SCALABLE MANUFACTURING METHODS FOR BIOMEDICAL MICROFLUIDICS

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ABSTRACT

As the delivery of healthcare has shifted from best clinical outcomes to best value outcomes, the promise of point-of-care diagnostics provides an avenue for faster and more accurate treatment, saving both time and money than traditional laboratory diagnostic testing. For example, the lateral flow pregnancy test, has provided an at-home diagnostic test that has greatly benefited many by rapidly and cheaply detecting the presence human chorionic gonadotropin (hCG) hormone in pregnant women. The need for new point-of-care diagnostics for disease detection and treatment has provided a strong opportunity for microfluidics. Microfluidics is the interdisciplinary field studying the manipulation of fluids in “microchannels”. Biomedical microfluidics has enabled the development of point-of-care diagnostic platforms that benefit from low-cost materials, small footprint, low reagent and sample volumes, and automation. However, while many microfluidic platforms have been developed, few have reached the market due to the inability to scale-up manufacturing. This dissertation contributes to the development of scalable rapid prototyping techniques to better translate microfluidic systems from the lab bench to the clinic. Chronic and idiopathic eye diseases are one facet of healthcare that could greatly benefit from microfluidic point-of-care diagnostic platforms to better preserve vision, reduce surgeries needed for large volume biopsies and personalize disease treatment. Three different scalable platforms have been developed to address this clinical need utilizing innovative microfluidic techniques such as centrifugal and paper-based flow control. These systems contribute to the field by: 1) providing a framework for scalable rapid prototyping of microfluidic devices using commercialization-friendly manufacturing methods and materials, 2) enabling the study of molecular diagnostic
analysis and complicated cell functions such as chemotaxis at very small time frames (<20 minutes), and 3) integrating diagnostic capabilities with necessary sample preparation techniques such as preconcentration using an innovative open-platform system. Altogether, this work improves the ability to accelerate the translation of research microfluidic diagnostic platforms to commercial products.
PREFACE

This thesis includes work described in the below publications by the author:


# TABLE OF CONTENTS

**ACKNOWLEDGEMENTS**

**ABSTRACT**

**PREFACE**

1. **INTRODUCTION**

2. **CRITICAL LITERATURE Review**

   2.1 **Point-of-Care Immunoassays**
   
   2.1.1 Treatment of Chronic Eye Disease
   
   2.2 **Point-of-Care Immunophenotyping**
   
   2.2.1 Idiopathic Eye Disease Diagnostics
   
   2.3 **Microfluidic Manufacturing**
   
   2.3.1 Soft Lithography
   
   2.3.2 Scalable Manufacturing Methods
   
   2.4 **The Maker Movement**
   
   2.5 **Centrifugal Microfluidics**
   
   2.6 **Paper-based Microfluidics**

3. **MATERIALS AND METHODS**

   3.1 **Microfluidic Sandwich Immunofluorescence Assay (sIFA)**
   
   3.2 **Point-of-Care Ocular Rapid Test (PORT)**
   
   3.2.1 Sedimentation Fundamentals
   
   3.2.2 Device and Assay Design
   
   3.2.3 Materials
   
   3.2.4 Device Fabrication
   
   3.2.5 Microsphere Preparation
   
   3.2.6 PORT Dose-Response Experiments
   
   3.2.7 Clinical Samples
   
   3.2.8 Image Analysis
   
   3.3 **Chemotaxis Sheath Flow Device (CSFD)**
   
   3.4 **Chemotaxis Immunophenotyping Device (CID)**
   
   3.4.1 Fabrication of Paperfluidic Device
   
   3.4.2 Gradient Measurement
   
   3.4.3 Cell Preparation
### 3.4.4 Cell Migration Experiment in Paperfluidic Device

### 3.5 Spinning Ocular Lymphocyte Immunophenotyping Device (SOLID)
- 3.5.1 Device and Setup Manufacturing
- 3.5.2 Vitreous Biopsy Model
- 3.5.3 Running the Device

### 3.6 Do-It-Yourself Microfluidics
- 3.6.1 Materials Selection
- 3.6.2 Tools
- 3.6.3 Design
- 3.6.4 Cut
- 3.6.5 Assemble

### 4. RESULTS AND DISCUSSION

- **4.1 Sandwich Immunofluorescence Assay (sIFA)**
- **4.2 Point-of-care Ocular Rapid Test (PORT)**
- **4.3 Chemotaxis Sheath Flow Device (CSFD)**
- **4.4 Chemotaxis Immunophenotyping Device (CID)**
  - 4.4.1 Characterizing the paper gradient
  - 4.4.2 Cell Studies
- **4.5 Spinning Ocular Immunophenotyping Device (SOLID)**
  - 4.5.1 First Design – Passive Sedimentation
  - 4.5.2 Final Design – Paper Filtration
    - 4.5.2.1 Device Characterization and Optimization
    - 4.5.2.2 Proof of Concept

### 5. CONCLUSIONS AND RECOMMENDATIONS

### 6. EXPERIMENTAL ACKNOWLEDGEMENTS

### 7. NOMENCLATURE

### 8. REFERENCES

### 9. APPENDIX

- **A.1 Future Recommendations**
- **A.2 Characterization Studies**
1. INTRODUCTION

The field of point-of-care (POC) microfluidic diagnostics has been predominantly addressed in academia using elastomer devices composed of polydimethylsiloxane (PDMS) and manufactured using clean room techniques such as soft lithography. Over the past few decades, hundreds of novel microfluidic point-of-care diagnostic platforms and applications have been published in peer reviewed journals; however, few have reached market. Even with large investments from government and industry in both Europe and North America, a surprisingly few lab-on-a-chip (LOC) based diagnostic tests have translated to a commercial product [1,2]. Yet the market for these devices is expected to grow from $1.6 billion in 2013 to $3.6 - $5.7 billion by 2018 [3,4].

One of the greatest hurdles in the microfluidics POC market is the inability for scalable manufacturing of PDMS and soft lithography methods [5]. PDMS is a high-cost material compared to the cost-efficient thermoplastics that can be rapidly manufactured via injection molding or hot embossing. Yet, the direct conversion of PDMS to alternative materials is rarely trivial as PDMS architecture is often too high resolution for traditional manufacturing methods. PDMS as a material also has some disadvantages for commercial products as the polymer will age over time and suffers certain inefficiencies that, while can be minimized through passivation or functionalization, are difficult to eliminate completely. These disadvantages reduce the potential for technology transfer as the diagnostic products may not be able to maintain high enough precision or reproducibility [1].
A technology map developed by Chin et al. shows how none of the major players in the microfluidic in-vitro diagnostics market use PDMS in their products, rather using plastic, glass, or paper materials instead [6-8]. Key challenges preventing the further expansion of the market are the lack of standardization and integration of current microfluidic POC diagnostic platforms [7,9]. Standardization is necessary to improve quality control and enable scalability of microfluidic platforms; and, integration is required to fully automate assay steps without complicated external equipment, signal readout modalities, or sample preparation steps. It is necessary for improved collaboration between academia and industry to overcome these challenges.

A variety of disease treatments could gain much from microfluidic POC diagnostic platforms. For example, one-size-fits-all treatments for chronic eye disorders, such as wet age-related macular degeneration and diabetic retinopathy, are often inappropriate for a large subset of patients and are rarely rectified early on. These inefficiencies in treatment are due to the large biopsy volume required for traditional laboratory testing that necessitates invasive surgery to acquire. There is a need for a point-of-care system to process smaller biopsy volumes, which may be collected with minimally invasive methods. Idiopathic ocular diseases, such as uveitis and primary intraocular lymphoma (PIOL), suffer a similar diagnostic problem. As the symptoms of these two diseases are identical, but require drastically different treatments as one is an infection while the other is a cancer, respectively. The gold standard diagnostic is taking a biopsy of ocular fluid from the larger vitreous humor chamber and determining invasive cell types within the eye. However, the integrity of the biopsy sample degrades rapidly once collected and the delay in analysis to transfer the sample to a laboratory causes few conclusive diagnoses to
be made. There is a need for a way to rapidly phenotype the invasive cell content at the point-of-care.

This dissertation discusses various methods to meet these clinical needs using scalable manufacturing methods and materials. Section 2 reviews the literature of traditional and alternative microfluidic manufacturing methods as well as review a few clinical examples related to ocular disease, which would benefit from new methods. Section 3 discusses the materials and methods used to produce scalable, point-of-care diagnostic technologies for both chronic and idiopathic eye disease, and how one can produce these devices on their own in makerspaces. Section 4 highlights the outcomes of these new microfluidic platform technologies and discusses their impact. Finally, Section 5 will provide conclusions and suggestions of future work to further these tools and methods to truly transition from research-scale to commercial-scale technologies.
2. CRITICAL LITERATURE REVIEW

Point-of-care testing has been of great interest as the focus of healthcare shifts from the best clinical outcomes to the best value outcomes. Common point-of-care testing methods such as immunoassays (Section 2.1) and immunophenotyping (Section 2.2) enable clinicians to rapidly and cheaply diagnose patients, such as those suffering from chronic or idiopathic ocular diseases. Microfluidic platform technologies are well suited to meet this need; however, only a rare few have reached the market. The transition from polymer materials and clean room manufacturing to low-cost thermoplastics, adhesives, and paper materials and makerspace manufacturing has enabled scalable rapid prototyping (Sections 2.3 and 2.4). Centrifugal- and paper-based microfluidics have provided an avenue to overcome loss in resolution from these new prototyping methods (Section 2.7 and 2.8).

2.1 Point-of-Care Immunoassays

Point-of-care (POC) testing has been steadily increasing since its introduction 40 years ago [2]. With the rising costs of healthcare, the focus of healthcare delivery has switched from best outcomes to the best value (outcome/cost) [10]. The World Health Organization (WHO) guidelines for point-of-care testing is to produce devices and products that are ASSURED: Affordable, Sensitive, Specific, User-friendly, Rapid & Robust, Equipment-free, and Delivered directly to the patient [11]. The utilization of simple chemistries, automated interfaces, and low reagent and sample volumes has shown microfluidics to hold great potential for POC testing [12]. Many in depth reviews
have shown the promise of microfluidic technology to meet these demands [13]. Point-of-care testing with microfluidic devices has shown growth in both developing and developed countries. Many existing diagnostic systems require advanced infrastructure, stable electricity, large sample and reagent volumes, highly trained technicians, dedicated facilities, and long sample-to-result times. The ruggedness and reliability of microfluidic technologies has been shown to be well suited to overcome these challenges and bring high quality diagnostics to low resource settings [14]. These low resource settings require point-of-care testing as it may be the only method for testing disease in a population. In contrast, more developed settings seek to take advantage of the increased value from point-of-care testing [15].

POC immunoassays have been a popular topic in microfluidics as quantification of target molecules for a variety of applications including clinical diagnostics, proteomics, pharmaceutical research and basic research [16-18]. Thus, there has been great attention to microfluidic immunosensor platforms using a variety of methods (e.g. optical, electrochemical, mechanical) [19]. And with the discovery of new biomarkers to diagnose and characterize a wide variety of diseases, microfluidic immunoassays have become more important [20].

The conventional immunoassay done in laboratories is the enzyme-linked immunosorbent assay (ELISA), which requires trained technicians to perform an hours long assay, even with the help of pipetting robots. Chin et al. developed the mChip to miniaturize and automate this process in a small chip that reduced cost, footprint, the need for trained technicians and improved chemistry kinetics to speed up the sample-to-result time [21]. Many other groups have developed similar automated microfluidic
processes with a variety of alternative methods [19]. Still, even with all the success of
POC immunoassay technology enabled by microfluidics, clinical studies have shown
drawbacks in sensitivity and specificity due to lack of robustness and complicating
factors found in common biological samples [22]. Systems need to be simplified and
well optimized to handle real world conditions. The key challenge in the clinic today
with POC immunoassay technology is developing microfluidic systems with equal or
superior sensitivity and specificity to common ELISA techniques [23].

2.1.1 Treatment of Chronic Eye Disease

An example of a clinical need that would benefit from the development of commercial
POC microfluidic diagnostics is the treatment of chronic eye disease. Diabetic
retinopathy (DR) and age-related macular degeneration (AMD) are collectively the
leading cause of blindness in the western world [24-26]. Both of these diseases are
characterized by aberrant angiogenesis within the eye (Figure 1).
Figure 1. Eye anatomy and disease. (A) Components and compartments of the human eye. Image adapted from [27] (B) Impact of aberrant angiogenesis on the retina. Image adapted from [28]

In the U.S. alone, approximately 5 million adults suffer from DR and 10 million from AMD. As the proportion of American adults aged 65 and over grows from its current level of 13% of the population to the expected level of 19% by 2030 [29], the incidence of both age-related diseases is expected to climb sharply. The abnormal angiogenesis in DR and wet AMD can be treated by inhibiting vascular endothelial growth factor (VEGF), secreted by leaky blood vessels. This inhibition is accomplished by injecting monoclonal antibodies against VEGF directly into the vitreous body of the eye (Figure 2) every 4-6 weeks, often for life. These injections are invasive and carry a small risk of bacterial infection or retinal damage (~1 in 1500 injections) [30]. Prior to receiving an injection, patients perform visual acuity tests, which consists of reading letters of varying sizes on a chart. Cross-sectional images via optical coherence tomography (OCT) are also
routinely obtained. The treatment regimen with respect to dosage and frequency of drug administered is typically modulated using visual acuity measurements and OCT images. There is currently no analytical measurement-based dosing capability, which is significant considering that anti-angiogenic drugs are expensive, costing Medicare nearly $2 billion for AMD treatment in 2010 alone [31].

