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Los Angeles

Rapid Concentration of Bacteria Using Microfluidic Magnetic Ratcheting

A dissertation submitted in partial satisfaction of the requirements

for the degree of Doctor of Philosophy

in Biomedical Engineering

by

Oladunni Adeyiga

2017

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ABSTRACT OF THE DISSERTATION

Rapid Concentration of Bacteria Using Microfluidic Magnetic Ratcheting

by

Oladunni Adeyiga

Doctor in Philosophy in Biomedical Engineering

University of California, Los Angeles, 2017

Professor Dino Di Carlo, Chair

Rapid identification and treatment of infectious agents is an essential part of the treatment plan that is provided in the care of those patients who have an infection. This allows the reduction in hospital length of stay, cost of care, and ultimately results in better care given to hospitalized patients. In addition, the rapid identification of resistant bacterial species has been identified as a national priority. At this time, there are various mechanisms used to identify infectious agents. These mechanisms include culture with biochemical phenotypic methods in order

to identify organisms that preferentially metabolize different substrates. However, the length of time for recovering infectious agents by the use of culturing is often lengthened due to the presence of fastidious organisms or the presence of antimicrobials that are frequently given empirically to patients. In addition, these factors often impede the recovery of infectious agents.

In this project, I use a combination of modalities, including centrifugation with filtration and microfluidics, to address the need for rapid identification of bloodstream infection. These methods are applied to formulate a technique that can be used to rapidly separate and enrich bacteria present within a suspension of fluid. This technique can be used for the purpose of identifying bacteria that cause bloodstream infection. In this case, using *Staphylococcus aureus* as a prototype organism, I present a system that can be used to rapidly separate and enrich bacteria by using a magnetic ratcheting on a microfluidic chip.

The dissertation of Oladunni Adeyiga is approved.

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2017

To God be the glory.

To Jesus Christ, my Lord and Savior.

To Dr. Olanrewaju Adeyiga, my father, the smartest man, and the best doctor that I know.

To Olori Mosekunola Adeyiga, my mother, the smartest, most beautiful, and sophisticated lady
that I know.

To all of my family.

I love you.

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- Gossett, Daniel R., Henry TK Tse, Keisuke Goda, **Oladunni Adeyiga**, Travis A. Woods, Steve W. Graves, Otto O. Yang, and Dino Di Carlo. (2012) "*Leukocyte Mechanophenotyping by Deformability Cytometry.*" MicroTAS
- Sandoz, Patrick A., Ahmet F. Coskun, Aram J. Chung, Westbrook M. Weaver, **Oladunni Adeyiga**, Delaram Khodadadi, Aydogan Ozcan, and Dino Di Carlo. (2012) "*Digital Readout Platform for Water-in-Oil Droplet Immunoassays Running on a Cell-Phone for Point of Care Viral Load Sensing.*" Proceedings of the 16th International Conference on Miniaturized Systems for Life Sciences and Chemistry.
- James Che, Hector Enrique Muñoz, Victor Yu, Jonathan Lin, **Oladunni Adeyiga**, and Dino Di Carlo. (2015) "*An Integrated Microfluidic Platform for High-Throughput Label-Free Purification and Mechanophenotyping of Circulating Tumor Cells.*" Proceedings of the 19th International Conference on Miniaturized Systems for Life Sciences and Chemistry.

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Chapter 1

New Technologies for Diagnosing Bloodstream Infection and Measuring Antimicrobial Resistance

1.

1.1 Chapter Summary

The ability to rapidly diagnose bloodstream infection would have a large impact on the clinical care of patients, particularly in the hospital setting. Also, President Obama outlined a national initiative directed at improving methods to combat antimicrobial resistance, which would benefit from an update in methodologies used to determine antimicrobial resistance. To that end, in this short review I highlight some of the newer technologies aimed at rapidly diagnosing bloodstream infection, which are able to generate results in a time frame that is shorter than the 48-72 hour time frame usually required by conventional blood culture techniques. In addition, I discuss some of the new methods designed for measuring antimicrobial resistance. There are several opportunities for growth in these areas, and I present a few here.

1.2 Introduction

The tools used for diagnostic clinical microbiology are due for a revolution(Heffernan and Fox 2014). Excitingly, many researchers and engineers, especially in the field of microfluidics, strongly agree, and are actively working to usher in a new era of diagnostic clinical microbiology(Afshari, Schrenzel et al. 2012). Several recent reviews have highlighted the potential impact of microfluidics in diagnostic testing of infectious diseases more generally

(Damhorst, Murtagh et al. 2015, Jung, Han et al. 2015, Su, Gao et al. 2015, Tay, Pavesi et al. 2016). Here I focus on one clinical syndrome that could greatly benefit from an upgrade in diagnostic techniques - bloodstream infection.

Given the morbidity and mortality associated with bloodstream infection, clinicians are quickly compelled to institute antimicrobial therapy, often empirically, at the first signs of systemic infection. This empiric antimicrobial therapy is given in an effort to minimize the morbidity and mortality that may result from untreated bloodstream infection. There is evidence that rapid antimicrobial therapy is extremely important in improving outcomes (Morrell, Fraser et al. 2005, Kumar, Roberts et al. 2006, Gaieski, Mikkelsen et al. 2010). What would be extremely valuable, however, would be the ability to rapidly give specific and directed antimicrobial therapy within hours of the first signs of systemic infection, rather than giving an empiric choice of therapy based on the clinician's most educated guess. As astute as clinicians can be, empiric therapy based on even the best educated guess will usually need modification, once the causative pathogen and its antimicrobial susceptibility has been determined. Furthermore, empiric antimicrobial therapy choices should be modified when updated information about the microbial pathogen, such as species identification or antimicrobial susceptibility, is available for several reasons. These reasons include the need to reduce the propagation of resistant pathogens, the need to reduce specific patient exposure to unnecessary broad spectrum antimicrobial drugs, and to increase the likelihood that broad spectrum antimicrobials will retain their effectiveness against resistant pathogens. Therefore, rapidly obtaining pathogen specific information in the diagnosis and treatment of bloodstream infection has the potential to revolutionize the practice of medicine in this area.

The current method of diagnosing bloodstream infection involves the use of blood culture (Washington and Ilstrup 1986, Klouche and Schröder 2008). Blood culture is performed in the following way: for an adult, usually 10 milliliters of blood is collected from the patient, which is then used to inoculate a blood culture bottle. In general, blood culture bottles will contain growth media and additional additives meant to enhance the recovery of microorganisms that could be present within the collected patient sample. The inoculated blood culture bottle is then monitored using continuous-monitoring blood culture systems. These systems detect the growth of microorganisms by detecting a change in amount of carbon dioxide or oxygen present within the blood culture bottle; a change in the pressure within a blood culture bottle is another technique by which these systems monitor for the growth of microorganisms. Once the growth of microorganisms has been detected, additional steps are taken to more specifically identify the organism, which frequently includes further subculture steps. Alternatively, ribosomal DNA sequencing can be performed to specifically identify a microorganism (Kolbert and Persing 1999). More recently, mass spectrometry has played an increasing role in microorganism identification with regard to diagnosing bloodstream infection. Notably, current methodologies and the majority of newer techniques being proposed as a method to diagnose bloodstream infection focus on a key aspect of detection: amplifying the signal that corresponds to the presence of bloodstream infection.

Given that current methodologies require at least 48 – 72 hours for pathogen identification, there is certainly room for improvement. In this short review, I present the progress in research and

development that is ongoing to solve this important need for more rapid tests. I also highlight some of the progress that has been made in the area of test development for determining antimicrobial susceptibility.

