DEVELOPMENT OF DIGITAL MICROFLUIDIC HETEROGENEOUS IMMUNOASSAYS

by

Alphonsus Hon-Chung Ng

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Doctor of Philosophy

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Abstract

Digital microfluidics (DMF) is an emerging liquid-handling technique in which picoliter to microliter-sized droplets are manipulated on a hydrophobic surface. The most common DMF systems actuate droplets through the application of electrical potentials on a generic \( (m \times n) \) array of insulated electrodes. The generic geometry and simple actuation scheme allows users to adopt a “programming” approach to microfluidics, calling a series of “functions” comprising various combinations of droplet operations (e.g. metering from reservoirs, splitting, merging, and mixing).

While this configuration is perfect for implementing homogeneous chemical and enzymatic reactions, this format is not an obvious match to applications that require functionalized surfaces. In particular, heterogeneous immunoassays, which require a solid support to immobilize antibodies or antigens, represent an important class of tests that can benefit from miniaturization and automation. Thus, this thesis describes the development, optimization, and validation two new digital microfluidic methods for performing heterogeneous immunoassays. The first method enables efficient on-chip separation and resuspension of magnetically activated particles. While previous embodiments of DMF/magnetic separations
have been limited by the particular format used (including suspension in immiscible oils), the method developed here was much more flexible, and was found to be very efficient at removing unbound reagents in the supernatant. The method was validated by application to quantifying a wide range of different analytes, including thyroid-stimulating hormone, 17β-estradiol, and rubella virus immunoglobulins. Based on this technique, we designed and built a shoebox-sized integrated instrument capable of performing immunoassays from sample-to-analysis with minimal manual intervention. The diagnostic performance of this system was evaluated using serum and plasma samples from a commercial anti-rubella mixed titer performance panel, yielding 100% sensitivity and specificity. The second method was given the moniker, “digital microfluidic immunocytochemistry in single-cells” (DISC), which can stimulate and fix adherent cells for single-cell immunoassays. The use of DMF facilitated rapid stimulation and fixation of cells, enabling the interrogation protein phosphorylation in the seconds to minutes timescale. Fixed and stained cells on the device were scanned using a microarray reader, which has the ability to resolve signaling networks in heterogeneous cell populations. Using this technique, we interrogated the phosphorylation state of PDGFR andAkt in NIH 3T3, MCF-7, and MDA-MB-231 cell lines resulting from various time, frequency, and concentration dependent PDGF-BB stimulation. These studies revealed detailed kinetic behaviour and population heterogeneity of PDGFR stimulation, and uncovered a possible Akt-dependent relationship to breast cancer cell aggressiveness. Unlike flow cytometry, this technique evaluates and visualizes adherent cells in situ, precluding the need for disruptive disassociation protocols. We propose that the new methods described in this thesis may eventually become useful for disease surveillance and diagnostics, and life science research.
Acknowledgments

This thesis was completed with the support of my mentors, colleagues, friends, and family—without your help, I would have never came this far.

I am especially grateful to my supervisor, Prof. Aaron Wheeler, for his guidance, unwavering support, and boundless patience. He created a unique environment that enabled me to pursue my own investigative path, foster deep collaborations and friendships, and grow as a researcher and an individual. Joining the Wheeler lab was one of the best things that happened to me.

To my research mentors, Professors Warren Chan, Craig Simmons, Christopher Yip, Julie Audet, and Shana Kelley, thank you for your guidance throughout these years—your expert critique has strengthened the quality of my work. A special thanks to Professor Samuel Sia for serving as my external committee member—your work has truly been an inspiration to me.

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I am grateful to colleagues who have trained me. Dean Chamberlain taught me everything I know about cell culture and biology. Lindsey Fiddes taught me how to fabricate channel-based PDMS devices. Mais Jebrail taught me how fabricate and operate DMF devices. Beth Miller showed me how to run surface immunoassays. Henry Lee and Yimin Zhou trained me on proper cleanroom procedures and helped debug numerous equipment problems in the cleanroom.

I am indebted to colleagues who have worked closely with me throughout the years—your friendship, selflessness, and ingenuity were the active ingredients in my dissertation. Ryan Fobel, thank you for spending so much of your time in developing and fine-tuning the droplet automation systems—your selflessness has made all our research easier. Irwin Eydelnant, thanks for teaching me to think outside the box and showing me that it is possible to integrate work, fun, and adventure. Kihwan Choi, I thank you for supporting me with your wisdom and friendship—your presence pulled me through very tough times in the lab. Dean, thanks for being a friend and
providing a much-needed guiding light in our biological investigations. Misan Lee, thank you for coming to my aid and pushing me through the finish line. Sam Au, thank you for constantly being a source of laughter and dedicating your time to characterizing on-chip cell health and pluronics—these components (including laughter) were fundamental to the success of all my projects. Betty Li, thanks for improving our fabrication pipeline and being my food buddy.

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>DMF</td>
<td>Digital microfluidics</td>
</tr>
<tr>
<td>DISC</td>
<td>Digital microfluidics in single-cells</td>
</tr>
<tr>
<td>DOE</td>
<td>Design of experiment</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid-stimulating hormone</td>
</tr>
<tr>
<td>E2</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>APTES</td>
<td>3-aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>DEX</td>
<td>Dextran</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-assembled monolayer</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-lysine</td>
</tr>
<tr>
<td>PAA</td>
<td>Poly (acrylic acid)</td>
</tr>
<tr>
<td>FFD</td>
<td>Fluid force discrimination</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive Protein</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Matrix metalloproteinase-8</td>
</tr>
<tr>
<td>ITP</td>
<td>Isotachophoresis</td>
</tr>
<tr>
<td>ITO</td>
<td>Indium tin oxide</td>
</tr>
<tr>
<td>EWOD</td>
<td>Electro-wetting on dielectric</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>DI</td>
<td>Dionized water</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>GO</td>
<td>Graphene oxide</td>
</tr>
<tr>
<td>PPO</td>
<td>Poly(propylene oxide)</td>
</tr>
<tr>
<td>PEO</td>
<td>Poly(ethylene oxide)</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NFDM</td>
<td>Non-fat dried milk</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variability</td>
</tr>
<tr>
<td>SBR</td>
<td>Signal-to-background ratio</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>POCT</td>
<td>Point-of-care testing</td>
</tr>
<tr>
<td>CAD</td>
<td>Computer-aided design</td>
</tr>
<tr>
<td>RMSE</td>
<td>Root mean square error</td>
</tr>
<tr>
<td>RV</td>
<td>Rubella virus</td>
</tr>
<tr>
<td>CRS</td>
<td>Congenital rubella syndrome</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light unit</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operating characteristic</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate-buffered saline</td>
</tr>
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Eq. 1.2 \[ F = L \gamma_{Lg} (\cos \theta - \cos \theta_0) = \frac{e_0 \varepsilon_e LV^2}{2d} \] ................................................................. 17

Eq. 1.3 \[ E(f, x) = \frac{L}{2} \left( x \sum_i e_0 \varepsilon_{e, i, \text{liquid}} V_i^2 \text{, liquid} (j2\pi f) + (L-x) \sum_i e_0 \varepsilon_{e, i, \text{filler}} V_i^2 \text{, filler} (j2\pi f) \right) \] .... 17

Eq. 1.4 \[ F(f) = \frac{\partial E(f, x)}{\partial x} = \frac{L}{2} \left( \sum_i e_0 \varepsilon_{e, i, \text{liquid}} V_i^2 \text{, liquid} (j2\pi f) \right) - \sum_i e_0 \varepsilon_{e, i, \text{filler}} V_i^2 \text{, filler} (j2\pi f) \] ... 18

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Eq. 3.1 \[
\dot{Y} = I + \beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \beta_3 \cdot X_3 + \beta_{12} \cdot X_1 \cdot X_2 + \beta_{13} \cdot X_1 \cdot X_3 + \beta_{23} \cdot X_2 \cdot X_3 \\
+ \beta_{11} \cdot X_1^2 + \beta_{22} \cdot X_2^2 + \beta_{33} \cdot X_3^2 + \beta_{122} \cdot X_1 \cdot X_2^2 + \beta_{133} \cdot X_1 \cdot X_3^2 + \beta_{211} \cdot X_2 \cdot X_1^2 \\
+ \beta_{233} \cdot X_2 \cdot X_3^2 + \beta_{311} \cdot X_3 \cdot X_1^2 + \beta_{322} \cdot X_3 \cdot X_2^2 
\]

Eq. 4.1 \[ \text{Sensitivity} = \frac{TP}{TP + FN} \] ................................................................. 79

Eq. 4.2 \[ \text{Specificity} = \frac{TN}{TN + FP} \] ................................................................. 79

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Overview of chapters

Chapter 1 provides an overview of microfluidic heterogeneous immunoassay platforms, with emphasis on digital microfluidic-powered immunoassays for analytes in biological liquids and cells. Sections of this introduction were published or accepted in three review articles:


Chapter 2 describes the development of digital microfluidic magnetic separation, a technique that enables efficient on-chip separation and resuspension of magnetically activated particles. Using this technique, we developed methods for non-competitive and competitive particle-based immunoassays, using thyroid-stimulating hormone (TSH) and 17β-estradiol (E2) as model analytes, respectively. The techniques developed here serve as the basis for the work described in chapter 3 and 4. This work was published in Analytical Chemistry:


Chapter 3 describes the development and characterization of a shoebox-sized integrated instrument capable of performing particle-based immunoassays from sample-to-analysis with minimal manual intervention. This instrument features (a) a 90 Pogo pin interface for digital microfluidic control, (b) an integrated (and motorized) photomultiplier tube (PMT) for chemiluminescent detection, and (c) a magnetic lens assembly which focuses magnetic fields into a narrow region on the surface of the DMF device, facilitating up to 8 simultaneous digital microfluidic magnetic separations. This work was published in Analytical Chemistry and was featured on the cover of the issue:

Chapter 4 describes the evaluation of DMF-powered diagnostic tests for infectious disease. Building on work described in chapter 2 and 3, immunoassays were developed with integrated sample preparation for the detection of rubella virus (RV) IgG and IgM. The performance (sensitivity and specificity) of these assays was evaluated using serum and plasma samples from a commercial anti-rubella mixed titer performance panel. This work has been submitted for publication:

• Ng, A.H.C.; Lee M.; Choi K.; Fischer A.T.; Robinson J.M.; Wheeler A.R. “A Digital Microfluidic Platform for the Detection of Rubella Infection and Immunity” (Submitted)

Chapter 5 presents a new method, called digital microfluidic immunocytochemistry in single-cells (DISC), which can precisely stimulate and fix adherent cells in the seconds to minute timescale for single-cell immunoassays. A manuscript describing this work is currently in preparation:

• Ng, A.H.C.; Chamberlain, M.D.; Wheeler, A.R. “Digital Microfluidic Immunocytochemistry in Single Cells” (In preparation)
Overview of author contributions

Prof. Aaron Wheeler provided guidance, expertise, and direction to all the work described in this thesis.

In the work described in chapter 2, I conceived, implemented, and characterized the digital microfluidic magnetic separation method. Using this method, Dr. Kihwan Choi (a postdoctoral fellow in the Wheeler lab) and I developed and characterized magnetic particle-based immunoassays for estradiol and thydroid stimulating hormone. Our collaborators at Abbott Diagnostics, Drs. Robert Luoma and John Robinson, contributed to helpful discussions regarding reagent composition and assay optimization.

Building on technology developed in chapter 2, chapter 3 represents a collaborative effort between scientists and engineers at the University of Toronto and Abbott Diagnostics for the design, construction, and characterization of an integrated instrument for DMF immunoassays. Ryan Fobel (a graduate student in the Wheeler lab) and Carl Oleksak (a research engineer at Abbott) designed and implemented the software and electronics of the instrument. Dr. David Chang-Yen, Elroy Pearson (instrument scientists at Abbott), and I designed and implemented the magnetic separation system. Lyle Yarnell (a mechanical engineer at Abbott) and Carl Oleksak designed and assembled the mechanical components of the instrument. I carried out the numerical simulations for the magnet design and characterized the performance of the magnet. Dr. Kihwan Choi carried out the full factorial immunoassay experiments. Together, Dr. Kihwan Choi and I characterized the performance of the PMT. I analyzed and interpreted the immunoassay data. Dr. Andrew Fischer (a scientist at Abbott) and Prof. Julie Audet (University of Toronto) contributed to helpful discussion for data analysis and interpretation. Drs. Robert Luoma and John Robinson contributed to helpful discussions regarding instrumentation design.

I conceived and designed the work described in chapter 4. Misan Lee (then an undergraduate student in the Wheeler lab) and I developed and optimized the immunoassays for RV IgG and IgM. Together, Dr. Kihwan Choi, Misan Lee, and I carried out DMF immunoassays on the patient samples. Drs. Andrew Fischer and John Robinson contributed to helpful discussions regarding assay optimization and data interpretation.
Dr. Dean Chamberlain (a research associated in the Wheeler Lab) and I conceived and designed the DISC method described in chapter 5. Dr. Dean Chamberlain provided biological guidance and cultured cells for the experiments. I optimized the DISC method, carried out the experiments, and analyzed the data. Together, Dr. Dean Chamberlain and I interpreted the data.
Chapter 1

1 Microfluidics and Immunoassays

This thesis describes the development, optimization, and validation of digital microfluidic methods for performing heterogeneous immunoassays. The following sections constitute a review of topics that are critical to understanding the context of this work, including immunoassays (1.1), microfluidic immunoassay configurations (1.2), fluid handling modalities for implementing microfluidic immunoassays (1.3), and digital microfluidics (1.4).

1.1 Immunoassays

The immunoassay is a technique that exploits the sensitivity and specificity of antibody-antigen interactions for the detection of relevant analytes. This analytical technique is widely used for the quantification of proteins and small molecules in a number of different fields such as medical diagnostics, proteomics, pharmaceutical research and biological research. Immunoassays can be classified into main two types: heterogeneous and homogeneous. In heterogeneous immunoassays, antibodies are immobilized on a solid support and interact with the antigen at the boundary layer. In this format, unbound antibodies and other reagents can be easily removed. In homogeneous immunoassays, antibodies interact with antigens in solution. In this case, the bound and unbound antibodies are discriminated based on physical or chemical changes arising from the binding event. Heterogeneous and homogeneous immunoassays can be further divided into competitive and non-competitive modes. In competitive mode, target antigens (from the sample) compete with exogenous labeled antigens for a limited number of antibody binding sites. Thus, the generated signal is inversely proportional to the antigen concentration. This mode is particularly important for small antigens with limited number of binding sites. In non-competitive mode, antigens are captured by an excess of antibodies and are detected after subsequent binding of a second set of labeled antibodies that bind to the antigen at a different epitope (binding site). This forms a “sandwich” immunoassay, in which the signal is proportional to the antigen concentration. This mode is only compatible with large analytes (>1000 Da) that have more than one epitope.
Since its conception in the 1960s, the immunoassay has grown in demand as the pace of discovery of new disease biomarkers has increased, warranting the development of fully automated robotic test instruments—the first of which were manufactured by Boehringer-Mannheim and Abbott in the 1980s.\textsuperscript{1} Self-standing, fully automated immunoassay systems currently represent the gold standard for in vitro diagnostics; however, there are several drawbacks that limit their effectiveness. One drawback is the requirement of large sample volumes, which limits their clinical utility. For example, in disease diagnostics, it would be ideal to assay a single sample of a patient’s serum for many different disease markers—this can pose problems when 100-200 μL of sample is required for each test. Another drawback is the time and cost required for each assay. Analysis can take up to several hours because analyte molecules must diffuse across long distances before they encounter antibodies on the solid phase. Since these systems rely on robotic instrumentation for fluid handling, they require at least $100,000 for equipment, $10,000 per sq. ft. for yearly overhead expenses, and skilled technicians for maintenance and operation.\textsuperscript{8} Consequently, these instruments are only feasible in centralized facilities such as hospital reference laboratories, to which samples are transported after collection. With the growing cost of health care, there is a need for decentralized immunodiagnostic tools capable of providing fast, quantitative results in the clinic or at the bedside. The availability of such tools can enable early diagnosis, decrease hospital stays, and eliminate transportation and administrative expenses.

The need for decentralized immunodiagnostics has prompted the development of portable assays, and many researchers addressed this challenge by miniaturizing immunoassays using microfluidics.\textsuperscript{9} The most common microfluidic paradigm relies on networks of enclosed micron-dimension channels.\textsuperscript{10-12} At these small scales, fluids exhibit laminar flow—i.e., fluidic streams flow parallel to each other and mixing occurs only by diffusion.\textsuperscript{13} Miniaturizing immunoassays using microfluidics offer at least three advantages over conventional methods:\textsuperscript{7} (1) The smaller dimensions of microfluidics can reduce diffusion times, resulting in faster analysis.\textsuperscript{14} (2) The small volumes used in microfluidics reduces the consumption of expensive reagents and precious samples, and makes it compatible with finger-pricked blood samples, eliminating the need for phlebotomist and lowering the cost per assay. (3) The fluid handling in microfluidics can be automated with simple, compact instrumentation, reducing the size and operating cost of test equipment.\textsuperscript{15,16} These advantages and others can potentially enhance the clinical utility of
automated immunoassays while making them more feasible in a decentralized near-patient setting. However, despite many advances in the development of microfluidic technologies, few have materialized into fully functional integrated devices that provide real clinical value.\textsuperscript{17} This realization has been hampered by hurdles in the development of practical microfluidic methods for sample collection, world-to-chip interfaces, sample pre-treatment, long-term preservation of reagents, and working with complex specimens.\textsuperscript{18} Another challenge is system integration—often research is focused on development of individual microfluidic components rather than toward a fully functional, integrated device. As a result, more money and effort is required to ensure that different critical components can work together.\textsuperscript{17-18}

1.2 Microfluidic Immunoassay Configurations

Microfluidic immunoassays have been implemented in both heterogeneous and homogeneous configurations. In heterogeneous configurations, antibodies are either immobilized on the surface of the microfluidic device or on micron-dimension beads ("microbeads") embedded in the device. In homogeneous configurations, bound and unbound antibodies are mostly discriminated by their electrophoretic ratio mobility in microchannels.

1.2.1 Heterogeneous surface-based immunoassays

\textit{Antibody immobilization}

The method of antibody immobilization on the microfluidic substrate can dramatically influence the performance of surface-based immunoassay. The substrate can be made from materials such as glass, polydimethylsiloxane (PDMS), poly(methyl methacrylate) (PMMA), silicon, silicon nitride, polystyrene and cyclic polyolefin; the pros and cons the various substrates have been reviewed elsewhere.\textsuperscript{7,13,19} The simplest form of immobilization is physical adsorption of antibodies/antigen onto the surface.\textsuperscript{20-22} Physical adsorption can reduce antibody binding activity because of unfavourable orientation, steric hindrance, and denaturation.\textsuperscript{19} In response to these challenges, many covalent and bioaffinity immobilization strategies have been developed for immunoassays in microfluidic platforms. Yakovleva et al.\textsuperscript{23-24} compared different strategies for covalent attachment of antibodies and bioaffinity proteins (protein A or G) on silicon substrates and found that covalent attachment with long flexible linkers such as polyethyleneimine (PEI)\textsuperscript{25} and dextran (DEX)\textsuperscript{26} are more favourable than shorter linkers such as 3-
aminopropyltriethoxysilane (APTES). These linkers circumvent problems associated with antibody accessibility and steric limitations. Other strategies of immobilization include the use of self-assembled monolayers (SAMs),\textsuperscript{27-28} lipids,\textsuperscript{29-31} DNA,\textsuperscript{32-33} poly(ethylene glycol) (PEG), polyacrylate chains,\textsuperscript{34} protein G hydrogels,\textsuperscript{35} and nanofibrous membranes.\textsuperscript{36-37} To preserve the functionality of immobilized antibodies, sugars,\textsuperscript{38-39} sol-gels,\textsuperscript{40} and desiccants\textsuperscript{41} have been used to protect antibodies against denaturation. Recently, Wen et al.\textsuperscript{42} developed a novel antibody immobilization method on PMMA surface that enhanced antibody binding efficiency and capture activity. In this work, biotin-poly(L-lysine)-g-poly(ethylene glycol) (biotin-PLL-g-PEG) was used as a surface linker to minimize the repulsive force between antibody and surface. The PMMA surface was activated by oxygen plasma and grafted with poly (acrylic acid) (PAA) to add functional carboxyl groups. The carboxylic groups formed electrostatic interactions with biotin-PLL-g-PEG and PLL-g-PEG molecules and biotinylated protein A was linked to the surface via NeutrAvidin bridges (Figure 1.1A). Protein A is a bioaffinity protein that selectively immobilizes the Fc regions of the antibody, enabling favourable orientation. The non-adhesive PEG chains on the surface prevented denaturing of the NeutrAvidin and reduced nonspecific adsorption of other proteins. This strategy resulted in higher antigen capture efficiency than PEI covalently linked to protein A. In addition, the long-branched chain of biotin-PLL-g-PEG combined with PLL-g-PEG helped distribute protein A on the surface to minimize steric hindrance and enabled efficient binding of antibodies.

**Analyte delivery and washing**

Innovative strategies for analyte delivery and washing can improve the sensitivity, duration, and limit of detection of surface-based immunoassays. Although diffusion distances in microchannels are significantly smaller than those in conventional microtiter well plate formats, analytes can still be transport-limited in microchannels at low sample concentrations.\textsuperscript{43} One can conceivably reduce the dimensions of the devices even further—but this increases fluidic resistance to impractical levels. To circumvent this problem, Hoffmann et al.\textsuperscript{44} developed a flow confinement method for rapid delivery of small sample volumes to capture antibodies. In flow confinement, a sample flow is joined with a perpendicular makeup flow of water or sample medium. Under laminar flow conditions, the makeup flow confines the sample into a thin layer above the sensing area and increases its velocity. Another strategy to improve analyte capture is to integrate mixing elements in the microfluidic device.\textsuperscript{45} Golden et al.\textsuperscript{46} demonstrated that
embossing patterned groves on the microchannel can increase immunoassay sensitivity by at least 26%. The grooves induced fluid mixing in the channel which enhanced delivery of analyte to the capture zone and prevented the depletion of analytes at the boundary layer.

Another strategy to improve the performance of surface-based immunoassays is the use of active forces. Mulvaney et al. developed a washing technique termed fluid force discrimination (FFD) to significantly reduce non-specific adsorption and achieved limits of detection of attomolar concentrations. In FFD assays, analytes captured on the surface are labeled with antibody-coated magnetic beads. Subsequently, non-specifically bound beads are removed by applying shear forces. The density of beads that remains bound is proportional to the analyte concentration and can be determined with either optical counting or magnetoelectronic detection of the magnetic labels. In another example, Morozov et al. exploited active forces (electric, magnetic, and mechanical) to achieve a zeptomole detection limit within three minutes (Figure 1B). First, electric fields were generated to electrophoretically draw the analytes to the surface and promote capture. Second, antibody-coated magnetic beads were flowed into the channel while being attracted to the surface with a magnet, causing them to slide over the surface. Finally, the non-specifically bound beads were removed by shear forces, similar to FFD.

1.2.2 Heterogeneous microbead-based immunoassays

Microbeads are frequently used in microfluidic immunoassays as they offer a dramatic increase in surface-area-to-volume and serve as a simple mechanism to reproducibly deliver antibodies to desirable locations. On the other hand, microbeads have the risk of adsorbing to device surfaces, clogging channels, increasing flow resistance, and scattering light. Microbeads can be either magnetic or non-magnetic—this often determines the method of implementation in the microfluidic device.

Non-magnetic microbeads

In non-magnetic microbead-based immunoassays, a physical retention micro structure is necessary to facilitate the removal of unbound analyte or antibodies. Kitamori and co-workers pioneered the use of antibody-coated polystyrene beads trapped by a dam structure for heterogeneous immunoassays. Subsequent work by other groups has included variations in detection strategy, fluidic modality, and microbead material. In a related configuration,
discrete microbeads can be immobilized in arrayed micro structures which enable simultaneous
detection of multiple analytes.\textsuperscript{70-74} As an alternative to physical retention, microbeads can be
immobilized by dielectrophoresis\textsuperscript{75-76} or electrostatic forces.\textsuperscript{77-78} The need for microbead
immobilization can be avoided with special detection mechanisms involving
immunoagglutination,\textsuperscript{79-84} resistive-pulse sensors,\textsuperscript{85} deflection velocity sensors,\textsuperscript{86-87} or microflow
cytometry.\textsuperscript{88}

Several unique bead-based immunoassay implementations have been developed to
address limitations in conventional assays. In one example, Yang et al.\textsuperscript{89} used superporous
agarose beads as a solid support for enhanced detection of goat IgG. Here, the porous beads were
covalently conjugated to protein A which immobilized the capture antibody in a favourable
orientation. The porosity of the beads lowered the fluidic resistance and increased the effective
surface area, thereby enhancing the sensitivity of the assay. In another example, Shin et al.\textsuperscript{90}
implemented a solid phase extraction strategy to increase the sensitivity of a competitive
immunoassay for C-reactive protein (CRP). In this work, CRP was captured by antibody-coated
microbeads packed against a frit and subsequently was eluted in an acidic buffer. This technique
improved the sensitivity by 20-fold which facilitated the use of an inexpensive on-chip
photodiode for detection. A similar strategy was successfully employed by Peoples et al.\textsuperscript{91} to
detect CRP in human serum and cerebrospinal fluid. In a very unique design, Wang et al.\textsuperscript{92}
demonstrated a nanofluidic based electrokinetic preconcentrator in a bead-based immunoassay
format (1, Figure 1.1C). In this work, antibody-coated polystyrene beads were trapped by a dam
structure just before the nanofluidic preconcentrator (2, Figure 1.1C). When a field is applied
across the nanofluidic channels, an ion depletion region is created which can be used to trap
analytes in solution. If the ion depletion force is balanced by an external flow, the analytes can
be accumulated in the vicinity of the microbeads to enhance antibody-alyte interactions. After
30 minutes of pre-concentration, the immunoassay sensitivity was improved by 500-fold. The
duration of pre-concentration can be used to modulate the dynamic range which enables the
analysis of protein concentrations that vary over many orders of magnitude.
Figure 1.1: Microfluidics immunoassay configurations

(A) Schematic of antibody immobilization strategy using biotin-PLL-g-PEG and biotin-Protein A linked by NeutrAvidin. (Reprinted from 42, with permission from Elsevier.)

(B) 1: Electrophoretic attraction of charged analytes to antibody array from bulk phase. 2: Magnetic attraction of magnetic bead labels to antibody array and removal of non-specifically bound labels by shear force. 3: Dark-field images magnetic bead label on antibody array at various capture time (min). (Reprinted with permission from 49. © 2007 American Chemical Society.)

(C) 1: Schematics of the nanofluidic preconcentration device. The middle sample channel is connected...
to the U-shaped buffer channel by a nanochannel array. 2: Bead loading, immunosensing and preconcentration procedure. (92– Reproduced by permission of The Royal Society of Chemistry) (D) 1-3: Magnetic retention microfluidic device with two permanent magnets (iii, iv). 4: Optical image of the self-assembled magnetic chains. 5: Fluorescent image of bead from off-chip incubation protocol. 6: Fluorescent image of bead from full on-chip protocol (higher intensity). (With kind permission from Springer Science+Business Media: 93) (E) 1: Schematic of gel-electrophoresis device. (%T - total acrylamide and %C - bis-acrylamide cross-linker). 2: On-chip loading and enrichment of antibody and analyte. 3: Electropherograms and gel-like plots show that enrichment increased signal of complex. (94, © 2007 National Academy of Sciences, U.S.A.)

*Magnetic microbeads*

The use of magnetic microbeads in microfluidic platforms for immunoassays is an emerging trend, as it eliminates the need for physical retention micro structures. In typical magnetic bead-based immunoassays, antibody-coated magnetic beads are immobilized on the device surface or directly on an integrated electrochemical sensor for the duration of the assay. In this case, the full utility of the beads is not realized because the beads are localized during antibody-antigen interaction. Ideally, microbeads should be dispersed or resuspended to reduce the diffusion distances between analyte and antibody. On the other hand, immobilizing/resuspending magnetic beads at different stages of the assay requires more sophisticated fluidic handling. To circumvent this problem, Lacharme et al. developed a unique magnetic bead retention technique. In this scheme, microchannels were formed with periodic changes in cross sectional width for the purpose of magnetic bead retention (1-2, Figure 1ID). In the presence of a homogenous magnetic field applied perpendicularly to the channel axis, magnetic beads self-assemble in chains along the channel (3-4, Figure 1ID). This magnetic bead retention strategy facilitates highly efficient mixing and enhanced antibody-antigen interaction. Using this system, two immunoassay protocols for the detection of mouse monoclonal antibodies were compared. In the first protocol, capture antibody and analyte were incubated off-chip, while exposure to the detection antibody was performed on-chip. In the second protocol, the complete immunoassay was executed on-chip. The full on-chip protocol was faster, consumed fewer reagents, and was more sensitive compared to the off-chip incubation protocol (5-6, Figure 1ID).