**Figure 2.** Intravitreal administration of anti-VEGF drugs such as Lucentis and Avastin is the standard of care to treat DR and AMD. This is an invasive procedure that must be performed in 4-6 week intervals, often for life. Image adapted from [28]

A significant subset of DR and AMD patients, termed non-responders, do not respond to anti-VEGF drugs. While virtually all patients of DR and AMD initially benefit from anti-VEGF drug injections, between 40-60% of AMD patients eventually stop responding and experience a relapse in deterioration of their eyesight [32]. These patients likely have reduced intraocular levels of VEGF, which may explain why anti-VEGF drugs fail to retain their effectiveness. However, without a rapid and quantitative test, this conclusion cannot be drawn at present. As a result, the current approach in treating these patients is to sequentially try all available anti-VEGF drugs and then consider laser-based treatment for ablation of neovascularized retinal tissue in an attempt to obtain a treatment
effect. If intraocular levels of VEGF could be easily measured in the clinic, such non-responders could be identified much earlier based on absence of VEGF, avoiding unnecessary, potentially risky and expensive anti-VEGF drug injections.

2.2 Point-of-Care Immunophenotyping

POC testing of the immune status of compromised or diseased patients provides rapid pivotal knowledge to treating infectious disease and immune dysfunction. Real-time measurements of immune status are essential in disease diagnosis, prognosis, evaluating efficacy of therapeutics and personalizing treatment [33]. The proportions, numbers, and cytokine production of immune cells can provide diagnostic information for a wide variety of infections, malignancies, and autoimmune disorders [34]. Thus, there is a need for a method to immunophenotype patient samples in a rapid and accurate manner. A review of recent techniques has shown how microfluidics can provide reliable and multiplexed immunophenotyping capabilities to characterize immune cells [35]; however, few of these technologies have matured to the market.

The gold standard for immunophenotyping is flow cytometry analysis. Briefly, cells are fluorescently labeled with specific antibodies containing a fluorescent tag and individually interrogated by a laser scanner that counts the presence of a fluorescent tag on each cell that passes. A multitude of microfluidic devices have been developed using the same mechanism at a reduced size and cost than the expensive standard capital equipment [36-38]. Beyond flow cytometry, microfluidics has developed a number of alternative techniques to perform cell sorting and separations from biological samples.
An example of one of these alternative techniques is using antibody coated magnetic beads and magnetic fields to rapidly displace and isolate target cells in a microfluidic channel [40]. A commercially available example is the Alere PIMA CD4 test that takes whole blood and tests for CD4+ T cells for HIV diagnostics and has shown comparable results to standard flow cytometry with a simple cartridge and reader system [41]. Some groups have even developed more complex microfluidics capable of doing even more than flow cytometry. Chen et al. developed a microfluidic device capable of isolation, enrichment, and stimulation of immune cells along with the ability to perform an on-chip immunoassay to quantify released cytokines using membrane filtration and a flow-based ELISA [42]. Finally, with the onset of droplet microfluidics, which can encapsulate single cells in tiny droplets, high throughput fluorescence and magnetic cell sorting can also be achieved on-chip as well [43].

2.2.1 Idiopathic Eye Disease Diagnostics

A clinical need for a point-of-care microfluidic immunophenotyping platform is for the diagnosis of idiopathic eye disease. Idiopathic ocular diseases such as uveitis and primary intraocular lymphoma (PIOL) require immediate and accurate diagnosis to prevent irreparable loss of vision and mortality, respectively. Uveitis is a general term for inflammation within the uvea, the region of the eye between the sclera and the retina (Figure 1). The exact cause of uveitis often cannot be determined; however, the inflammation associated with uveitis manifests itself as an invasion of T-lymphocytes within the eye. Approximately 2.3 million Americans suffer from uveitis, and the disease is responsible for 10-15% of the blindness in the U.S. [44]. The presence of uveitis is
especially important to diagnose early as an underlying systemic disease (i.e. autoimmune disease) is the root cause in 25-50% of patients [44]. When a patient shows symptoms of nonspecific, chronic uveitis and resistance to treatment, then PIOL may be the true cause. PIOL is a type of cancer that is often related to lymphoma somewhere within the primary nervous system [45]. Because of its gradual onset and ability to mimic other ocular diseases (e.g. uveitis), delays of up to two years are common before a diagnosis of PIOL is reached, leading to a two-year survival rate of 39% [46,47]. For this reason, PIOL is commonly referred to as masquerade syndrome [45].

Current methods to diagnose PIOL and uveitis include polymerase chain reaction (PCR), ELISA, cytology, and flow cytometry. PCR can be used to detect uveitis only if it is pathogen related [48]. ELISA has been used to analyze cytokine content of the vitreous humor. However, while cytokine ratios of IL-10/IL-6 and IL-10/IFNγ can be helpful as an initial screening to rule out PIOL from uveitis, no definitive diagnosis can be made [49]. Cytologic evaluation can be used to identify common cell morphologies; however, cells in the vitreous biopsy are fragile and undergo morphological changes within 60 minutes ex vivo [50]. Flow cytometry has been the gold standard for diagnosis as it provides phenotypic cellular information diagnosing PIOL as an invasion of B-lymphocytes in the eye and uveitis as an invasion of T-lymphocytes. However, due to low cell number and fragility of the biopsy sample, the current reported diagnostic yield of flow cytometry is around 20% [51].

Accurate and rapid detection of uveitis and PIOL in early stages prevents scarring of eye tissue and permanent vision loss by allowing treatment to begin earlier. Disease management can be personalized based on cellular content, avoiding ‘broad’ treatments
that may be harmful to the patient. Finally, label-free sorting requires little to no preprocessing steps, which is especially significant when dealing with a fragile sample. The diagnostic tool can also be developed further to characterize the biopsy cells for other markers allowing for more targeted treatments and confident diagnosis.

2.3 Microfluidic Manufacturing

While many clinical needs would be met with microfluidic POC diagnostics, current rapid prototyping methods for microfluidics used by researchers are not scalable for commercial use. Traditional PDMS microfluidics use a technique known as soft lithography introduced by the Whitesides group [52,53]. These methods have been used extensively over the past couple decades in academic labs to rapidly prototype microfluidic devices. And while there are many benefits to this manufacturing method, it is not easily scalable. Alternative rapid prototyping materials and methods are required to improve commercial potential of these devices.

2.3.1 Soft Lithography

Soft lithography methods create master molds from photolithography followed by curing of a prepolymer (PDMS) on top of the mold master, where after curing, a PDMS negative stamp of the mold is created and bonded irreversibly to glass (Figure 3). Soft lithography techniques has been proven useful in microfluidics under a wide range of applications from channel fabrication to pattern generation [54]. The key benefit of soft lithography methods is the ability to rapidly prototype [55]. The technique is ideal as
feature resolution can match the micron feature sizes used in biology. The PDMS polymer provides an ideal candidate for microfluidic devices as is nontoxic, widely available, transparent, hydrophobic, gas permeable, and moderately stiff [53]. Oxidized PDMS surfaces can be irreversibly bonded together by a spontaneous dehydration of SiOH groups and PDMS can be passivated and functionalized through various chemistries for high efficiency molecular assays. A wide variety of geometries, layering, and unit operations are possible using soft lithography [53].

**Figure 3. Soft Lithography Overview.** (Left) Device master fabrication is completed by spinning photoresist on a silicon wafer and exposing to UV under a mask containing the desired channel geometry. After, the unreacted photoresist is removed through a developing solution to leave behind the chosen pattern. (Right) Device fabrication is completed by pouring a prepolymer (i.e. PDMS) over the mold followed by curing, removal by cutting, bonding to a substrate, and punching of access holes. Image adapted from [56] under the creative commons public license.
However, the soft lithography method used to create these molds suffers from the nature of the complicated, costly and time consuming pour, cure, cut and bond process as opposed to the simple injecting molding process where a mold is filled, polymer rapidly cured, and part ejected. And while contract manufacturers such as FlowJEM (Ontario, Canada) and SIMTech Microfluidics Foundry (Singapore) can provide a low-cost molds for a fee, the design process is slowed down waiting for molds to be manufactured and shipped. PDMS devices serve the prototyping market well; however, their inability to be scaled up in manufacturing and deterioration with age prevent the material from being widely commercialized [5].

2.3.2 Scalable Manufacturing Methods

Alternative rapid prototyping methods to photolithography have been reviewed previously [57]. For example, laser cutting can be used to cut microfluidic channels in double-sided pressure sensitive adhesive (PSA), to directly ablate microfluidic channels in polymer materials, and even to create molds for PDMS from laser cut adhesive [58-60]. Plotter cutting, also known as xurography, uses a drag knife printer to cut microfluidic designs from laminate and masking films [61-63]. Xurography has even been expanded to directly cut microfluidic channels in PDMS and cyclic olefin copolymer films [59,64]. While both of these methods do not provide the superior resolution of photolithographic methods, the use of thermopolymers, paper, and laminate substrates allows for more direct translation to scalable manufacturing methods such as hot embossing and injecting molding to be used to translate a finished prototype to a commercial product.
2.4 The Maker Movement

The emergence of these new alternative manufacturing methods has been augmented with the growth of the ‘Maker Movement’ that has enabled non-experts to take part microfluidics through makerspaces. The maker movement refers to the rapid growth of do-it-yourself and do-it-together projects involving collaboration through shared community space. This community space is often referred to by a variety of names such as hackerspaces, fablabs, and makerspaces; and, their purpose is to provide a do-it-yourself facility for product designers to create, invent, and learn using a variety of tools such as 3D printers, CAD software, electronics, and hardware supplies. The primary impact of makerspaces is the access to capital intensive manufacturing tools, a cost likely prohibitive for individuals but capable to be shared throughout the makerspace membership. The key advantages of makerspaces is promoting product design, innovative thinking through collaboration of interdisciplinary fields and reducing prototyping costs [65]. In the field of microfluidics, makerspaces are an attractive alternative to traditional clean rooms, which require specialized facilities, exceptionally high user costs, and costly equipment for highly trained individuals (Figure 4). Moving microfluidic system fabrication from the clean room to makerspaces will widely open the field to other disciplines. Makerspaces also provide the additional benefit of manufacturing complementary microfluidic components such as 3D-printed spinners for centrifugal devices, alignment rigs for multi-layered device building, and even common laboratory equipment [66]. The access to human capital also provides a strong boon to
makerspaces where a diverse community of individuals from varying backgrounds can provide unique prototyping assistance.

![Image of Kostas Clean Room facility and MIT-Lincoln Technology Office Innovation Laboratory makerspace](image)

**Figure 4.** (Left) Kostas Clean Room facility: photo by Matthew Modoono/Northeastern University (Right) MIT-Lincoln Technology Office Innovation Laboratory makerspace

The recent growth of makerspaces in industry and academia have created shared rapid prototyping spaces containing equipment such as laser cutters, plotter cutters, 3D printers, computer numerical control (CNC) micromilling machines, machine shops, electronics lab, and relevant hardware and software. Detailed protocols analyzing plotter cutting and laser cutting and printing manufacturing methods provide detailed technical information to help develop microfluidics from these systems [67,68]. Microfluidic prototyping in a clean room required sufficient technical expertise in photolithographic methods; whereas, maker spaces can allow anyone to rapidly prototype microfluidic platforms. Common types of microfluidic platforms well-suited for makerspaces are centrifugal and paper-based microfluidics as they only require spinning or passive flow to manipulate fluid.
2.5 Centrifugal Microfluidics

Centrifugal microfluidics uses centrifugation to manipulate fluid flow in a device. Centrifugal microfluidic platforms have been of interest to industry for over 50 years since the development of the centrifugal analyzer from Oak Ridge National Labs [69,70]. Since then, many companies such as Sony DADC and Gyros AB have developed commercial platforms as disc technology became more prevalent in point-of-care diagnostic applications. Further enabling the field, Ramachandraiah et al. demonstrated how centrifugal disc technology can be made compatible with a standard Sony DVD drive that has been converted to a laser scanning microscope [71].

A European industry partnership has even developed one of the first centrifugal microfluidics for analyzing biological samples called the Bio-Disk Platform, which has been able to perform complex assays in a multiplexed format on a single disc taking advantage of rotational symmetry and the centrifugal field [72]. Reviews of unit operations possible on centrifugal microfluidic platforms have shown the promise for disc based devices to automate all necessary assay steps such as aliquoting, valving, mixing, separating, and detection for a wide array of bioanalytical techniques from PCR to immunoassays [73,74]. For example, Burger et al. discuss how magnetic and geometrical traps can separate cells based on size, density and even surface expression [75]. The marketability of these platforms has been proven due to their more integrated nature than traditional lab-on-a-chip for simple use at the clinic [76].
2.6 Paper-Based Microfluidics

Paper-based microfluidic devices have gained a great deal of popularity as new materials, device fabrication methods, and unit operations have been developed to transform standard dipsticks into complete analysis systems [77,78]. The key advantages of paper-made devices are low cost and pumpless function [79,80].

Paper microfluidics or “paperfluidics” has regained popularity with new fabrication methods and innovative unit operations. The commercial potential comes from the ability to use passive capillary forces to move fluid and the extremely low cost of substrates and manufacturing [81]. Traditionally, paper microfluidics was commonly used as a lateral flow test such as the at-home pregnancy test. However, new innovations over the past decade have enabled paper to perform more complex functions. The melting of patterned wax on paper has allowed the creation of membranes and channel boundaries on paper devices [82-84]. Additional modifications such as patterned UV treatment have allowed further control over fluid flow characteristics [85]. Unit operations of paperfluidic devices such as valving, flow switching, and timing have allowed paperfluidic devices to perform the vast majority of unit operations offered by traditional pump flow through a device [86]. Paper microfluidics has even been used to create chemical gradients, hydrodynamic focusing, and dilution regions [80,87]. For example, Warren et al. developed an integrated paperfluidic platform capable of identifying synthetic urinary biomarkers to monitor disease [88].

