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Optical Systems for Integration with Microfluidics

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

Doctor of Philosophy

in

Electrical Engineering (Applied Physics)

by

Jessica M. Godin

Committee in charge:

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2010
The dissertation of Jessica M. Godin is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2010
Dedication

This dissertation is dedicated to my family.
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Publications


My thesis research has focused on means of integrating optical systems into microfluidic chips, specifically for the creation of lab-on-a-chip flow cytometers. The benefits of microfluidics are perhaps most often applied to biological assays, which frequently employ optical readout of fluorescence or light scatter. By integrating the optical system onto the microfluidic chip, we can facilitate chip interfacing while ensuring optical alignment to a tiny sample. Integrated optical systems also offer the ability to collect light from a localized area, allowing for the collection of true angular light scatter (which carries much information about cells) and can furthermore significantly improve the signal to noise ratio (SNR) relative to simple fiber or
waveguide based approaches to integrated light collection. This work explores both
the unique challenges and advantages encountered when creating optical systems
integrated with mold-replicated microfluidic devices.

The first contribution presented is the demonstration of fluid-filled lenses
integrated alongside microfluidic channels using a slab waveguiding structure. The
use of fluid represents an important tradeoff between lens power and Fresnel
reflections. The creation of a slab waveguiding structure is critically important to
control light losses when utilizing lens systems for light collection.

The second contribution in this work is the demonstration of a microfluidic
chip employing a number of lenses to perform both localized excitation of the samples
as well as light collection from localized areas defined by a specific angular range.
Sample coefficients of variation (CVs) ranged from 9-16% for a single bead
population, far exceeding previously-published CVs of 25-35%.

The last contribution is an atypical approach to optical systems based on the unique
advantages offered by microfabricated architectures, namely small sizes and close
proximities to the sample. Using only custom-shaped total internal reflection (TIR)
based components and light blocking elements, we create a device that can achieve
forward light scatter CVs of 8-28%. The device is able to clearly distinguish 5 μm, 10
μm, and 15μm beads based on forward and side scatter. The results from this vastly
simplified optical system show great promise towards reaching the performance
metrics of the commercial cytometer.
Chapter 1

Introduction

Flow cytometry is based on the optical interrogation of individual samples in fluid flow, making it an ideal test bed for miniaturized optofluidic technologies. Lab-on-a-chip flow cytometry has been under development for more than a decade. While numerous advances have been made towards reliable flow control and optical detection, much work remains to be done. In this work, I present the development of integrated optical systems that can be easily fabricated alongside fluidic systems. Two main approaches will be highlighted: integrated lenses and the use of an exclusion-based approach to optical design. Results from flow cytometry chips incorporating each of these systems are benchmarked against commercial devices and compared to similar devices in the literature. Strong advances will be demonstrated towards the development of a robust, easily-integrated optical system for light scatter detection.
1.1. Microfluidics

The field of microfluidics is poised to revolutionize biomedical research and healthcare by enabling a wide range of low-cost, portable, and/or highly automated devices. A number of excellent resources exist for in-depth discussion of the field of microfluidics [1-2], so only a brief overview of some relevant matter will be covered here. In general, microfluidics leverages the fabrication technologies of the microelectronics industry to create compact devices with tiny, tightly-controlled features. These small features, in turn, enable tight control over what goes on within the microfluidic chip. The Reynold’s number of flow in a pipe is defined as the ratio of inertial forces to viscous forces, and is defined as

\[ R_e = \frac{\rho V^2}{\mu V} = \frac{\rho V L}{\mu} \]  

(1.1)

where \( \rho \) is the fluid density, \( \mu \) is the dynamic viscosity of the fluid, \( V \) is the average fluid velocity, and \( L \) is a characteristic linear dimension (in the case of a square pipe, the pipe diameter). The Reynold’s number can be rewritten as

\[ R_e = \frac{QD_H}{\nu A} \]  

(1.2)

where \( Q \) is the flow rate, \( \nu \) is the kinematic viscosity \( \left( \frac{\mu}{\rho} \right) \), \( D_H \) is the hydraulic diameter of the pipe (four times the cross-sectional area divided by the perimeter of
the pipe), and $A$ is the cross-sectional area of the pipe. The resulting dimensionless number indicates whether the flow tends to be dominated by viscous forces (i.e. laminar flow) or inertial forces (i.e. turbulent flow). The laminar flow regime is generally in the realm of $Re<2000$, while the turbulent flow regime is roughly $Re>4000$. Microfluidic channels (especially those presented in this work) tend to have channel dimensions on the order of $100 \mu m$. The Reynolds’s number for such devices indicates that flow will be laminar at flow rates even above $10 \text{ mL/min}$; that is, laminar flow is all but guaranteed in microfluidic devices.

The benefits of laminar flow include diffusion-only mixing and predictable flow. Such effects have a number of implications for the creation of devices that can manipulate small volumes of fluid in a highly-controlled manner. As a result, the field of microfluidics has exploded in the last decade. Integrated chips have been demonstrated that can pump liquids [3], repeatably and reliably mix fluids [4], culture cells [5], and even perform digital logic operations[6-9].

In addition to laminar flow, microfluidic platforms offer the benefits of small-volume usage. Both mass transfer and thermal transfer will occur much more rapidly on such small volume scales, allowing their use as passive means of altering a system. Thermal cycling or diffusion of nutrients for cell growth and signaling, for example, are more readily performed on such scales. In addition, many assays, both for clinical testing as well as basic research, currently require large volumes of samples. On the flip side, most research subjects (e.g. mice) and critically ill patients cannot readily
give large volumes of such samples (e.g. blood). Here again microfluidics finds itself a strong application, enabling thorough analysis of tiny volumes of fluid, even into the nanoliter regime.

Further aiding the push towards microfluidics, as mentioned before, is the ability to leverage many of the pre-existing microfabrication techniques to create devices that are not just tiny, but perhaps mass-producible as well. This enables such devices to be made at a low cost, and may further allow such devices to be disposable in nature. All of this provides the driving force behind the consideration of microfluidics as a platform to enable point-of-care medicine, one day allowing for a variety of medical instruments to exist not just in isolated testing laboratories, but in clinics around the world, even in some of the most remote and resource-poor settings.

Each individual component developed for microfluidic platforms represents more than just that accomplishment in and of itself; it also represents a step towards fully-integrated, highly-automated, low-cost lab-on-a-chip devices. A flow cytometer on a microfluidic chip is an exciting prospect by itself, however it holds the potential to be much more. Combined with technologies for metering and mixing fluids, for incubating cells, and for sorting samples in fluid flow, the chip can rapidly become a highly-automated, fully-functioning miniaturized laboratory. Such devices would increase reliability and repeatability of assays and results, reducing the possibility for human error as well as the chances of biological contamination. The goal of this work, then, is not just to advance technologies for making microfluidic flow cytometers, but
further to add to a growing body of knowledge to facilitate the development of such highly integrated, highly-automated miniaturized laboratories.

1.2. Flow Cytometry

A number of excellent learning materials also exist to cover flow cytometry in great depth [10-12], so once again only an overview of the device and some relevant points will be discussed in this thesis. In flow cytometry, individual cells are optically identified as the flow single-file through the device. A light source (typically a single-wavelength laser) interrogates each passing cell. Angular light scatter and fluorescence (from antibody-labelled or stained cells) are used to determine the cell’s identity. The statistical data collected from a number of cells (typically thousands to tens of thousands of cells) is used to understand disease state, treatment progression, sample population dynamics, etc. In some machines, a downstream system will remove samples of interest from the flow for future analysis, collecting these samples into a tube or relocating them into wells on a plate. A schematic representation of a typical flow cytometry device is shown in Figure 1.1.
For accurate optical identification of single samples, light must be collected only from a single cell at a time with enough fidelity to ensure accurate interpretation. This means that the interrogation conditions of each cell must be identical to that of the last (light intensity, beam width, cell location in detection area). Also, light must be collected from a highly localized area to prevent cross-talk, and collected background light must be low enough to allow the resolution of a clear signal.

1.2.1. Flow Control

Flow control must be achieved to ensure single-sample detection (few coincidences). The flow control must also ensure that each sample is passing through the same location and flowing at the same speed, to ensure that identical samples yield the identical signals. Any variations in flow velocity or illumination intensity will create false variations in the resulting data. Although some flow cytometers use a jet-
in-air system for fluid flow, most employ a small-volume flow cuvette with a circular cross-section of approximately 100-500 µm diameter or a square cross-section of similar edge length. It is well-known that fluid flow in a pipe results in a parabolic cross-section due to viscous effects at the pipe wall (i.e. Pouiselle flow). As a result, samples traveling through the flow chamber along different flow lines would possess different flow velocities. As discussed above, this would result in unacceptable signal variations. To combat this issue, some means of flow control must be established to ensure that all samples pass through a single location in the chamber (generally the center) to reduce the sample velocity distribution.

Flow control is generally achieved via hydrodynamic focusing. In this approach, the sample flow enters the center of the flow chamber (the ‘core’ flow, surrounded on all sides by a higher pressure fluid flow (the ‘sheath’ flow). As a result, the core flow is confined to a narrow region (generally 10-25 µm for typical sample diameters) in the center of the flow channel, resulting in a very narrow distribution of sample velocities. It should be noted that this approach requires a substantial volume of sheath fluid, and that this fluid must be optically clean so as not to add noise to the system.

1.2.2. Optical System

The fluidic system (see Section 1.2.1) delivers individual sample to the interrogation region, a small area (e.g. 20 µm x 60 µm) illuminated by the interrogation source. Flow cytometers generally have 1-3 illumination sources, often a
488 nm source for fluorescence, sometimes a source in the 600-700 nm band (e.g. 630 nm) for some fluorophores requiring higher-wavelength excitation, and increasingly often a laser in the 500-600 nm range (e.g. 561 nm) for moderate-wavelength excitation. Other laser choices, such as <488 nm excitation (i.e. violet laser diodes, or VLDs) are being employed to further expand the available pool of fluorophores [13]. Often the highest-wavelength source will be utilized for angular light scatter, as the angular variation in light scatter becomes stronger with longer wavelengths.

Typically both angular light scatter and fluorescence are collected. Forward scatter (FSC) and side scatter collection (SSC) are the nearly ubiquitous angular scatter parameters. Directly across from the interrogation source, a beam stop will be used to absorb the remaining (unscattered) illumination beam, and by default any light scattered at low angles to the optical axis. Light scattered at slightly larger angles (3-20°) around the optical axis comprise the FSC collection. This line collects a solid angle ~0.2-1.3 sr (accounting for the beam block). The side scatter collection generally shares an optical path with the fluorescence line. The axis of this collection line will be orthogonal to the illumination axis (thus this line is often referred to as orthogonal light scatter). In fluorescence collection (FL), solid angle collection is key, and thus the optics shared by the SSC line and the FL line generally have a large numerical aperture (NA), ranging anywhere from 0.4-1.2 (23° to >50°· half angle), i.e. a collection solid angle of 1.9 sr to >7 sr. For the collection of various fluorescence signals, dichroic mirrors and wavelength-selective filters will be used to remove the illumination light and selectively collect over the desired fluorescence bands. The
number of fluorescence bands collected varies from device to device. A simple cytometer might collect over three bands (i.e. FL1, FL2, and FL3) using only 1-2 lasers, while a high-end device might collect over 12 fluorescence bands by utilizing three or more laser sources.

As a particle passes through the illumination beam, an increase in light intensity will be recorded on the various collection lines. The relative intensity is indicative of the particle’s identity. Forward scatter is indicative of the size and refractive index (or, effectively, the size of the nucleus) of the sample [10]. Side scatter carries information about the granularity of the cell [10]. Fluorescence will give information about the presence and quantity of various cellular components or antibodies that have been stained or labelled with some form of fluorophore.

Each collection line is routed to a single photodetector. Typically the FSC collection location sees a much higher intensity signal that the other lines, thus this line often uses a silicon photodetector. For SSC detection, some devices use silicon photodiodes, while others (especially older models) use higher-gain, low-noise photomultiplier tubes (PMTs). Basically all FL lines will use PMTs as detectors. Signals from each detector will be collected by a data acquisition system. Information about each passing sample (an ‘event’) will be recorded, such that each sample creates a vector of data of pulse areas and/or heights for each detection line. The resulting data matrix generated by the entire sample volume can be analyzed for statistical
information about the sample (e.g. absolute or relative sizes of subpopulations within the sample, degree of protein expression, etc).

1.2.3. Analyzing Cytometry Data

The data from each individual analyte can be visualized on a scatter plot, displaying values for any two selected parameters of interrogation (e.g. FSC and FL1, FSC and SSC, etc). This allows for ‘cluster differentiation’, the identification of subgroups within the sample population. Figure 1.2 shows an example of cytometry data from human white blood cells and platelets.

![Panleucogating Technique](image.jpg)

**Figure 1.2** Panleucogating. (left) CD45 and orthogonal scatter are used to gate all leukocytes (A), including lymphocytes (B), monocytes (C), and granulocytes (D). (right) The results of gate A are plotted on a CD4-Scatter plot and enabling the isolation of CD4+ lymphoid cells (E). The information from these plots gives absolute CD4+ cells and relative CD4+ % among both lymphocytes (B) and leukocytes (A) Figure reproduced with permission from the American Society for Microbiology [19].

Initial identification of a cluster can facilitate differentiation of sub-clusters through a method referred to as ‘gating’. Two parameters will be used to identify a
population, and that population will then be plotted on another scatter plot using 1-2 other parameters. In this way, a new, smaller sub-cluster may be identified. For example, in Figure 1.2(a), fluorescent tags on CD45 antibodies are used along with side scatter to identify the major leukocyte subsets (monocytes, lymphocytes, and granulocytes) from a sample of blood (once the red blood cells have been removed or lysed). The data points from gate A are replotted as fluorescently-tagged CD4 vs orthogonal scatter in Figure 1.2(b). By gating out only the leucocytes, an accurate count of CD4+ lymphocytes (without sample contamination) is made possible.

1.2.4. Applications

Flow cytometry has a wide range of applications, which are discussed in a variety of resources [10-12]. The monitoring of HIV/AIDS has been dubbed the ‘killer application’. Monitoring the status of the immune system allows clinicians to identify the point at which antiretroviral treatment will be needed. This helps reduce unnecessary usage of costly drugs, which becomes especially important in resource-poor settings where such drugs are in limited supply (and likewise patients have very limited resources available to access them). Flow cytometry is often used in routine bloodwork, and has found vast applications in basic research. Sorting cytometry also has many applications, among them the isolation of rare cells (e.g. stem cells or circulating tumor cells) for further study. In summary, there is no shortage of applications for flow cytometry, and the advent of low-cost, small volume cytometry would further fuel the field.
1.2.5. The Importance of Light Scatter

Angular light scatter measurements in flow cytometry are based on the principles of Mie Scattering, the formalism that describes light scattering when the size of the scatterer is on the order of the wavelength of the light source [14]. Analytic solutions exist for only simple cases, such as homogenous spheres. A number of numerical solutions have also been determined for various special cases [15-16]. Figure 1.3 shows the Mie Scattering solution for a 1, 5, 15, and 35 µm homogenous sphere of refractive index 1.57, surrounded by a material of refractive index 1.33, under 488 nm illumination. It can be seen that at low angles (i.e. near zero) there is a clear relationship between size and scattering intensity. This relationship does exist at 90° as well, however the intensity change with bead diameter is less pronounced at this location. As it turns out, at this location, light scatter intensity is much more sensitive to internal structure (granularity) of the scatter [15]. The two commonly used light scatter parameters in flow cytometry are forward scatter (FSC; approximately 3-20°) and orthogonal, or side scatter (SSC; centered about 90°), as discussed in Section 1.2.2.
Figure 1.3 Angular scatter intensity profiles for various polarization states for individual 1, 5, 15, and 35 µm polystyrene beads in water (from lowest to highest) [17].

While most of the information garnered from flow cytometry ultimately comes from the fluorescence measurements, the light scatter measurements are extremely important from a practical standpoint, as they are often used as a gating parameter, as demonstrated by Figure 1.2. In fact, as shown in Figure 1.4, light scatter alone can differentiate the major white blood cell subsets, providing label-free (i.e. nearly cost-free) data. This is especially important when considering applications in resource-poor settings, however these parameters remain important even in more routine research and testing. Section 14 discusses the development of microfluidic flow cytometers. Often times, the importance of including light scatter is overlooked, in part due to geometry considerations. The work presented in this thesis specifically considers methods of creating optical systems on microfluidic chips for light scatter collection.
Figure 1.4 Clusters of leukocytes and platelets (P) identified by light scatter alone. The populations include lymphocytes (L), monocytes (M), and granulocytes (G). Image reprinted with permission from Wiley [18].

1.3. Optofluidics and Flow Cytometry

Optofluidics is the field encompassing the synergy of both optics and fluidics, existing at the interface of micro-optics and microfluidics. The term encompasses a broad range of devices, from fluid-tunable waveguides and lenses [18,20] to lab-on-a-chip microscopes [21]. As stated prior, the flow cytometer is a device incorporating both optics and fluidics, with seemingly limitless applications in biology, medicine, and other fields. As such, the device is an excellent testbed for platforms that seamlessly integrate both optics and fluidics.

The creation of a practical microfluidic flow cytometer necessitates the development of (a) excellent fluidic control on the microscale, (b) a means of creating robust optical systems alongside the aforementioned fluidic systems, and (c) a means
of interfacing such a chip with the outside world. Optionally, such development would likely include other functionalities, such as reagent metering, cell sorting, and similar enabling technologies.

In this work, the emphasis is on (b), the development of micro-optical systems that can be seamlessly integrated alongside microfluidics in a tiny, low-cost package. The goal is to develop a system that is sophisticated enough to create the quality optical systems required by at least basic flow cytometers, yet simple enough to potentially be mass-fabricated and portable in nature. Monolithically integrated optical systems would eliminate the issues of optical alignment, increasing portability and reducing the need for maintenance. In addition, many such chips could be interfaced by a single ubiquitous chip reader (encompassing the pumps, lasers, detectors, and data acquisition system, for example), enabling the development and use of a number of application-specific chips without requiring multiple hardware units. Lastly, incorporating both the optics and the fluidics onto a small, disposable chip could significantly improve the device’s ease-of-use, as much of the troubleshooting would consist of simply replacing the chip itself. Individual laboratories and clinics (rather than core facilities) would find great use for such simplified, low-cost devices, enabling rapid test results, time-based studies, and many other uses not possible when the cytometer is a higher-cost tool located in a shared facility.
1.3.1. Miniturized and Integrated Optical Systems

Initially, microfluidic chips were looked at effectively as a replacement for the flow cuvette of the cytometer; all of the optical systems remained essentially the same, and often this is still the case [22-24]. Unfortunately, utilizing a bulk optical system drastically reduces the benefits of miniaturizing the fluidic platform by overshadowing the gains of miniaturization. Ideally, the optical system would scale down in size with the fluidic system while maintaining both the low system cost and the possibility for mass production. Additionally, reducing the size of the optical system would reduce the total optical path length, which may reduce absorption losses, an important consideration in fluorescence measurements, as well as other losses. To address these issues, some researchers considered collecting light in close proximity to the channel by using lower-cost, small photodetectors such as avalanche photodiodes [25-26] placed above or below the chip, making a more compact device. This approach generally won’t work for multi-parameter detection, however, limiting its utility for flow cytometers.