1.2.3 Homogeneous capillary electrophoresis-based immunoassays

In homogenous configurations, bound and unbound antibodies have been discriminated by differences in diffusion characteristics, isoelectric point, fluorescence polarization, fluorescence resonance energy transfer, and enzyme activity. But the most popular form of
homogeneous immunoassay is based on capillary electrophoresis (CE), in which immune complex and free antibodies are discriminated based on their electrophoretic mobilities. CE-based microfluidic chips have become very popular because of their compatibility with electrokineic fluid manipulation, rapid electrophoretic separation, and enormous potential for multiplexing. Harrison and co-workers\textsuperscript{4, 111-114} pioneered the development of microfluidic CE for immunoassays. In their most elaborate design, they integrated six functional microfluidic CE on a single chip and achieved simultaneous quantification of anti-estradiol and ovalbumin in less than one minute.\textsuperscript{114} Subsequent development from other groups focused on implementing electrochemical detection,\textsuperscript{115-117} increasing throughput,\textsuperscript{118-119} and miniaturizing device footprint.\textsuperscript{120} In a unique application, Kennedy and co-workers\textsuperscript{121-124} developed high-throughput CE-based devices for long-term on-chip monitoring of insulin secretion from islet of Langerhans. Notably, they developed integrated strategies to continuously perfuse fresh reagents and electrophoresis buffers to extend the operation time of their CE devices for up to 24 hours.\textsuperscript{121}

Recently, gel-electrophoresis-based microfluidic immunoassays have gained attention because of their enhanced ability to discriminate between bound and unbound analytes.\textsuperscript{94, 125-128} In contrast to non-sieving CE microfluidic chips, gel electrophoresis can separate molecules based on electrophoretic mobility and molecular weight. This is particularly important for large analytes since their charge-to-mass ratios are very similar to those of the corresponding immune complexes. To ensure that the immune complexes will remain intact during gel electrophoresis, the sieving gels must be non-denaturing; examples of such gels include methylcellulose\textsuperscript{127} and polyacrylamide.\textsuperscript{128} Using different compositions of polyacrylamide gels, Herr et al.\textsuperscript{94} developed a gel electrophoresis-based immunoassay microchip that integrated sample pretreatment (mixing, incubation, and enrichment) and electrophoresis to rapidly quantify matrix metalloproteinase-8 (MMP-8) in saliva. The microchip operates on three photopatterned polyacrylamide elements: (1) a large pore-size gel for sample loading and preparation, (2) a size-exclusion membrane for sample enrichment and mixing; and (3) a small pore-size gel for electrophoretic separation of bound and unbound MMP-8 (1, \textbf{Figure 1.1E}). First, the antibodies and analytes were electrophoretically loaded against the size-exclusion membrane for a fixed duration. Then, the sample plug was eluted off the membrane for electrophoretic separation. The enrichment process enhanced the antibody-antigen interaction by minimizing diffusion distances (2, \textbf{Figure 1.1E}).
As a result, a longer enrichment time resulted in a higher immune complex signal (3, Figure 1.1E).

1.3 Fluid Handling Modalities for Microfluidic Immunoassays

Conventional immunoassays require sophisticated fluid handling steps at various stages of the assay.129 Thus, analogous fluidic operations have been developed for microfluidic immunoassays. Most microfluidic systems are based on continuous fluid flow in networks of enclosed microchannels, and the active forces that drive these fluids fall under three major categories: pressure, passive, and electric. These channel-based fluid handling schemes are powerful techniques, each with unique capabilities that can be exploited to improve the performance or versatility of immunoassays.

1.3.1 Pressure-driven fluid handling

Pressure-driven flow is the most popular fluidic modality for immunoassays in microfluidic platforms. In its simplest form, pressure-driven flow can be created with either i) a vacuum pump by opening an inlet to atmospheric pressure and applying vacuum at the outlet or ii) by applying positive pressure at the inlet and opening the outlet to atmospheric pressure.13 Pressure-driven flow can also be controlled by thumb-actuation57,130-131 and chemical reactions132 in devices targeted for low cost point-of-care analysis. In contrast to electrokinetic flow, this modality is compatible with a wide range of substrate materials and solvent compositions—even non-conductive solvents and conductive substrates.19 However, pressure-driven flow has a parabolic velocity flow profile which causes sample plug dispersion and peak broadening, rendering it less attractive for separations.133 Moreover, channel dimensions cannot be too small because high pressures are required to counter the fluidic resistance in such channels.13

*Pneumatic valves*

Recently, pressure driven flow based on integrated pneumatic valves has become popular for immunoassays because such systems are well-suited for integration and automation.134-140 This paradigm relies on mechanical elastomeric valves that are formed by multilayer soft-lithography with PDMS.141 These structures can be used to isolate reagents and samples from each other for storage or reactions. A mixing component can be formed by placing several pneumatic valves in a circular loop.129 Also, by arranging at least three pneumatic valves in a row, peristaltic pumps
are formed for fluid propulsion. These micro pumps are well-suited for sequential reagent delivery, making them a good fit for immunoassays. Using pneumatic valves, Kong et al. constructed an integrated microfluidic chip for high-throughput analysis of clenbuterol. This microchip was integrated with 36 normally-closed pneumatic valves which facilitated the delivery of reagents and isolation of reaction mixture. The valves were fabricated by sandwiching a PDMS membrane layer between a fluidic channel layer and a pneumatic control layer (Figure 1.2A). The valves were controlled by adjusting the pressure in the pneumatic layer using an external diaphragm vacuum and compressor which were controlled by computer regulated solenoids. A competitive immunoassay was performed on the surface of the detection region and detected using laser induced fluorescence. Although pneumatic valve devices have an impressive potential for throughput and automation, fabrication of multilayer devices is not simple and the operation of these devices require external vacuum pumps and compressors.

Centrifugal fluid handling

Centrifugal-based microfluidic platforms are typically formed from round substrates (often matching the footprint of compact discs, CDs) containing channels and microchambers that rely on spin frequency to drive fluid movement. Fluid movements between microchambers are typically gated by capillary valves or hydrophobic valves. By spinning the disc with a motor, the centrifugal force overcomes the capillary or surface forces of these valves, enabling fluid to be pumped sequentially from the centre of CD to the edge with increasing spin frequency. Like all pressure-driven flow techniques, centrifugal flow devices are insensitive to physiochemical properties of fluids such as pH, ionic strength, or chemical compositions. Because of their geometries, these devices are easily adapted to existing optical detectors and well-suited to do multiple assays in parallel. Various fluidic functions such as valving, decanting, calibration, mixing, metering, sample splitting, and separation, can be implemented on such platforms; the ability to implement blood separation on-chip is particularly attractive. There are several examples of CD-based immunoassays implemented in either bead-based or surface-based heterogeneous formats. In one approach, Lee et al. developed a centrifugal bead-based immunoassay for the detection of antigen or antibody of Hepatitis B virus (Figure 1.2B). The device was capable of plasma separation; to control the flow between chambers, the authors used an innovative valve strategy based on the melting of ferrowax. Ferrowax comprises paraffin wax with embedded iron oxide nanoparticles which enable faster melting of the wax in
the presence of low intensity laser light. These valves can be either normally-closed or normally-open, depending on the requirement of the procedure. The immunoassay is fully automated—after injecting a blood sample, the disc was inserted into a programmable blood analyzer that has an integrated detector, servo motor, and laser diode for valve control.

Figure 1.2: Fluid handling modalities for immunoassays in microchannel platforms
(A) Layout of the pneumatically driven integrated microfluidic device for surface-based immunoassay. (140 Reproduced by permission of The Royal Society of Chemistry). (B) Centrifugal driven Disc design detailing layout and function. Blue numbers represent normally-close valves and red numbers denote normally-open valves. (105 Reproduced by permission of The Royal Society of Chemistry). (C) Concept of capillary force driven one step immunoassay. 1: sample is separated from blood, 2: sample redissolves deposited detection antibodies, 3: sandwich immunoassay formed, and 4: unbound antibody and analyte removed. (41 Reproduced by permission of The Royal Society of Chemistry) (D) Schematic of electrokinetically driven preconcentration of protein sample in a gel-electrophoresis channel. (E) Fluorescent images of electrokinetic sample injection and sample stacking. (Reprinted with permission from 127. © 2007 American Chemical Society.)
1.3.2 Passive capillary force fluid handling

Passive fluid handling is becoming popular because of portability, low dead volume, ease of operation, and low power consumption. 133, 147 Although passive microfluidic immunoassays have been implemented using degassed PDMS by Hosokawa et al. 148-150, the most prevalent and versatile passive driving force is capillary force. 21, 41, 67, 151-154 In a particularly interesting example, Gervais et al. 41 developed a one-step, simple to use microfluidic immunoassay platform that is passively driven by capillary forces. The microfluidic device is comprised of a sample collector, delay valves, flow resistors, detection antibody (dAb) deposition zone, a reaction chamber with immobilized capture antibodies (cAb), a capillary pump, and vents (Figure 1.2C). The flow rate of the device depends on the total flow resistance and capillary pressure which are determined by the intricate microstructure of the capillary pumps, sample collector, delay valves and flow resistors. 155 The capillary valve and sample collector were designed to minimize flow resistance and maximize capillary pressure. The delay valves were designed to minimize the risk of entrapping air by consolidating the flow stream from the sample collector before progressing to the flow resistors. The flow resistor is a convenient component which can be easily added or removed in the design to modulate flow rate. Toward a one-step immunoassay, all necessary reagents were integrated in the device. In the deposition zone, dAb were deposited by inkjet; these dAb were then redissolved as fluid flowed through the device during the assay. In the reaction chamber, the PDMS surface is patterned with cAbs to facilitate the capture of incoming analyte. The addition of a blood sample to a loading pad triggers a cascade of precise fluidic events, resulting in a completely autonomous heterogeneous immunoassay. Capillary force is also used in lateral flow devices (also known as “paper microfluidics”) which produce qualitative or semi-quantitative results for primary screening at point of care; the pros and cons of these devices are reviewed elsewhere. 156-157

1.3.3 Electrokinetic flow

In microchannels, electric forces for flow are generated by electrophoretic and electroosmotic interactions of applied fields with ionic species in the fluid. 19 In electrophoresis, charged molecules are manipulated in the presence of electric field by electrostatic forces. In electroosmosis, a layer of fluid enriched in solvated ions is attracted to the oppositely charged channel walls; in the presence of electric field, the solvated ions and their waters of hydration are driven toward the oppositely charged electrode while dragging the bulk fluid via viscous forces.
to form a uniform plug-like flow. The direction of this flow can be controlled by applying appropriate voltage polarity to the channel reservoirs and/or changing the net charges on the channel surface. Consequently, electrokinetic flow does not require valves or pumps and is amenable for automation. The use of this fluid modality for surface-based heterogeneous immunoassays was pioneered by de Rooij and co-workers and further popularized by Li and coworkers. Recent simulations by Hu et al. suggest that electrokinetically driven immunoassays have better reaction kinetics than pressure-driven assays because of the uniform plug-like velocity profile afforded by electroosmosis. Although electrokinetic flow has many salient features, there are strict requirements for buffers and reagents used in the channels and materials used for device construction. For example, fluids used in such systems must be conductive; however, if the ionic strength is too high, Joule heating can undermine the performance of the assay. This often precludes the use of biological liquids such as blood and urine. Furthermore, the device substrate material should be non-conductive to prevent electrical breakdown.

In addition to fluid transport, electrokinetic forces can be used for electrophoretic separations and sample stacking. Electrophoresis is a useful phenomenon that forms the foundation of CE-based homogeneous immunoassays, where antigen and immune complexes are discriminated by their electrophoretic mobility. The sensitivities of CE-based immunoassays can be enhanced by a sample stacking technique called isotachophoresis (ITP). Using this technique, Mohamadi et al. developed a highly sensitive immunoassay for quantifying human serum albumin (HSA) in a gel electrophoresis-based microfluidic device (Figure 1.2D, E). In this work, a mixture of fluorescently labeled HSA and its immune complex with a monoclonal antibody was pre-concentrated by ITP and resolved by electrophoresis in a methylcellulose solution. ITP pre-concentration was facilitated by the inclusion of high-mobility ions in the leading electrolyte (LE) and low-mobility ions in the trailing electrolyte (TE), which were respectively loaded in the buffer waste reservoir (BW) and buffer reservoir (BR) (1, Figure 1.2D). The sample mixture was electrokinetically injected into the channel from the sample reservoir (SR) by applying high electric potential at sample waste reservoir (SW) (2, Figure 1.2D). Subsequently, a sample plug was injected in the orthogonal separation channel by changing the potential at the reservoirs (4, Figure 1.2D). During ITP stacking, the disparate electric field intensities in the TE, sample plug, and LE caused the sample plug to focus in a
narrow band (3-5, Figure 1.2D). Concomitantly, the HSA and immune complex formed distinct bands by electrophoretic separation (6, Figure 1.2D). An 800-fold signal enhancement was achieved with respect to control experiments without ITP pre-concentration. Figure 1.2E shows fluorescent images of electrokinetic sample injection and sample preconcentration by ITP. The full process of injection, pre-concentration, and separation were controlled by an automated sequential voltage switching program. Subsequent work on ITP-CE-based immunoassays by Satomura and coworkers\textsuperscript{161-162} integrated all assay steps on chip by using ITP to efficiently mix antibodies and analyte in addition to pre-concentration and separation.

Although microchannel schemes are useful for many applications, their flexibility and reconfigurability is limited because fluid streams are restricted to the confines of the channel walls. Also, since microchannels are serial by nature, they are difficult to use for controlling many different reagents simultaneously. These drawbacks can make it extremely challenging to integrate several different microchannel functions in one system.

1.4 Digital Microfluidics

In an emerging, channel-free paradigm of electric fluid manipulation called digital microfluidics (DMF), droplets are manipulated on an open surface in contrast to continuous flow in channels. In DMF, droplets of reagents and samples are manipulated on an array of electrodes which are insulated by a hydrophobic dielectric layer. By applying sequences of AC or DC electric potentials between ground and actuation electrodes, droplets can be driven to move, merge, split, and dispense from reservoirs by a combination of electrostatic and dielectrophoresis forces (Figure 1.3A).\textsuperscript{163-165} Since droplets are manipulated on relatively generic 2-D arrays of electrodes, droplet operations can be reprogrammed without changing device design. This flexibility makes them suitable for a wide range of applications, especially those requiring long, multistep protocols such as immunoassays. The following sections address sub-topics that are critical to understanding this thesis: DMF theory (1.4.1), the effect non-specific adsorption on DMF (1.4.2), and DMF immunoassays (1.4.3).

1.4.1 DMF theory

Two common configurations of digital microfluidic devices are depicted in Figure 1.3B: single-plate\textsuperscript{166} (also referred to as open), and two-plate\textsuperscript{167-169} (closed) devices. In the two-plate format,
droplets are sandwiched between two substrates patterned with electrodes. Typically, the top plate consists of a continuous ground electrode formed by a transparent, conductive, indium tin oxide (ITO) layer. The bottom plate houses an array of actuation electrodes. In the one-plate format, droplets sit on a single substrate bearing both actuation and ground electrodes. In both cases, an insulating dielectric layer covers the bottom plate electrodes and all surfaces are coated with a hydrophobic coating. Two-plate devices are operated either in air or in immiscible oil; the latter strategy is useful for reducing the resistive friction and thus the voltage necessary for droplet movement.\textsuperscript{168} While the operational principles for one- and two-plate devices are similar, two-plate devices enable a wider range of operations, including dispensing, moving, splitting, and merging.\textsuperscript{168-169} One-plate devices are incapable of splitting and dispensing, but they are well suited for preparative applications in which analytes are recovered after droplet actuation.

Forces that affect droplet movement can be divided into driving and resistive forces. Theoretical methods for estimating the driving forces have been reported by several groups. Early attempts were based on a thermodynamic approach using the Young-Lippman equation:\textsuperscript{168-170}

\textbf{Eq. 1.1} \quad \cos \theta = \cos \theta_0 + \frac{\varepsilon_r \varepsilon_0 V^2}{2 \gamma d} \\

where $\theta$ and $\theta_0$ are the static contact angles with and without applied voltage respectively, $\varepsilon_r$ is the relative permittivity of the dielectric, $\varepsilon_0$ is the permittivity of free space, $V$ is the applied voltage, $\gamma$ is the liquid-filler media surface tension and $d$ is the dielectric thickness. In this approach, droplet movement is assumed to occur because of capillary pressure that results from asymmetric contact angles across the droplet. It can be shown that the driving force, $F$, is equal to:\textsuperscript{170}
\[ F = L \gamma_{LG} \left( \cos \theta - \cos \theta_0 \right) = \frac{\varepsilon_0 \varepsilon_r L V^2}{2d} \]

where \( L \) is the length of the contact line overlapping the actuated electrode. This approach is based on experiments of sessile droplets and a phenomenon called electrowetting on a dielectric (EWOD),\(^{171-172}\) which has led to numerous references in literature of an “EWOD”-based force.\(^{168-170}\) It should be stressed that the driving force in DMF is a result of electrostatic forces and that large contact angle change is not a requirement for droplet movement, but rather the wetting is an observable effect of the forces acting on the droplet.\(^{173-177}\) The thermodynamic (or “EWOD”) approach is limited in that it requires knowledge of the advancing and receding contact angles and/or estimation of these values based on the Young-Lippman equation. It also fails to explain the liquid-dielectrophoretic force which is predominant at high-frequencies and/or for dielectric liquids.\(^{173-175}\)

A more direct and generalized approach for estimating the force on a droplet uses a circuit representation of the DMF device (Figure 1.3B) and either the Maxwell stress tensor\(^{173-175}\) or an electromechanical framework.\(^{176}\) Following the electromechanical derivation, the amount of energy, \( E \), capacitively stored in the system is calculated as a function of frequency and droplet position along the x-axis (the direction of droplet propagation), assuming that the cross-sectional area of the drop can be approximated as a square with sides of length \( L \).\(^{176}\)

\[ E(f, x) = \frac{L}{2} \left( x \sum_i \frac{\varepsilon_0 \varepsilon_r \text{liquid} V_{\text{liquid}}^2}{d_i} (j 2 \pi f) + (L - x) \sum_i \frac{\varepsilon_0 \varepsilon_r \text{filler} V_{\text{filler}}^2}{d_i} (j 2 \pi f) \right) \]

where \( \varepsilon_r \text{liquid} \), \( V_{\text{liquid}} \), and \( \varepsilon_r \text{filler} \), \( V_{\text{filler}} \) are the relative permittivity and voltage drop for the liquid and filler fluid portion of the electrode respectively, and \( d_i \) is the thickness of layer \( i \). The \( i \) subscript represents one of the dielectric, top and bottom hydrophobic, or liquid/filler layers. The change in energy as \( x \) goes from 0 to \( L \) is equivalent to the work done on the system, and therefore differentiating \textbf{Eq. 1.3} with respect to \( x \) yields the driving force as a function of frequency:
A critical frequency, $f_c$, can be calculated for each device geometry/liquid combination. Below this frequency, the estimated force reduces to that predicted by the thermodynamic (or “EWOD”) method. In this regime, the force acting on the droplet is driven by charges accumulated near the three-phase contact line being electrostatically pulled toward the actuated electrode, and its magnitude depends almost exclusively on the capacitive energy stored in the dielectric layer. Above the critical frequency, a significant electric field gradient is established within the droplet which results in a liquid-dielectrophoretic force pulling the droplet towards the activated electrode. In this case, the force is weighted by the difference in permittivity between the liquid and filler medium. For a dielectric liquid in an aqueous filler media, negative dielectrophoresis can be used to push the droplet away from an actuated electrode. Force estimates for some common lab reagents using typical device parameters are given in Figure 1.3C. It can be seen that at low frequencies (below ~10 kHz), forces on the order of tens of micro-Newton can be applied to a wide range of fluids using a driving voltage of 100 $V_{\text{RMS}}$. Moving liquids that have both low conductivity and permittivity can require prohibitively high voltages even at DC, although this situation can sometimes be ameliorated by mixing in a second liquid to adjust the overall electrical properties.

In addition to the driving electrostatic force acting on the drop, there exists 1) a shear force between the drop and the plates and 2) a viscous drag force because of displacement of the filler fluid, both of which impede drop motion. The shear force results in a threshold voltage, $V_{\text{min}}$, that must be overcome before drop displacement is initiated. Although Equ. 1.4 predicts an actuation force that scales with the square of the applied voltage, this relationship breaks down at what is commonly referred to as the voltage saturation limit. The exact mechanism for saturation is still the subject of debate, though it is thought to be linked to the contact angle saturation reported in the electrowetting literature, and possible explanations include gas ionization at the contact line, charge trapping in the dielectric layer, ejection of satellite microdroplets, and a zero solid-liquid interface tension limit at the contact line.
Figure 1.3: Digital microfluidics (DMF) theory
(A) Droplet operations in DMF, including mixing, splitting, merging, and dispensing from reservoirs. (B) Side-view schematics of two-plate and one-plate DMF devices. The two-plate device includes an overlaid circuit model. A top-view schematic of the two-plate device is shown below the side-view schematic. The circuit parameters depend on the cross-sectional area of the droplet overlapping with the activated (red) electrode, $A = xL$. (C) Driving Force estimation for a two-plate DMF device operating on phosphate-buffered saline (PBS), deionized (DI) water, toluene and methanol. Forces are based on a 1 mm$^2$ electrode size, 6 μm of Parylene-C as a dielectric, 235 nm of Teflon-AF ® as a hydrophobic layer, an inter-plate spacing of 150 μm, and an applied voltage of 100 V$_{RMS}$ for a range of frequencies (100 Hz to 1 MHz).
1.4.2 Preventing protein adsorption

As mentioned in section 1.4.1, the most versatile and prevalent device format for digital microfluidics is the two-plate configuration, in which droplets are sandwiched between two parallel substrates patterned with electrodes (Figure 1.3B). Typically, the bottom-plate houses an array of actuation electrodes (formed from any conductive material) covered by an insulating dielectric layer (e.g., Parylene C, Silicon nitride, PDMS, or SU-8). These electrodes are referenced to a continuous ground electrode in the top-plate made from ITO, and spacers are used to create a fixed gap between the top- and bottom-plates. Critically, both plates are coated with a fluorinated hydrophobic coating (e.g., Teflon AF® from DuPont, Cytop® from Asahi Glass, or FluoroPel® from Cytonix) to minimize the friction experienced by aqueous droplets during actuation. By applying a sequence of preprogrammed voltages (Figure 1.4A), this device configuration affords a wide range of droplet operations including metering droplets from reservoirs, splitting (Figure 1.4B), merging (Figure 1.4C), and mixing. Unfortunately, the necessity of using a hydrophobic layer makes it challenging to work with droplets that contain proteins. When proteins in aqueous solution encounter a hydrophobic solid surface, it is often thermodynamically favourable for the proteins to become immobilized on the surface via hydrophobic interactions. As such, any contact between the liquid droplet and the hydrophobic surface will promote protein adsorption, causing the surface to become hydrophilic. When this happens to a sufficient degree, droplet transport is impeded. For example, the maximum movable concentration of bovine serum albumin (BSA) in a two-plate device (operated in air) is a mere 0.005 mg/mL. This low concentration is not adequate for most immunoassay applications, which often require at least 3 orders of magnitude higher protein content (i.e., 5 mg/mL or greater). To enable transport of increased concentrations of proteins (up to 10 mg/mL BSA), Freire et al. developed a modified device geometry that pulls the droplet away from the surface. This “field dewetting” configuration does not support essential fluidic operations such as droplet splitting or metering droplets from reservoirs, but it may be useful for some simple applications.

To operate DMF devices without sacrificing the capability to perform essential droplet operations, the two most effective techniques used to prevent protein adsorption involve 1) encapsulating droplets in a non-conductive, immiscible liquid, or 2) doping additives into the aqueous droplets themselves. In the first method, devices are filled with a low-viscosity fluid
(typically silicone oil); in this configuration (Left, Figure 1.4D), droplets (which must be immiscible with the filler) are encapsulated by a film, which prevents droplets from directly contacting the hydrophobic surface. Using this approach, Srinivasan et al.\textsuperscript{190} demonstrated the successful manipulation of droplets containing various protein-rich droplets from physiological sources including whole blood, serum, plasma, urine, saliva, sweat, and tears. In general, the stability of the oil film around each droplet decreases with increasing protein concentration because of the decrease of liquid-oil interfacial tension. To enhance the stability of the oil film, a lipophilic surfactant such as Triton X-15 (0.1\% w/w) can be added to the oil to further decrease its surface tension, and therefore increase the liquid-oil interfacial tension. Polack et al.\textsuperscript{191} demonstrated that this strategy enables reliable manipulation of droplets containing up to 75 mg/mL of lysozyme. In addition to preventing protein adsorption, the oil-filled configuration has many other advantages including low actuation voltage and minimal droplet evaporation (particularly useful for applications requiring high-temperatures, e.g., polymerase chain reaction). Conversely, the presence of oil imposes a viscous drag on the droplet, which can increase the power required\textsuperscript{192} and limit the speed of droplet movement. Indeed, if a droplet moves too fast through the oil phase, the droplet may inadvertently split or fragment. To address these and other limitations, Bassard et al.\textsuperscript{193} developed a hybrid technique, in which aqueous droplets are encapsulated in a thin oil shell and transported on a device that is predominantly filled with air (Middle, Figure 1.4D). This aqueous-oil core-shell configuration supports all fundamental droplet operations, while lowering viscous drag and eliminating the fabrication and packaging required to confine the silicone oil in the device. Because of its low volatility, silicone oil may be incompatible with applications that require in situ drying of droplets (e.g., to isolate proteins for crystallization experiments or for dissolution in a different solvent). To circumvent this problem, Fan et al.\textsuperscript{194} developed a technique to remove the oil in a core-shell droplet by bringing the droplet into a reservoir of hexane. After the oil is dissolved in hexane, the droplet is moved out of the reservoir and the remnant hexane is rapidly evaporated, leaving behind the bare aqueous droplet. As an alternative, Aijian et al.\textsuperscript{195} used fluorinated fluids (instead of silicone oil) to generate the core-shell droplet configuration. Because fluorinated liquids are predominantly immiscible with aqueous (and organic) solvents and are relatively volatile, they can facilitate droplet movement and subsequently be removed by evaporation at room temperature, enabling users to deliberately dry/crystalize samples as needed.
Figure 1.4: Two-plate digital microfluidic configurations

(A) Graphical representation of pre-programmed voltage sequence (left) and corresponding frames from a video (right) depicting metering of droplets from reservoirs. Frames from videos depicting (B) splitting of a droplet and (C) merging and mixing of droplets. Dyes were added to droplets to enhance visibility. (D) Methods for preventing protein adsorption include the oil-filled configuration (left), core-shell configuration (middle), and oil-free configuration (right) with miscible additive.

