 20 deionized water. Connect it to a filter (Pall Corporation, 0.2 μm Tuffryn® membrane), tubing (Tygon®, 0.020-inch inner diameter), and metal inserts. Clamp it into place on a syringe pump.
  2. Let the pump run at 5-10 µL/min until you see droplets forming at the ends of the tubing. Plug the metal inserts into outlets of the device, merging the droplets from the tube with
droplets over the outlets. For Mask Design 1, load into one of the outlets. For Mask Design 2, load from both outlets.

3. Position and secure the device under the microscope (Zeiss Axio Imager M1M) equipped with a 12-bit CCD camera (LA Vision Imager QE) and fluorescent light source (EXFO X-Cite® 120).

4. Observe. Increase the flow rate for faster bubble elimination. Let the priming solution fill inside the device for 20 minutes.

- **Cell sample**
  Similar to microsphere sample but prime the device with ethanol, DI water, and 1% bovine serum albumin (BSA) solution in this order. For the ethanol, flush the device until bubbles disappear. Then flush with DI water to get rid of ethanol traces and flush with BSA for 20 minutes.

**Device Set-up:**

1. Load sample into a 1 mL syringe (Becton, Dickinson and Company) and load filtered DPBS into two 10 mL syringes (Becton, Dickinson and Company). Connect to filters (Pall Corporation, 0.2 µm Tuffryn® membrane; only for the DPBS syringes), tubing (Tygon®, 0.020-inch inner diameter), and metal inserts. Clamp it on syringe pumps.

2. Let the pump run at 5-10 µL/min until you see droplets forming at the ends of the tubing. Stop the pump. Plug the metal inserts into outlets of the device, merging the droplets from the tube with droplets over the outlets.

3. Run the syringe pumps (For Mask Design 1, 2.5, 0.5, and 1.25 µL/min proportional to the corresponding inlet channel widths. For Mask Design 2, 5µL/min for the sample and 15 µL/min for the buffer). Set the sample syringe pump such the syringe is vertically pointing the ground. Observe and adjust flow rates if needed.

4. Unless indicated otherwise, fluorescent images in the DAPI, FITC, and TRITC channels are recorded at 100 µsec, 1 msec, and 1 msec exposure time respectively.

**Clean-up:**

1. When the experiment is finished, disconnect the syringes from the device.

2. Flush out the plastic syringes a few times before throwing them away. Keep the metal inserts for future use.
Image Processing and Data Extraction:
Micro-Manager 1.4 open source microscopy software was used to control the CCD camera for image recording. The fluorescent images were recorded as monochrome images. The recorded time-lapsed images of particle exit positions were analyzed in MATLAB® R2014a, adapting the codes from the guidelines in [13]. The processing steps will be summarized here. First, a median filter was applied to a stack of time-lapsed images in order to find the background. The median filter calculated the median values from the current frame and the next nine frames. The choice of median values was because the particles were sparse enough that the median values were the background. Each current frame was then subtracted by the calculated background. A 100-pixel-wide region of interest in the difference image stack was determined manually from the corresponding bright-field image, by determining the positions toward the back of the DLD array in the image. These positions of the channels were then used to crop the difference image stack before the image stack was turned into a binary stack by thresholding. The exit positions of the particles were extracted from centroids of the detected objects in the binary stack. Accumulated exit positions of particles over all frames in the stack were plotted as a histogram. Note that, for each frame, we closely monitored the background, the current frame, the difference image (current frame - background), cropped binary image (difference image after thresholding) as well as the positions and number of detected particles. We learned that because of the median filter and the difference filter, these image processing and data extraction steps were robust that no other noise filtering step was required. Only the thresholds needed to be adjusted for an image stack recorded with different exposure time.

2.4 Results and Discussion
2.4.1 Early Observations for the Improvement of the Experimental Protocol
In this section, we discuss some challenges we have encountered in DLD experiments, and how we have improved and finalized the experimental protocols shown in the Materials and Methods chapter.

From my experience, clogging is the main challenge in DLD devices as it can deviate fluid flow and sample particle trajectories inside the device causing an irregular pattern of velocity profiles, as well as reduce separation efficiency and accuracy. Clogging is caused by multiple factors and at different locations of narrow channels. Locating and identifying the source of clogging is
important to minimizing the issue. From observation, there are two regions in DLD devices that are regularly clogged (Figure 2-10). The first region is the narrow feeding channel, which is easily clogged by clusters of particles or large chunks of PDMS or dust that may enter with the solution. The second region is at the first few rows of the DLD array that are adjacent to the openings of the inlet channels. Basically, these first few rows of the DLD array act as a fluid filter. Clusters of particles or junk larger than the gap width will get stuck while smaller particles can pass through. To minimize the number of clusters of sample particles, different approaches are combined. Most microspheres used in this study have carboxyl groups on the surface such that each particle would repel other particles of the same charge. Samples are also typically pre-filtered by 5 µm, 10 µm, or 35 um filters via manually applied pressure. Furthermore, priming and buffer solutions in loaded syringes are filtered by a 0.2 µm pore size filter before entering the Tygon tubes and the devices to prevent any large particles from entering the two bottleneck regions. Also, for microsphere samples 0.1% Tween-20 is added to the buffer solutions to reduce formation of microsphere clusters.

Nonspecific binding between the sample and PDMS walls can take place and becomes sites of particle accumulation over time in the microfluidic channel. In particular, microchannels with arrays of obstacles, such as DLD, increases the surface area of exposed PDMS, increasing chances of non-specific binding. Therefore, device priming is a necessary step before any experiments as the primer will coat exposed PDMS surfaces, reducing nonspecific binding of the samples. For microsphere experiments, the devices are primed by flushing 0.1% tween-20 in DI water through device outlets for five minutes. For cell experiments, devices are flushed with ethanol or isopropanol alcohol (IPA) to wet PDMS surfaces, followed by deionized water to clean out the alcohol and bovine serum albumin (BSA) to coat the PDMS surfaces. BSA can be filled or flushed in devices for twenty minutes before experiments begin. During this priming process, any bubbles generated inside the devices must be eliminated by applying high pressure from fluid flow to force the bubbles out through gas-permeable PMDS walls or through inlets and outlets. The complete procedure can be found in the Materials and Methods chapter.
2.4.2 Results from Mask Design No.1 Devices

After we fabricated the first set of devices (Mask Design No. 1), we first wanted to test basic functionality by using a single-Dc device with at least two bead sizes to observe both bump mode and zigzag mode. As a reminder, this device was designed such that particles larger than 5 μm would get bumped along the entire array and exit through the top outlet channel while smaller particles would zigzag a few times and exit through the bottom outlet (Figure 2-11).

![Figure 2-10 Common regions of clogging in devices.](image1)

Figure 2-11 Expected trajectories of particles with different sizes in Device D4.

We have independently injected 1, 2, 4.5, 6, and 10 μm functionalized polystyrene microspheres into Device D4; however, only zigzag mode was observed for all bead sizes (data not shown). In
hope of observing bump mode behavior in Device D4, we selected microspheres with even larger diameters (15 µm red-orange polystyrene microspheres) and they were injected into the devices. Figure 2-12 shows the resulting trajectories taken by 15 µm red-orange polystyrene microspheres in Device D4 under TRITC channel and fluorescein flow tracer of the buffer solutions under FITC channel. The fluorescein tracer was mixed with the buffer and injected into the two buffer inlet channels in order to ensure the expected laminar flow and the balanced flow rates between the three inlets. We chose to mix the fluorescein tracer with the buffer instead of the sample because we have previously observed fluorescein tracer in the sample bled through into TRITC channel and overpowered the red-orange bead trajectories. There are two key observations from these results.

First, both zigzag mode and bump mode of travel were observed for the 15-µm-diameter microspheres, even though their diameters were significantly larger than the target critical diameter of 5 µm. Upon further speculation, we conjectured that the particles undertook two modes of motion because of the sidewall effects. A closer look at the “zigzag” trajectory in Figure 2-12B revealed that the two zigzag motions happened at particular locations of the device where there was space for flow right next to the bottom sidewall, which have been completely obstructed by the upstream microposts attached to the channel wall (Notice the decreasing gap width between the bottom sidewall and the first column of microposts as we looked from the rows after which zigzag actions occurred to a few rows upstream where the gaps starting to disappear in Figure 2-12A). This flow needed to come from adjacent unobstructed stream; therefore, a certain amount of fluid was drawn from next fluid stream to consistently feed this empty region. This effect could locally alter the designed flow profile along the gap and draw particles larger than the critical diameter into zigzag motion. Evidence from particle trajectories in Figure 2-12 supported this conjecture. In Figure 2-12D,F, the particle appeared to be operating in bump mode; however, that was not what happened. These particles were not being drawn into zigzag motion because they entered the DLD array at slightly upper locations along the gap and did not start to negotiate the post until they passed the points where zigzag mode was forced on particles. Notice existence and non-existence of the repeated scallop shape on both sides of the bead trajectories of Figure 2-12D,F compared to those of Figure 2-12B. To elaborate, the bead trajectory in Figure 2-12D shows four upward concave curves in the first four rows of microposts, followed by straight line (no curve on both side) for a few rows and then downward
concave curves. Similarly, the bead trajectory in Figure 2-12F shows one small upward concave curve in the first row of microposts, followed by straight line (no curve on both side) and then downward concave curves. On the other hand, the bead trajectory in Figure 2-12B shows small downward concave curves in the first four rows of microposts, followed by straight line (no curve on both side) and then more downward concave curves. This evidence led us to assume that the bead in Figure 2-12D hit the bottom of the third-column micropost for a few rows when entered the DLD array while the bead in the beads in Figure 2-12B hit the top of the second-column micropost when entered, being only one of the three beads that negotiating the post at the first row. The bead in Figure 2-12F entered in-between and did not hit either the bottom of the third-column microposts or the top of the second-column microposts. In other words, they were starting at streamlines different from the aberrant one which was most adjacent to the microposts.

Second, although some microspheres were bumped all the way through the DLD array, they still unfavorably ended up in the bottom outlet channel. The reason behind this effect was embedded in the figures of fluorescein flow tracer. That is, a fraction of the fluid flow from the upper inlet channel had to flow out through the bottom outlet forcing any particles in the solution stream (fluorescein negative stream) to also flow out via the lower outlet. There were two factors that could make an impact on this effect: relative inlet flow rates and outlet geometry. Due to asymmetry of the inlet channel designs, the lateral position of the solution stream needed to be controlled by a combination of flow rates supplied to each of the inlet channels. The most ideal flow rate combination yielded constant fluid velocity in the direction of the flow, along the width of the DLD chamber at the entrance, meaning the flow stream from each inlet did not force the stream widths from other inlets to expand or shrink from the inlet opening width once entering the DLD chamber. According to the Figure 2-12(A,C,E), the flow in this setup has proven to be laminar and almost ideal as the width of the fluorescein positive and negative streams correspond to the widths of the inlet channels where the streams entered the DLD chamber. Because of the geometry of the inlets and outlets, the solution streamline deviated slightly from the middle of the outlet chamber and exited the chamber below the intended lateral exit position. This could be fixed by designing a new outlet geometry accounting for this deviation.
Figure 2-12 Fluorescein flow tracer showing trajectories of the two buffer channels (A,C,E) and the corresponding 15-µm microsphere trajectories (B,D,F) observed in Device D4. The resulting images are representative from two repeated experiments with different Device D4.
Mask Design No. 1 was designed with the goal of minimizing the device footprint so that it could be readily integrated with the IDS devices. Particularly, the width of the DLD chamber was minimized by having an asymmetric inlet geometry and by having only a few columns of microposts in the array. However, this came with a high cost for there was not a large-enough design space and little tolerance for errors due to model or fabrication variations.

Because of the narrow chamber width, particles of interest traversed very close the chamber sidewall and were influenced by the undesired sidewall effects. Also, there were no repeats of the micropost array as a fail-safe mechanism to account for fabrication defects, any irregular activities e.g. clogging, or the stochasticity introduced in deterministic separation process by particles randomly entering at different lateral positions along the gap even with the focused laminar flow and not necessary negotiating the post at the first row of microposts.

2.4.3 Results from Mask Design No. 2 Devices

After we have experimented with Mask Design No. 1 devices, we concluded that a few changes needed to be made on Mask Design No.1 in order to design a successful DLD array. The main changes were 1) number of columns of the microposts were increased and the sidewall geometry was modified such that the sidewall effects were reduced, 2) the array for each Dc was repeated three times to improve robustness. These changes were applied to the design and we named it Mask Design No. 2. The device of interest on this mask was the chirped array with desired critical diameters of 6.65, 9.27, 11.85, and 14.39 μm, as depicted in Figure 2-13. Zone 0 to Zone 5 and the expected trajectories were also shown in the figure. The zoning was to help identify the regions in the lateral position where the beads of different sizes were expected. From here on, the 6 zones corresponding to ones depicted in the schematic would be used as reference for lateral positions inside a chirped array device. Except for zone 0 where no beads were expected and Zone 5 where particles larger than ~14.39 μm were expected, each zone (Zone 1-4) had the width equivalent to three times the pitch size (λ). The zone width of 3λ was to account for the expected entering position which were 3λ wide and also account for each Dc having three repeats of array. Traveling through an array of three repeats for a particular Dc, the particles were expected to either zigzag and end up with zero lateral position or bump and be laterally displaced by 3λ. The expected trajectories will be explained in the next paragraph.
To characterize the size separation performance of the DLD array, the device was first injected with polystyrene microspheres of three sizes independently, namely bright blue 4.5 μm, yellow-green 10 μm, and red-orange 15 μm. The expected trajectories for the selected bead sizes were shown in Figure 2-13 as blue (4.5 μm), green (10 μm), and red (15 μm) solid lines. The size distribution of the three microsphere populations was measured individually by the coulter counter to be 4.367 ± 0.289 μm, 10.15 ± 0.292 μm, 15.28 ± 0.38 μm respectively. The resulting size distributions are normalized and plotted together in Figure 2-14.
To clarify, the 4.5 μm, 10 μm, and 15 μm bead populations were run individually in three devices of the same design on different days. The exit positions of beads were recorded in movies and extracted by MATLAB®. The resulting exit position histograms for each bead population were selected from experiments on the same device to represent the results and plotted in Figure 2-15(A,B). Figure 2-15B shows bead exit positions from a repeat experiment taken the same day in the same device as those of Figure 2-15A. From the exit position histograms, the corresponding normalized estimated size histograms were calculated from the ideal exit-position and expected size relation (Figure 2-16B) and plotted in Figure 2-15 (C,D). The total number of detections for the 4.5 μm, 10 μm, and 15 μm size group were 58, 27, and 21 in Figure 2-15A; and 58, 67, and 41 in Figure 2-15B. Zoning in Figure 2-15(A,B) is depicted as number zero to five separated by dashed lines, which corresponded to the zoning which was discussed in Figure 2-13. Recall that each of zone 1 to zone 4 has the width of three pitches. Furthermore, ideally particles must not exit from zone zero as that would mark negative lateral displacement, which is not expected to occur for particles in bump mode nor for particles in displacement mode.