1.3.2. Fiber-Based Solutions

By the 1980’s, the cytometry community had started to make use of optical fibers in their machines and had even begun to consider the possibility of replacing conventional bulk optics with optical fibers [27]. Many in the microfluidic flow cytometry research community also began to investigate the use of alternative optics. As shown in Figure 1.5, Pamme et al. used fibers held at fixed angles above the fluidic channel to collect light scatter at 15° and 45° while using a lamp for sample
illumination. Light scattering CVs were typically 25-30%; quite an achievement for a microfluidic device but considerably larger than the expected 5% variation that might be expected from a benchtop device [28]. The authors attributed this large signal distribution to effects such as scattering from sidewall roughness, illumination beam imperfections, and cross-talk. Chabinyc et al. also employed fibers, integrating them on the chip by first clamping them to the mold in close proximity to the microfluidic channel and then pouring PDMS to make the mold replica around them [26]. The glass-PDMS adhesion during polymer curing yielded a robustly-integrated optical fiber that required no index matching fluid. The method still requires some alignment and assembly, a problem which is often avoided by the use of fiber sleeves. In this approach, the mold includes a channel into which the fiber is inserted after polymer curing and epoxy-fixed in place [29]. The fiber sleeve approach can also avoid the use of index matching liquids by filling the airspace instead with a curable liquid, often the very same heat-curable polymer used to make the device itself [30].

![Figure 1.5](image-url)  
**Figure 1.5 (a)** Light scatter data from a microfluidic chip.  **(b)** Light was collected at two angles using optical fibers. Figure reproduced by permission of the Royal Society of Chemistry [28].
1.3.3. Integrated Waveguides

Waveguides offer similar light-confinement capabilities to optical fibers but in a more robustly integrated fashion. Such chips would directly interface with light sources and detectors, making for a simple testbed that readily allows for chip changes. On-chip waveguides can be used to direct light to targets, such as detectors within microns of the illuminated samples without the difficulties of alignment, epoxy-fixing, or breakage. On-chip waveguides fabricated by oxide deposition [30] or ion exchange [31] have been applied to on-chip detection systems. Anisotropic silicon etching has been also utilized to form metalized silicon waveguide grooves capped by metal strips. In this application, optical coupling between the waveguides and microchannels is achieved by reflection from the end-facets of the waveguides [32]. Many these methods, however, require complex and lengthy fabrication process, and often the materials are not well-suited for biological applications or for visible light.

Figure 1.6 (a) Top-down image of an integrated polymer waveguide, and (b) a side image of the waveguide demonstrating confinement of fluorescence. © 2004 IEEE. [34].

Monolithic integration of polymer waveguides made by PDMS with microfluidic channels is an attractive approach due to simple channel sealing, device
robustness, good material optical properties, and precise alignment of waveguides and microchannels [33]. V. Lien demonstrated monolithic integration of microfluidic channels and waveguides in PDMS-based devices [34]. A higher refractive index PDMS (n=1.42) was injected into core channels surrounded by cladding layers of lower refractive index PDMS (n=1.407). This cost-effective method demonstrates simple prealignment and enables optical coupling between the channels and waveguides. Figure 1.6(a) and (b) show, respectively a prealigned waveguide structure and a side view of a device waveguide emitting fluorescent light [33]. Since the waveguides and the microchannels are self-aligned by photolithography during the mold fabrication, no fine alignment using microscope translation stages or micro-positioners is needed during fabrication. Bliss et. al. similarly demonstrated a liquid optical waveguide by injecting high refractive index liquid PDMS prepolymer into the prealigned microfluidic channels [30].

Whitesides’ group has been working on liquid-core/liquid-cladding (L2) optical waveguide, which consists of a liquid core fluid with high refractive index and liquid cladding fluid with lower refractive index [35]. They used deionized water (nd = 1.335) as the cladding fluid and CaCl2 (aqueous, nd = 1.445) as the core fluid inside the PDMS-based microfluidic channel. A stream of core fluid is released into the center of the stream of cladding fluid, and light is guided within the higher-index core fluid. By using different fluids, the numerical aperture of the waveguide can be modified by changing the refractive index contrast.
These different schemes of moving the optical systems onto the microfluidic chip have several advantages, particularly for the light collection system. Close-proximity detection can theoretically allow for lower loss and higher-NA light collection due to the effectively ‘immersed’ optical system (no on-chip air gaps). The whole system may also become smaller and more compact. Tung et al. demonstrated the use of integrated fibers to collect light from multiple angles, an important feature of flow cytometry to help enable multi-parameter detection [29]. Exploiting the ability to put a large angle between the illumination axis and the detection line can help minimize the intensity of light scatter reaching fluorescence detectors. The approach can be modified to instead collect light scatter from several angles. For example, orthogonal light scatter could now be collected, something very difficult to achieve by off-chip optics. Chips with integrated illumination and detection lines seemed to offer greater possibility for mass-production and utility, eliminating the problem of aligning off-chip optics after chip replacement or system movement.

Waveguide and fiber based systems can help reduce the size of the device and facilitate interfacing, but their gains have some limitations. The lack of a collimated interrogation source is problematic for uniform, localized sample excitation; similarly, the use of fibers or waveguides for light collection doesn’t provide the same localized, high NA light collection used in traditional benchtop flow cytometers [29]; most of the light collected by a fiber originates from locations other than the cell. Indeed, many of these problems were pointed out some years ago in the context of fiber-based optical systems for benchtop cytometers [27]. The issues of cladding mode propagation, high
levels of background light collection, and fluorescence induced in the materials are just as applicable to the above-mentioned fiber- and waveguide-based miniaturized systems as they were to a bulk system.

1.3.4. On-Chip Lenses

In recent years, research has been working to more exactly replicate the flow cytometry optical system on a chip by including some form of lenses to allow for light control. On-chip lenses could allow for a collimated interrogation beam, yielding more reproducible results and reducing cross-talk. The levels of background light collection can be greatly reduced with a properly designed optical system. Additionally, lenses can be used to increase the numerical aperture of light collection from the cell, further improving device sensitivity. In fiber- and waveguide-based systems, the diverging light path suggests the need for some form of optics for collimation.
Figure 1.7 (a-c) Optical fibers illuminate a microfluidic channel with a characteristically diverging beam. (d-f) Apertures are used to provide angular restriction, achieving the approximately collimated beam shape desired for illumination. Image reproduced with permission from the American Chemical Society [37].

Many researchers have focused their attention on the chip for improving the optical system performance. The first integrated lenses in microfluidic flow cytometry were seen on the interrogation line. Camou et al curved the face of the fiber sleeve, almost like a lensed fiber, to create lenses with a radius of curvature as high as 70 μm with an aperture the width of an optical fiber [36]. This idea was explored further in microfluidic chips for absorbance measurements by Ro et al., giving a clear demonstration (see Figure 1.7) of the advantageous collimating effect of lenses on fiber ends, but also pointing out the dependence on relative fiber position [37]. Wang et al demonstrated a device employing the more performance-stable waveguide analog of this idea [38]. As shown in Figure 1.8(a), the end facet of a waveguide was given lens-like curvature to help localize the excitation beam in the fluidic channel. The
authors measured light scattering CVs of 26.6-29.7%. In this device, the waveguiding structure shown in Figure 1.8(b) is cumbersome to fabricate, and while the results were among the best published at the time, the device CVs are high and most bead populations shown in Figure 1.8(c) would not be readily discriminated by forward scatter. In summary, each of the above works helped to localize the excitation beam in the fluidic channel, recognizing and acknowledging the critical role of that the optical system plays in the functionality of a flow cytometer, however much work remains to be done in terms of improving device results.

Figure 1.8 (a) Microfluidic flow cytometer incorporating an integrated waveguide with a lensed facet for illumination beam shaping. The waveguide structure is created as shown in (b). (right) Forwards scatter distributions for various bead sizes. Figure reprinted by permission of the Royal Society of Chemistry [38].

Seo and Lee took the on-chip lens concept to the next level, employing multiple lenses to shape the illumination beam in order to maximize the resulting fluorescence intensity of a sample in a microfluidic channel (see Figure 1.9) [39]. This
work demonstrates an important improvement: the use of ‘free space’ optics on a microfluidic chip. The lenses are not tethered to the end of a fiber or waveguide; they can be placed anywhere on the chip. With such a technique, researchers can start to recreate an optical system on a chip. It should be noted that creating lenses for light collection systems pose a slightly harder problem than lenses for interrogation systems due to the potential for large losses of light in the vertical dimension after only a short travel distance (e.g. across the microfluidic channel). This issue will be addressed in greater detail in Chapter 3, where work is demonstrated to enable such lenses to be used not just in illumination systems, but also in light collection systems.

**Figure 1.9** (a) Schematic and (b) microscope image of microfluidic devices integrating air-filled lenses for illumination beam shaping. Reprinted with permission from Elsevier [39].

In addition to such two-dimensional systems, some attempts have been made to fabricate three-dimensional lenses [40]. The authors used a diffusion mask to scatter the collimated light from a traditional mask exposure system. The resulting features had sidewalls that curved inwards, thus the molded lenses were convex in nature. This technique helps to keep light from escaping above or below the optical path; however the curvature of the lens is not currently well-controlled with this approach. With
further development, three-dimensional lenses could reduce or eliminate the need for a
slab-waveguided system, and may be able to increase the numerical aperture of
collection in the vertical dimension.

1.3.5. Motivation for This Work

While much of the above-mentioned work shows great promise and
advancement towards integrated optical systems (namely for flow cytometry), several
key problems remain.

Perhaps first and foremost, no system had been able to demonstrate reasonable
light scattering CVs, even for polystyrene beads. This indicates a lack of adequate
flow control as well as the presence of a signal-to-noise ratio (SNR) issue. CVs.
According to Steen, most flow cytometers are set up such that angular light scatter
intensity levels are more than sufficient for detection of subwavelength particles;
detection is often limited not by signal intensity, but rather by background collection
[40]. The implication, then, is that simple waveguides or fibers likely are not enough;
a true optical system must be developed. Localized light collection is important; the
system must collect light from the location of the interrogated sample and reject light
from other locations. Such localization of collection is imperative for adequate signal-
to-noise ratios in the collection system. This is especially true in light scatter
measurements, as wavelength filters cannot be employed to reduce stray light.

Another problem frequently faced in optical interrogation on microfluidic
chips is a lack of robustness or ease of manufacturing. Many of the devices are made
by combining a few pieces to make a system, resulting in a final device that is not feasibly portable or mass-fabricated. In addition, many microfluidic flow cytometry devices suffer from impossibly low throughput. While it is true that multiplexing will make up for some of these downfalls, the individual devices themselves must be capable of achieving some practical benchmarks (e.g. 1,000 samples/sec).

This work aims to develop an elegant, integrated solution to introducing optical systems to microfluidic chips. The work looks to develop a chip that can operate at a reasonable throughput and improve upon the scattering CVs already demonstrated in the literature. The emphasis is on monolithic integration, truly preserving the potential for low-cost mass-fabrication.

In this thesis, all devices are created monolithically by replica-molding in an optically-transparent polymer. Chapter 2 will discuss the details of fabrication for such devices, including several issues that pertain to the inclusion of optics in such devices. In Chapter 3, devices integrating optical systems based on fluid-filled lenses will be demonstrated, including a device for microfluidic flow cytometry. In Chapter 4, a system of integrated optics based on an exclusion-based design approach is presented, again including light scattering results from a microfluidic flow cytometer with an integrated optical system created in this manner. A final summary of this work and future research directions will be given in Chapter 5.
Acknowledgements

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References


[17] Image created by MieCalc at http://www.lightscattering.de/MieCalc/eindex.html


Chapter 2

Tools and Processes for Device Fabrication, Data Acquisition, and Data Analysis in Microfluidic Flow Cytometers with Integrated Optical Systems

Integrating optical systems into microfluidic chips requires an additional level of consideration not previously necessary for fluidics-only chips. For integrated optics, the feature sidewalls will generally become the optical facets, thus devices will require greatly-improved methods for creating silicon molds. In addition, integrated optical systems that require any significant track length (>100-300 µm) with require some method of preventing vertical light losses. While three-dimensional lenses would solve this issue, the lenses themselves are rather impractical to create, thus it was determined that some form of a waveguiding structure would ultimately be necessary. These two considerations are critical for the practical continuation of this work.
In addition, a number of elements of the testing and analysis systems need to be reconsidered for practical integration of optical systems. This includes methods of optically interfacing with the device (in this work, fiber interfacing), creating a test setup to measure and record such optical systems, and determining a method of comparison between the microfluidic flow cytometer and the benchtop flow cytometer. This Chapter presents the key tools, methods, and processes that were developed for designing, fabricating, testing, and analyzing optofluidic devices.

2.1. Designing Optical Devices

In order to create devices with integrated optics, the optical qualities of the material must be known. Previous devices had largely been created based on optical fibers or integrated waveguides. In these cases, the exact refractive index is not as critical; however, when working with refractive optics (lenses) the material qualities become much more important. The first task, then, was to choose an appropriate materials set and measure the optical properties of those materials. Soft lithography using polydimethylsiloxane (PDMS) was chosen for rapid, low-cost prototyping. Optical properties were measured by an Abbe refractometer and adjusted through experimentation. With this knowledge of the material’s optical properties, device design can begin. The key properties of the devices (namely waveguide numerical aperture) will be fixed by the materials choice, and the design will largely revolve around those parameters and the chosen angles for light scatter collection.
2.1.1. Refractive Index and Dispersion

For optical devices, the refractive index and dispersion characteristics of the device material are critically important. For this reason, Gelest and Nusil brand polymers were favored in this work, as their refractive index (and in the case of Nusil products, the dispersion characteristics) were known and publicized. Conversely, a number of varying results have been published for the refractive index of standard Sylgard 184, with the quoted refractive index ranging from 1.41 to 1.43 [1-2].

As discussed in Chapter 3, a slab waveguide was needed to allow for integrated lens systems in the PDMS devices. This resulted in a three-material system: (1) a vertical cladding material (1.41; Gelest OE 41), (2) a material to serve as the core for the integrated waveguides (1.42 Gelest OE 42), and (3) a polymer with a refractive index larger than the cladding material but smaller than the waveguide core material. This last material was created by a custom mixing of another PDMS polymer, Sylgard 184 (Dow Corning). Measurements of the refractive index were accomplished using a simple commercially-available Abbe refractometer. Figure 2.1 shows the dispersion curves for several mix ratios. Measurements were also taken for the waveguide core and cladding materials for comparison, to ensure that the refractive index of the custom material fell between the constraints of the commercially-available material, even if global errors in the measured values were present. In later experimentation (see Chapter 3) it became clear that there were in fact minor errors in the measurement, however, for the reasons stated above these errors were readily accommodated.
Figure 2.1 (a) Measured dispersion curves for Gelest OE41 and several custom mixtures of Sylard 184 (Dow Corning). (b) Measured dispersion curves for Gelest OE41 and OE42 PDMS.

PDMS was generally mixed according to the manufacturer’s directions, except for in the creation of the slab waveguide layer for devices containing two-dimensional lenses. As previously stated, for this layer a custom mix ratio was employed to achieve the desired refractive index. Table 2.1 lists the PDMS and PDMS-like materials used in this work, including their mix ratios and approximate ($n_d$) refractive indices.

2.1.2. Optical Simulations

For optical simulations, relevant material data is specified using either (a) the refractive index at the sodium d line ($n_d$) and the Abbe number ($V$) of the material (as $n_d:V$) or (b) a table of wavelengths and measured refractive index. The Abbe number is defined as

$$ V = \frac{n_D - 1}{n_F - n_C} \quad (2.1) $$
where $n_D$, $n_F$, and $n_C$ are the refractive indices of the material at the Fraunhaufer D, F, and C wavelengths (i.e. 589.2 nm, 486.1 nm, and 656.3 nm, respectively). For the lens fluid, a dispersion plot is supplied by the manufacturer (SantoLight or Nusil), thus the second method was used. For our specially mixed PDMS (Sylgard 184, Dow Corning) as well as our standardly-mixed PDMS polymers (Gelest OE41, Gelest OE42), a refractometer was used to determine $n_d$ and $V$. Comparisons between simulations and experimental results from our proof-of-concept device have demonstrated the validity of our measurement methods for the lens fluid and our specially-mixed polymer. Table 2.1 also lists the optical values used for Code V simulations.

### Table 2.1
Polymer mixing data and optical properties. Properties were either specified Polymer mixing data and optical properties.

<table>
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<tr>
<th>Material</th>
<th>Weight A/Base : WeightB/Crosslinker</th>
<th>Approximate $n_d$</th>
<th>Simulation Parameters Used $n_d$:V or [$\lambda$, $n(\lambda)$]</th>
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<td>1.41:63</td>
</tr>
<tr>
<td>Sylgard 184</td>
<td>10:3</td>
<td>1.415 (M)</td>
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<td>(Custom)</td>
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<td>NA</td>
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<td>1:1</td>
<td>1.42 (S/M)</td>
<td>1.4250:63</td>
</tr>
<tr>
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<td>411 589 833 1306 1550 1.5997 1.5674 1.5558 1.5474 1.5457</td>
</tr>
</tbody>
</table>

#### 2.1.3. Waveguide Numerical Aperture and Solid Angle

Device design revolved around the goals of each lens system (e.g. collection over a 15° in-plane angle) and the numerical aperture of the embedded waveguides.
In Chapter 3, the designs utilize integrated lenses to relay light from the sample to the integrated collection waveguide, minimizing contribution from non-sample sources. In Chapter 4, custom optical elements are utilized to collect light from the sample while reducing the collection of noise from other areas on the chip.

The NA of the embedded polymer-core, polymer calculated waveguides can be calculated from the typical equation for numerical aperture,

\[ NA = n \sin \theta = \sqrt{n_2^2 - n_1^2} \]  

(2.2)

For devices with the slab waveguide configuration (Chapter 3) operated at ~630 nm, \( n_2 \sim 1.425 \) (Gelest OE42, the waveguide core), and in the plane of the device \( n_1 \sim 1.415 \) (Sylgard 184 custom mix; the slab waveguide), while in the vertical dimension \( n_1 \sim 1.41 \) (Gelest OE 41; the thick cladding layers). Thus in the plane of the device the numerical aperture is ~0.17, yielding a half-angle collection of 6.8°. The vertical NA is ~0.12, for a collection NA of ~4.8°. For devices without the slab waveguide configuration (Chapter 4) operated at 488 nm, the waveguide cores are \( n=1.43 \) while the device body is \( n=1.419 \) and the upper cladding is \( n=1.415 \). Thus the NA for these devices is 0.11 for a collection half angle of 4.3°.