In the second class of techniques, an additive is included in the droplet composition to prevent proteins from interacting with the surface (Right, Figure 1.4D). For example, Perry et al. demonstrated the use of graphene oxide (GO) additives to enable the movement of droplets containing up to 0.26 mg/mL of BSA in oil-free devices. The GO molecules act as nano sponges for proteins—if the concentration of GO is at least double that of BSA, then there is sufficient GO to bind the protein molecules in solution, preventing them from adsorbing onto the device surface. On the other hand, there is no indication whether proteins can be released from GO after transport, or how adsorption to GO affects protein activity. The most established additive for preventing protein adsorption in digital microfluidics is Pluronics. This family of triblock copolymers is formed from a (relatively hydrophobic) poly(propylene oxide) (PPO) chain flanked by two (relatively hydrophilic) poly(ethylene oxide) (PEO) chains (PEO-PPO-
PEO). Amphiphilic molecules (bearing both hydrophobic and hydrophilic residues) are known to reduce protein and cell adsorption to hydrophobic surfaces; in the many different varieties of Pluronics, this property is tuned by varying the lengths of the PPO and PEO components.\textsuperscript{198} Adding low concentrations (0.08 \%) of Pluronic F127 (with PEO/PPO/PEO average chain length of 100/65/100), Luk et al.\textsuperscript{187} demonstrated movement of droplets containing up to 50 mg/mL of BSA in oil-free devices. In a more systematic study, Au et al.\textsuperscript{199} tested a series of eight Pluronic surfactants using cell culture media (containing 10\% bovine serum) as a model fluid. Consistent with previous observations,\textsuperscript{198} the authors observed that Pluronics with longer hydrophobic PPO chains are better at reducing protein adsorption during droplet movement. Specifically, Pluronics with less than 30 PPO units (F38, L35, and L44) fail to enable translation of droplets containing 10\% fetal bovine serum, while Pluronics with greater than 30 PPO units (F68, L64, L62, L92, and P105) supported movement for hundreds of droplet operations. To exploit the benefits of multiple approaches, some groups have implemented more than one technique in a device. For example, Vergauwe et al.\textsuperscript{200} and Li et al.\textsuperscript{201} employed both oil core-shell configuration and Pluronic additives for IgE immunoassays and the culture of embryos, respectively.

### 1.4.3 DMF immunoassays

When I began my thesis work, the advantages of digital microfluidics for immunoassays were relatively unexplored—there were only four reports of immunoassays on DMF, all of which rely on oil to prevent non-specific adsorption. In the earliest approach, Rastogi and Velev\textsuperscript{202} described immunoassays for IgG and ricin, using the agglutination of antibody coated latex and gold particles. A droplet suspended in fluorinated oil on a single plate device served as a container with a controlled evaporation rate; the pattern assumed by the antibody coated latex and gold particles as the droplet evaporated was indicative of the quantity of antigen present in the sample. This method reduced sample volumes and limits of detection compared to commercially available methods, but the readout may be difficult to standardize or automate for high-throughput analysis. In another example, Sista et al.\textsuperscript{103,203} demonstrated the use of DMF for magnetic bead-based heterogeneous immunoassays to quantify human insulin and interleukin-6 (IL-6) (Figure 1.5A, B). The full immunoassay protocol was implemented in six steps: (1) a droplet containing a mixture (prepared off-chip) of magnetic beads with antibodies, reporter antibodies and blocking proteins was merged and mixed with a droplet of analyze on-chip; (2) the pooled droplet was shuttled on six set of electrodes for two minutes to allow for antibody-
antigen binding (1, Figure 1.5A); (3) the reaction mixture was delivered over the magnet to immobilize the magnetic beads (2, Figure 1.5A); (4) the unbound supernatant was removed by splitting the excess liquid from the beads (3, Figure 1.5A); (5) unbound molecules were further washed by passing five droplets of wash buffer over the magnetic beads (4, Figure 1.5A); and (6) using the interfacial tension of the receding edge of the droplet, the magnetic beads were moved away from the magnet and resuspended for detection. For detection, the enzymes on the reporter antibodies catalyzed a chemiluminescent signal that was detected by an integrated photomultiplier tube. Figure 1.5B shows frames from a movie demonstrating bead washing by removal of excess supernatant. To demonstrate the clinical applicability of this approach, the same group performed troponin I immunoassays using whole blood samples, obtaining analyte recoveries ranging from 77-108%. In the most recent example (prior to my thesis work), Vergauwe et al. explored the use of paramagnetic nanoparticles as solid support for IgE immunoassays. The authors demonstrated that nanoparticles can be dispensed by DMF with reproducible densities (CV 2.83%), which is critical for any analytical application. To perform the IgE immunoassays, one droplet containing magnetic nanoparticles (modified with Anti-IgE) and fluorescently labeled IgE aptamer (instead of reporter antibodies) was merged and mixed with a second droplet containing non-labelled IgE sample. Magnetic bead washing was performed in a similar manner as described above. Although a full calibration curve was not shown, the authors demonstrated an ability to detect IgE at concentrations as low as 150 nM.

Figure 1.5: Digital microfluidic immunoassay
(A) Protocol for heterogeneous magnetic bead-based immunoassay on a digital microfluidics platform. (B) Video sequence of magnetic bead washing by removing the excess supernatant on chip. (Reproduced by permission of The Royal Society of Chemistry).
The digital microfluidics immunoassays, described above, used either fluorinated oil or silicone oil to prevent non-specific adsorption and enable droplet manipulation. Although oil-filled systems require lower voltages for droplet actuation and prevent evaporation of droplets, they also have several disadvantages that can limit their usability:

- The analyte of interest (e.g. protein or small molecule) may partitioning into oil phase, which can adversely affect the assay performance, and contaminant the surrounding medium.

- Oil-miscible liquids/reagents such as methanol or octane, which are commonly required in liquid-liquid/solid-liquid extraction protocols, are incompatible in oil-filled systems.

- Applications or analysis techniques that require droplet drying on surface (e.g. matrix-assisted laser desorption/ionization mass spectrometry [MALDI-MS]) are incompatible with oil-filled systems.

- Techniques that require interactions with device surface, such as nucleotide hybridization or electrochemical detection, may be hindered by the oil barrier surrounding the droplet.

- The oil barrier can also restrict exchange of gases between the droplet and the atmosphere, which is unfavourable for applications that involve living cells.

- From a practical standpoint, the use of oil requires sophisticated device packaging that prevents oil from leaking out of the device.

For these reasons, as described in this dissertation, I took on the challenge of developing the first DMF immunoassays without the use of oil (i.e., droplets will be surrounded by air). This required the development of immunoassay protocols that relied on Pluronics to enable droplet movement, and the development a technique that facilitated efficient on-chip separation and resuspension of magnetically activated particles (chapter 2). These methods were further enhanced with the construction of an integrated instrument, design and numerical simulation of a new magnet system, and optimization of assay protocols by design of experiments (chapter 3). Further, I evaluated the diagnostic performance (sensitivity and specificity) of my DMF immunoassay technique by benchmarking it against conventional laboratory methods (chapter 4). Finally, leveraging on the experience gained from DMF immunoassays and DMF cell-culture
techniques established in the Wheeler lab, I developed techniques for DMF in-cell immunoassays, which have the ability to analyze signaling pathways (phosphorylated proteins) in single cells (chapter 5). Overall, I propose that this thesis represents an important step toward the development of (potentially portable) DMF-powered immunoassays, which may someday be useful for (a) decentralized diagnosis infectious diseases, and/or (b) high-time resolution basic studies evaluating the behaviour of individual cells.
Chapter 2

2 Digital Microfluidic Magnetic Separation for Particle-based Immunoassays

We introduce a new format for particle-based immunoassays relying on digital microfluidics (DMF) and magnetic forces to separate and resuspend antibody-coated paramagnetic particles. In DMF, fluids are electrostatically controlled as discrete droplets (pL to μL) on an array of insulated electrodes. By applying appropriate sequences of potentials to these electrodes, multiple droplets can be manipulated simultaneously and various droplet operations can be achieved using the same device design. This flexibility makes DMF well-suited for applications that require complex, multistep protocols such as immunoassays. Here, we report the first particle-based immunoassay on DMF without the aid of oil carrier fluid to enable droplet movement (i.e., droplets are surrounded by air instead of oil). This new format allowed the realization of a novel on-chip particle separation and resuspension method capable of removing greater than 90% of unbound reagents in one step. Using this technique, we developed methods for non-competitive and competitive immunoassays, using thyroid-stimulating hormone (TSH) and 17β-estradiol (E2) as model analytes, respectively. We show that, compared to conventional methods, the new DMF approach reported here reduced reagent volumes and analysis time by 100-fold and 10-fold, respectively, while retaining a level of analytical performance required for clinical screening. Thus, we propose that the new technique has great potential for eventual use in a fast, low-waste, and inexpensive instrument for the quantitative analysis of proteins and small molecules in low sample volumes.

2.1 Introduction

The immunoassay is a technique that relies on antibody-antigen interactions to quantify relevant analytes in applications such as medical diagnostics, pharmaceutical research and biological research. As mentioned in chapter 1, the measurement is effected using either a competitive or a non-competitive assay mode. In competitive mode, exogenous labeled antigens compete with unlabeled antigens from the sample for binding sites on the capture antibodies. In non-competitive mode, labeled antibodies and capture antibodies bind to the target antigens from the
sample at different epitopes, forming an antibody-antigen-antibody complex. In both assay modes, capture antibodies are typically immobilized on a solid support such as on the surface of microtitre well-plates or micron-dimension particles (i.e., “microparticles”). Schematic representations of microparticle-based competitive and non-competitive immunoassays are shown in Figure 2.1A.

Since its conception in the 1960s, the immunoassay has grown in demand as the pace of discovery of new disease biomarkers has increased, driving the development of fully automated immunoassay analyzers in the 1980s. These systems represent the current gold standard for immunodiagnostics; however, there are several drawbacks that limit their effectiveness. One drawback is the requirement of large sample volumes, which limits their clinical utility. For example, in disease diagnostics, it would be ideal to assay a single sample of a patient’s serum for many different disease markers—this can pose problems when 100-200 μL of sample is required for each test. Another drawback is the cost and time required for each assay. Because these systems rely on robotic instrumentation for fluid handling, they are expensive to maintain and operate, restricting their use to wealthy laboratories and centralized facilities. Analysis can take up to several hours because analyte molecules must diffuse across long distances before they encounter antibodies on the solid phase. A potential solution to these drawbacks is the miniaturization of immunoassays using microfluidics. The smaller dimensions of microfluidics can reduce diffusion times and reagent consumption, resulting in faster analysis and lower cost per assay. In addition, fluid handling in microfluidics can be automated with simple, compact instrumentation, reducing the size and operating cost of test equipment.

In the past decade, significant effort has been focused on developing microfluidic immunoassays with varying configurations and fluid handling schemes. Among the wide range of assay configurations, the use of microparticles as solid support is attractive as this approach offers a dramatic increase in the surface-area-to-volume ratio and serves as a simple mechanism to reproducibly deliver antibodies into a microfluidic device. Paramagnetic particles that respond to external magnetic fields are particularly appealing as they can be easily immobilized without the need for microfabricated structures for physical retention in the device. The magnetic forces exerted on paramagnetic particles can also enhance mixing, focus particles for immunoagglutination assays, and serve as a detection mechanism for immunoassays. While most fluid handling schemes in microfluidic immunoassays rely on continuous fluid flow or
droplets within networks of enclosed micron-dimension channels (i.e., microchannels), an alternative droplet-based fluid handling scheme called digital microfluidics (DMF) is growing in popularity. In DMF, fluids are electrostatically controlled as discrete droplets (pL to μL) on an array of insulated electrodes. By applying a series of potentials to these electrodes, droplets can be made to merge, mix, split, and dispense from reservoirs. Since droplets are manipulated on a generic array-geometry, multiple droplets can be controlled simultaneously and droplet operations can be reprogrammed without changing device design. This flexibility makes DMF suitable for applications that require complex, multistep protocols such as immunoassays.

To date, two DMF methods for magnetic particle-based immunoassays have been reported. In the first approach, Sista et al. demonstrated non-competitive immunoassays for insulin, interleukin-6, and troponin I using 1.05 μm diameter paramagnetic particles. In these methods, a solution containing a mixture of magnetic particles, labeled antibodies, and blocking proteins was prepared off-chip and then dispensed, merged and mixed with a droplet of analyte on-chip to form antibody-antigen complexes. Subsequently, a "serial dilution" method was used to wash unbound reagents from magnetic particles. To prevent non-specific adsorption of proteins onto the device surface, the device was filled with an immiscible carrier fluid (1.5 cSt silicone oil), forming a barrier between the droplet and the surface. In the second approach, Vergauwe et al. explored the use of 15 nm diameter paramagnetic particles for non-competitive immunoassays for IgE. The authors also employed the serial dilution method (as above); but rather than completely filling the device with silicone oil, each of the aqueous sample and reagent droplets were encapsulated by thin oil shells.

The microparticle immunoassay techniques reported previously represent important milestones, but they suffer from some limitations. First, both techniques describe non-competitive immunoassays only; competitive immunoassays (which are important for many clinical applications) have never been reported in digital microfluidic systems. Second, both techniques require significant off-chip sample and reagent preparation. Third, both approaches make use of an oil carrier fluid. Oil is useful for DMF systems in that it a) enables droplet actuation at lower voltages, b) reduces the amount of surface-adsorption, and c) significantly reduces the droplet evaporation rate. But there are also several disadvantages of using oil, including: 1) proteins in sample and reagent droplets may adsorb to the water-oil interface; 2) oil is problematic for integration with other on-chip functions including cell culture;
based assays, and liquid-liquid/solid-liquid extraction; specialized device packaging is required to prevent leaks.

Here, we report a new format for DMF magnetic particle-based immunoassays, with several improvements relative to the techniques reported previously. First, we report the first competitive immunoassay implemented by digital microfluidics, for 17β-estradiol (E2). [To illustrate the versatility of the technique, we also developed a non-competitive assay for thyroid-stimulating hormone (TSH).] Second, the new method allows for each of the required assay reagents to be injected individually (with no pre-mixing), such that the entire assay can be performed on-chip. Third (and perhaps most importantly), the method reported here was designed to not require the use of an oil filler fluid; i.e., the droplets in this method are surrounded by air. Although oil-free DMF systems have been used to manipulate magnetic particles for other applications, this work represents the first oil-free DMF method for particle-based immunoassays. This change in format allowed the realization of a novel on-chip particle separation method, representing a significant improvement to the particle separation methods used previously. We propose that the new technique has great potential for use in a fast, low-waste, and inexpensive instrument for the quantitative analysis of proteins and small molecules in low sample volumes.

2.2 Experimental

2.2.1 Reagents and materials

Unless otherwise specified, reagents were purchased from Sigma Chemical (Oakville, ON). Deionized (DI) water had a resistivity of 18 MΩ•cm at 25°C. Pluronic L64 (BASF Corp., Germany) was generously donated by Brenntag Canada (Toronto, ON). E2 and TSH well-plate ELISA kits were purchased from Calbiotech (Spring Valley, CA).

2.2.2 Reagent formulations used on-chip

Immunoassay reagents used on-chip included analyte standards, antibody coated paramagnetic microparticles, enzyme conjugated reporters, chemiluminescent substrate, and human serum matrix. The analyte standards and particles were adapted from ARCHITECT immunoanalyzer reagent kits obtained from Abbott Laboratories (Abbott Park, IL). TSH analyte standards in TRIS buffer with protein stabilizer and mouse monoclonal Anti-β TSH coated particles in
ARCHITECT particle diluent (ARCHITECT TSH reagent kit 7K62) were used for TSH immunoassays. E2 analyte standards in TRIS buffer with protein stabilizers and rabbit monoclonal Anti-E2 coated particles in ARCHITECT particle diluent (ARCHITECT E2 reagent kit 7K72) were used for E2 assays. Horse-radish peroxidase (HRP) conjugated goat polyclonal Anti-TSH, was purchased from Fitzgerald Industries (Acton, MA). E2-HRP (conjugated via 6-CMO), was purchased from BiosPacific (Emeryville, CA). SuperSignal ELISA Femto chemiluminescent substrate, comprising stable peroxide (H$_2$O$_2$) and luminol/enhancer solutions, was purchased from Thermo Fisher Scientific (Rockford, IL).

Most reagents and solutions were from kits (as listed above). Prior to use, these reagents were supplemented with Pluronic L64 (0.05% v/v). Three custom reagents (not from kits) were also used, including wash buffer, particle diluent, and conjugate diluent. DMF-compatible wash buffer (pH 7.7) was formed from Tris-base (0.35 g/L), Tris-HCl (1.10 g/L), NaCl (8.367 g/L), and L64 (0.05% v/v). DMF-compatible particle diluent (pH 10) was formed from Tris-base (6.1 g/L), NaCl (5.8 g/L), Sucrose (136 g/L), BSA (1% w/v), Thimerosal (0.05% w/v), and L64 (0.05% v/v). DMF-compatible conjugate diluent (pH 7.4) was formed from Tris-Base (1.9 g/L), Tris-HCl (13.2 g/L), NaCl (17.5 g/L), BSA (1% w/v), cold fish gelatin (0.1% w/v), Thimerosal (0.05% w/v), and L64 (0.05% v/v). TSH and E2 conjugate solutions were formed by dissolving Anti-TSH-HRP (2 µg/mL) and E2-HRP (1 µg/mL), respectively, in conjugate diluent.

### 2.2.3 Microparticle preparation

Prior to introducing onto digital microfluidic devices, magnetic particles were processed off-chip in three steps: (1) washing (for TSH and E2 particles), (2) blocking (for TSH particles only), and (3) suspending in DMF-compatible buffer (for TSH and E2 particles). In step (1), particles in ARCHITECT particle diluent were immobilized in an Eppendorf tube using a permanent magnet, the diluent was removed, and the particles were washed twice with DI water. For step (2), particles were suspended in blocking buffer (pH 7.8), formed from Tris-base (0.30 g/L), Tris-HCl (1.2 g/L), NaCl (8.76 g/L), 0.01% (w/v) Tween-20, and 3% (w/v) Sigma-brand non-fat dry milk (NFDM), incubated for 1 hour, and then washed twice in DI water. For step (3), particles were suspended in DMF-compatible particle diluent (recipe above) at ~3.0×10$^8$ particles/mL.
2.2.4 Device fabrication and operation

Clean room reagents and supplies included MF-321 photoresist developer from Rohm and Haas (Marlborough, MA), CR-4 chromium etchant from Cyantek (Fremont, CA), AZ-300T photoresist stripper from AZ Electronic Materials (Somerville, NJ), Teflon-AF from DuPont (Wilmington, DE), acetic acid from Caledon (Georgetown, Ontario), Parylene C dimer and Silane A174 from Specialty Coating Systems (Indianapolis, IN).

Digital microfluidic devices, each comprising a bottom-plate and top-plate (Figure 2.1B), were fabricated in the University of Toronto Emerging Communications Technology Institute (ECTI) cleanroom facility, using transparent photomasks printed at 20,000 DPI (Pacific Arts and Designs Inc., Markham, ON). The bottom-plates of DMF devices bearing an array of electrodes were formed by standard photolithography and wet etching. Briefly, chromium- (200 nm thick) and photoresist-coated glass substrates (2” × 3” ×1.1 mm) (Telic Co., Santa Clarita, CA) were exposed to UV light through a photomask using a Suss MicroTec mask aligner (29.8 mW/cm², 10 seconds). The exposed substrates were developed in MF-321 (3 min) and post-baked on a hot plate (125°C, 1 min). The developed substrates were etched in CR-4 (3 min) and the remaining photoresist was stripped in AZ300T (5 min). After forming electrodes, the substrates were primed for parylene coating by immersing in silane solution (2-propanol, DI water, A-174, and acetic acid 50:50:1:2 v/v/v/v, 10 min) and curing on a hot-plate (80°C, 10 min). After rinsing and drying, devices were coated with ~7 µm of Parylene C (vapor deposition) and ~200 nm of Teflon-AF (spin-coating, 1% w/w in Fluorinert FC-40, 2000 rpm, 60 s), and post-baked on a hot-plate (165°C, 10 min). The polymer coatings were removed from contact pads by gentle scraping with a scalpel. The top-plates of DMF devices were formed by coating Teflon-AF (~200 nm, as above) on unpatterned indium-tin oxide (ITO) coated glass substrates (Delta Technologies Ltd, Stillwater, MN). Devices were assembled with an unpatterned ITO glass top-plate and a patterned bottom-plate separated by a spacer formed from two pieces of Scotch double-sided tape (3M, St. Paul, MN) with total spacer thickness of 180 µm.

The device design featured an array of 116 actuation electrodes (2.25 × 2.25 mm ea.) connected to 10 reservoir electrodes (4.5 × 4.5 mm ea.) (Figure 2.1C). The actuation electrodes were roughly square with interdigitated borders (140 µm peak to peak sinusoids) and inter-electrode gaps of 30-80 µm. The pitch of the actuation electrodes (2.25 mm) enables the
alignment of electrodes to standard micro-plate readers (96, 384, or 1536 well-plate format). Unit droplet and reservoir droplet volumes on these devices were ~800 nL and ~3.5 µL, respectively.

Figure 2.1: Immunoassays and digital microfluidics
(A) Scheme of non-competitive and competitive immunoassays using magnetic particles. (B) 3D Schematic and side view of a DMF device, which features a moveable magnet underneath the device for particle immobilization. (C) Top view of DMF device, showing the position of the magnet, 10 reservoirs to accommodate reagents, 96 actuation electrodes to carry out the immunoassay protocol, and dedicated regions for assay and detection steps.

To drive droplet movement, AC potentials (~150 V_{RMS}, 10 KHz) were generated by a function generator (Agilent Technologies, Santa Clara, CA) and a high-voltage amplifier (TREK,
A sine wave output was applied between the top-plate (ground) and sequential electrodes on the bottom-plate via the exposed contact pads. Reagents were delivered to their respective reservoirs by simultaneously applying driving potential to a reservoir electrode and pipetting the reagent adjacent to the gap between the bottom and top plates (Figure 2.1C). Waste and unused reservoir fluids were removed with KimWipes (Kimberly-Clark, Irving, TX). Unit droplets were dispensed from reservoirs by actuating a series of adjacent electrodes as described previously. To perform a mixing operation, a unit droplet was shuttled in a circular motion across four electrodes; more electrodes were used for larger droplets. During sample or reagent incubation, droplets were continuously mixed to minimize non-specific adsorption on device surface. Droplet actuation was monitored and recorded by a CCD camera mounted with a lens.

2.2.5 Magnet movement

A magnet manifold was formed by removing selected wells from a stripwell microplate. A 5/8” diameter × 1/4” thick, N48 Grade, 15.3 lb pull force Neodymium magnet (Emovendo Magnets & Elements, Petersburg, WV) was positioned in the manifold; the missing wells form a groove that delineates allowable magnet positions. In experiments, a DMF device was positioned on top of the manifold (Figure 2.1B, C). Magnet position was controlled manually using a metal clip, which when moved, caused the magnet to move to the desired position.

2.2.6 Estimation of magnetic force

The force on a magnetic particle inside a magnetic field is determined by the volume of the particle (V), the magnetic susceptibility of the particle (χ), and the magnetic flux density (B). Assuming that the particle is in a non-magnetic medium with negligible susceptibility (e.g., air), the force on the particle is given by:

\[ F = \frac{V \cdot \chi}{\mu_0} (B \cdot \nabla)B \]

where \( \mu_0 \) is the permeability of free space.

During magnetic separation in the work reported here, a neodymium (NdFeB) magnet disc (5/8” diameter × 1/4” thick, relative permeability \( \mu_r = 1.05 \), remnant field strength \( B_r = 1.38 \)
T) is positioned 1.40 mm below an 800 nL droplet of particle suspension (1.40 mm is the thickness of the microfluidic device). When magnetic force is applied, the particles in the droplet focus into a pellet on the surface of the device, the volume of which is estimated to be the volume of one particle multiplied by the number of particles in the droplet. This configuration can be represented by a geometric model comprising a disc (magnet) and sphere (pellet), where the bottom-edge of the magnet is the x-y-z origin and the pellet is 7.75 mm above the origin (Figure 2.2A). The magnetic flux density and its gradient were numerically calculated using finite element analysis software, COMSOL (Burlington, MA; Model: 3D, Magnetic Fields, No Currents) (Figure 2.2B).

Assuming that the microparticles have magnetic susceptibility $\chi = 1.05$, the magnetic force on the pellet (z-component, directed towards the magnet) was calculated as a function of pellet position (horizontal and vertical) and particle density. In the first calculation, the horizontal position of the pellet was varied from the center of the disc to a distance 15 mm away (Figure 2.2C). Here, the vertical distance of the particles from the magnet was fixed at 1.40 mm, and the particle density was fixed at $3.0 \times 10^8$ particles/mL (the minimum density observed experimentally for successful separation of a pellet from a moving droplet). As expected, the maximum force is experienced for particles positioned at the edge of the magnetic disc, ~7.9 mm from the center. This force, 470 $\mu$N, represents the minimum force required to overcome the interfacial force of the droplet. In the second calculation, the vertical distance of the pellet was varied from the magnet surface to a distance 3 mm away (Figure 2.2D). As expected, the force decreases as the pellet was moved farther away from the magnet. In the final calculation, the particle density of the solution was varied (Figure 2.2E). Since volume is proportional to force, as indicated by Equation 2.1, the force on the pellet increases linearly with higher particle density.

### 2.2.7 Measuring droplet volume change

Droplet volume change was evaluated on-chip using an impedance-based measurement circuit attached to the device top-plate (i.e., the ITO-coated slide) as described previously. Before assembling the digital microfluidic device, a 1 $\mu$L droplet of wash buffer was pipetted on the assay region of the device. Immediately afterward, the device was assembled by as described above, sandwiching the droplet between two plates. The capacitance of the droplet was measured
every minute for 30 minutes and normalized to the initial capacitance (at 0 minutes). Three normalized capacitance measurements were averaged for each point in time and plotted as a function of time (**Figure 2.3**).

**Figure 2.2**: Numerical simulations of magnetic flux density of magnet disc and force on pellet of paramagnetic particles
(A) Geometric model of magnet and pellet. (B) Intensity of magnetic flux density normal (heat map) and direction of field lines (black arrows). (C) Magnitude of z-component force as a function of horizontal distance from magnet center. (D) Magnitude of z-component force as a...
function of vertical distance from magnet surface. (E) Magnitude of z-component force as a function of particle density.

![Graph showing normalized droplet capacitance over time.](image)

**Figure 2.3: Normalized droplet capacitance over time.**
After 30 minutes of incubation inside a device, droplets of wash buffer only reduced volume by ~5% as measured by capacitance, which correlates with droplet volume. Error bars are ±1 S.D from three replicates.

2.2.8 Magnetic particle washing protocol

A wash-test assay was developed to evaluate the washing efficiency of the serial dilution{	extsuperscript{103,203}} and supernatant separation methods. A wash-test suspension was prepared by resuspending Anti-β TSH particles in TSH conjugate solution at ~3.0×10^8 particles/mL. For serial dilution washing, DMF was used to merge and mix a droplet of wash-test suspension with a droplet of wash buffer. Next, the magnet was positioned such that the particles were immobilized to one side of the pooled droplet, the droplet was split into two daughter droplets, and the droplet not containing particles was moved to waste. After the magnet was removed, the droplet containing the particles was mixed to resuspend the particles in solution. For supernatant separation washing, particles in a droplet of wash-test suspension were first immobilized by the magnet and DMF was used to actuate the supernatant droplet away from the magnet to waste, leaving the particles immobilized on the device surface. Next, the magnet was removed, the particles were reconstituted in a droplet of wash buffer, and the droplet was mixed to resuspend the particles in solution. For both techniques, the washing procedure was repeated four times; after each wash step, the supernatant waste droplet was collected by removing the top plate. Using a pipette, the waste droplet was diluted in 50 µL of wash buffer and transferred to a well in a transparent 96-well plate. To this mixture, 100 µL of TMB reagent from a Calbiotech TSH ELISA kit was added to each well.
After incubating for 3 minutes at room temperature, the reaction was stopped by adding 50 µL of Stop Solution from the ELISA kit. Within 15 minutes, the absorbance at 450 nm was measured with a Sunrise microplate reader (Tecan, Durham, NC) in “Normal” read mode. Three replicates were evaluated for each condition.

2.2.9 On-chip immunoassay protocol

Using the supernatant separation method (described above) for particle separation and resuspension, an eight-step protocol was developed to implement on-chip immunoassays. (1) A droplet containing paramagnetic particles was dispensed from a reservoir and separated from the diluent. (2) Three droplets containing sample were dispensed and delivered to the immobilized particles for resuspension and incubation for 6 minutes. (3) The particles were washed four times in wash buffer and separated from the supernatant. (4) One droplet of conjugate solution was dispensed and delivered to the immobilized particles for resuspension and incubation for 2 minutes. (5) The particles were washed four times in wash buffer, two times in H₂O₂, and separated from the supernatant. (6) One droplet of H₂O₂ was dispensed and delivered to the immobilized particles for resuspension and incubation for 2 minutes. (7) The droplet of H₂O₂/particle suspension was merged and mixed with one droplet of luminol/enhancer solution. (8) The pooled droplet was incubated for 1 minute and the chemiluminescence was detected with a well plate reader (Pherastar, BMG Labtech, Cary, NC). To prepare for measurement, the device was affixed with tape to an empty 96 well Stripwell plate so that each droplet was aligned to a well. The device/plate assembly was inserted into the plate reader and evaluated with focal height = 8.6 mm, gain = 3600, using the LUM module.