According to Figure 2-15(A,B), we see that larger particles are preferentially observed in the higher-numbered exit positions, and there is a clear separation between the 10μm and the 15μm microspheres. Ideally, if we assume that particles entered the DLD chamber inside the middle 3λ range (lateral entering position) and that all particle motions inside the DLD array are as predicted in theory, the 4.5μm, 10μm, and 15μm microspheres are expected to exit at zone 1, zone 3, and zone 5, respectively (Figure 2-13), with the size distributions predicted by the coulter counter in Figure 2-14. Experimentally, the lateral exit positions of particles in each group were distributed over at least two zones and there is no clear separation between 4.5μm and 10μm microspheres as the exit position histograms of 4.5 and 10μm microspheres overlapped. Figure 2-15A and Figure 2-15B represent results taken from the same device at different times. Except for that of 4.5μm microspheres, the 10μm and 15μm movies for data in Figure 2-15A were recorded before the ones in Figure 2-15B. Although larger number of particles might still be needed to definitely conclude this argument, we suspected that, as time passed, there was generally a higher chance of particles clogging and flow profile deviation, resulting in a decrease of separation efficiency since there was more overlap between the exit position histograms of different bead sizes in Figure 2-15A than in Figure 2-15B. Below we will investigate further the
potential reasons why we observed 10µm microspheres in zone 1 of Figure 2-15A and also 15µm microspheres in zone 0, 2, and 3 of Figure 2-15B.

In order to gain insight into the deviations in exit position observed in Figure 2-15(A,B), we compared the predicted and observed distributions of exit position as we vary the starting position in the array. The exit position ranges for the experimental data from Figure 2-15A are shown along with the theoretically predicted exit position ranges in Figure 2-16 as we vary the starting input position from ideal (Figure 2-16A,C,E) to allowing up to 6λ added variation in inlet position (Figure 2-16E). These variations could result from nonideal entering positions or any nonidealities inside the array.

Figure 2-15 Results from single-sized microsphere characterization studies. (A),(B) Particle exit position histograms and (C),(D) corresponding size histogram estimated from particle exit positions. BB, YG, and RO represent fluorescent spectra of 4.5, 10, and 15 µm particles. (E) Size distribution of particles as measured by coulter counter. Blue, yellow, and red represent 4.5, 10, and 15 µm particles respectively.
Figure 2-16 Error models based on $\lambda$ scale and corresponding size-exit position relations. (A,B) Ideal condition, (C,D) $\pm 2 \lambda$ errors, (E,F) $\pm 3\lambda$ errors. (Left column) Equivalent errors from entering position ranges of particles as indicated in blue. (Right column) Red shades depict theoretical size-exit position relationship based on errors accounted for by entering position. Black shades indicate experimental size-exit position relationships based on the experimental means and standard deviations.
We observe that none of the three experimental size-exit position relationships fit inside the expected range for the ideal case (Figure 2-16B). However, in Figure 2-16D, the experimental exit position ranges of the 4.5 µm and the 15 µm particles fit inside the expected range of the ±2λ case. That means that one potential explanation for the lateral exit position errors for the 4.5 µm and the 15 µm particles is that they entered the array along a wider range than the expected 3λ range, or, if the entrance positions were within the ideal range, they could run into non-idealities inside the array that replicated the effect of entering along a wider range (such as clogging). ±2λ errors are acceptable for this particular experimental set up because there were only three tested, narrowly distributed particle sizes that were far apart in size, and there was rooms for this type of error because each zone has a repeat of three arrays and the chosen tested particle sizes fall into zones which were far apart from each other by 3λ. Some of the 10 µm microspheres; however, deviated more than ±3λ error (Figure 2-16F) in lower boundary direction, resulting in overlap of the histograms of lateral positions of the 4.5 µm and the 10 µm particles.

To further investigate the cause of the deviations in the exit positions of the 10 µm particles, the three individual aberrant trajectories of 10 µm diameter particles exiting through zone 1 from data in Figure 2-15(A,green) are shown in Figure 2-17. Note that the contrast of these greyscale images have been adjusted so that the trajectories could be observed. All 10 µm microspheres were expected to enter the array in zone 1, bump laterally into zone 2 for the first set of first critical diameter arrays, namely 6.65 µm, since they are significantly larger than the critical threshold. Due to the size distribution of the particles, we expected the particles to either go through the second critical diameter array in zigzag mode or bump mode and end up in zone 2 or zone 3. For the last two critical diameters, the microspheres were expected to zigzag and remain within their zones. Because of the limited field of view (FOV) of the 5X objective, only the arrays of the last three critical diameters, namely 9.27, 11.85, and 14.39 µm were captured. While the majority of the particles entered the FOV close to the top edge of zone 2, got bumped by a few arrays and exited through zone 2 or zone 3, these three aberrant trajectories undesirably entered the FOV through zone 1, zigzagged through the array and exited the FOV within zone 1. Unfortunately, movies for the FOV of the first critical diameter did not record any trajectories that exited FOV through zone 1, but I suspect that either these aberrant particles entered the array through zone 0 and were bumped into zone 1 by the first three arrays of 6.65 µm critical diameter or they entered, zigzagged, and exited via zone 1 as if the particles were smaller than 6.65 µm.
According to the recorded trajectory movie, the second case was more likely because these aberrant trajectories (Figure 2-17(left)) had fairly low contrast with the background compared normal trajectories (Figure 2-18). The ratio of pixel intensity of particle trajectory to background were less than 5:1 for aberrant trajectories and at least 10:1 for normal trajectories. That was either because particle moved fast and resident time was small or the particles moved at the same speed but they were smaller or fluoresced less. The first speculation was less likely because the flow velocity was controlled by syringe pump, and the flow velocity in zone 1 should not differ much from the other zones. All evidence suggested that the aberrant trajectories were formed by particles which were smaller than the expected 10 µm diameter, potentially due to trajectories that were bleed-through from the previous runs of bright-blue 4.5 µm microspheres.

Similar to the study of the aberrant 10 µm particle trajectories, the aberrant 15 µm particle trajectories are shown in Figure 2-19. 15 µm particles were expected to enter via zone 1, bump laterally through all 12 arrays of four different critical diameters, and exit via zone 5. There were two observed causes of deviation. Top figures showed trajectories of particles which entered the FOV via zone 1 instead of the expected zone 2, and zigzagged around bead-clump obstacles a few times, resulting in ± 5λ-6λ errors. Bottom figures showed that some aberrations were results of sudden release of previously bound microspheres toward the back of the arrays. The nonspecific binding of particles in zone 0 and zone 1 were not recorded, but were assumed to occur between the two recorded movies. In the first 15-µm microsphere movie, only the clumps toward the back of zone 4 and zone 5 were starting to form and the movie ends before any nonspecific binding sites in zone 0 and zone 1 were formed. From experience, I suspected that these clumps in zone 4 and 5 were results of particle-particle interaction that took place in the array (not shown.
in Figure 2-19) because the 15 µm diameter particles were about 0.75 of the gap width. Thus, if two particles entered the same gap at the same time that could result in one particle being bumped and the other zigzagged as the particles themselves forced each other to be in certain streamlines along the gap leading to different paths and traps at unexpected regions. Since both aberrant and normal trajectories had approximately equal contrast and line width, I did not speculate that the deviations were caused by difference in true particle sizes, but by the above conjecture or by particles going around clogs.
Figure 2-17 Detected aberrant trajectories of 10µm microspheres from Figure 2-15A data. (Left) As recorded, (Right) After contrast enhancement. Zone 0 to zone 5 were separated by red solid lines.
Figure 2-18 Normal trajectories of 10 μm microspheres from Figure 2-15A data as recorded.

Figure 2-19 Aberrant trajectories of 15 μm microspheres from Figure 2-15B data.
After characterization with single-sized rigid polystyrene microspheres, we tested our devices with BA/F3 cells. Figure 2-20(A,B) showed the observed lateral exit position histograms from two repeats of cell trajectories in the same device on the same day. The total number of detected cells in Movie 1 and 2 were 100 and 56, respectively. Similar to the previous study, the exit positions were separated into six zones according to ideal experimental condition. The BA/F3 cells, whose diameters were roughly normally distributed between 9 to 16 μm (Figure 2-20 (C,D)), exited through zone 2 to 5 with majority (86% in (C) and 91.7% in (D)) of the population in zone 3 and zone 4, as expected. This is comparable to the fraction of the cell population with diameter between 9.27 and 14.39 μm (i.e., population in zone 3 and zone 4) as measured by the coulter counter (86.5%). The size histograms estimated from the lateral exit positions and measured via Coulter Counter are shown in Figure 2-20 (C,D). The size distribution of cells in
the second set of experiments was narrower than that of cells in the first set of experiments and was on the smaller side of the size distribution measured in the first set of experiments and by the coulter counter, potentially because of a bias in the size distribution of the cell population over time. From experience with microspheres, 15 μm polystyrene microspheres suspended in DPBS solution settled down faster than the 10 μm particles in the same condition. The sample-loaded syringe was pointed down toward to the ground. It was possible that larger cells settled more quickly and were preferentially drawn out into the DLD devices earlier, before the second movie was recorded. Therefore, due to this change in size distribution of cells, cells only exit through zone 2, 3, and 4, but not through zone 5.

Finally, to demonstrate separation of two different populations of particles, we mixed together 6 μm microspheres and BA/F3 cells and separated them in the device (Figure 2-21). As expected, the 6 μm particles are found at lower exit positions (corresponding to smaller size) than the cells, which are larger in size (Figure 2-21A). The total numbers of detected 6 μm microspheres and BA/F3 cells are 24 and 124, respectively. Figure 2-21B shows that measured size distribution of particles with the coulter counter overlapped with the size distribution estimated from the recorded lateral exit positions. Note that the large peak at 4 μm was likely contamination in the coulter counter measurement.

A small number of cells exited through zone 1, likely similar to the red-orange 15 μm aberrations. Figure 2-22 shows this abnormal behavior. Figure 2-22A shows the front part of the DLD array where arrays with the smallest critical diameter were located. It depicted what was expected to also happen in the previous independent 15 μm microspheres and cell studies, but was not captured before. Particles went around obstacles at the front of the array either through the top or through the bottom side of the obstacles. The trajectories on the top side were thin and they showed zigzag properties while the ones on the bottom side were thicker and depicted the desired bump mode of motion. The thin trajectories of cells circumventing next to the top of obstacles and later zigzagging in Figure 2-22A suggested that these cells behaved as if they were smaller than 6.65 μm, the smallest critical diameter. On average, the width of the aberrant trajectories was estimated to be one third of the normal ones. If the flow speed was constant everywhere in the array, the width of the particle trajectory should be directly related to the particle size, and this would imply that the aberrant trajectories were formed by particles smaller
than 5 µm in diameter, supporting the claim of the true size of these aberrant particles. Note that the histogram of the mixed sample showed significant amount of the particles whose diameters were smaller than 5 µm. This was possible because 1) cells have wide distribution in size or 2) the detected particles could have been small fragments of stained cells. Furthermore, Figure 2-22A shows that the clog at the front of the array shifted the lateral position of the entering normal cell particles down. This implied that the trajectories could also be unfavorably shifted up laterally by the clog with a few λ errors such that, for example, a 10 µm particle could be shifted up and exited the frame of Figure 2-22A in zone 1 where a particle with \(D_c < 6.65\) µm would be expected, or worse, exited the frame in zone 0, decreasing the separation efficiency in our system. This claim was also implicitly supported by the unexpected trajectory in Figure 2-22B. Since the unexpected trajectory was as bright as the normal ones, the particle creating that trajectory was expected to be between 6.65 and 14.39 µm in diameter. That particle, however, entered the frame of Figure 2-22B via zone 1 instead of the expected zone 2 or 3. That means that the particle could have been shifted up by the clog, cancelling the \(3\lambda\) lateral displacement downward that could have been gained by the bump mode through the three repeats of the first \(D_c\).

![Figure 2-21 Separation of 6 µm microspheres and BA/F3 cells based on size. (A) exit position histogram (B) Corresponding estimated size and measured size histogram. (C) Corresponding estimated size distribution only.](image)
Figure 2-22 Example of aberrant cell trajectories from Figure 2-21A data.
2.5 Conclusions

We have designed, fabricated, and characterized a functional chirped DLD array which could be one of the sensor modules in our integrated cell analysis platform. The goal of this DLD module is to measure size of individual particles. Therefore, the designed chirped array described in this chapter was aimed for accuracy and precision of size-based displacement such that the detected lateral positions of particles can be accurately converted to their estimated size. We have shown that our fabricated DLD device could perform size separation of beads and cells with particles exiting the correct zone with accuracy of 100% in 6 out of 9 runs, and with up to 11.3% of unexpected trajectories in 3 out of 9 runs. Of particular interest are the trajectories that deviated from the expected path. Evidence from snapshots of these aberrant trajectories suggested that these non-idealities took place due to undesired factors such as clogging or particle-particle interactions, and rarely pointed to the array design parameters. Non-idealities from external factors can be reduced by careful control of experiments and redundancy of arrays, especially minimizing clogging-prone sites such that clogging has less impact on the designated lateral displacement, at the price of larger device footprint.
Chapter 3 : Lensless Digital In-Line Holographic Microscope

3.1 Introduction

An innovative two-step lensless imaging system, which is now known as holography, was introduced by Dennis Gabor in 1948 [26]. Since its conception, the process involves recording of object diffraction patterns and wavefront reconstruction from the recording. Gabor recognized that when an object is illuminated by a single coherent point source, the scattered wave from the object interferes with the unscattered reference wave from the point source, allowing the amplitude and phase information of the object wavefront to be encoded as intensity on the recording material. Ultimately, an image of the original object can be optically reconstructed from the recorded interference pattern, which is also known as a hologram, meaning a total recording [27].