1.3 Technologies in Translation for Clinical Use

Outside of academic research, much of the focus has been on molecular analysis approaches such as PCR to enable the faster diagnosis of bloodstream infection (Ecker, Sampath et al. 2010, Lebovitz and Burbelo 2013). The ability to successfully directly detect pathogens present within patient blood samples without the need for incubation in culture would be a very significant addition to our means of diagnosing bloodstream infection. To that end, several technologies in this area have been undergoing commercial development to assist with rapid diagnosis of bloodstream infection (Stevenson, Pandor et al. 2016). These include the LightCycler®SeptiFast, SeptiTest®, and VYOO® systems.

In this first example, the developers for SeptiFast® have created a multiplexed probe PCR based detection system that is based on work first reported by Lehman and colleagues in 2008 (Lehmann, Hunfeld et al. 2008, Lehmann, Alvarez et al. 2009). In this work, authors analyze whole blood samples with a series of steps, which includes blood sample preparation, DNA amplification with real-time PCR and the use of specific hybridization probes to recognize PCR products, and computer software mediated automated identification of PCR products generated from microorganism species and controls within the sample. Sample preparation is performed with mechanical lysis using ceramic beads in a MagNA Lyzer® instrument. This is

followed by DNA purification where a protease and a chaotropic buffer(Salvi, De Los Rios et al. 2005) are both used, to release nucleic acid material and protect released DNA from blood DNAses. Following this, after adding an internal control, DNA within the mixture is purified with a solid-phase extraction method using a spin-column with a glass fiber insert that is subjected to serial wash and centrifugation steps, to complete the sample preparation steps. DNA within the prepared sample is then amplified using real-time PCR, with universal and specific primers targeting a particular genomic region that has been shown to have a higher PCR amplification success rate(Schoch, Seifert et al. 2012). Finally, the authors use software designed to identify pathogen based on the kinetics of the PCR products generated from each pathogen.

The *SeptiFast*[®] platform is currently manufactured by Roche Diagnostics, and run using the LightCycler[®], their real-time PCR instrument (Dark, Wilson et al. 2012). The two modalities have been combined as the LightCycler[®]*SeptiFast* system, which, to date, has been evaluated in 41 phase III clinical trials (Dark, Blackwood et al. 2015). While these studies were of variable quality, the platform has been shown to have higher specificity than sensitivity, with a specificity of 0.86 (95% CI 0.84-0.89) and a sensitivity of 0.68 (95% CI 0.63-0.73), when compared with conventional blood culture. In another study, authors suggest that the platform can be used to predict disease severity in the diagnosis of patients with sepsis(Ziegler, Josefson et al. 2014). The developed platform is described as having the ability to detect twenty-five pathogen species present within 1.5 milliliters of whole blood in less than 6 hours.

In this next example, The SeptiTest[®] system, manufactured by Molzym, Bremen, Germany, is a platform employing a sample preparation step that reportedly enriches microbial nucleic acid material from human nucleic acid material, in an effort to enhance the signal that results from the presence of microbes in the sample (Gebert, Siegel et al. 2008, Wellinghausen, Kochem et al. 2009, Wellinghausen, Siegel et al. 2009). In this case, by utilizing chaotropic conditions generated within the collected sample, human cells are selectively lysed, after which, enzyme mediated digestion of human DNA and extracellular pathogen DNA occurs, followed by sedimentation of intact pathogen cells with the aid of centrifugation (Linow). Intact pathogen cells then undergo enzymatic degradation for the purpose of pathogen DNA purification, which can be completed using a bind-wash-elute method, or any other DNA isolation kit. Purified pathogen DNA is then identified with a universal 16S rRNA gene-based PCR assay.

The LOOXSTER[®]/VYOO[®] system has a similar approach, where microbial nucleic acid material is enriched from mammalian nucleic acid material, to improve test sensitivity (Sachse, Straube et al. 2009, Bloos, Sachse et al. 2012). For this platform, the sample is subjected to a conventional DNA extraction method such as chemical lysis with a physical disruption technique. Enrichment of pathogen DNA from human DNA is achieved by affinity chromatography using a protein that will preferentially bind motifs characteristic of pathogen DNA (Horz, Scheer et al. 2008). After these sample preparation steps, a multiplex PCR assay is run on the prepared sample, and species are identified gel electrophoresis. With a similar approach, another platform is able to detect common causative pathogens of bloodstream infection, in addition to three drug resistance genes (Carrara, Navarro et al. 2013). In this prospective observational study, authors compared blood culture with three PCR based assays

available commercially(Schreiber, Nierhaus et al. 2013). Whereas blood culture was positive in 26% of collected samples, the evaluated PCR assays were able to detect pathogens in 12%, 10%, and 14% of collected samples.

PCR can also be coupled with electrospray ionization mass spectrometry (PCR/ESI-MS) in order to address the issue of quickly diagnosing bloodstream infection (Jordana-Lluch, Carolan et al. 2013, Laffler, Cummins et al. 2013, Vincent, Brealey et al. 2015). The use of PCR/ESI-MS allows the mass of each PCR amplicon to be determined and the specific and clear calculation of the nucleotide base composition, so that organism identification can occur without the use of organism specific PCR primers.(Ecker, Sampath et al. 2008, Baldwin, Howe et al. 2009, Ecker, Massire et al. 2009, Hall, Sampath et al. 2009, Wolk, Blyn et al. 2009, Kaleta, Clark et al. 2011). The IRIDICA BAC BSI Assay, which is a modification of this technique, involves using a higher volume of a collected blood sample to increase pathogen detection sensitivity and further optimization of the PCR conditions used for the assay. This platform can be used for the broad identification of fungal candida and bacterial species (Jordana-Lluch, Giménez et al. 2015, Metzgar, Frinder et al. 2016).

In this final example, superparamagnetic particles that have been functionalized with a pathogen specific target, allow detection of pathogens in bloodstream infection such as fungal candida species(Pfaller, Wolk et al. 2016). The platform described here, the T2MR technology(Neely, Audeh et al. 2013, Mylonakis, Clancy et al. 2015), is offered by T2 Biosystems. The T2MR machine uses a miniaturized, magnetic resonance based diagnostic to detect pathogens that are

bound to functionalized superparamagnetic particles. By applying the same technique that allows magnetic resonance imaging to be obtained for medical radiology imaging, the designers leverage the difference in signals generated in the presence of an applied magnetic field by aggregation of superparamagnetic particles bound to the pathogen of interest and water molecules present in whole blood.

Rapid determination of antimicrobial susceptibility would also be a key improvement in treated diagnosed infections, such as bloodstream infection. With regards to antimicrobial susceptibility testing, Choi and colleagues have developed a technique where images showing the morphological changes that bacteria exposed to various antimicrobials undergo are recorded. The recorded images are then analyzed to rapidly evaluate the antimicrobial susceptibility of tested microorganisms. This analysis is termed SCMA, or single cell morphological analysis (SCMA). This is an improvement upon their prior reported technique, the microfluidic agarose channel (MAC) system, where, instead of obtaining single cell morphology data, the area occupied by bacteria is calculated, to determine whether or not bacteria are growing. In the SCMA design, morphology data is collected instead. After performing time-lapsed microscopy on bacteria within agarose, the images of bacteria that have been exposed to various antimicrobials are acquired and analyzed, to obtain morphological data corresponding to growth, that correlates with antimicrobial susceptibility testing performed using the broth microdilution method (Choi, Yoo et al. 2014). This is in contrast to more conventional techniques, which depend on a more macro level analysis such as optical density measurements of bacterial suspension turbidity, which reflects the number of organisms present in solution.