Ultimately a higher waveguide NA would be desirable, especially for the slab waveguided devices. While the in-plane light collection in these devices can theoretically be made to match the NA of systems for commercial devices, the lower slab waveguide NA will ultimately hurt device performance by lowering the total
signal collected. Higher index materials were considered, and some success was found using LS-6946 (Nusil), an optical elastomer similar to PDMS. This polymer was extremely viscous (35,000-40,000 cP), necessitating some thinning with toluene, hexane, or a similar solvent [4]. Slab waveguides were successfully created with this material as the slab core, raising the vertical collection half angle to nearly 15°. To do this, a two-material system was adopted, in which the waveguide cladding, rather than the waveguide core, was filled after demolding. In this way, the integrated waveguides would see the same refractive index contrasts vertically and horizontally, and the entire system would be reduced to two materials (instead of three). The main problem with this approach was issues with mold replica fidelity; for example, if thin waveguide cores did not replicate well bonding problems would ensue, resulting in significant light losses. This was especially problematic for long waveguides. As with the custom Sylgard mixture, LS-6946 also exhibited a tendency to bond to molds if the molds were not first ‘primed’ with another, more standard PDMS. Ultimtaely, it seemed the materials chosen were not sufficiently rigid to use the filled-cladding approach, however this approach would likely be more desirable in other less flexible materials set choices.

### 2.2. Device Fabrication

Microfluidic devices are often prototyped in polydimethylsiloxane (PDMS) using the soft lithography techniques pioneered by the lab of Professor George Whitesides [1]. In this technique, the devices are created from mold-replicated
structures created in PDMS. This allows for low-cost, rapid prototyping of devices with small, precise features. Features as small as 20 nm have been successfully replicated with good fidelity in PDMS [3].

PDMS is an optically transparent silicon-based organic polymer. The chemical formula for PDMS is $\text{CH}_3[\text{Si(\text{CH}_3)_2\text{O}}]_n\text{Si(\text{CH}_3)_3}$, where $n$ is the number of monomers in the chain. The siloxane backbone provides structural integrity while maintaining flexibility. The methyl groups help keep the material chemically inert and give it a hydrophobic surface. PDMS is typically sold in two parts that crosslink when mixed to form the final polymer. Various proprietary formulations of PDMS exist to give different material and optical properties.

Each of the devices demonstrated in this work is made in PDMS polymer; however, device operation is not dependant on any specific properties of PDMS aside from the refractive index and optical transparency. Similar devices could be created in different materials via alternative fabrication processes, such as injection molding or direct laser writing.

While the details of device fabrication change from run to run, or from design to design, the general steps are conserved. The process occurs as follows:

- Mold Creation (Section 2.2.1.1-2.2.1.2)
- Mold Replication (Section 2.2.2-2.2.3)
- Cleaning, Hole Punching, and Device Bonding (Section 2.2.4-2.2.5)
• Aperture Filling (Section 2.3.1)

• Waveguide Filling and Fiber Insertion (Section 2.3.2)

• Lens/Optic Filling (Section 2.3.1)

• Fluidic Interfacing (Section 2.4)

• Device Testing (Section 2.5)

• Data Processing and Analysis (Section 2.6)

2.2.1. Soft Lithography

Figure 2.2 illustrates the principles of soft lithography. A pattern is printed to be used as a ‘mask’. In this work, both low-cost transparency prints (minimum feature 10 µm) and high-resolution e-beam written masks (minimum feature <1 µm). The pattern of the mask is transferred to the mold via lithography (for polymer-on-silicon molds) or lithography and etching (for etched silicon molds). Both processes were utilized in this work.
2.2.1.1. Photoresist Molds

Photoresist-on-silicon molds are often used as molds for soft lithography. Molds were created using standard SU-8 50 (Microchem) processing techniques. The mask was exposed onto 50-70 µm thick SU-8 50 and developed to create the mold patterning. After the standard post-exposure bake, the molds were hard-baked at 200°C for 1 hour to impart additional structural strength.

2.2.1.2. Silicon Molds

An important part of the development of processes to integrate optical systems into microfluidics is the ability to create optical-quality interfaces in microfluidic devices. Typical SU-8 molds possess neither sharp corners nor vertical sidewalls. In addition, sidewall roughness is an important consideration for integrated optics. One
estimate of the maximum allowable surface roughness for optical-quality performance is given by the Marechal criterion:

\[ \sigma_{\text{RMS}} < \frac{\lambda}{10} \]  

(2.3)

For devices utilizing 488 nm light, this means \( \sigma_{\text{rms}} \leq 35 \text{nm} \). Molds created by typical SU-8 processes would not be sufficient to reach this goal. Post-processing, such as wet etches, can help mitigate these issues [1]. Highly directional etches, such as the Bosch Process, can help address sidewall verticality and structural rigidity, however again sidewall roughness (\( \sigma_{\text{rms}} \sim 500 \text{ nm} \)) is highly problematic due to the cyclical nature of the process [5].

Cryogenic etching offers a highly directional means of etching silicon while maintaining low-roughness sidewalls. In this process, a plasma is ignited in the presence of SF\(_6\) and O\(_2\) under vacuum (~12 mTorr), creating oxygen radicals (for passivating) and F radicals (for etching). The low temperature (<100°C) ensures low-energy ions, providing a directional etch. Because of the ion directionality, the sidewall passivation layer does not need to be robust to prevent etching. Under the right conditions, the F radicals will be able to remove the passivation layer on the wafer surface and etch the silicon while the sidewalls are continually protected by a thin passivation layer [6]. Temperature control of the substrate is critical for uniform etching and maintaining low ion energies, thus backside helium flow is utilized to maintain substrate cooling.
To perform the cryogenic etching, an Oxford P100 was used. Proper conditions to achieve a smooth, vertical sidewall were determined through a series of calibration runs. The process window can be determined with the assistance of the Black Silicon Method [6-7]. In this approach, the point of ‘black silicon’, or ‘grass’ formation is located as a starting point. This phenomenon occurs as the point where the etch is extremely directional, thus micro-masking effects are significant enough to form the ‘grass’, which is observed as a blackening of the silicon etch surface. From this point, the relatively known manner in which variables affect the process (gas flow rates, chamber pressure, chamber temperature, RF and ICP power) are used to manipulate the process to reach a point just shy of black silicon formation. The goal of parameter manipulation is to provide a slight undercut to the etch, such that as ‘grass’ is formed, it is eventually cut away, maintaining a relatively smooth etch surface. An example of black silicon formation via micromasking is shown in Figure 2.3.

![Black silicon ('grass') formation from cryogenic etching at incorrect conditions.](image)

**Figure 2.3** Black silicon ('grass') formation from cryogenic etching at incorrect conditions.

It was determined that chamber conditions were critically important to etch repeatability. The etching of various materials other than silicon, especially glass or
polymers, appeared to destabilize the process window. It is highly recommended that, for cryogenic etching, a dedicated insulator is purchased for the machine to be used only with cryogenic etching, while another insulator is used for standard etch processes. This dedicated insulator should only be used with a limited group of materials (e.g. only silicon and chrome). A cleaning/priming run prior to cryogenic etching was also found to improve etch reliability. For this work, the above recommendations were critical to process stabilization and reliability.

Etch reliability was also found to depend heavily on the wafer clamping conditions. Incomplete clamping is a frequent occurrence, resulting from debris on the wafer holder or wafer, and is exacerbated by the small overlap area between a standard 4” wafer and the wafer holder. Helium leakage into the chamber results in (a) temperature nonuniformities across the wafer, which translate into etch nonuniformities, (b) chamber pressure destabilization (affecting etch quality), and (c) helium particles in the chamber, which appear to directly interfere with the plasma and etch conditions. In general, excess helium pressure is indicative of a clamping issue, and will generally render a substrate unusable. Ultimately, a custom-made wafer clamp was created to provide the process stability necessary for repeated use of cryogenic etching.

A metallic etch mask must be used with cryogenic etching to (a) provide robustness against the etch itself, (b) prevent thermal issues, such as cracking and delamination, and (c) prevent contamination of the etch chamber. For this work, 10 nm
of titanium followed by 50 nm of nickel was e-beam evaporated onto a silicon wafer, using 1500 nm of photoresist (NR-9 1500PY, Futurrex) as a liftoff mask. After liftoff, the substrate was solvent cleaned in the typical manner. Oxygen plasma cleaning was utilized to remove any remaining organic residue. Lastly, etch quality was found to improve when the substrate was briefly dipped in buffered oxide etch to remove the oxide layer on the substrate [6]. Here great care must be taken to reduce expose time in order to ensure that the etch mask is not significantly attacked by the acid cleaning.

**Table 2.2** Process parameters for cryogenic etching of silicon wafers.

<table>
<thead>
<tr>
<th>Process Parameter</th>
<th>Process Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helium Pressure (wafer cooling)</td>
<td>6-7.5 mTorr</td>
</tr>
<tr>
<td>Chamber Pressure</td>
<td>12 mTorr</td>
</tr>
<tr>
<td>O₂ Flow Rate</td>
<td>6 sccm</td>
</tr>
<tr>
<td>SF₆ Flow Rate</td>
<td>40 sccm</td>
</tr>
<tr>
<td>RF</td>
<td>8 W</td>
</tr>
<tr>
<td>ICP</td>
<td>1200 W</td>
</tr>
<tr>
<td>Process Time</td>
<td>40-50 min</td>
</tr>
</tbody>
</table>
Etching was performed in a commercial ICP (Inductively Coupled Plasma) System (P100, Oxford Instruments). Table 2.2 lists the process conditions for an etch depth of 60-70µm using a metallic mask as detailed above. For the reasons stated above, these parameters tend to drift over time, however, they provide a useful starting point for process tuning.

A white-light interferometer (WYKO NT110, Veeco) was used to characterize the roughness of the resulting sidewalls. A small portion of an etched device (generally a waveguide) was cleaved near the sidewall and mounted sideways on the interferometer. A 20x objective was used for the measurement. A smaller portion of this viewing area was utilized for RMS roughness calculations, as large-scale features could falsely increase the measurement (e.g. large-scale ridges due to 10 µm roughness present in the photomasks, which can be eliminated when using higher-quality e-beam written photomasks). Figure 2.4 shows results from interferometry
measurements on a cryogenically-etched waveguide sidewall. The measured RMS roughness ($\sigma_{\text{RMS}} = 30 \text{ nm}$). Figure 2.5 shows several samples of mold-quality etches.

![Figure 2.5 Images of mold-quality etches. Images (a) and (c) show a rounded and cornered edge, respectively © 2008 IEEE [9]. (b) A bird's-eye view of a mold including lenses and waveguides © 2009 IEEE. [10]](image)

### 2.2.2. PDMS Molding

As mentioned above, PDMS replicas of the mold are used to create devices. Reactive species on the constituent elements of uncured PDMS will bond irreversibly to glass, and thus will also bond with oxide on a silicon wafer. To block this bonding and allow for replica demolding, silicon molds (both polymer-on-silicon and etched silicon) are treated with a fluorinating agent, effectively blocking potential interaction sites. For this work, either chlorotrimethylsilane or (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-tricholosilane (Sigma Aldrich, United Chemical) was used for surface passivation. By placing both the passivation agent and the silicon mold under vacuum (in this work, ~100 kPa for >5 minutes), the vapor pressure of the passivation agent is exceeded, allowing it to react with any available bond sites on the mold.

After surface passivation, degassed PDMS prepolymer is poured onto the mold. After pouring onto the mold, the uncured polymer is degassed again, to remove any remaining bubbles, and the polymer is cured at the appropriate temperature specified
by the manufacturer. After curing, the molded portion can be cut away from the excess PDMS to be used in subsequent bonding steps.

2.2.3. Slab Waveguide Fabrication

Slab waveguide development was necessary for confinement of light in the vertical dimension when utilizing in-plane lenses for light shaping and collection. Discussion and details of slab waveguide fabrication is discussed in Chapter 3.

2.2.4. Replica Bonding

Cured PDMS can be bonded irreversibly to glass or to another piece of PDMS by properly activating both surfaces. Activation involves the formation of reactive silanol (-OH) groups on the surfaces to be bonded. In this work, two methods of PDMS-PDMS bonding were utilized. In early portions of the lens-based work, oxygen plasma was used for surfaced activation. A UV-Ozone cleaner was used to generate oxygen plasma in the presence of oxygen (pressure of 10 psi, flow rate of 0.3 scfh). For the majority of the devices detailed in this work, a UV-Ozone (UVO) Cleaner (UVO Cleaner Model 42, Jetlight) was used for surface activation. The device was operated for three minutes with an oxygen flow of 0.3 scfh. The internal tray was set high (close to the bulb) with the PDMS pieces to be bonded bond-side up. After treatment, the pieces were put together to form the device, and placed in an 80°C oven with a weight (200g) on top for >5 hours. The resulting bond is extremely strong, able to withstand pressures of over 300 kPa [11]. After bonding under proper conditions, the interface between the two pieces is no longer visible (see Figure 2.6).
Devices were diced and sonicated in isopropanol, followed by water, and then dried prior to bonding. Access holes for the optical elements were often punched prior to pre-bond cleaning, to reduce the likelihood of debris in the optical path.

Figure 2.6 Device cross-section showing nearly-invisible interface after bonding.

2.2.5. Multi-Layer Alignment

Some devices detailed in this work had patterning on both of the PDMS replicas used to create the final device. In these cases, alignment of the two replicas was necessary before bonding. Initially, methanol was used to facilitate movement of one replica across the surface of the other replica. This approach has been reported in the literature, and showed semi-reliable success for this work as well [11]. Ultimately, it was found that the surface activation could withstand several placements and replacements without noticeably affecting bond quality. As a result, double-patterned devices (such as those in Chapter 4) were generally fabricated by taking several attempts at alignment under a stereoscope (0.7-3x magnification). Lifting and pushing or pulling on corners, rather than peeling off and re-placing the entire replica, can facilitate final placement.
2.3. Optical System Interfacing

Optical elements on the device are created by the choice of element geometry and by the choice of material used to fill the element. The cycles of fabrication and testing of individual optical devices was accelerated by the use of optical fibers for interfacing with the on-chip waveguides. While the initial integrated lens prototype chips (see Section 3.4) required time-consuming alignment of an optical fiber to the on-chip waveguide, subsequent devices incorporated a fiber sleeve for rapid, repeatable interfacing with fiber optics.

2.3.1. Filling Apertures, Waveguides, and Optics

There are numerous ways to fill chambers in the device. The preferred method involved removal of air from the spaces via the application of vacuum pressure the device, subsequent filling of the chamber, and (for waveguides) finally inserting the fiber into the fiber sleeve manually.

First, holes must be punched to access the lines to be filled. For waveguides, these holes can be punched along the fiber sleeves, so that they do not interfere with the optical performance of the chip. For apertures, the holes will not interfere so long as they are not near the aperture edges. Punching should occur before cleaning and bonding (Section 2.2.3) to reduce the possibility of debris in the optical path. The device itself is then placed under vacuum (<100 kPa) for an extended period of time (>20 min). After returning to atmospheric pressure, degassed PDMS (generally Gelest OE 42 for waveguides, Sylgard 170 for apertures, or Nusil LS-6257 for solid lenses) is
dropped onto the device to cover the access holes. Pushing a luer stub into the hole to help coat the sidewalls to start polymer flow and can facilitate more rapid filling. Within minutes, the chambers should be fully filled. At times, additional exposure to vacuum pressures would be needed to complete chamber filling.

Direct-pressure filling was used when the preferred method of vacuum-assisted filling might disrupt other areas of the device, such as when filling lens chambers after the curing of polymer waveguide/fiber complexes. This approach works well in PDMS due to the fact that PDMS is air-permeable. A syringe is filled with the liquid or uncured polymer of choice. Steady pressure is applied, and over time the air is forced to leave the chamber, allowing the polymer to fill the space. This method was often used for liquid-filled lenses.

2.3.2. Fiber Insertion

For waveguide/fiber interfacing, the final step is manual insertion of fiber optics with the help of a stereoscope. These fibers were inserted and fixed into place during fabrication to ensure consistent results with repeated use. By inserting the fibers into the uncured waveguides, the fibers are nearly index-matched to the waveguides, avoiding the formation of a lossy air interface.
Figure 2.7 Integrated fiber sleeves facilitate interfacing with integrated waveguides for prototype testing. Note the alignment of the fiber core to the waveguide.

Figure 2.7 shows an integrated fiber sleeve. The sleeve is created in the same manner as a fluidic channel or a waveguide, and thus has the same height as these features. The width is larger (100-150 µm) to accommodate the larger diameter of an optical fiber. The sleeve is attached to the end of a waveguide channel, and terminates on the other side in a cutting guide. When the device is diced for bonding, the guide is cut open. This is where the fiber will be inserted after waveguide filling.

Fibers are cut, stripped, cleaned, and cleaved prior to insertion. In this work, most fibers are multimode step-index glass fibers, with a numerical aperture (NA) of 0.22. For the devices with exclusion optics, (see Chapter 4) fiber with an NA of 0.20 was utilized for better matching to the NA of the integrated waveguides. The device is placed on a clean glass slide (2” x 3”) for stability. The fibers are then inserted and secured in place with scraps of cured PDMS and epoxy.
It was observed that Gelest OE 42 had a tendency to develop bubbles even after curing, especially in the vicinity of the fiber. An example of this problem is shown in Figure 2.8. This may be the result of incomplete curing due to the surrounding PDMS device body, or perhaps from delamination as a result of thermal or mechanical stress on the device during use. Likely any movement of the fiber optic exacerbates the issue, thus fixing the fiber optic via epoxy or similar was expected to help resolve this issue. Regardless of the origin of bubble formation, it was found that by covering the interface with additional polymer or epoxy, bubble formation in the waveguides was significantly reduced. A small reservoir was created by scrap PDMS at the edge of the device, around the location of the fiber sleeve. This served as structural support onto which to epoxy the fibers, as well as functioning as the boundaries of a pool to fill with excess polymer up to the height of the insertion point to prevent bubble formation. This approach, which was found to significantly improve prototype yield, is shown in Figure 2.9.
Waveguide facets varied in size throughout the works discussed in this thesis, however each waveguide always terminated with a diameter of 50 µm (tapering down to this diameter, if necessary). This diameter was chosen to match the diameter of the cores of the multimode fibers used in this work. The fibers themselves were chosen to closely match the numerical aperture of the integrated waveguides. Initially, a step-index fiber of NA=0.22 was utilized (ASF50/125Y, Thor Labs); later graded-index fiber of NA=0.20 was used (GIF50, ThorLabs).

2.4. Fluidic Interfacing

Fluidic chambers were interfaced in a variety of methods throughout this work. Generally speaking, a hole must be punched to access the fluidic channel, and some method of tubing interfacing devised to create a leak-free system. The preferred method utilizes commercially-available biopsy punches (Harris Unicore) to create clean, standard-sized holes. Appropriately sized tubing was then directly inserted into the access holes. For a biopsy punch diameter of 1 mm, tubing of ~0.03 in outer diameter is a good match. Replica thickness should be at least 2-3 mm to ensure a
strong hold on the tubing. Fluidic access holes were often punched after bonding and filling to prevent contamination with undried polymers.