The original Gabor holography was an in-line system intended to circumvent a magnetic lens aberration problem in electron microscopy as it avoided the usage of the magnetic lens altogether. In-line geometry described a system where the reference wave is in general alignment with the object wave, in contrast to off-axis geometry, where there is an angle between the reference and object waves. In the 1960s and afterwards, the concepts and technology for holographic systems have been significantly improved. Typically, holography is implemented with photochemical media as the recording material and an optical procedure for reconstruction. Digital holography, on the other hand, replaces photochemical media with an electronic imaging sensor, and replaces optical reconstruction with digital computation.

There are several digital in-line holographic systems for microscopy applications, also known as digital in-line holographic microscopes (DIHM), all of which can be exploited for three-dimensional imaging [28]. We are particularly interested in the system introduced by the Ozcan group because of its large field of view and simplicity for the imaging set-up [80]. A hologram of each object recorded from this system contains rich information and can be used as signature to distinguish different object types even without reconstruction [49]. Therefore, we will implement the system with two approaches. The first approach attempts to validate imaging capability of our system. For the first approach, holograms of static objects are reconstructed and compared to either microscopic images or manufacturer datasheets of the objects. The second approach aims to tackle the problem of tracking particles as they traverse the integrated microfluidic platform.
For the second approach, time-lapsed holograms of objects in a device of interest are detected and tracked with no reconstruction. Without reconstruction, computation procedures are simplified and the operating time is minimized.

3.2 Theory
3.2.1 Recording
Digital in-line holographic set-ups for coherent and partially coherent illumination are illustrated in Figure 3-1 [44]. For coherent illumination, coherent light from a laser is focused onto a pinhole. The pinhole, whose diameter is a few times the wavelength of the laser beam, acts as a lowpass spatial filter and emits a coherent spherically divergent field. For partially coherent illumination, a light emitting diode (LED) is butt-coupled to a pinhole in order to increase the spatial coherence of the LED source. Since the intensity of the LED light is inversely proportional to the square of the distance from the source, the LED is placed adjacent to the pinhole to ensure that more LED light is incident on the object. The spherical wave emitting from the pinhole acts as a secondary point source and is directed onto the object. Each object diffracts, absorbs, and
refracts the incoming light according to its size, morphology, subcomponents, and refractive index. The interference patterns between the light diffracted from the object and the unscattered light from the secondary source are then recorded on an optoelectronic sensor array such as a complementary metal oxide semiconductor (CMOS) or a charge-coupled device (CCD) camera. For in-line holography in general, the object of interest is assumed to be highly transmissive or only weakly scattering, such that a portion of the wavefront from the secondary point source can transmit through the object plane without being scattered and acts as a reference wave.

Let us assume that the primary source emits light at wavelength $\lambda$ and that the pinhole is an aperture with diameter $d$. The object is positioned at distance $z_1$ from pinhole and at distance $z_2$ in front of the optoelectronic sensor array.

For conventional in-line holography, $z_2$ is generally chosen to be larger than $z_1$ in order to yield lateral magnification of the object at the recording plane.
\[ M = \frac{z_1 + z_2}{z_1} \quad (3-1) \]

\( z_1 \) is typically a few millimeters while \( z_2 \) is on the order of centimeters. The numerical aperture (NA) of the system [30] is defined as

\[ NA = n \cdot \sin(\alpha) \quad (3-2) \]

where \( n \) is refractive index of the propagating medium and \( \alpha \) is half angle of illumination. If we assume air as propagating medium (\( n=1 \)) and assume small angle of illumination (\( \sin(\alpha) = \alpha \)), NA can be written as

\[ NA = \alpha. \quad (3-3) \]

*Assume the image sensor array width is \( W \) and recording geometry as in*

\[
\begin{align*}
\text{Object Plane} & \quad \text{Detector Plane} \\
\text{Pinhole Plane} &
\end{align*}
\]

Figure 3-2, NA is given by

\[ NA = \frac{W}{\sqrt{\left(\frac{W}{2}\right)^2 + (z_1 + z_2)^2}}. \quad (3-4) \]
The lateral resolution is limited by the Rayleigh criterion and is defined as
\[ \delta x \geq \frac{\lambda}{2NA} \] (3-5)
and the depth resolution or depth of field (DOF) is given as
\[ \delta z \geq \frac{\lambda}{2(NA)^2} \] (3-6)
Therefore, the lateral and the depth resolution can be improved by decreasing the wavelength of the primary source or by increasing NA. According to (3-4), increasing the image sensor width or decreasing \( z_1 \) and \( z_2 \) can increase NA and enhance the lateral and depth resolution, potentially at the cost of reduced lateral magnification.

Let \( A_{\text{ref}}(x,y) \) represents the unscattered reference light at the detector plane.
\[ A_{\text{ref}}(x,y) = |A_{\text{ref}}(x,y)| \exp[j\phi(x,y)] \] (3-7)
where \( \phi(x,y) \) represents the phase of \( A_{\text{ref}}(x,y) \). And let \( A_{\text{obj}}(x,y) \) represents the weakly scattered light from the object at the detector plane, which is to be reconstructed.
\[ A_{\text{obj}}(x,y) = |A_{\text{obj}}(x,y)| \exp[j\psi(x,y)] \] (3-8)
where $\psi(x,y)$ represents the phase of $A_{obj}(x,y)$. Since all recording materials only respond to the light intensity, the recorded interference intensity pattern at the camera can be expressed as in Equation (3-9), and can be simplified in two ways as in (3-10) and Equation (3-11).

$$I(x,y) = |A_{ref}(x,y) + A_{obj}(x,y)|^2$$

$$= |A_{ref}(x,y)|^2 + |A_{obj}(x,y)|^2$$

$$+ 2|A_{ref}(x,y)||A_{obj}(x,y)| \cos(\theta - \psi)$$

$$= |A_{ref}(x,y)|^2 + |A_{obj}(x,y)|^2$$

$$+ A_{ref}^*(x,y)A_{obj}(x,y) + A_{ref}(x,y)A_{obj}^*(x,y)$$

Equation (3-10) shows explicitly how phase information of the object scattered wave is encoded in the recorded holographic intensity pattern [27]. Equation (3-11) shows how mathematical terms can be linked to optical features of the hologram [43]. The first two terms are background DC intensity as the first term represents intensity of the reference wave while the second term represents the classical diffraction pattern of the object. We refer to the last two terms in Equation (3-11) as the holographic diffraction patterns since they are interference terms between the unscattered reference wave and the scattered wave from the object. The third term represents an optical field proportional to the original object scattered wave, which emerges from the virtual image of the original object at a negative distance $z_2$ from the recording plane. The fourth term is proportional to a conjugate of the original object scattered wave and can be used to reconstruct a real image of the original object at a positive distance $z_2$ from the recording plane. The virtual image and real image separated by distance $2z_2$ are referred to as the twin image, because both contain equivalent information about the object. Contrary to off-axis holography, for an in-line holographic system recorded information from the DC background, object image, and twin image are centered on the hologram axis as is implied in Equation (3-11) because there is no angle between the object and reference beams. Therefore, with an in-line recording geometry, the object and twin image are optically inseparable. When the real image is brought to focus, the out-of-focus virtual image ensues. The opposite happens when the virtual image is brought to focus. This problem is known as the twin-image problem. Common DIHM techniques for twin image elimination and reconstruction of the desired object image with quality comparable to that obtained from conventional optical microscopes will be discussed in the next section.
3.2.2 Wavefront Reconstruction

Several methods have been developed to minimize the effects of the twin image in DIHM systems [44]. In one of the main methods, a number of recorded holograms with varying phase-shifted reference wave are required to eliminate the DC terms and one of the twin image terms. Another variation requires two recorded holograms at different planes. For the purpose of explaining the main optics concepts behind wavefront reconstruction of DIHM systems in this thesis, we consider a simple reconstruction method that requires only one recorded hologram. This method is useful in cases where the twin image is considered to be out of focus and its energy spreads out over the recorded hologram to the extent that it can be assumed to be negligible after numerical processing, as in the case of far-field holography [45]. The details of this method are described in this section.

Equation (3-11) suggests that in order to reconstruct the object image, one needs to remove the DC terms and either one of the real image and virtual image terms such that only one term which is proportional to the object scattered wave is left. Barton [81], [82] proposed to remove the reference wave as much as possible by creating a contrast image from the hologram

$$I_{\text{cont}}(x,y) = I(x,y) - I_{\text{ref}}(x,y)$$  \hspace{1cm} (3-12)

where $I_{\text{ref}}(x,y)$ is the intensity image of the reference wave and is equal to $|A_{\text{ref}}(x,y)|^2$ and can be obtained from averaging the hologram with an n-pixel square window or by removing the object and rerecording. Equation (3-12) becomes:

$$I_{\text{cont}}(x,y) = |A_{\text{ref}}(x,y) + A_{\text{obj}}(x,y)|^2 - |A_{\text{ref}}(x,y)|^2$$  \hspace{1cm} (3-13)

$$= |A_{\text{obj}}(x,y)|^2 + 2A_{\text{ref}}^*(x,y)A_{\text{obj}}(x,y) + |A_{\text{ref}}(x,y)A_{\text{obj}}^*(x,y)|^2$$  \hspace{1cm} (3-14)

Barton [81], [82] also proposed to remove other unwanted terms by smearing out energy in the reconstructed wave.

$$I_{\text{cont}}(x,y) = \frac{I(x,y) - I_{\text{ref}}(x,y)}{\sqrt{I_{\text{ref}}(x,y)}}$$  \hspace{1cm} (3-13)

$$= \frac{|A_{\text{obj}}(x,y)|^2 + A_{\text{ref}}^*(x,y)A_{\text{obj}}(x,y) + A_{\text{ref}}(x,y)A_{\text{obj}}^*(x,y)}{|A_{\text{ref}}(x,y)| + |A_{\text{ref}}(x,y)|}$$  \hspace{1cm} (3-14)
Since the object is assumed to weakly scatter, it follows that the amplitude of the object scattered wave is much lower than the amplitude of the reference wave, $|A_{obj}(x, y)| \ll |A_{ref}(x, y)|$. As a result, Equation (3-14) can be simplified to

$$I_{cont}(x, y) = \frac{A_{ref}^*(x, y)A_{obj}(x, y)}{|A_{ref}(x, y)|} + \frac{A_{ref}(x, y)A_{obj}^*(x, y)}{|A_{ref}(x, y)|}$$

(3-13)

The object image can then be reconstructed from the generated contrast image in Equation (3-13) by a propagation algorithm as the object image will be in focused while the twin image will be defocused. Recall that we only consider the cases where, after numerical propagation, the twin image will be defocused and smeared out to the extent that it can be neglected. Therefore, the desired object image can be retrieved without the twin-image effects.

One commonly used approach for propagation calculation is the Helmholtz-Kirchhoff transform. Readers may refer to [27] [83] and [28] for basic scalar diffraction theory and other numerical propagation approaches. The Helmholtz-Kirchhoff transform does not use Fresnel approximation and can be applied to variations of the DIHM setup. The reconstructed complex image at the effective distance $z_{eff}$ from the recording plane by the Helmholtz-Kirchhoff integral can be written as

$$U(x, y, z_{eff}) = \iint I_{cont}(x_H, y_H, 0)P(x - x_H, y - y_H, z_{eff})dx_Hdy_H$$

(3-15)

where $I_{cont}(x_H, y_H, 0)$ is the pre-processed contrast image at the recording plane in Equation (3-13), and $P(x, y, z)$ is the spatial impulse response of the system, given by

$$P(x, y, z) = \exp[(x^2 + y^2 + z^2)^{1/2}]$$

(3-16)

$z_{eff}$ is the effective image reconstruction depth corrected for point-source effects given by

$$z_{eff} = \frac{(z_1 + z_2)}{z_1}z_2$$

(3-17)

The convolution Equation (3-15) is optimally processed by applying the convolution theorem and utilizing two fast Fourier Transforms (FFT) and one inverse FFT transform.

$$U(x, y, z_{eff}) = F^{-1}\{F[I_{cont}] \times F[P]\}$$

(3-18)

Where $F$ represents the Fourier transform.
This reconstruction method has been shown to effectively eliminate the twin image and recover the desired object image from the recorded hologram for different samples including microspheres [42], [45], the sectioned head of a fruit fly [39], and mammalian carcinoma cells [38].

Although DIHM systems have some limitations, such as the restriction for the object to be weakly scattering and the need to perform image reconstruction from the recorded hologram, DIHM have several advantages over conventional light microscopes. A DIHM recording setup is simpler and more adaptable than conventional microscopes because DIHM is implemented with fewer optical components and the image magnification in DIHM is achieved by the geometry of the setup rather than alignment of objective lenses. The image resolution of the system can be improved by changing the geometry of the setup, increasing the spatial and temporal coherence of the source, or by replacing the optoelectronic sensor with one of finer pixel resolution. Most importantly, a single recorded hologram from a DIHM system contains both the amplitude and phase information of the object. It can be used to reconstruct a two-dimensional object image with resolution comparable to a microscope image at any cross-sectioned plane within the depth of field of the DIHM system. Because of the system’s large depth of field, a stack of two-dimensional reconstructed images at different planes along the holography axis can be combined to obtain a three-dimensional structure of the object.