In summary, new strategies are being employed to bring microbiological diagnostics into another era. With regard to antimicrobial susceptibility testing, the ability to rapidly perform single cell analysis, and to correlate cell characteristics with the effect of an antimicrobial on each cell, has the potential to allow clinicians to more completely understand and quantify the presence of antimicrobial resistance at the single cell level. This would be an improvement upon current measurements, which more reflect population dynamics at large. With regard to diagnosing bloodstream infection, by focusing on amplifying the signal present that corresponds with the presence of microorganisms in blood, such as microorganism nucleic acid material, the reported technologies are an improvement as compared with blood culture, and can hopefully increase our ability to detect bloodstream infection, even in cases where microorganism viability is compromised. With the use of blood culture, there is a need for organisms to remain viable so that they adequately grow, thus allowing the amplification of the number of organisms present so as to facilitate their detection. With these new described molecular techniques, the emphasis is on enriching and amplifying microorganism nucleic acid material by PCR without requiring the organism to be viable. Thus, organism detection can still occur, even when the organism is not viable.

In the highlighted technologies, the general approach is the same, although there is some variation in each component of the overall platform process. The exception is with PCR/ESI-MS, where mass spectrometry is now applied for the purpose of identifying nucleic acid amplification products, instead of for the purpose of identifying protein fragments, where mass

spectrometry is customarily applied. While PCR is a valuable molecular diagnostic tool, the performance of this method is dependent on the capability of the primer employed for each particular assay. The choice of PCR primer typically requires prior knowledge of the nucleic acid sequence in the genomic region where amplification is desired. A platform that could amplify the presence of microorganisms without the need for culture or even the use of nucleic acid primers, such as with PCR, would be extremely valuable.

1.4 Emerging Technologies in Research and Development.

Given that there are still opportunities for improvement with regard to technologies currently in stages of clinical translation, this is an area of active research within academia. There are key opportunities for development, for instance, in terms of increasing the yield of detection, defining other parameters by which we can accurately and precisely identify bloodstream infection, and furthering our ability to describe and understand of how antimicrobial agents impede the growth and activity of pathogenic microbes and may impact other elements of the inflammatory response. In fact, several early-stage technologies for diagnostic clinical microbiology are emerging from academic research institutions.

These emerging technologies focus on several arenas, including high-throughput blood processing, high sensitivity pathogen detection, and antibiotic susceptibility testing. Researchers are working to increase processing rates for large volume samples in order to increase the yield of a positive test indicating the presence of bloodstream infection without the use of blood culture. In addition, there is much research and development into technologies that increase

sensitivity of pathogen detection. Also, several reported works focus on creating new methodologies for the rapid determination of antimicrobial susceptibility. This area of work has especially gained increased attention, especially given the national call to action in the battle to combat the development of antimicrobial resistance(Obama 2014).

1.4.1 High-throughput Sample Processing.

In this section, the highlighted technologies make use of droplet microfluidics, inertial microfluidics, and dielectrophoresis in order to perform high throughput sample processing. Given that current methods involve obtaining 10 milliliters of blood to test for bloodstream infection with blood culture, the capability of a platform to rapidly process a patient sample with high volume becomes imperative. One approach to improve the speed with which bloodstream cultures are identified, (Kang, Ali et al. 2014) uses a technique called integrated comprehensive droplet digital detection (IC 3D). This technology is designed to identify the presence of single cells present in diluted whole blood within four hours compared to the current time for blood culture positivity which can range from 8-48 hrs.

There are three components for this technology: a microfluidic droplet generator, a particle counter system, and a bacterial detection method that utilizes DNAzyme sensor technology. The use of a microfluidic droplet generator allows for rapidly processing of a large volume of blood into small volume liquid droplets. Generating small liquid volume droplets is useful for the purpose of increasing the likelihood that a single bacteria cell will be isolated within a small volume, leading to a high effective concentration of bacteria within each occupied droplet such

that reaction with the sensor molecule leads to a high concentration of signal isolated within the drop. These conditions will have the effect of increasing the sensitivity of detection and increasing the signal to noise ratio for detection of the presence of a bacteria. Diluted blood is mixed with a solution that contains DNAzyme sensor solution and bacterial lysis buffer, which is then processed by a microfluidic droplet generator. DNAzyme sensors that detect the presence of bacteria can be detected using a 3D particle counter able to reliability count single fluorescent particles. Importantly, the capability of the sensor molecule to sensitively detect the presence of specific types of lysed bacteria will increase the accuracy of the technology.

The IC3D technology's use of a DNAzyme sensor molecule to differentiate *E. coli* strains from two mammalian cell lines leads to a selective and specific signal. The authors perform further comparison experiments with other clinically relevant gram negative organisms, such as *C. freundii* and *K. oxytoca*. Here, while they are able to show a statistically significant difference in fluorescence intensity between the measured organisms, the magnitude of the difference in fluorescence intensity is small between organism types, showing the potential for cross reactivity, among different organism types.

Another approach makes use of inertial microfluidic separation (Hou, Bhattacharyya et al. 2015) to purify bacteria from background cells. The authors in this work combine inertial microfluidics with a nucleic acid based method of detection in order to detect pathogens spiked into diluted whole blood. An attractive feature of this platform is the use of a label-free method

to separate smaller sized bacteria from larger sized blood cells, which are abundantly present in whole blood and certainly can obscure the presence of small sized bacteria.

This method is label free in that it does not require an affinity based technique, such as antibody binding, to perform the separation. Instead, the separation of these different sized particles is achieved by exploiting some of the unique physics exclusive to microfluidic fluid flow. For this work, authors employed the physics of inertial microfluidics to allow for particle separation, where particle movement across streams that occurs within fluid flowing through a microfluidic channel is distinctive for each sized particle. The knowledge of these unique physics informs the authors' creation of geometric designs for fluid channels that can segregate smaller and larger cells. In this case, the authors explore and determine the optimal microfluidic channel geometry that efficiently performs this separation, and also determine the optimal whole blood sample preparation necessary for use with their chip.

The microfluidic chip designed in this study has a spiral configuration, with two ports at the inlet and two ports at the outlet. Two types of fluid are introduced into the each inlet port: a diluted whole blood sample that has been spiked with bacterial pathogens, and a sheath fluid. From one outlet port fluid is removed that contains separated red and white blood cells; from the other outlet port, a fluid containing bacteria, along with some platelets, is removed. After introducing blood containing bacterial pathogens at the inlet, the authors demonstrate that their chip is able to recover several types of bacteria at the outlet port, including *E. coli*, *P. aeruginosa*, *E. faecalis*, and *S. aureus*. The authors take the bacteria containing-fluid collected from the outlet port,

perform an additional off-chip concentration and bacteria lysis steps, and then apply use of the NanoString technology in order to detect the bacteria of interest.

The NanoString detection system involves the use of nucleic acid sequence reporter molecules that will bind complimentary nucleic acid sequence molecules present in the tested suspension(Geiss, Bumgarner et al. 2008). With the NanoString detection system, pre-constructed probes that contain capture and reporter regions for specific nucleic acid segments of interest are allowed to mix in suspension and hybridize in the first step. Then, after affinity purification is performed to remove unhybridized probes, the suspension of hybridized complexes is allowed to adhere to a surface onto which a capture agent has been coated. These hybridized complexes can then be visualized and counted, given that the reporter region of the probe has a fluorescent segment, and an applied electric field orients and elongates each hybridized complex. In contrast to PCR, the cyclic steps needed for nucleic acid amplification are not employed. Here, the authors use the NanoString technology to detect bacterial rRNA instead of mRNA, with the rationale that rRNA is present in higher amounts and will allow a lower level limit of detection for their approach.