Literature reviews have discussed the commercial potential of new paperfluidic devices gained from their low-cost, simple fabrication techniques as well as clinical applications with incorporated detection methods [89,90]. Manufacturing of paperfluidic
devices has been accomplished by a variety of methods including laser, ink, wax, and plotter cutter based methods [91]. 3D paperfluidic devices have been enabled in large scale manufacturing using modified bookbinding methods [92]. The combination of paper and centrifugal microfluidics have been shown to complement each other by providing competing paper capillary force and centrifugal force for more advanced fluid manipulation and separation [93].
3. MATERIALS AND METHODS

This section details the techniques utilized for microfluidic device fabrication and their use in biological sample analysis, ranging from traditional to scalable methods. First, chronic eye disease diagnostics are addressed by traditional microfluidic manufacturing methods (Section 3.1) and then scalable methods (Section 3.2). Second, idiopathic eye disease diagnostics are developed first with traditional soft lithography methods (Section 3.3) and then with scalable methods using paper (Section 3.4) and centrifugal microfluidics (Section 3.5). From all of these experiences, a guide to do-it-yourself microfluidics using laser and plotter cutting is provided in Section 3.6.

3.1 Microfluidic Sandwich Immunofluorescence Assay (sIFA)

The original platform to determine the best treatment for chronic ocular diseases was developed by Green et al. using soft lithography techniques to create a pillar array device. Images of the sIFA microfluidic device are shown in Figures 5a and 5b [94]. Figure 5a is a photograph of the device showing 12 independent fluidic channels and Figure 5b shows the interior detail of one channel where VEGF has been captured and illuminated with a fluorophore. Each channel contains vertical pillar structures to enhance the surface area of immobilized capture antibodies from a sample flowing from top to bottom or vice versa in Figure 5b. Microfluidic devices were fabricated as described previously [94].
Figure 5: Sandwich Immunofluorescence Assay (sIFA) (a) Photograph of multiple sIFA channels on a single PDMS device; (b) 3D schematic of single sIFA channel with pillar array; (c) Fluorescent microscope image of fluorescent capture by pillar elements coated with fluorescent capture antibody. Figure reprinted with author permission from [94].

To characterize the sensitivity and accuracy of the sIFA, we developed two sets of calibration standards. The first set utilized the microfluidic channel with a bare pillar array filled with solutions of Oregon Green fluorophore (A-6374; Invitrogen, Grand Island, NY) at concentrations of either 1, 5, 50 or 100 pmol/L [94]. These solutions were passed into each of the five channels on each microfluidic device manually with a syringe. We chose this approach since it allowed us to directly test signal at known fluorophore concentrations in the absence of complicating factors such as coating of the sIFA posts with antibodies and VEGF-antibody binding efficiency. A set of control chips (phosphate buffered saline only) was also fabricated so that background measurements could be obtained and subtracted from each sample. For each device, the mean and standard deviation from the five channels were computed. This was repeated 4 times for each concentration.
A second set of sIFA calibration standards were prepared where the posts were coated with anti-VEGF (MAB293, R&D Systems, Minneapolis, MN) capture antibodies. Solutions containing known concentrations of VEGF (293-VE, R&D Systems) were then passed through the microfluidic device using a syringe pump (PHD2000 microsyringe pump, Harvard Apparatus, Holliston, MA) and captured on the coated posts. A biotinylated detection antibody (BAF293, R&D Systems) and an Avidin-Oregon Green conjugate (A-6347, Invitrogen) were then sequentially passed through each channel of the device. A series of chips was fabricated using VEGF solutions of 1, 5, 10, 50, 100, 500 and 1000 pg/mL. This range was selected as it approximately covers the expected concentration range of VEGF in clinical vitreous fluid samples. A control microfluidic device (0 pg/mL) was also prepared, and each experiment was repeated 4 times for each concentration.

As an initial test to quantify the VEGF concentration from clinical fluid samples, we performed a limited trial on two intraocular fluid samples obtained during vitrectomy surgery. This project was conducted in compliance with all applicable Health Insurance Portability and Accountability Act regulations. Informed consent was obtained from all subjects on whom personal health identifying information was recorded in accordance with a Duke Institutional Review Board-approved protocol. These vitreous samples, diluted by balanced saline infusion fluid were obtained from eyes of patients who underwent planned vitrectomy at the Duke Eye Center. Samples were frozen and shipped to Northeastern University. Prior to analysis, these samples were concentrated using centrifugal filter devices (Amicon Ultra-15 10k Da membrane, Millipore, Billerica, MA)
from 50-200 mL total volume to between 1-3 mL. A single microfluidic chip containing 5 channels was prepared as above for each of the clinical samples.

All sIF A microfluidic devices were read using fluorescence microscopy. A Nikon Eclipse TE2000 inverted microscope with a charged-coupled device camera (CCD) was used, and images were taken with a constant gain (16X) and an exposure time of 0.5 seconds. Image analysis software (Nikon NIS Elements 2.1) determined the average fluorescence intensity for a rectangular region in the center of each channel. This measurement was made on the same z-plane for all devices, although fluorescence measurements showed negligible variation at different z-planes.

3.2 Point-of-Care Ocular Rapid Test (PORT)

The second platform to determine the best treatment for chronic ocular diseases was developed using scalable microfluidic manufacturing techniques. The Point-of-care Ocular Rapid Test (PORT) uses fundamental sedimentation principles (Section 3.2.1 and 3.2.2) in a device composed of double sided adhesive with laser cut microfluidic channels sandwiched between plastic discs (Sections 3.2.3 and 3.2.4). Leveraging bead-based ELISA chemistry (Section 3.2.5), the PORT device was set up to quantitatively measure intraocular VEGF levels from model and clinical samples (Sections 3.2.6 – 3.2.8).

3.2.1 Sedimentation Fundamentals

The method employed by the PORT device uses sedimentation fundamentals as previously described by Schaff et al. [96]. Briefly, a sample containing an analyte of
interest is mixed with antibody-coated microspheres and detection antibody. This mixture is transferred into a microfluidic channel and deposited on top of a fluid with density greater than that of the bulk sample but less than that of the microspheres (Figure 6). An incubation period allows linking between the microspheres and detection antibody when the analyte is bound to the former. When a centrifugal force is applied, the dense microspheres will travel through the density medium away from the sample region to the bottom detection region. The detection region will then generate a fluorescent signal whose intensity is proportional to the concentration of the analyte in the original sample.

**Figure 6. Microsphere Sedimentation Assay Principles** (1) Each region on the microfluidic channel is loaded sequentially from highest to lowest density. Sample is mixed with detection reagents including antibody coated microspheres, biotinylated detection antibody and streptavidin-conjugated enzyme. (2) A short incubation period of 10 min enables formation of a sandwich complex, including coated microspheres, target analyte, and an enzyme. (3) A moderately fast centrifugation efficiently separates and washes sandwich-complexed microspheres from unbound detection reagents. Bound enzyme on the pelleted microspheres will then reduce the fluorogenic substrate and thereby convert colorless, inactive substrate particles to active and fluorescent particles.
The sedimentation rate of spherical microspheres (Equation 1) is based on Stokes’ law:

\[ U_s = \frac{2}{9} \frac{\rho_m - \rho_f}{\mu} g R^2 \]  

(1)

where \( U_s \) is the sedimentation velocity, \( \rho_m \) is the density of the microsphere, \( \rho_f \) is the density of the fluid, \( \mu \) is the viscosity of the fluid, \( g \) is the acceleration due to gravity or centrifugation, and \( R \) is the microsphere radius.

Equation 1 illustrates how this approach is a simple, but very effective method to separate complexed assay microspheres from unbound assay reagents. The radius of unbound detection antibody molecules is approximately three orders of magnitude smaller than that of the microspheres. Per Equation 1, the sedimentation rate of the microspheres is, therefore, six orders of magnitude less than that of the microspheres (as \( U_s \) is proportional to \( R^2 \)). The sedimentation of a microsphere through a density medium also provides an efficient washing mechanism as any molecules not bound to the microspheres will encounter resistance to sedimentation within the density medium and become trapped. Once the microspheres are pelleted to the bottom of the channel, the only detection reagents present will be the ones bound to the microsphere through the sandwich complex.
3.2.2 Device and Assay Design

The approach shown schematically in Figure 6 is carried out in wells fabricated within plastic disks. The wells are radially aligned with the detection regions located near the disk periphery. As described above, the detection region is composed of a dense, colorless, fluorogenic substrate, which becomes fluorescent by hydrolysis in the presence of an enzyme. The density medium is dextran in buffer and the sample region contains a mixture of analyte-laden sample, antibody-coated microspheres, biotinylated detection antibody and streptavidin-conjugated enzyme. The incubation step (second step in Figure 6) allows time for the sandwich complexes to form, connecting the microspheres to the enzyme only if the sandwich is completed by the presence of bound analyte. Finally, sedimentation will pellet the microspheres to the detection region while leaving excess enzyme label and unbound antibody in the sample region. Once the microspheres are pelleted, the bound enzyme will rapidly trigger hydrolysis of the fluorogenic substrate converting substrate molecules from an optically inactive state to an active fluorescent state.

3.2.3 Materials

Dynabeads Antibody Coupling Kit and VEGF Human ELISA Kit were obtained from Life Technologies (Carlsbad, CA). VEGF\textsubscript{165} Standard, VEGF Biotinylated Polyclonal Antibody, and VEGF Monoclonal Antibody were obtained from R&D Systems (Minneapolis, MN). Dextran (~10 kDa MW), Resorufin \( \beta \)-D-galactopyranoside fluorogenic substrate and streptavidin-\( \beta \)-galactosidase enzyme, potassium chloride (KCl),
magnesium chloride (MgCl$_2$) and Tween-20™ were obtained from Sigma-Aldrich (St. Louis, MO). Phosphate buffered saline (PBS; 1x, without calcium or magnesium), bovine serum albumin (Fraction V), dimethyl sulfoxide (DMSO), Amicon Ultra-15 10kDa membrane filter units and tris base were purchased from Thermo Fisher Scientific (Waltham, MA). A rechargeable oscillating toothbrush was purchased from CVS Pharmacy (Boston, MA). Immunoassay disks and custom made disk spinner were purchased from Sandstone Diagnostics Inc. (Livermore, CA). 7x detergent cleaning solution was purchased from MP Biomedicals (Santa Ana, CA).

3.2.4 Device Fabrication

The Point-of-Care Ocular Rapid Test (PORT) centrifugal microfluidic device was fabricated using a Full Spectrum laser cutter at Sandstone Diagnostics (Livermore, CA). The PORT device consists of layers of acrylic sheet stock and pressure-sensitive adhesive (PSA) laser cut and sandwiched together to fully-enclose the PSA fluidic channels. Fluidic channels are cut into the center PSA layer while the top acrylic layer contains one inlet and one air outlet.

Before device assembly, a cleaning protocol was implemented to remove microscale debris from all surfaces. An oscillating toothbrush was used with MP Biomedicals cleaning solution diluted to 1x to clean the top and bottom faces of the acrylic layers. Disks were dried using the custom disk spinner at the low setting to remove adherent cleaning fluid. Device cleaning and assembly were both performed in a biological safety
cabinet to ensure device cleanliness. Once a PORT device was fully assembled, it was packaged in a small, individual, zip-locked plastic bag until its use.

### 3.2.5 Microsphere Preparation

Dynabeads M-270 epoxy microspheres were washed with kit buffer and then incubated with 100 μg VEGF monoclonal antibody and kit incubation buffer overnight on a rotisserie (LabQuake) in an incubator maintained at 37°C for 24 hours. Microspheres were then blocked with kit blocking reagent according to kit instructions and washed several times according to the Dynabeads Antibody Coupling Kit instructions and brought to a final microsphere concentration of 10 mg/mL in kit standard buffer.

### 3.2.6 PORT Dose-Response Experiments

Density medium was prepared by adding dextran to PBS buffer containing 0.1% BSA and 0.005% Tween-20 to a final dextran concentration of 12.5% (w/v) dextran. Resorufin β-D-galactopyranoside fluorogenic substrate stock was reconstituted to a final concentration of 15 μg/mL in sterile DMSO. The detection medium was created by adding 0.1% v/v resorufin β-D-galactopyranoside stock and 25% (w/v) dextran to 100 mM Tris buffer with 2.0 mM KCl and 0.1 mM MgCl2. Both density medium and detection medium stocks were stored at 4°C.
Next, the VEGF standard was reconstituted to 100 μg/mL in PBS with 0.1% BSA. Serial dilutions were prepared at a ratio of 1:10 to make standard VEGF solutions at concentrations of 1 μg/mL, 100 ng/mL, 10 ng/mL, and 1 ng/mL. A negative control solution consisting of PBS with 0.1% BSA was also prepared. Next, streptavidin-β-galactosidase enzyme was reconstituted to 2 mg/mL in deionized water. A working dilution was then prepared by creating a 1% v/v streptavidin-β-galactosidase in PBS with 0.1% BSA.

To prepare samples, all assay reagents were mixed together. First, 35 μL of 1% antibody-coated microspheres solution was placed into a 0.5 mL microcentrifuge tube. A rare earth metal magnet was then used to remove the buffer supernatant from the microspheres. Next, 25 μL of VEGF standard sample (or control solution) was added followed by 5 μL of biotinylated VEGF antibody stock solution and 5 μL of streptavidin-β-galactosidase solution (working dilution). The resulting solution was then wrapped in aluminum foil and placed in the lab rotisserie to incubate for 10 minutes. Separate 40 μL aliquots of resorufin-dextran detection medium and density medium were also made for individual use.

A Nikon Eclipse TE2000 inverted microscope was prepared for resorufin fluorescence using a green-filtered fluorescent lamp and a red-filtered, charged-coupled device (CCD) camera. The CCD camera was set up with NIS-Elements imaging software to control the gain and exposure time for fluorescent imaging. With each individual disk, sample wells were analyzed, one at a time, in a randomly chosen fashion.