As shown in Figure 2.1C, each DMF device had four separate assay regions (for four individual assays) and a shared detection region. The assay regions were used for steps 1-5 of the protocol. After the final rinse, the particles were delivered to the detection region for steps 6-8 of the protocol. Between measurements, the detection region was rinsed by moving droplets of H₂O₂ over the electrodes. The device was disposed after four assays.

2.2.10 DMF immunoassays

Using the protocol above, a calibration curve was generated on-chip from standard solutions of TSH (0, 0.4, 1, 4, and 20 µIU/mL) or E2 (0, 10, 50, 500, and 1000 pg/mL). Four measurements
at each concentration were averaged and fitted to a linear equation (TSH) or a four-parameter logistic equation\textsuperscript{223} (E2). The limit of detection (LOD) was the concentration corresponding to the position on the curve of the average signal generated from blank measurements plus (for TSH) or minus (for E2) three times the standard deviation of the blank measurements.

2.2.11 Well-plate experiments

Calbiotech ELISA kits (with antibodies immobilized on the surface of transparent 96-well plates) for TSH and E2 were used following manufacturer’s instructions. Briefly, the absorbances at 450 nm were measured using a Sunrise microplate reader in “Normal” read mode, and calibration curves were generated and LODs determined as above.

2.3 Results and Discussion

2.3.1 Droplet movement and biofouling

In digital microfluidics, droplet movement is impeded by drag forces arising from differential surface energy. Many of the reagents used for immunoassays, including protein solutions and protein-coated particles, are susceptible to non-specific adsorption (or “fouling”), which locally increases the device surface energy, impeding the movement of aqueous droplets.\textsuperscript{188}

To alleviate problems of protein fouling, most researchers use either a) silicone oil filler fluid\textsuperscript{168} or b) Pluronic additives in reagent droplets.\textsuperscript{187} Although silicone oil filler fluid is useful for many applications, it is not a universal solution and has some disadvantages (as described in the introduction). The latter technique, employing Pluronic additives, obviates the need for oil. In this work, Pluronic L64\textsuperscript{199} was included at 0.05% (v/v) in all reagent and samples solutions to facilitate robust droplet operations without oil (this addition was found not to interfere with specific binding in well-plate or DMF immunoassays). After the addition of L64, most assay reagents were compatible with DMF actuation without further dilution or modification. The exceptions were the ARCHITECT particle diluent, conjugate diluent, and wash buffer, which did not move well even after the addition of L64. We speculate that there are some cross-effects between the (added) L64 and the (native) surfactants in these solutions. This problem was solved by developing custom DMF-compatible particle diluent, conjugate diluent, and wash buffer containing L64 as the only surfactant. All reagent formulations are detailed in the experimental section.
2.3.2 Development of on-chip washing and solvent exchange protocol

A key requirement in heterogeneous immunoassays is the ability to remove unbound reagents (e.g., antibodies or antigens) from the surface of the solid support. All of the magnetic particle-based immunoassays implemented in DMF reported previously\textsuperscript{103,200,203} have used a "serial dilution" method to wash unbound reagents from particles as depicted in Figure 2.4A. First, the particles are immobilized by an external magnet. Second, the droplet containing the particle suspension is diluted by merging with a droplet of wash buffer. Third, DMF is used to split the pooled droplet and remove the excess solution from the particles. This process is repeated until sufficient dilution is achieved to minimize the background noise of the assay. This washing technique is inherently inefficient because a) unbound reagents are slowly diluted over many washes, and b) particles are immobilized by the magnet throughout the wash process, which can trap unbound material between particles.\textsuperscript{203}

We hypothesized that we could improve upon the performance of the serial dilution method by using an alternative approach that allows for the near-complete separation of the supernatant solution phase from the particle solid phase in one step (similar methods were recently reported for different applications in digital microfluidics\textsuperscript{224} and other droplet/microfluidic formats\textsuperscript{225-226}). We proposed that this "supernatant separation" method (Figure 2.4B) would be capable of much more efficient isolation of particles from unbound reagents. In this method, a magnet causes particles to focus into a pellet and immobilize on the device surface, such that the pellet remains behind when the supernatant droplet is actuated away. After separation from the supernatant, the immobilized particles can be resuspended by removing the external magnet, passing a fresh droplet over the pellet, and shuttling the droplet in a circular motion across four electrodes.
Figure 2.4: Comparison of magnetic particle washing protocols
(A) Scheme (top) and video sequence (bottom) of serial dilution washing protocol. (B) Scheme (top) and video sequence (bottom) of supernatant separation washing protocol. (C) Comparison of the efficiency of serial dilution (red diamonds) and supernatant separation (blue circles) methods by measuring enzyme activity in the supernatant after each wash step. Error bars are ±1 S.D. from three replicates.

We developed a wash-test assay to evaluate the efficiency of both the supernatant separation and serial dilution methods. In each assay, a droplet containing anti-β TSH particles in anti-TSH-HRP solution was washed by one of the two methods, and the resulting waste droplet
was interrogated for peroxidase activity. This process (wash and measure) was repeated four times. To ensure an unbiased comparison, identical wash buffer volumes, particle suspension volumes, and particle densities were used for both methods (within the range of standard error). To equalize the conditions further, a modified version of the serial dilution method was used (removing the magnet during mixing and particle resuspension), which differs from the previously reported serial dilution methods\textsuperscript{103,200,203} in which particles are immobilized by the magnet throughout the wash process. Finally, in both methods evaluated here, the initial solution and wash solution had the same volume (i.e., 1:1 wash:initial); in previous work,\textsuperscript{203} larger ratios (e.g., 5:1) were found to increase the washing efficiency.

As shown in Figure 2.4C, the supernatant separation method is much more efficient than the serial dilution method. If we define 98% reduction in HRP activity relative to the activity in the initial droplet as an arbitrary benchmark for minimizing background signal of the assay, the supernatant separation method achieves this target within 2 washes, while the serial solution requires at least 6 washes. In fact, this analysis may understate the advantage of the supernatant separation method, as our modified version of the serial dilution method is apparently more efficient than the previously reported methods, in which 10-18 washes\textsuperscript{203} were required for a 1:1 wash, and 5 washes\textsuperscript{203} were required for a 5:1 wash. This is likely because our modified serial dilution method (in which the magnet is removed during mixing and particle resuspension) is less susceptible to the trapping of unbound enzymes between particles.

There are three requirements for successful implementation of the supernatant separation method: 1) sufficient magnetic force, 2) adjustable magnetic force, and 3) stable particle suspension. For the first requirement, the magnetic force immobilizing the particles must be stronger than the interfacial force pulling the particles into the droplet. For a single micron-dimension paramagnetic particle in the presence of a magnet, the interfacial force is 2-3 orders of magnitude higher than a typical magnetic force that might be applied in such a system.\textsuperscript{204} However, because the interfacial force is proportional to the particle radius and the magnetic force is proportional to the cube of the particle radius, the magnetic force is dominant for particles larger than a critical size. A pellet comprising many microparticles can satisfy this requirement, and in the system used here, pelleting and separation was observed to be possible for droplets containing at least $\sim 3.0 \times 10^8$ particles/mL. From numerical simulations, we estimate that this configuration corresponds to a magnetic force of $\sim 470 \, \mu N$ (Figure 2.2C). For the
second requirement, after the particles are separated from the origin droplet, the magnetic field strength should be adjustable to permit particle resuspension in a new droplet. In the system described here, this was accomplished by moving the magnet (horizontally) away from the device. For the final requirement, the particles should not dry or irreversibly aggregate during the time spent as a pellet on the surface. In the system reported here, when the origin droplet is removed, we observe that a small amount of liquid (approximated by imaging to be ~5% of the origin droplet volume) is retained by the hydrophilic particles. To minimize the potential for particle aggregation or drying, the particles are then resuspended immediately in a new droplet. Other factors that may determine particle stability include diluent composition and surface chemistry of particles. Finally, we propose that some combination of these three requirements makes oil-immersed digital microfluidic devices incompatible with the supernatant separation method, but more work is required to evaluate this hypothesis.

In the final analysis, the choice between the serial dilution method and the supernatant separation method likely depends on the application. The former method is less efficient, but can be implemented with a static magnetic field, making it attractive for instruments with low complexity and small footprint. The latter method is more efficient, but requires an adjustable magnetic field, making it better suited to larger, more complex instruments. In the work reported here, the supernatant separation method was used, and we propose that it will be useful tool for a variety of circumstances in which a movable permanent magnet (or an electromagnet with adjustable forces\textsuperscript{227}) is available.

### 2.3.3 Immunoassay protocol development

To demonstrate that DMF immunoassays can be adapted for a wide range of analytes, we developed a non-competitive immunoassay (the type of assay used in all previous DMF methods) and a competitive immunoassay (new for DMF) using thyroid-stimulating hormone (TSH) and 17β-estradiol (E2) as model analytes, respectively. The TSH immunoassay is used to evaluate thyroid function and diagnose thyroid disease, and thus is one of the most commonly performed immunoassays in clinical laboratories. The normal serum range of TSH is 0.4 to 3.0 µIU/mL, although this is frequently revised\textsuperscript{228-229}. Estradiol (E2), a small molecule steroid (272.38 Da), is the most potent natural estrogen in mammals. E2 serum levels for males,
premenopausal females, and postmenopausal females are 10 to 50 pg/mL, 30 to 400 pg/mL, and less than 30 pg/mL, respectively (reported ranges vary between different laboratories\textsuperscript{230}).

A number of competing requirements were evaluated in developing the methods reported here. In a traditional immunoassay, reagent volume, incubation duration, and reagent composition should be optimized to achieve the greatest signal to noise ratio while minimizing assay time and reagent use. But for the methods reported here, these parameters were also observed to affect the reliability of droplet manipulations on DMF; unreliable droplet movement should be avoided, as it leads to increased assay variability.\textsuperscript{222} Balancing these requirements led us to select a sample volume of three unit droplets (800 nL × 3 = 2.4 µL)—this provided significant signal improvement over one sample droplet, yet the volume was still small enough to be easily manipulated by DMF (volumes much larger than a unit droplet are more difficult to control reliably). Likewise, for each of the other reagents (wash buffer, particle suspensions, conjugates, and substrates), we used one unit droplet (800 nL) to minimize reagent consumption and to ensure reliability of merged-droplet manipulation. In preliminary experiments, a sample incubation of 6 minutes was sufficient to saturate the analyte on particle surfaces (data not shown). For this incubation time, sample droplets did not experience significant volume decrease due to evaporation. In fact, we observed that at least 30 minutes are required for a 1 µL wash buffer droplet to lose 5\% of its original volume (Figure 2.3). For chemiluminescent detection, the reagent manufacturer recommends mixing equal parts H\textsubscript{2}O\textsubscript{2} solution and luminol/enhancer solution prior to the assay; however, we found that the signal generated following this procedure exhibited high interassay variability (data not shown). Therefore, we developed an alternative procedure comprising two H\textsubscript{2}O\textsubscript{2} wash steps to remove leftover wash buffer solution and an on-chip mix step of the H\textsubscript{2}O\textsubscript{2} and luminol/enhancer solutions immediately prior to use. For the conditions evaluated, the chemiluminescence intensity was observed to saturate after 1 minute—this was chosen as the measurement endpoint.

Among all of the parameters, we found that the reagent composition is the most critical in determining the performance of the assay—we highlight two examples here: 1) In E2 immunoassays, the concentration of conjugated antigen (E2-HRP) is essential in determining the dynamic range and sensitivity of the assay. In particular, in initial studies using high E2-HRP concentrations, it was impossible to distinguish between different E2 sample concentrations (e.g., 50 and 500 pg/mL have similar signal). The performance of the assay greatly improved
when we determined the optimal (lower) concentration of 1 µg/mL. 2) TSH immunoassays are very sensitive to the non-specific adsorption of conjugated antibodies on the surface of magnetic particles. In initial TSH immunoassay experiments, the high background noise prevented consistent differentiation between 0 and 0.4 µIU/mL analyte. Initially, we tried adding protein, serum, or surfactant components to reduce non-specific adsorption, but several of these options compromised droplet movement reliability. To alleviate this problem, we developed a technique to block the TSH microparticles using non-fat dried milk\textsuperscript{231} prior to the assay, which significantly reduced the background noise without affecting droplet actuation.

2.3.4 Optimized immunoassay

Figure 2.5A illustrates the optimized immunoassay procedure for both (competitive) E2 and (non-competitive) TSH immunoassays by DMF. As detailed in the Experimental section, both assays used identical droplet actuation sequences and washing protocols, but with different microparticles and conjugate solutions. The highlights of this procedure are represented in six frames from a movie, shown in Figure 2.5B. First, magnetic particles were separated from solution. Second, the particles were resuspended in three droplets of sample and incubated for 6 minutes. Third, after 4 wash steps, the particles were resuspended in one droplet of conjugate (containing HRP labeled antibody or analyte) and incubated for 2 min. Fourth, after 4 wash steps, the particles were reconstituted in one droplet of H\textsubscript{2}O\textsubscript{2} and incubated for 2 min. Fifth, one droplet of luminol/enhancer solution was merged with the H\textsubscript{2}O\textsubscript{2} to activate enzymatically driven chemiluminescence. Finally, sixth, chemiluminescence was measured in a well plate reader with detection zone aligned to 96-well plate format.
Figure 2.5: Digital microfluidic immunoassay procedure
(A) Scheme of droplet-based on-chip immunoassay. The E2 and TSH immunoassays use the same droplet actuation sequences and supernatant separation technique for particle washing. (B) Video sequence of on-chip immunoassay procedure, showing two assays in parallel. 1) Magnetic particles are separated; 2) Sample reconstituted the particles and incubated; 3) After wash steps, droplet of conjugate reconstituted the particles and incubated; 4) After wash steps, droplet of H$_2$O$_2$ reconstituted the particles and incubated; 5) droplet of luminol/enhancer solution merged to activate enzymatic reaction; 6) Chemiluminescence is measured.

We carried out this protocol for various concentrations of standards to generate calibration curves for E2 and TSH as shown in Figure 2.6. Each curve has a dynamic range of approximately 2 orders of magnitude, and the error bars represent the standard deviation between four measurements performed on-chip. The E2 and TSH data have coefficients of variation (CVs) ranging from 9 to 21% and 5 to 14%, respectively. As listed in Table 2.1, these CVs are similar (but slightly elevated) relative to those observed for comparable assays carried out in multiwell plate format. We note that the DMF assays reported here were implemented semi-manually by applying voltage sequences to the device using hand-held voltage probes, and we attribute some of the variability observed to differences in droplet movement sequences and timing. We propose that this variability will be reduced upon implementation of automated fluid handling with feedback control (as has been observed previously$^{22,23}$).
Figure 2.6: Calibration curves for immunoassays implemented using digital microfluidics (A) 17β-estradiol (E2) competitive immunoassay. (B) Thyroid-stimulating hormone (TSH) non-competitive immunoassay. Error bars are ±1 S.D from four replicates.

The most important characteristic of immunoassays is the limit of detection (LOD). As listed in Table 2.1, the DMF assays had ~2-3x lower LODs relative to well-plate assays when calculated in terms of absolute amount, but were ~3-10x higher when calculated in terms of concentration. The LODs of the DMF system are suitable for a wide range of applications, including the prognosis and diagnosis of hypothyroidism\textsuperscript{229} (TSH > 3.0 µIU/mL) or ovarian cancer\textsuperscript{233-234} (E2 > 20pg/mL, depending on patient age). But for some applications, it may be useful to develop modified assays with lower LODs; this might be possible by increasing the amount of sample (e.g., analyte in multiple droplets of sample may be concentrated on the particles through several solvent exchanges until sufficient sensitivity is achieved). In addition, the use of a dedicated detector may help improve the assay sensitivity and CV—the calibration curves used here were obtained using a multi-purpose (fluorescent, luminescent, absorbance) well plate reader that is used regularly by a number of different users. Note that LODs and CVs were not reported for the DMF microparticle-based immunoassays reported previously.\textsuperscript{103,200,203}

There are two salient advantages of DMF for immunoassays: reduced consumption of reagent volumes and faster analysis time. As context for the former advantage (reagent consumption), a typical well-plate ELISA assay requires 50 µL sample, 100 µL conjugate, 900 µL wash buffer, and 150 µL substrate. In contrast, DMF immunoassay requires 2.4 µL sample, 0.80 µL conjugate, 6.4 µL wash buffer, and 1.6 µL substrate. This represents a 100-fold reduction in reagent consumption in the DMF method, from 1.2 mL to 11.2 µL. For the latter...
advantage (analysis time), sample incubation in well-plate ELISA kits requires 60 minutes, and particle-based immunoassay in ARCHITECT immunoanalyzers requires 18 minutes. In contrast, the DMF immunoassays only require 6 minutes of sample incubation time. This advantage is directly related to the reduction of reagent volumes—the same number of particles is packed into a smaller volume, increasing surface area-to-volume ratio and reaction kinetics.

<table>
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<th>Immunoassay System</th>
<th>Well-Plate ELISA Kit</th>
<th>DMF</th>
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</thead>
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<td>Sample Incubation Time</td>
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2.4 Conclusion

We have developed the first particle-based immunoassay on DMF without the aid of oil carrier fluid. This new format allowed the realization of a novel on-chip magnetic particle separation method capable of removing greater than 90% of unbound reagents in one step. Using this technique, we successfully developed the first DMF-driven competitive immunoassay (for 17β-estradiol), as well as a non-competitive immunoassay (for thyroid stimulating hormone). Compared to conventional techniques, this method reduced reagent volumes and analysis time by 100-fold and 10-fold, respectively, while retaining a level of analytical performance required for clinical screening. We propose that the new technique has great potential for eventual use in a fast, low-waste, and inexpensive instrument for the quantitative analysis of proteins and small molecules in low sample volumes.
Chapter 3

3 Automated Digital Microfluidic Platform for Magnetic Particle-based Immunoassays with Optimization by Design of Experiments

We introduce an automated digital microfluidic (DMF) platform capable of performing immunoassays from sample-to-analysis with minimal manual intervention. This platform features (a) a 90 Pogo pin interface for digital microfluidic control, (b) an integrated (and motorized) photomultiplier tube for chemiluminescent detection, and (c) a magnetic lens assembly which focuses magnetic fields into a narrow region on the surface of the DMF device, facilitating up to 8 simultaneous digital microfluidic magnetic separations. The new platform was used to implement a three-level full factorial design of experiments (DOE) optimization for thyroid stimulating hormone (TSH) immunoassays, varying (1) analyte concentration, (2) sample incubation time, and (3) sample volume, resulting in an optimized protocol that reduced detection limit and sample incubation time by up to 5-fold and 2-fold, respectively, relative to chapter 2. To our knowledge, this is the first report of a DOE optimization for immunoassays in a microfluidic system of any format. We propose that this new platform paves the way for a benchtop tool that is useful for implementing immunoassays in near-patient settings around the world, including community hospitals, physicians’ offices, and small clinical laboratories.

3.1 Introduction

The *in-vitro* diagnostic industry is dominated by robotic immunoanalyzers—the gold standard for high-throughput protein and small molecule quantitation. These instruments are capable of quantifying disease biomarkers from patient samples at clinically relevant concentrations at a rate of hundreds of tests per hour. Importantly, the throughput of these instruments allows developers to rapidly determine optimal assay parameters via a Design of Experiments (DOE) approach, leading to reduced assay development and optimization timelines. Unfortunately, robotic immunoanalyzers are large, complex instruments found only in well-funded centralized facilities such as hospital reference laboratories, to which patient samples are transported after collection. As health care costs continue to rise, the global *in-vitro* diagnostic
market is gradually shifting from centralized facilities to point of care testing (POCT). This market trend is facilitated by technological advances in nanomaterials, integrated sensors, and microfluidics. In particular, microfluidics is proving useful for the miniaturization of liquid handling, leading to the development of various microfluidic immunoassay systems and the commercialization of these platforms. To date, however, there have been no reports of DOE optimization of microfluidic immunoassays, likely because of a lack of automation, parallelization, and control.

While most miniaturized immunoassay systems rely on enclosed microchannels, several groups are exploring the use of an alternative open surface format, known as digital microfluidics (DMF). In DMF, fluids are electrostatically manipulated in discrete droplets (picoliter to microliter sized) on an open array of electrodes coated with a hydrophobic insulator. When appropriate sequences of potentials are applied to these electrodes, droplets can be driven to mix, merge, split, and metered from reservoirs. Droplet movement sequences can be reprogrammed to accommodate different experimental conditions (e.g. incubation time and volume) without the need for a new device design. The most established approach to DMF immunoassays uses magnetic particles for antibody solid support and relies on oil carrier fluid to enable aqueous droplet movement. Using this method, immunoassays for insulin, interleukin-6, troponin I, immunoglobulin E, and hypercoagulability panel have been implemented in benchtop digital microfluidic instruments with integrated fluorescent detection. These demonstrations, though few, represent important milestones towards DMF-powered near-patient analysis, or even (eventually) POCT.

A new approach to DMF immunoassays eliminates the need for oil carrier fluid and improves washing efficiency of magnetic particles using a technique called digital microfluidic magnetic separation. In chapter 2, we demonstrated the utility of this approach for quantifying 17β-estradiol and thyroid stimulating hormone (TSH) at clinically relevant levels, with 100-fold and 10-fold reductions in reagent volumes and analysis times relative to conventional systems. But this initial demonstration was carried out using a prototype setup, requiring significant manual involvement for assay implementation. Specifically, droplet sequences were delivered using handheld voltage probes, magnet position was manually adjusted for particle separation, and measurement of chemiluminescent signal was performed off-line in a commercial well-plate reader.
Here, we report a significant improvement over the method described in chapter 2, centered on the development and characterization of an automated platform capable of performing complete immunoassays with minimal manual intervention. This platform features a 90 Pogo pin interface for digital microfluidic control, an integrated (and motorized) photomultiplier tube (PMT) for chemiluminescent detection, and a new magnet assembly for particle separation. The latter, called a “magnetic lens,” focuses magnetic fields into a narrow region on the surface of the DMF device, facilitating up to 8 simultaneous digital microfluidic magnetic separations. This new platform was used to implement a three-level full factorial DOE optimization of immunoassays, varying (1) analyte concentration, (2) sample incubation time, and (3) sample volume. The resulting optimized protocol reduced detection limit and sample incubation time by up to 5-fold and 2-fold, respectively, relative to chapter 2. More importantly, these results establish digital microfluidics as a useful tool for implementing DOE optimization; to our knowledge, this is the first report of DOE optimization for immunoassays in any type of microfluidic system. We propose that the new platform has great potential for the quantitative analysis of disease biomarkers in various near-patient settings, including community hospitals, physicians’ offices, and small clinical laboratories.

3.2 Experimental

3.2.1 Reagents and materials

Unless otherwise specified, reagents were purchased from Sigma Chemical (Oakville, ON). Deionized (DI) water had a resistivity of 18 MΩ•cm at 25°C. Pluronic L64 (BASF Corp., Germany) was generously donated by Brenntag Canada (Toronto, ON).

3.2.2 On-chip immunoassay reagents

Reagent solutions used on-chip were either obtained from vendors or were custom-made in-house. Reagents from vendors include TSH standards and antibody coated paramagnetic microparticles from Abbott Laboratories (Abbott Park, IL), Horse-radish peroxidase (HRP) conjugated mouse monoclonal Anti-TSH from Abcam Inc. (Cambridge, MA), and SuperSignal ELISA Femto chemiluminescent substrate, comprising stable peroxide (H₂O₂) and Luminol-Enhancer solution, from Thermo Fischer Scientific (Rockford, IL). Custom DMF-compatible wash buffer and conjugate diluent were prepared as described in chapter 2. DMF-compatible
particle diluent (pH 10) was formed from Tris-base (6.1 g/L), NaCl (5.8 g/L), BSA (1% w/v), Thimerosal (0.05% w/v), and L64 (0.05% v/v). Prior to use, the TSH standards and chemiluminescent substrate were supplemented with Pluronic L64 at 0.05% and 0.025% v/v, respectively. TSH conjugate working solutions were formed by diluting HRP conjugated Anti-TSH (2 µg/mL) in conjugate diluent. The microparticle working solution was formed by resuspending microparticles in particle diluent at ~3.0×10⁸ particles/mL.

3.2.3 Device fabrication and operation

Digital microfluidic devices, each comprising a bottom plate and top plate, were fabricated in the University of Toronto Nanofabrication Centre (TNFC) cleanroom facility and were assembled as described in chapter 2. The bottom-plate device design featured an array of 80 actuation electrodes (2.2 × 2.2 mm ea.) connected to 8 reservoir electrodes (16.4 × 6.7 mm ea.) and 4 waste reservoir electrodes (16.4 × 6.4 mm ea.). The actuation electrodes were roughly square with interdigitated borders (140 µm peak to peak sinusoids) and inter-electrode gaps of 30-80 µm. Unit droplet and reservoir droplet volumes on these devices were ~800 nL and ~3.5 µL, respectively.

An automated platform (Figure 3.1) was designed and built to manage droplet operation, magnet and photomultiplier tube (PMT) position, and data collection. Droplet movement was controlled via the open-source Microdrop software and an Arduino-based (Smart Projects, Italy) high-voltage switching instrument described in detail elsewhere. A custom plugin for the Microdrop software was used to control the motors in the platform, read signals from their respective optical limit switches, and trigger PMT reading. Signals from the PMT were processed using a CT2 counter timer module (ET Enterprises, Sweetwater, TX) connected to a computer via RS232 interface and recorded (at a period of 1 second) using the associated EM6 counter timer software. An integrated LED and webcam assembly was used to monitor on-chip droplet movement prior to analysis. All hardware components fit in a benchtop form factor enclosure (approximate dimensions: 18 × 23 × 30 cm).

To load a DMF device onto the platform, the hinge-based enclosure is opened, and a DMF device is positioned on the device holder, connecting the top-plate with the grounded brass leaflets (Figure 3.2). When the device is secured in the holder, the enclosure is closed, and the Pogo pins are lowered onto the device (via motor and two optical limit switches) to form secure
electrical connection with the exposed contact pads in the bottom-plate. The electrical interface comprises a pair of 15 by 3 Pogo pin arrays with center-to-center spacing of 100 mils, capable of delivering 90 individual voltage inputs (Figure 3.1A). To drive droplet movement, a sine wave AC potential (100-120 V_{RMS}, 10 KHz) was applied between the top plate (ground) and sequential electrodes on the bottom-plate. Reagent reservoirs were filled by pipetting the reagent adjacent to the gap between the bottom and top plates and applying driving potential to a reservoir electrode. Waste and unused reservoir fluids were removed with KimWipes (Kimberly-Clark, Irving, TX). Unit droplets were dispensed from reservoirs, moved, merged, and mixed as described previously. To perform a mixing operation, a unit droplet was shuttled in a circular motion across four electrodes; more electrodes were used for larger droplets.

Figure 3.1: Integrated platform for digital microfluidic particle-based immunoassays
(A) Photo of automation setup (opened orientation) with labels showing the DMF device and Pogo pin interface. (B) Cross-sectional Computer aided design (CAD) rendering of integrated PMT in default (left) or measure (right) states. (C) Cross-sectional CAD rendering of motor controlled magnet in default (left) or separation (right) states. The automation setup is in closed orientation in B and C.

3.2.4 Measurements with integrated PMT

The automated control system includes an integrated Hamamatsu H10682-110 PMT with + 5 V DC source. The bias voltage and discrimination level for the PMT were preset by the manufacturer to optimal values. Using a motor and two optical limit switches, the vertical position of the PMT can be adjusted to either the disengaged state (default) or the engaged state.
In the disengaged state, the PMT is positioned ~5 cm above the DMF device and the sensor orifice is protected from ambient light by a shutter. In the engaged state, the shutter is opened and the PMT is positioned several hundred micrometers above the DMF device, allowing the collection of light emitted from the surface of the device.

**Figure 3.1B**

**Figure 3.2: DMF device holder**

*Top:* Computer aided design (CAD) rendering of DMF device on device holder with top-plate connected to grounded brass leaflet and magnet is engaged. **Bottom:** Schematic showing magnet position relative to DMF device and the approximate separation zone (in orange) created by the magnetic lens.