Finally, the authors propose use of a Transcriptional Susceptibility Score, as a quickly obtainable score that correlates with the antimicrobial susceptibility of an organism. The score is determined by first performing a short incubation of specific bacteria with an antimicrobial of interest. Then, the authors lyse bacteria and detect the mRNA present in solution with the NanoString technology. The authors are able to show that their devised Transcriptional

Susceptibility Score correlates with antimicrobial susceptibility measured using conventional culture techniques. This element of analysis adds an additional benefit to the proposed technology. The author's use of the NanoString technology addresses concerns such as the low levels of microorganisms often present in bloodstream infection. However, given that RNA detection with the NanoString assay is performed after centrifugation and lysis of the bacteria rich solution obtained from processing using the designed chip, further work is necessary if this process is to be completely contained on-chip.

In another example, authors design a microfluidic chip where dielectrophoresis is the label-free method used to extract pathogens of interest (Cai, Xiao et al. 2014). In contrast to the approach of Hou and colleagues, where some process elements are performed off chip, in this system, diluted whole blood is loaded onto the chip, where pathogen extraction occurs. A wash step is performed, after which, pathogens are extracted, and a PCR based method of detection is used to identify extracted pathogens on chip.

These highlighted technologies are excellent examples of the work that can be performed using a multidisciplinary approach. The platforms ultimately resulting from these technologies that are translated into the clinical arena can surely have a large impact on the rapid diagnosis of bloodstream infection because of their ability to process large volumes on chip, and their demonstrated use to detect clinically relevant microorganisms. In addition, in each approach, the authors incorporated clinically relevant organisms and scenarios, demonstrating the applicability of the designed platforms in addressing the needs of clinical practitioners. These noted successes

in this research focus area should hopefully inspire even more innovation that aims to solve this complex problem of rapidly acquiring pathogen specific information from large volumes of blood in diagnosing bloodstream infection.

1.4.2 Improved Pathogen Detection Sensitivity.

In approaching this problem, researchers have looked at detecting compounds, such as proteins, nucleic acid, or other chemicals, exclusively produced by particular infectious agents, which may be present in an amount that is either easier to detect or more abundant in the clinical samples collected from patients with a suspected infection. Another strategy has been to develop more sensitive reporter molecules. To increase sensitivity, one approach (Alatraktchi, Johansen et al. 2016), focuses on detecting a virulence factor of *P. aeruginosa*, pyocyanin, which is exclusively secreted by this organism. That this chemical is exclusively secreted by *P. aeruginosa* makes the detection of this chemical extremely useful for selective diagnosis of *P. aeruginosa* infection. Whereas the detection of the molecule is commonly performed with the use of spectrophotometry, obtaining reliable spectrophotometry sample measurements requires pretreatment of the sample to be tested, in order to reduce the presence of background noise. Pretreatment is necessary, given that pyocyanin is a redox active compound. The authors describe the detection of this molecule using amperometric electrochemical detection, where this chemical is detected by sensing a change in ions with the use of an electrical current. This proposed technique has been designed to give a better signal to noise ratio that would result in the ability to detect the presence of *P. aeruginosa* that is present in low amounts.

The work described here was performed where pyocyanin was measured in artificial sputum. However, further work is required in order to transfer this technique for detecting this pyocyanin in patient samples such as human blood and respiratory samples, unless pre-testing sample preparation steps occur. Respiratory samples such as sputum can be highly viscous and complex, in terms of the presences of various substances such as glycoproteins, immunoglobulins, and inflammatory cells. Whole blood is even more complex, given the sheer abundance of cells and breath of proteins present, as well as other compounds such as lipids and carbohydrates, which can also be redox active compounds. This example features an important direction in refining our ability to diagnose infection: the technique of measuring biomarkers that correlate with the presence of infection.

In the next two techniques, authors design detection systems involving different reporter materials. The development of materials that can enhance our ability to detect markers associated with infection would advance the field. In one case, a unique detector system where cubic retroreflectors are modified with an antibody specific to the pathogen of interest was used(Garvey, Shakarisaz et al. 2014). In this case, E. coli and MS2 virus was the target, and a sandwich type ELISA assay was performed with the functionalized cubic retroreflectors within qPCR tube reaction vessels that also contained antibody-modified tube caps. Immobilized cubes present within reaction vessels are imaged and counted. The authors describe additional modifications that can be made in order to make this a multiplex assay.

In another work, researchers employ microarray technology using functionalized nanotubes, to allow the detection of multiple pathogens on one platform. The authors here (de la Rica, Pejoux et al. 2010) describe the use of antibody-conjugated peptide nanotubes; where the specific pathogens of interest bind to nanotubes that have been modified to have an antibody to the particular pathogen.. The complexes consisting of modified nanotube bound to pathogens quickly settle onto the surface of the chip, where transducers have been patterned. As these complexes settle, a change in impedance occurs, generating a signal that can be used for pathogen detection. In this case, given that the functionalized nanotubes are not patterned onto the chip surface, functionalized nanotube-pathogen complexes that develop and settle onto the chip surface can be gently washed away. In this manner, there is the potential for chip reuse, which can be a cost and resource-saving measure.

Nucleic acids have also been assayed using loop-mediated isothermal amplification, or LAMP, to assist with increasing pathogen detection sensitivity. In contrast to conventional PCR, which requires temperature cycling for its process, LAMP offers rapid amplification of nucleic acid sequences present within the sample of interest at a single temperature, thus eliminating the need for a thermal cycler, which is necessary for conventional PCR methods. In this reported study, authors developed an integrated microfluidic electrochemical DNA (IMED) chip, for the purpose of detecting *Salmonella* serovars from the whole blood of infected mice. (Patterson, Heithoff et al. 2013). After introducing whole blood with LAMP reagents onto the IMED chip, LAMP is performed, and the products of amplification are detected using designed E-DNA probes that have been attached to a downstream region of the chip. The chip was designed so that minimal processing steps are needed, with the intention that it may be used in the point of care setting in a

variety of commercial applications. In general, by investigating the utility of more sensitive reporter molecules, or looking at compounds that also correlate with the presence of infection, our ability to more accurately and specifically diagnosed infection will be enhanced.

1.4.3 Detection of Antimicrobial Susceptibility.

Much work is being conducted to advance our ability to quickly determine antimicrobial susceptibility. Given that current methods for determining antimicrobial susceptibility testing take time can take 24 hours or more to determine results, or can provide conflicting results across modalities, new methodologies for determining the ability of various antimicrobials to impede the growth of or to kill specific organisms are being developed. Current methodologies usually rely on an increase in the optical density of a suspension containing the microorganism of interest. This increase in optical density corresponds to increase in organism cell population, which then leads to the conclusion that the antimicrobial of interest has no effect on organism growth, and that the organism of interest is resistant to that antimicrobial. Currently, routinely performed measurements yield the minimum inhibitory concentration, whereas the minimum bactericidal concentration for a particular compound can be obtained with additional testing. Minimum inhibitory concentration measures are currently used in interpreting the antimicrobial susceptibility of various organisms. To develop new methodologies for determining antimicrobial susceptibility of various organisms, an understanding of the mechanisms of antimicrobial resistance is needed. For example, what are the mechanisms involved that allow a particular organism to continue to replicate in the presence of a drug that should impair this ability (MacDougall 2011, Curello and MacDougall 2014, Narayanan, Johnson et al. 2016)?

With this knowledge base, specific detection approaches can be applied to identify molecular markers underlying these mechanisms.

Instead of imaging turbidity to identify susceptibility, some have taken the approach of viewing the single-cell division events as a way of determining antimicrobial susceptibility testing more quickly. Several strategies have been employed to achieve this. In the microfluidic agarose channel (MAC) system, the growth behavior, in the form of the proportional growth rate of single bacteria cells immobilized with agarose is microscopically observed using time lapse microscopy as these cells are exposed to different concentrations of antimicrobials(Choi, Jung et al. 2013). Additionally, microfluidic devices have also been used to obtain single-cell growth data that can be used to build a pharmacodynamics model for bacteria exposed to different gradients of antimicrobials (Hou, An et al. 2014).