Fluid channels on the PORT disks were loaded in three stages. First, 1 μL of resorufin-dextran detection medium was injected into one well and spun down at the
9000 rpm for 20 seconds with the custom disk spinner to ensure that all the fluid went into the bottom. Secondly, 4 μL of dextran density medium was added and spun at 9000 rpm for 1 second to stack on top of the detection medium. Finally, 4 μL of the incubated sample was added and spun at 6500 rpm setting for 30 seconds, which caused the microspheres to pellet completely at the bottom of the detection region. Disks were then placed on the microscope stage and the area directly behind each microsphere pellet was imaged using the 10x objective. The microsphere pellet was then exposed for 8 minutes to a full-fluorescence lamp to induce background photobleaching after which an image was taken at every minute exactly.

3.2.7 Clinical Samples

Due to the invasive nature of isolated aqueous tap biopsies and the convenience of obtaining vitreous samples during already scheduled vitrectomy surgery, vitreous aspirates were used in place of anterior chamber fluid as VEGF levels correlate between these two chambers of the eye [95,97,98]. Discarded vitreous aspirates obtained from patients undergoing vitrectomy surgery at the Duke Eye Center were obtained via an Institutional Review Board (IRB) approved protocol. A vitrectomy is a surgical procedure wherein the gel-like vitreous humor that makes up the vitreous body of the eye is extracted using a device that cuts the gel into small fragments and withdraws them from the eye while a substitute water-based saline fluid is infused into the eye at the same rate as vitreous fluid is removed to maintain the hydrostatic pressure of the eyeball. The vitrectomy fluid sample volume was typically 100-200 mL, and comprised a mixture of vitreous fluid and infusion fluid. The large sample volume variability resulted from
variable amounts of infusion fluid that was admixed with the vitreous fluid aspirate. For this study, vitrectomy samples collected in the operating room were frozen and shipped on dry ice to Dr. Murthy’s lab for analysis.

Prior to analysis, the biopsy samples were pre-concentrated using centrifugal filter devices with a 10 kDa cutoff membrane from 50 – 200 mL total volume to between 1 – 3 mL. Concentration factors were recorded as the ratio of start to final volumes and vitreous samples were kept in a -20°C freezer. Experiments using vitreous samples followed the protocol described above under “PORT Dose-Response Experiments” with the biopsy sample in place of the VEGF standard.

3.2.8 Image Analysis

Microscope images were analyzed using the tools provided in the NIS-Elements image analysis software. Using the small circle tool, fluorescence intensity measurements were made directly behind the microsphere pellet and at the far bottom-right hand corner as a background measurement. The fluorescent intensity measurement was taken as the numerical difference between the background and target measurement. A final measurement was produced by averaging all fluorescent intensity measurements after 5 minutes to get a “steady” state value.

3.3 Chemotaxis Sheath Flow Device (CSFD)

The second clinical need of diagnosing idiopathic eye disease between uveitis and PIOL was first developed using soft lithography techniques to create a sheath flow
device. The PDMS devices were designed in AutoCAD (Autodesk) and sent to a contract manufacturer (FlowJEM, Toronto, Canada) to produce a mask, SU-8 master and multiple pre-bonded and cleaned devices. The device had three inlets and two outlets and is shown schematically below in Figure 7.

![Schematic design of the CSFD device](image)

**Figure 7: Schematic design of the CSFD device.** Left side represents the inlets where cells entering the center inlet are sheathed by the edge inlets and cells are sorted into the right outlets.

Syringe pumps (PHD2000 microsyring pump, Harvard Apparatus) were connected at both the inlet and outlet and only the sheath inlets and outlets actively pumped or vacuumed, respectively, at rates between 1 to 5 µL/min. And, Jurkat cells were obtained from ATCC and incubated in Corning T-75 flasks at 37°C with 5% CO₂ in RPMI 1640 culture medium with L-Glutamine, 10% fetal bovine serum and 10 U/mL penicillin-streptomycin.
3.4 Chemotaxis Immunophenotyping Device (CID)

The second platform to diagnose idiopathic ocular diseases such as uveitis and primary intraocular lymphoma was developed using scalable microfluidic manufacturing techniques. The Chemotaxis Immunophenotyping Device (CID) was created as a paper-based microfluidic device (Section 3.4.1) that could produce a rapid and stable chemical gradient (Section 3.4.2). Primary immune cells were prepared (Section 3.4.3) and experiments were setup to monitor and analyze cell migration on the device (Section 3.4.4).

3.4.1 Fabrication of Paperfluidic Device

The procedures for manufacturing the paper device require only hand-cut pieces of Whatman’s conjugate pads (Fusion 5 and Standard 17; GE Healthcare Life Sciences, Pittsburg, PA) and a standard 25 mm x 75 mm glass microscope slide (ThermoFisher). Briefly, sheets of Fusion 5 and Standard 17 conjugate pads were cut into rectangular pieces of 1 cm x 2 cm and 2 cm x 2 cm, respectively. The Standard 17 squares act as chemoattractant reservoirs while the Fusion 5 acts as the bridge for the gradient. The paper device was assembled on the standard microscope slide. The paper is placed in the format shown in Figure 8.
Figure 8. Schematic showing the paper device. (A) Two paper devices, one dry and one wet with blue and red food coloring showing a sharp gradient in the center. (B) Visual representation of the paper device containing B-cell attractant 1 (BCA-1) and stromal-derived factor 1 alpha (SDF-1α) acting on fluorescent cells. The inset image shows the visualization of cells through the Fusion 5 paper membrane.

3.4.2 Gradient Measurement

Gradient testing was completed using the above paper device and wetting each side with 100 μL of fluorescent Dextran conjugated with Oregon Green® 488 or Texas Red® simultaneously and analyzing the meeting point using a fluorescence TE2000 Nikon inverted microscope with fluorescein (480 ± 30 nm/535 ± 40 nm) and rhodamine (540 ± 25 nm/605 ± 50 nm) filters. Gradient testing solutions were composed of complete, supplemented cell media containing fluorescent dextran (5 μM, MW = 10kDa, Life Technologies, Carlsbad, CA) of similar molecular weight to the tested chemokine (MW of SDF-1α = 8.8 kDa). Each side of the device contained dextran with a differently conjugated fluorophore. Each solution was pipetted simultaneously on either side of the device and imaging began as soon as the solutions met on the paper bridge. Images were
taken using NIS-Elements software every minute for 20 minutes with both red and green filters for Texas Red® and Oregon Green® conjugated dextran, respectively. Images were then analyzed using ImageJ software by averaging pixel intensity across the fluorescent images. The gradient profiles at time of 0 and 20 minutes were compared using a Kolmogorov-Smirnov test for similarity at $\alpha = 0.05$.

3.4.3 Cell Preparation

Human peripheral blood pan-T cells were obtained directly from StemCell Technologies (Vancouver, Canada). Lymphocytes were incubated on a 24-well plate in supplemented culture medium (RPMI-1640 with L-Glutamine, 10% premium FBS and 10 U/mL penicillin-streptomycin). Cells were also incubated with Dynabeads® Human T-Activator CD3/CD28/CD137 and 5 ng/mL IL-2, all purchased from Life Technologies, to trigger and maintain cell activation as per the manufacturer’s protocol. Cells were maintained with fresh media and cytokines every 2-3 days and experiments were only performed 7-10 days following initial activation.

3.4.4 Cell Migration Experiment in Paperfluidic Device

The microfluidic device was prepared by first coating the bottom of a 60 mm Falcon petri dish (ThermoFisher, Waltham, MA) with a solution of 5 μg/mL fibronectin (Life Technologies) in PBS. After one hour incubation, the petri dish was rinsed out with sterile PBS and subsequently blocked with a solution of PBS with 2% BSA for one additional hour.
An aliquot of approximately one million T lymphocytes was stained with the cell tracking dye carboxyfluorescein succinimidyl ester (CFSE, Life Technologies) following manufacturer’s protocol. Once the staining was complete, cells were suspended in supplemented culture medium to a final volume 100 μL and kept in a cell incubator until use. Assay solutions were then prepared by aliquoting supplemented culture medium with and without 1 μg/mL stromal-derived factor 1 alpha (SDF-1α, Life Technologies).

An aliquot of 10 μL dyed cells was placed in the center of the petri dish. After 30 seconds, 100 μL of warmed supplemented cell medium was added on top of the cells. The dish was then covered for 15 minutes to allow cells time to attach to the fibronectin. Next, 150 μL of cell medium with or without SDF-1α was added to each reservoir square of the paper device. A mark was made on the slide to indicate where the two fluids collided on the paper which bridged the two reservoirs. The paper device was then placed face-down on the fibronectin-coated petri dish. The gradient area was aligned directly over the cells. The entire paper device in the fibronectin-coated petri dish was placed on a heated microscope stage (37°C) with carbon dioxide injection (5%). The gradient was lined up on a Zeiss Axiovert 200 M microscope and five to ten locations were manually selected along the gradient containing multiple single cells in each frame. A mercury-xenon lamp was used to trigger fluorescence with a 3 second exposure time and images at every location were captured every minute for 20 minutes with the AxioVision software. Sets of images were grouped and cell tracks were analyzed using a custom Matlab script described in another publication [99]. This process is similar to the one schematically shown in Figure 8.
3.5 Spinning Ocular Lymphocyte Immunophenotyping Device (SOLID)

The third platform to diagnose idiopathic ocular diseases such as uveitis and primary intraocular lymphoma was also developed using scalable microfluidic manufacturing techniques. The Spinning Ocular Lymphocyte Immunophenotyping Device (SOLID) was established as a full system to process large biopsy volumes with a custom spinning stand and a disc device with microfluidic channels laser-cut from double-sided adhesive (Section 3.5.1). A large volume vitreous biopsy model was created using both microspheres and cells (Section 3.5.2) and a protocol was developed to process and image captured cells (Section 3.5.3).

3.5.1 Device and Setup Manufacturing

The rotating platform is composed of a custom built aluminum stand with integrated EC 90 flat 90 mm diameter, brushless, 90 watt motor with hall sensors and ESCON 50/5, 4-Q servocontroller from Maxon Motor. Depressions on top of the spinning aluminum stand allow a vacuum seal to form between the device and the stand, connected directly to a 230 VAC VWR vacuum gas pump. A custom, Plexiglas waste collection chamber is placed on top of the aluminum stand. Finally, a second custom, Plexiglas outer stand is placed on top to safely contain any leaks or spills during operation. A photograph of the rotating platform is shown in Figure 9a.
Figure 9. Overview of setup and SOLID filtration device. (a) Photo of custom built setup, device stands on rotating aluminum stand connected to a motor and controller. Blue suction line keeps disc device connected to stand. Discarded fluid is collected by the waste collector and outer case is added for safety. (b) Exploded view of device without 50 mL centrifuge tube top (c) Schematic of particles being captured by the device during spinning. Flow is triggered by the centrifugal force of the spinning disc on the fluid and particles are filtered by the paper.

The SOLID device is composed of pre-cleaned, laser cut, polypropylene, double-sided, pressure-sensitive adhesive (PSA) tape (#90880 ARcare) and Whatman’s Fusion 5 paper sandwiched between two polycarbonate (PC), DVD flat-shape, 0.6 mm thickness, 120 mm diameter discs with no stacking ring and a flat stamperholder (Axxicon). The bottom disc has a standard 15 mm diameter center hole while the top disc has a custom 22.5 mm diameter center hole to better match the diameter of the sample tube (26 mm inner diameter) above. Briefly, a 150 mm square piece of PSA was laser cut with a Hypertronics HT6340 Synrad Laser cutter to have four microfluidic channels. A laminator was used to bind one side of the cut PSA to a PC disc. For the filtration SOLID design, four pieces of Fusion 5 paper were manually placed in each channel before placing the top disc on top using a custom disc aligner stand. Pressure was then
applied using a hand roller to seal the device together. Next, an 18.5 mm diameter sticker (#9964 3M) was placed over the smaller diameter disc to block leakage from the sample tube. Afterwards, two-component Araldite super glue was placed on the open end of a 50 mL Falcon centrifuge tube (Fisher) and placed upside-down over the center of the disc and left for 12 hours to seal. Finally, a hand saw was used to cut off the closed side of the centrifuge tube. A schematic of the device is shown in Figure 9b and 9c.

3.5.2 Vitreous Biopsy Model

Vitreous biopsy models were created with either fluorescent microspheres or cells (Jurkat or Raji). Jurkat and Raji cells were obtained from ATCC. All cells were incubated in Corning T-75 flasks at 37°C with 5% CO₂ in supplemented culture medium (RPMI 1640 with L-Glutamine, 10% fetal bovine serum and 10 U/mL penicillin-streptomycin). First, a dilution of microspheres or cells were pipetted into Eppendorf tubes with concentrations ranging from 1-5 million particles/mL in a total volume of 1 mL. Just before an experiment, microspheres or cells were injected into a larger volume of PBS or pre-warmed culture media to a total volume of 20 mL to model the high volume and low particle number expected from a vitreous biopsy.