2.5. Device Testing

Figure 2.10 shows a schematic of a typical test setup. A laser is used to bring light onto the chip, and for cytometry chips detectors are used to collect light scatter. The detector signals are amplified, collected by an acquisition block (BNC 2210, National Instruments), and digitized by a data acquisition card (PCI-6251 M Series, National Instruments). Signals were logged using commercial software (Signals Express, National Instruments). The specific lasers and detectors used for testing evolved with the chip design.
Most optical interfacing was performed via fiber optic, as discussed in Section 2.3. Initially, bare fiber splices (ULTRA splice, Siemens) were used to connect the fibers inserted into the device to fibers leading to the detectors. Eventually longer fibers were utilized, eliminating the need for the splice. Bare fiber adapters (F-AM-FC, Newport) connected any bare fibers to the detectors themselves via a fiber connection adapter (SM1FC, Thor Labs). The device, which had been affixed to a glass side (see Section 2.3) is placed on a microscope (TE2000-U, Nikon). The laser and detector fibers are then connected appropriately. The microscope is not necessary for device operation, but facilitates troubleshooting (e.g. clogged channels, poorly focused flow, etc) and helps develop an understanding of device operation.
2.6. Data Processing

To best compare results from the microfluidic devices with results from the commercial devices, it is preferable to perform analysis of both data sets on the same software. This is important both to ensure that gating is performed in a similar fashion for both samples, as well as to facilitate clear, convincing side-by-side comparison of data sets. We chose to perform data analysis (i.e. gating, CV calculations, etc) on FlowJo (Treestar, Inc). The performance of such software is far superior to basic gating, especially when using features such as auto-gating. The downside to this approach is that effectively all cytometry analysis software (such as FlowJo) is only able to receive input in ‘.FCS’ (Flow Cytometry Standard) format. This format is composed of details concerning the operating conditions of the machine (header information) as well as a list of ‘events’ (i.e. the intensity values on each channel for each passing sample). Thus in order to utilize commercial analysis software, a method must be devised to (a) convert a plot of intensity vs time to a list of events, and (b) convert an event list to .FCS format.

To convert our acquired signal to an event list, custom Matlab code was employed. The code evolved from run to run, however the core functions remain the same. In the first step, an ASCII version of the data (.txt file) was read by Matlab and reformatted into small portions to reduce the memory strain of processing. The data is a list of intensity values for each data channel at each time point. The Matlab code performs the following steps:
1. DC baseline removal

2. Identification of peaks on 1\textsuperscript{st} channel

3. Identification of nearby peaks on 2\textsuperscript{nd} channel

4. Recording of various event parameters

5. Export of event list in .txt format

Baseline removal is generally accomplished by the application of a high-pass finite-impulse response (FIR) filter of order 2000 with a cutoff frequency of 90 Hz. A high-pass filter is also occasionally employed to reduce high-frequency noise in the signal. Next, a search algorithm identifies local maxima on channel 1 (extinction or FSC, depending on the device) with a peak intensity magnitude greater than some user-defined threshold. This threshold is generally set just above the noise floor to ensure that low-intensity signals are not overlooked. Better gating will be performed down the line via FlowJo. Once a peak is identified on channel 1, the Matlab code checks the surrounding area to ensure that this point is the true local maximum. Once the true local maximum is identified, the corresponding nearby peak on channel 2 (15\degree scatter or SSC, depending on the device) is located.

Once a peak is found on both channels, the event data is recorded. Event data consists of a number of parameters that were used to develop the algorithm and learn from the devices. The main parameters used to evaluate device performance are (1) peak locations, (2) maximum peak magnitudes, (3) peak widths, (4) peak areas, (5)
spacing between peaks on the two channels. A matrix of event parameters is exported in .txt format.

The last step before data analysis is conversion of the event list (.txt format) to cytometry format (.FCS format). This was accomplished using A2FCS, an ASCII to FCS conversion program included in the free Mean Fluorescence Intensity (MFI) program available online (copyright Eric Martz) [12].

2.6.1. Light Scatter: Evaluating Performance

While the method of quantifying fluorescence performance in flow cytometers is rather well-established (e.g. approaches such as defining the Molecules Equivalent Soluble Fluorophore, or MESF) [13-15], there is no clear analog for light scattering measurements. For this reason, results from various lab-on-a-chip cytometers performing light scatter measurements are often difficult to interpret and compare. In this work, I have routinely asserted that the best metric is to measure the coefficient of variation (CV) for a number of standardly-sized beads. The coefficient of variation is defined as

\[ CV = \frac{\sigma}{\mu} \]  (2.4)

where \( \sigma \) is the standard deviation of the population and \( \mu \) is the mean. This statistic effectively quantifies the spread of the data. Effectively all populations will have some spread. In the case of cytometry data, we can separate a population’s CV into two components: the *intrinsic* and the *extrinsic* contributions. The intrinsic CV is the
true CV of the bead standards; that is, the actual variations in bead diameter. This can also be considered in relation to the measurement metric. For example, part of the intrinsic CV may be the actual variation in refractive index, which in turn relates to an actual difference in measured light scatter intensity. On the other hand, the extrinsic CV is the contribution to the CV resulting from variations in the measurement system, for example flow rate variations, illumination intensity variations, etc. While the true intrinsic CV can never really be known, we can estimate this CV by either the manufacturer’s specifications or by using a ‘gold standard’ measurement, such as a commercial cytometer, and comparing our own results to this metric.

The population CV is far more telling of a cytometer’s performance than any single-application result, for example it’s ability to distinguish beads and cells, or some similarly arbitrary choice. In terms of application-specific tests, the white blood cell differential is perhaps the only other clearly meaningful result that can be presented, as this is a widely understood and highly relevant application of light scattering in flow cytometry.

2.7. Summary

In this Chapter, a number of important developments were made to enable the design, fabrication, and testing of the devices that will be presented in the next two Chapters. A materials system was chosen and characterized to allow for design and fabrication of optical devices and devices with integrated slab waveguides. In order for optical systems to be integrated alongside fluidic systems, a practical method of
creating such systems must be devised. Cryogenic etching was shown to be one such method, facilitating the creation of robust, optical-quality molds for both the optical and fluidic features in a reliable, monolithic fashion. In addition, the methods of fabricating and testing microfluidic devices with integrated optics were outlined in the remainder of this Chapter. This work all forms the basis for the devices and results that will be presented in Chapters 3 and 4.

References


Chapter 3

Fluid-Filled Lenses in Polymer Microfluidic Devices

This chapter describes the development of a system for creating fluid-filled lenses in polymer-based microfluidics, showing the results of two such devices, including a microfluidic cytometer employing integrated lenses. The goal is to make strides towards the creation of a robust, integrated optofluidic chip for light scattering measurements in flow that can ultimately replace the bulk optics and fluidic systems to enable the creation of a compact, low-cost, potentially portable flow cytometer.

In Section 3.1, the design considerations for such fluid-filled lenses are discussed, including some details of the fabrication of the slab waveguide and the integrated lenses. Section 3.2-3.3 discuss the design and testing of the first proof-of-concept device to demonstrate the feasibility and performance of our system of designing and fabricating integrated liquid-filled lenses. Section 3.4 demonstrates the first microfluidic flow cytometer with integrated liquid-filled lenses for illumination and the collection of extinction and light scatter signals. After discussion of the results
obtained from this device, Section 3.5 discusses the future outlook for this work, including some of the practical considerations that ultimately led to the development of the exclusion-based approach to the issue of localization in integrated optical systems, as discussed in Chapter 4.

3.1. Design of Integrated Lenses and Slab Waveguides

The development of integrated fluid-filled lenses in an effort to address the issues discussed in Chapter 1: the need for a means of shaping the light path to allow for both uniform single-sample excitation as well as localized light collection. Monolithically-fabricated lenses allow for integration of the optical system into the microfluidic chip, creating a system in fixed alignment to the microfluidic channel. As discussed in Chapter 1, a few forms of integrated lenses had been previously demonstrated [1-3]. In this work we focus on solving the two major problems observed with other approaches: (1) substantial reflective (Fresnel) losses due to the high refractive index contrast and inability to anti-reflection coat (Section 3.1.3) and (2) substantial loss of light in the direction perpendicular to the chip due to the cylindrical nature of integrated lenses (Section 3.1.3). Slab-waveguided, liquid-filled lenses offer a solution to both of these issues, offering a much more practical means of including such optical elements.

3.1.1. Materials Considerations

As mentioned above, previous demonstrations of integrated lenses typically possess a high refractive index contrast, such as an air/PDMS interface [1] or an
SU8/air interface [3]. Such high index contrasts allow for high-powered (fast) lenses, which can help to keep the total system track length lower. Unfortunately for such tiny, embedded lenses, a clear tradeoff exists between lens power and reflective losses due to a lack of anti-reflection coating. Fresnel reflection losses at near-normal incidence can be calculated as

$$R = \left( \frac{n_1 - n_2}{n_1 + n_2} \right)^2$$

(3.1)

The above-described loss is for a single surface; such losses rapidly increase as additional surfaces are added. Figure 3.1 shows a plot of Fresnel losses as a function of refractive index contrast for an optical system consisting of 1-4 lenses. The proposed liquid-filled lenses will have an index contrast of 0.27, while air/PDMS lenses will have an index contrast of 0.4, and air/SU8 lenses will have an index contrast of 0.6 or larger. For systems with more than a single lens, the benefit of using a low index contrast materials system quickly becomes obvious. From Fresnel reflection losses alone, a two-lens air/PDMS system will have higher losses than a liquid/PDMS lens system, even if that lower-powered system requires twice as many lenses to accomplish the same results. These losses do not take into account scattering losses based on surface roughness, which would even further exacerbate the issue. Thus the use of a low-powered lens system will generally be a necessary tradeoff to maintain acceptable reflective losses.
Figure 3.1 Fresnel reflection losses as a function of refractive index contrast for systems consisting of 1, 2, 3, or 4 lenses.

The fluid-filled lenses detailed in this chapter employ commercially-available optical-quality fluids to create a moderate index contrast (SL-5267, Santolight) [4]. The dispersion curve for this fluid is shown in Figure 3.2. Later work further demonstrated the feasibility of using a more stable, lower refractive index optical gel (LS-5257, Nusil) [5], as well as the feasibility of using a highly stable polymer-filled (i.e. solid) lens of a similar refractive index (LS-6257, Nusil) [6].
Figure 3.2 Dispersion curve for SantoLight SL-5267, the optical fluid used to fill the integrated lenses [4].

3.1.2. Optical Simulations: Beyond the Paraxial Approximation

Imaging systems are typically designed in the paraxial regime, where sources are much smaller than the lens aperture and located far from the lens. When working on the scale of microns to millimeters, however, light exiting a multimode waveguide (or even a single mode waveguide) can no longer be approximated as a point source. Similarly, light scattered or emitted from samples on the scale of several microns are also not well-approximated as a point source at such length scales. Thus to design a quality paraxial imaging system for this sort of source, we would need to extend to
track lengths (and lens diameters) to much greater dimensions than would be desired for a compact lab-on-a-chip device. The systems created in this work are thus non-paraxial systems.

The goal of illumination systems is to create a small illumination volume of relatively uniform intensity. Complete collimation is both difficult to achieve and not fully necessary; angular divergence of ±3-4° is acceptable for this device. The goal of light collection systems is to collect light at the location and with the numerical aperture appropriate for the collection parameter chosen (e.g. FSC, SSC, etc). Collection systems are created to have a long focal distance (low NA), and the collection waveguides will be placed at the location where the diameter of the ray bundle has decreased down to 50 µm, allowing it to fit inside of the waveguide.

Ray-tracing simulations are used to design all of the lens-based optical systems involved in this work (Code V, Optical Research Associates). For simplicity, basic lens systems are laid out in only two dimensions, as the lenses employed are all cylindrical. Three-dimensional simulations are considerably more computationally intensive and do not give any information useful for designing the system (however these simulations can yield useful quantitative information for analyzing the final system).

Waveguide illumination sources are simulated as an array of point sources spanning the diameter of the waveguide (in this work, 50 µm), with each point source possessing the NA characteristic of the materials chosen for the core and cladding
For the device in this chapter, the in-plane numerical aperture of the waveguides is 0.12 (±4.8). Lens systems were then designed to shape the emitted light such that at some location the diameter would be relatively small (~20-50 µm) and the majority of incoming rays would approach at low (<5°) angles.

Collection systems were designed by first creating a point source with the intended numerical aperture of collection, and then creating a system to relay that light to a spot with the same (or smaller) diameter as the integrated waveguides (50 µm), with an incidence angle no larger than that which would be permitted by the waveguide NA (i.e. 4.8° from normal). Systems of 2 lenses were laid out and then optimized (manually or automatically) to allow the desired rays to satisfy total internal reflection (TIR) requirements within the waveguide.

### 3.1.3. Development of an Integrated Slab Waveguide

While integrated lenses can be used to control light in the plane of the device, the creation of optics would result in significant light losses if created by typical microfluidic device fabrication methods. As noted before, each feature created by soft lithography is two-dimensional, thus the integrated lenses are cylindrical (rather than spherical). As a result, the lenses do not act on the z-component (vertical component) of the light propagation vector. As stated before, light exiting the integrated waveguide is diverging (both in the plane of the device and vertically), and thus without some intervention much of this light will be lost while traveling in the device.
body. This is especially problematic when integrating lenses, as the transit distances become much longer than those of simple waveguide-only devices.

This concern has been recognized in the literature in the past; however generally in work involving integrated lenses either no solution was proposed to remedy the issue [1] or the proposed solutions were lacking in robustness [3]. The desired outcome for this work was to create a slab waveguide core, a region sandwiched between lower-index cladding regions and capable of incorporating higher-index embedded waveguides for illumination and collection. This requires knowledge of at least the relative refractive index of each material involved, and preferably knowledge of the dispersion curve. Furthermore, the approach needed to be consistent with typical PDMS mold-fabrication approaches to microfluidics.

The slab waveguide fabrication method devised for this work involved spinning a thin layer of moderate-index polymer onto the device mold, then pouring a thick low-index PDMS layer on top of the slab waveguide to create the upper cladding. Bonding to another low-index layer to create the bottom cladding layer and seal the channels would finish the device. Initially each of the two sides of the device had a slab waveguide region, however a single slab waveguide on the featured side of the device was equally adequate for confinement. A third, high refractive index PDMS would be used to fill the waveguides. To create a truly robust and integrated device, it must be possible for each of the three materials to bond to one another, suggesting that
we work within the PDMS family of polymers (it was this choice dictated the aforementioned lens material choices).

There are two major methods of bonding PDMS to PDMS: cure-bonding and surface activation bonding. Surface activation bonding is used to bond two fully-cured pieces of PDMS to one another. Cure bonding is the process of either bonding either two partially-cured pieces of PDMS to one another or bonding a cured portion of PDMS to an uncured or partially cured portion of PDMS [7]. Bonding liquid prepolymer to cured PDMS can give unreliable results. Because the slab waveguide would be subjected to fluidic pressure, even localized delaminations would not be acceptable. On the other hand, surface activation bonding could prove difficult, as the upper surface of the spin-coated slab waveguide would not be entirely flat. Thus the ability to cure-bond was extremely helpful to the creation of the slab waveguide via a reliable, relatively simple process. The slab waveguide PDMS was spin-coated onto the silicon mold master, partially cured for a short time (~10 minutes), and then covered in a thick layer of the cladding prepolymer. The partial curing of the slab waveguide was necessary to ensure a clean step in refractive index. The result of this process is shown in Figure 3.3 a robust interface that demonstrated a clear refractive index change.
Figure 3.3 Microscope image of a slab waveguide, viewed from the side. Upper layer is low index, middle layer is high index, and darkest lower layer is just air (slab has been cut open for imaging).

The slab waveguide is created from a custom mixture of Sylgard 184 (Dow Corning; see Section 2.1) to yield a refractive index of $n_d=1.415$, just between the index of the waveguide cores ($n_d=1.42$) and the device cladding ($n_d=1.41$). It was found that ‘priming’ the mold with liquid prepolymer PDMS mixed in the typical mixture ratios was often necessary to prevent the custom-mixed PDMS from bonding to the silicon wafer itself. This effect appeared to be independent of silanizing treatment of the silicon mold master, perhaps the result of the atypical mixture ratio employed in the custom material. After only a single ‘priming’ (curing PDMS on the wafer, then peeling off), a mold was found to be amenable to use with the custom polymer for an indefinite number of molding events.


A device with a single lens was designed, fabricated, and tested to demonstrate the concept of a fluid-filled lens in PDMS. This device demonstrated the first PDMS-PDMS slab waveguiding structure to enable the use of longer track lengths, permitting
the inclusion of two-dimensional lenses in microfluidic devices. Results from this device were utilized to adjust measured values for the refractive index of the customized PDMS mixture used in the slab waveguide. This would ensure that optical simulations for later, more complex optical systems would be correct.

3.2.1. Device Design and Layout

The proof-on-concept device was designed using Code V. A schematic of the resulting design is shown in Figure 3.4. Light is coupled onto the device from a waveguide (nd=1.42; Gelest OE42). Upon exiting the waveguide, it enters the slab waveguide (cladding: nd=1.41; Gelest OE41, and core: nd=1.415; Sylgard 184 custom blend, Dow Corning). The light diverges (expands) and is incident upon the fluidic lens (nd=1.67; Nusil LS-5267). The lens is used to focus the light into the fluidic channel. The lens has an aperture of 1 mm and a width of 600 µm. The lens is spherical, with a radius of curvature of 690 µm.
The above-described development of a method of integrating a very thin (~50 µm) slab waveguide into the device fabrication process was critical to the practical inclusion of integrated lenses. The resulting numerical aperture in the vertical dimension of the slab waveguide is 0.12. The optical system track length (waveguide facet to fluidic channel) employed in the simple proof-of-concept device is 17.8 mm. Without a slab waveguide, more than 95% of the light would have been lost due to divergence in the vertical dimension. Complete optical systems (illumination and collection) would likely involve even longer track lengths, making them incredibly
impractical for use without the development of this sort of robust, integrated slab
waveguide for vertical light confinement.

3.3. Demonstration of Focusing

The proof-of-concept device was fabricated in PDMS by the methods
described in Section 2.2 using a mold created by the SU-8 process. After filling and
curing the integrated waveguide, the device end was sliced off with a razor blade to
allow access to the waveguide. The result is a rather rough entrance facet, however,
this proved sufficient for the observation of focusing in the device. A bare fiber optic
(SMF2850, Corning) was aligned to the waveguide facet to couple light into the
device for observation. A 5 mW, 650 nm semiconductor laser was used to illuminate
the device. A free-space fiber coupling system coupled laser light into the 50 µm core
of a portion of bare multimode fiber. The other end of the fiber was manually aligned
to the exposed waveguide facet.

Ideally, no light should be seen when viewing the device from above, making
observation of focusing in the device rather difficult. Only orthogonally-scattered
light will be viewed from above. Because of the close core diameter (50 µm diameter
circular cross-section vs 50 µm x 50 µm square cross-section) and a numerical
aperture mismatch between the fiber optic and the integrated waveguide (0.22 vs 0.12),
a significant portion of the illumination light entered the device without coupling first
into the waveguide. The lens will not focus this light, however this light does create
difficulties in clear, direct observation of the focused light. Light both from the
waveguide and from around the waveguide would be incident on randomly located scattering centers, especially at the rough fluidic channel wall, reducing the clarity of the observation.