The described approaches represent what can become a new paradigm in terms of understanding how what the impact of antimicrobial therapy is at the single cell level. Current methodologies, such as techniques where suspension turbidity is measured as a marker of antimicrobial resistance, obtain measures that very likely are more representative of population dynamics instead of single cell behavior. The analysis of single cell behavior could potentially lead to more detailed studies of the cell to cell interactions that occur between inflammatory cells and pathogenic organisms in the presence of various antimicrobials, which may lead to interesting findings, and also inform drug development in this area. The detection of genes from within single cells, and the proteins that are produced from them, give clinicians important information

regarding whether a drug will or will not be effective against a particular pathogen. The capability of studying single cell behavior in cells exposed to various antimicrobials offers a new frontier in this area of clinical microbiology.

1.5 Conclusions and Future Direction

The described innovation currently ongoing in the area of diagnosing bloodstream infection and determining antimicrobial susceptibility reflects the high level of interest in developing new tools to address these issues in diagnostic microbiology. With regard to diagnosing bloodstream infection, given the complexity of this problem, a multidisciplinary approach will be necessary in order to make significant strides in this area. In terms of designing more tools for the purpose of performing antimicrobial susceptibility testing, there is much interest and development in this area, which will ultimately lead to progress and results. There are exciting developments on the horizon, with technology emerging that will allow the diagnosis of bloodstream infection within 24 hours, without the need for blood culture, and new knowledge with regard to understanding and measuring the way that microbial drug resistance patterns occur.

As stated above, the current method of diagnosing bloodstream infection involves the use of blood culture. 10 milliliters of blood is taken from a patient, which is then used to inoculate a blood culture bottle which is monitored using continuous-monitoring blood culture systems. These systems detect the growth of microorganisms by detecting the presence of carbon dioxide or oxygen or a change in pressure within a blood culture bottle. Both current methodology and

the majority of newer techniques being proposed as a method to diagnose bloodstream infection focus on this aspect of detection: amplifying the signal that corresponds to the presence of bloodstream infection.

In this dissertation, I focus on developing a technique to improve upon the methodology currently used in the diagnosis of bloodstream infection. Specifically, I explore a method to perform rapid process of a large volume of blood in order to separate microorganisms present within blood from other blood components, to facilitate rapid identification. As previously stated, a large volume of blood is usually obtained to increase the likelihood of detecting the presence of microbes within the collected sample. Given the low numbers of bacterial cells that may be present with a collected blood sample from a patient with suspected bloodstream infection, the collection of a large volume of blood increases the yield of blood culture. To that end, the ability to process a blood sample of large volume is an important component in solving this problem. Thus far, technologies within the field of microfluidics that have been developed which perform large volume separation for this purpose will need to be scaled up in order to process a typical volume of blood collected to diagnose bloodstream infection. Performing a separation step is valuable, in that oftentimes clinicians are compelled to administer empirical antimicrobial therapy to patients with suspected bloodstream infection. Therefore, blood samples from patients with this suspected diagnosis contain compounds that reduce the yield of recovery for microorganisms. In addition, other components of blood, such as complement proteins and white blood cells, will certainly reduce the recovery of microorganism by performing their function: the targeted destruction of invading microbes.

I also develop a method for concentrating the number of microorganisms present within a liquid suspension, without the use of culture, by using microfluidics. Concentrating the number of microorganisms present within a liquid suspension allows for the amplification of the signal that indicates the presence of infection. As has been described, the techniques currently used within the field of microfluidics focus on nucleic acid amplification techniques. In contrast to the nucleic acid techniques described, the method used here employs the use of magnetic forces in order to achieve separation and concentration of bacterial cells, and is performed on chip. Using a methodology called microfluidic magnetic ratcheting, magnetically tagged entities present within a liquid suspension are concentrated from within a sample loading patch into a collection patch on chip, without the need for additional wash steps or additional microbial culture steps. Using this microfluidic technique can greatly enhance efforts to improve upon current methods used to diagnose bloodstream infection.

In summary, this dissertation focuses on two aims. The goals of Aim 1 are to: 1) Separate microorganisms from blood components and 2) Perform this separation in a short amount of time, while processing a large sample volume. The goals of Aim 2 are to: 1) Rapidly concentrate bacteria present within a liquid suspension using microfluidics and 2) Characterize the performance of the microfluidic chip designed to rapidly concentrate bacterial cells of interest.

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1.7 Tables

Table 1: Bloodstream Infection Diagnostic Methods

Bloodstream Infection Diagnostic`	Signal Detection/Amplification Method	Microbial Detection and Identification Method	Turn Around Time	Advantages	Disadvantages
Conventional Blood Culture	Microbial cell multiplication in growth media.	Biochemical phenotypic sub-culture methods. MALDI.	48 – 72 hours	Clinical Experience. Broad microbe applicability.	Difficulty with fastidious organisms.
Multiplex PCR	Nucleic acid amplification.	Fluorescent probes.	< 6 hours	Ability to detect common bloodstream infection pathogens.	Limited by primers used for nucleic acid amplification.
PCR/ESI MS	Nucleic acid amplification.	Electrospray Ionization Mass Spectrometry.	< 6 hours	Potential for lower limit of detection.	Potential for increased unit operational cost.
Functionalized Nanoparticles coupled with Magnetic Resonance	Detection of nuclear magnetic resonance signals of aggregate nanoparticles and water within the tested sample	Species specific probes that are used to functionalize superparamagnetic particle surface.	< 6 hours	Benchtop unit. Ability to use same instrument for additional testing needs.	Limited by probes used to bind pathogens of interest.

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Chapter 2

Centrifugation and Filtration as a Method for Sample Preparation in Medical Diagnostics for Infectious Diseases

2.1 Chapter Summary

Centrifugation and filtration are common benchtop techniques in a medical diagnostic laboratory, for the purpose of preparing a sample for further downstream analysis. With regard to the use of medical diagnostics in infectious diseases, it is important to appreciate how the techniques of centrifugation and filtration are incorporated into sample preparation, because they have the potential to enhance or reduce the final yield of a particular diagnostic test. This is especially important in the development of diagnostics for infectious diseases, where, depending on the sample of interest, the microbe of interest may be hard to isolate. In this chapter, I review centrifugation and filtration methods that have been used for the purpose of medical diagnostic sample preparation in infectious diseases. I also describe a technique that I developed for the purpose of Aim 1 of this dissertation: to 1) Separate microorganisms from blood components and 2) Perform this separation in a short amount of time, while processing a large sample volume.

2.2 Introduction: Use of Filtration in Medical Diagnostic Sample Preparation

There are many instances where filtration is used as a method to prepare medical samples for analysis. Considering that often specific analytes or cells of interest need to be further characterized for an accurate diagnosis to be made, it naturally follows that filters have been

incorporated in the sample preparation process in order to separate analytes of interest from background material that will obscure analysis. During the process of filtration, a suspension containing various analytes, compounds, or cells, which will collectively be referred to as solutes, is allowed to pass through a material that allows the passage of one or more solute while preventing the passage of the remaining solutes within the suspension. The material being used in the process described would be referred to as the filter. Many types of materials can be used as a filter, examples of which would include granular material such as sand and silica(McDowell-Boyer, Hunt et al. 1986), or membranes that have been created from varying types of compounds. With regards to the types of membranes used for filtration, membrane material properties such as hydrophobicity or hydrophilicity, membrane pore size, and the size of the surface area to be used for filtration are all important factors to consider when determining the best membrane to be used for the filtration process desired(Ahmad, Leo et al. 2015).