3.5.3 Running the Device

After motor calibration with the ESCON studio software, a completed microfluidic device was placed on the free-spinning aluminum stand and the vacuum was turned on to seal the device down. After the waste chamber was placed, the vitreous biopsy model was poured into the device. The outer case was then placed over the setup and the motor
was started by pressing and holding a manual switch with an acceleration of 20 rpm/s to minimize the torque requirement of the platform. Unless otherwise stated, all experiments were performed by spinning at 1000 rpm with 45 seconds on, 5 seconds off, and 30 seconds on to ensure all fluid had been processed. When cells were used, a second step adding 1 mL of FxCycle PI/RNase or Calcein AM prepared to manufacturer’s protocol in warmed media was added and spun down for 5 seconds. Once all spinning was complete, the outer and waste containers were removed and the vacuum was switched off to remove the device. The sample tube was then manually removed from the top of the device and the bottom disc was placed under a Nikon Eclipse Ti-E microscope connected to a Lumencor Sola SE II light engine with Texas Red and FITC filters and images were taken along the paper filters in each of the 4 channels on the device using ProgRes CapturePro software. Fluorescent images going from the proximal edge of the paper to the distal edge were stacked up manually and inputted into ImageJ software. A “Plot Profile” was built from fluorescent signal from the proximal to distal end of the paper in each device channel. Further information about the image analysis technique used can be found in Figure 10.
Figure 10. Plot Profile Measurement Method. Fluorescent particle distributions were determined by stacking multiple images along a linear path of the filter paper. Images were aligned using reference markings between images. Images were then analyzed in ImageJ using the rectangular “Plot Profile” tool to integrate mean fluorescence measurements along a rectangular path shown in yellow.

3.6 Do-It-Yourself Microfluidics

The below section offers insight to development of microfluidic platforms using simple materials and manufacturing equipment often found in makerspaces. The guide provides advice for materials selection (Section 3.6.1) and tool selection (Section 3.6.2). The process for do-it-yourself microfluidics in makerspaces is then detailed in a Design-Cut-Assemble process in Sections 3.6.3 – 3.6.5.
3.6.1 Materials Selection

Selecting a plastic for an application can depend on a variety of factors such as optical clarity, solvent resistance, and available manufacturing methods to name a few. While this section focuses on acrylic, other options such as polystyrene may be desired. However, due to the inability of polystyrene to be cut on a conventional laser cutter, a specialty contract manufacturer such as Axxicon (http://axxicon.com/) may be required. The downside of these manufacturers is often the requirement of large bulk orders of custom parts to make a profit. Acrylic, on the other hand, is a widely available plastic available in a variety of shapes, thicknesses, colors, and coatings from companies such as McMaster-Carr (www.mcmaster.com) and can be cut with a laser cutter. For laboratories without a laser cutter, materials can be shipped pre-cut by laser cutting services such as Ponoko (www.ponoko.com) at a low cost with no minimum order.

Selecting a tape adhesive can be a daunting task especially with the expansive selection from 3M (www.3m.com) and Adhesives Research (www.adhesivesresearch.com). The key considerations for selecting a tape are 1) manufacturing considerations, 2) tape thickness, and 3) cost/availability. For manufacturing a plastic device held together by double-sided tape with laser or plotter cut channels, the tape will need to be ‘Double Lintered’, meaning both sides of the adhesive have a liner. While tape converter companies such as Converters Inc. (www.converters.com) offer to add a second liner, large minimum orders can be cost prohibitive. Converters can be avoided by purchasing tapes that already come with liner on both sides. The second consideration of tape thickness can also help guide the type of tape needed. Transfer tapes are double sided adhesives composed entirely of adhesive
material whereas traditional double-sided adhesive have a carrier layer coated on both sides with adhesive. Thus, transfer tapes are better suited for thinner applications (<50 μm); whereas, double-sided adhesives are suited for thicker applications (50 – 200 μm).

The final consideration is cost and availability of double sided adhesives as the minimum order direct from 3M or Adhesives Research are typically on the range of 1500 foot rolls and cost upwards of $10,000. Both of these companies do offer samples of their products or their products can be purchased in smaller amounts from a distributor such as Grainger (www.grainger.com) and Amazon.com (www.amazon.com) pending on availability. Table 1 contains a list of all the adhesives used by the authors along with comments to best help guide tape selection.
Table 1. Table of recommended double-sided adhesives for microfluidics from 3M and Adhesives Research.

<table>
<thead>
<tr>
<th>Product Description</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARseal 8026 – Clear Silicon Transfer Tape (25 micron)</td>
<td>-Cuts well</td>
<td>-Very difficult to peel and place (too thin, no carrier layer)</td>
</tr>
<tr>
<td>ARcare 90445 – Clear Polyester Double-Sided Adhesive Tape (81 micron)</td>
<td>-Popular in microfluidics</td>
<td>-Burn products may effect PCR and similar reactions</td>
</tr>
<tr>
<td>ARcare 92848 – While Polyester Double-Sided Heat Sealing Tape (97 micron)</td>
<td>-Tape seal improves with heat instead of pressure</td>
<td>-Not translucent</td>
</tr>
<tr>
<td>ARcare 92712 – Clear Polyester Double-Sided Adhesive Tape (48 micron)</td>
<td>-Cuts well</td>
<td>-Difficult to peel and place (too thin, very sticky)</td>
</tr>
<tr>
<td>ARcare 90106 – Clear Polyester Double-Sided Adhesive Tape (142 micron)</td>
<td>-Serves well as a single-sided tape</td>
<td>-Opaque liner cuts oddly on laser cutter (burn products)</td>
</tr>
<tr>
<td>ARseal 90880 – Polypropylene Double-Sided Adhesive Tape (142 micron)</td>
<td>-Easiest to cut</td>
<td>-Only available in one thickness</td>
</tr>
<tr>
<td>3M 9964 – Clear Polyester Diagnostic Microfluidic Medical Tape (60 micron)</td>
<td>-Easy to cut</td>
<td>-Single-sided adhesive</td>
</tr>
<tr>
<td>3M 9965 – White Polyester Double-Sided Tape (90 micron)</td>
<td>-Bioassay compatible</td>
<td>-White (not translucent)</td>
</tr>
<tr>
<td>3M 9969 – Adhesive Transfer Tape (60 micron)</td>
<td>-Easy to cut</td>
<td>-Can be difficult to place</td>
</tr>
<tr>
<td>3M 468MP – Adhesive Transfer Tape (130 micron)</td>
<td>-Easy to cut</td>
<td>-Not targeted for microfluidic platforms</td>
</tr>
<tr>
<td></td>
<td>-Widely available from distributors (Amazon)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-Provides initial repositionability on plastics</td>
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</table>
Selecting a paper substrate is entirely dependent on the context for its application as a filter, fluid reservoir, gradient generator or imaging substrate. GE Healthcare Life Sciences’s Whatman line (www.gelifesciences.com) offers a wide variety of paper substrates with thicknesses appropriate for plastic/tape microfluidics (50 – 500 µm). Table 2 contains a list of all of the paper substrates used by the authors along with comments to best help guide paper selection.

Table 2. Recommended Whatman paper substrates available from GE Healthcare.

<table>
<thead>
<tr>
<th>Paper Substrate</th>
<th>Good for:</th>
<th>Bad for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard 14 and 17 – Glass fiber</td>
<td>-Holding large volumes of fluid</td>
<td>-Fluorescence microscopy (high background)</td>
</tr>
<tr>
<td>Fusion5 – Proprietary Single-Membrane Matrix</td>
<td>-Fluorescence microscopy (low and uniform background)</td>
<td>-Holding large volumes of fluid</td>
</tr>
<tr>
<td>CF1, CF3, CF4, CF5, CF6, CF7 – Cotton Linter Material</td>
<td>-When you need a specific thickness -Fluid transfer</td>
<td>-Fluorescence microscopy (non-uniform background)</td>
</tr>
<tr>
<td>CF2 – Cellulose Fiber</td>
<td>-Applications that require sturdy paper</td>
<td>-Does not excel in any particular area</td>
</tr>
<tr>
<td>Grade 470</td>
<td>-Blotting paper and gelatinous samples</td>
<td>-Fluid transfer</td>
</tr>
</tbody>
</table>

3.6.2 Tools

Laser and plotter cutting are the two methods examined in this report for cutting microfluidic channels in tape, paper, and plastic. Both of these cutting methods are similar in workflow by feeding a substrate into each to be cut by either a laser or knife. Laser cutters have the benefit of non-contact cutting and higher resolution. These benefits come at the expense of higher capital equipment costs, requirement for a vacuum pump to clear out debris and fumes, and potential burn products created during the
Plotter cutters (also commonly referred to as vinyl cutters) are significantly cheaper, require no pumping, and leave no burn products. With the growing popularity of makerspaces in both academia and industry, many facilities now have these manufacturing capabilities already available in a shared space.

For example, the VLS 4.60 Universal Platform Series laser cutter (http://www.ulsinc.com/products/vls460/) sells standard with the manufacturer’s suggested retail price of $22,500 and additional options such as a filtration system are extra. CAD designs from a variety of programs such as AutoCAD, Solidworks, and CorelDRAW can be imported to the laser cutter’s software and instantly cut with pinpoint thickness dependent on proper focusing. Additional details can be read from the manufacturer’s webpage.

An example of a plotter cutter is the Graphtec CE-40 Vinyl Cutter (www.graphteccorp.com). The product sells on Amazon.com for $1,195 including software and cutting knife. With an addressable resolution of 10 µm, the plotter cutter can accept CAD designs from Adobe Illustrator, CorelDRAW, or their own software Graphtec Studio. Additional details can be read from the manufacturer’s webpage.

3.6.3 Design

Once the materials have been chosen, a computer-aided drawing (CAD) file is required to guide the laser or plotter cutting process. Designs constraints are typically few except for the feature size, which must be as large as your cutter’s resolution. Some specific practical constraints for design are the following:
(1) Carefully consider your choice of design software for the cutter being used. While almost any software can produce files that can be converted to many types, some laser and plotter cutters can directly convert designs from software such as AutoCAD, Solidworks and CorelDRAW.

(2) Ensure there are no repeated lines in the design or two cuts will be made.

(3) Plastic is usually not gas permeable, air vents must be included to allow fluid to freely flow through the channels. The lack of air vents can block flow and effect flow patterns (which may be purposefully leveraged). A recommended size for air vents is 0.9 mm diameter, a size big enough to easily remove unwanted material.

(4) Do not forget to cut inputs into the top layer, a recommended size is 1.6 mm diameter to fit a pipette.

(5) Consider channel volume as the best way to determine how big to design your microfluidic chambers. Consider your input volume and then create designs that allow that volume to be properly processed through the device at each step.

(6) If paper is going to be embedded into a tape device, a recommended method is creating a perfect-fit “cast” for the paper. It is recommended to have the paper slightly thicker than the tape thickness to prevent escape. A laminator or laminating roller will squeeze the paper in place.
3.6.4 Cut

Once your materials have been selected and your design complete, it is time to select a manufacturing method. While this guide focuses on laser and plotter cutting, 3D printing and CNC-micromilling machines are potential viable alternatives. However, for the most simple and rapid manufacturing, laser and plotter cutters provide the simplest interface for users with a material and CAD file. To help select a manufacturing method, Table 3 below highlights the key differences between laser and plotter cutters.

Some considerations while using these cutting methods:

(1) Keep your material as flat as possible during cutting; for laser cutting, use painter’s tape on the edges to prevent a substrate from blowing away; for plotter cutting, use an adhesive backing to flatten out a substrate during cutting.

(2) It may require some trial and error of laser power and knife force profiles in order to properly cut through your substrate. Use machine manuals to help make a best guess and edit appropriately. The ideal cut would cut cleanly through your substrate for paper and plastic and only cut through the first liner and adhesive layer of double-sided PSA while keeping the bottom liner intact. The latter scenario is important to prevent feature ‘droop’ when adhering to another layer.

(3) This process is iterative, design changes may be required for proper manufacturing. While a design may look good on CAD, the order of the cuts made may cause a feature to blow or skew outside of the target area. Consider multiple cuts or incomplete cuts that can be manually completed to overcome these issues.
Table 3. Key differences between laser and plotter cutting for microfluidics.

<table>
<thead>
<tr>
<th>Laser Cutter (Universal VLS 4.60)</th>
<th>Plotter Cutter (Graphtec CE6000-40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Easy-to-use</td>
<td>Requires some optimization [67]</td>
</tr>
<tr>
<td>Expensive ($22,500)</td>
<td>Low Cost ($1,195)</td>
</tr>
<tr>
<td>50 micron resolution</td>
<td>200 micron resolution</td>
</tr>
<tr>
<td>Tight Corners</td>
<td>Overcut Corners</td>
</tr>
<tr>
<td>Produces burn products</td>
<td>No burn products</td>
</tr>
<tr>
<td>Cuts plastic, tape, paper</td>
<td>Cuts tape and paper only</td>
</tr>
</tbody>
</table>

3.6.5 Assemble

Once all the layers are cut, they must all be brought together. These below quick protocols provide insight for the best results.

Tape-to-Plastic:

(1) Ensure the plastic is clean, the removal of liner covering can cause dust to build up from electrostatic interactions. A simple cleaning protocol would be to use a mild detergent and a sonic toothbrush to directly clean the surface, followed by a wash and dry with gas or microfiber cloth. Be careful about using harsh organics which may damage or reduce the optical clarity of the plastic

(2) Remove all ‘cut’ features from the tape using tweezers (removing both the top tape liner and adhesive layer only)

(3) Peel of top tape layer in one continuous motion if possible, use tweezers to help for complicated areas

(4) Place tape upside down and line up over plastic (use dowels or an alignment rig to assist) and press down lightly, unpeel and repeat the process if alignment is off

(5) Use a laminator or smooth laminating roller (McMaster-Carr #7533A12) to apply heavy pressure to seal tape to plastic
Paper-in-Tape:

(1) Once the tape ‘cast’ has been laid down, used tweezers and a gloved finger to position the cut paper substrate into the fitting

(2) If another layer of plastic or tape is going to be added on top, remove the second liner from the tape while holding the paper down with tweezers then press the second layer of plastic or tape down

(3) Use a laminating roller on the area immediately surrounding the paper first to produce a seal and prevent bubble formation near the paper which could cause leakage

Tip: A great paper/tape combo is ArSeal 90880 (142 µm) and Fusion5 (approximately 250 µm)

This full process is depicted schematically below in Figure 11.
Figure 11. Design, Cut, Assemble. The three steps of the DIY microfluidics manufacturing using a laser cutter and laminating roller to put together a disc device equipped with microfluidic channels.
4. Results and Discussion

This section presents results from experiments performed with the full suite of microfluidic devices described in the preceding section. The microfluidic immunoassays and immunophenotyping diagnostics developed by traditional manufacturing (Sections 4.1 and 4.3) and scalable manufacturing (Sections 4.2 and 4.4 - 4.5) illustrate the potential for POC testing of ocular diseases.