Despite the benefits of the conventional DIHM system mentioned above, a byproduct intrinsic to the geometry devised to achieve magnification in conventional DIHM is its small field of view. One adaptation of the DIHM system known as Lensless Ultra-wide-field Cell Monitoring Array platform based on Shadow imaging (LUCAS) was developed to provide larger field of view [46], [47]. This system can be optimized to acquire a field of view that is equivalent to the area of the optoelectronic sensor array used in recording. With the current technology, one can choose CMOS sensors with FOV on the order of tens of square millimeters or CCD sensors with FOV on the order of a square centimeter, which can satisfy our need for an imaging platform with a FOV large enough to observe particles in a square-centimeter-scale integrated cell analysis platform.
3.2.3 Lensless Ultra-wide-field Cell monitoring Array platform based on Shadow imaging (LUCAS)

The LUCAS system, first introduced in 2007, is a lensfree cell monitoring platform that relies on recording of classical diffraction pattern (i.e. shadow) of each cell onto an optoelectronic sensor array [46]. A sample is placed between an optical source such as an incoherent white light source or laser beam and an optoelectronic sensor array. The distance between the sample and the sensor array was set to be less than 200 μm in order to maximize the signal-to-noise ratio on the sensor plane. This system was able to improve the FOV to more than two orders of magnitude larger than that of conventional light microscopes without mechanical scanning, at the expense of spatial resolution. In 2008, the same group made a slight change to their LUCAS system that still maintained the large FOV while significantly improving the spatial resolution [49]. This system was named Holographic-LUCAS. The change was their use of a 100-μm pinhole butt-coupled to the primary light source in order to spatially filter the light beam and allow the holographic diffraction patterns as well as the classical diffraction patterns of cells to be recorded. Holographic-LUCAS is similar to a conventional DIHM system. Holographic diffraction patterns recorded with Holographic-LUCAS were encoded with phase information from the sample that was normally lost in incoherent LUCAS; thus, holographic diffraction patterns contained richer information about detailed features of the sample. Even without image reconstruction, different particle types including red blood cells, neutrophils, platelets, and multiple-sized polystyrene beads can be distinguished in the recorded hologram. Holographic-LUCAS was further developed and equipped with wavefront reconstruction in [80].

There are a few key differences between Holographic-LUCAS and conventional DIHM. First, the sample is positioned closer to the recording plane in Holographic-LUCAS. In Holographic-LUCAS, the sample is placed at about 2 to 5 cm from the pinhole and approximately 1 to 2 mm from the recording plane. Recall from Equation (3-1),

\[ M = \frac{z_1 + z_2}{z_1} \]  \hspace{1cm} (3-19)

The effective imaging FOV of the system is defined as

\[ f = \frac{A}{M^2} \]  \hspace{1cm} (3-20)

where A the active area of the sensor array.
The Holographic-LUCAS configuration allows holograms to be recorded with unit fringe magnification (M~1), resulting in FOV equivalent to the entire active area of the sensor array (f~A). As discussed in section 3.2.1, in conventional DIHM, the sample is usually positioned closer to the pinhole for fringe magnification of more than 10 (M>10), which reduces the FOV of the system by two orders of magnitude. Second, it has been shown theoretically and experimentally that with this configuration incoherent illumination through a large aperture can still yield a holographic diffraction pattern if each individual object is coherently illuminated. That is, in Holographic-LUCAS the coherence diameter of the system only needs to larger than the size of each individual object rather than the size of the entire sensor array. Monochromatic LED has been used as the primary source to successfully record holograms in Holographic-LUCAS. Because the system requires less spatial and temporal coherence, the diameter of the pinhole used in Holographic-LUCAS (50-100 μm) can be a few orders of magnitude larger than that used in conventional DIHM (2-3 μm). Using larger pinholes also enhances the energy efficiency of the primary light source by allowing more light to pass through, and eliminates the need for a sophisticated optical alignment tools. Third, some conventional DIHM systems claim to be lensfree since the objective lenses are used to focus light from the primary source into the pinhole and do not contribute to image formation. Holographic-LUCAS is truly lensfree as it does not make use of any objective lens. Finally, because the sample is placed close to the recording plane, the energy of the twin image does not get smeared after numerical propagation. Phase-retrieval algorithms are thus needed to reconstruct images. Two phase-retrieval techniques will be discussed in the Materials and Methods section.

3.3 Materials and Methods

3.3.1 Test Sample

To test the performance of our imaging system, polystyrene microspheres of several sizes were used, including 2 μm (Polysciences, Inc., Catalog# 19814—diameter 1.93±0.05 μm, CV 3%), 3 μm (Polysciences, Inc., Catalog# 17134—diameter 3.0±0.06 μm, CV 2%), 4 μm (Polysciences, Inc., Catalog# 64070—diameter 4.172±0.173 μm, CV 4.1%), 6 μm (Polysciences, Inc., Carboxylate, Catalog# 17141—diameter 6.081±0.195 μm), and 10 μm (Polysciences, Inc., Catalog# 17136—diameter 9.606±0.763 μm). Polystyrene beads had simple spherical structures that represented the structure of suspension cells. Polystyrene beads were diluted in deionized water to the final concentration of ~10^4 particles/mL. Then 6 μL of the microsphere solution was
pipetted onto a clean glass coverslip. A small drop of glycerol was pipetted onto the center of another clean glass coverslip. The coverslip was inverted so that the glycerol side was down and carefully pressed on the bead coverslip. The sealed sample was let dry and could be used for two weeks if kept clean.

Other than simple spherical structures, we are interested in the image quality of the reconstructed fine features of adherent NIH/3T3 mouse embryonic fibroblast cells. The NIH/3T3 cells from the parental cell line of the ATCC cell bank (ATCC* CRL-1658 TM), which has been continually maintained in our laboratory according to the ATCC protocol [84], were cultured on sterilized removable-chamber microscopy glass slides. The cell culture media consists of ATCC-formulated Dulbecco's Modified Eagle's Medium with high glucose content (Catalog No. 30-2002), bovine calf serum (10% v/v), L-glutamine (2% v/v) and penicillin/streptomycin (1% v/v). After the desired concentration of cells was reached, the glass slide was detached from the removable chamber and washed with phosphate buffered saline (PBS). The adhered NIH/3T3 cells were fixed by immersing the glass slide into 4% formalin for 30 minutes and washed in PBS twice. The glass slide was then sealed with a clean coverslip in the same manner as described for the microsphere sample.

For tracking experiments, we observed BA/F3 cells as they flowed through a deterministic lateral displacement array (DLD) with our lensless holographic microscope. The DLD fabrication process is discussed in section 2.3.1 and in Appendix A: Fabrication Process Flow. The DLD experiment protocol is detailed in Appendix B: DLD Experiment Protocol. BA/F3 cells are cultured in RPMI-1640 media with 1% Sodium Pyruvate, 1% MEM Non-Essential Amino Acids, 1% Penicillin Streptomycin and 10% Fetal Bovine Serum.
3.3.2 Imaging Setup

![Diagram of digital in-line holographic microscope](image)

**Figure 3-3** Schematic of digital in-line holographic microscope as developed by [80].

**Figure 3-4** An implementation of the digital in-line holographic microscope.

For our on-chip imaging, we implemented a lensfree holographic microscopic system with a partially coherent source derived from Holographic-LUCAS (Figure 3-3 and...
Figure 3-4) [80], which consists of three main components, namely a light-emitting diode (OSRAM Opto Semiconductors Inc., Part# LYE65B—center wavelength: 587 nm, bandwidth: 15 nm), a pinhole (Thorlab—diameter 50±3 μm (Part# P50S) or 100±4 μm (Part# P100S)), and an optoelectronic sensor array (CMOS chip, Micron Technology, Model: MT9P031—pixel size 2.2 μm, active imager size 5.70 mm x 4.28 mm). The sample to be imaged is placed between the pinhole and the sensor array. For our system, the pinhole-to-sample distance is $z_1 \sim 2$ cm. The sample-to-sensor distance is $z_2 < 2$ mm. The pinhole is butt-coupled to the LED to increase the coherent length of the LED so that it is larger than the size of objects of interest. To elaborate, the coherent length of a typical LED is $L_{coh} = \frac{c}{\Delta \nu}$, where $c$ is the speed of light in vacuum and $\Delta \nu$ is the LED spectral bandwidth (i.e. FWHM) in Hertz. When the LED is butt-coupled to a D-diameter pinhole, the coherence length at the plane with distance $z_1$ away is $D_{coh} = \frac{\lambda_0 z_1}{D}$, where $\lambda_0$ is the LED center wavelength. For our system, $L_{coh} \approx 23 \mu$m without a pinhole and $D_{coh} \approx 235 \mu$m with a 50 μm-diameter pinhole. Note that $L_{coh}$ is on the same order of magnitude as micro-objects of interest while $D_{coh}$ is on an order of magnitude larger than $L_{coh}$, and that is enough to get a hologram signature of micro-scale objects. Hologram images were recorded with a 40-80 ms exposure time.

For convenience in handling of samples for our large-FOV DIHM system, three designs of substrate holders were 3D printed via Makerbot Replicator 2X and Ultimaker 2 Extended+. Makerbot Replicator 2X enjoyed better positioning precision (XY: 11 μm, Z: 2.5 μm) than the Ultimaker 2 Extended+ (XY: 12.5 μm, Z: 5 μm). Nevertheless, Ultimaker 2 Extended+ provided 2.6 times the build volume and came with swappable nozzles and the minimum layer resolution of 20 μm compared to the Makerbot Replicator 2X with a 0.4 mm nozzle and the layer resolution of 100 μm. We used the two 3D printer models based on availability as both sufficed the needs to construct simple substrate holders. The schematics of the 3D-printed substrate holders are shown in Figure 3-5. Different designs were used for different types of substrates. The main consideration for all designs was to minimize the gap between the sample and the CMOS sensor. As a result, the thickness of the substrate holders was restricted to the minimum. The first two designs (Figure 3-5A&B) were for the viewing of static samples on coverslips and on glass slides, respectively. While the two designs were sufficient for the corresponding substrates, we found that the extended rectangular support of the second design to be too thin and flimsy to use.
with microfluidic chips. Therefore, a third design (Figure 3-5C) was implemented with slightly larger thickness to increase the steadiness of the holder for heavier substrates and could be used for different substrates. Extended beams on top of Figure 3-5A&B were to help secure the substrates; however the beams were not included in Figure 3-5C as they restrict the position and orientation of the substrates.

**Figure 3-5 Schematics of the substrate holders for (A) coverslip substrate, (B) glass slide substrate, and (C) multipurpose.**

### 3.3.3 Image Reconstruction
Two iterative phase-retrieval techniques were recommended along with the recording set-up [80] and will be summarized here for completeness. The two methods have three things in common. First, both methods are restricted to objects with finite support. This constraint is an important piece of information for reconstruction. It is easily satisfied as real objects have finite sizes. Second, raw holograms are upsampled by a factor of four to six by cubic spline interpolation before applying the phase-retrieval techniques. Upsampling increases spatial frequency of the holograms and can contribute to more accurate object support and sub-pixel resolution of the
final reconstructed images. Accurate object support is crucial for achieving accurate reconstruction with small convergence time. Third, for both methods the angular spectrum approach is used to computationally propagate the optical field from one plane to another. To elaborate, the Fourier transform of the optical field at the original plane is multiplied by the transfer function of propagation through linear, isotropic media with refractive index, n:

\[
H_z(f_x, f_y) = \begin{cases} 
    e^{j2\pi z^2/\lambda} \sqrt{1 - \left(\frac{\lambda f_x}{n}\right)^2 - \left(\frac{\lambda f_y}{n}\right)^2}, & \sqrt{f_x^2 + f_y^2} < \frac{n}{\lambda} \\
    0, & \text{otherwise}
\end{cases}
\]

where \( f_x \) and \( f_y \) are the spatial frequencies. The product is then inverse transformed to attain the optical field at the plane distance \( z \) away from the original plane.

**Method 1** is categorized as one of the Interferometric Phase-Retrieval techniques, which is based on knowledge of the twin-image effects and is applicable only to recorded holograms where the holographic diffraction pattern dominates. That is, images of recorded holograms propagated through distance \( \pm z_2 \) from the recording plane contain duplicate information about the objects. After the recorded hologram is propagated through distance \( -z_2 \) from the recording plane (Figure 3-6B, \( z = 0 \)), there exists a focused virtual image inside a finite object support overlapped with a defocused real image (fringes) that spreads out inside and outside of the object support (Figure 3-6A). On the other hand, if the recorded hologram is propagated through distance \( +z_2 \) from the recording plane, the real image of the object is focused inside the object support while the virtual image is defocused (Figure 3-6C). Therefore, the twin image can be eliminated by filtering out either the focused virtual image at distance \( -z_2 \) (inside the red object support outline of Figure 3-6A) or the focused real image at distance \( +z_2 \) (inside the red object support outline of Figure 3-6C) before propagating the edited optical field to distance \( +2z_2 \) or \( -z_2 \), respectively. Since information from the defocused twin image that lies inside the object support are also deleted in this process, the lost information needs to be iteratively recovered by assuming an initial estimate of the lost information and propagating back and forth between the two planes at distance \( \pm z_2 \). Detailed steps of this method are summarized below.

1) Propagate the hologram at the detector plane to the object plane by a distance \( +z_2 \) to obtain the initial wavefront (\( U_{rec} \)) and determine the object support by thresholding.
2) To filter out the focused real image inside the object support, delete the values in the object support (S) by setting them to a constant value as initial guess.

\[ U_{z_2}^{(1)}(x, y) = \begin{cases} 
U_{rec}(x, y), & x, y \notin S \\
\bar{U}_{rec}, & x, y \in S 
\end{cases} \]

where \( U_{z_2}^{(1)} \) represents the field at distance \(+z_2\) from the detector plane in the first iteration. \( \bar{U}_{rec} \) denotes the mean value of \( U_{rec} \) within the object support.