### 3.2.5 Detector characterization

The performance of the PMT in the automated system was evaluated against that of a well-plate reader using an on-chip homogeneous HRP assay with SuperSignal chemiluminescent substrate. Using digital microfluidics, one droplet each of HRP standard solution (0, 26, 52, 130, or 260 µU/mL in DPBS supplemented with 0.05% v/v L64) and chemiluminescent substrate (equal parts of H₂O₂ and Luminol-Enhancer) were dispensed from their respective reservoirs and merged together. The pooled droplet was mixed for 40 seconds, driven to the detection area, and the chemiluminescence was measured after 2 minutes using (a) the integrated PMT with the settings described in the experimental section, or (b) a commercially available well plate reader (Pherastar, BMG Labtech, Cary, NC), with the settings described in chapter 2. Three measurements were collected for each HRP concentration, and were averaged and fitted to a
linear equation. The limit of detection (LOD) for this assay was the concentration corresponding to the position on the curve of the average signal generated from blank measurements plus three times the standard deviation of the blank measurements.

### 3.2.6 Magnetic lens and modeling

The automated control system includes a motor driven magnet assembly positioned beneath the center of the device (Figure 3.1C and Figure 3.2). The magnet assembly, called a “magnetic lens,” comprises a neodymium magnet bar (3" × 1/2" × 1/4" thick, relative permeability $\mu_r = 1.05$, remnant field strength $B_r = 1.32$ T) flanked by two steel (AISI 1080) machined armatures (Figure 3.3A). Using a motor and two optical limit switches, the vertical position of the magnet can be adjusted to either the disengaged state (default) or the engaged state (for particle separation). In the disengaged state, the magnet is positioned ~3.8 cm below the device. In the engaged state, the magnet is positioned ~150 μm below the device.

Magnetic forces exerted on particles by the new magnetic lens (described above), and the magnet system used in chapter 2 (i.e., a 5/8" diameter × 1/4" thick neodymium disc with relative permeability $\mu_r = 1.05$ and remnant field strength $B_r = 1.38$ T) were modeled using finite element analysis software (COMSOL, Burlington, MA; 3D Model with Magnetic Fields and No Currents). Magnetic particles are assumed to have diameters of 5 μm, magnetic susceptibility $\chi = 1.05$, and a pellet of particles is represented geometrically as a sphere with volume equal to the volume of one particle multiplied by the number of particles. For example, for a particle density of $3.0 \times 10^8$ particles/mL and a droplet volume of 800 nL, the pellet has a radius of 155 μm. The distance between the particle pellet and the magnet was assumed to be 1.4 mm, which takes into account the thickness of the bottom plate (~1.15 mm) and the tolerance of setup. The magnetic force on the pellet (both y-component and x-component) was calculated as a function of horizontal and vertical pellet position.
Figure 3.3: Comparison of forces for the magnetic lens (new design) and the magnet disc (previous design).

(A) Schematic of magnetic lens and numerical modeling of magnetic flux density normal (heat map with red = high field, blue = low field) and field lines. (B) Schematic of magnet disc and numerical modeling of magnetic flux density normal (heat map) and field lines. Force on particle
pellet (directed vertically towards magnet) as a function of (C) horizontal position from magnet center and (D) vertical position from magnet surface.

3.2.7 Pilot immunoassays and DOE analysis

Using DMF magnetic separation for reagent exchange and particle washing as described in chapter 2, pilot on-chip immunoassays for DOE analysis were implemented in seven steps: (1) A droplet containing paramagnetic particles was dispensed from a reservoir and separated from the diluent. (2) 1, 3 or 5 droplets (representing 0.8, 2.4, or 4 µL after merging) of TSH standards (0, 0.4, or 4 µIU/mL) were dispensed, delivered to the immobilized particles, and mixed for 3, 6, or 9 minutes. (3) The particles were washed four times in wash buffer and separated from the supernatant. (4) One droplet of HRP conjugate solution was dispensed, delivered to the immobilized particles, and mixed for 2 minutes. (5) The particles were washed five times in wash buffer; after the fifth wash, the particles were kept suspended in wash buffer and queued for analysis. (6) The particles were separated from the wash buffer, resuspended in one droplet of H₂O₂, and this droplet was merged and mixed (for 40 seconds) with one droplet of luminol-enhancer solution. (7) The pooled droplet was incubated for 100 seconds, and the chemiluminescent signal was recorded using the integrated PMT. In these assays, two experiments were run simultaneously and four replicates were evaluated for each condition. The ratio of the chemiluminescent signal from each sample relative to the average signal generated from the blank (0 µIU/mL TSH) was collected for each sample volume and sample incubation time. These measured signal-to-background ratio (SBR) responses were fitted via centered polynomial regression using a modified second-order model:

$$
\hat{Y} = I + \beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \beta_3 \cdot X_3 + \beta_{12} \cdot X_1 \cdot X_2 + \beta_{13} \cdot X_1 \cdot X_3 + \beta_{23} \cdot X_2 \cdot X_3 \\
+ \beta_{11} \cdot X_1^2 + \beta_{22} \cdot X_2^2 + \beta_{33} \cdot X_3^2 + \beta_{122} \cdot X_1 \cdot X_2^2 + \beta_{133} \cdot X_1 \cdot X_3^2 + \beta_{211} \cdot X_2 \cdot X_1^2 \\
+ \beta_{233} \cdot X_2 \cdot X_3^2 + \beta_{311} \cdot X_3 \cdot X_1^2 + \beta_{322} \cdot X_3 \cdot X_2^2
$$

Eq. 3.1

where \( \hat{Y} \) is the predicted response, \( I \) is the constant term, \( \beta_i \) is the coefficient (effect) of factor \( X_i \), \( \beta_{ij} \) is the coefficient of the 2-factor interaction \( X_i \cdot X_j \), \( \beta_{ij} \) is the coefficient of the quadratic factor \( X_i^2 \), and \( \beta_{ij} \) is the coefficient of the linear \( \times \) quadratic interaction factor \( X_i \cdot X_j^2 \). Statistical analysis, model construction, and data interpretation were performed using the JMP 10 statistical analysis package (S.A.S. Institute Inc., Cary, NC).
3.2.8 Optimized immunoassays

The optimized immunoassay method was similar to that of the pilot protocol, with three changes. First, in step #1, optimized assays used 5 droplets of TSH standards (0, 0.4, or 4 µIU/mL) and the particles were mixed for 3 minutes. Second, in steps #5-6, the particles were washed four times in wash buffer, separated from the supernatant, and chemiluminescent reaction was initiated immediately. Finally, in step #7, chemiluminescent substrate and particles were mixed for 4 minutes and the signal was collected after 10 seconds using the integrated PMT. Four measurements at each concentration were averaged and fitted to a linear equation. The limit of detection (LOD) for this assay was the concentration corresponding to the position on the curve of the average signal generated from blank measurements plus three times the standard deviation of the blank measurements.

3.3 Results and Discussion

3.3.1 Automated immunoassay platform

We designed and built an integrated platform capable of performing immunoassays with minimal manual intervention (Figure 3.1A). The platform comprises three core components: a Pogo pin interface for digital microfluidic control, an integrated photomultiplier tube (PMT) for chemiluminescent detection, and an adjustable magnet for particle separation. These components fit in a light-tight, shoebox-sized enclosure; in the absence of a chemiluminescent reaction, the photon count rate inside the enclosure was less than 50 counts per second, within the specifications of a typical dark count rate for this PMT.

The use of Pogo pins as an electronic interface to control droplet movement provided several improvement over previous approaches. First, the new interface allowed us to distribute 90 individual high voltage inputs on a 3 by 2 inch DMF device (i.e., 15 inputs per square inch), allowing for much higher density than for configurations reported previously (4-7 inputs per square inch). Second, compared to slot connectors, device loading using Pogo pins is more straightforward—after placing a device in the device holder, the Pogo pins are lowered (via motor) to automatically form secure electrical contacts. Third, the Pogo pin interface is compatible with a wide range of substrate material and thickness. Although the
current project uses bottom-plate substrates with \( \sim 1.15 \) mm thickness, this same interface is compatible with thinner or thicker substrates (data not shown).

We evaluated the performance of the integrated PMT by employing digital microfluidic homogeneous enzyme assays\(^{252}\) using horse radish peroxidase (HRP) and luminol-\( \text{H}_2\text{O}_2\)-based chemiluminescent substrate. In these assays, HRP standards and substrate solution were dispensed and mixed using digital microfluidics, generating a chemiluminescent product with light intensity proportional to the HRP concentration. As a comparison, these assays were also analyzed using a commercial well-plate reader used in chapter 2. As shown in Figure 3.4, the calibration curves measured by both methods were linear and had a dynamic range of at least 2 orders of magnitude. Importantly, the coefficients of variation (CVs) for the measurements generated using the integrated PMT (0.56 to 9.3\%) are lower than those from the well-plate reader (0.63 to 21\%). In addition, the integrated PMT method had a slightly lower limit of detection (LOD) compared to that of the well plate reader (4.88 vs. 7.97 \( \mu \text{U/mL} \)). We speculate that the improved performance is related to the reproducible positioning of the detector and device (in one package), in contrast to manual insertion of devices into a well-plate reader.
Figure 3.4: Characterization of detectors
Calibration curves for homogenous HRP assay using H$_2$O$_2$/Luminol detected by (A) integrated PMT and (B) well-plate reader.

3.3.2 Magnet design and modeling

Although digital microfluidic magnetic separation is proving to be a useful and efficient method for the extraction and clean-up of specific analytes, its successful implementation requires an adjustable magnet that can induce sufficient force on particles inside a droplet.$^{250}$ In chapter 2 and previous work,$^{224,250}$ this requirement was satisfied by an off-the-shelf neodymium magnet disc which was introduced or removed from the DMF device during magnetic separation or resuspension, respectively, facilitating up to two simultaneous digital microfluidic magnetic separations. We attempted to improve on the performance of this format by designing a new system that can satisfy several competing requirements: (1) simple to fabricate, assemble, and automate, (2) increased number of parallel assays, (3) narrower area of effect such that magnetic
induction is minimal outside of the separation zone, and (4) stronger magnetic field gradient
inside the separation zone for faster separations and greater flexibility in the distance between the
magnet and the device. After several design iterations (data not shown), we finalized a unique
magnet assembly design, we call the “magnetic lens,” comprising a neodymium magnet bar
flanked by two steel machined armatures that act as field directors (Figure 3.3A). This
arrangement focuses the magnetic field to the tips of the two triangular steel poles, forming a
strong field gradient at the apex of the assembly. When this magnet assembly is positioned under
a DMF device along its width (Figure 3.1C), a separation zone is formed across the middle of
the electrode array (Figure 3.2). The magnetic lens design has several salient advantages over
magnet discs used in chapter 2, as described below.

The first advantage of the magnetic lens is the localization of the field to a narrow area,
minimizing unintended particle attraction away from the separation zone (e.g., in the magnetic
particle reservoir). As shown in the numerical simulations (Figure 3.3C and Figure 3.5), the
vertical and horizontal components of the force originating from the magnetic lens acting on a
particle pellet (located 1.4 mm above the magnet) fall off rapidly as the particle pellet is moved
away from the center of the magnet—these forces become negligible (< 35 µN) at greater than 4
mm from the center. In contrast, the magnetic fields in the system used in chapter 2 (i.e., an
unmodified magnet disc) cover a wide region of the device, often causing unintended particle
attraction outside the separation zone. For example, according to numerical simulations, a
particle pellet positioned 10 mm from the center of a magnetic disc (i.e., not in a position in
which trapping is desired) experiences vertical and horizontal forces of ~50 µN and ~100 µN,
respectively. This phenomenon is exacerbated when the disc is moved horizontally to transition
between engaged and disengaged state as in chapter 2. In the new magnet design reported here,
this problem is circumvented because the magnet is moved vertically by engaging a motor
(Figure 3.1C).
Figure 3.5: Horizontal force on particle pellet
Numerical simulations (blue squares: magnetic lens, red circles: magnetic disc used in chapter 2) of force on particle pellet (directed horizontally towards magnet center) as a function of (A) horizontal position from magnet center and (B) vertical position from magnet surface.

The second advantage of the magnetic lens is the induction of a stronger magnetic force on the particles in the separation zone relative to that of the magnet disc. In particular, the vertical force increases exponentially as the vertical distance between the particle pellet and magnetic lens decreases (Figure 3.3D). This distance is limited to the thickness of the device substrate and the separation between the device and the magnet. In chapter 2, it was determined that a minimum vertical force of \(~470\ \mu\text{N}\) was required to effectively immobilize the particles for separation, which was achievable only when the magnetic disc made direct contact with the bottom of the device. In contrast, with the magnetic lens system introduced here, magnetic separation is achievable with a \(~150\ \mu\text{m}\) gap between the magnet and the device. As in the inset
in Figure 3.3D, this configuration corresponds to a vertical force of ~600 µN. From a design standpoint, this gap allowance is desirable for preventing the magnet from colliding with the device.

The third advantage of the magnetic lens system is the facilitation of parallel magnetic separations. As shown in Figure 3.6, when the magnet is engaged, magnetic particles inside eight droplets pellet on the surface of the device above the magnet. The immobilized particles can then be separated from the supernatant by moving the droplets, leaving eight pellets of particles on the device surface. Subsequently, the particles can be collected and resuspended by disengaging the magnet, moving a droplet of exchange reagent over the particles, and mixing the droplet. In the future, the multiplexing could be increased with a longer magnet or smaller electrode design. In contrast, when using the magnetic disc geometry used in chapter 2, it is difficult to achieve more than two separations, with no possibility for expansion.
3.3.3 Design of experiments analysis for TSH immunoassays

An immunoassay for thyroid stimulating hormone (TSH) was chosen as a test-case for the new system, given the wide prevalence of this assay in routine clinical testing for thyroid disease. To determine the optimal assay parameters for this new integrated platform, we implemented a $3^3$ full factorial design of experiments (DOE) for TSH immunoassays. Although there are more efficient methods such as the central composite design, the full factorial design represents
an elaborate test-case, suitable for the evaluation of the new automated platform. As a starting point, we varied three factors: sample volume (\textit{vol}: 1, 3, and 5 pooled unit droplets), sample incubation time (\textit{inc}: 3, 6, and 9 minutes), and concentration (\textit{con}: 0, 0.4, and 4 \(\mu\)IU/mL). With replicates, this translated to a total of 108 discrete experiments, a number that would have been very tedious using the non-automated system described in chapter 2.

The three-level DOE design allowed us to estimate linear, nonlinear, and interaction effects of all of the tested factors.\textsuperscript{237} The mid-levels for \textit{vol} and \textit{inc} were selected to be the same as the optimal parameters in the prototype setup from chapter 2 (3 unit droplets and 6 minutes). A pilot immunoassay protocol (described in the experimental section) was used in these experiments, in which TSH is quantified using antibody coated magnetic particles, HRP conjugated detection antibody, and luminol-H\textsubscript{2}O\textsubscript{2}-based chemiluminescent substrate. The signal-to-background ratios (SBRs) from these assays were used as the measured response for DOE optimization.

The results from the full factorial experiment were evaluated in three steps: (1) model construction and validation, (2) identification of significant factors, and (3) prediction of optimal assay parameters. In the first step, the SBR was fitted with a modified second-order model (\textbf{Equation 3.1}), which included linear, quadratic, and interaction factors.\textsuperscript{256-257} However, this initial fit resulted in residual values (i.e., differences between actual and predicted SBR) that are not distributed normally, suggesting that a response transform was required to meet the fundamental assumptions of standard least square multiple regression. A Box-Cox plot confirmed that a power transform of \(~0.5\) minimized the sum of squares error, and therefore, a square-root transform was applied to the SBR. The transformed response, Sqrt\(\text{(SBR)}\), was refitted with \textbf{Equation 3.1} and the fit quality was assessed using several diagnostic tests: (a) according to Shaprio-Wilk test and normal probability plot, the residual values were normally distributed (\textbf{Figure 3.7A}), (b) as shown in \textbf{Figure 3.7B}, the predicted Sqrt\(\text{(SBR)}\) was well correlated with the actual Sqrt\(\text{(SBR)}\), (c) the R\textsuperscript{2} and adjusted R\textsuperscript{2} values were 0.90 and 0.88, respectively, and (d) the factors in the model have a significant effect (p-value \(<0.0001\)) on the Sqrt\(\text{(SBR)}\) response as determined by analysis of variance (ANOVA). In the second step, we identified the significant factors (via ANOVA) and estimated their coefficients. The non-significant factors that are not implicated in significant interactions were discarded in the final model. As shown in \textbf{Figure 3.7C}, the remaining factors were sorted by decreasing t-ratio (i.e.,
the ratio of the coefficient estimate to its standard error), with the most substantial effects at the top. Not surprisingly, the factors con and con*vol have significant positive coefficient estimates—increasing TSH in the assay results in higher Sqrt(SBR). However, this increase eventually plateaus at higher con or vol as predicted by the negative coefficient quadratic factors (con*con and vol*vol). Interestingly, several interaction factors involving sample incubation time (vol*vol*inc, vol*inc, and con*inc) have negative coefficient estimates, suggesting that longer sample incubation time, coupled with high amount of TSH, reduces the Sqrt(SBR). In the final step, the completed model, constructed from factors and coefficients in Figure 3.7C, was used to predict the optimal sample volume and sample incubation time for TSH immunoassays. The maximum response was achieved at 4 µIU/mL TSH concentration using 5 droplets of sample and 3 minute sample incubation time. These parameters were found to produce optimal response at lower TSH concentrations, which is crucial for assay sensitivity. For example, at 0.4 µIU/mL TSH concentration, the response surface increases for higher sample volume and lower sample incubation time, achieving maximum response at 5 droplets and 3 minute sample incubation (Figure 3.7D).

Interestingly, these DOE data suggest that the new integrated platform allows for faster analyte binding to magnetic particles relative to the initial prototype setup in chapter 2. This improvement is likely related to the rapid, continuous droplet mixing during sample incubation for the new setup—such uniform mixing was impossible to achieve using handheld voltage probes in the prototype setup. A detailed comparison of the new integrated platform and the prototype setup is included in Table 3.1.
Figure 3.7: Design of experiment (DOE) analysis for TSH magnetic particle immunoassay
(A) Normal probability plot of the residuals and W test indicates that the residuals are normally distributed. (B) Actual versus predicted plot superimposed with confidence curves (0.05). The plot correlates model predictions to actual data; the P-value, $R^2$, adjusted $R^2$, and Root Mean Square Error (RMSE) for the correlation are indicated below the plot. (C) Coefficient estimates for centered polynomial regression sorted by decreasing t-ratio; the bar graph plots the t-ratios and the vertical lines define the 0.05 significance level. (D) A three-dimensional signal-to-background ratio response surface at 0.4 µIU/mL TSH concentration showing the interaction between sample incubation time and number of sample droplets (heat map with red = high response, blue = low response).

### Table 3.1: Comparison of prototype setup and the new integrated platform

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<thead>
<tr>
<th>Prototype Setup in Chapter 2</th>
<th>New Integrated Platform Reported Here</th>
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<tr>
<td>Property</td>
<td>Effect</td>
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<tr>
<td>Manual particle separation using magnet disc</td>
<td>-Two separations per magnet</td>
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<td></td>
<td>-Labour intensive to manually move magnet</td>
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<td></td>
<td>-Separation failure caused by undesired magnet positioning</td>
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<tr>
<td>Detection by commercial well-plate reader</td>
<td>-Device position in well-plate is prone to variability (CV: 0.63-21%), causing reduced sensitivity</td>
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<td>(LOD: 7.97µU/mL HRP)</td>
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<tr>
<td>Droplet actuation using handheld high voltage probes</td>
<td>-Labour intensive and prone to variability</td>
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3.3.4 Optimized immunoassay

Using the results of the DOE analysis, we developed an optimized immunoassay protocol with improved performance relative to chapter 2. In pilot immunoassays, we observed high variability between replicates, with CVs ranging from 7 to 40%. This variability was caused by a flaw in the measurement procedure, in which samples assayed in parallel experience different wait times.
prior to measurement. Since there is a single, stationary PMT, droplets containing completed particle immunoassays must queue for analysis until the detection area becomes vacant. As a result, in the pilot immunoassays, the particles that are second in the queue must wait two extra minutes in the wash buffer relative to the first set of particles (and so on). Interestingly, this extended wash buffer incubation caused a drop in chemiluminescent signal of up to 45%. To circumvent this problem, we developed a new measurement procedure, in which wash buffer is immediately removed after the final wash step. Subsequently, the particles are resuspended in luminol-H₂O₂ substrate and mixed for 4 minutes. After mixing, the chemiluminescent signal becomes saturated, creating a stable measurement window (~1 min) for all particles to be analyzed sequentially. Using this new measurement procedure and the optimized parameters from DOE analysis (5 pooled sample droplets and 3 minute sample incubation), immunoassays were implemented for various concentrations of TSH (0, 0.4, and 4 µIU/mL) on the integrated platform. As shown in Table 3.2, the integrated platform exhibited better performance when compared to the prototype setup in chapter 2. The reproducibility of the integrated platform is substantially better, with CVs ranging from 6 to 10%, and the integrated platform had 3-fold or 5-fold lower LOD when calculated in terms of absolute amount or concentration, respectively. This improvement in LOD (to 0.15 µIU/mL) is critical, as it provides the capability to screen for hyperthyroidism (TSH < 0.3 µIU/mL). Finally, the sample incubation time was reduced by 2-fold because of the enhanced mixing offered by the integrated platform.

<table>
<thead>
<tr>
<th>Immunoassay System</th>
<th>Prototype Setup in Chapter 2</th>
<th>Integrated Platform</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coefficient of Variability</td>
<td>9-21%</td>
<td>6-10%</td>
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<tr>
<td>Limit of detection (absolute)</td>
<td>2.0 nIU</td>
<td>0.6 nIU</td>
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<tr>
<td>Limit of detection (concentration)</td>
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<tr>
<td>Sample Volume</td>
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<tr>
<td>Sample Incubation Time</td>
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</tbody>
</table>

### 3.4 Conclusion

We developed an automated digital microfluidic platform for magnetic particle-based immunoassays. This platform features a 90 Pogo pin interface for digital microfluidic control, an integrated detector for chemiluminescent detection, and a new magnet assembly that facilitates up to 8 simultaneous digital microfluidic magnetic separations. Most importantly, the new
platform allows for the implementation of DOE optimization of immunoassay performance. As a test case, a three-level full factorial DOE analysis enabled the development of an optimized protocol that reduced detection limit and sample incubation time by up to 5-fold and 2-fold, respectively. We propose that this new platform has great potential for the quantitative analysis of disease biomarkers at various near-patient settings world-wide.
Chapter 4

4 A Digital Microfluidic Platform for the Detection of Rubella Infection and Immunity

While disease surveillance for infectious diseases such as rubella is important, it is critical to identify pregnant patients at risk of passing rubella to their offspring, which can be fatal and/or can result in congenital rubella syndrome (CRS). The traditional centralized model for diagnosing rubella is cost-prohibitive in resource-limited settings, representing a major obstacle to the prevention of CRS. As a step toward decentralized diagnostic systems, we developed a digital microfluidic (DMF) diagnostic platform, which possesses the flexibility and performance of automated immunoassay platforms (used in central facilities), in a small form factor with reduced sample volumes and costs. DMF immunoassay methods were developed with integrated sample preparation for the detection of rubella virus (RV) IgG and IgM. The performance (sensitivity and specificity) of the assays was evaluated using serum and plasma samples from a commercial anti-rubella mixed titer performance panel. The new platform can perform all essential processing steps including sample aliquoting for four parallel assays, sample dilution, and IgG blocking. Testing of performance panel samples yielded diagnostic sensitivity and specificity of 100% and 100% for both RV IgG and IgM, rivaling the performance of lab-based RV testing. These assays are fast (~30 min for four parallel assays), have excellent reproducibility (<10% average coefficient of variability), and require only a few microliters of sample and reagents. This work establishes DMF-powered immunoassays as being useful for the diagnosis of an infectious disease. The platform features laboratory-quality performance and flexibility, while reducing instrumentation form factor, cost of assays, and sample volume requirements.

4.1 Introduction

Vaccine-preventable infectious diseases such as measles, mumps, and rubella continue to be a global threat because many developing countries have low or non-existent immunization coverage. Although rubella is manageable for most adults and minors (rash, fever, and flu-like symptoms), rubella infection during pregnancy has significant risk of causing fetal death and/or a
debilitating suite of defects known as congenital rubella syndrome (CRS). This disease is particularly pernicious as asymptomatic rubella infection in adults is common, meaning that without testing, many cases of CRS (including more than 100,000 infants born each year) are not diagnosed until after delivery.

As is the case for many infectious diseases, screening for rubella susceptibility, immunity, and infection is challenging, requiring the selective detection of rubella virus (RV)-specific IgG (RV IgG) and IgM (RV IgM). Because the test components of RV IgM immunoassays can cross-react with other biogenic proteins (e.g., RV IgG or rheumatoid factor IgM), there is a significant occurrence of false negative or false positive results depending on the quality of the assays and the experience of the operators. Thus, although there are portable tests available, rubella is almost always diagnosed in central facilities using qualified laboratory techniques. In resource-limited settings, transportation from rural communities to remote laboratories is often cost-prohibitive, meaning that rubella infections (and attendant cases of CRS) routinely sweep through rural populations without testing or treatment.

Given the challenges described above, there is great enthusiasm for moving to decentralized systems for disease diagnosis and surveillance. This model requires portable assays, and the microfluidic (or “lab on a chip”) community has addressed this challenge primarily through systems that rely on fluid flow through enclosed microchannels or lateral flow through a paper-based absorptive matrix. Microchannel-based systems have excellent analytical sensitivity and proven diagnostic performance in resource-limited, but they often require extensive ancillary equipment to operate (e.g. pumps, flow meters, valves, etc.) and intricate device fabrication for integrated sample preparation. In contrast, paper-based systems can be simple and inexpensive to manufacture and use, but adequate analytical performance in such devices has only been demonstrated for a few analytes, and multistep liquid manipulation with uniform flow profiles requires clever material manipulation and device design.

An alternative to (channel-based or lateral) fluid flow for miniaturized analysis systems is a technology known as digital microfluidics (DMF), in which sample and reagents are manipulated as discrete droplets on a hydrophobic surface. DMF systems actuate droplets through the application of electrical potentials on a generic \((m \times n)\) array of insulated
electrodes—a format that enables software-reconfigurable, concurrent droplet operations including merging, mixing, splitting, and metering from reservoirs.\textsuperscript{169} DMF devices can be cost-effectively fabricated on paper by inkjet printing\textsuperscript{282} and can be operated using simple and compact instrumentation\textsuperscript{248,283} with no need for pumps, interconnects, valves, or fittings. Although these advantages have motivated the development of DMF-powered immunoassays for small-molecule and protein biomarkers,\textsuperscript{247,250,284-285} DMF immunoassays have never (to our knowledge) been used for diagnosis of infectious diseases.

Here, we report the development of DMF-powered RV IgG and RV IgM immunoassays, with a focus on evaluating the diagnostic performance of DMF immunoassays relative to laboratory methods. These assays rely on rubella virus-immobilized magnetic particles to capture the analyte from the sample and enzyme-linked detection antibodies to transduce analyte binding events to chemiluminescent signal – all assay steps, from sample to analysis, are implemented by DMF. In particular, this represents the first report of an immunoassay capable of differentiating IgM from IgG by digital microfluidics, which is an important step in the diagnosis of many infectious diseases. Although these results were collected in a laboratory, they were executed using a ~shoebox-sized instrument prototype, and we predict that later generations will be suitable for portable testing in the field. We propose that this is a useful step towards the development of decentralized diagnostic tools that will be instrumental for diagnosis of rubella and other infectious diseases.

4.2 Experimental

4.2.1 Reagents and materials

Unless otherwise specified, reagents were purchased from Sigma-Aldrich (Oakville, ON). Deionized water had a resistivity of 18MΩ•cm at 25°C. Pluronic L64 (BASF Corp., Germany) was generously donated by Brenntag Canada (Toronto, ON).

Most immunoassay reagents were adapted from the ARCHITECT rubella IgM and IgG assay kits from Abbott Laboratories (Abbott Park, IL), including RV IgG calibrators, RV IgM cutoff Calibrator 1 (Cal 1), RV IgM pre-treatment reagent containing goat anti-human IgG, and virus-coated paramagnetic microparticles. Reagents from other vendors include Superblock Tris-buffered saline and SuperSignal ELISA Femto chemiluminescent substrate, comprising stable
peroxide (H₂O₂) and luminol-enhancer solution, from Thermo Fischer Scientific (Rockford, IL), anti-rubella mixed titer performance panel (PTR201-00-0.5) from SeraCare Life Sciences (Milford, MA), and defibrinated sheep blood from Quad Five (Ryegate, MT).