4.1 Sandwich Immunofluorescence Assay (sIFA)

Approaching the clinical need for determining treatment efficacy of DR and AMD, quantitative measurements of VEGF levels in the eye can serve as metrics for patient stratification. It is known that VEGF levels within the vitreous humor correlate with those within the comparatively more accessible aqueous humor, the front-most region of the eye [95,97,98]. In order to meet the clinical need described above, the technical requirement is therefore accurate and fast measurement, in minutes, of VEGF concentration in aqueous humor samples, which are typically 50-100 μL in volume. The gold standard in protein quantification is the plate-based ELISA, where extremely low detection limits and large dynamic ranges can be achieved. However, this method requires multiple manual pipetting steps and long incubation times (typically multiple 30 min intervals). New approaches in protein assays based on bead technologies, such as the systems offered by Luminex and Illumina, have coupled ultrasensitive and robust reagents with automated analyzers that do not require manual steps beyond sample loading. However, these new systems are specifically designed for multiplexed assays.
wherein multiple proteins (typically large panels) must be quantified in a high-throughput manner. As a result, these systems are far more capital-intensive (typical system cost is >$40k) and require expensive reagents. Moreover, even these systems are not capable of generating a readout of protein concentration in the timescale of ~15 min between sample loading and instrument readout. Hence between the reasonably priced but labor-intensive plate-based ELISA kits and the high-end bead based systems, there exists a technological gap where the clinical need lies for AMD and DR treatment, as well as other diseases.

The simple sandwich immunofluorescence assay (sIFA) device previously described by our group has shown promise to VEGF quantification using a low volume biopsy sample [94]. An extension of this work sought to further develop this platform through the development of a dedicated reader system to automate signal readout [101]. During development, the PDMS microfluidic sIFA platform was shown to lack robustness with high variability in similar measurements. Figure 12 below shows sample data taken both from a standard 96-microwell plate ELISA kit and from the sIFA device.
Figure 12. Comparing data from standard ELISA to sIFA. (Left) Sample data taken from plate ELISA using kit VEGF standards. (Right) Sample data taken from the sIFA device using ELISA kit VEGF standards. Error bars are present on both plots (n = 3).

The higher variability of the sIFA device may be attributed to a multitude of factors including the protocol complexity and materials used. One source of error may be from user-error when performing the surface chemistry functionalization of the sIFA device as well as running all of the sample and reagent steps through the device. The multitude of steps requiring fresh reagents and properly functioning engineering equipment (i.e. syringe pumps) can cause difficulty in variability if not all steps are performed identically in each device. A second source of error comes from inefficiencies from the material itself. PDMS microfluidic devices have been shown to be dissimilar in function as level of curing and elastomer-to-initiator ratio can affect surface functionalization.
4.2 Point-of-care Ocular Rapid Test (PORT)

To overcome problems with the PDMS device, VEGF standards in PBS were analyzed with the PORT system. When individual samples were analyzed with the fluorescent microscope, the images had three qualities of interest (Figure 13). First, the microsphere pellet appears as a dark cloudy area with a halo-like effect around it. Such an effect occurs because fluorescent substrate is generated in the interstitial fluid of the pellet in addition to the region behind the pellet. Note that by design, the microsphere pellet area is itself not fluorescent (except for the 1 μg/mL VEGF sample) as the microspheres themselves occlude the substrate fluorescence. In contrast, the 1 μg/mL VEGF sample had such a high analyte concentration that the fluorescence could be viewed from the pellet. The primary reason for fluorescence occlusion is the limitation of a two-dimensional field of view which blocks fluorescent particles behind the first microsphere layer. The paramagnetic iron oxide content of the Dynal beads also makes them especially opaque at visible light wavelengths. The second area of interest is the fluorescent signal diffusing from the microsphere pellet through the detection medium. This area was taken to be our target area for signal measurement as the amount of fluorescent substrate diffusing from the pellet is directly proportional to the number of VEGF complexes present on the microspheres (Figure 13b-c). Finally, past the immediate diffused fluorescent substrate we observed the background fluorescence of the detection area. In this area, photobleaching reduces the background to a steady level after 5 minutes (Figure 13b). We arbitrarily chose the 5 min mark to view the fluorescent signal, as the rate of signal drop was not as pronounced after that and the difference in signal between target and background was consistent. When we measured the
fluorescent intensity both at the target and background locations, the brightness decreased rapidly over the first few minutes, until a quasi-steady state was reached (Figure 13c). The approximate steady state was achieved when the particles that diffused through the measurement area was equal to the diffusion of particles out of the area. The fluorescent lamp of the microscope was continuously illuminated to trigger photobleaching, and thereby improve the dynamic range by removing background fluorescence. This consistent photobleaching caused the slight dip in fluorescent intensity; thus, we subtracted a background point to better stabilize our results. Repeatability studies were also performed with three samples all containing 1 μg/mL of VEGF (Figure 13d). The three samples were loaded on the same disk and the disk was spun on the microscope stand and each channel was read approximately every minute. The fluorescence measurements in Figure 13d represent the difference between the target and background measurements and the horizontal curve fit lines represent the “steady” state fluorescence intensity. When all three trials were averaged, the final measurement was 194 ± 3 arbitrary units (standard error applied).
Figure 13. “Steady”-State Signal (a) Brightfield images of the PORT device, channels are filled with blue food coloring dye. (b) Series of zoomed-in fluorescent images of a channel containing 100 ng/mL VEGF sample over 6 minutes (scale bar = 250 µm). (c) Sample graph of fluorescence measurements taken directly behind the microsphere pellet and in the far background of the detection region. (d) shows the progression of brightness over three samples, background corrected, containing the same amount of VEGF standard.

The fluorescence over time of different VEGF concentrations is shown in Figure 14a. As the concentration of VEGF increases, so does the fluorescence over time until the measurement stabilized after 5 minutes. While the measurement was not perfectly steady-state due to the very slow rate of diffusion in the thin channel, an accurate estimate can nevertheless be obtained. The 1 µg/mL VEGF measurement shows a
plateau first and then decreases slightly over time; this is attributed to the saturation level of the optical detector.

The VEGF dose calibration curve from the PORT device is shown in Figure 14b. A polynomial curve proved to be the best fit for the data. There are technological detection limits both at the high end and low end as it is difficult to determine the differences between very dark or very bright images without changing the gain or exposure time used to take the measurements. The small error bars confirmed that a temporal “steady” state was reached in each sample from the averages of measurements between 5 and 8 minutes.

The comparison of the PORT system to a commercially available plate-based ELISA kit is shown in Figure 14c-d. The comparison was made by taking three patient samples (all of which had negligible baseline VEGF levels based on ELISA and PORT) and spiking them with VEGF standard in a double-blind manner. One researcher prepared VEGF samples and randomly injected between 0.1 and 1 mL of the three VEGF standards (1 μg/mL, 100 ng/mL, and 10 ng/mL) into 1 mL of each sample. A second researcher then tested all the random samples and results were compared between researchers. In the case of the PORT device, all three random values lie very close to the best-fit curve generated with the standards, as shown in Figure 14c. The 96-well plate ELISA (Figure 14d), as expected, also shows strong agreement between the standard samples and the random samples’ true values; although the random sample values were slightly, but consistently, lower than the sample standards. Along with the plate ELISA, the PORT system shows the ability to differentiate over three full orders of magnitude of VEGF concentrations and the low and high random samples fit well on the curve.
Furthermore, the PORT system’s measurements are consistent with that of the ELISA for both standards and random samples.

Figure 14. Evaluation of PORT Device (a) Background and zero subtracted measurements of fluorescence in the PORT device for different VEGF standards over time. Averages are taken temporally at assumed “steady”-state from 5 to 8 minutes. These averages are represented by the horizontal best-fit lines in the graph. (b) Averaged data from “steady”-state measurements plotted versus standard concentration to make a dose-response calibration curve with a polynomial fit, where error bars represent the standard error of all fluorescence measurements taken from 5 to 8 minutes for each concentration. (c) Three spiked patient samples (red squares) were tested in a double-blind study and plotted on the dose-response calibration curve (blue diamonds), the final VEGF concentrations of these spiked random samples are 292 ng/mL, 40.7 ng/mL, and
2.49 ng/mL; (d) 96-well plate ELISA kit results with the same VEGF standards (blue diamonds) and spiked random samples (red squares) as Figure 14c.

In summary, we have described a sensitive immunoassay technique in which the sandwich ELISA methodology has been adapted into a two-step method via sedimentation principles; the first step is mixing and loading of assay reagents and the second step is the assay itself. The relatively simple sedimentation principles applied herein can wash and separate excess label as the microspheres travel through the microfluidic channel to the detection region. The detection and density media can also be preloaded into the CD as the high surface tension and very small air boundary greatly limits both diffusion and evaporation. On the front-end, a test sample only requires addition of assay reagents before loading onto the disk. Loading is followed by a short incubation time of 10 min, a 30 second spin for washing and pellet formation, and a 5 min period prior to readout. The total time from sample to readout is around 16-18 min, far more rapid than the typical 4 hour Novex® plate ELISA and 3.5 hour Luminex® bead-based ELISA. This time frame is compatible with POC analysis in a clinical setting. Also, compared to current 96-well plate ELISA methods, our method circumvents the need for multiple hour-long incubations and wash steps. Our method also has a greater dynamic range than plate ELISA allowing better quantitative analysis for patients with especially low or high ocular VEGF. The method described is also portable as the PORT device completely contains all assay parts and has a much smaller footprint than the ELISA plate method which requires a designated lab bench. The PORT method also requires only a single microliter of sample; while the ELISA plate requires 100 microliters of sample at a minimum.
The potential for a mature, ocular point-of-care immunoassay requires dedicated and less improvised equipment than used for this paper. There is a need to develop a robust, high performance device with integrated fluorescent measurement capabilities. For example, integrating a custom-designed fluorescent optics system with the disk spinner device will eliminate the need for a fluorescence microscope, and make the assay system truly portable. Also, the PORT system will require automation for sample metering, motor control, and fluorescence measurement. The addition of the optics system and automated steps will drastically reduce sample-to-measurement time and keep measurements consistent and accurate. The ability to analyze samples quickly will also allow for multiple channels to be measured virtually simultaneously as a difference in seconds between measurements may be considered equivalent for the same time point. In this work, fluorescence intensities of sample wells were measured one at a time due to the inability to rapidly switch channels on the Nikon microscope. An automated system will also need to generate calibration curves each day and possibly even with each individual disk to ensure that changes in intensity levels resulting from optical equipment are taken into account.

The major contribution of this work lies in the use of a fluorescent substrate for signal amplification. In the approach described herein, each enzyme molecule is capable of generating hundreds of fluorescent substrate molecules per second. While the present work focuses specifically on VEGF, the concept can be applied broadly to any immunoassay target. Applying such amplification leverages major design strengths of the disk-based immunoassay system originally described by Schaff et al., namely the speed and simplicity of the sedimentation-based approach with avoidance of mixing or
valving components on-chip and external flow or electrical and magnetic interfaces [96]. Furthermore, as samples can be introduced directly into wells via pipetting, there is no tubing or pumping required.

In the context of our ultimate goal for a point of care system for ophthalmologic use, the approach described herein has the potential to provide fast readouts of VEGF concentration from small volumes (under 50 μL) ocular fluid samples that can be collected from the anterior chamber in eyes with DR and AMD. Such capability would allow ophthalmologists to provide customized doses of anti-VEGF therapeutics and, equally importantly, quickly identify those patients who would not respond to such therapeutics.

4.3 Chemotaxis Sheath Flow Device (CSFD)

Cell immunophenotyping of invasive cell types in the eye can also provide valuable diagnostic information for ocular disease. Specifically, uveitis and primary intraocular lymphoma (PIOL) can be diagnosed by the identification of either T or B cells in a vitreous humor biopsy sample, respectively [48]. There is a need for a diagnostic tool that can be deployed at the point-of-care to immediately allow patients to receive the proper treatment and avoid misdiagnosis from cell morphological changes \textit{ex vivo} after an hour [50].

The chemotaxis sheath flow device (CSFD) was developed to investigate and leverage chemotaxis phenomena of T and B cells in a chemical gradient with target chemokines. The device was developed in PDMS as a microfluidic 2D sheath flow with two exits.
Preliminary experiments were able to display proper envelope flow; however, many repeatability issues showed up as debris in the sample or from the PDMS device caused on-chip clogging (Figure 15). It was determined early on due to lack of robustness and the low flow speed to allow time for chemotaxis was an undesirable solution for the point-of-care. Alternative methods would be required using alternative materials and techniques.

Figure 15. Chemotaxis Sheath Flow Device (CSFD) (left) 2D sheath flow schematic showing T (Jurkat) and B (Raji) cells swimming toward their respective chemoattractant: B-Cell Chemokine (BCA-1) and Stromal Derived Factor 1 alpha (SDF-1α). (right) insert showing a microscope image of PDMS clogging device near outlet.
4.4 Chemotaxis Immunophenotyping Device (CID)

The entirely new approach to cell immunophenotyping using chemotaxis on paper provided a simple and rapid chemical gradient generator capable of examining chemotaxis in very short timeframes (20 minutes). The CID device is capable of functioning without the need for pumps nor expensive staining antibodies. The paperfluidic device was first characterized (Section 4.4.1) and then cell studies were performed for proof of concept (Section 4.4.2).