3) Propagate the field by a distance \(-z_2\) and set the region outside the object support to a constant value. This step ensures that any remaining out-of-focus real image is eliminated.

\[ U_{-z_2}^{(i)}(x, y) = \begin{cases} 
D - \frac{D - U_{-z_2}^{(i)}(x, y)}{\beta}, & x, y \notin S \\
U_{-z_2}^{(i)}(x, y), & x, y \in S 
\end{cases} \]

\( U_{-z_2}^{(i)}(x, y) \) represents the field at distance \(-z_2\) from the detector plane after \( i \)th iteration. \( D \) is the mean value outside the object support on the \(-z_2\) plane. \( \beta \) is a relaxation parameter which is normally set to 2 or 3. As \( \beta \) increases, fewer iterations are required until convergence, but there will be less immunity to background noise.

4) Propagate the field back to the object plane by a distance \(+2z_2\). We now obtain a better estimate for the previously deleted region inside the object support and we have to make sure that the region outside the object support remains the same.

\[ U_{z_2}^{(i+1)}(x, y) = \begin{cases} 
U_{z_2}^{(1)}(x, y), & x, y \notin S \\
U_{z_2}^{(i+1)}(x, y), & x, y \in S 
\end{cases} \]

5) Repeat step 3 and 4 until the final image converges (i.e. the intensity values of \( U_{-z_2}^{(i)} \) do not change with the next iteration). The image usually converges within 10 to 15 iterations.
Method 2 is a Non-Interferometric Phase-Retrieval technique. This method does not treat the recorded image as a hologram but rather as an intensity of any diffracting optical field. It is applicable to any recorded images whether the holographic diffraction patterns dominate or not. This technique tries to recover the phase information of the object that is lost during recording process by iteratively propagating between the recording plane and the object plane separated by
distance $z_2$ and imposing known conditions. As the lost phase information is recovered, the twin image is eliminated.

1) Take the square root of the recorded hologram intensity in order to obtain the amplitude of the field at the detector plane.
2) Assuming the phase of the field at the detector plane to be zero as an initial guess, propagate the field to the cell plane at $-z_2$. Determine the object support by thresholding the intensity of the field at the object plane.
3) Replace the complex field values outside the object support by a background value while preserving the complex field inside the object support,

$$U_{-z_2}^{(i+1)}(x,y) = \begin{cases} 
  m \cdot D_{-z_2}(x,y), & x, y \notin S \\
  U_{-z_2}^{(i)}(x,y), & x, y \in S
\end{cases}$$

where $D_{-z_2}$ denotes the square root of the background intensity (the recorded hologram with the same setup in an absence of objects) propagated to the cell plane and

$$m = \frac{\text{mean}(U_{-z_2}^{(i)}(x,y))}{\text{mean}(D_{-z_2}(x,y))}.$$  

4) Propagate the field to the detector plane by a distance $+z_2$. The field now has a better non-zero phase value but the amplitude of the field should not change. Replace the amplitude of the field with square root of the original recorded hologram intensity.
5) Repeat step 2 to 4 until the final image at the cell plane converges. The image usually converges within 15 iterations.

Both methods were implemented in MATLAB® 2014a. Reconstruction quality for the two methods will be shown and compared in the next section.

3.3.4 Parallel Processing of Large Scale Image Reconstruction

Since each individual object in the hologram recorded with our lensless holographic microscope is formed by the local object scattered wave and the local reference wave, we argued that the recorded holograms can be divided into distinct blocks and these blocks are then processed by the phase-retrieval techniques in parallel in order to decrease processing time of the large-scale image (~5Mpixels). The MATLAB® function “blockproc” is used to facilitate this process. The blockproc function allows users to choose block size, border size, and padding method.
Operation time and effects of these parameter choices are discussed in the Results and Discussion section.

3.3.5 Tracking
As a second approach to implementing the lensless holographic microscope setup, we attempted to detect and track unstained BA/F3 cells inside our deterministic lateral displacement array devices. Since particle sizes are reflected in their lateral positions, the region of most interest inside a DLD device, which is toward the back of the array, is recorded. Holograms are recorded at the frame rate of 4 frames per second. In order to decrease processing time, cells are detected and tracked directly from the recorded time-lapsed holograms rather than from a movie of reconstructed images. The tracking process is decomposed into three main steps. First, we apply a temporal difference filter on the recorded time-lapsed holograms, filter out noise, and generate a movie of time-lapsed binary images of detected cells. MATLAB® code for this step is adapted from [13]. The difference filter calculates the mean of the first ten frames of the time-lapsed holograms and subtracts the calculated background from each recorded hologram in the movie. The difference images are enhanced in contrast with “imadjust” and smoothed by a Gaussian filter. After morphological operations such as “imdilate” and “imerode” are applied to further remove the noises with small areas, binary images are generated by thresholding. Any detected objects with area larger than 500 pixels are removed and assumed to be false positive objects. In the second key step, centroid coordinates of the detected objects are extracted from the generated binary movies. This step utilizes functions in MATLAB® Computer Vision System Toolbox [85]. “ForegroundDetector” computes a foreground mask from a background model trained with the first three initial video frames. “BlobAnalysis” is implemented to analyze any connected regions (blobs of detected objects) from the foreground mask in each frame and computes the centroids for those regions. The third step associates the detected centroids to the corresponding objects over time and generates tracks of centroids that belong to the same objects, superimposed on the original video. The code for this step is modified from the Multiple Object Tracking Tutorial by Student Dave [86]. In the original code, for each frame, a Kalman filter predicts the next position of the object from its previous position and predicted motion. A cost matrix then computes the distances between all previous positions, predicted positions, and detections on the current frame. The Hungarian algorithm assigns detections to the most likely tracks based on minimal distance between the detections and the previous positions. If the cost (i.e. distance) of
assigning a detection to a track is too high, then the predicted position gets assigned instead. Any detection that has not been assigned starts a new track. This is equivalent to a new object entering the region of interest. Any track with no new assignments will be removed after a certain number of frames as the object is assumed to have exited the region. Previous detections that belong to the same track are connected by lines and are shown connected to the current detection. The connected lines disappear once the object exits from the region of interest. We made two adjustments to the original code. The first adjustment is to take only the detections into consideration and ignore the predictions. The second adjustment includes direction of motion into account when the cost function is computed. Since we know \textit{a priori} that objects always travel inside the device in certain directions, we can assign an infinite cost to any unlikely directions of motion.

3.4 Results and Discussion

3.4.1 Image Reconstruction

\textbf{Small-Scale Reconstruction}

Polystyrene Beads

To test the performance of our lensless holographic microscope and validate the implemented phase-retrieval algorithms, a sample of 10 μm polystyrene beads sandwiched between two coverslips was imaged with our holographic microscope and with a conventional microscope (Zeiss Axiovert Z1) equipped with a 12-bit CCD camera (LA Vision Imager QE). Polystyrene beads were used as the first test target because they were spherical, representing a simple geometrical shape and the structure of suspension cells. Small-scale images (157x157 pixels equivalent to \(\sim 0.12 \text{ mm}^2\)) were cropped out from the full-scale recorded holograms (2592x1944 pixels equivalent to \(\sim 24.40 \text{ mm}^2\)) in ImageJ and were applied to the two phase-retrieval algorithms described in section 3.3.3 in MATLAB®. The full-scale recorded hologram showing three cropped regions of interest is illustrated in Figure 3-8. For each region of interest, the exploded views of the small-scale reconstructed images from Method 1 and Method 2, together with the 10X objective microscope image are shown for comparison. From the full-scale recorded hologram, we observed holograms of individual 10 μm beads as well as clusters of beads and dust particles. Holograms of individual 10 μm beads were distinguishable from the others because of their quantity and uniformity. The size distribution of the 10 μm bead sample was known from the manufacturer datasheet to be 9.606 ± 0.763 μm, which was considered to be
narrow as the standard deviation was equivalent to one third of the pixel size. With this information, we could expect the holograms of the individual 10\(\mu\)m beads to be uniform in fringe patterns and we could expect these patterns to be of large quantity in our full-scale recorded hologram. Three regions of interest in the full-scale hologram were cropped and reconstructed, using the two phase-retrieval methods separately. Each region contained two holograms of what we assumed to represent two individual 10\(\mu\)m beads. This assumption was validated qualitatively and quantitatively. Qualitatively, structures and arrangements of presumed 10\(\mu\)m beads in the 10X microscope images of same regions corresponded well to those in the reconstructed images from both methods. Furthermore, the two methods were able to reconstruct particles of smaller sizes and of unknown origins that were present in the microscope images. These small particles were estimated to be approximately 2.41 \(\mu\)m in diameter from manual measurement of particles in microscope images by using ImageJ. Quantitatively, mean particle sizes from six particles shown in the insets of reconstructed images were estimated from known pixel size of the CMOS to be 10.42±0.52 \(\mu\)m in Method 1 and 9.84±0.63 \(\mu\)m in Method 2. Compared to the bead manufacturer data, these estimates were well within two standard deviations of the mean. The estimates of bead diameters were calculated from the averages of the major axis lengths and the minor axis lengths of the individual beads after thresholding. The same threshold was used for each method. Mean particle size of the same six beads from the microscope images was computed from manually measured sizes in ImageJ to be 9.84±0.36 \(\mu\)m, corresponding to the estimates from the reconstructed images. Although bead size estimates from the reconstructed images subtly depended on the choice of threshold, we estimated and included mean particle sizes to confirm the coarse sizes of the beads. The bead size estimates together with microscope images validated the performance of our holographic microscope and of the two phase-retrieval methods for simple spherical particles.

There are a few noteworthy observations and constraints in our holographic microscope. First, currently our phase-retrieval algorithm only works for square images, requiring the recorded holograms to be cropped as a preprocessing step. Second, there are two parameters for our phase-retrieval algorithms that need to be manually observed and adjusted: the threshold value for generating the object support mask and the effective propagation distance between the object plane and detector plane. Unless stated otherwise, for all of the image reconstruction in this thesis, the effective propagation distance and the intensity threshold value were determined as
described below. Movies of reconstructed images at varying propagation distances are created to facilitate the process of determining the effective propagation distance. A montage from the movies of reconstructed images at varying propagation distances is shown in Figure 3-7. The propagation distances in this montage were from \( z = 0.42 \text{ mm} \) to \( z = 0.80 \text{ mm} \), with a step of 0.02 mm. Note that to optimize the time to check for the effective propagation distance of a certain sample, only the propagation algorithm was applied to the hologram at the recording plane without further processing. While the effective propagation distance was easier to be estimated visually with the movies of reconstructed images, we could still observe the trend of the object images being focused into smaller object support area as the propagation distance increased toward \(~0.58 \text{ mm}\) and being defocused into larger object support area as the propagation distance increased beyond \(~0.58 \text{ mm}\). Therefore, we visually determined the effective propagation distance to be \(~0.58 \text{ mm}\). This number corresponded to the expected distance equivalent to the sum of the sensor lid thickness (\(~0.4 \text{ mm}\) and the coverslip thickness (no. 1.5: 0.16 - 0.19 mm). The minimum propagation distance was typically set to \(~0.40 \text{ mm}\) since this was the distance between the CMOS sensor lid and the CMOS sensor active surface. The step increment could be set to larger values for coarse adjustment or to smaller values for fine adjustment as in the case of conventional microscopes. Future improvements for determining the effective propagation distance are discussed in the conclusions and future directions section.
Figure 3-7 Montage of the images propagated through varying distances from the recording plane where $z = 0$. As an example, the propagation distances in this montage were from $z = 0.42$ mm to $z = 0.80$ mm, with a step of 0.02 mm. Red rectangle indicates the visually determined effective propagation distance.

The default threshold value is set to be half of the average intensity. When the default threshold fails to generate a proper object support mask, the intensity values inside and outside the object are inspected and used to estimate the new threshold. For example, nonuniform background illumination was observed in Figure 3-8, where the right side of the frame was brighter than the other area. The same threshold value could be used for Region 2 and Region 3 that were next to each other and in the equally dim background. However, the threshold value needed to be increased for Region 1 which located in the bright background. Figure 3-9 shows 1D intensity plots across the centroids of the top and the bottom beads in the three regions of interest from the two methods as previously shown in Figure 3-8. Note that for comparison, the intensity values from Method 1 were square rooted to match the magnitude of the intensity values from Method 2. According to Figure 3-9, while there was more contrast between the bead and the background in the intensity plots from Method 2, the intensity plots reconstructed with Method 1 were more smoothened out enveloping the crooked plots from Method 2, particularly in background. Nevertheless, the shapes of the intensity plots from Method 1 corresponded to those of the plots from Method 2 and the two methods provided similar reconstructed images qualitatively and
quantitatively. Since phase-retrieval Method 1 and Method 2 could achieve similar reconstruction quality, we chose to use only Method 1 in future experiments and in all of the studies afterwards in this thesis. Method 1 was favored for not requiring the recording of a background image without a sample while the recording of the background image without sample was recommended for Method 2 to improve the reconstruction quality.

Figure 3-8 Full-scale hologram recorded with our holographic microscope showing three cropped regions of interest. The insets show small-scale reconstructed images from Method 1 and Method 2, compared to recorded images from 10X objective microscope.
Figure 3-9 One-dimensional intensity plots across the centroids of the top and the bottom beads in the three regions of interest from the two reconstruction methods as previously shown in Figure 3-8.