Alumina particles (diameter ~0.3 µm) were utilized to enable higher-resolution visualization of the focusing effect of the integrated fluid-filled lens [9]. A device was created in which 0.3 µm alumina particles were injected into the microfluidic channel. As shown in Figure 3.5, this allowed visualization of optical localization within the fluidic channel, and provided a means of measuring the diameter of the focus at the microfluidic channel (~150 µm). These measurements can then be quantitatively compared to simulation results.

![Figure 3.5 Alumina particles in the microfluidic channel provide a quantitativo means of characterizing the effects on the integrated fluid-filled lens. © 2006 IEEE. [9].](image)

By visualizing the light path and the focal spot, we can both confirm device performance and also verify simulation parameters. While the dispersion curve of the
fluid filling the lens (LS-6257, Nusil) is published, the dispersion curve for the custom blend of PDMS (Sylgard 184, Dow Corning, see Section 2.1) was not verified. Measurements with a simple refractometer gave a good approximation to the curve, however the results shown in Figure 3.5 provide a means to verify this data and adjust accordingly. The measured diameter of the light beam at the first sidewall of the microfluidic channel was used to adjust the value of nd for the custom mixture. The original design was intended to bring the light to a focus in the fluidic channel. In the experiment, overfocusing is observed (the focus occurs beyond the microfluidic channel). The simulation was readily corrected by adjusting the manner in which the waveguides were simulated (originally as a point source an appropriate distance from the waveguide facet to create a 50 µm wide beam; now an array of point sources as discussed prior). Small changes were also made to the dispersion characteristics of the materials.

Another test device was created to verify the refractive index correction. Alumina particles were suspended in methanol, and before the device was bonded a small volume of this suspension was manually placed between the lens and the fluidic channel using a pipette. The result is high-density area of tiny scattering centers, predominantly confined to the area between the lens and the fluidic channel. These scattering centers again allow visualization of the light path from above (via microscope). This enables direct comparison of the experimentally-observed beam shape and the simulated effects of the lens. Figure 3.6(a-b) shows a comparison
between the experimental results and predictions from the adjusted simulation. This visualization is made possible by the alumina particles shown in Figure 3.6(c).

![Figure 3.6](image)

**Figure 3.6** Experimental images (a) can be compared with simulated ray trace results (b) by depositing alumina particles between the lens and the fluidic channel. Adapted figure copyright 2006 American Institute of Physics, reprinted with permission from [8].

Lastly, the practical utility of the light localization was demonstrated by flowing polystyrene beads through the microfluidic channel and monitoring the light intensity scattered at 90° from the illumination path. Figure 3.7 shows a series of images taken by a CCD camera as a 5 µm bead passes through the illuminated area of the channel. While passing through this area (Figure 3.7 a-c), the bead is plainly illuminated, scattering light perpendicularly towards the CCD sensor. After passing through this area, however, the bead is only dimly visible (Figure 3.7 d). This localized excitation is what we desire for flow cytometry.
Figure 3.7 Images of 5 µm polystyrene bead scattering light as it traverses the microfluidic channel. The particle is visible while it passes through the focus of the lens (a-c), but it is only barely visible outside of this region (d-e). Figure copyright 2006 American Institute of Physics, reprinted with permission from [8].

3.4. Multi-lens Flow Cytometer

Having developed a platform for integrating lenses alongside microfluidic channels, the next goal was to create an entire optical system on a chip for performing both illumination and detection in flow cytometry. A number of strong advances were made in this area, however at the same time a number of potential downfalls of this approach were elucidated throughout this work. Ultimately, it became clear that lenses may not offer the ideal approach to the optical system. This work is presented both to detail some of the significant accomplishments that were made in developing
the capabilities to create moderately complex optical systems in an integrated fashion as well as to benchmark the performance of such systems to better describe the subsequent change in direction that this work would take. Shows an image of the device presented in this section.

![Microfluidic chip](image)

**Figure 3.8** Microfluidic chip with integrated fluidic channels, polymer-filled waveguides, and fluid-filled lens systems. In this device the features are black for improved visualization.

### 3.4.1. Lensed Device Fabrication

Again, our microfluidic cytometry chip (now with an integrated optical system for flow cytometry based on fluid-filled lenses) was fabricated in PDMS. The device is comprised of a fluidic system for sample flow and an optical system for sample illumination and interrogation at three locations: extinction, large-angle forward scatter, and backscatter (described below). As before, light travels in the thin polymer core region of a slab waveguide (\(n_d=1.415\); custom-mixed Sylgard 184, Dow Corning), which is cladded by two thicker polymer regions (\(n_d=1.41\); Gelest OE41). The thin core region contains all of the monolithically fabricated features of the device,
such as lenses, channels, and waveguides (nd = 1.42, Gelest OE42; nd=1.67, Nusil LS-5267), while the thick cladding regions restrict light travel to the core region and make the device easier to handle.

3.4.2. Device Layout

The device layout is shown in Figure 3.9. The device includes an illumination segment, directly followed by a detection segment for extinction (EX). Additional detection segments are located at ±15° from the extinction detection line for the collection of scattered light (15° scatter). High numerical aperture, large-angle scatter detection segments are located at 160° in either direction of the forward detection line, but were not used in this work. Each of the 6 resulting waveguide ends has a 50 μm diameter waveguide extending from it. Each waveguides centrally intersects a roughly 100 μm diameter fiber sleeve (see Section 2.3.2) which terminates into one of two large rectangular fiber entrance portions (one on either side of the device), each of which is shared by 3 fiber sleeves.
Each side of the device layout includes channels connecting the lens spaces to allow them to be simultaneously filled with lens fluid, which terminate in two reservoirs (top and bottom of the device) for channel access after fabrication. The waveguides are similarly connected to allow them to be filled with a higher-index PDMS. The device also has a fluidic channel, flanked to either side by sheath flow channels, both at the top and bottom of the device (so that the device can be run in either direction). All six of these access points terminate in reservoirs, and the central (cell flow) line reservoirs taper into the channel to facilitate flow. Figure 3.10 shows the ability of the sheath flow fork structure to localize sample flow to the center of the flow channel.
Figure 3.10 Demonstration of hydrodynamic focusing using a forked structure. Dye is flowing through the center (sample) channel, while water flowing through the two side (sheath) channels confines the flow to the center of the channel © 2006 IEEE [10].

3.4.3. Optical Design

The device was designed to operate using a 630 nm illumination source. In testing, a brighter, lower-noise source was required. Ultimately an 830 nm source was used for testing.

Figure 3.11 Optical simulation showing illumination line design. The illumination light is coupled onto the chip via the waveguide (left). Two lenses are used to focus the light to a small area in the microfluidic channel (right) for single-sample illumination.

Figure 3.11 shows the optical simulation used to design the optical system to shape the illumination light. In the original design, the illumination line begins with a
50 µm diameter waveguide with an NA (at 630 nm) of 0.18. Two lenses are used to focus the light down to create an interrogation zone beam width of approximately 20-30 µm, enabling single-sample detection. When operated at 830 nm, this interrogation width expands to approximately 50 µm.

Table 3.1 Optical system specifications for illumination line.

<table>
<thead>
<tr>
<th>Surface #</th>
<th>Name</th>
<th>Y Radius</th>
<th>Thickness</th>
<th>Glass</th>
<th>Y-Semi Aperture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dummy Surface</td>
<td>Inf</td>
<td>0.000</td>
<td>Air</td>
<td>n/a</td>
</tr>
<tr>
<td>2</td>
<td>Waveguide Source</td>
<td>Inf</td>
<td>0.181</td>
<td>1.4150:63.00</td>
<td>0.025</td>
</tr>
<tr>
<td>3</td>
<td>Waveguide Exit to PDMS</td>
<td>Inf</td>
<td>0.500</td>
<td>1.4150:63.00</td>
<td>0.025</td>
</tr>
<tr>
<td>4</td>
<td>Lens</td>
<td>0.5</td>
<td>0.300</td>
<td>1.6679:21.59</td>
<td>0.3000</td>
</tr>
<tr>
<td>5</td>
<td>PDMS</td>
<td>-0.5</td>
<td>1.750</td>
<td>1.4150:63.00</td>
<td>0.3000</td>
</tr>
<tr>
<td>6</td>
<td>Lens</td>
<td>0.55</td>
<td>0.300</td>
<td>1.6679:21.59</td>
<td>0.3000</td>
</tr>
<tr>
<td>7</td>
<td>PDMS</td>
<td>-0.6</td>
<td>2.450</td>
<td>1.4150:63.00</td>
<td>0.3000</td>
</tr>
<tr>
<td>8</td>
<td>Fluidic Channel Wall</td>
<td>Inf</td>
<td>0.025</td>
<td>Water</td>
<td>0.0250</td>
</tr>
</tbody>
</table>

Figure 3.12 shows the optical simulation used to design the collection lines. For these lines, a two-lens system is designed to collect light emitted at ±7° from a point in the microfluidic channel (located along the system optical axis) and relay it to the collection waveguide. The collection lines were located along the illumination axis for extinction collection, as well as at locations rotated ±15° about the sample point (see Figure 3.9). The total solid angle of collection is approximately 0.04 sr. The system parameters can be found in Table 3.2.
Figure 3.12 Optical simulation showing detection line design. Light originating from the fluidic channel (left) is collected and relayed to the collection waveguide (right) via two lenses. The detection lines are placed along the interrogation beam axis (for extinction collection at 0°) and at ±15° from the optical axis (for scatter collection).

Table 3.2 Optical system specifications for detection lines.

<table>
<thead>
<tr>
<th>Surface #</th>
<th>Name</th>
<th>Y Radius</th>
<th>Thickness</th>
<th>Glass</th>
<th>Y-Semi Aperture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cell/Channel</td>
<td>Inf</td>
<td>0.000</td>
<td>Air</td>
<td>n/a</td>
</tr>
<tr>
<td>2</td>
<td>Channel Wall</td>
<td>Inf</td>
<td>0.025</td>
<td>Water</td>
<td>0.3000</td>
</tr>
<tr>
<td>3</td>
<td>PDMS</td>
<td>Inf</td>
<td>2.450</td>
<td>1.4150:63.00</td>
<td>0.3000</td>
</tr>
<tr>
<td>4</td>
<td>Lens</td>
<td>0.6</td>
<td>0.300</td>
<td>1.6679:21.59</td>
<td>0.3000</td>
</tr>
<tr>
<td>5</td>
<td>PDMS</td>
<td>-0.55</td>
<td>1.750</td>
<td>1.4150:63.00</td>
<td>0.3000</td>
</tr>
<tr>
<td>6</td>
<td>Lens</td>
<td>0.5</td>
<td>0.300</td>
<td>1.6679:21.59</td>
<td>0.3000</td>
</tr>
<tr>
<td>7</td>
<td>PDMS</td>
<td>-0.5</td>
<td>0.650</td>
<td>1.4150:63.00</td>
<td>0.3000</td>
</tr>
<tr>
<td>8</td>
<td>Waveguide Entrance</td>
<td>-0.5</td>
<td>0.250</td>
<td>1.4150:63.00</td>
<td>0.2500</td>
</tr>
</tbody>
</table>

3.4.4. Test Conditions

To characterize the microfluidic cytometer’s performance relative to that of a benchtop cytometer, a suspension of polystyrene beads of known diameter is used. A solution of 4.8 µm polystyrene beads (Bangs Labs) was run through both our own microfluidic cytometer as well as a commercial cytometer (FACScan, Becton Dickinson). For the benchtop cytometer, a few drops of bead solution were suspended in FACSFluid (Becton Dickinson) and run at low speed (12 µL/min sample flow rate) through the cytometer.

For the microfluidic cytometer, beads were suspended at roughly 105+ bead/mL in a 15% sucrose solution made with 0.22 µm-filtered DI water. The
sucrose solution (~12.5° Bx) is used to density-match the solution to the polystyrene beads (density ~ 1.05 g/cm³). The solution was sonicated for homogeneity and run through the micro-cytometer at 1 μL/µl flanked by 0.22 μφιλερεδ FACSFluid from the two sheath channels at 11 μL/µl total flow rate. Fluid flow is accomplished using two syringe pumps (NE-1000, New-Era Pump Systems), one for the sample flow and one for the two sheath flow inlets. The channels are interfaced with silicon tubing (0.020” inner diameter and 0.083” outer diameter, Cole-Parmer) via the metal tubing removed from a 23 ga luer stub (McMaster-Carr).

During testing, illumination is provided by a 150 mW, 830 nm laser diode (DL8032-001, Sanyo) powered by a diode laser driver (LDC-3724, ILX Lightwave) in a temperature controlled mount (Series 700, Newport) whose output was free-space coupled into a 50/125 μμ optical fiber via an objective (F-L10B, Newport). This fiber is attached to the illumination port fiber of the device using a mechanical splice (multimode ULTRASsplice, Siemens). Signals from both the extinction line and the 15° scatter line were detected via off-the-shelf PiN detectors (DET 30A, Thor Labs). The signals are sent to amplifiers (PDA 6424, ILX Lightwave) connected to a BNC adaptor (BNC-2110, National Instruments) then recorded via data acquisition card (PCI-6251 M-Series, National Instruments) at an acquisition rate of 5 kHz. Flow focusing is visually confirmed via top-mounted CCD. Acquired data is first processed by custom Matlab routines, then analyzed by commercial cytometry software (FlowJo, TreeStar Inc.). See Section 2.6 for more details.
3.4.5. Test Results

Figure 3.13 Sample of data from microfluidic cytometer after Matlab processing. Scatter peaks and extinction dips are marked with squares and circles, respectively. The resulting event list is analyzed by FlowJo.

Figure 3.14(a) shows a dot plot of 15° scatter intensity vs. the absolute value of the extinction intensity drop for each event. Figure 3.14(b) shows a similar dot plot for the same beads when run through the FACScan commercial flow cytometer. The corresponding histograms can be found in Figure 3.16. FlowJo used the data in these plots to calculate the coefficient of variation (CV) for each parameter, a measure of the homogeneity of the sample and resolution of the device.
Figure 3.14 Scatter plots show results from 5 µm beads for (a) our microfluidic device and (b) a commercially available device. The multiple populations seen on the commercial device are doublets, triplets, etc.

Figure 3.15 Histograms of intensities from microfluidic device for (a) extinction signals and (b) 15° scatter signals.
3.4.6. Performance Analysis

The measured parameters for each cytometer cannot be directly compared. The standard configuration for commercial cytometers is discussed in Section 1.2.2. In brief, ‘forward scatter’ refers to light scattered in the forward direction (nominally 2-15° from the forward direction). Light from the illumination beam is prevented from reaching the detector by a beam stop. ‘Side scatter’ refers to light scattered around 90° from the forward direction (orthogonal light scatter). The parameters measured in the microfluidic device, however, are extinction (the intensity of light that is blocked) and 15° scatter.

Classically, extinction measures only the illumination beam (in systems that measure extinction there is no beam block). As beads or cells pass through the beam they block light, and the resulting dip is seen on the detector. The resulting measurement is similar to slit-scanning due to the small illumination beam size and low beam divergence, thus the pulse width becomes the meaningful measurement [11].
In our microfluidic system, the illumination beam is approximately 50 µm and light is collected over a 15° angular field of view, thus the collected light is both scattered light and illumination beam (the effect of the scattered light often visible but small due to the significantly higher intensity of the illumination beam). With this arrangement, pulse magnitude, rather than pulse width, is the more meaningful parameter. The light scatter line in the microfluidic cytometer consists of light collected over a 15° range centered at 15° from the forward direction. This collection range has a portion that is expected to look like forward scatter in a benchtop device (7-15°) and a portion that might be expected to bear more resemblance to large-angle scatter (15-22°). In summary, some care must be taken when comparing individual measured parameters between the commercial and microfluidic devices in order to properly understand the results.

The most quantitative means of comparing the device performance is by comparing the coefficients of variation (CVs) for each sample population run through the two devices. This data can be found in Table 3.3. The CVs are determined both by the size distribution of the beads (intrinsic CV) and the uniformity of measurements made by the device (device CV). In this work, the CVs measured by the FACScan cytometer are intended to serve as benchmark CVs for the bead sample used. For completeness, the robust CV is also displayed. This is defined by FlowJo as the spread which encloses the central 63% of the events, or roughly one standard deviation. This statistic will be affected less by outliers.
Table 3.3 Coefficients of variation (CVs) for identical bead populations, as measured by the microfluidic device and by the commercial device.

<table>
<thead>
<tr>
<th></th>
<th>Microfluidic Device</th>
<th>CV</th>
<th>Robust CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extinction</td>
<td>9.50%</td>
<td>8.49%</td>
<td></td>
</tr>
<tr>
<td>15° Scatter</td>
<td>14.90%</td>
<td>12.50%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FACScan Cytometer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward Scatter</td>
<td>10.50%</td>
<td>8.70%</td>
<td></td>
</tr>
<tr>
<td>Orthogonal Scatter</td>
<td>15.10%</td>
<td>16.20%</td>
<td></td>
</tr>
</tbody>
</table>

The bead manufacturer quotes an intrinsic CV of approximately 7.8% [11]. The FACScan measured a forward scatter CV of 10.5% and a side scatter CV of 15.1%. The larger side scatter CV is not too surprising, given the sensitivity of large-angle scatter to small variations in size for symmetric particles [7]. The lower side scatter intensity relative to the forward scatter is expected from Mie Theory.

As shown in Table 3.3, the microfluidic cytometer measured a CV of 9.5% for extinction and 14.9% for 15° scatter. The 15° scatter CV is a bit higher than the forward scatter CV of the FACScan, but not larger than the side scatter CV. The detection line geometry may contribute to the larger CV, and lack of vertical flow focusing may also play a part. The CV values compare very favorably with typical values of 25-30% from similar scattering experiments performed by microfluidic flow cytometers [3,13]. Our significantly reduced scattering CVs demonstrate the sensitivity of the device afforded by the inclusion of an optical system.
There are several notable differences between the dot plots (see Figure 3.14) generated by the FACScan and the microfluidic flow cytometer. First, there is a clear correlation between the extinction and the 15° scatter measurements ($r=0.72$). This strong correlation is clearly not present between the forward scatter and side scatter measurements of the FACScan. Since extinction intensity would be expected to be an indicator of bead size and the 15° scatter has some significant forward scatter character to it (also an indicator of size in the case of beads), this is to be expected. It does indicate that in future devices it will be preferable to modify the design such that the measured parameters yield more orthogonal data sets.

It is also noted that the microfluidic flow cytometer exhibits a much lower throughput than the FACScan. While benchtop cytometers achieve throughputs of 10,000 beads/sec, in this work our cytometer was operated at a throughput of less than 10 beads/sec. In this work, our illumination beam was rather large compared to commercial devices (~50 µm vs. ~20 µm). This forced the use of a low-density bead suspension to prevent multiple bead passings (doublets). Subsequent designs should work to improve upon this constraint. Data acquisition rate (and thus flow rate) was also kept low to reduce the size of the data sets created while achieving sufficiently long data runs to yield statistically meaningful results; however, higher acquisition rates would also allow for higher throughput.
3.5. Conclusions and Future Outlook

In this Chapter, we have demonstrated two highly-integrated devices employing on-chip fluid filled lenses to create integrated optical systems. The creation of integrated slab waveguides and the use of fluid-filled lenses with a moderate refractive index contrast are the two key advances that make practical such systems of integrated two-dimensional optics. The first device created was used as a test vehicle for these technologies, and helped to validate our optical modeling. The second device created was a two-parameter scatter-based flow cytometer. Results from the device for 5 µm polystyrene beads show CVs a bit higher than those measured by a benchtop flow cytometer, but substantially lower than those measured by other microfluidic devices with integrated optical systems.