Custom digital microfluidic-compatible wash buffer and conjugate diluent were prepared as described in chapter 2 and 3. Conjugate working solutions for RV IgG or IgM assays were formed by diluting horse-radish peroxidase (HRP) conjugated goat polyclonal anti-human IgG (21 ng/mL) or HRP conjugated goat anti-human IgM (23 ng/mL) in conjugate diluent, respectively. Pretreatment working solution was formed from a 3× dilution of IgM pretreatment reagent in conjugate diluent. The microparticle working suspension was formed in an Eppendorf tube by removing the original diluent (with particles immobilized using a neodymium magnet), washing the particles twice in Superblock, and resuspending the washed particles in Superblock at 1.5 × 10⁸ particles/mL (~10× the stock concentration). Simulated whole-blood samples were formed from a 1:1 mixture of RV IgG calibrator and sheep blood. Prior to running immunoassays, calibrator solutions, simulated whole-blood samples, or patient samples (from performance panel) were diluted 10× in Dulbecco’s phosphate buffered saline (DPBS) containing 4% BSA. All reagents used on digital microfluidic devices or “on-chip” were supplemented with Pluronic L64 at 0.05%.

4.2.2 Device fabrication and operation

The custom automation system in chapter 3 was used to manage droplet operation, magnet and photomultiplier tube (PMT) position, and data collection (Figure 4.1A). Digital microfluidic devices, comprising a top plate and bottom plate, were fabricated in the University of Toronto Nanofabrication Centre cleanroom facility and were assembled as described in Chapter 3 (Figure 4.1B). The bottom plate device design featured an array of 80 actuation electrodes (2.54 × 2.54 mm) connected to reservoir electrodes for sample and reagent storage, and waste removal. Unit droplet volumes on the actuation electrodes were ~900 nL, as determined by the area of each actuation electrode and the gap spacing (~140 µm) between the top plate and bottom plate.

Droplets were actuated by applying a preprogrammed sequence of driving voltages (80-100 V_RMS 10 kHz sine wave) between the top plate (ground) and electrodes in the bottom plate through a Pogo pin interface (90 pins). For on-chip particle separation, a motorized magnet
system called a magnetic lens is positioned ~150 µm underneath the device. In this activated state, >600 µN of magnetic force is sufficient to focus particles into a pellet, immobilizing them on the surface (Figure 4.1C). Using digital microfluidic actuation, the supernatant can be separated from the particles, provided that the immobilization force exceeds the minimal threshold (~500 µN) required to overcome the surface tension of the droplet. The particle pellet is resuspended by deactivating the magnetic lens (i.e. magnet is positioned 3.8 cm below the device) and moving and mixing a droplet over the particles (Figure 4.1D).

Figure 4.1: Automated digital microfluidics platform
(A) Left: Photo of automation system (front view) showing the position of electrical inputs from the DropBot and external motors that control the photomultiplier tube (PMT) and enclosure. Right: Cross-section computer aided design rendering showing the interior components of the system, including the PMT, the magnetic lens, and the motor system that controls the position of the magnetic lens. (B) Three-dimensional schematic and side view of digital microfluidic device positioned above the magnetic lens. (C) Video sequence of digital microfluidic magnetic separation of paramagnetic particles in parallel rubella virus IgM and IgG immunoassays. (D) Video sequence of resuspending and mixing of particles in wash buffer solution. Red arrows indicate the direction of droplet movement.

To measure on-chip chemiluminescence, the reaction droplet is moved to the center of the electrode array and a motorized PMT is positioned several hundred micrometers above the device to collect light from the droplet. When the PMT is inactive (i.e., the PMT is positioned
5 cm above the device), an integrated LED and webcam assembly is used to monitor on-chip droplet movement. Three design measures were implemented to prevent users from damaging the PMT, including (1) two sensors to ascertain the position of the PMT, (2) a shutter mechanism to prevent light from saturating the sensor when it is not in use, and (3) deep groves in the enclosure to ensure that the instrument is light-tight during measurements.

### 4.2.3 Rubella virus IgG immunoassay

RV IgG antibodies were detected on-chip using an indirect ELISA comprising seven steps. (i) A droplet of virus-coated paramagnetic particle suspension (1.8 µL) was dispensed from a reservoir and the particles were separated from the diluent. (ii) A droplet of sample (1.8 µL, containing RV IgG) was dispensed, delivered to the particles, mixed for 3 minutes, and separated from particles. (iii) The particles were washed in four successive droplets of wash buffer (i.e., 4×1.8 µL), each time mixing and then separating from the supernatant. (iv) A droplet of HRP conjugate solution (1.8 µL, containing HRP conjugated anti-human IgG) was dispensed, delivered to the particles, and mixed for 2 minutes. (v) Step iii was repeated. (vi) One droplet each of H2O2 (900 nL) and luminol-enhancer solution (900 nL) were dispensed and merged with the particles; the solutions and particles were mixed for 2 minutes. (vii) The average chemiluminescent signal over 10 seconds was obtained using the integrated PMT. In these assays, four parallel tests were run simultaneously (i.e., steps i-vii were performed in parallel for four samples). Applying this protocol, a calibration curve for RV IgG was established in four replicates (intra-assay), using calibrator solutions containing 0, 5, 10, 15, 75, and 250 IU/mL of RV IgG. The limits of detection (LOD) and quantitation (LOQ) for this assay were the concentrations corresponding to the position on the calibration curve of the average signal generated from blank measurements plus 3 (LOD) or 10 (LOQ) times the standard deviation of the blank measurements. A given set of calibration data were used for repeated experiments until a new batch of devices or magnetic particles were introduced, or when the control sample measurements were observed to be out of range. In practice, this allowed the use of each set of calibration data for more than 200 experiments over 50 days.

### 4.2.4 Integrated on-chip dilution and RV IgG immunoassay

As shown in Figure 4.2, samples were diluted on-chip in three iterating steps. (I) One droplet of sample (1.8 µL) and one droplet of diluent (1.8 µL, DPBS 4% BSA) were dispensed, merged and
mixed. (II) The pooled sample droplet (2× diluted) was split into two sub-droplets. (III) One sample sub-droplet was stored for later analysis, while the second sample sub-droplet was used to repeat steps I-III until the desired dilutions were achieved. For example, 4 repetitions generates four droplets with 2×, 4×, 8×, and 16× dilution of original sample. Following dilution, RV IgG immunoassays were performed (using the seven-step procedure described above) by delivering the diluted samples to virus-coated particles. When the particles were washed, the regions on the device used for dilution and sample storage were also washed with additional wash buffer to prevent cross-contamination.

An integrated protocol (including dilution in four iterations of steps I-III followed by RV IgG immunoassays in steps i-vii) was evaluated using a RV IgG calibrator and a simulated whole-blood sample (1:1 mixture of RV IgG calibrator and sheep blood), both containing 250 IU/mL of RV IgG. Here, on-chip dilution was performed to generate droplets containing 15.6, 31.3, 62.5, and 125 IU/mL of RV IgG. For each sample (calibrator and whole-blood), the protocol was repeated on three different devices (i.e., three inter-assay replicates).

**Figure 4.2: On-chip sample serial dilution**
Video sequence (frames 1-8) illustrating an on-chip process to form a series of four droplets with 2×, 4×, 8×, and 16× dilution of original sample Red dye added for visualization.

### 4.2.5 Off-chip rheumatoid factor IgM screening

A rheumatoid factor IgM ELISA (ORGENTEC Diagnostika, Mainz, Germany) was used (following manufacturer’s instructions) to screen for rheumatoid factor IgM in the RV IgG calibrators and RV IgM Calibrator 1. In these experiments, at least two replicates were evaluated for each sample by absorbance, measured at 450 nm (reference 650 nm) using a Sunrise microplate reader (Tecan, Durham, NC) in “Accuracy” read mode. The rheumatoid factor IgM
concentration of the samples were estimated from a calibration curve, generated by fitting the optical density of the ELISA calibrator solutions to a 4-parameter logistic equation.

### 4.2.6 RV IgM immunoassay

The RV IgM immunoassay is identical to the seven-step protocol described above, except that the conjugate solution contains HRP conjugated anti-human IgM (instead of IgG) and the sample is pre-treated with anti-human IgG (prior to step i). In the pretreatment procedure, a droplet of sample (1.8 µL) and a droplet of pretreatment reagent (0.9 µL) were merged and mixed for 7 minutes. The assay was evaluated with and without pretreatment using four test samples: (a) 0 IU/mL RV IgG calibrator (control), (b) 250 IU/mL RV IgG calibrator (“IgG”), (c) 1:1 mixture of Cal 1 and 0 IU/mL RV IgG calibrator (“IgM”), and (d) 1:1 mixture of Cal 1 and 500 IU/mL RV IgG calibrator (“IgM + IgG”). For each sample, four intra-assay replicates were performed for each condition tested. The results (for IgG, IgM, and IgG + IgM) were reported as fold change, which was obtained by normalizing the signal intensity of each assay to the average signal intensity of the respective control experiments.

Applying the integrated RV IgM protocol (including both pretreatment and immunoassay), the cutoff calibration data was established in three replicates, using unmodified Cal 1. In addition, the background signal of the RV IgM assays was evaluated in four replicates (intra-assay), using a 0 IU/mL RV IgG calibrator.

### 4.2.7 Anti-rubella mixed titer performance panel

The diagnostic accuracy of DMF RV IgG and IgM immunoassays was estimated using 25 plasma/serum samples (PTR201-01 to 25, SeraCare Life Sciences). Serum or plasma samples were thawed and aliquoted into single-use vials for DMF immunoassays. Working solutions including microparticles, conjugates, and chemiluminescent substrates were made fresh each day from stock solutions.

DMF immunoassays for the panel specimens were completed over a span of one month by three different operators. Each assay was carried out in 3 to 4 replicates in parallel, and operators were blinded to the reference result until all of the data were collected. The DMF assay results were reported as signal-to-cutoff ratios (s/co), calculated by normalizing the average signal intensity of each assay to the average signal intensity of the respective cutoff
control. The cutoff controls for RV IgG and IgM assays are the 10 IU/mL RV IgG calibrator and the RV IgM cutoff Cal 1 (both included in the respective kits), respectively. A DMF s/co ≥ 1 is interpreted as positive, a DMF s/co in the grayzone (0.69-0.99 for IgG and 0.75-0.99 for IgM) is interpreted as equivocal/indeterminate, and a DMF s/co below the grayzone (< 0.69 for IgG and < 0.75 for IgM) is interpreted as negative.

4.2.8 Evaluation of diagnostic accuracy

The diagnostic sensitivity and specificity were calculated for the DMF immunoassays using the Abbott EIA as a reference standard test. The sensitivity is the probability that a truly infected individual will test positive, and is given by Equation 4.1

\[ \text{Sensitivity} = \frac{TP}{TP + FN} \]

where \(TP\) is the number of true positives and \(FN\) is the number of false negatives. The specificity is the probability that a truly uninfected individual will test negative, and is given by Equation 4.2

\[ \text{Specificity} = \frac{TN}{TN + FP} \]

where \(TN\) is the number of true negatives and \(FP\) is the number of false positives. The 95% confidence intervals for sensitivity and specificity were calculated using Equation 4.3

\[ p \pm 1.96 \times \sqrt{\frac{p(1-p)}{n}} \]

where \(p\) is sensitivity or specificity and \(n\) is the total number of samples from infected \((TP+FN)\) or uninfected \((TN + FP)\) subjects, respectively.\(^{286}\) If the calculated sensitivity or specificity is equal to 1, a p-value of 0.999 was used, as recommended by WHO\(^ {287}\) and others.\(^ {273-274}\)
4.3 Results and Discussion

4.3.1 Digital microfluidic instrumentation

Motivated by the need to identify patients at risk for congenital rubella syndrome (CRS), we developed digital microfluidic (DMF) immunoassays for rubella virus-specific immunoglobulins, representing the first application of DMF to implement a test for an infectious disease. The challenging nature of this application, which includes the need to distinguish between RV IgG and RV IgM, necessitates a sophisticated system capable of handling a large number of reagents and performing a complex series of processing steps—thus, these assays are almost always performed in centralized laboratories. To achieve similar levels of processing sophistication in a miniaturized instrument, we adapted an in-house designed computer-controlled instrument from chapter 3 powered by DropBot, an open-source digital microfluidic (DMF) automation system.

The shoebox-sized instrument comprises a motorized photomultiplier tube (PMT) for chemiluminescent detection, an electronic interface to the DropBot for droplet movement, and a magnet assembly (called a “magnetic lens”) for particle separation (Figure 4.1A). Movement of protein-containing droplets through an air-filled device (Figure 4.1B), which allows for more efficient particle-concentration and analyte washing than the more common methods relying on oil-filled devices as shown in chapter 2, is facilitated by the inclusion of Pluronic additives in samples and reagents, which reduces protein adsorption and limits cross-contamination.

During particle separation, particles in the droplet are immobilized on the surface in a region above the magnet, referred to as the separation zone (Figure 4.1C). Subsequently, the droplet is actuated away to waste, leaving behind a particle pellet. For particle resuspension, the magnetic lens is deactivated, and a new droplet is actuated over the particle pellet and mixed in a circular motion; this is sufficient to break up the particle pellet and completely reconstitute the particles in suspension (Figure 4.1D).

The DMF instrumentation and devices described here are prototype designs employed to demonstrate the diagnostic performance of DMF immunoassays. We propose that later generation designs may be suitable for portable testing in the field [i.e., low cost, user friendly, battery-powered, and with compact form factor]. These new designs will likely benefit from
the development of low-cost device fabrication methods,\textsuperscript{282} simple instrumentation,\textsuperscript{248,283} and integrated electrochemical sensors.\textsuperscript{289-290}

4.3.2 RV IgG Immunoassay and sample dilution

Whether acquired naturally or induced by vaccination, the persistence of RV IgG antibody confers protection against rubella infection. The WHO interpretation of RV IgG serological results is dependent on the antibody level: a sample is positive if antibody level is greater than 10 IU/mL, equivocal if it is between 5 and 10 IU/mL, and negative if it is less than 5 IU/mL. Individuals with antibody levels below 10 IU/mL are susceptible to clinical illness upon exposure to rubella virus. As such, testing women for RV IgG before conception or at their first antenatal visit can help minimize the risk of congenital rubella syndrome (CRS).

Using the particle separation and resuspension techniques described above, we developed a seven-step DMF indirect ELISA for RV IgG, in which samples or reagents are aliquoted to the particles for four parallel assays (Figure 4.3). As shown in Figure 4.4A, RV IgG antibodies present in the sample bind to the rubella virus-coated paramagnetic particles. After incubation and washing, HRP-conjugated anti-human IgG is added to form immunocomplexes. Following another incubation and wash cycle, hydrogen peroxide and luminol-enhancer solutions are added to the particles, and the resulting chemiluminescence is measured as relative light units (RLUs). As described in chapter 3, this assay procedure, including determination of diluent compositions, reagent concentrations, incubation times, droplet operations, and chemiluminescent signal generation, were developed from design of experiment optimization. The use of chemiluminescence is particularly advantageous because very few optical components are required (e.g. no excitation source, filters, lenses, etc.)—this improves assay sensitivity, allows for smaller footprint, and improves ruggedness.
Figure 4.3: On-chip sample loading and aliquoting
Video sequence (frames 1-6) illustrating an on-chip process of loading and aliquoting four samples to the magnetic particles in RV IgG immunoassays.

The assay described here was designed for detection of RV in patient serum and plasma samples. Thus, to optimize and calibrate the method, we used commercial calibrators designed to simulate human serum (buffer containing 4% BSA). As shown in the calibration curve (Figure 4.4B), a direct relationship exists between the RLUs detected by the integrated PMT and the amount of RV IgG in the calibrators. The calibration curve had a dynamic range of approximately 2 orders of magnitude, the intra-assay coefficients of variability (CVs) ranged from 0.3 to 6.5%, and the LOD and LOQ of the assay were 0.03 IU/mL and 0.1 IU/mL, respectively (well below the clinical cutoff levels described above). Each set of four assays requires ~25 min to complete.

In some instances, samples with high analyte concentration must be diluted prior to analysis to facilitate accurate quantitation; this technique is relatively trivial for central laboratories, but is not commonly used in portable tests. Hence, we evaluated whether the DMF platform can reliably perform a range of dilutions on samples containing 250 IU/mL RV IgG, which is beyond the linear range of the calibration curve (Figure 4.4B). Briefly, it is straightforward to dilute such samples on-chip – for example, to form four droplets of 125, 62.5,
15.6, and 31.3 IU/mL (Figure 4.2). Subsequently, RV IgG immunoassays can be performed on the diluted samples in parallel.

The assays described here were designed for application to patient serum and plasma samples (validation results described below). But for portable analysis in the field, it would be useful if the assay could also be used with whole blood, negating the requirement of phlebotomy and fractionation. To evaluate this possibility, simulated whole-blood samples (calibrator mixed with sheep’s blood) were diluted on-chip and tested using the methods described above; as shown in Figure 4.4C, the inter-assay CVs for these assays were only marginally higher in the blood samples (10 to 15%) compared to the control samples (6 to 9%). Because of dissimilarities in matrix composition, the signal intensities of the calibrator samples differed significantly (P < 0.05) from the samples containing high blood content (i.e., 1/8 and 1/4 dilutions of blood). But when these samples were diluted further (i.e., 1/16 and 1/32 dilutions of blood), the results for blood and calibrator samples could not be distinguished (P > 0.05). These observations suggest that sample dilution may be useful for alleviating interferences arising from the blood matrix for application in the field, provided that the dilution does not reduce the analyte concentration below the LOQ of the assay.
Scheme and procedure of rubella virus (RV) IgG immunoassay on paramagnetic particles. Calibration data (red diamonds) and curve (dotted line) for RV IgG immunoassay implemented in the digital microfluidic system. Comparison of RV IgG immunoassay results for samples in calibrator solution (gray) and blood (white) for dilutions formed on-chip. Each value represents the mean +/- standard deviation from 3 replicates. Statistical significance (P <0.05*) was evaluated by Student’s two-tailed t-test.

4.3.3 RV IgM Immunoassay and IgG blocking

While testing for RV IgG (described above) is useful for determining susceptibility and immune status, the critical test for diagnosing rubella infected patients (including the identification of pregnancies at-risk for CRS) is the detection of RV IgM. Unlike RV IgG, there is no agreed-upon cutoff level for RV IgM to distinguish between negative or positive test results. As such, assay vendors develop their own cutoff calibrators based on serological testing of specimens obtained from normal and infected donors. In the assays reported here, the RV IgM Calibrator 1 (Cal 1) from the Abbott ARCHITECT rubella IgM kit was used to define the cutoff between positive and negative/equivocal results.

Similar to the RV IgG immunoassays described above, the on-chip RV IgM assays rely on rubella virus-immobilized paramagnetic particles to implement indirect ELISA. In an ideal assay, RV IgM antibodies from the sample will bind to the particles, and the detection antibodies (HRP-conjugated anti-human IgM) will bind to the RV IgM to form immunocomplexes (Figure 4.5A, panel 1). However, there are at least two potential sources of errors in this assay scheme caused by the presence of RV IgG and/or rheumatoid factor (RF) IgM in human serum. If the sample contains RV IgG, these antibodies will compete for binding sites on the particles, leading to false negative results (Figure 4.5A, panel 2). If the sample contains both RV IgG and RF IgM, immunocomplexes will form on the particles, leading to false positive results (Figure 4.5A, panel 3). Complicating matters, vendor-specific calibrators, which are formed from pooled human sera, often contain varying amounts of RF IgM or other cross-reactive species, depending on the source. To ascertain the presence of RF IgM in the calibrators used here, they were tested using an off-chip ELISA kit (Table 4.1)—the RV IgG calibrators have low levels of RF IgM (1.4 to 1.85 IU/mL), while the RV IgM cutoff calibrator has high levels of RF IgM (27.0 IU/mL).

Fortunately, errors related to the presence of endogenous RV IgG and RF IgM (Figure 4.5A) can be alleviated by pre-treating the sample with exposure to exogenous anti-human IgG
prior to analysis, which serves to block unwanted binding of RV IgG (and RV IgG/RF IgM complexes) onto the virus-laden particles.\textsuperscript{261-263} Emulating the ARCHITECT rubella IgM assay procedures, we developed an on-chip pretreatment method, in which a droplet of sample (1.8 µL) is mixed with a droplet of pretreatment reagent (900 nL, containing goat anti-human IgG) for 7 minutes before analysis by on-chip ELISA. To evaluate this method, we used test samples (formed from calibrators) containing RV IgG (“IgG”), RV IgM (“IgM”), or both RV IgG and IgM (“IgG + IgM”). As expected, the “IgG” and “IgM” sample results were unaffected by the pretreatment (Figure 4.5B) because these samples did not contain high concentrations of RF IgM and RV IgG (Table 4.1). In contrast, the pretreatment significantly (P = 0.0003) suppressed the non-specific signal arising in the combined “IgG + IgM” sample, which (as per Table 4.1) contains substantial levels of RV IgG (250 IU/mL) and RF IgM (~14 IU/mL). Thus, accurate diagnosis of rubella infection requires IgG blocking, which is straightforward to implement in digital microfluidics.

\textbf{Figure 4.5: Rubella virus IgM immunoassay}
(A) Scheme of rubella virus (RV) IgM immunoassay (panel 1) and potential interferences or non-specific interactions arising from the presence of RV IgG (panel 2) and rheumatoid factor IgM (panel 3). (B) Bar graph illustrating the effect of on-chip sample pretreatment (gray bars) relative to no pretreatment (white bars) in RV IgM immunoassays. (C) Diagnostic cutoff calibrator signal for RV IgM immunoassays implemented in digital microfluidics with sample pretreatment. Each value represents the mean +/- standard deviation from 3 to 4 replicates. Statistical significance (P <0.05*) was evaluated by Student’s two-tailed t-test.

<table>
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<th>Calibrator</th>
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<tr>
<td>A - 0 IU/mL RV IgG</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>B - 5 IU/mL RV IgG</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>C - 15 IU/mL RV IgG</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>D - 75 IU/mL RV IgG</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>E - 250 IU/mL RV IgG</td>
<td>1.3 ± 0.0</td>
</tr>
<tr>
<td>F - 500 IU/mL RV IgG</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>1 - cutoff RV IgM</td>
<td>27.0 ± 2.0</td>
</tr>
</tbody>
</table>

4.3.4 Evaluation of diagnostic accuracy

The diagnostic accuracies of the digital microfluidic RV IgG and IgM immunoassays were estimated using 25 plasma/serum samples from a commercial rubella mixed titer performance panel (Figure 4.6). These are undiluted aliquots of plasma or serum collected from individual donors between 1994 and 1995 by the vendor, used by diagnostic laboratories and manufacturers to evaluate their rubella tests. For each sample, the vendor provided reference results (signal-to-cutoff values and test interpretation) from tests performed by the vendor and independent reference laboratories (between 1995 and 1996) using several leading ELISA platforms at that time. In this study, the Abbott EIA test was chosen as the reference standard because test results were provided for both RV IgM and IgG.

In estimating diagnostic performance, all 25 samples were divided and tested for both RV IgG and IgM in three to four replicates to ascertain assay reproducibility. Typically, four parallel immunoassays, requiring approximately 25 (for RV IgG assays) or 35 minutes (for RV IgM assays), were carried out in single- (only RV IgG or IgM in quadruplicates) or duplex- (both RV IgG and IgM in duplicates) format. The average intra-assay CV (range) was 4.6% (0.4-17.5) for RV IgG assays and 7.5% (1.5-13.4) for RV IgM assays. In the RV IgG assays (Figure 4.6A), the DMF tests identified 23 positives, 0 equivocal, and 2 negatives, which represents perfect
agreement with the Abbott EIA results. The diagnostic sensitivity and specificity (with 95% CIs) were 100% (98.6-100) and 100% (95.5-100), respectively (Table 4.2). In the RV IgM assays (Figure 4.6B), the DMF tests identified 5 positives, 1 equivocal, and 19 negatives, which represents close agreement with the Abbott EIA results (two mismatches: one DMF-equivocal/Abbott-positive, and one DMF-positive/Abbott-equivocal). Sensitivity and specificity are not defined for equivocal results, so we estimated these parameters in three ways: (i) 100% (96.8-100) and 100% (98.5-100), respectively if the equivocal results were omitted; (ii) 100% (97.4-100) and 100% (98.4-100), respectively if the equivocal results were interpreted as positive; and (iii) 80% (44.9-100) and 95% (85.4-100), respectively if the equivocal results were interpreted as negative (Table 4.3).

The measured s/co for all 25 samples tested are shown in Figure 4.6C. For the two mismatches in the RV IgM assay, the DMF values (s/co = 0.9 and 1) were at or near the cutoff value (s/co = 1). Thus, the discrepancy between the DMF and the standard results may be caused by a combination of assay variability (CV < 15%) and sample degradation of the 20-year-old specimens. In any case, mis-matches between positive and equivocal are the “best” kind to observe (rather than mismatches with negative). In standard practice, all patients who test positive or equivocal are retested to ascertain final diagnosis, which is then used to determine the course of action for pregnancies at risk of CRS.292
Figure 4.6: Performance of rubella virus IgG and IgM immunoassay on commercial plasma/serum panel

Plots of signal-to-cutoff (s/co) values for digital microfluidic (DMF) rubella virus (RV) IgG (A) and IgM (B) immunoassays from RV mixed titer panel (Seracare PTR201). Each DMF s/co value represents the mean from 3 to 4 replicates and is categorized as positive (pos), equivocal (equ), or negative (neg) based results from a leading commercial RV antibody immunoassay (Abbott EIA) determined by the supplier. DMF s/co values in the shaded region was interpreted as equivocal. The inset plots show the receiver operating characteristic (ROC) curves for DMF assays (red lines) and random guesses (dashed lines). The area under the ROC curve (AUC) is reported in each plot. (C) Table comparing the results of the DMF and Abbott EIA assays. The s/co and interpretation of the Abbott EIA assays were supplied by Seracare. Shaded box indicates discrepancy in DMF assay with Abbott EIA.
Table 4.2: Cross tabulation of RV IgG immunoassay results

<table>
<thead>
<tr>
<th>RV IgG</th>
<th>Abbott EIA</th>
<th></th>
<th></th>
<th>TP+FP</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMF</td>
<td>Positive</td>
<td>Equivocal</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Equivocal</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>FN+TN</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>TP+FN</td>
<td>23</td>
<td>FP+TN</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3: Cross tabulation of RV IgM immunoassay results

<table>
<thead>
<tr>
<th>RV IgM</th>
<th>Abbott EIA</th>
<th></th>
<th></th>
<th>TP+FP</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMF</td>
<td>Positive</td>
<td>Equivocal</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Equivocal</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>FN+TN</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>0</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>TP+FN</td>
<td>4</td>
<td>FP+TN</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

4.4 Conclusion

The performance of the new DMF test (Figure 4.6) rivals that of central laboratory-based RV testing. This is not surprising, as DMF (like automated immunoassay platforms) is automated and software programmable, allowing for integration of operations like aliquoting, dilution, or IgG blocking as needed in an immunoassay workflow. More importantly, when compared to the only “rapid” rubella IgM test that we are aware of for which the manufacturer reports sensitivity and specificity (Alere ImmunoComb® Rubella IgM, sensitivity/specificity 87%/99%), the DMF method has the added value of built-in, automated replicates and digital readout (i.e., no user interpretation of results). Of course, additional testing must be done to further validate the diagnostic performance of the new method, but these initial results suggest that a portable DMF-based system, perhaps combined with inexpensive, paper devices, may represent a useful new tool for identification of patients at risk for CRS in low-resource settings. More generally, we propose that similar methods might be useful for distributed diagnostics for a wide range of infectious diseases.
Chapter 5

5  Digital Microfluidic Immunocytochemistry in Single Cells

We describe the development of a new method, called digital microfluidic immunocytochemistry in single-cells (DISC), which can stimulate and fix adherent cells for single-cell immunoassays in situ. Digital microfluidic (DMF) was used to automate protocols for cell culture, stimulation, and immunocytochemistry. The use of DMF facilitated rapid stimulation and fixation of cells, enabling the interrogation of protein phosphorylation in the seconds to minutes timescale. Fixed and stained cells on the device were scanned using a microarray reader, which provided the capability to resolve signalling responses in heterogeneous cell populations. Using this technique, we interrogated the phosphorylation state of PDGFR and Akt in NIH 3T3, MCF-7, and MDA-MB-231 cell lines, evaluating different time-, frequency-, and concentration-dependent effects of PDGF stimulation. These studies revealed detailed kinetic behaviour and population heterogeneity of PDGF receptor stimulation. In particular, the DISC platform was able to capture the early dynamics of receptor phosphorylation with 15 second resolution. Interestingly, 10 second stimulation of cells with low concentrations of ligand was sufficient to commit >25% of cells to activate Akt, downstream of the receptor. Furthermore, the DISC platform was able to show that breast cancer cell lines have dramatically different numbers of hyper-responders to PDGF stimulation and the number of hyper-responders may correlated to the cancer cells phenotype. Unlike flow cytometry, this technique evaluates and visualizes adherent cells in situ, eliminating the need for disruptive disassociation protocols. We propose that DISC may be an important new technique for probing temporal dynamics of signaling in single cells for a wide range of applications.