With regard to sample preparation, an important aspect to keep in mind is the volume of fluid that needs to be processed in order to prepare the sample for analysis. In terms of samples for medical diagnostics, collected sample volume size can vary widely, depending on the clinical scenario. For example, a typical respiratory sample could have a volume of 2-3 milliliters, whereas a sample obtained from the abdominal cavity of a patient with liver failure could have a volume of nine liters. For this reason, it is important to appreciate the techniques available for performing filtration for different amounts of sample volumes. Filtration can be achieved on a nano and micro scale, with sub-milliliter volumes, or macro scale, where larger volumes are processed. Filtration can be performed on a size-exclusion or size-inclusive basis, where solutes of interest within a suspension are collected, whereas other components of the suspension are not

collected, based on solute size. Also, several classes of forces can be used to generate the flow of a fluid suspension for which filtration is needed. Some examples of the classes of forces frequency used in filtration include gravitational, centrifugal, hydrostatic, and oncotic forces. The combination of several factors, including the characteristics of the filter chosen, the characteristics of the fluid suspension to be filtered, and the characteristics of the solute desired to be collected from the fluid suspension to be filtered will all determine the ultimate chosen technique for sample preparation.

Several techniques have been described where filters are used to assist with medical samples for infectious diseases(Kim, Johnson et al. 2009). For example, in an effort to increase the sensitivity of smear microscopy, a common method for evaluating patient respiratory samples for the presence of *Mycobacterium tuberculosis*, the use of a small membrane filtration technique has been described(Jones-López, Manabe et al. 2014). This filtration system uses a cylindrical manifold that is then connected to a single vacuum. Sample tubes are attached to manifold via a screw-on port adapter that contains two reservoirs. This allows for two separate filtration and concentration steps: first, through a nylon net pre-filter, with a 30 micron pore size, which is then followed by an Isopore™ membrane filter, a polycarbonate screen filter with a 0.8 micron pore size. The system is able to accommodate 50 milliliter centrifuge tubes. In this study, respiratory samples up to 30 milliliter in volume were processed, and the maximal reported processing time was approximately 5 min. The purpose of the apparatus was to concentrate bacteria onto a filter that could then be visualized under a microscope to determine if bacilli were present.

In another example, strains of *Alicyclobacillus* are discriminated with horizontal attenuated total reflection (HATR) accessory with Fourier transform infrared (FT-IR), which is performed on bacteria that has been pelleted using centrifugation (5000 rpm for 5 min), and then air dried onto a nitrocellulose membrane filter (Chin, Laksanasopin et al. 2011). There has also been work to adapt the dried blood spot technique with the use of filters. In this work (Li, Henion et al. 2012), authors adapt a device originally designed to determine patient homocysteine levels from whole blood to be used for sample preparation in order to allow further adaptation of the dried blood spot technique. The filtration process here is a passive filtration process with the use of a two-layer polymeric membrane substrate; the final goal is to separate erythrocytes from whole blood to allow for analysis of plasma. There has been some suggestion that the dried blood spot technique could be applied to human immunodeficiency virus (HIV) point of care diagnostics in the developing world (Cassol, Salas et al. 1992); ways to modify this technique to allow use of this application to occur would be very useful.

In microfluidics, much has been reported with regard to performing filtration of patient blood samples on chip. This microfluidic cell sorting polydimethylsiloxane (PDMS) chip (Kang, Ali et al. 2014), performs continuous-flow separation of leukocytes from blood samples using a crossflow filtration design. The 2-layer chip contains microfluidic chips on the top and bottom, with PDMS microfiltration membranes in between, in order to separate leukocytes from erythrocytes using size-exclusion filtration. This chip is able to process whole blood at a flow rate of 1 milliliter·hr⁻¹. In this work (Songjaroen, Dungchai et al. 2012), a microfluidic paper-based analytical device has been developed, but for use with small volumes. These authors (Davies, Kim et al. 2012) report a microfluidic filtration system, a poly(methyl

methacrylate) (PMMA) device, that is used to purify extracellular vesicles from whole blood. The device consists of PMMA open cross-channel microfluidic chip onto which membranes created by the UV polymerization of photo-patterned porous polymer monoliths (PPM) present in solution have been patterned. The device uses two modes of operation, pressure-driven filtration, at a flow rate of $1.0 \text{ microliters} \cdot \text{min}^{-1}$, and electrophoresis-driven filtration, where filtration of 240 microliters of blood occurred over 2 hours.

A technique to perform high speed microchip electrophoresis using a 96 well ultrafiltration membrane has also been reported(Primack, Flynn et al. 2011). In another technique(Thorslund, Klett et al. 2006), a hybrid PDMS device that has incorporated within it a polypropylene filter, which can accommodate a volume of 20 microliters, was developed for the purpose of whole blood filtration to allow for testosterone testing. Finally, the use of use transverse-flow microfilter devices has been described(Crowley and Pizziconi 2005). Here, authors fabricated these devices using silicone wafers onto which micro-channels were fabricated using standard photolithographic techniques coupled with plasma etching. The chip was tested using small volumes of bovine blood: 5 microliters.

2.3 Challenges Unique to the Use of Filtration in Infectious Diseases

Medical Diagnostic Sample Preparation in Bloodstream Infection.

The previously described methods are useful for the problems they were designed to address, but would need adaptation if they are to be applied to the clinical problem to diagnosing

bloodstream infection. For example, given the abundance of cells present within whole blood, membrane fouling that can occur should be characterized and addressed (Xue, Lu et al. 2014). In addition, it is important to consider the biocompatibility of the filter to be used, pore size, and, perhaps, the shape of the pathogen to be recovered. Any one of these factors will certainly impact the recovery of the infectious agent of interest from whole blood samples. In the case of bloodstream infection, the current standard is to collect 10 milliliters of blood, which is the accepted optimal amount to increase sensitivity of blood culture in identifying bloodstream infection. Given the large volume used, rather than using a microfluidic system to achieve large scale filtration, it may be useful to perform this process off chip. To solve this problem, I propose creating a system that would allow accommodation of this large amount of fluid. In this case, the proposed system would allow for selective whole blood cell lysis along with filtration.

The work addressed in this chapter focuses on Aim 1 of the dissertation. The goals of Aim 1 are to: 1) Separate microorganisms from blood components and 2) Perform this separation in a short amount of time, while processing a large sample volume. The chosen methods for this section of my dissertation work include lysing the blood cells present within whole blood, without lysing the bacteria of interest. This lysis step is necessary, so as to reduce the effect of fouling that will occur as a result of the high abundance of blood cells present within whole blood. This is then followed by centrifugation and filtration of the lysate using a benchtop centrifuge. The centrifugation and filtration step involves the use of a custom designed 50 milliliter conical tube insert that has a membrane incorporated into it to allow size-exclusion filtration. By performing a centrifugation and filtration step, any blood cells that were not lysed should be separated from the bacteria of interest, allowing further purification of these bacteria.

2.4 Selective Whole Blood Cell Lysis

Several agents have been described in the literature as potential agents that can be used for whole blood cell lysis (Bossuyt, Marti et al. 1997). In terms of the blood cells present within whole blood, for this work, it is important to consider both erythrocytes and leukocytes. In the whole blood of healthy male patients, the expected erythrocyte concentration would be $4.86 \times 10^{12}/L$, and the expected leukocyte concentration would be $6.12 \times 10^9/L$ (d'Onofrio, Chirillo et al. 1995, Targher, Seidell et al. 1996, McNerlan, Alexander et al. 1999). Erythrocytes are by far the most abundant cell present within whole blood; therefore, it would make sense to target the effectiveness of any potential lysing agent for this cell. Collectively, saponins are known to be natural glycosides with the ability to lyse erythrocytes (Waite, Jacobson et al. 2001, Podolak, Galanty et al. 2010, Weng, Thakur et al. 2011, Yu, Chen et al. 2012). Additionally, while saponins have been shown to have antibacterial potential, there have been some reports showing saponin to have a lack of cytotoxic effect on *E. coli* (Arabski, Wegierek-Ciuk et al. 2012). For this reason, saponin was used as the lysing agent in the lysing solution for these experiments.