The devices presented in this Chapter demonstrate simple, monolithic integration of optical systems alongside microfluidic systems. Optical systems for both illumination and collection are created in fixed alignment with the fluidic systems, offering the possibility of a robust, highly functional optofluidic chip with reduced alignment concerns. Optical elements in the collection systems can improve device SNR, improving collections CVs for light scatter and potentially for fluorescence as well. While this work made significant advances in integrated optics for flow cytometry, several key issues with this approach remain to be addressed in future work.
3.5.1. Three-Dimensional Flow Focusing

The cytometry device presented, like nearly all microfluidic cytometers demonstrated to date, could greatly benefit from the use of three-dimensional flow focusing. While light collection systems in microfluidics certainly have SNR issues that need to be addressed, there is little doubt that a significant portion of the CV increase in microfluidic devices (relative to commercial devices) is due to insufficient flow control. This had been a secondary concern for the work demonstrated in this Chapter; however, future devices will certainly require additional consideration of this matter.

3.5.2. The Need for True FSC and SSC

Results from the cytometry device in this chapter demonstrate the need for the creation of optical systems that truly address the interests of the end user: the collection of forward scatter and side scatter. The device in this chapter, like many other microfluidic cytometers, did collect angular light scatter, but at non-traditional locations. For the cytometry community to view these devices as potentially useful (let alone to actually consider using them), there must be a clear way to compare the performance of these devices to the performance of the already-used devices. The best means of accomplishing this benchmarking is to look both qualitatively and quantitatively at the resulting scatter plots. Unless the detection parameters are very similar, such comparisons will be impossible. Each potential application would have to be considered individually, as scattering varies from one sample type to another. For example, if the microfluidic device is able to resolve different bead populations,
based on 15° and 45° light scatter, that does not guarantee that it can resolve different white blood cell populations. Researchers and clinicians know that these populations can be resolved using forward and orthogonal light scatter, however they will generally have no idea what to expect from 15° and 45° light scatter. Most end users are not involved in the pioneering research to discover means of differentiating cell populations based on light scatter; they simply wish to use this knowledge to run their assays. For the sake of benchmarking performance and attracting the attention of future end users, the microfluidic flow cytometry devices we create should deliver results that look as similar as possible to the results from today’s commercial devices. In future work (see Chapter 4), the inclusion of forward scatter and orthogonal scatter will be a primary concern in design geometry.

3.5.3. Persistent Concerns with Surface Reflections

Perhaps the greatest problem associated with integrated lenses remains the issue of surface reflections. In this work, fluid-filled lenses were used to reduce the index contrast such that these reflections would be significantly lowered relative to other approaches. The use of a solid lens, rather than a fluid-filled lens, would be more practical. We demonstrated successful polymer filling of lenses, thus, upon heat curing, creating a solid lens [14]. Even with these considerations, however, reflections due to refractive index contrast alone remain a concern. Ideally, the interfaces in the device would be as low contrast as possible; however, as stated before this results in low-powered optics, necessitating very long track lengths. The goal of our integrated optical systems for scatter collection is, again, to collect light from a localized area (i.e.
the cell, not the surrounding regions). In Chapter 4, we consider a method of localizing light collection without the need for multiple interfaces with significant refractive index contrast.

Acknowledgements

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References


Chapter 4

A New Approach to Integrated Optics: Custom Waveguides and Light Blocking Elements

In this chapter, I will demonstrate a microfluidic flow cytometer that achieves localized light collection without the use of lenses. In this device, an exclusion-based approach was taken to the optical design; that is, while including the signal was certainly a main goal of the design, the focus was on excluding noise from the areas surrounding the sample. The exclusion is affected by the exploitation of both shallow-incidence reflection and refraction to redirect noise and prevent coupling to the collection waveguides. This new design approach, used to create two new devices, will be discussed in Section 4.1. Fabrication and testing of the device are discussed in Section 4.2, and the results are shown in Section 4.3. Section 4.4 considers the
implications and future directions for this work, and Section 4.5 summarizes the work presented in this Chapter.

4.1. Design Approach

Microfluidic chips have a number of unique benefits over traditional bulk optics and fluidics; in this work, the ability to create small features in close proximity to the fluidic channel within microfluidic chips will be exploited. Rather than designing a traditional optical system to perform the task of localized light collection, this work considers ways to use the design flexibility afforded by single-step fabrication to create simpler optical structures to perform these same tasks in new ways. The focus shifts from refractive manipulation to the exploitation of the natural angular selectivity of total internal reflection and the angularly selective loss mechanisms of reflection. The design of these new systems will shift away from optimization of traditional lens-based optical systems, instead turning back to develop a new basic starting geometry for later optimization.

In Chapter 3, I presented microfluidic chips with integrated lenses in order to localize light collection. As shown in Chapter 1, the use of simple fibers or waveguides for collecting light results in a poor signal-to-noise ratio due to the fact that such TIR-based optical elements will intrinsically collect from a cone of light characteristic of the element’s numerical aperture, and the further the sample is from the element the worse the SNR becomes. The problem, then, can be looked at not as an issue of signal collection (indeed previous work clearly demonstrates that signals
can be collected by such simple schemes), but rather an issue of noise exclusion. This is the focus of the design presented in this chapter.

In the signal exclusion approach, the goal is to create a system that can selectively collect or reject light based on its point of origin. There are two main approaches we will take to excluding light: (1) directly blocking, as by absorption and (2) redirecting unwanted light, as by refraction or reflection.

Directly blocking noise is readily accomplished by the use of apertures, baffles, and similar elements. Such elements have been demonstrated in microfluidic devices by filling chambers with ink or other opaque substances to reduce stray light [1]. For this work, another commercially-available PDMS polymer (Sylgard 170, Dow Corning) was employed. This allowed for liquid-filling of chambers, yielding solid-filled components after heat curing. The result is a robust integrated light blocking element. The attenuation capability of this material was measured to be approximately 98% for 100 µm thickness (see Appendix 1)
Direct light blocking is not sufficient to fully perform the task of noise exclusion in the microfabricated device due to a number of geometry constraints. Ideally, a tiny aperture could be placed directly in front of the sample, with the collection waveguide right behind it, and all outside noise would be eliminated. Realistically, the aperture hole is too tiny to mold-replicate well, especially given the resulting aspect ratios the result from the typical feature heights needed (>50 µm). In addition, one cannot practically place the aperture so close to the sample; the sample is located within the fluidic channel, thus the aperture will need to be located a safe distance from the channel wall. Light blocking elements, then, can be used for ‘coarse’ noise exclusion, but some sort of fine-tuning will be necessary.

To give higher spatial resolution to the light exclusion system, refraction and reflection are employed. The idea is to use a combination of (a) the location at which light is incident on an element and (b) the angle at which light is incident on an
element to effectively give information on the location from which the light orginiated (i.e. sample or surrounding), and thus exclude or include the light on this basis. Waveguides are used to collect light, but their shapes will be modified to create a collection system that has a minimal effect on the signal (in terms of possible reflective and scattering losses) while significantly reflecting or redirecting the unwanted light from other areas (i.e. noise). The design consisted of two phases: an ‘inclusion’ design, and the subsequent addition of ‘exclusion’ elements.

![Image of microfluidic cytometry chip with integrated optical system created by exclusion-based design.](image)

**Figure 4.2** Image of microfluidic cytometry chip with integrated optical system created by exclusion-based design.

### 4.1.1. Inclusion Design

In the inclusion design, the locations and dimensions of the collection waveguides were chosen to include light scattered over the angular ranges desired to be collected. Modifications were made to the basic geometry to allow for true FSC and SSC to be collected, which would allow for direct comparison to benchtop devices. A beam dump was included in the design to make possible the collection of FSC
(rather than extinction). Basic light blocking elements (baffles) were included in the design, however the majority of the exclusion approach would come in the next design phase. All features (except for the SSC optic filling line and the flow-focusing chevron features) are created monolithically, and are approximately 60 μm tall.

4.1.1.1. Fluidic Channel Configuration

The flow cytometer is an inherently three-dimensional device; that is, the illumination/FSC line is perpendicular to the SSC line, and both of those lines are perpendicular to the flow stream. This is extremely problematic when trying to replicate the functionality of the device in a two-dimensional architecture (i.e. mold-fabricated microfluidic devices). For this reason, no previous work had demonstrated integrated SSC collection combined with either integrated illumination or integrated FSC collection. In our device, the illumination waveguide is not perpendicular to the flow channel; an 16° offset from perpendicular exists between the structures. The result is that the angle space around the perpendicular from the illumination direction is opened up, allowing for light collection. Thus this small offset allows for the functionality of a three-dimensional structure to be created on a two-dimensional platform.
Three-dimensional flow focusing is known to be of great importance in reducing population variation in flow cytometry, as changes in sample positioning will equate to changes in travel velocity. This effect is increasingly important as sample size decreases. In an attempt to address this issue, the final design ultimately included the typical lateral hydrodynamic flow focusing (as shown in Figure 3.10), and also mimicked the chevron-shaped patterns demonstrated by the Ligler lab for focusing in the vertical dimension (as shown in Figure 4.3b) [3]. As shown in Figure 4.3(a), flowing dye through the sample channel and viewing the device through the side confirms improvements in vertical flow confinement. All branches of the fluidic channel are 50 µm wide and roughly 70 µm tall, converging into a single 100 µm wide channel. The four chevrons structure were approximately 25-35 µm in depth and 50 µm thick, with an edge-to-edge spacing of 70 µm.

4.1.1.2. Illumination Configuration

The configuration of the optical elements is shown in Figure 4.4. A 50 µm waveguide is used to bring illumination light onto the chip. For this chip, 488 nm
illumination would be used. Again, this small change (488 nm instead of the previously-used 830 nm) is quite important, allowing for direct comparison to the commercial flow cytometer. The waveguide dimensions were again chosen to match the core dimensions of the bare fiber that would be used to interface with the waveguide. Because this device involved no lenses, and thus only small distances would be traversed between light guiding elements, no slab waveguide was used. This allowed for a two-material system (Gelest OE41 and Gelest OE42). The result is a larger refractive index contrast for the waveguides, increasing the numerical aperture from 0.12 to 0.17, significantly improving coupling to the fiber optics. Lower NA fibers (0.20 instead of 0.22) were also purchased (GIF50, Thor Labs).

![Figure 4.4](image)

**Figure 4.4** (a) Microscope image of the inclusion device. The fluidic channel (i) is illuminated by the illumination waveguide (ii). The light path (iii) traverses the channel, illuminating the FSC and SSC collection locations (red circles) at from primarily different directions (red arrows). (b) After the channel, the light is confined by the beam dump (iv), allowing the collection of FSC to the left and SSC to the right. The light blocking elements (v) help enable localized light collection.

The illumination beam created by the waveguide is a diverging source, not the collimated illumination light typically desired for flow cytometry. This commonly
occurs in microfluidic devices, however it creates problems when attempting to define angular light scatter collection. In particular, the ‘forward’ direction (generally ~3-15°) for FSC is difficult to discern when the interrogation source has an angular range of ±7°. In an effort to combat this problem, the edges of the interrogation beam are chosen as the locations from which to collect light scatter. At these locations, the angular range of the illumination light is much smaller (approximately 3-6° range), making the definition of the direction of the illumination light much more clear. The directions considered to be the primary direction of the illumination beam at each of the two interrogation locations are shown as arrows in Figure 4.4(a).

It should be noted that this approach does significantly reduce the effective illumination intensity; a large portion of the light is ‘thrown away’ in this configuration, however, it does allow for angularly-resolved light scattering measurements. The other result of this configuration is that the FSC and SSC collection regions are designed to be distinct points on the device, separated by approximately 70 µm. This will result in a minor throughput decrease (< 2x) relative to commercial devices, as typical beam widths in commercial devices are 30-60 µm. This is not a great concern, as no microfluidic cytometer has been demonstrated to date that has been capable of approaching the throughput limits of commercial devices. On the other hand, this split configuration may ultimately enable single-detector light collection, reducing the cost and complexity of the end device.
4.1.1.3. Beam Dump Inclusion

After traversing the microfluidic channel, the illumination beam was coupled into a beam dump to reduce interference with the FSC collection. The beam dump is a slightly lensed waveguide (radius of curvature = 133 µm). This lensing helps to slightly reduce the divergence of the beam, allowing for an 2° taper to the beam dump waveguide. This, in turn, allows for the physical placement of the FSC waveguide by creating enough ‘dead space’ on the chip to allow for a structurally sound wall to exists between the two waveguides. Without this modification, it would not be possible to fit a separate FSC waveguide onto the chip. The inclusion of the beam dump also ensures the removal of the illumination light from the device, reducing possible scatter contributions from this source, which would be much greater in intensity than actual light scattering signals. Lastly, this element also provides a means of blocking or redirecting some light originating from closer to the SSC collection point, further aiding the attempt to create a system of localized light collection.

4.1.1.4. Light Scatter Collection Waveguides

The locations of the light scatter collection waveguide facets were chosen to bring them as far back from the interrogation point as possible while still ensuring collection over their full numerical aperture in the vertical dimension (as no slab waveguide was included in the design). The width of the waveguides was chosen to be just large enough to not restrict the collection of the angular range of light scatter desired. Any larger waveguides would simply collect more noise.
The FSC waveguide was designed to collect light scattered over an angular range of approximately 3-12° from the forward direction at the FSC illumination point. To further ensure the removal of the illumination beam (which would be much higher intensity than the light scatter signal), the axis of the FSC waveguide was rotated by 3°, so that the largest possible angle accepted by the system would be just a bit above the largest angle emitted by the interrogation waveguide, helping to ensure no illumination light would couple into the waveguide. The overall solid angle of collection is approximately 0.03 sr. The front facet is 118 µm in diameter, located 1.032 mm from the interrogation point. The SSC waveguide was designed to collect light scattered over an angular range of 82-98° from the forward direction at the SSC interrogation point. The overall solid angle of collection is approximately 0.06 sr. This waveguide is 209 µm in diameter, and is located 182 µm from the SSC interrogation point. Each waveguide tapers down (<1° angle) to 50 µm to interface with a fiber optic via a fiber sleeve (see Section 2.3).

4.1.1.5. Light Blocking Elements

Some basic light blocking elements were needed to supplement the geometrical light collection constraints imposed by the waveguide placement. As stated prior, these elements were filled with an opaque PDMS polymer to create solid-filled elements to block stray light. Due to their relatively high reflectivity, they were these elements are only suitable for blocking low-intensity stray light (as opposed to, for example, absorbing the illumination beam as in a beam stop for a standard cytometer).
The range of light scattering angles collected by the FSC waveguide was defined by the size, location, and tilt of the collection waveguide. The numerical aperture did not directly limit the collection, but rather dictated the choices for the aforementioned parameters. Some light originating from points to the right of the FSC interrogation point would be directed away from the waveguide due to the presence of the beam dump, which extends lower than the facet of the FSC waveguide. Light originating from the left of the FSC interrogation point, which is the direction in which collection was biased due to the waveguide axis tilt, had no such obstruction. There is a good deal of physical space between the waveguide facet and the fluidic channel in this region, permitting the inclusion of an opaque region to prevent stray light collection from this region. The edge of this light blocking region extends to the edge of the intended collection cone for the FSC waveguide, pulling away as the block approached the facet to allow for a structurally sound PDMS wall between the two features (which are each initially fabricated as empty spaces).

The range of light scattering angles collected by the SSC waveguide was primarily limited by the waveguide numerical aperture, and the size and location were thus chosen accordingly. The waveguide was thus open on both sides to the inclusion of light originating from points other than the SSC interrogation point. Such stray light was far more likely to originate from points to the left of the SSC interrogation point, as this is where the illumination source was located. A light blocking element was included on this side to combat this problem. No such element was included to the right of the waveguide as the physical space between the waveguide facet and the
fluidic channel was rather small, and any such element would have to extend a ways backwards along the device to allow for the creation of a filling reservoir that would not interfere with other elements on the device. The resulting long, thin sidewalls would likely have become failure points, where the filling contents of one element (waveguides, fluidic channels, or light blocking features) could leak into the chambers intended to become other elements.

4.1.2. Exclusion Design

The second design phase involved the addition of exclusion-based optics to the prior inclusion-focused design. The focus of this design phase was primarily on reducing the noise contribution from particles as they passed through the illumination beam between the two interrogation points. This device represents a first pass at redefining the geometry of integrated optics to create simpler, yet fully effective systems.

![Figure 4.5](image.png)

**Figure 4.5** (a) Microscope image of the microfluidic device. (b) Scale schematic of device showing light scatter (‘FSC’ and ‘SSC’) collected by waveguides from interrogation centers (two black circles in channel). Note that light originating from between these centers is incident on an angled facet, resulting in large reflection or refraction losses. (c) Typical fork-style lateral hydrodynamic focusing and (d) chevron-based vertical focusing are used to confine sample flow [4].
Tapered features added to the previously flat-faceted waveguides perform light exclusion based on the location and angle at which light is incident on the waveguide. These features can be seen in Figure 4.5. The tapered features are designed to enclose the identical collection angle range as the previous inclusion-based design. The cone of light will now enter the optical system much earlier, however, through a tiny flat facet close to the flow channel. The goal is that, for the scattering signal, little difference will exist between the previous inclusion-based design and the new design with the exclusion optic. The difference between the designs is mostly seen only by the unwanted light.

4.1.3. Waveguide Collection and Angular Mie Scatter Intensity

To understand the action of the exclusion optics, it is helpful to first consider the locations from which we expected stray light to originate, as well as the relative magnitude of these signals. The primary source of ‘stray’ light we attempt to remove from the collection system is light scatter at angles we do not wish to collect and/or light scatter originating from locations we do not wish to collect from. It is reasonable to assume that the vast majority of this scatter will originate from the locations in the channel that are illuminated by the illumination waveguide; i.e. the region between the FSC interrogation point and the SSC interrogation point. We recognize that the illumination will be brightest in the center of this region, tapering off towards the edges. Thus, the areas from which we intend to collect our signal, the outer extremes of the interrogation beam, will certainly experience lower-intensity illumination than the areas from which our excess signal will be generated. The expectation is that this
will be mitigated (a) by the relative signal intensities of Mie scattering (i.e. low angle scatter is much brighter than larger-angle scatter) and (b) by the relatively high reflective losses that occur for near-grazing incidence upon the tapered walls.