NIH/3T3 Cells

To further test the performance of our imaging system, we studied fine features of adherent NIH/3T3 mouse embryonic fibroblast cells. Fibroblast cells are typically morphologically heterogeneous and elongated in shape. Figure 3-10A depicts a 10X objective microscope image of two NIH/3T3 cells. The two NIH/3T3 cells had different elongated triangular shapes and
either two or three filopodia radiating from the main structures. The mean side length of the main triangular cell bodies of the two cells were 16.53 and 27.24 μm. The thin filopodia of both cells were approximately 1 μm wide. In the background, there were salt crystal-like residues that were believed to originate from the PBS. Figure 3-10B shows a holgram of the two NIH/3T3 cells recorded with our holographic microscope. From the hologram, we observed two main structures of cells without the legs. The background of hologram appeared to be noisy which was likely to be caused by salt residues present in the conventional microscope image. The reconstructed image from the cropped hologram of cells was illustrated in Figure 3-10C. Fine-featured filopodia were not present in the reconstructed image. The equilateral triangular structure of the cell at the top of the figure in the reconstructed image showed morphological resemblance to the corresponding cell in the conventional microscope image. Although the cell at the bottom of the reconstructed image was not similar to the one in the microscope image, the obtuse isosceles triangular structure of the cell in the reconstructed image was noticeable. The difference in reconstruction quality between the two cells was partly attributed to noise from undesired residues and partly to the generated mask for object support from the noisy hologram, which was able to cover the entire top cell but only a small region of the bottom cell (Figure 3-10D). Furthermore, the object support mask for structures with fine features was more difficult to generate compared to simple spherical structure. From visual observation of the processed images in a step-by-step running of the object support mask algorithm, we learned that after thresholding, the cell at the bottom of the image was detected as two separate objects with smaller areas. After more noise filtering, the top of the two small detected objects was completely removed while the bottom of the two small detected objects shrank. The separation of detected objects of the bottom cell could be vaguely observed from Figure 3-10C. The threshold value was selected to be on the stringent side of the spectrum as it was necessary to remove background noise.
Large-Scale Reconstruction

We have validated the performance of our imaging system with small-scale image reconstruction. Next, we are interested in studying how threshold values can affect size measurement, and determining the resolution of the our system. As we use the MATLAB® function blockproc to process large-scale images, we will also explore the effects of block sizes and padding methods on the quality of reconstructed images. These studies of interest require statistics from large data sets obtained from large-scale reconstructed image.

Effects of Threshold Values on Particle Size Measurement

From small-scale reconstruction studies, we observed that bead size measurements obtained processed images depend on the choice of threshold values. To investigate the nature of size measurement dependence on threshold values, we imaged sandwiched samples of 6 μm, 3 μm and 2 μm beads, respectively, with a conventional microscope and with our imaging system. The purpose of observation with the conventional microscope was to ensure equal distribution of particles in the FOV and not to compare images particle-by-particle between the two imaging modalities as in the small-scale studies. For the purpose of this study, large-scale images (450x450 pixels, equivalent to ~1 mm²) were cropped from the recorded holograms and applied to the phase-retrieval algorithm Method 1. Particles were detected with varying threshold values and particle diameters were calculated from the detected areas. All operations were performed in MATLAB®.
Figure 3-11 shows microscope images of the 6 \( \mu \)m, 3 \( \mu \)m and 2 \( \mu \)m beads at different magnifications. According to the images, it has been ensured that particles of interest were of significant quantity and were uniformly distributed.

<table>
<thead>
<tr>
<th>Diameter</th>
<th>2.5X Objective</th>
<th>5X Objective</th>
<th>10X Objective</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 ( \mu )m</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>3 ( \mu )m</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>2 ( \mu )m</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>

*Figure 3-11 Microscope images of 6 \( \mu \)m, 3 \( \mu \)m, and 2 \( \mu \)m beads with different magnifications.*

As previously mentioned, the diameters of beads in an image reconstructed from a recorded hologram were calculated from the areas of detected objects in the corresponding object support mask, which relied on the threshold values. Histograms of bead diameters for certain threshold values were generated to show the distributions of bead populations in diameters (bottom row of Figure 3-12 (6 \( \mu \)m), Figure 3-13 (3 \( \mu \)m), and Figure 3-14 (2 \( \mu \)m)). To calculate the mean diameters of the 6 \( \mu \)m, the 3 \( \mu \)m and the 2 \( \mu \)m bead populations in the reconstructed images, the two-term Gaussian model (also known as ‘gauss2’ in MATLAB®; the red curve in the bottom row of Figure 3-12 (6 \( \mu \)m), Figure 3-13 (3 \( \mu \)m), and Figure 3-14 (2 \( \mu \)m)) was used to fit the
histogram data and the mean diameter was estimated from the centroid of the peak of the Gaussian model with higher counts (vertical dashed lines in the bottom row of Figure 3-12 (6 μm), Figure 3-13 (3 μm), and Figure 3-14 (2 μm)). The gaussian model fit the peaks of the data curve and the number of terms determines the number of peaks we tried to fit. We used a Guassian model because we assumed the beads to have Gaussian distribution in size, which was often the case in nature. By visual observation, the two-term model was more robust and fit the histogram data better than the one-term model; therefore, a two-term model was used. Curve fitting was deemed necessary because according to the examples of histograms in the bottom row of Figure 3-12 (6 μm), Figure 3-13 (3 μm), and Figure 3-14 (2 μm), the distributions of bead diameters had the shape of Gaussian distributions, sometimes with a long tail or a cutoff on one side. We suspected that the detected objects with diameters on the extreme ends of the spectrum (long tail) might not reflect the beads of interest but was noise that was introduced in the recording and the thresholding processes. This noises was detected as objects that could alter the true mean of the bead diameter distribution. Therefore, to calculate the mean diameter from the histogram data, a curve fitting of a two-term Gaussian model would render the estimated mean diameter more robust to this noise.

Figure 3-15 plots the estimated mean diameters for varying threshold values from the reconstructed images of the 6 μm, 3 μm and 2 μm beads. With the known statistics of bead sizes from the manufacturer datasheets, we could calibrate the threshold values such that the estimated means of bead sizes from the histograms agreed with the means of the bead sizes from the datasheets. With this criteria, the suitable threshold value for the reconstruction of the 6-μm-beads image (manufacturer specification: 6.081μm±0.195) was 0.85 which corresponded to an estimated mean diameter of 5.91 μm. Note that the threshold values of 0.84 and 0.86 yielded the estimated mean diameters of 5.51 and 6.23. With similar criteria, for the 3 μm beads (manufacturer specification: 3.0±0.06 μm) and 2 μm beads (manufacturer specification: 1.93±0.05 μm), the suitable thershold values were 0.665 and 0.882, respectively.

Furthermore, as expected, linear trends between extract bead size and threshold value were observed, meaning the measured particle diameters increased as the threshold values increased. The reason for this has to do with how the size is extracted from the images. Reconstructed images were stored as intensity matrices. Pixels with higher intensities were brighter. In our
holographic microscope and other bright-field recordings, microscale beads were typically dark in the center and gradually get brighter as the pixels of the beads were further away from the center. Therefore, the measured particle diameters were expected to increase as the threshold values increased. Reconstructed images and particle diameter histograms of the 6 μm, 3 μm, and 2 μm bead samples, corresponding to three selected threshold values are shown in Figure 3-12, Figure 3-13, and Figure 3-14, respectively. Increasing detected bead areas and the calculated bead sizes resulted from increasing threshold values could be clearly observed from the white boundaries depicting detected outlines of the beads in the insets of, Figure 3-13, and Figure 3-14.

![Reconstructed images and particle diameter histograms](image)

Figure 3-12: Reconstructed images and particle diameter histograms of 6 μm bead sample, corresponding to three selected threshold values. The insets show exploded views of reconstructed bead images superimposed with (white) boundaries of detected beads as were set by the threshold values. The number of bins for the histogram is 40. The fitted curves on the histograms were calculated with the two-peak Gaussian model as explained in the text.
Figure 3.13 Reconstructed images and particle diameter histograms of 3 μm bead sample, corresponding to three selected threshold values. The insets show exploded views of reconstructed bead images superimposed with boundaries of detected beads as were set by the threshold values. The number of bins for the histogram is 40. The fitted curves on the histograms were calculated with the two-peak Gaussian model.

Figure 3.14 Reconstructed images and particle diameter histograms of 2 μm bead sample, corresponding to three selected threshold values. The insets show exploded views of reconstructed bead images superimposed with boundaries of detected beads as were set by the threshold values. The number of bins for the histogram is 40. The fitted curves on the histograms were calculated with the two-peak Gaussian model.
Spatial Resolution of the Holographic Microscope

In an attempt to measure the spatial resolution of our holographic microscope, we used image processing to calculate the minimum distance between two detected particles that were near each other and still distinguishable from each other. Two computational techniques were devised to compute the minimum distance (Figure 3-16). For the first technique, we computed the distance between centroids of two detected particles and subtracted it by the radii of the two particles. The minimum distance was regarded as the spatial resolution of our system. For the second technique, the spatial resolution was computed from the minimum distance between boundaries of two particles. For both techniques, particles of interest were constrained to detected particles of equivalent diameters between 0.67 and 1.33 times the mean diameter and of eccentricity less than 0.6. Eccentricity values range between 0 and 1, where 0 corresponds to a circle while 1 corresponds to a line segment. The two constraints were applied to ensure that the particles in this analysis were beads.

Reconstructed images of 6 μm and 4 μm beads were used in this study. As the first test case, we looked at two cropped regions (450x450 pixels) of the reconstructed 6 μm bead images. Data is not shown. We applied the two techniques to compute the minimum distance between any two particles and found that the minimum distances obtained from the two techniques were similar (3.97 and 4.06 μm for the first region; and 4.68 and 4.68 μm for the second region). Thus, we proceeded with the second technique alone. In the next study, large-scale reconstructed images (1800x1800 pixels, equivalent to 15 mm²) of the 6 μm and 4 μm bead samples were processed to compute the minimum distance between any two beads by calculating the shortest distance.
between the boundaries of two beads. Figure 3-17 shows a histogram of detected bead diameters of the reconstructed 6 μm bead images and the location of the minimum distance at three different magnifications. The blue asterisks in the figure depict the centroids of the two particles that were closest to each other and still were distinguishable from the other. The minimum distance was calculated to be 3.45 μm. Figure 3-18 shows similar results with the reconstructed 4 μm bead image. The minimum distance calculated from the 4 μm bead image was 2.12 μm. From this analysis, we determine that the spatial resolution of our holographic microscope was at least 2.12 μm, which was reasonable compared to pixel size of the CMOS sensor of 2.2 μm.

Figure 3-16 Two techniques used to compute the spatial resolution of our imaging system. The minimum distance of all distance values computed from the two techniques is regarded as the spatial resolution.
Minimum Distance for 6 um Bead Sample
Manufacturer Mean: 5.75 um

Our System
z = 1.4 mm
threshold = 0.85
Histogram Mean = 5.99 um
Minimum Distance = 3.45 um

Figure 3.17 Histogram of detected bead diameters of the reconstructed 6 μm bead images (top-right) and the location of the minimum distance at three different magnifications in the object support mask (middle row) and in the reconstructed image (bottom row).
Figure 3-18 Histogram of detected bead diameters of the reconstructed 4 μm bead images (top-right) and the location of the minimum distance at three different magnifications in the object support mask (middle row) and in the reconstructed image (bottom row).

Processing Time

MATLAB®’s distinct block processing (blockproc function) was applied along with the phase-retrieval Method 1 for large-scale image reconstruction in order to decrease the image reconstruction time. Blockproc operates by dividing an input image into blocks, applying a function to the distinct blocks, and piecing back the results from each block to form the output image. A parallel mode could be selected to allow MATLAB® to run in parallel mode, distributing the processing of each block to multiple MATLAB® sessions [87].

In order to compare the processing times of the reconstruction algorithm with and without blockproc, images of different sizes were cropped from the recorded 6 μm bead hologram and were input into phase-retrieval Method 1 with and without blockproc. Processing times with and without blockproc for image sizes of 750 pixels to 9000 pixels with step of 750 pixels were
recorded and plotted in Figure 3-19. The images were upsampled by a factor of five before being cropped. It is important to note that all cropped images were square, and the image sizes refer the side lengths of the squares. As expected, we observed a parabolic increase of the processing time as the image size increased for the image processed with blockproc, since the number of pixels in the image increased as the square of the pixel side length. The processing times without using blockproc were slightly smaller than the processing times using blockproc for image sizes smaller than approximately 1117.5 pixels. The processing times without using blockproc were slightly larger than those using blockproc for the image sizes between 1117.5 and 6000 pixels (the processing time without blockproc remained below 1.05X of the processing time with blockproc). At the largest image sizes of 6750, 7500, and 8250 pixels, the processing time without the use of blockproc leapt to approximately 2X, 10X, and 13X the processing time with blockproc. The processing times without blockproc for the image sizes of 9000 pixels were not recorded as the image was too large to be reconstructed without using blockproc. This study was performed with an Intel® Core™ i7 CPU unit.

Figure 3-19 Plots of operation times with and without blockproc for image sizes of 750 pixels to 9000 pixels with step of 750 pixels.
Effects of Padding Methods and Block Sizes on Quality of Reconstructed Images in Distinct Block Processing

In MATLAB®’s distinct block processing, there are a few parameters that, if changed, can affect the quality of the reconstructed images. Two parameters of interest are padding methods and block sizes.