To determine the optimal amount of lysing agent to use, experiments were conducted by incubating whole blood with varying concentrations of saponin mixtures (see Figure 1). The goal was to determine the optimal concentration of saponin to use that would allow for maximal lysis of erythrocytes and minimal lysis of bacteria. Saponin mixtures were made using PBS as the base solvent. To determine the effect of saponin concentration on red cell lysis, whole blood was spiked into mixtures with varying concentrations of saponin, and allowed to incubate for 30

minutes. Then, by measuring the number of erythrocytes present at baseline and the number of erythrocytes present after spiking, the effect of each concentration of saponin on the lysis of erythrocytes was determined. The number of erythrocytes present before and after spiking was measured by performing hemocytometer counts of erythrocytes present in each mixture. In Figure 1, the results of these experiments are shown. There is a dramatic drop of the number of erythrocytes that remain in each mixture at a concentration of 0.175% (w/v) saponin.

Next, to determine the effect of saponin concentration on the number of bacteria cells, experiments where bacteria in liquid suspension was allowed to incubate with varying concentrations of saponin mixtures were performed (see Figure 2). Saponin mixtures with different weight based concentrations were made in the same manner as in experiments performed to determine saponin effect on the lysis of erythrocytes, using PBS as the base solvent. *S. aureus* was grown in liquid media and allowed to reach the mid-log phase of growth. Next, aliquots of bacteria in liquid suspension was mixed with saponin mixtures, and allowed to incubate for 30 min. Then, an aliquot of the saponin-liquid bacteria solution was plated onto Tryptic Soy Agar plates, which were then incubated for 24 hours at 37 degrees C. Following this, each colony forming unit of bacteria was counted from each plate. In Figure 2, the results of these experiments are shown. An analysis of variance was performed, to determine if there was a statistical difference between the means of each group. There results indicate that there is no statistical difference in the number of *S. aureus* colony forming groups recovered from each condition.

Finally, to determine the effect of saponin concentration on the growth curve of bacteria, experiments where bacteria in liquid suspension was allowed to incubate with varying concentrations of saponin mixtures over a 24 hour period (see Figure 3). Again, saponin mixtures with different weight based concentrations were made in the same manner as in experiments performed to determine saponin effect on red blood cell lysis, using PBS as the base solvent. *S. aureus* from frozen stock was grown in liquid media overnight, after which, a small aliquot was added to each saponin solution, within a 96 well plate. The 96 well plate was allowed to incubate for 24 hours at 37 degrees C rotating at 237 rpm within a microwell plate reader. The optical density of each solution was measured by the microwell plate over the course of 24 hours. In Figure 3, the results of these experiments are shown. Interestingly, these results show there is no decrease in the growth rate of *S. aureus* allowed to incubate with saponin, and that there may be a level of augmentation of growth that occurs. Based on these experiments, an optimal concentration of 0.18 % (w/v) saponin was used for further experiments (Figure 4).

2.5 Macroscale Filtration.

2.5.1 Design Process for Making the Centrifuge Insert.

Given the volume of liquid that needs to be accommodated for this process, a method to quickly process this amount of fluid in a short period of time was desired. In addition, it would be useful for the proposed method to integrate with equipment and material currently in use in laboratory settings. The centrifuge insert was designed to be compatible with a benchtop 50 milliliter conical tube. After several design iterations with drawings created using AutoCAD®,

and several design prototypes created with 3D printing, a final centrifuge insert design was fashioned using Delrin[®] (Figure 5). Delrin[®] is durable, autoclavable, and biocompatible. It also will allow minimal adhesion for bacteria, to minimize loss of bacterial cells. The insert was designed to accommodate a total of 20 ml of fluid, so as to accommodate 10 ml of human blood (the optimal amount of blood needed to increase yield of blood cultures) with lysis buffer that has been added at a 1:1 ratio, resulting in 20 ml of fluid. The centrifuge insert consists of two pieces with a male and female component. Adding a lysis buffer significantly reduces the burden of blood cells that will require filtration, thus reducing membrane fouling. In order to allow filtration of undesired larger blood cells that are not lysed, a 5 micron hydrophilic filter is juxtaposed between the male and female centrifuge pieces, along with an O-ring (added to create a water-tight seal).

2.5.2 Experiments with Centrifuge Insert.

To first demonstrate that the assembly of the centrifuge insert has a water-tight seal, experiments were performed where a hydrophobic membrane was used to prevent passage of water. These experiments confirmed that this was a water tight seal, in that there was no passage of water. The final assembly of the centrifuge insert is displayed in Figure 6. In order to determine the ability of the centrifuge insert to perform size-exclusion filtration of particles with a diameter greater than 5 microns, further experiments were conducted. Here, solutions of water containing 10 micron beads were subjected to the filtration process. These solutions were meant to simulate whole blood. The filtration process is as follows: the centrifuge insert was assembled as shown in Figure 6a. The entire assembly was then inserted into a 50 milliliter conical tube. After creating a solution, this was then poured into the interior of the centrifuge insert. Finally, the

entire assembly, which consisted of a 50 milliliter conical tube containing the centrifuge insert filled with a solution of water with 10 micron beads, was run in a centrifuge at 1000 rpm for 5 minutes. The concentration of 10 micron beads in the solution filling the centrifuge insert before centrifugation was compared to the concentration of 10 micron beads in the filtered solution. These results of these experiments are shown in Figure 7. Here, it can be seen that almost 100% of the 10 micron beads have been retained by filter. The filter in this case is a nylon filter with a 5 micron pore size. Moving forward, the centrifuge assembly set up included the McMaster-Carr O-ring, and 5 micron nylon filter.

Finally, to determine the yield of recovery of bacteria present in the filtrate produced after lysis and filtration of whole blood using this system, another set of experiments were conducted. In these experiments, whole blood with bacteria added was subjected to whole blood lysis by incubating the bacteria spiked whole blood solution with a saponin solution of 0.18% (w/v) for 30 min. This solution was then introduced into the centrifuge insert filtration system for filtration, using a centrifuge at 1000 rpm for 5 minutes. In this case, *S. aureus* was the bacteria used. Aliquots of each solution was grown tryptic soy agar plates, which were incubated for 24 hours at 37 degrees C. Colony counts of *S. aureus* present before incubation with the lysis solution, after incubation with the lysis solution, and in the filtrate solution. These results of these experiments are shown in Figure 7. A significant amount of bacteria was lost to recovering, possibly from over-aggressive lysis or membrane fouling. In fact, by the end of the process, there was an average of 75% loss of bacteria, as compared to the amount present in bacteria spiked blood at the beginning of the process.

2.6 Conclusions and Future Direction

Using the described approach of performing centrifugation and filtration in order to purify blood samples for the purpose of isolating and identifying the pathogens in bloodstream infection was not successful. There was significant loss of bacteria at each step in the process. On average, there was a 75% loss of bacteria present in whole blood that was spiked with bacteria prior to being processed using this technique. If this technique is to be used, it will be necessary to perform further optimization at each step of the process, including whole blood lysis and centrifugation with filtration.