4.4.1 Characterizing the paper gradient

A wide variety of Whatman’s absorbance and conjugate pads were tested for both the fluid reservoirs and bridge pieces. Five different types of paper were tested: glass fiber (Standard 14 and Standard 17), cotton linter (CF1, Grade 470), cellulose (CF2), and proprietary membrane matrix (Fusion 5). Autofluorescence was measured with an inverted, fluorescence Nikon TE2000 microscope using a xenon-mercury fusion lamp and fluorescein (480 ± 30 nm/535 ± 40 nm) and rhodamine (540 ± 25 nm/605 ± 50 nm) filter sets. Autofluorescence was very high for both colors on all cotton linter and cellulose papers. And while there was low autofluorescence on the glass fiber paper, microbundles of fibers in the paper appeared too similar to fluorescent cells. The proprietary single membrane Fusion 5 was chosen as the bridge piece for its low and uniform background fluorescence for both colors. All papers were then cut into a 2 cm x 2 cm size square and then 250 μL of fluid was injected on top of them. Only the glass fiber papers were able to retain the fluid without leakage and the fluid was still wet to the touch after 30 minutes at room temperature. The glass fiber paper Standard 17 was
chosen for the reservoir pieces as it was the thickest glass fiber paper. The dimensions of
the paper were selected to conveniently fit into a 60 mm Petri dish for prototyping.

The analysis of the gradient on paper is shown below in Figure 16. The
autofluorescence of the paper was virtually nonexistent compared to the fluorescent
dextran intensity. Dextran is a biologically inert, water soluble protein that comes in
many sizes based on the number of linkages in its structure. The 10 kDa dextran used in
this experiment provides a good model for the chemokine SDF-1α (8.8 kDa) as they are
roughly the same size and can be considered to have similar diffusion characteristics.

Also, the Fusion 5 substrate is a uniform, hydrophilic material that should have low
binding affinity for both Dextran and SDF-1α. Over the course of 20 minutes, there
appeared to be some relaxing of the gradient; however, the effect is very minimal. Due to
the non-gaussian nature of the data, two-sample, Kolmogorov-Smirnov (K-S) tests were
performed on both the red and green distributions to determine if the gradients changed
shape over time. K-S tests are a useful alternative to the paired t-test as no normality
assumption is made [102]. Both the green and red distributions were not considered
statistically different by the K-S test with α = 0.05. Of interest to note is the gradient at
the time of zero minutes. On the device, the gradient stretches over 3 mm, whereas the
time-dependent, 1D solution to Fick’s Equation produces a step-function. A reason for
the irregular nature of the gradient produced is the complex, 2D boundary where two
fluids meet do not allow for the simplifications required for 1D gradient modeling.

However, estimating the diffusion coefficient to $1.7 \times 10^{-6}$ cm² s⁻¹ for 10 kDa molecules,
the gradient should only stretch out a on the scale of microns over 20 minutes, providing
a quasi steady-state gradient over the 3 mm gradient area for the length of the experiment.
Results from the gradient generation show a stable soluble gradient of chemokine is maintained for 20 minutes. With such a sharp gradient taking place only over 2 mm, cells directly under the gradient will more easily notice the change in chemokine concentration. Current gradient generating devices show similar gradient size [103]. The ease of use of paper provides a very simple mechanism that does not require the use of syringe pumps or any additional equipment to maintain the gradient.

4.4.2 Cell studies

The results for the cell studies are shown in Figure 17. Studies for control (n = 257 cells) and gradient (n = 158 cells) conditions were made over 3 independent trials for which 8 positions were selected for time-lapse microscopy. For all trials, cells under the
gradient condition had more directed movement than the cells in the control condition. Analyzing the cell tracks in Figure 17, there appears a bias in both the control and chemokine conditions for average cell displacement in the positive y-direction. Also, only cells in the chemokine gradient condition showed a bias toward the positive x-direction (towards the chemokine source). We postulate that movement in the positive y-direction is due to the wicking of fluid to the side of the paper bridge while under observation. Notably, cells in the gradient condition had significantly greater ($p << 0.01$) overall displacement than the cells in the control.

Analysis of cell migration under the gradient shows cells do move preferentially toward a chemoattractant when present. In fact, cells showed much smaller displacements overall when a chemoattractant was not present. In the absence of chemoattractant, cells migrate in a slower and random fashion. This type of migration is referred to as chemokinesis, as opposed to chemotaxis. However, there is a general bias in both gradient and control conditions that cells move toward the edge of the paper (positive y-direction in Figure 17). We believe this is due to a small level of convective movement of the medium due to evaporation at the edges of the bridge paper piece. This preferential evaporation may be due to the paper device being sandwiched between the Petri dish and the glass microscope slide where only the edges of the paper are exposed to air and the effect of the majority of images taken being near the center mark on the upper part (positive y-direction) of the paper. This perpendicular current, caused by evaporation, may be enough to trigger a bias. It is important to note that with the bias present, a majority of the cells in the gradient condition still can be seen moving towards the gradient.
Figure 17. Analysis of cell migration of the paper device. (a and b) Cell tracks accumulated from 3 independent experiments for both (a) gradient and (b) control conditions. The gradient chemokine source is located in the positive x-direction. (c) Analysis of average net displacement toward the chemokine source for both control and gradient conditions showing significantly greater cell movement for the gradient sample ($p << 0.01$).

Analysis of the chemotactic indices of the control and gradient conditions are shown in Figure 18. Chemotactic index is defined as the cosine of the angle between the direction of the displacement of the cells and the direction up the gradient [104]. The control condition has a sharp normal distribution of chemotactic index focused between -0.2 and 0.2. The gradient condition has a much more skewed distribution peaking at 0.8 to 1. Average analysis of these distributions shows that the control condition has no bias towards any direction; while the gradient condition biases cells significantly more.
towards the chemokine source. The chemotactic index shown for the gradient condition in Figure 18c falls within error of the chemotactic index reported by Lin et al. for the same cell type and chemokine in a microfluidic flow chamber [103]. However, their use of a flowing gradient with conventional microchannels creates a different environment for the cells adding shear effects and replenishing media, which differs from our static paper-based approach. These differences may explain discrepancies in chemotactic data between methods as shear effects may affect migration distance and speed.

![Figure 18. Distributions and analysis of chemotactic indices](image)

**Figure 18. Distributions and analysis of chemotactic indices** of the (a) control and (b) gradient conditions. (c) Shows a significant difference in chemotactic index ($p << 0.01)$ in the control and gradient conditions.

The use of such a simple device without complicated engineering equipment requires a certain level of control for consistency. We examined variability between chemotaxis condition trials by averaging the means of the chemotactic indices between the three trials and calculating the standard error the mean between trials. Variability between trials was less than 30%, typical of lymphocyte migratory behavior, while we observe statistically significant differences between the control and chemotactic conditions. One potential cause of such variability is the small dilution of cells which the gradient is
placed over. The cell gradient protocol differed from the fluorescent dextran protocol as, once the gradient on the paper was established, it was placed over a liquid bath of 100 μL media containing cells. The presence of this fluid could cause dilution of the chemokine and potential broadening of the gradient. To mitigate gradient broadening and dilution effects, we chose the concentration of 1 μg/mL of SDF-1α as it experimentally provided the most consistent results. The potential broadening of the gradient was minimized by reducing the cell dilution to only 100 μL; however, even this low amount may trigger some variability as reported between trials. Another source of error may be due to the marking method used to show where the gradient is created on the paper device. The marking method was created to make up for the error in asymmetrically pipetting media and chemokine onto each side of the paper device simultaneously. The microscope software uses this marking to locate a starting point and cells above that point are measured around that location both in the x and y directions to broaden the field of view and limit the variability between trials.

Literature has shown that current microfluidic chemotaxis devices are limited in their use due to the requirement for special engineering skills and tools not normally available in a biology lab such as a syringe pumps and plasma cleaner [105]. The paper device described in this work bypasses any need for additional equipment or skills for preparation. Also, currently used materials such as polydimethylsiloxane (PDMS) and soft hydrogels add cost and time to producing devices [105]. A paper device provides the additional benefit of simple assembly which can be achieved by scissors and hand placement. Current microfluidic schemes to study chemotaxis use gradient generators such as the ‘Y’ and network devices [103,106]. These devices provide instant gradients;
however, also have the drawback of requiring highly precise external flow control and the potential for flow-induced shear stress. In addition, these devices are fragile to any small flow obstruction which can cause gradient skew. Flow-free devices have shown some success but can require hours to reach a pseudo steady state gradient. The paper device described herein avoids all these drawbacks and provides a simple solution to gradient generation within seconds.

All of the presented results together show a great potential for paper being used as a gradient generator for chemokine studies both in the lab and eventually the point-of-care. Further work on this system will develop a competing chemokine gradient for separating and identifying cell types specific to particular chemokines. This rapid, low-cost immunophenotyping method could provide an effective diagnostic tool for clinicians as an alternative to flow cytometry and histology. Key further steps for such translation will include temperature control and CO$_2$ injection system along with optics and software to automatically track cell movement.

4.5 Spinning Ocular Immunophenotyping Device (SOLID)

While the CID device meets the basic criteria for a POC test, the necessity of sample preconcentration forces an additional step to be performed before analysis. Ideal POC platforms have all of the sample preparation steps integrated and automated into the device itself for true “plug-and-play” functionality. The SOLID device integrates sample preparation steps required for large, dilute volume biopsy samples, such as vitreous humor aspirates. The SOLID device was developed in two iterations; the first design
utilized only centrifugal forces to capture and stain cells (Section 4.5.1), while the second design integrated paper into the disc format to achieve the same goals (Section 4.5.2).

4.5.1 First Design – Passive Sedimentation

The first version of the spinning ocular immunophenotyping device was developed as an investigation to determine if centrifugal force itself could separate cells directly from fluid. The geometry of the device was designed in an “S” shape which would force the fluid to travel first to the distal end of the disc, then against the centrifugal force back to the center, before entering a third final chamber which ejects the fluid outside the device. The hypothesis was that the sedimentation force on the cells (Equation 1 – Section 3.2.1) could overcome the radial viscous flow between the two parallel annual plates (Equation 2) as the flow travels from the distal end of the disc to the proximal end: [107]

\[ v_r = \frac{z(h - z)\Delta P}{2\mu r \ln(r_2/r_1)} \]  

Where \( v_r \) is the flow velocity, \( z \) is the height in flow \((0 < z < h)\), \( h \) is the channel height, \( r \) is distance from center of disk of flow, \( r_2/r_1 \) is ratio of inner to outer disk radius, \( \hat{f} \) is the flow direction vector and \( \Delta P \) is the pressure difference over the channel. Assuming the particles move with the fluid, at the maximum velocity \((z = h / 2)\), the flow velocity was determined to proportional to the centrifugal force and the height of the channel whereas the sedimentation velocity (taken from Equation 1) was strictly proportional to the centrifugal force:
\[ v_r \propto h g \]  \hspace{1cm} (3)

\[ v_s \propto g \]  \hspace{1cm} (4)

Thus, it was determined that a thin enough channel would allow for cells to sediment within the channel as fluid passed. However, COMSOL modeling and preliminary experiments proved that the required channel thickness for this phenomenon to occur was too small and slow for practical use, and very few cells were captured using this method (Figure 19).

Figure 19. Passive Sedimentation Technique (Left) photograph of the SOLID on top of the custom spinning platform filled with blue food coloring dye, (Right-Upper) COMSOL simulation of fluid speed through SOLID channel, units are in m/s (Right-Lower) COMSOL simulation of T and B cells passing through
4.5.2 Final Design – Paper Filtration

While sedimentation principles could not be practically manipulated to capture cells passively out of flow, a second channel design was created which integrated a paper filter to capture cells as fluid passed through. This new device was characterized and optimized (Section 4.5.2.1), before proof-of-concept experiments were performed (Section 4.5.2.2).

4.5.2.1 Device Characterization and Optimization

The device was first characterized to optimize manufacturing, geometry and analysis time. Separate radial channels provided the best fit for the paper. A 140 µm thickness tape was chosen as it best sealed the Fusion 5 paper (<200 µm compressed) and provided faster processing times of 20 mL PBS buffer than thinner adhesives. Processing time for 20 mL of PBS was tested on the final design at different speeds and results are tabulated in Table 4. Speeds greater than 1000 rpm were not tested as the motor began to heat up under heavier load. The speed of 1000 rpm provided a safe running speed for the system while processing 20 mL of fluid in approximately one minute. The “g” force that the cells feel at 1000 rpm and 4.5 cm from the center of rotation where the cells first meet the paper is approximately 50 × g. For reference, this force magnitude is half of the 100 × g force recommended by ATCC for routine passaging of T and B lymphocytes. Note that this device is being designed for aqueous body fluids with minimal cell content, which have significantly simpler processing requirements relative to more complex fluids such as blood.
Table 4. Rotational speed and corresponding processing time of 20 mL of PBS in the device. All speeds have a ramp acceleration of 20 rpm/s.