The relative intensities of Mie scatter will also play an important role in the relative intensities of the signal we wish to collect and the excess signal. Figure 1.3 shows a sample plot of Mie scatter intensities for several polystyrene beads. It can be readily observed the light scatter in the forward direction will far exceed the intensity of light scatter at larger angles. Thus, while samples positioned towards the center of the illumination area may receive the highest illumination intensity, the majority of that scatter will be in the forward direction. If we can reduce the intensity of light entering the collection waveguides from the forward directions, we can reduce the contribution from the unwanted signals. For the FSC line, these considerations will be particularly important. For the SSC line, this concern with the collection of non-directional scatter is not as critically important. Typical commercial devices will collect SSC with a numerical aperture of >0.4, typically >0.6. This equates to a collection half angle of 15-25° (or more). Our system will be designed to collect light with a half-angle of 8°, thus our source can vary in directionality by ±10-15° without significantly disrupting the character of the collected light relative to the angles collected by some commercial devices. The main concern for the SSC line is restricting the collection of true noise (sidewall scatter, etc) rather than unwanted signal. The larger the NA of the collection system, however, the more likely the collection of such stray light, thus there will still be a tradeoff between collection NA
and the collection of noise. Thus while excess signal may not affect the character of SSC in the same way that it affects the FSC signal, we will wish to restrict the location from which both lines collect light in order to restrict stray light collection.

### 4.1.4. Effects of Reflection and Refraction

In the exclusion design, light originating from between the two interrogation points will generally be incident on a tapered portion of the exclusion optic, resulting in significant reflection and/or redirection by refraction. As can be seen from Figure 4.5, as the origin of light moves away from either of the interrogation points, the location at which that light will be incident on the exclusion optics rapidly becomes a tapered portion of the exclusion optic. Because the taper is designed to just barely enclose the intended collection cone, light from just outside this angular range will be incident on the exclusion optic at grazing or near-grazing incidence angles.

The polarization-dependent Fresnel equations for light reflection based on refractive index contrast and angle of incidence can we written as

\[
R_s = \left( \frac{n_1 \cos \theta - n_2 \sqrt{1 - \left( \frac{n_1 \sin \theta}{n_2} \right)^2}}{n_1 \cos \theta + n_2 \sqrt{1 - \left( \frac{n_1 \sin \theta}{n_2} \right)^2}} \right)^2
\]

(4.1)
\[ R_p = \left( \frac{n_1 \left[ 1 - \left( \frac{n_1}{n_2} \sin \theta \right)^2 - n_2 \cos \theta \right]}{n_1 \left[ 1 - \left( \frac{n_1}{n_2} \sin \theta \right)^2 + n_2 \cos \theta \right]} \right)^2 \]  

(4.2)

where \( R_s \) and \( R_p \) are the reflection coefficients for the s and p polarization states, and \( \theta \) is the angle of incidence of the light originating in a material with refractive index \( n_1 \) and impinging upon the material with refractive index \( n_2 \). A plot of reflection coefficient as a function of incidence angle can be found in Figure 4.6. It is seen that regardless of polarization, the reflected portion of light will rapidly increase as the incidence angle approaches grazing incidence. As a result, light originating from very close to (but outside of) the interrogation region will be largely reflected by the exclusion optic (20%-80%, depending on angle of incidence).

**Figure 4.6** Percent Fresnel reflection as a function of angle of incidence (relative to normal incidence). At an interface with \( n_1=1.41 \) and \( n_2=1.42 \). For incidence >82° from normal (i.e. approaching grazing incidence; i.e. approaches 90°), the reflected portion of the light rapidly increases.

The exclusion features for both the FSC line and the SSC line are very similar; the major difference is that the FSC exclusion optic is merged into the waveguide,
while the SSC optic is disconnected from the waveguide and split into two regions. Isolation is easier to achieve in the FSC optic due to its orientation with respect to the tilt of the fluidic channel. For a small displacement of the sample further down the fluidic channel, a larger angular shift (relative to the optic axis) is seen by the FSC line than the SSC line. In this way, spatial resolution is more easily achieved by the FSC line than by the SSC line. To improve spatial resolution, the SSC optic is split into two regions, helping to ensure more of the excess light is incident on an angled facet. In addition, the optic is separated from the waveguide to allow filling with a higher refractive index material (nd=1.57; LS-5257, Nusil). The resulting reflection as a function of angle of incidence (relative to normal) is shown in Figure 4.7. In this material system, a higher percentage of light incident at grazing angles will be lost, however there is small a tradeoff in reflective losses of the signal as well. For the SSC line, which would otherwise collect significantly more noise, this tradeoff is necessary.

**Figure 4.7** Percent Fresnel reflection as a function of angle of incidence (relative to normal) at an interface with n1=1.41 and n2=1.57. Higher reflections occur in this material system than in the previous lower-contrast system for the same angle of incidence. Higher reflection losses also occur, however, at the flat facet where the signal enters the waveguide.
For both the SSC and the FSC optics, the removal of scattering originating from areas other than the interrogation centers will not be complete. Reflection losses will help reduce contributions from non-interrogation locations, and refraction at the tapered faces should reduce the likelihood of coupling to the collection waveguides. The optics should thus ensure that the peak measurement occurs at or near the locations of interest. As long as the majority contribution occurs at or near the designated interrogation centers, the measurement will be localized and will be based on the more directional illumination light that exists at the edges of the illumination beam, and the collection line will collect light from a more localized area than would be possible with a simple flat-facet waveguide structure. The main goal is to meet or exceed the performance of a lens-based system, thereby creating a simplified integrated optical system.

4.1.5. Final Design

The FSC line has a 3° taper (symmetric about the axis of the optic). The entry facet is 28 μm in diameter, located 200 μm from the FSC interrogation point. The SSC optic tapers at 6° (symmetric about the axis). It is split in half, with the closer entry facet having a diameter of 21 μm 200 μm from the SSC interrogation point, and the lower entry facet having a diameter of 58 μm located 549 μm from the SSC interrogation point. This 200 μm gap exists between the optic and the collection waveguide to ensure a structurally sound wall between the two features.
Considering the dimensions employed in this device, it can be easily appreciated that this approach is made possible only by the integrated, microfabricated approach to the device architecture. Good spatial resolution can be achieved because small movements along the fluidic channel result can be made to result in a significant change in the location at which the light is incident on the optic (i.e. flat or angled facet). This drastic change is made possible by the creation of extremely small entry facets created in very close proximity to the interrogation points. As these facets are moved further from the interrogation points, the spatial resolution of the system rapidly decreases. Thus this lensless approach to localized light collection is only possible in an integrated, microfabricated system.

4.2. Fabrication and Testing

4.2.1. Fabrication

Devices are fabricated using the typical soft lithography methods outlined in Chapter 2. A printed transparency (CAD/Art Services, Bandon, Oregon, USA) at 20,000 dpi is used as a photomask. Using standard lithography practices, the pattern is transferred to a layer of 60 µm thick SU-8 50 photoresist (MicroChem Corp.). The body of the device is made from Gelest PDMS (nd~1.41; OE41, Gelest Inc). After curing, two replicas (from the mold and from a flat) are bonded with UV/Ozone cleaner (UVO-Cleaner 42, Jetlight Inc.). The baffles are filled with black-colored PDMS (Sylgard 170, Dow Corning). Waveguides are filled with another transparent Gelest PDMS, this time of a higher refractive index (nd~1.42, OE42, Gelest) using
vacuum and subsequent pressure and capillary action forces. Cut, cleaved, and cleaned fibers are inserted into the fiber sleeve portion of the device to aid in coupling light into the device. Holes are punched to access the fluidic channels directly with Tygon tubing.

4.2.2. Experimental Conditions

Spherotech size calibration beads (PPS-6K, Spherotech, Inc.; this work uses only the 5 µm, 10 µm, and 15 µm beads) were used to benchmark performance against commercial device. For tests of the inclusion-based device, these beads were suspended in a 12.5° Brix sucrose solution with a few drops of surfactant (Tween-20, ICI Americas, Inc.) to prevent agglomeration. For tests of the exclusion-based device, the beads were simply suspended in 0.22 µm filtered DI water, as it was found that for short runs, the Tween and sucrose were not necessary. The sample solution is pumped into the device via a syringe at a rate of 12 µL/min for the inclusion-only device, and 10 µL/min for the exclusion device. For each device, flow from a single syringe feeds the sheath flow channels at rate of 90 µL/min. In both cases, the sheath fluid was 0.22 µm filtered DI water.

In the inclusion device, the flow channel was 50 µm in diameter, while in the exclusion device the channel was widened to 100 µm to allow for the vertical flow focusing features. Thus the expected equivalent flow velocity in the 100 µm x 60 µm channel (neglecting the parabolic flow profile) is approximately 52 cm/sec in the inclusion device and 28 cm/sec in the exclusion device.
For the microfluidic devices, the interrogation region is illuminated with a 488 nm laser (Cyan 40 mW, Newport Corporation) with a multimode fiber-coupled output (OzOptics, Canada). The output fiber interfaces with bare multimode fiber (GIF50, Thor Labs) inserted into the fiber sleeve of the illumination line via a bare fiber adapter (FC connected, Newport). After these connections, the total power entering the device is substantially less than the 40 mW the laser emits. Fibers carrying collected light (FSC and SSC lines) interface with detectors via a fiber adapter (SM1FC, Thor Labs). Signal from the FSC line is detected via a battery-biased silicon detector (DET36A, Thor Labs) and amplified (PDA 6424, ILX Lightwave) before acquisition. Signal from the SSC line is detected by a PMT. For the inclusion device, a PMM02 (Thor Labs) was utilized. This PMT had a built-in amplifier with a 20 kHz bandwidth. For the exclusion device, a lower-noise H10425 (Hamamatsu) was used, amplified by an external preamplifier with a 20 kHz bandwidth and 105 gain (C7319, Hamamatsu). Both systems (photodiode and PMT) connected via BNC to a connector block (NI BNC 2110, National Instruments), allowing signal capture by a data acquisition card (NI PCI-6251, National Instruments) using off-the-shelf software (Signal Express, National Instruments). Acquisition occurs over 3 channels (FSC, SSC, EXT) at 60 kHz for the inclusion device, and at 50 kHz for the exclusion device.

For comparison, the same beads were run through a commercial flow cytometer (C6, Accuri). The commercial device was operated at its ‘slow’ flow setting, i.e. sample flow at 14 µL/min and a core size of 14 µm. The standard 488 nm
illumination source was used for light scatter in the commercial device. In this device, detection for both FSC and SSC is performed by silicon photodiodes.

4.3. Results and Analysis

4.3.1. Inclusion Device

A sample of the FSC and SSC signal pulses from the inclusion device are shown in Figure 4.8. A total of 27,209 events were recorded in a 4 minute data run, for an average throughput of 113 samples/sec. Distinct peaks can be seen on both the FSC and SSC line.

![Figure 4.8](image)

**Figure 4.8** Processed signal trace from FSC (above) and SSC (below) lines. Each trace shows a 5 \( \mu \text{m} \) bead (left peak), two 10 \( \mu \text{m} \) beads (center peaks), and a 15 \( \mu \text{m} \) bead (right peak).

Figure 4.9(a) shows FSC CVs obtained by manually gating the FSC histogram. The SSC histogram is shown in Figure 4.9 (b). The SSC CVs were obtained by
manually creating two-dimensional regions for each of the three clusters on the scatter plot (shown in Figure 4.9 (c)), then gating the resulting single-cluster SSC histogram. This approach was used as the SSC data for the device exhibited significant overlap between clusters
Figure 4.9 Data plots for a sample of 5, 10, and 15 µm beads from the inclusion device, including FSC height histogram (a), SSC height histogram (b), and scatter plot (c).
Table 4.1 Bead CVs obtained from inclusion device.

<table>
<thead>
<tr>
<th></th>
<th>FSC CV (µF Inclusion)</th>
<th>SSC CV (µF Inclusion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 µm</td>
<td>28.3%</td>
<td>38.8%</td>
</tr>
<tr>
<td>10 µm</td>
<td>11.9%</td>
<td>26.9%</td>
</tr>
<tr>
<td>15 µm</td>
<td>8.6%</td>
<td>21.3%</td>
</tr>
</tbody>
</table>

4.3.2. Exclusion Device

A sample of the FSC and SSC signal pulses from the exclusion device are shown in Figure 4.10. A total of 12,985 events were recorded in a nearly 5 minute data run, for a throughput of 43 beads/sec.

Figure 4.10 (left) Close-up of a signals from a single bead (scaled to display together), showing a slight time delay between the FSC peak and the SSC peak. (right) Signals recorded over two seconds of data show a large number of beads passing through the device. [4]
Figure 4.11(a-b) shows FSC area histograms obtained by utilizing the autogating feature to gate the distinct bead regions. For the data from the microfluidic device, a simple quadrant gate was also employed to remove the contribution from low-level noise. The SSC histograms are shown in Figure 4.11 (c-d). The scatter plots and gating regions are show in Figure 4.11 (e-f). Table 4.2 summarizes the data obtained by this device and the commercial device.
Figure 4.11 Data plots for a sample of 5, 10, and 15 µm beads from both our exclusion device (left column) and the commercial device (right column), including FSC area histograms (a,b), SSC height histograms (c,d), and scatter plots (e,f). The FSC separation on the microfluidic device is quite clear. The SSC separation is less clear on both devices [4].
Table 4.2 Beads CVs obtained by exclusion device.

<table>
<thead>
<tr>
<th>Size (µm)</th>
<th>FSC CV (µF exclusion)</th>
<th>FSC CV (commercial)</th>
<th>SSC CV (µF exclusion)</th>
<th>SSC CV (commercial)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>18.3%</td>
<td>4.47%</td>
<td>37.5%</td>
<td>23.6%</td>
</tr>
<tr>
<td>10</td>
<td>13.2%</td>
<td>2.86%</td>
<td>34.2%</td>
<td>17.5%</td>
</tr>
<tr>
<td>15</td>
<td>9.7%</td>
<td>3.21%</td>
<td>27.7%</td>
<td>11.9%</td>
</tr>
</tbody>
</table>

4.4. Discussion

The light scattering CVs presented in this work are easily among the best light scattering CVs for integrated optical elements. No other authors have demonstrated results integrated side scatter alongside integrated illumination, and few have demonstrated true forward scatter. Typical CVs from devices with integrated light scatter range from 25%-30% [5, 6]. Side scatter is typically not included due to geometry constraints. When it is included in the design, actual CV results have not been published, likely due to the inherent problems of collecting from this lower-intensity line with sufficient SNR to resolve samples reliably. The results achieved in this work exceed the results of our previous lens-based design due to the geometry-based design focusing on the exclusion of noise.

The FSC SNR of the exclusion device improved by roughly an order of magnitude relative to the inclusion device, as shown in Table 4.3. This suggests that more localized excitation was in fact achieved, reducing excess light collection and thereby lowering background noise. For the 5 µm CVs, the exclusion device performs...
significantly better (CVs reduced by nearly 30%). This is likely due in part to improved flow confinement, however this clearly has a lot to do with SNR improvements. The FSC CVs for 15 µm and 10 µm beads are actually quite similar between the inclusion and exclusion design. This suggests that the light blocking elements already in place in the exclusion design play a key role in reducing stray light collection. Basically, these signals are strong enough that the SNR was not so critical. The data from the 5 µm beads, however, shows a significant improvement resulting from the improved SNR.

Table 4.3 Signal-to-noise ratios for each detection line for inclusion and exclusion devices, calculated for 10 µm beads.

<table>
<thead>
<tr>
<th></th>
<th>FSC Line SNR</th>
<th>SSC Line SNR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inclusion Device</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Exclusion Device</td>
<td>40</td>
<td>3</td>
</tr>
</tbody>
</table>

For the SSC line, the CVs for 15 µm and 10 µm beads actually worsened, while again the CV for the 5 µm beads showed an improvement. As shown in Table 4.3, the SNR for this line saw no significant change. This is likely due, in part, to the splitting of the optic, which reduced the overall collection range of the detection line. In addition, the rather viscous material used to fill the SSC optic (LS-5257, Nusil) had a strong tendency to develop small bubbles, further reducing the total light collected. The increased CVs may also be due, in part, to the increased collection NA in the vertical dimension. In the inclusion device, the waveguide facets were located further
from the sample location, thus the angular range collected was quite a bit lower than in the exclusion device. As a result, differences in sample positioning may have had a greater impact in the exclusion-based device.

The initial design was largely geometry-based. It was clear that light paths other than the intended collection path existed, however it was expected that the relative contributions from those paths would be lower based on the larger scattering angles that those contributions represented. In practice, it appears that those contributions were larger than expected. The calculated separation distance between the FSC and SSC location appears to be closer to 30 $\mu$m, rather than the intended 70 $\mu$m. This difference likely arises due to increased contributions from the center of the illumination beam to the FSC line, effectively moving the FSC collection location towards the SSC collection location. It also seems likely that both interrogation points were located a bit too wide relative to the actual illumination beam, further exacerbating the issue of relative intensity by lowering the intensity of light originating from the intended collection point.

The issue of excess FSC collection from the SSC point should be easily remedied with slight changes to the current design. The current left sidewall taper appears to be too shallow of an angle, resulting in some TIR coupling of light from non-collection locations closer to the SSC collection point. As it turns out, the left hand baffle appears to sufficiently block light originating from the left of the FSC collection point, thus the left hand sidewall design can focus on excluding light
originating from the right. With this in mind, the left hand sidewall taper should be made such that the wall is near-orthogonal to incoming light approaching from non-collection locations. This means utilizing an outward flare, rather than an inward taper, for this sidewall. Some portion of a light blocking element will need to remain beneath the waveguide flare to block light from the left hand side. The idea is shown in Figure 4.13. Again, this design will not affect the light originating from the intended collection, but will focus on preventing other light from coupling into the waveguide. The design modification improves the relative collection ratio between the green (signal) and red (noise/cross-talk from 60 µm away) from ~1:1 (in the original design) down to ~40:1 (in the new design concept). Even at a distance of 15 µm, the green:red collection ratio will be ~2:1.
Figure 4.12 (a) FSC exclusion design presented in this work. Green source is light from the intended collection area, while blue and red light demonstrates noise sources at ±60 μm away. (b) A simple redesign concept for the FSC line, which reduces collection from the red noise source by 35x.

Our device is able to resolve 2-3 μm beads (data not shown), however it is not clear that all of the beads in flow were well resolved, so these results were not included. This was, in part, due to the lower SNR on the SSC line. In the case of the SSC line, a larger collection NA would also be expected to benefit the signal strength. Typical commercial devices utilize the same high NA collection optics for SSC as they do for fluorescence, thus the total solid angle collected in our device is significantly lower than that of a commercial device. The resolution of SSC collection in commercial devices, however, is often limited not by total signal collection but
rather by noise collection [7]. This suggests that the total solid angle typically collected for SSC is somewhat excessive, and could be reduced if paired with sufficient stray light reduction. Thus while the solid angle collected in our device should be increased, the increase likely does not need to be as drastic as the current disparity between the collection solid angle of this device and commercial devices might suggest. Utilizing a core material of higher refractive index for the waveguide, such as Nusil LS-6257, could increase the solid angle of collection. This would certainly require the use of additional light blocking elements to offset the increase in the collection of light from other areas in the device, but this seems like the simplest means of improving both the in-plane and vertical CV. A redesign to eliminate the need for the split optic would also help to reduce unnecessary losses. A flaring of the right hand taper of this waveguide should provide similar improvements to those shown for the FSC line.