5.1  Introduction

Elucidating the mechanisms that regulate cell function and fate requires the measurement of signalling events in response to perturbation. These mechanisms can be difficult to study as they involve networks of diverse biochemical reactions that occur in a range of timescales. For example, early signalling events such as cell surface-receptor activation occur within seconds to
minutes after stimuli, while the subsequent activation of intracellular signalling molecules and their translocation to the nucleus occurs in minutes to hours. It is important to investigate such effects in detail, as a given signalling ligand or molecule can lead to diverse cellular responses depending on the frequency, concentration, and duration of the stimulus. Finally, most of what is known about cell signalling has been gleaned from evaluating the average responses from large populations of cells; however, each individual cell can encode and decode important information through the dynamics of cell-specific signalling. Thus, there is a great need for tools capable of dissecting the mechanisms and dynamics of single-cell signalling with high temporal resolution.

There are several platforms that have been recently developed to evaluate single-cell signalling dynamics, relying on either flow-based microfluidics or flow cytometry. Microfluidic systems enable the automated delivery of chemical stimulant to cells, and the resulting cellular responses can be imaged in real-time using green fluorescent protein (GFP) reporters, fluorogenic calcium chelators, or fluorescence resonance energy transfer (FRET) via live cell microscopy. The use of microfluidics represents a significant milestone in the study of cellular temporal dynamics, and has led to several seminal findings in yeast signalling pathways. However, there are a few limitations to these systems. First, to achieve fast switching of chemical stimulant (allowing for fine time resolution), high flow rates are needed, which (unfortunately) can increase shear stress, cause bubble formation (which can cause cell detachment), reduce viability, and result in unintended flow-stimulated signalling. Second, the serial nature of flow-based microfluidics typically requires that systems incorporate integrated PDMS-based valves to prevent cross-talk between cell culture chambers. This means devices can be tedious to set up and operate, and great care must be taken to enable cell attachment, prevent adsorption of biomolecules, and minimize toxicity of uncured PDMS. Third, since single cells are imaged one at a time, real-time microscopy has inherently low throughput relative to flow cytometry. Finally, the use of fluorescent fusion proteins requires genetic modification of the cells, which is not suitable for tracking most post-transcriptional modifications such as phosphorylation.

The alternative to microfluidics for probing single-cell signalling dynamics, flow cytometry, is a powerful technique that has been used for several decades and has recently been adapted to measure phosphorylation events in signal transduction. Despite these advances,
flow cytometry suffers from three key limitations. First, it requires a relatively large number cells compared to other techniques. Second, cells must be in a disaggregated, single-cell suspension. Therefore, adherent cells or solid tumor samples must be mechanically or enzymatically dissociated into a single-cell suspension, which can disrupt native cell-cell and cell-matrix connections and cause unintended perturbation of cell signalling. Third, before flow cytometry is used for analysis, significant sample preparation is required including, cell stimulation (for various durations and/or concentrations of stimuli), fixing, permeabilization, blocking, and staining. This is a labour-intensive and expensive process that requires numerous manual pipetting steps in well plates, representing a major bottleneck in analysis workflow.

To address the challenges described above, we report here a new method called digital microfluidic immunocytochemistry in single cells (DISC), which can automate all the sample preparation steps required to perturb and analyze early-stage signalling in adherent single cells \textit{in situ} (Figure 1). This system has a similar ability to rapidly change ligand concentrations as channel microfluidic systems but without high levels of shear stress. It can also generate single cell population data similar to flow cytometry but in an automated fashion with \textit{in situ} adherent cells. This method relies on three technologies: digital microfluidics, immunocytochemistry, and laser scanning cytometry. Digital microfluidics (DMF) is a non-flow channel based technique that electrostatically manipulates discrete droplets (pL to μL-sized) of reagents with little to no dead volume, on an open surface (wall-less) bearing an array of insulated electrodes. Multiple droplets can be automatically moved in parallel by applying pre-programmed sequences of potentials to the electrodes, enabling the delivery of versatile stimulation patterns to cells with media switching in the order of seconds. DMF is used to seed, and culture the adherent cells, deliver stimuli, and carryout immunocytochemistry.\textsuperscript{210,321} These processes are enabled using microfabricated hydrophilic sites in the device top-plate for rapid and precise metering of reagents to the cells cultured in self-contained droplets known as virtual microwells (Figure 1A,B).\textsuperscript{322} Because the top plate (bearing the cells) is in the format of a typical microscope slide, the fixed and stained cells can be readily imaged via laser scanning cytometry in a microarray reader\textsuperscript{323} for quantitative analysis of proteins in single cells (Figure 1C). The images are processed by image analysis software to identify the cells and extract quantitative measurements of fluorescence intensity and cell size (Figure 1D).
Using the DISC method, we studied the phosphoinositide 3-kinase (PI3K)/Akt signalling pathway downstream of platelet derived growth factor (PDGF) stimulation of PDGF receptor (PDGFR). PDGFR belongs to the receptor tyrosine kinase (RTK) family of cell-surface receptors, which regulate key cellular processes including proliferation, differentiation, metabolism, and migration.\textsuperscript{324} In particular, PDGFR activates several well-characterized signalling cascades involved in embryogenesis and angiogenesis, and is implicated in several types of cancer such as glioblastoma multiforme.\textsuperscript{325} As shown in Figure 1E, upon PDGF binding, the auto-phosphorylation of PDGFR forms the binding sites for PI3K which phosphorylates the lipid PI(4,5)P to PI(3,4,5)P. The PI(3,4,5)P lipid then recruits Akt to the plasma membrane, allowing for its phosphorylation (activation) and subsequent regulation of cell survival, proliferation, and migration. The PDGFR/Akt signalling pathway was used to develop and validate the DISC platform for studying signalling cascade dynamics and the heterogeneity of cellular responses. These studies revealed detailed kinetic behaviour and population heterogeneity of PDGF receptor stimulation. In particular, the DISC platform was able to capture the early dynamics of receptor phosphorylation with 15 second resolution. Interestingly, 10 second stimulation of cells with low concentrations of ligand was sufficient to commit >25% of cells to activate Akt, downstream of the receptor. Furthermore, the DISC platform was able to show that breast cancer cell lines have dramatically different numbers of hyper-responders to PDGF stimulation and the number of hyper-responders may correlated to the cancer cells phenotype. We propose that DISC may be an important new technique for probing temporal dynamics of signaling in single cells for a wide range of applications.

5.2 Experimental

5.2.1 Reagents and materials

Unless otherwise specified, reagents were purchased from Sigma Chemical (Oakville, ON). Deionized (DI) water had a resistivity of 18 MΩ•cm at 25°C. 10% Pluronic F68 solution, Dulbecco modified Eagle medium (DMEM), and Dulbecco’s Phosphate-Buffered Saline (DPBS) with no calcium and magnesium (-/-) were purchased from Life Technologies (Carlsbad, CA). Antibodies were purchased from Santa Cruz Biotechnology (SCB) (Dallas, TX) and Cell Signaling Technologies (CST) (Danvers, MA). Clean room reagents and supplies included S1811 photoresist and MF-321 photoresist developer from Rohm and Haas (Marlborough, MA),
CR-4 chromium etchant from Cyantek (Fremont, CA), AZ-300T photoresist stripper from AZ Electronic Materials (Somerville, NJ), Teflon-AF from DuPont (Wilmington, DE), and Parylene C dimer and Silane A174 from Specialty Coating Systems (Indianapolis, IN).

5.2.2 Macroscale cell culture

NIH 3T3 cells were grown in DMEM containing 100 U/mL penicillin G and 100 µg/mL streptomycin supplemented with 10% calf serum (CS) in a humidified incubator at 37°C with 5% CO₂. The MCF-7 and MDA-MB-231 cell lines were grown in DMEM containing 100 U/mL penicillin G and 100 µg/mL streptomycin supplemented with 10% fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO₂. With the exception of the cell density optimization studies, all DMF experiments were seeded with a cell density of 500 000 cells/mL.

5.2.3 On-chip reagent composition

On-chip cell culture reagents including culture media (10% serum [CS or FBS] and 0.05% v/v Pluronic F68), starving media (0.5% serum [CS or FBS] and 0.05% v/v F68), and serum-free media (0.05% v/v F68), were formed in DMEM containing 100 U/mL penicillin G and 100 µg/mL streptomycin. Immunocytochemistry reagents including fixing solution (FS) (4% paraformaldehyde and 0.05% v/v F68), wash buffer 1 (WB1) (0.05% v/v F68), wash buffer 2 (WB2) (0.05% v/v Brij 35), blocking solution (BS) (1% bovine non-fat dried milk and 0.05% v/v Brij 35), and permeabilization solution (PS) (0.2% v/v Tween-20), were formed in DPBS. Primary antibody staining solutions for Akt [comprising pan Akt (Mouse mAb, 1:50, CST) and p-Akt S473 (Rabbit mAb, 1:200, CST)], PDGR-β [comprising PDGR-β (Mouse mAb, 1:50, CST) and p-PDGR-β Y857 (Rabbit pAb, 1:200, SCB) or p-PDGR-β Y1021 (Rabbit pAb, 1:200, SCB)], and GAPDH (Rabbit pAb, 2µg/mL, SCB) were formed in BS. Secondary antibody staining solution, comprising Anti-Mouse IgG Alexa Fluor 647 (Fab2, 1:500, CST) and Anti-Rabbit IgG Alexa Fluor 555 (Fab2, 1:500, CST), was formed in BS. Secondary antibody staining solution for signal/background optimization, containing the Anti-Rabbit IgG Cy3 (Goat pAB, 1:100, Life Technologies), was formed in BS.

5.2.4 Device fabrication and operation

Digital microfluidic devices, each comprising a bottom plate and top plate (Figure 1), were fabricated in the University of Toronto Nanofabrication Centre (TNFC) cleanroom facility, using
transparent photomasks printed at 20,000 DPI (Pacific Arts and Designs Inc., Markham, ON). The bottom plates of DMF devices bearing an array of electrodes were formed by photolithography and wet etching, as described previously. After forming electrodes, the substrates were primed for parylene coating by immersing in silane solution (2-propanol, DI water, and A-174 50:50:1 v/v/v, 10 min) and curing on a hot plate (80°C, 10 min). After rinsing and drying, contact pads of the devices were covered with dicing tape (Semiconductor Equipment Corp., Moorpark, CA). Next, the devices were coated with ~7 µm of Parylene C (vapor deposition) and ~200 nm of Teflon-AF (spin-coating, 1% w/w in Fluorinert FC-40, 2000 rpm, 60 s), and post-baked on a hot plate (165°C, 10 min). The polymer coatings were removed from contact pads by peeling off the dicing tape.

The top plates of DMF devices, bearing hydrophilic sites for cell culture, were formed by a fluorocarbon lift-off technique on indium-tin oxide (ITO) coated glass substrates (Delta Technologies Ltd, Stillwater, MN). Prior to fabrication, ITO substrates (3” × 1”) were immersed in a cleaning solution comprising DI water, NH₄OH, and H₂O₂ (5:1:1 v/v/v) on a hot plate (30 min, 80°C). After thorough rinsing with DI water and drying with nitrogen gas, S1811 was spin-coated (3000 RPM, 45 seconds) on ITO substrates and baked on a hot plate (2 min, 95°C). The photoresist-coated ITO substrates were exposed to UV light through a photomask using a Suss MicroTec mask aligner (29.8 mW/cm², 10 seconds). The exposed substrates were developed in MF-321 (3 min), rinsed with DI water, and dried under a gentle stream of nitrogen. Subsequently, the developed substrates were exposed (without photomask) to UV light (29.8 mW/cm², 10 seconds). After forming the photoresist patterns, the substrates were spin-coated with Teflon-AF (1% wt/wt in FC40, 4000 RPM, 30 sec), baked on a hot-plate (2 min, 165°C), and immersed in acetone until liftoff occurred (~ 5-10 sec), exposing defined patterns of ITO hydrophilic sites or “lift-off spots”.

Prior to assembly of a DMF device, one pair of top and bottom plates was sterilized by immersing in 70% ethanol (10 min) and then air dried. The hydrophilic sites (exposed ITO) on the top plate were aligned visually to the center of the electrode array on bottom plate, and the two plates were joined by a spacer formed from two pieces of Scotch double-sided tape (3M, St. Paul, MN) or double coated white polyester diagnostic tape 9965 (3M, St. Paul, MN) with total spacer thickness of ~180 µm. The bottom plate device design featured an array of 80 actuation electrodes (2.2 × 2.2 mm ea.) connected reservoir electrodes for reagent storage and waste
removal. The actuation electrodes were roughly square with interdigitated borders (140 µm peak to peak sinusoids) and inter-electrode gaps of 30-80 µm. Unit droplet and reservoir droplet volumes on these devices were ~900 nL and ~6 µL, respectively. The top plate design featured a row of 8 lift-off spots (1.75mm diameter, 4.4 mm pitch). The virtual microwells dispensed on these lift-off spots were ~400 nL.

An in-house designed computer-controlled instrument powered by DropBot, an open-source digital microfluidic (DMF) automation system, was used to manage and record droplet operations. Droplets were actuated by applying a preprogrammed sequence of driving voltages (80-100 V$_{\text{RMS}}$ 10 kHz sine wave) between the top plate (ground) and electrodes in the bottom plate through a Pogo pin interface (90 pins). Reagents were loaded to their respective reservoirs by pipetting the reagent adjacent to the gap between the bottom and top plates and actuating the reservoir electrodes. Once inside the reservoirs, droplets were actively dispensed by electrostatically stretching the droplets from the reservoirs followed by splitting (Figure 1A). In addition to active dispensing, the devices also relied on a phenomenon known as passive dispensing. To initiate passive dispensing, a source droplet is translated across a vacant lift-off spot and a sub-droplet or “virtual microwell” is spontaneously formed because of hydrophobic-hydrophilic interactions. Once a virtual microwell is formed, subsequent passive dispensing will displace the contents of the virtual microwell with the contents of the new source droplet. Waste and unused fluids were removed by delivering them to reservoirs containing KimWipes (Kimberly-Clark, Irving, TX).

5.2.5 Digital microfluidic cell culture and immunocytochemistry

Digital microfluidics was used to automate the protocols required for cell culture and immunocytochemistry including cell seeding, starving, ligand stimulation, fixation, permeabilization, blocking, staining, and washing. To seed cells, three unit droplets (2.7 µL) of cells in culture media were passively dispensed on each vacant lift-off spot, forming virtual microwells of ~400 nL. The device was inverted and incubated in a homemade humidified chamber at 37°C for 24 hours, allowing cells to settle and adhere to the hydrophilic sites. In the next eleven steps, a sequence of reagents and wash buffers was sequentially passively dispensed on each virtual microwell; unless otherwise stated, droplet operations and incubations were performed in room temperature. 1) Cells were starved by passively dispensing 5.4 µL of starving
media and incubating at 37°C for 24 hours. 2) The starving media was rinsed by passively dispensing 5.4 µL of serum-free DMEM. 3) Cells were stimulated by passively dispensing two unit droplets (1.8 µL) containing PDGF-BB in serum-free DMEM; the volume, concentration, frequency and duration was varied depending on the experiment. 4) Immediately after stimulation, cells were fixed by passively dispensing 2.7 µL of FS and incubating for 10 minutes. 5) The FS was rinsed by passively dispensing 5.4 µL of WB1. 6) The fixed cells were permeabilized by passively dispensing 2.7 µL of PS and incubating for 10 minutes. 7) The PS was rinsed by passively dispensing 8.1 µL of WB2. 8) The rinsed cells were blocked by passively dispensing 2.7 µL of BS and incubating for 1 hour. 9) The blocked cells were stained with primary antibodies by passively dispensing 2.7 µL of primary antibody solution and incubating overnight in 4°C. 10) The unbound primary antibodies were removed by passively dispensing 5.4 µL of WB2. 11) The primary antibodies were stained by secondary antibodies by passively dispensing 2.7 µL of secondary antibody solution and incubating 2 hours.

Subsequently, the top plate was dissembled from the device, rinsed in a falcon tube containing 10x dilution of DPBS containing 0.5 % Tween-20 for 30 seconds, rinsed in another falcon tube containing DI water for 30 seconds, and air dried in the dark. In time series experiments, droplet sequences in step 3 were staggered so that step 4 was performed simultaneously for all virtual microwells in one device—this prevents cells from being exposed to paraformaldehyde fumes in step 3.

5.2.6 Microarray scanner analysis

Top plates bearing stained cells were analyzed in an Axon GenePix 4000B microarray scanner (Sunnyvale, CA). Typically, the scanner was set at 5 µm resolution, 0 µm focus, with 532 nm and 635 nm excitation lasers set at 100% power and the two PMT potentials set at 500 V. These settings were adjusted accordingly to maximize signal without saturating the PMTs. For example, in some instances, the power of the 532 nm laser was decreased to 10% to prevent signal saturation. In other instances, the PMT voltage for the Cy5 channel was increased to 600V when the signal was weak. The resulting images were cropped in ImageJ and analyzed using the open-source CellProfiler software package (http://www.cellprofiler.org/). A custom pipeline was developed, including image cropping, identifying single-cell regions of interest from whole Akt or PDGFR-R fluorescent images (Cy5 channel), measuring sizes of the cells, and measuring
fluorescence intensities of both phosphorylated (Cy3 channel) and non-phosphorylated (Cy5 channel) Akt or PDGFR-R. To identify cells, the software was instructed to detect 15-100 µm diameter objects using the Otsu Global thresholding method (two classes, weighted variance, 0.7 threshold correction factor). For each cell, the fluorescence intensity from the phosphorylated protein was normalized to the fluorescence intensity from the corresponding whole protein (phosphorylated + non-phosphorylated) to account for differences in single-cell protein expression level. To express as fold change, the each data point was further normalized to the average signal from negative control cells on the same device, which were exposed to a droplet containing only serum-free DMEM (i.e., no stimulation).

5.2.7 Washing efficiency

The washing efficiency of passive dispensing was evaluated using a soluble fluorescent tracker. First, an on-chip calibration curve was generated using various concentrations of fluoresceinamine (5 µM, 0.5 µM, 0.05 µM, 0.005 µM, and 0 µM) in WB1. These solutions were passively dispensed on vacant lift-off spots to form ~400 nL virtual microwells. The fluorescent intensities in the virtual microwells were measured using a Typhoon 9400 (GE Healthcare Bio-Sciences, Pittsburgh, PA) scanner using 520 BP 40 emission filter, blue laser, +3mm focus, and 450 V PMT voltage. Second (in separate experiments), 5 µM fluoresceinamine was passively dispensed onto vacant lift-off spots and measured as above (wash 0). Next, two unit droplets (1.8 µL each) of WB1 were passively dispensed across each liftoff site and the resulting (diluted) virtual microwells were measured as above (wash 1). This was repeated three times (wash 2, 3, and 4), and the fluorescence intensities were extrapolated to fluoresceinamine concentration using the calibration curve. Finally, the concentrations were normalized to the maximum concentration and plotted against the wash number.

5.2.8 Optimization experiments

The method was optimized for cell density and signal/background ratio using a variation of the 11-step protocol on NIH-3T3 cells. In cell density optimization experiments, prior to step 1, cell suspensions at different densities (10³, 10⁴, 10⁵, 10⁶ cells/mL) were seeded and cultured overnight as described above. The cells were imaged in brightfield using 20× objective on a Leica DM2000 microscope (Leica Microsystems, Inc., Concord, ON, Canada). Steps 1-3 were omitted, and in steps 9 and 11, cells were stained with GADPH primary antibody and anti-Rabbit
IgG Cy3 secondary antibody. For signal/background optimization, prior to step 1, cell suspensions at $10^6$ cells/mL were seeded and incubated overnight as above. Steps 1-3 were again omitted, and in steps 9 and 11, cells were stained with GAPDH primary antibodies and anti-Rabbit IgG Cy3 secondary antibodies. Control experiments were identical, except that BS was used in place of primary antibodies in step 9. In subsequent experiments, cells were seeded at a density of 500,000 cells/mL, yielding approximately 150 to 300 cells in each virtual microwell.

5.2.9 Dose-response data

PDGF-BB dose-response curves were collected for PDGFR (both Y857 and Y1021) and Akt using the 11-step protocol on NIH-3T3 cells. In step 3, 1.8 µL droplets of PDGF-BB (0, 3.125, 6.25, 12.5, 25, 50, 200 ng/mL) were passively dispensed to virtual microwells and incubated for 2 minutes (for PDGFR) or 5 minutes (Akt) prior to continuing with step 4. In step 9, cells were labeled with primary antibodies for PDGR-β (Mouse) and p-PDGR-β (Rabbit) or for Akt (Mouse) and p-Akt (Rabbit). In step 11, cells were stained with secondary antibodies including anti-Mouse IgG Alexa Fluor 647 and anti-Rabbit IgG Alexa Fluor 555. To determine the Hill coefficient, the average signal from negative control cells was subtracted from the average signal from cells stimulated by various concentration of PDGF-BB on the same device. The background subtracted data was normalized to the highest average response on the same device. The normalized, background subtracted data was fit to a Hill function:

\[
y = \frac{x^n}{K^n + x^n}
\]

where $y$ is the proportion of activated phosphorylation site, $x$ is the concentration of PDGF-BB, $K$ is the concentration of PDGF-BB required for half maximal phosphorylation, and $n$ is the Hill coefficient, describing the cooperativity of ligand induced activation of receptor.

5.2.10 High and low concentration time series

High and low stimulation concentration/time series responses were collected for PDGFR Y857 and Y1021 using the 11-step protocol on NIH-3T3 cells seeded. In step 3, 1.8 µL droplets of 3.125 or 50 ng/mL PDGF-BB were passively dispensed to the virtual microwells and incubated for various durations (0, 30, 45, 60, 75, 90, 105, or 120 seconds) prior to continuing with step 4. In step 9, cells were labeled with primary antibodies for PDGR-β (Mouse) and p-PDGR-β
(Rabbit); in step 11, cells were stained with secondary antibodies including anti-Mouse IgG Alexa Fluor 647 and anti-Rabbit IgG Alexa Fluor 555.

5.2.11 Inhibitor time series

Inhibitor time series responses were collected for PDGFR Y857 using a variation of the 11-step protocol on NIH-3T3 cells. In step 2, the starved cells were rinsed in serum-free DMEM supplemented with 10 mM sodium orthovanadate, and in step 3, cells were stimulated with 1.8 µL of 3.125 ng/mL PDGF-BB supplemented with 10 mM sodium orthovanadate and were allowed to incubate for various durations (0, 120, 180, 240, 300, 360, or 420 seconds) prior to continuing with step 4. In control experiments, sodium orthovanadate was not included in the rinsing and stimulation reagents. In step 9, cells were labeled with primary antibodies for PDGR-β (Mouse) and p-PDGR-β (Rabbit); in step 11, cells were stained with secondary antibodies including anti-Mouse IgG Alexa Fluor 647 and anti-Rabbit IgG Alexa Fluor 555.

5.2.12 Ligand renewal time series

Ligand renewal time series responses were collected for PDGFR Y857 using a variation of the 11-step protocol on NIH-3T3 cells. The standard step 3 was replaced with step 3a and step 3b. In step 3a, cells were stimulated with 1.8 µL of 3.125 ng/mL PDGF-BB and were allowed to incubate for 90 seconds. In step 3b, cells were stimulated again with 1.8 µL of 3.125 ng/mL PDGF-BB and were allowed to incubate for an additional duration (30, 90, 150, 270, or 330 seconds) prior to continuing with step 4. In control experiments, only step 3a was performed with 1.8 µL of 3.125 or 6.25 ng/mL PDGF-BB, followed by step 4. In step 9, cells were labeled with primary antibodies for PDGR-β (Mouse) and p-PDGR-β (Rabbit); in step 11, cells were stained with secondary antibodies including anti-Mouse IgG Alexa Fluor 647 and anti-Rabbit IgG Alexa Fluor 555.

5.2.13 Ligand removal

Ligand removal responses were collected for PDGFR Y857 and Akt using a variation of the 11-step protocol on NIH-3T3 cells seeded. The standard step 3 was replaced with step 3i and step 3ii. In step 3i, cells were stimulated with 1.8 µL of 3.125 ng/mL PDGF-BB and were allowed to incubate for 10 or 30 seconds. In step 3ii, ligand was depleted by delivering 1.8 µL of serum-free medium and cells were allowed to incubate for an additional duration (110 or 90 seconds, for
PGDFR or 290 or 270 seconds, for Akt) prior to continuing with step 4. In control experiments, the standard step 3 was performed, either with serum-free medium (negative control) or 3.125 ng/mL PDGF-BB (positive control), followed by incubation for 2 min (for PDGFR) or 5 min (for Akt) prior to continuing with step 4. In step 9, cells were labeled with primary antibodies for PDGR- β (Mouse) and p-PDGR- β (Rabbit); in step 11, cells were stained with secondary antibodies including anti-Mouse IgG Alexa Fluor 647 and anti-Rabbit IgG Alexa Fluor 555.

5.2.14 Cancer cell time series

Time series responses were collected for Akt using the 11-step protocol on MCF-7 and MDA-MB-231 cancer cells lines. In step 3, 1.8 µL droplets of 50 ng/mL PDGF-BB were passively dispensed to virtual microwells and incubated for various duration (0, 1, 2, 3, 4, 5, 6, or 7 minutes) prior to continuing with step 4. In step 9, cells were labeled with primary antibodies for Akt (Mouse) and p-Akt (Rabbit); in step 11, cells were stained with secondary antibodies including anti-Mouse IgG Alexa Fluor 647 and anti-Rabbit IgG Alexa Fluor 555.

5.2.15 Statistical analysis

A one-way analysis of variance with Bonferroni post-test analysis was applied to compare means between multiple time points. Data were considered statistically significant at p<0.05. All analysis was done with GraphPad Prism software (GraphPad, La Jolla, CA).

5.3 Results

5.3.1 DISC development and optimization

DISC was developed to address the limitations in current techniques that are used to evaluate signalling in single cells. The DISC device consists of two parallel plates that are 180 µm apart (Figure 1A,B). The bottom has an array of electrode that act as reagent reservoirs and can manipulate droplets of reagents to perform immunocytochemistry assays. The top plate contains 8 hydrophilic sites or “lift-off spots” which are used to form virtual microwells (VM). The VMs contain the cells that are stimulated, fixed and labeled with antibodies for phosphorylated proteins of interest (Figure 1B). The top plate is then removed from the device and analyzed by a microarray scanner (Figure 1C). The images generated are analyzed using CellProfiler to calculate the ratio of each phosphorylated protein to total receptor in each cell (Figure 1D).
Figure 5.1: Digital microfluidic immunocytochemistry in single cells (DISC)

(A) Top-view schematic of a digital microfluidic device used for cell culture, stimulation, and immunocytochemistry. The expanded view illustrates the two primary fluid-handling operations, active and passive dispensing, required for metering and delivering reagents to cells cultured in virtual microwells. (B) Side-view schematic showing adherent cells cultured on the patterned top plate in a virtual microwell. The cells are sequentially treated with ligand and then probed for protein phosphorylation by immunocytochemistry. (C) Cartoon and microarray image illustrating how the top-plate (a 3 by 1 inch slide bearing fixed and stained cells) is transferred from the device to a microarray scanner for laser scanning cytometry. (D) Images and data illustrating how the scans are processed by CellProfiler (open-source cell image analysis software) to identify individual cells and extract quantitative data. For each cell, the fluorescent intensity from the phosphorylated protein is normalized to the fluorescent intensity from the corresponding whole protein (phosphorylated + non-phosphorylated) to account for differences in expression level. (E) Cartoon illustrating the mechanism of signal transduction in PDGFR/PI3K/AKT pathway.
The DISC platform enables the ability to perturb and analyze cells at high temporal resolution, an important property that most analysis systems are not capable of. In the DISC technique, we found that approximately 4 seconds is required between two reagent exchanges (Figure 2A). This hypothetically allows us to stimulate cells (droplet one) for 4 seconds before fixing with formaldehyde (droplet two) with minimal shear stress. A related capability is the ability to perform very efficient reagent exchange — using fluorescent tracers, we found that one reagent exchange can displace ~93% of the original solution in the virtual microwell, while two exchanges can displace >99% (Figure 2B).