<table>
<thead>
<tr>
<th>Speed (rpm)</th>
<th>Time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>&gt;900</td>
</tr>
<tr>
<td>500</td>
<td>150 ± 10</td>
</tr>
<tr>
<td>750</td>
<td>91 ± 6</td>
</tr>
<tr>
<td>1000</td>
<td>56 ± 5</td>
</tr>
</tbody>
</table>

Fusion 5 paper was chosen as it provided the lowest and most uniform fluorescent background of various Whatman papers tested previously [108,109]. Additional testing was performed on the Fusion 5 matrix to determine the effect of nonspecific binding of 1 μg/mL anti-IgE conjugated with phycoerythrin to the paper and results are shown in Figure 20. At a gain of 1.0 and exposure time of 5 seconds a weak fluorescent background can be seen on the dry paper in the device. Addition of PBS reduced the scattering and the addition of a large amount of anti-IgE antibody conjugated with phycoerythrin then tripled the fluorescent signal present. An after-wash with 5 mL of PBS returned the paper within 12% of its previous background fluorescence level.
Figure 20. Analysis of fluorescence intensity on Fusion 5 paper under different conditions. (a) Dry, (b) Wet with PBS, (c) Stained with 1 μg/mL anti-IgE conjugated with phycoerythrin [Ab*], (d) same as (c) but washed after with 5 mL PBS, (e) Analysis of average fluorescent intensity of the four different conditions pictured (a-d). All scale bars for microscope images are 100 μm.

Device complexity was kept to a minimum by utilizing simple and readily available materials, manufacturing, and methods. Standard polycarbonate discs used to make DVDs, double-sided pressure sensitive adhesive, and filter paper are low cost materials that are widely accessible. These materials provide multiple advantages over classic PDMS and glass materials which are fragile, sharp, less available, and more expensive to make. Laser cutting and lamination provide faster, simpler and cheaper manufacturing than traditional soft lithography methods employed in microfluidic device manufacturing. And the use of centrifugation to provide all the forces necessary forgoes the need for complicated engineering equipment such as syringe pumps. Finally, the disc device also allows the user to place the device directly on a fluorescent microscope for imaging when complete.
4.5.2.2 Proof of Concept

Our first proof-of-concept experiment processed a large solution (20 mL) containing fluorescent microspheres. Microsphere capture was tested on the device using fluorescent polystyrene and melanin microspheres of 5 and 10 µm sizes, respectively. Analysis plots are shown in Figure 21 (the method used to produce the plots are shown in Fig. 10). Figure 21a shows 10 µm microspheres focused at the first 250 µm of the paper while their concentration reduces until virtually nothing is seen past 1000 µm. The 5 µm microspheres, on the other hand, can penetrate much further into the paper, are more evenly distributed and reduce their numbers gradually further into the paper. When 10 µm microspheres are washed with 100 mL of PBS as shown in Figure 21c, the beads penetrate further into the paper matrix and are more spread out than without the wash. Comparing all three conditions together in Figure 21d shows how washing spreads out the 10 µm microsphere distribution and how the smaller 5 µm beads have greater penetration length in the 3 mm thick paper. The increased fluorescent signal seen from the 5 µm microspheres can be attributed to the fluorescent intensity of the green dye than the red dye making comparisons in absolute concentrations difficult. Looking at the distal end of the paper, virtually no fluorescence was seen for any condition showing that 3 mm was long enough to capture all the microspheres. It is also important to note that the wash of 100 mL, the higher end volume of vitreous aspirates, still had captured microspheres even though their distribution was more spread out.
Figure 21. Sample images and distributions of different size particles in the filter paper. The method used to produce distribution graphs is shown in Figure 10. Each line arbitrarily represents one of the four pieces of filter paper on a device. (a) 10 μm microspheres, (b) 5 μm microspheres, (c) 10 μm microspheres after 5 washes of 20 mL PBS, (d) Average penetration data from (a-c)

The next proof-of-concept experiment examined on-chip staining of T cells using a live cell stain. Once the vitreous biopsy model as described in the Materials and Methods section was processed in the device, a calcein AM stain was prepared in 1 mL of culture medium as per the manufacturer’s instructions and spun for a few seconds into the device and incubated for 10 minutes. The device’s four channels were then imaged using the
image stacking method shown in Figure 10. Figure 23a shows cells are, captured, alive and expressing the stain. It is also of note to see the general gradient of fluorescence from the proximal edge of the paper (top left corner) to the distal end. The appearance of a gradient may be attributed to cells not in focus on the paper and being more concentrated at the inner edge than further away. Figure 23b shows differences in the cell capture and/or staining in each channel of the disc. A potential reason why cells may have preferred one channel over another include asymmetric fluidic resistances causing fluid to enter the some channels preferentially over others as can be seen by the differing fluorescent maximum intensities (Figure 23b) and microscope images (not shown). Other effects may include the preferential distribution of calcein stain or asymmetric cell capture on the paper away from the middle of the paper. Unstained cells were not visible under the microscope due to the occlusion of the paper. Additional stains would have to be applied for cell death or presence of a particular surface marker.

Figure 23. **Proof-of-concept on-chip staining of T cells.** (a) Calcein AM stained Jurkat cells caught in paper. (b) Fluorescence intensity of paper from the center of the four channels on a device to the distal end. Scale bar is 100 μm.
A major consideration for this platform is particle loss through the Fusion 5 matrix. With a nominal pore size of 11 µm (specified by the manufacturer), this large pore size allows rapid ejection of fluid at the expense of smaller cells being able to travel through the matrix. We used a paper thickness of 3 mm to force cells to travel a long distance before escaping. Manipulation of this pore size could allow for a physical cell separation for dealing with more complex samples in the future. Table 5 shows testing results for a T and B cell line with a size between 10 – 15 µm where the majority of cells are captured near the proximal edge of the paper (left side). All waste from these experiments was collected, spun down to 100 µL and counted manually on a hemocytometer counting slide. While very few cells were counted from waste, an additional analysis was performed using a known, low number of 10 µm fluorescent microspheres in 20 mL of PBS and counting the absolute number of microspheres captured on-chip rather than collected in waste. Results are shown in Table 6. While low capture percentages were reported, pipetting error and counting error with creating such low microsphere concentrations can explain this reduced capture. These disadvantages do not greatly diminish clinical relevance as diagnosing idiopathic eye disease from vitreous aspirates only requires identification of the dominant invasive cell type, not absolute count [48]. Also, the analysis of a vitreous aspirate (50 - 100 mL) in general has been shown to have a higher diagnostic yield than smaller sampling (1 mL) from the vitreous humor [110].
Table 5. Analysis of cells dyed with FxCycle PI/RNase staining solution recovered from the waste chamber. After a known number of input cells in 20 mL warmed media were processed in the device, all ejected fluid was collected and spun down to 100 μL then counted by hand using a hemocytometer counting slide.

<table>
<thead>
<tr>
<th>Input (# Microspheres)</th>
<th>Captured (# Microspheres)</th>
<th>% Captured</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>1000</td>
<td>237 ± 17</td>
<td>23.7 ± 1.7</td>
</tr>
</tbody>
</table>

Table 6. Analysis of cells dyed with FxCycle PI/RNase staining solution recovered from the waste chamber. After a known number of input cells in 20 mL warmed media were processed in the device, all ejected fluid was collected and spun down to 100 μL then counted by hand using a hemocytometer counting slide.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Input (cells/mL)</th>
<th>Collected from Waste (cells/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat</td>
<td>2.72 x 10⁴</td>
<td>&lt; 200</td>
</tr>
<tr>
<td>Raji</td>
<td>5.42 x 10⁴</td>
<td>400</td>
</tr>
</tbody>
</table>
5. CONCLUSIONS AND RECOMMENDATIONS

This work has developed new robust platform technologies that have the potential for commercialization as in-vitro diagnostics. The further development of these tools and use at the ophthalmology clinic and other venues have the potential to accomplish improved patient outcomes, personalized treatment, and reduced cost and delay in diagnosis and proper treatment.

The PORT device demonstrates how centrifugal principles can be combined with enzyme–fluorogenic substrate pairs in a sensitive, single-marker immunoassay that can provide a readout within 16–18 min from sample to result. The work represents an important step in the development of an integrated microfluidic platform for ophthalmologists to determine dosing and treatment regimens for the eyes of patients with DR or AMD and to identify patients who will not respond to standard therapies. Future work of the PORT device includes the integration of the device into a customized reader which could automate all of the spinning steps as well as the imaging and imaging analysis.

The CID platform presents a paperfluidic device capable of generating and self-maintaining a chemotactic gradient for cell migration studies. The paper device provides an extremely low-cost and rapid alternative to current platforms that require complicated fabrication techniques, long time scales, and fine fluid control with syringe pumps. By simplifying gradient generation, cell migration can be studied in biology laboratories without the need for special engineering tools or skills and hopefully at the point of care for diagnostics. Future work for the CID platform would be development of an automated method to concentrate the cellular content of vitreous biopsy aspirates and
analysis on the paperfluidic device. Also, multiple cell types would need to be tested on the platform to confirm that competing gradients can be used to immunophenotype cells.

The SOLID system demonstrates an approach to integrate centrifugation with analysis of a large volume (20 mL) sample containing a small number of cells ($10^4 – 10^6$ cells) for immediate imaging. By utilizing commercialization-friendly manufacturing methods and materials, this framework could help provide for a truly plug-and-play system where the clinician simply inputs the patient sample and without any additional steps a readout is given. Future work of the SOLID system will examine staining methods for specific cell immunophenotyping, improvements to automation and include optics to provide a “black box” for point-of-care analysis. In addition, while cells may not be easily extracted from the device, on-chip lysis may be possible as an additional step and nucleic acid may be able to be flushed out for downstream analysis. Modifications to device design can also be implemented to change channel geometry to improve the concentration in smaller channels or use multiple paper filters with different capillary size for sequential capture. There is strong potential to incorporate this system into an optico-centrifugal platform as described by Burger et al.[111]. And an even further end goal would be to incorporate this technology into a modified standard DVD drive used as a fluorescent laser scanning microscope, similar to the one described by Ramachandraiah et al. [112]. Integration of sample preparation and analysis on a high throughput (20 mL/min) microfluidic device via centrifugation is well suited for the point-of-care.

Together these technologies have taken advantage of new, scalable rapid prototyping methods that do not rely on elastomer materials nor photolithography methods. The big vision to continue this work would be to develop more methods to rapidly convert soft
polymer microfluidic devices into scalable systems. To accomplish this goal, new microfluidic methods need to be developed to overcome the challenges of working at a reduced feature resolution (>50 μm). For example, single cell analysis requires both a capture and selective release of a target cell for analysis. The need for miniaturization of this process has provided a unique opportunity for microfluidics to provide a high-throughput screening of the rich information that can be gained through single-cell analysis. Many microfluidic devices have been produced for this purpose using traditional methods; however, almost none have been commercialized [113,114]. And while exceptions exist, such as the Fluidigm system, rarely is soft lithography scaled up enough for a commercially viable product. The application of scalable rapid prototyping methods to single cell analysis could provide the needed push to allow the development of new commercial platforms for applications from basic research to personalized medicine.
6. EXPERIMENTAL ACKNOWLEDGEMENTS

Gratitude is expressed to Erica Bortoff and Aaron Medley for their hard work in cleaning and manufacturing many centrifugal devices for testing. Juliette Kassas is thanked for her efforts in cell culture and experimental preparation for cell chemotaxis studies. Mark Lalli is acknowledged for his efforts in writing custom MATLAB code to track fluorescent cell movement and analyzing cell populations. Mary Amasia and Indradumna Banerjee are also thanked for their training help in using the laser cutter and laminator equipment at the Kista Electrum Building.

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7. NOMENCLATURE

Explanations appear in order as presented within the dissertation text.

POC     Point-of-Care
PDMS    Polydimethyl Siloxane
LOC     Lab-on-a-Chip
PIOL    Primary Intraocular Lymphoma
PSA     Pressure Sensitive Adhesive
CAD     Computer-Aided Drawing
CNC     Computerized Numerical Control
WHO     World Health Organized
ELISA   Enzyme-linked Immunosorbent Assay
DR      Diabetic Retinopathy
AMD     Age-related Macular Degeneration
VEGF    Vascular Endothelial Growth Factor
OCT     Optical Coherence Tomography
HIV     Human Immunodeficiency Virus
PCR     Polymerase Chain Reaction
sIFA    Sandwich Immunofluorescence Assay
PORT    Point-of-care Ocular Rapid Test
MW      Molecular Weight
DMSO    Dimethyl Sulfoxide
PBS     Phosphate Buffered Solution
BSA     Bovine Serum Albumin
CCD     Charged-Coupled Device
IRB     Institutional Review Board
CSFD    Chemotaxis Sheath Flow Device
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CID</td>
<td>Chemotaxis Immunophenotyping Device</td>
</tr>
<tr>
<td>BCA-1</td>
<td>B-Cell Chemoattractant 1</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>Stromal-Derived Factor 1 alpha</td>
</tr>
<tr>
<td>SOLID</td>
<td>Spinning Ocular Lymphocyte Immunophenotyping Device</td>
</tr>
<tr>
<td>PC</td>
<td>Polycarbonate</td>
</tr>
</tbody>
</table>
8. REFERENCES


94


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APPENDIX

A.1 Future Recommendations

To continue this work on scalable manufacturing methods of microfluidics, additional studies of materials and manufacturing methods should be explored. For materials, additional studies of optical properties, solvent resistance, surface chemistry affinities, biofouling, and other surface modifications should be further explored to allow engineers to not only select the best material but tune the material properties to best suit their application. Additional materials should also be considered outside of standard industrial thermoplastics, adhesives, and papers such as softer plastics and 3D-printed materials. For manufacturing methods, additional studies should be performed on manufacturing using methods such as micro-CNC or 3D printing. These studies should focus on the capabilities of these methods to cut precise channels with high resolution based on appropriate materials for those techniques. Also, large scale manufacturing methods should also be studied such as hot embossing, injection molding and die cutting to determine the limitations of those methods in comparison to the rapid prototyping methods discussed in this dissertation.
A.2 Characterization Studies

Helpful characterization studies of materials and methods have been shown in literature and are listed here to assist in scalable microfluidic manufacturing:

Materials – Plastics


**Materials – Adhesives**


**Methods – Laser Cutting**


**Methods – Plotter Cutting**


**Methods – Micromilling**

Methods – 3D Printing