Under the current flow rates, the device throughput is limited to ~400 samples/sec (to ensure a coincidence loss of 1% or less). This limit could be increased somewhat by increasing flow velocity. Results were observed for this device at total flow rates as high as 200 µL/min (calculated throughput of 800 samples/sec), however this is currently the approximate limit of detection sensitivity for this device. As discussed above, further reducing background noise from rough sidewalls and fabrication imperfections should significantly increase this throughput. In addition, the distance between the two interrogation points can be further reduced if a more collimated illumination source is created (e.g. lower NA illumination waveguide),
allowing for further throughput increases by decreasing the total pulse width per sample.

4.5. Conclusion

The work presented in this chapter represents a new direction for considering integrated optical systems. The key idea is to emphasize not the signal strength alone, but the signal-to-noise ratio. The focus, then, is on restricting collection to a localized area by blocking light originating from other locations. The result is both reduced stray light collection (from sidewalls, debris in the fluid flow, etc) as well as improved angular resolution.

These devices demonstrate integrated illumination as well as integrated light scatter collection for two parameters. This is an especially notable achievement due to the need to reduce a typically three-dimensional system (fluid flow, illumination, orthogonal scatter) to an effectively two-dimensional (planar) system when FSC, SSC, and illumination are all desired on-chip. The inclusion of FSC and SSC is a key modification to accommodate the true interests and needs of end users. The FSC CVs measured from this device range from 18.3% down to 9.7% (5 µm to 15 µm diameter beads). This compares quite favorably with FSC CVs from previously demonstrated microfluidic devices, which generally range from 25%-30% (1 µm to 9 µm diameter beads) [5, 6].

The relatively strong performance of the inclusion device is quite promising; this is attributed to the use of simple light blocking elements to improve localization.
The 10x improvement in the FSC SNR ratio of the exclusion device is a very promising result for the exclusion-based approach. Similar improvements in the SSC line would result in a very capable device. The localization capabilities of reshaped optics combined with light blocking elements could be particularly powerful with a collimated or near-collimated illumination source, providing a more directional, uniform interrogation.

The work presented in this chapter represents something of a proof-of-concept for the exclusion-based design, however the results presented are among the best published for integrated optics. The results from these simple designs already exceed the results from previous lens-based designs. The designs exploit the microfabricated platform and the close proximity of the optical components to the fluidic components to take a new approach to optics on the chip level. The promising results demonstrated in this chapter suggest that the approach has great potential to impact the way that integrated optical systems are designed.

Acknowledgements

Portions of Chapter 4 have been submitted for publication, 2010, J. Godin and Y.-H. Lo. The dissertation author is the first author of this paper.

References


Chapter 5

Conclusion and Future Directions

Microfabrication opens the door to a new realm of possibilities for lab-on-a-chip technologies. The ability to make miniaturized devices, the highly-predictable laminar flow regime in which microfluidics operates, and the ability to seamlessly integrated several types of components or functionalities hold promise to revolutionize both medicine and biological research. Miniaturized flow cytometers are only the tip of the iceberg.

The shift from bulk devices to integrated microfluidic chips is a significant change that perhaps requires an equally significant adjustment in the ways in which engineers approach the design of these devices. The field of microfluidics has already seen the start of this shift. Specialized microluidic approaches to separations [1]-4, mixing [5-7], and flow confinement [8-10] have been developed. Indeed, the basic assumptions about the physics of the fluidic systems have recently been coming into question, as it becomes increasingly evident that some typical assumptions (such as
Stoke’s flow) are not necessarily valid in many cases [11]. While such changes in the ways that we think about creating fluidic systems may initially provide stumbling blocks for the field of microfluidics, ultimately they result in greater development of our physical understanding of the world, arming us with new tools with which to tackle a host of interesting problems.

Just as the field of fluidics has needed to adapt and broaden to accommodate the unique field of microfluidics, integrated micro-optics will bring some evolution to the field of optics in terms of integrated devices. The underlying physics will not shift to the same degree, as the relative scale of the devices is still quite large with respect to the wavelength of light (the field of nanophotonics is already driving that more significant shift). On a lesser scale, however, the field of micro-optics will drive changes in how optical designers approach optical systems on integrated platforms.

There are a number of important features of integrated optical systems that set them apart from typical free-space optics. Integrated systems are often immersion systems. Integrated optical elements cannot (at this time, and likely at any point) be anti-reflection coated in a reliable fashion. These optical systems have unique fabrication needs. Creating very small, precisely-shaped features for miniaturized optics certainly presents a significant challenge. At the same time, the ability to create highly customized two-dimensional optical systems, with lenses or optical elements of nearly any conceivable shape, provides a significant advantage over traditional systems. The ability to mass-fabricate and optical system in fixed alignment is
certainly an advantage of microfabricated optical systems. The extremely short path lengths of microfabricated systems provide both a benefit (in terms of losses) and often a challenge (in terms of system design).

In this work, I sought means to create practical integrated optical systems. The goal was to create a simple, robust chip through relatively practical fabrication methods that could be capable of performing localized excitation and localized light collection. The integrated lenses demonstrated in Chapter 3 provided a practical means of fabricating an optical system on a chip using the more traditional imaging optics approach. The work presented in Chapter 4 aims to recreate this same functionality by exploiting the exclusion abilities of elements that guide light by total internal reflection in conjunction with the close proximity of the optical elements to the location from which we wished to collect light.

While the flow cytometer (and most other optically-based assays) is traditionally created using imaging optical systems, the chip-based architecture may suggest a nonimaging approach to the optical system. The design flexibility afforded by the integrated approach allows for the creation of extremely atypical component shapes that can be ideal for taking a reflection or TIR-based approach to both light collection and the formation of an illumination beam. This approach is further aided by the ability to bring components very close to the cells under interrogation, and is almost necessitated by the desired path length reduction. Nonimaging approaches are used for light collection and shaping in a number of other fields, such as solar
concentration and LCD backlighting, thus a number of texts in this field are available [12-13]. Indeed, some recent works in microfluidics have exploited similar concepts to those employed in Chapter 4 [14]. The need is not, then, to reinvent a field, but to apply this knowledge and these approaches to devices where such approaches have not generally been used in the past.

Much work remains to be done in order to create truly low cost, integrated optofluidic chips for flow cytometry. The best method of fabricating devices on a mass-scale has yet to be identified. While PDMS provides an excellent prototyping material, it may not be suitable for many devices due to issues such as adsorption [15], autofluorescence (though the autofluorescence of PDMS is better than many other common chip fabrication materials) [16], and the lack of a large-scale PDMS device foundry. Another persistent issues remains the problems of devising a reliable means of flow focusing, one which does not depend on flow rates or properties of the sample. In the realm of optics, integrated optics still do not receive a great deal of attention. Most devices continue to use simple fiber-based approaches, or entirely off-chip approaches. With further development systems that can be easily fabricated and easily integrated, the shift towards integrated optics may take a stronger hold. Many devices could benefit from integrated optics, however, not all device designers care to develop a background in optics. The field of microfluidics has become more mainstream by developing a sort of pick-and-choose toolbox of functionalities, such as valves [17-18] and mixers [5-7]. In the same way, developing an optical toolbox, such as waveguides
[19-21], baffles and apertures [22-23], lenses [24-30], and filters [31-33], will lower the barrier for entry into the realm of integrated optics.

The development of more sophisticated designs for both imaging and non-imaging light collection systems provides an interesting challenge for future work. While one suggestion was demonstrated in Chapter 4, there are many other ways to collect light from a localized location using simple elements with a low refractive index contrast (low Fresnel reflections). In addition, it would be interesting to see this same technique eventually utilized for fluorescence collection. For example, one can envision creating the waveguides for this approach by using the dyes employed for integrated filters. Exclusion optics may also be well-suited to spatial-temporal coding approaches to data multiplexing [4, 34].

The most exciting applications for microfluidics involve the integration of many features, the true lab-on-a-chip devices; similarly, some of the most exciting applications for integrating optical systems involve the automation of studies conducted on such chips. Integrating cell culturing, reagent metering, and sample analysis onto a single chip promises to deliver the sort of highly repeatable results rarely, if ever, found in standard bulk-volume approaches to sample preparation and analysis. Many biological analysis tools utilize optical interrogation (generally fluorescence-based), thus by integrating the actual optical systems onto the chip, one can automate not just the process by which data is created, but also the process by which that data is collected.
By bringing the optical system onto the chip, a single, more standardized chip reader can be created to interface with countless specialized or custom-designed chips. Such chips could be disposable or reusable, depending on the needs of the user and the experiment. This one-size-fits-all approach to the interfacing hardware could bring this ubiquitous device to many research labs, enabling individual labs to do work previously only performed in core facilities. A multi-day study that would previously be difficult (if not impossible) to run in a shared facility could be readily performed; and more importantly, with integrated detection and readout, such experiments could be automated. Researchers can spend more time designing experiments and less time running them, accelerating the pace of laboratory research.

The field of microfluidics is poised to change not just the face of medicine, but the face of research as well. Integrating optical systems alongside microfluidics will further open new areas for exploration.

**References**


Appendix 1

Sample Data Processing Matlab Code

The following code reads in data of the format:

<table>
<thead>
<tr>
<th>Time</th>
<th>FSC Intensity</th>
<th>SSC Intensity</th>
<th>EXT Intensity</th>
</tr>
</thead>
</table>

and plots this data, finds peaks, and records a new list of ‘events’ to be converted to .FCS format

----------------------------------------------------------------------------------------------------

close all
clear all

%----------Enter filename--------%
filename1 = ['form1 2010-8-5_5.txt'];
%-----------------------------%

for s=1:size(filename1,1)
foldername = filename1(s,1:size(filename1,2)-4);
Data1=[]; Event2=[]; Data1Filtered=[];
samplesize=inf; %read all data points
%------Settings------------%  
savedata = 'Y';  % 'Y' to save, 'N' to not save  
samplingrate = 50000;  % Data sampling rate  
pulsewidthFS = 7;  % set minimum pulse width (in # data points)  
pulsewidthSS = 2;  
Lfwd = .007;  
Lside = .005;  

window = 50;  % Window to check for max  
beadspacing = 100;  % normally 100 (50 for resampled)  
peakdiff = 20;  % normally 20 (4 for resampled)  

%------Read in Data-------%  

fid = fopen(filename1(s,:), 'r');  
Data1 = fscanf(fid, '%g %g %g %g', [4, samplesize]);  
Data1x = Data1';  
Data1 = [];  
% Re-order according to order data was taken in  
Data1(:,1) = Data1x(:,1);  
Data1(:,2) = Data1x(:,4);  
Data1(:,3) = Data1x(:,2);  
Data1(:,4) = Data1x(:,3);  
clear Data1x  
fclose(fid);  

%------Filtering-----------%  

% Read in filter factors (high-pass stopbands) FF1 and FF2  
FF1 = 2000;  

% Rough mean subtraction  
Data1Filtered(:,1) = Data1(:,1);  
Data1(:,2) = Data1(:,2) - mean(Data1(:,2));  
Data1(:,3) = Data1(:,3) - mean(Data1(:,3));  
Data1(:,4) = Data1(:,4) - mean(Data1(:,4));  

% Fine-tune with high-pass filter  
b2 = fir1(FF1,[.003], 'high');  
hd2 = dfilt.dffir(b2);  
Data1Filtered(:,2) = filter(hd2,Data1(:,2));  
clear b2  
reset(hd2);  

b2 = fir1(FF1,[.003], 'high');  
hd2 = dfilt.dffir(b2);
Data1Filtered(:,3)=filter(hd2,Data1(:,3));
clear b2
reset(hd2);

b2 = fir1(FF1,[.003], 'high'); hd2 =dfilt.dffir(b2);
Data1Filtered(:,4)=filter(hd2,Data1(:,4));
clear b2
reset(hd2);

extmean=mean(Data1Filtered(:,4));

%Clear up some memory
clear Data1

%--------END Filtering--------------%

%----------------Find all local maxima in FWD and SIDE DET data-------%
%-------- Writes a matrix of EVENTS for scatter plot analysis --------%

index=10000;
q=1;
r=2;

Event2(1, :)=[0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0];

while index < (size(Data1Filtered,1)-100000)

%Check all FSC points above Lfwd
if Data1Filtered(index,2)>Lfwd

%Re-initialize variables
positionleft=[];
positionright=[];
positionmax=[];
search=[];
positionleftFS=[];
positionrightFS=[];
positionmaxFS=[]; positionmaxBS=[];
searchFS=[];
maxval=[]; maxvalFS=[]; maxvalBS=[];
positionleftmost=[];
positionrightmost=[];

end
L_back = .01;
localmax = 0;

% Locate local maximum (search at most 'window' left and right, recenter)
searchFS=index;          % search FS is original start point
checklocation=searchFS;
fscmax=0;
pos=[];

if any(Data1Filtered(checklocation+i:checklocation+i+25,2) > Data1Filtered(checklocation,2))
    [y,pos]=max(Data1Filtered(checklocation+i:checklocation+i+25,2));
    checklocation = searchFS+pos;
else
    end
fscmax=1;

if fscmax==1  % Continue only if a true local max was found
    searchFS=checklocation; % searchFS holds FSC max position

    % Check left and right for zero position
    while any(Data1Filtered(checklocation:checklocation-5,2)>L_fwd)
        checklocation=checklocation-1;
    end
    positionleftFS=checklocation;

    checklocation=searchFS;
    while any(Data1Filtered(checklocation:checklocation+5,2)>L_fwd)
        checklocation=checklocation+1;
    end
    positionrightFS=checklocation;

    % Continue ONLY if pulsewidth is wide enough
    if (positionrightFS-positionleftFS)>pulsewidthFS

    %%%% %%%% %%%% Continue ONLY if distance to last bead large enough
    if positionleftFS-Event2(r-1,10)>beadspacing

    % Find nearest SSC local max
%Find local max (within +/- 2 FSC widths)
checklocationleft=searchFS-2*(positionrightFS-positionleftFS);
    %FSC max less 1 pulse width
checklocationright=searchFS+2*(positionrightFS-positionleftFS);
[x,point]=max(Data1Filtered(checklocationleft:checklocationright,3));
checklocation=point+checklocationleft;

%Check left and right for end positions of SS
search=checklocation;
while (Data1Filtered(search,3)>.25*Lside)
    search=search-1;
end
positionleft=search-1;

search=checklocation;
while(Data1Filtered(search,3)>.25*Lside)
    search=search+1;
end
positionright=search+1;

[maxvalEXT,positionmaxEXT]=
    min(Data1Filtered(positionleftFS:positionrightFS,4));
[maxval,positionmax]= max(Data1Filtered(positionleft:positionright,3));
[maxvalFS,positionmaxFS]= max(Data1Filtered(positionleftFS:positionrightFS,2));

%Correct FSC using beam dump baseline
localmean(r,1)=mean(Data1Filtered(positionleft-10:positionleft+2,4));
localEXTmean=mean([mean(Data1Filtered(positionleft-20:positionleft,4))
    mean(Data1Filtered(positionrightFS:positionrightFS+20,4))]);

%Record Event info
Event2(r,1)=r;
Event2(r,2)=positionright-positionleft;   %SS Width
Event2(r,3)=maxval;                 %SS Max
Event2(r,4)=positionleft+positionmax;  %SS pos'n of max
Event2(r,5)=maxvalFS;              %FS Max
Event2(r,6)=abs(sum(Data1Filtered(positionleft:positionright,3)));   %SS Sum
Event2(r,7)=abs(sum(Data1Filtered(positionleftFS:positionrightFS,2))); %FS Sum
Event2(r,8)=positionrightFS-positionleftFS;       %FS Width
Event2(r,9)=positionleftFS;                  %FS left
Event2(r,10)=positionrightFS;                %FS right
Event2(r,11)=positionleftFS+positionmaxFS;    %FS Max pos'n
Event2(r,12)=10000*(Event2(r,11)-Event2(r,4)); %p-p distance
Event2(r,13)=positionright;
Event2(r,14)=positionleft;
Event2(r,15)=sum(Data1Filtered(positionleftFS:positionrightFS,2));  %FSC Area
Event2(r,16)=sum(Data1Filtered(positionleft:positionright,3));        %SSC Area
Event2(r,17)=maxvalEXT-localEXTmean;
Event2(r,18)=localEXTmean;
Event2(r,19)=positionleft+positionmaxEXT;
Event2(r,20)=maxvalEXT;  %literal value
r=r+1;

index = positionrightFS+1;
else index = index+1;
end
else index = index+5;
end
else index = index+1;
end
else index = index+1;
end
else index = index+1;  %remove here
end
end

%FSC Histogram
handle31=figure(31);
h = findobj(gca,'Type','patch');
set(h,'FaceColor','m','EdgeColor','m');
hist(Event2(:,5),500);
xlabel('FSC')
ylabel('Counts')

%SSC Histogram
handle32=figure(32);
h = findobj(gca,'Type','patch');
set(h,'FaceColor','m','EdgeColor','m');
hist(Event2(:,3),50000);
xlabel('SSC')
ylabel('Counts')

if savedata=='Y'
%Print events to file
fid = fopen(fullfile(foldername,'_events.txt'),'wt')
fprintf(fid,'#g\t%g\t%g\t%g\t%g\t%g\t%g\t%g\t%g\t%g\t%g\n', Event2);
exported=size(Event2)
fclose(fid);
end
%-----------------------Plots to help analyze settings-----------------------%

% Plot Data with Data Markers
figure(7)
clf(7)
hold on
order=1000;

%Plot Data
Data1Filtered(order:size(Data1Filtered,1),1)=order:size(Data1Filtered,1);
plot(Data1Filtered(order:size(Data1Filtered,1),1),
     .5*Data1Filtered(order:size(Data1Filtered,1),3), 'r')
plot(Data1Filtered(order:size(Data1Filtered,1),1),
     Data1Filtered(order:size(Data1Filtered,1),2), 'b')

%Plot Markers - FS max/location of center
for q=1:size(Event2,1)
    plot(Event1(q,4), scale1/scale2*Event1(q,3), 'ko');
%Plot Markers - FS max/location of peak
%Plot Markers - SS width at SS max
    plot(Event2(q,9), 0, 'ro');
%Plot Markers - SS max
    plot(Event2(q,10), 0, 'ro');
    plot(Event2(q,13),0.01, 'kd');
    plot(Event2(q,14),0.01, 'kd');
end

low=0; normal=0;
for q=1:size(Event2,1)
    plot(Event2(q,4), .5*Event2(q,3), 'bs'); %SS max/location of center
    plot(Event2(q,11), Event2(q,5), 'ms'); %FS max/location of center
end

%-----------------------Plots-----------------------%

%Scatter Plots
handle15=figure(15);
clf
hold on
plot(Event2(:,5), Event2(:,3), 'r.'
title('FS vs SS')
xlabel('FSC')
ylabel('SSC')
end
Appendix 2

Sylgard 170 Transmission Properties

The transmission of a 100 µm sheet of Sylgard 170 was measured twice, with control measurements between to ensure that the setup was not disturbed. The following plots were used to determine the transmission properties of the material.
Original spectrum data:

![Spectrum with and without Aperture Material](image)

The following data was calculated by subtracting the source data from the data with the aperture material (i.e. Sylgard 170) to determine the transmission spectra.