One problem in the images reconstructed with blockproc was undesired fringes on the image border (Figure 3-20A). This was expected as the holograms were cut off at the border, and could be mitigated by using blockproc’s built-in Name-Value Pair Argument, ‘PadMethod’. The padding method determines how blockproc pads the image boundary [87]. Three options are available including ‘X’, ‘replicate’, and ‘symmetric’. ‘X’ pads the image boundary with scalar values, X. By default, blockproc pads image borders with zeros (X=0). For ‘replicate’ option, blockproc repeats border elements of the image. ‘Symmetric’ pads the image with a mirror reflection of itself. Figure 3-20B and Figure 3-20C show the reconstructed images using ‘replicate’ and ‘symmetric’ padding methods, respectively. Border fringes subsided in the Figure 3-20B and Figure 3-20C. We concluded that the border problems in the reconstructed images could be similarly suppressed with either replicate or symmetric padding methods.
To find the appropriate block size to be used in blockproc, we ran a few trials and observed that the choices of block size was important as it had an impact on the reconstruction quality. Block sizes refer to the size of the block. For the purpose of this analysis, we used only square blocks.
and block sizes refer to side lengths of the square blocks. Large-scale holograms (450x450 pixels) of 6 μm bead sample were reconstructed using blockproc with block sizes of 250, 450, 750, 1125 pixels. It is important to note that holograms were upsampled by factor of 5 and became 2250 pixels by 2250 pixels before blockproc was applied; therefore, the block sizes were specified after upsampling. Figure 3-21 shows the resulting images that were reconstructed with different block sizes. Discontinuities of intensity were observed at the borders of each block in all reconstructed images. Because there were a larger number of blocks in the reconstructed images with block sizes of 250 and 450 pixels, more discontinuities were apparent. Also, image reconstruction of beads near the border was not complete. This was observed from fringe residues that remained in the reconstructed images for all the block sizes. Furthermore, the reconstructed bead image with 250 pixel block size showed less contrast between the beads and the background than the other block sizes, which was not desirable. This effect was likely caused by the fact that, for a particular bead inside a block, there was not enough coverage of the defocused image of the bead within the 250 pixel block size. Therefore, some necessary pieces of information about the bead would not be taken into account in the reconstruction inside this particular block. This effect could be reduced by adding border pixels to each block in order to provide the missing information, but it would be at the cost of larger effective block size and increasing processing time. In the case of 1125 pixel block size, fringe patterns were observed inside each distinct block but they were not prominent in the other cases. This may be caused by the fact that larger blocks contained more beads; therefore, there was more interference between the hologram of each bead in the same block, reducing the effectiveness of the phase-retrieval algorithm. It seemed that we needed a block size that was not too large or too small such as 450 and 750 pixel block size in order to cover enough area for reconstruction and to provide qualitatively acceptable images. In order to choose between the acceptable 450 and 750 pixel block sizes, the processing time and the discontinuities between blocks of the two block size choices were considered. The processing times to reconstruct a 2250x2250 pixel image with 450 and 750 pixel block sizes were 35.95 seconds and 38.17 seconds, respectively. If we quantified the discontinuities between all the blocks by the length of the all the borderline between those blocks, for a 2250x2250 pixel image, there would be 4 times as much discontinuities in 450 pixel block size than 750 pixel block size. The fewer discontinuities between blocks in 750 pixel block size outweighed the 6% lower processing time for the 450 pixel block size. Thus, a block size of
750 pixels was selected and used throughout this thesis where distinct block processing of large-scale images were performed since it resulted in acceptable image reconstruction quality.

Figure 3-21 Reconstructed images of 6 μm beads as the input block sizes vary. The block sizes refer to side lengths of the square blocks.

Reconstruction of Microwell Devices

Besides bead and cell samples, we were interested in testing the performance of our imaging system with large microscale structures, specifically the features of PDMS microfluidic devices. Joseph Kovac’s microwell device [88] was imaged with our holographic microscope. A microwell device was selected as a test target representing PDMS microfluidic devices because it contained arrays of simple cylindrical wells of varying sizes. Although these structures were negatives of each other, imaging of microwells could offer some insights into how cylindrical posts in a deterministic lateral displacement device would look like. Patched microscope images and the reconstructed
images of the microwell device are shown in Figure 3-22.

Figure 3-22.
Figure 3.22 Patched microscope images and the reconstructed images of the microwell device.
Although all the main well structures were observable, the edges of all the microwells appeared to be blurry in the reconstructed images, deteriorating the images of the smaller wells specifically. The number labels along the side of the microwells were decipherable in the reconstructed images. The microscope image, the recorded hologram, and the reconstructed images showing an exploded view of the device labels are illustrated in Figure 3-23. As expected, the images of holograms with more crowded features (small microwells) were harder to reconstruct that those with more empty spaces (large microwells and labels). This was because of one of the constraints of a holographic imaging system, which is that the object needs to be weakly scattering so that the holographic diffraction pattern will dominate over the classical one.

![Figure 3-23](image)

*Figure 3-23 The microscope image, the recorded hologram, and the reconstructed images showing two exploded views of the microwell device.*

### 3.4.2 Tracking

As a second approach to implement a holographic imaging platform, we attempted to track BA/F3 cells as they traversed across a microfluidic platform based only on the raw unreconstructed holograms. In this study, we imaged a region in a microfluidic platform where a
deterministic lateral displacement device adjoined another microfluidic channel (Figure 3-24). For the purposes of this study, we restricted the scope of the analysis to investigating whether tracking of cells by tracing the cell holograms recorded with our holographic imaging setup was possible. To emphasize, a tracking algorithm would be applied to the time-lapse holograms without image reconstruction. As an example of resulting trajectories and to point out the errors generated by the tracking algorithm, the first 12 frames, which contained 3 real tracks, were taken from an 88-frame video, which contained 6 real tracks in total, in order to create a montage. The montage of the cell trajectories detected with our tracking algorithm is shown in Figure 3-25. This montage was generated from movie frames which were recorded at 4 frames per second. That is, each frame was recorded at 0.25 seconds after the previous frame. The cells were expected to travel from the bottom left to the top right inside the channel of the device. The montage depicts a few flaws in our tracking algorithm that need to be improved. Two of the main problems were false positive and false negative detection of cells. In the second frame, for example, one of the two detections was a false positive. This false positive persisted through the third frame. Because the false positive in the third frame was closer to the detected cell in the fourth frame than the correct detected cell in the third frame, the detected cell in the fourth frame was assigned to the false positive, generating a false positive trajectory. False negative (missing a true cell detection) was most apparent in the twelfth frame (3.00 seconds), where the cell exiting the frame on the top right of the device was not detected and that cell’s trajectory disappeared before expected, unlike the green track which represented a cell gradually travelled through the straight channel adjacent to the curved channel before it disappeared. False positive problem occurred partly because our detection algorithm required the first few frames as training frames. Therefore, the algorithm might not have been trained well and might not function properly in the first few frames. False negative problem most likely arose from the stringent noise filters, which were necessary for reducing the false positives. Nevertheless, generated trajectories in the fifth frame to the eleventh were accurate compared to manual visual tracking. This example montage was chosen as it reflected both the success and the weaknesses of the algorithm in small number of frames. The tracking algorithm was quite robust as similar results were obtained from running the algorithm with three videos of 320 frames and more than 20 real tracks. The detection algorithm in the tracking process may still need some improvements but we were able to show that tracking of cells with time-lapsed holograms recorded with our lensless holographic imaging
setup was promising. Future improvements to the tracking algorithm will be discussed in the Conclusions and Future Directions chapter.

Figure 3-24 Schematic of a microfluidic platform. The red rectangle depicts the region recorded by the CMOS sensor.

Figure 3-25 Montage of the BA/F3 cell trajectories in a microfluidic platform detected with our tracking algorithm. The red circles depict the current location of the cells while the colored line segments represent the cell trajectories. The vertices of the colored line segments represent all detected previous locations of the cells. The line segments disappeared once the cell of that trajectory left the frame.

3.5 Conclusions

In this chapter, we have implemented a lensless holographic microscope introduced by [80] and we have measured its imaging performance by qualitatively comparing small-scale reconstructed images of static samples of size-varying polystyrene beads and adherent NIH/3T3 cells to the corresponding microscope images. Parallel block processing was implemented to improve the
computation time of the phase-retrieval algorithm for large-scale images. With the large-scale reconstructed images and larger sample size of bead data, we were able to study the effects of thresholding on the measured particle sizes as well as determine the spatial resolution of our system. We have also optimized the choices of the built-in parameters such as block sizes and padding methods of the MATLAB® blockproc function to improve the reconstruction quality of our images. In addition, large-scale image reconstruction has been performed with recorded holograms of a PDMS microfluidic device and the main features of the microfluidic device were visible. It was with the large-scale reconstructed images that the true potential of a lensless holographic microscope was revealed. Finally, we investigated and demonstrated the feasibility of tracking cells in a microfluidic platform by tracing cell holograms.
Chapter 4 : Conclusions and Future Directions
This thesis illustrates the possibility of an integrated platform consisting of a microfluidic device, the deterministic lateral displacement (DLD), and a lensless imaging system, the partially coherent digital in-line holographic microscope (DIHM). We have modelled, fabricated, and tested the DLD as well as implemented and validated the DIHM system. After the DLD and the DIHM system were separately tested, they were partially integrated at the end of Chapter 3 where we tracked particles in a DLD device from time-lapsed holograms recorded with the DIHM system. In this chapter, we discuss the improvements that could be made to the DLD and the DIHM system. We conclude with the future directions toward the envisioned intrinsic cytometry.

4.1 Size Separation by Deterministic Lateral Displacement
For the purpose of intrinsic cytometry, an accurate, reusable, clog-free size-separating device is desired. We have shown that our fabricated DLD device could perform size separation of beads and cells with accuracy of 100% in 6 out of 9 runs and with up to 11.3% of unexpected trajectories in 3 out of 9 runs. The recorded movies of particle trajectories presented evidence that the non-idealities arose from undesired factors such as clogging or particle-particle interactions, and not from the accuracy of the critical diameter calculation. The main challenge that our DLD design encountered in Mask Design No. 1, and to a lesser extent in Mask Design No.2, was clogging of particles over time, especially at the front of the micropost array for all particles and in the back of the array where large 15 µm beads were expected to go through the gaps.

Clogging, which occurred at different locations and with different degrees of severity, had different undesirable effects on the functionality of the device. At the low spectrum of severity, as in the case when a few particles attached themselves to the PDMS micropost wall without fully stopping the flow through the gap, it could alter the local flow profile of the clogged sites, potentially changing the effective critical diameter locally in the nearby regions and reducing the accuracy of the size separation. In our current integrated platform, the FOV of the CMOS sensor would not allow observation of the active DLD arrays, but only the readout of the size measurement from the lateral exit positions of the particles if one wanted to track particles across at least two intrinsic-property modules. If clogging took place inside the active area of the DLD
and was not detected, the size results derived from the lateral exit positions could be inaccurate. To a more severe degree, clogging at important locations such as the sample inlet bottleneck or the front of the micropost arrays could obstruct the flow and deviate the particle trajectories, or worse, physically prevent the particles from entering and exiting the device altogether. This would significantly hinder the expected size separation of particles and result in reduced recovery. Furthermore, clogging compounds over time, as clogging either to a small or to a severe degree could lead to more clogging.

One approach to minimize clogging and the inaccuracy issues arising from clogging that has been mentioned in the literature is to change the shape of the microposts [78], [89]. As mentioned in section 2.2.3, circular posts contain large surface area where the surrounding flow becomes zero in velocity, trapping the incoming particles. Different post shapes such as triangular and streamlined may be implemented instead of circular, as the post surface area with surrounding zero-velocity flow reduces. Another approach, which might be less effective overall, is to increase the number of micropost rows for the smallest threshold at the front of the array. This is to ensure that particles smaller and larger than the first threshold are separated to a great extent even if clogging takes place at the front of the array. Increasing the number of rows at the front of the array will reduce the effect of clogging on the entering lateral positions of the particles and contribute to larger lateral separation of particles during the first threshold section.

4.2 Lensless Digital In-Line Holographic Microscope

Computational imaging is a necessary tool for our integrated platform because it can provide images of a large-scale region from one recording without mechanical scanning. The selected DIHM system implemented with the CMOS sensor has a FOV of tens of square millimeters, and is estimated to enable the optical reading and tracking of particles on an integrated microfluidic platform across up to two sensor modules. Since the FOV of the system is equivalent to the active area of the optoelectronic sensor array, the FOV can be easily improved to square centimeters by replacing the current CMOS sensor with an appropriate large-format sensor. A square-centimeter FOV is estimated to allow at least three sensor modules of the microfluidic platform to be observed. As the light intensity is inversely proportional to the square of the distance, another improvement to the imaging setup that would be required for an increase in the FOV is the power of the light source. Currently, the light source of our imaging setup is a yellow LED butt-coupled to a pinhole. The LED has a large viewing angle of 60 degree, which means
that only a limited portion of the generated light pass through the pinhole and an unnecessarily large portion radiates elsewhere. As a preliminary step, a lens may be inserted between the LED and the pinhole to collect more light from the primary source and focus it through the pinhole. If that change does not provide sufficient light intensity through the pinhole and onto the CCD array, then a different primary source such as a laser diode or a more powerful LED with narrow viewing angle is needed. An increase of light intensity from the source (i.e. the reference wave) asserts the dominance of the holographic diffraction patterns over the classical diffraction patterns, increasing the signal to noise ratio of the holograms. Furthermore, as the light intensity from the source increases, the time required for integration of the light intensity over each pixel is reduced, increasing the upper limit for recording frame rates.

In our first approach to DIHM, static images of beads, cells, and a microwell device were reconstructed from the recorded holograms with phase-retrieval algorithms and compared to conventional microscope images. We have implemented two phase-retrieval techniques and continued using only one of them as comparable reconstructed images from the two techniques were obtained. Distinct block processing was implemented with the chosen phase-retrieval algorithm to reconstruct large-scale images with reduced operation time. There are a few improvements that can be made to the phase-retrieval algorithm. Currently, the algorithm only takes square images as input and the other two input parameters, \( z_2 \) and threshold values have to be manually fine-tuned. Changes can be made to the codes such that rectangular images are acceptable as input as well, and the holograms would not have to be cropped before reconstruction. The \( z_2 \) is approximated from the auto-generated movie of holograms propagating through different potential \( z_2 \) distances. Subjective visual judgement of object sharpness in the movie frames is required to determine \( z_2 \) where the object image appears to be most focused. This process can be automated if the object is a pure amplitude or a pure phase object, as has been theoretically proven [28]. For a pure amplitude object, only the optical field at the focal plane is real and the sum of the absolute values of the complex image array is minimum when the field is in focus. For a pure phase object, the optical field has a constant magnitude across the frame at the focus and the sum of the image array is maximum. The threshold values for creating the object support mask are adaptive to the average image intensity. When adaptive thresholding is applied with distinct block processing, the threshold values are adaptive to the average intensity within the block. This local thresholding performs well with nonuniform illumination.
background. However, visual judgement is still required as sometimes objects of interest are not properly detected and an inaccurate object support mask can severely deteriorate the quality of the reconstructed image. Other object detection may be improved with more sophisticated detection techniques such as feature-based detection or cascade training detection [90], [91].