As a first test, a constitutively expressed protein, GADPH, was stained and evaluated on-chip for various seeding densities of fibroblasts ranging from \(10^3\) to \(10^6\) cells/mL (Figure 2C). The results demonstrated that \(10^5\) cells/mL to \(10^6\) cells/mL is sufficient for analysis, yielding approximately 150 to 300 cells in each virtual microwell (area of 2.4 mm\(^2\)). This preliminary test allowed us to 1) confirm that single-cell detection is possible and 2) ensure that a sufficient signal (relative to background) can be achieved. As shown in Figure 2D, the GADPH signal (measured by labeling with \(1^\circ\) and \(2^\circ\) antibody) was significantly (at least 5x) higher than background signal (measured by labeling with \(2^\circ\) antibody only). This sensitivity improves further in the single cell analysis since the background noise outside of the cells is ignored. Compared to 96 well-plate assays, the DISC method uses 60 to 150x fewer cells (15,000 cells/well vs. 150-300 cells/virtual microwell) and significantly less working volume (200 µL vs. 400 nL). Although, we are interrogating fewer cells in the DISC platform, the ability to perform single cell analysis enables insight into cell population distribution.

5.3.2 DISC for cell signalling

To validate DISC as a method to study signal transduction we investigated the well-characterized PDGFR/PI3K/Akt pathway (Figure 1E). We profiled two PDGFR tyrosine phosphorylation sites (Y857 and Y1021) and one Akt serine phosphorylation site (S473) in NIH 3T3 fibroblasts arising from stimulation by various concentrations of PDGF.
Figure 5.2: Characterization of DISC method

(A) Time-stamped movie sequence showing passive dispensing of two successive droplets to a virtual microwell—approximately 4 seconds is required between two reagent exchanges. (B) A plot showing the efficacy of passive dispensing for removing fluoresceinamine from virtual microwells as a function of the number of rinse droplets. Error bars are +/- S.D. from 3 technical replicates. (C) Brightfield microscopy and microarray scans of NIH 3T3 fibroblasts seeded at various densities. (D) Bar graph of GAPDH staining (1° and 2° antibodies) and control (2° antibody only) showing the signal-to-background ratio of the optimized DISC protocol. The insets at the top are representative microarray scans. Error bars are +/- S.D. from 4 technical replicates.

Cell responses were first probed at the “bulk” or “average” level (with no differentiation between individual cells. As expected, there was a dose dependent increase in receptor phosphorylation with stimulation using increasing concentrations of PDGF-BB (left, Figure 3A,B). As shown, the phosphorylation of receptor tyrosine 857 and 1021 sites starts at a PDGF concentration of 3.13 ng/ml and increased with increasing concentrations of PDGF until 50 ng/ml of PDGF where it plateaus. Using this data the Hill coefficient was calculated to be 1.73±0.33 ($R^2 = 0.88$) and 2.13±0.44 ($R^2 = 0.89$) for Tyr-857 and Tyr-1021, respectively,
meaning that the receptor experiences cooperative ligand binding. These data are in line with the previously calculated Hill coefficient of 1.55 for the total tyrosine phosphorylation of the receptor. \(^{327}\) Again probing cell behaviour at the bulk level, the activation of the PDGFR was observed to lead to downstream activation of Akt (left, Figure 3C). Interestingly, the downstream response is amplified – for example, when stimulated with 3.125 ng/mL PDGF there is a ~22% activation of the receptor, while at the same stimulation the Akt activation is ~71%. This is consistent with the findings of Park et al.\(^{327}\), who observed that only 10% of maximum receptor phosphorylation is needed to achieve a 50% activation of Akt. Overall, the results shown in Figure 3 (left, A-C) are consistent with previous observations, suggesting that the DISC platform does not alter (or disrupt) the expected behaviour of PDGFR signalling pathways.

Data similar to that described above can be obtained using traditional methods of measuring phosphorylation states in cell populations, such as Western blots. But DISC was designed to dig deeper, evaluating cell-to-cell variability at the single-cell level. The phosphorylation states in individual cells can be presented as histograms (middle, Figure 3) and scatter plots (right, Figure 3) to highlight different aspects of the results. The histogram plots highlight the distribution of the cellular response. At ligand concentration of zero, all of the cells had the same basal level of phosphorylation, as expected. Stimulation with 3.125 ng/mL of PDGF caused phosphorylation of the receptor, shown by the rightward shift in the histograms, but the level of activation of the cells was still very similar between individual cells at both receptor phosphorylation sites (middle, Figure 3A,B). When PDGF concentration was increased to 50 ng/mL, there was increase in the distribution of phosphorylation response by the individual cells as shown by the increasing width of the histograms with both sites. The scatter plots can be used to easily identify important outlier populations such as hyper-responders that are shown in the middle panels of Figure 3 or non-responders. This information about how the individual cells respond to PDGF stimulation is lost with traditional western blot techniques.
Figure 5.3: Activation of PDGFR/PI3K/AKT pathway by PDGF-BB in NIH 3T3 cells
Dose-response plots of (A) PDGFR Y857 and (B) PDGFR Y1021 phosphorylation 2 min after stimulation, and (C) AKT phosphorylation 5 min after stimulation. Data points are expressed as fold changes with respect to cells exposed to a droplet containing only serum-free DMEM (i.e., no stimulation). The ‘Average’ plots (left) are average response +/- S.E.M. from an average of all of the cells in 3-4 virtual microwells performed on different days. Representative ‘Histograms’ (middle) and single cell ‘Scatter plots’ with population mean +/- 1 S.D. (right) reveal the effect of PDGF-BB dosage on cell population distribution. Histogram distributions were offset vertically for comparison.
5.3.3 DISC for time-resolved cell signaling

DISC was developed to allow for high temporal resolution of cell signaling processes – with stimulations as fast as 4 seconds prior to fixing and analysis. This is particularly useful when evaluating fast processes such as receptor phosphorylation. We evaluated PDGFR tyrosine phosphorylation using high and low ligand concentrations (Figure 4A,B). At high PDGF concentration (50 ng/mL) there is rapid phosphorylation of the receptor that has a maximal phosphorylation level at 75 sec. and 60 sec. after stimulation for the phosphorylation sites Tyr-857 and Tyr-1021, respectively; however, at the low PDGF concentration (3.125 ng/mL) there is a relative low steady state level of receptor phosphorylation at both the measured phosphorylation sites over time (Figure 4A,B). This behaviour is similar to what Park et al. reported, but in their work, the earliest time point was 2 min, preventing them from finding the time of maximal receptor phosphorylation.

The low level of receptor is interesting, and we wondered if this was due to signal regulation by phosphatase activity or if it was caused by low ligand to receptor ratio. To test this, the receptor was stimulated in the presence or absence of orthovanadate. As shown in Figure 4C, the inhibitor made little difference in the phosphorylation pattern, suggesting that the low level of phosphorylation is caused by low ratios of ligand-to-receptor and not phosphatase activity. Given this assumption, we hypothesized that adding a second stimulation of PDGF ligand should increase the phosphorylation of the receptor. As shown in Figure 4D, after a second bolus of 3.125 ng/mL was introduced at 90 sec., the phosphorylation of Tyr-857 did indeed increase to an intermediate level (shaded), between the level of phosphorylation arising from single stimulations at t=0 with 6.25 ng/mL (black) or 3.125 ng/mL (white) PDGF ligand.

The DISC platform can also be used to perform the converse experiment, in which ligand is washed away. For example, after an initial stimulation with 3.125 ng/mL PDGF for 10 seconds or 30 seconds, delivery of a ligand-free media droplet can rapidly remove most of the ligand (assuming that 93% of ligand is removed as in Fig. 2b, this translates to ~0.2 ng/mL PDGF in the virtual microwell left behind). These schemes, as well as negative control (no stimulation) and positive control (stimulation with no wash) are depicted as traces (i)-(iv) in Figure 4E. To determine if the wash steps alter receptor activation, receptor Y857 and Akt phosphorylation states were analyzed at 2 and 5 minutes, respectively for conditions (i)-(iv). As shown in Figure
Figure 4F, removing the ligand decreases the relative phosphorylation of receptor and Akt, but does not abolish the response completely. Moreover, the fast ligand wash (after 10 second stimulation) resulted in greater decrease in phosphorylation compared to the slow ligand wash (after 30 second stimulation). The data in Figure 4 (D, F, G) demonstrate the ability of the DISC to both add and remove ligand, allowing complex stimulation patterns to be studied. Interestingly, these observations suggest that a 10 or 30 seconds exposure of limiting amounts of ligand was sufficient to commit >25% or >43% of cells to activate Akt downstream, 5 minutes later.
Figure 5.4: Time-resolved DISC results for phosphorylation by PDGF-BB ligand stimulation in NIH 3T3 fibroblasts.

Plots showing (A) Y857 and (B) Y1021 phosphorylation in cells stimulated for 0-120 sec with high (50 ng/mL, squares) or low (3.125 ng/mL, circles) concentrations of PDGF-BB. (C) Plot showing Y857 phosphorylation in cells stimulated for 0-420 sec with 3.125 ng/mL PDGF-BB in the presence (inh+, circles) or absence (inh-, squares) of phosphatase inhibitor sodium orthovanadate. (D) Plot showing Y857 phosphorylation as a function of time in cells stimulated
with 6.25 ng/mL PDGF-BB at only t=0 (black), 3.125 ng/mL PDGF-BB at t=0 and again at t=90 seconds (shaded), or 3.125 ng/mL PDGF-BB at only t=0 (white) [+- S.E.M from at least 5 wells (virtual microwells) performed on different days; *p<0.05, **p<0.01, ***p<0.001]. (E) Plots of four stimulation schemes representing cells treated with (i) 0 ng/mL PDGF-BB (negative control), (ii) 3.125 ng/mL with wash at 10 seconds after stimulation, (iii) 3.125 ng/mL with wash at 30 seconds after stimulation, and (iv) 3.125 ng/mL without wash (positive control). Histograms showing the corresponding response to each stimulation (i-iv) for Y857 phosphorylation at 2 min (F) and Akt phosphorylation at 5 min (G). The dotted line in (G), at 1.5 p-Akt S473 fold change, represents the threshold of Akt activation, chosen such that the negative control has >95% of cells inactivated. The percent values listed in (G) indicate percentage of cells with inactive Akt. Data in kinetic plots (A-C) are average fold changes with respect to zero (no stimulation) +/- S.E.M from at least 3 wells (virtual microwells) performed on different days. Histogram distributions (F-G) were generated from at least 3 different day replicates, and were offset vertically for comparison.

As a final application for DISC, we evaluated the ability to study the heterogeneous signaling responses in different cell types, a topic of particular importance in the understanding of cancer biology and chemotherapy or drug efficacy.295 We used DISC to interrogate two breast cancer cell lines (MCF-7 and MDA-MB-231) by treating the cells with high concentrations (50 ng/mL) of PDGF ligand. As shown in Figure 5A, the MCF-7 cells are generally unresponsive to treatment, with very few cells having greater Akt phosphorylation than the baseline. In contrast, the MDA-MB-231 cells responded consistently after 3 minutes of PDGF stimulation, with a wide spectrum of Akt phosphorylation within the individual cells. Interestingly, PDGF has been implicated in the inducement of cancer phenotypes (i.e., nuclear translocation of β-catenin, cell proliferation and DNA incorporation) in MDA-MB-231, but not in MCF-7 cells.328 The results observed here suggest that enhanced Akt activation in response to PDGF-BB may be the mechanism that drives the cancerous phenotype of MDA-MB-231. Furthermore, this study demonstrates that the DISC system can be used to study the heterogeneity of the cellular response to different stimuli.

5.4 Discussion

In the experiments reported here, three adherent cell types were evaluated; but in principle, the technique should be compatible with any adherent cells that can be grown in virtual microwells (which can include primary cells).321 DISC has the flexibility to stimulate the cells with discrete droplets of ligand or other agonists/antagonists at different concentrations and/or time periods. Because this system uses discrete droplets, it has the advantage of requiring small volumes of ligand and reagents, making it ideal for use with expensive reagents. The DISC platform does
not require pumps, which makes set up straightforward, and more importantly, results in very low shear stress (i.e., weakly adhered cells and cells grown in hydrogels with low viscoelastic modulus can be washed with negligible cell loss or damage), in contrast to constant flow microfluidics. Also although the effects of electrostatic actuation on cell health were not explicitly evaluated here, the cells behaved as expected when stimulated with PDGF (left, Figure 3) and previous studies have reported that DMF actuation has negligible effects on cell phenotype when probed for targeted (qPCR for stress-related genes) and untargeted (cDNA microarrays) expression changes. These studies suggest that DMF does not alter cellular responses due to the electrostatic actuation—this is not surprising as a majority of the electrical field drops across the insulating layer rather than the droplets containing cells. The Beebe group however has shown that microfluidic channels can alter the expression of some genes and metabolism of the cells when compared to macroscale tissue culture. This may be due to the differences in surface area to volume ratio or other factors. Although the studies with the DISC platform did not show any large scale alterations in signal transduction, we cannot rule out the possibility that the microscale nature of the virtual microwells may affect some aspects of cell function.

Figure 5.5 Probing Akt phosphorylation in breast cancer cell lines stimulated by PDGF-BB
Single-cell scatter plots showing Akt (S473) phosphorylation status in (A) MCF-7 and (B) MDA-MB-231 cell lines stimulated for 0-7 min with 50 ng/mL of PDGF-BB. Error bars are population mean +/- S.D. and scatter plots were generated from at least 3 different day replicates.

DISC provides the capability to analyze single cells, allowing for the generation of histograms and scatter plots of protein phosphorylation state. This has the advantage of generating flow-cytometry-like-data, but for adherent cells evaluated in situ. Thus, cells do not
have to be detached from the surface of the plate, which can disrupt cell surface markers and remove any spatial information about how cell-cell interaction affects cell signalling.

DISC was validated by probing the dynamics of the PDGFR/PI3K/AKT pathway. The results reported here are in line with the model of PDGFR activation developed by the Haugh group, suggesting that DISC operation does not introduce bias during the measurement of signal transduction. The single-cell resolution of the data enabled determination that the phosphorylation of the receptor has an increase in variation as the concentration of the PDGF increases, suggesting that there is a broadening of the individual cellular responses (middle, right, Figure 3A,B). This increase in variability passes down the signalling cascade, as is shown with the increased broadening of the phosphorylation of Akt when compared to the receptor (middle, right, Figure 3C). The single cell data also shows an amplification of the signalling transduction, where the Akt phosphorylation levels are more similar to each other at different concentrations of PDGF than the phosphorylation of the receptor at different concentrations. This information about single-cell signal propagation cannot be determined using traditional techniques such as Western blots that can only measure average responses of the system. It is increasingly being realized heterogeneous responses by cells is important in many forms of cancer.

Finally, DISC makes it convenient to distribute temporally varying concentrations of reagents to individually addressable virtual microwells for different periods of time, making it attractive for studying complex signalling events (Figure 4D,F,G). We propose that DISC may be an important new technique for probing temporal dynamics of signaling in adherent single cells for a wide range of applications.
Chapter 6

6 Conclusions and Future Directions

6.1 Conclusions

Prior to carrying out the work described in this thesis, the digital microfluidic immunoassay was still a relatively nascent technique. Most previous work was focused on protocol development and proof of principle demonstration of analytical performance with modest amounts of quantitative data. These early systems were predominantly implemented in oil-filled DMF devices which relied on a dilution-based particle washing procedure, requiring upwards of 16 wash steps to remove unbound reagents in the supernatant. Through the work presented here, we have made significant contributions to (1) the development of an oil-free DMF immunoassay method with enhanced particle washing efficiency, (2) the design and construction of integrated instrumentation to minimize manual intervention and improve assay throughput and performance, (3) the evaluation of DMF immunoassay performance (sensitivity and specificity) for the diagnosis of an infectious disease, and (4) the development of DMF immunoassay methods for analysis of in-cell signaling proteins.

Chapter 2 describes a new particle-based immunoassay on DMF without the aid of an oil carrier fluid to enable droplet movement (i.e., droplets are surrounded by air instead of oil). This new format allowed the realization of an on-chip particle separation method called digital microfluidic magnetic separation, capable of removing greater than 90% of unbound reagents in one step. Using this technique, we developed methods for non-competitive and competitive immunoassays, using thyroid-stimulating hormone (TSH) and 17β-estradiol (E2) as model analytes, respectively. This work represents the first competitive immunoassay implemented using digital microfluidics. We showed that, compared to conventional methods, the new DMF approach reduced reagent volumes and analysis time by 100-fold and 10-fold, respectively, while retaining a level of analytical performance required for clinical screening. This work has become an important touchstone for the Wheeler Laboratory, which is now (in work outside the context of this thesis) employing digital microfluidic magnetic separation for a diverse array of
applications including enzymatic reactions, solid phase extraction, high abundant protein depletion, pull-down assays, and solid phase peptide synthesis.

Building on the digital microfluidic magnetic separation and immunoassay protocols in chapter 2, chapter 3 describes the development of a DMF-powered integrated instrument capable of performing immunoassays from sample-to-analysis with minimal manual intervention. This platform features (a) a 90 Pogo pin interface for digital microfluidic control, (b) an integrated (and motorized) photomultiplier tube for chemiluminescent detection, and (c) a magnetic lens assembly which focuses magnetic fields into a narrow region on the surface of the DMF device, facilitating up to 8 simultaneous digital microfluidic magnetic separations. The new platform was used to implement a three-level full factorial design of experiments (DOE) optimization for TSH immunoassays, varying (1) analyte concentration, (2) sample incubation time, and (3) sample volume, resulting in an optimized protocol that reduced detection limit and sample incubation time by up to 5-fold and 2-fold, respectively, relative to the methods described in chapter 2. Importantly, this represents the first report of a DOE optimization for immunoassays in a microfluidic system of any format.

Leveraging the methods and instrumentation developed in chapter 2 and 3, chapter 4 describes the evaluation of the DMF particle-based immunoassays for the diagnosis of rubella in donor samples. Specifically, assay protocols were developed to include integrated sample preparation for the detection of rubella virus (RV) IgG and IgM. The performance (sensitivity and specificity) of the assays was evaluated using serum and plasma samples from a commercial anti-rubella mixed titer performance panel. Testing of performance panel samples yielded diagnostic sensitivity and specificity of 100% and 100% for both RV IgG and IgM, rivaling the performance of lab-based RV testing. This represents the first report of an immunoassay capable of differentiating IgM from IgG by digital microfluidics, which is an important step in the diagnosis of many infectious diseases. These assays are fast (~30 min for four parallel assays), have excellent reproducibility (<10% average coefficient of variability), and require only a few microliters of sample and reagents.

Chapter 5 describes the development of a new method, called digital microfluidic immunocytochemistry in single-cells (DISC), which can stimulate and fix adherent cells for single-cell immunoassays. Digital microfluidic (DMF) was used to automate protocols for cell
culture, ligand stimulation, and immunocytochemistry. The use of DMF facilitated rapid stimulation and fixation of cells, enabling the interrogation protein phosphorylation in the seconds to minutes timescale. Fixed and stained cells on the device were scanned using a microarray reader, which has the ability to resolve signaling networks in heterogeneous cell populations. Using this technique, we interrogated the phosphorylation state of PDGFR and Akt in NIH 3T3, MCF-7, and MDA-MB-231 cell lines resulting from various time, frequency, and concentration dependent PDGF-BB stimulation. These studies revealed detailed kinetic behaviour and population heterogeneity of PDGFR stimulation, and uncovered a possible Akt-dependent relationship to breast cancer cell aggressiveness. Unlike flow cytometry, this technique evaluates and visualizes adherent cells in situ, precluding the need for disruptive disassociation protocols. Since most liquid handling steps are implemented by digital microfluidics, this technique minimizes the labour-intensive steps that are often required for phosphorylation studies using flow cytometry.

6.2 Future Directions

The development of new methodologies is a long and exciting journey filled with many obstacles that require perseverance, ingenuity and open-mindedness to overcome. For many researchers, their journey ends at the proof-of-principle stage, in hopes that an industrial entity will pick up the baton and bring it to the finish line. This is a fine path to take, as it allows researchers to focus their efforts in generating novel ideas and, perhaps to invent a technique with the importance of PCR or ELISA. On the other hand, more and more researchers are interested in pushing their invention to the end-user by leading translation activities (clinical trials/field testing) and/or spinning off companies from the laboratory.

6.2.1 DMF magnetic particle-based immunoassay

As a result of the work described in this thesis, the DMF magnetic particle-based immunoassay has become one of the most widely implemented techniques in the Wheeler lab, with ongoing projects that focus on integration with sample preparation or on-chip detectors. Although this technique is more refined than many other techniques developed in the Wheeler Lab, it is still very much at the proof-of-principle stage. The next phase should involve field testing with at least 200 patient samples, which would give us a better estimate of diagnostic performance. However, before practical field testing can be performed, the current prototype instrument will
need to be upgraded to become field-ready – i.e., low cost, user friendly, battery-powered, and with compact form factor. Design changes to consider include an inexpensive detector, lighter construction materials, simpler device fabrication, integrated amplifier, a hand-free reagent loading platform, and an electromagnet system with no motors. Some of these design changes may be leveraged from recent developments in low-cost device fabrication methods, simple electronic actuation systems, and integrated electrochemical sensors.

Although it is recommended that one should specialize in the testing one disease in the early stages of assay development, I propose that in this case, it makes sense to develop assay protocols for measles IgG and IgM (to accompany the existing assays for rubella, described in chapter 4) so that a multiplexed measles and rubella testing platform can be realized. There are at least three reasons to develop such a platform. First, measles continues to be a critical problem, with regular outbreaks in areas that are not immunized, and even a resurgence of outbreaks in North America. Second, the vaccination of rubella is often performed simultaneously with measles, making a multiplexed measles and rubella test much more valuable from a disease surveillance standpoint. Third, patients infected with measles or rubella have very similar symptoms (e.g. fever and rash) and it is often necessary to identify the source of infection so that patients can receive appropriate treatment or care.

To initiate these next steps, we were recently awarded funding from Grand Challenges Canada to develop measles and rubella testing instrumentation based on digital microfluidics. This grant entails assay development and refinement for measles and rubella, instrument design and construction, and field testing of these instruments with the help of our collaborators in Vietnam. Through this work, we hope to learn firsthand the difficulties associated with implementing these assays in an environment with limited infrastructure. And through this experience, we will be evaluate the feasibility of our instrument and learn how to make it more field-ready and accessible for non-experts.

6.2.2 DMF immunocytochemistry in single-cells

Because the DISC method is a brand new technique, its advantages, for the most part, have not been exploited. We propose that DISC is a potentially important new tool for single-cell analysis of cell signaling, and may be widely useful for high-time resolution interrogation of heterogeneous cells populations and applications in cancer diagnostics.
One particularly attractive avenue for the study of population heterogeneity is to investigate the effects of cell-cell interaction and paracrine and autocrine signaling. The DISC method described in this thesis uses circular hydrophilic ITO spots of 1.75 mm diameter, which allows cells to randomly adhere and spread within the spot. However, the designs of these hydrophilic sites can be easily extended to restrict the attachment of cells such that they can be interrogated as single-cells (without being in contact with others). DMF devices bearing hydrophilic spots on the order of 10 to 20 µm diameter have been reported previously, and I propose that it would be straightforward to generate well-defined microscale hydrophilic sites within a larger virtual microwell. This design would allow exquisite control of cell placement—for example, in a virtual microwell, cells could be positioned such that they are touching or that they are 5, 10, 15, 20, or 25 µm away from the nearest neighbour. I propose that new methods coupling this type of top-plate design with DISC and CellProfiler algorithms that can perform nearest neighbour analysis, will form the basis of a robust and powerful platform to study how cell-cell interactions affect the way they respond to external stimuli.

Another possible avenue for the DISC method is in cancer diagnostics. A relatively new technique called single cell network profiling (SCNP) is becoming popular in cancer biology. SCNP is a flow cytometry-based technique that can quantify phosphorylation of proteins inside cells following perturbation. This technique has been used for ‘personalized medicine’ applications to collect signaling profiles of malignant cells in cancer patients and to categorize their phosphorylation patterns in relation to the aggressiveness and drug sensitivity of the patients’ tumors. As more data is gathered on these cancer cell profiles, this data can ultimately be used to predict patient outcome and drug sensitivity. For example, when this technique is applied to evaluating blood cells in cancer patients, the phosphorylation of the STAT3/STAT5 pathway can be correlated with patient outcomes, and this data may be useful in predicting outcomes in other patients. However, as described in chapter 5, while flow cytometry is a powerful technique, it is limited by labour-intensive sample preparation and by the cost and size of the instrumentation—these drawbacks will likely make the proposed diagnostic/prognostic assays inaccessible to many cancer patients. I propose that the DISC method has the potential perform these assays with similar performance to flow cytometry, but with the added benefit of being faster, cheaper, and less labour-intensive. As demonstrated in chapter 5, this method can automate steps required for cell culture and immunocytochemistry
and can produce flow cytometry-like histograms using an inexpensive microarray reader. A good starting point would be to use DISC to replicate a common SCNP experiment that is typically done in flow cytometry, and compare their performances.

Finally, the DISC method is also still very much a proof-of-principle technique. Before it can be more widely used, several challenges must be addressed pertaining to digital microfluidics in general. There is a need for 1) new methods to resist the effects of biofouling (perhaps more effective additives or fouling-resistant coatings), 2) simpler device fabrication (mitigated to some extent by printed electronic devices\textsuperscript{282,339}), and 3) simpler or off-the shell electronic control systems (mitigated to some extent by open-source software/hardware platforms\textsuperscript{248}). For the method to truly be useful to the life sciences community, an easy-to-use, all-in-one integrated system facilitating humidity, temperature, and gas control, droplet operation, and analysis must be developed.
References

(71) Jokerst, J. V.; Raamanathan, A.; Christodoulides, N.; Floriano, P. N.; Pollard, A. A.; Simmons, G. W.; Wong, J.; Gage, C.; Furmaga, W. B.; Redding, S. W.; McDevitt, J. T. *Biosensors and Bioelectronics* **2009**, *24*, 3622-3629.


(103) Sista, R. S.; Eckhardt, A. E.; Srinivasan, V.; Pollack, M. G.; Palanki, S.; Pamula, V. K. *Lab on a Chip - Miniaturisation for Chemistry and Biology* **2008**, 8, 2188-2196.


(137) Luo, Y. Q.; Yu, F.; Zare, R. N. *Lab on a Chip* **2008**, *8*, 694-700.


(201) Rastogi, V.; Velev, O. D. Biomicrofluidics 2007, 1, 14107.
(220) Pamme, N. Lab on a chip 2006, 6, 24-38.
(236) Mullin, R. Chemical and Engineering News 2013, 91.
(238) Kalorama Information The World Market for Point of Care Diagnostics 2012.
(242) Raamanathan, A.; Simmons, G. W.; Christodoulides, N.; Floriano, P. N.; Furmaga, W. B.; Redding, S. W.; Lu, K. H.; Bast, R. C., Jr.; McDevitt, J. T. Cancer Prevention Research 2012, 5, 706-716.
(245) Escobar, J.; Bartsch, M. S.; Patel, K. D. Lab on a Chip 2012, 12, 2452-2463.
(260) WHO. 2012.
(264) Alere(TM).
(266) Reef, S. E.; Strebel, P.; Dabbagh, A.; Gacic-Dobo, M.; Cochi, S. Journal of Infectious Diseases 2011, 204, S24-S27.

(287) WHO *Operational characteristics of commercially available assays to determine Antibodies to HIV-1 and or HIV-2 in human sera*; Geneva, 1999.

(288) Fu, E.; Yager, P.; Floriano, P. N.; Christodoulides, N.; McDevitt, J. T. *Pulse, IEEE* 2011, 2, 40-50.


(292) Tipples, G. A. *Journal of Infectious Diseases* 2011, 204, S659-S663.