As our second approach to large-area imaging, trajectories of suspension BA/F3 cells inside a microfluidic platform were extracted from the recorded time-lapsed holograms with no image reconstruction. We have shown that tracking without image reconstruction was possible because the cell holograms were detectable. The tracking algorithm was implemented with three key steps as discussed in Chapter 3: object detection, centroid extraction, and track assignment. For object detection, a difference filter was applied to the time-lapsed holograms to detect any changes from frame to frame. The difference image output was noisy with salt and pepper noise and with noise due to flow fluctuations. To minimize these noise sources, filters with stringent criteria, e.g. size and intensity thresholds, were applied to the difference images, resulting in missed detections (false negatives) in a few cases. Although missed detections were not desirable, they were preferred over false positive detections, which could seriously deteriorate the assignment of tracks. For centroid extraction, a few first training frames were required to train the extraction algorithm. The consequence of this requirement was that centroid extraction for the first few training frames might not be accurate. This issue could be avoided by recording movies of cells with large number of frames so that the results in the first few frames could be neglected. For track assignment, the detected objects from the current frame were assigned to the detected objects from the previous frame based on a minimum distance between objects of the two frames. Two constraints that were ranked above the minimum distance assignments were the likely direction of motion and the maximum possible distance. This algorithm performed well when compared with visual judgement except in the case where false positives were detected that were closer to the previous object location than the true current position of the object, leading to incorrect assignments. This problem could be minimized if the false positives were minimized.

4.3 Future Directions of the Intrinsic Cytometry
The envisioned intrinsic cytometry takes a complex heterogeneous cell sample as an input and reads out multiple intrinsic properties of individual cells as the output. With the intrinsic cytometry as an interrogatory tool, multiple intrinsic properties of individual cells can be investigated and used to potentially form a diagnostic database to help identify the target cell
types in a complex mixture. Our proposed approach incorporates the advantages of two
developed systems to form an integrated intrinsic cytometry platform: the microfluidic system
and the DIHM system. Microfluidic systems can extract information about intrinsic properties of
cells and encode that information into the relative positions or the positions relative to time,
which can be optically read out and computationally decoded. The DIHM system is a tool to
monitor cell position relative to time inside the microfluidic system and read out that encoded
information on a large scale.

In this thesis, a prototype was made to probe the plausibility of our proposal. We have shown
with a conventional microscope that a DLD device could extract size information of cells and
encode the information into the form of relative lateral positions. We have also shown that the
implemented DIHM system could track positions of individual cells over time inside a
microfluidic platform. Although we have yet to decode the size information from the tracked
positions of cells with the DIHM system, we believe this processing step to be straightforward
and we hope to show that our proposed approach for making an intrinsic cytometer is plausible.
We estimate that up to two sensor modules for the microfluidic platforms can be monitored with
the current DIHM configuration and can be extended to three or more modules if small changes
are made to the DIHM system. In future work, we will incorporate an isodielectric separation
technique [11] into our microfluidic platform to extract the intrinsic polarizability information of
individual cells and encode it in the relative lateral positions. The polarizability and the size
information of individual cells will be decoded and linked by the tracking algorithm in our
DIHM system. Data of the two intrinsic properties of the target and non-target cell samples will
be collected to form a database and we will evaluate if these two key intrinsic properties are
sufficient to identify the target cell types from a complex mixture of sample or what other
intrinsic parameters may be needed to utilize the information from this untapped area.

4.4 Contributions

Microfluidics have been integrated with the traditional digital in-line holography and the large-
FOV digital in-line holography, mostly to facilitate sample transferring in and out of the
observation areas. Rarely has microfluidics been integrated with DIHM for its full functionality,
namely particle separations. In this thesis, we have demonstrated observation of size separation
and tracking of particles in a microfluidic deterministic lateral displacement array by a large-
FOV DIHM system from raw time-lapsed holograms without image reconstruction. The large-FOV DIHM allows us to investigate particle separation in a field of view that is 10 times larger than the 10X objective of the conventional microscope. DLD extracts particle size information and encodes it into relative lateral position. A large FOV can be useful for designing a DLD with larger lateral dimension, either to increase the lateral separation distance between critical dimensions or to enhance the size separation range, both of which can improve the separation performance of the DLD. More generally, a large FOV is useful for designing any microfluidic device that laterally separates particles based on their intrinsic properties and does not have restrictions on the device’s lateral dimension.

This work also proves that our approach to implement the envisioned intrinsic flow cytometry is plausible. The system has the potential to provide an even larger FOV if we substitute the current CMOS sensor with a larger-format optoelectronic sensor array. Although future works are required to demonstrate, an observation system with ~2 μm resolution and a large FOV is promising to be implemented with an integrated microfluidic platform since a larger area of the platform at any point in time can be monitored with a single recording, without mechanical or optical scanning. This benefit provides us with a large footprint for the designing of the integrated microfluidic platform, which is not possible with the conventional microscope.
Appendix A: Fabrication Process Flow
Microfluidic Channel Pattern on 6” Silicon Wafer
Starting Materials:
- 150-mm-diameter, 650-μm-thick Silicon wafers (WaferNet, Inc., San Jose, CA)

Photomask: Front Range Photomask, 7”x7”, .12” thick (~3.05mm)

Facility: MIT Technology Research Lab

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Machine</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Dehydration bake</td>
<td>Hotplate 300</td>
<td>200°C, 30 min</td>
</tr>
<tr>
<td>1</td>
<td>Adhesion Layer</td>
<td>SU-8 spinner</td>
<td>SU-8 2002(2 μm thickness), Dispense ~6ml SU-8 (1ml per inch diameter)</td>
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<td></td>
<td></td>
<td>Hotplate 2 (95°C)</td>
<td>Hold at 95°C for 1 min; cool to ambient</td>
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<td></td>
<td></td>
<td>EV1</td>
<td>Flood exposure for 10 seconds</td>
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<tr>
<td></td>
<td></td>
<td>Hotplate 2 (95°C)</td>
<td>Hold at 95°C for 2 min; cool to ambient</td>
</tr>
<tr>
<td>2</td>
<td>SU-8 Spin</td>
<td>SU-8 spinner</td>
<td>SU-8 2015(20 μm thickness): Dispense ~6ml SU-8</td>
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<td></td>
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<td></td>
<td>Ramp to 500 rpm at 100 rpm/sec acceleration and hold for 5-10 seconds</td>
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<td></td>
<td></td>
<td>Ramp to 2250 rpm at 300 rpm/second and hold for total of 30 seconds</td>
</tr>
<tr>
<td>3</td>
<td>Prebake</td>
<td>Hotplate station</td>
<td>Slow ramp from 60°C to 95°C, hold at 95°C for 4 min; cool to ambient</td>
</tr>
<tr>
<td>4</td>
<td>UV expose</td>
<td>EV1</td>
<td>Flow chamber mask, 10 seconds. 30 μm separation</td>
</tr>
<tr>
<td>5</td>
<td>Post-expose bake</td>
<td>Hotplate station</td>
<td>Slow ramp from 60°C to 95°C, hold at 95°C for 5 min; cool to ambient</td>
</tr>
<tr>
<td>6</td>
<td>Develop</td>
<td>SU-8 hood</td>
<td>~ 3-5 min soak in PM Acetate from Pass-Thru</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spray with PMA and IPA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dry with Nitrogen gun</td>
</tr>
<tr>
<td>Step</td>
<td>Task</td>
<td>Location</td>
<td>Instructions</td>
</tr>
<tr>
<td>------</td>
<td>------</td>
<td>----------</td>
<td>-------------</td>
</tr>
<tr>
<td>7</td>
<td>Hard bake</td>
<td>Hotplate 150°C</td>
<td>Leave the SU-8 in the hotplate for ~30 minutes up to 2 hours. The purpose is to make remaining solvent evaporate.</td>
</tr>
<tr>
<td>8</td>
<td>Silanize</td>
<td>Acid/Base Hood</td>
<td>Put 3-4 drops of trichloro octadecl silane into aluminum foil cup in vacuum jar. Put wafer in wafer holder in the jar. Close jar, turn on vacuum for 5-10 min. Turn vacuum off, let it sit for ~30 min.</td>
</tr>
<tr>
<td>9</td>
<td>Mix and degas PDMS</td>
<td></td>
<td>Weigh the PDMS (Polydimethylsiloxane, Sylgard 184) pre-polymer components in a 10:1 ratio. Mix well. Utilize approximately 70 grams of the PDMS. Degas under vacuum (desiccator) until no bubbles appear in the mixture (20-30 minutes). Make sure the PDMS mixture does not foam out of the container.</td>
</tr>
<tr>
<td>10</td>
<td>Pour PDMS</td>
<td></td>
<td>Place the SU-8 master wafer into aluminum foil. Carefully pour the PDMS into the foil to minimize bubbles.</td>
</tr>
<tr>
<td>11</td>
<td>Cure PDMS</td>
<td></td>
<td>Cure the PDMS on a hotplate at 80°C for 2 hours. After curing, the wafer with the PDMS is stable and may be stored for months.</td>
</tr>
<tr>
<td>12</td>
<td>Remove the mold</td>
<td></td>
<td>Carefully peel off the PDMS mold from the SU-8 master, and cut out individual devices with a razor.</td>
</tr>
<tr>
<td>13</td>
<td>O₂ plasma glass slide and PDMS mold</td>
<td></td>
<td>Clean glass slide and PDMS mold to be bonded with tape. Place a clean glass slide, and the PDMS mold with the surfaces to be bonded facing up in the plasma chamber. Turn on a magenta-colored “air plasma” and hold for about 30 seconds.</td>
</tr>
<tr>
<td>14</td>
<td>Assemble devices</td>
<td></td>
<td>Place the glass slide on a clean surface. Carefully place the PDMS onto the glass slide starting on one edge and as to avoid trapping air bubbles. Press down on the PDMS to ensure a good bond to the glass.</td>
</tr>
</tbody>
</table>
Appendix B: DLD Experiment Protocol

Materials:

1. Assembled DLD device
2. Two to five 10 ml plastic syringes (Becton, Dickinson and Company)
3. Two 1 mL plastic syringes (Becton, Dickinson and Company)
4. Tubing (Tygon®, 0.020-inch inner diameter)
5. Adapters for tubing and syringes
6. Five 25 mm syringe filters (Pall Corporation, 0.2 µm Tuffryn® membrane)
7. Solution of polystyrene microspheres or cells

Sample preparation

- Microsphere sample
  8. Dispense 100 µL bead solution from bottle into a 1 mL Eppendorf with 900 µL of 0.1% tween® 20 DPBS solution. Keep track of bead concentration. The proportion of beads to buffer can be adjusted according to bottle concentration. Vortex bead container before dispensing.
  9. Centrifuge the Eppendorf according to bead size.

<table>
<thead>
<tr>
<th>Bead Diameter (µm)</th>
<th>G – Force (cm/s²)</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>594</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>334</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>120</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>53</td>
<td>2</td>
</tr>
</tbody>
</table>

10. Carefully drain the liquid until only the beads are left in the bottom.
11. Dilute the washed solution to the final concentration of 10⁵ to 10⁶ particles/ml.
12. Take 100 µL out for coulter counter analysis.

- Cell sample
  9. Load cells suspended in cell culture media into a 15 mL Falcon tube.
10. Centrifuge at 1500 rpm for five minutes. In the meantime, thaw fluorescent stain, Calcein AM, in water bath for 2 minutes.
11. Gently drain the media out of the Falcon tube without discarding the cells.
12. Dispense 1 mL of DPBS-T and 1 µL of thawed Calcein Am into the tube. Vortex.
13. Wrap the tube with aluminum foil to prevent exposure to light. Wait for 30 minutes.
14. Dispense a few drops of sample onto a glass slide for observation under microscope.
15. Take 100 µL out for coulter counter analysis.

Device Pre-conditioning:

- Microsphere sample
  5. Load a 10 mL syringe with 0.1% Tween® 20 deionized water. Connect it to a filter, tubing, and metal inserts. Clamp it into place on a syringe pump.
  6. Let the pump run at 5-10 µL/min until you see droplets forming at the ends of the tubing. Plug the metal inserts into outlets of the device, merging the droplets from the tube with
droplets over the outlets. For Mask Design 1, load into one of the outlets. For Mask Design 2, load from both outlets.

7. Position and secure the device under the microscope (Zeiss Axio Imager M1M) equipped with a 12-bit CCD camera (LA Vision Imager QE) and fluorescent light source (EXFO X-Cite® 120)

8. Observe. Increase the flow rate for faster bubble elimination. Let the priming solution fill inside the device for 20 minutes.

- Cell sample
  Similar to microsphere sample but prime the device with ethanol, DI water, and 1% bovine serum albumin (BSA) solution in this order. For the ethanol, flush the device until bubbles disappear. Then flush with DI water to get rid of ethanol traces and flush with BSA for 20 minutes.

Device Set-up:

5. Load sample into a 1 mL syringe and load filtered DPBS into two 10 mL syringes. Connect to filters (only the DPBS syringes), tubing, and metal inserts. Clamp it on syringe pumps.

6. Let the pump run at 5-10 µL/min until you see droplets forming at the ends of the tubing. Stop the pump. Plug the metal inserts into outlets of the device, merging the droplets from the tube with droplets over the outlets.

7. Run the syringe pumps (For Mask Design 1, 2.5, 0.5, and 1.25 µL/min proportional to the corresponding inlet channel widths. For Mask Design 2, 5µL/min for the sample and 15 µL/min for the buffer). Set the sample syringe pump such the syringe is vertically pointing the ground. Observe and adjust flow rates if needed.

8. Unless indicated otherwise, fluorescent images in the DAPI, FITC, and TRITC channels are recorded at 100 µsec, 1 msec, and 1 msec exposure time respectively.

Clean-up:

3. When the experiment is finished, disconnect the syringes from the device.

4. Flush out the plastic syringes a few times before throwing them away. Keep the metal inserts for future use.
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