For example, adding sodium polyanethol sulfonate (SPS) could assist in improving the performance of the lysing solution. SPS is an anticoagulant; this compound enhances the recovery of bacteria from the blood samples of patients with suspected blood culture(Eng 1975). It has also been shown to have an inhibitory effect on *in vitro* growth of *Neisseria meningitides*, though this effect was not present for *Staphylococcus aureus*(Eng and Iveland 1975). While it has been noted to decrease complement mediated hemolysis of red blood cells (Myers, Hamati et al. 1992), it may inhibit complement pathway mediated killing of microorganisms that occurs *in vitro*(Palarasah, Skjoedt et al. 2010). This addition of this compound may assist in augmenting the yield of bacteria that remain in solution after performing whole blood lysis. Further experiments could be performed to determine whether this would be a successful addition to the current lysing solution, and, if so, what optimal concentration should be used.

In addition to the experiments outlined above, further experiments could be performed in order to determine the optimal centrifuge parameters to be used for the centrifuge insert. It is also possible that membrane fouling is responsible for the low recovery of bacteria, and the use of a different membrane consisting of different material could improve this. To measure this, it would be important to determine the effect of the filter on removing white blood cells, which would likely be the main factor in membrane fouling. Note, a ratio of 1:1 was used in terms of the volume of lysis solution used for the volume for the volume of blood processed. Varying this ratio may also reveal a ratio that would also improve the recovery of bacteria. Any combination of the proposed investigations could improve the yield of this proposed method. Alternatively, if it is possible to perform the overall objective of isolating bacteria from whole blood without the use of centrifugation and filtration, efforts should focus on the methodology that could perform that process.

2.7 Acknowledgements

I would like to acknowledge the Boelter Hall Machine shop, who assisted in machining the final Centrifuge Insert, and also, the 2015 Bioengineering Capstone Group C students, who contributed the computerized drawing of the centrifuge insert (Figure 5a).

2.8 Figures

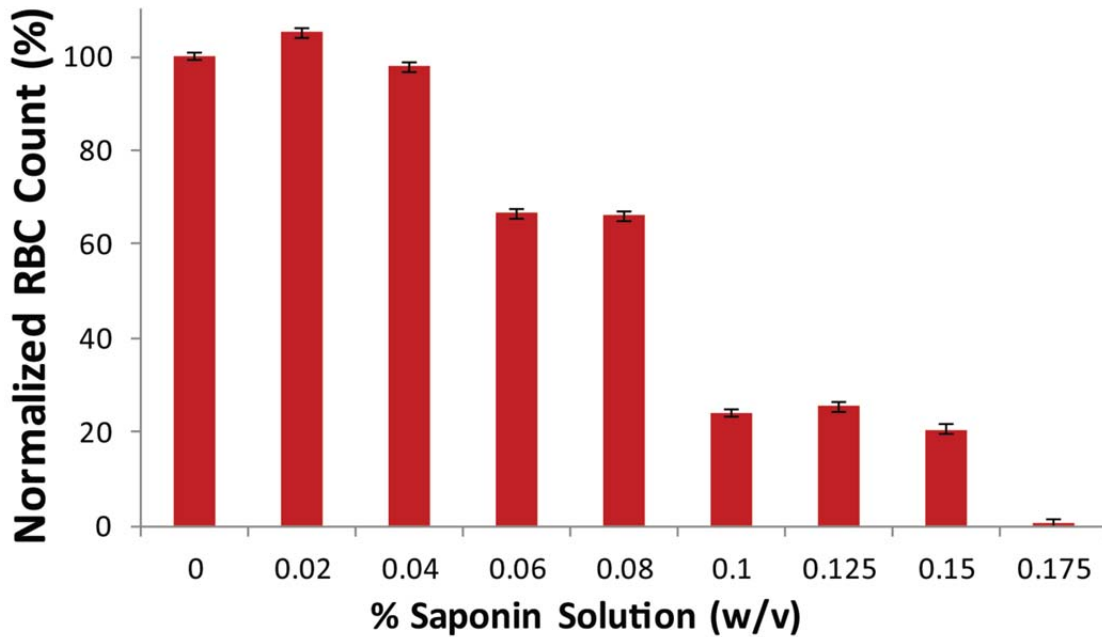


Figure 1: Effect of Saponin on Red Blood Cell Lysis. A normalized red blood cell count (RBC) calculated for each condition where whole blood is allowed to incubate with a saponin mixture. The normalized red blood cell count is a ratio of the number erythrocytes, or red blood cells (RBCs), present in solution after incubation with each saponin mixture compared with the number of erythrocytes present in whole blood before lysis. There is a significant decrease in the number of erythrocytes remaining in solution after incubation with a saponin concentration of 0.175% (w/v). Three trials were performed for each condition.

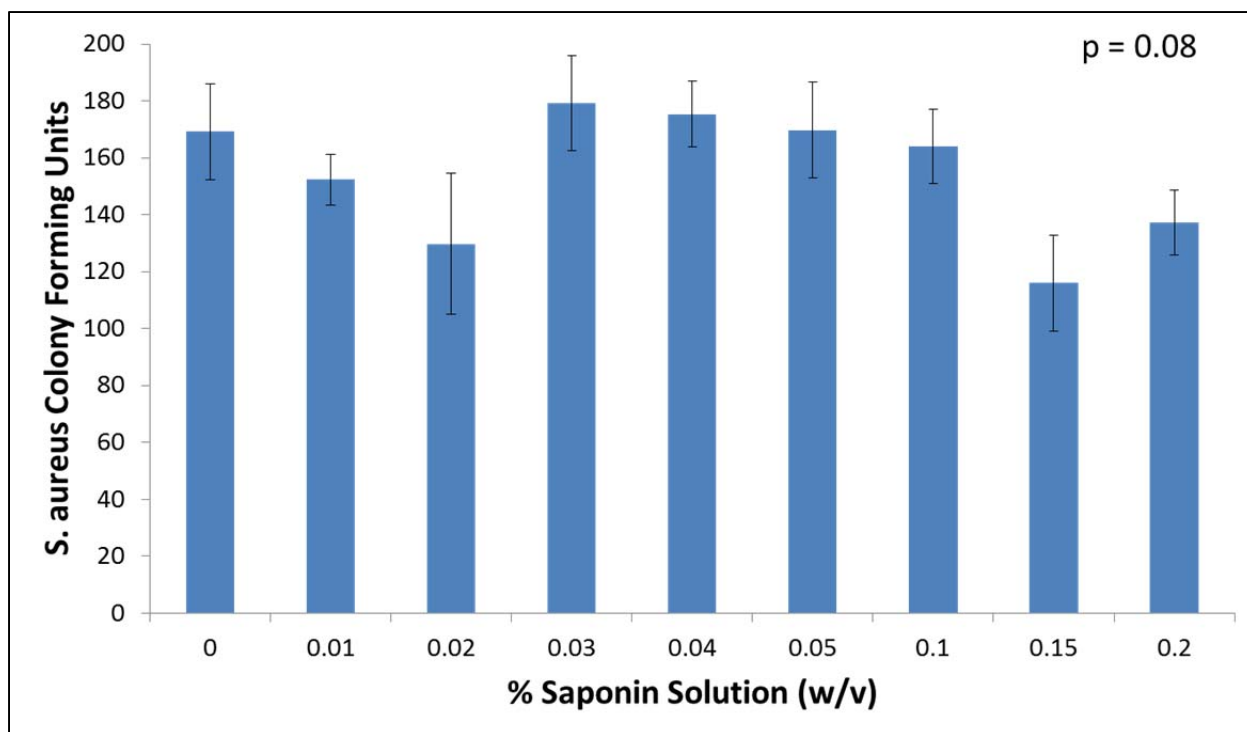


Figure 2: Measuring the Cytotoxic Effect of Saponin on *S. aureus*. The number of *S. aureus* colony forming units recovered after exposure to varying concentrations of saponin is displayed. The number of colony forming units recovered for each condition is similar in magnitude. Three trials were performed for each condition.

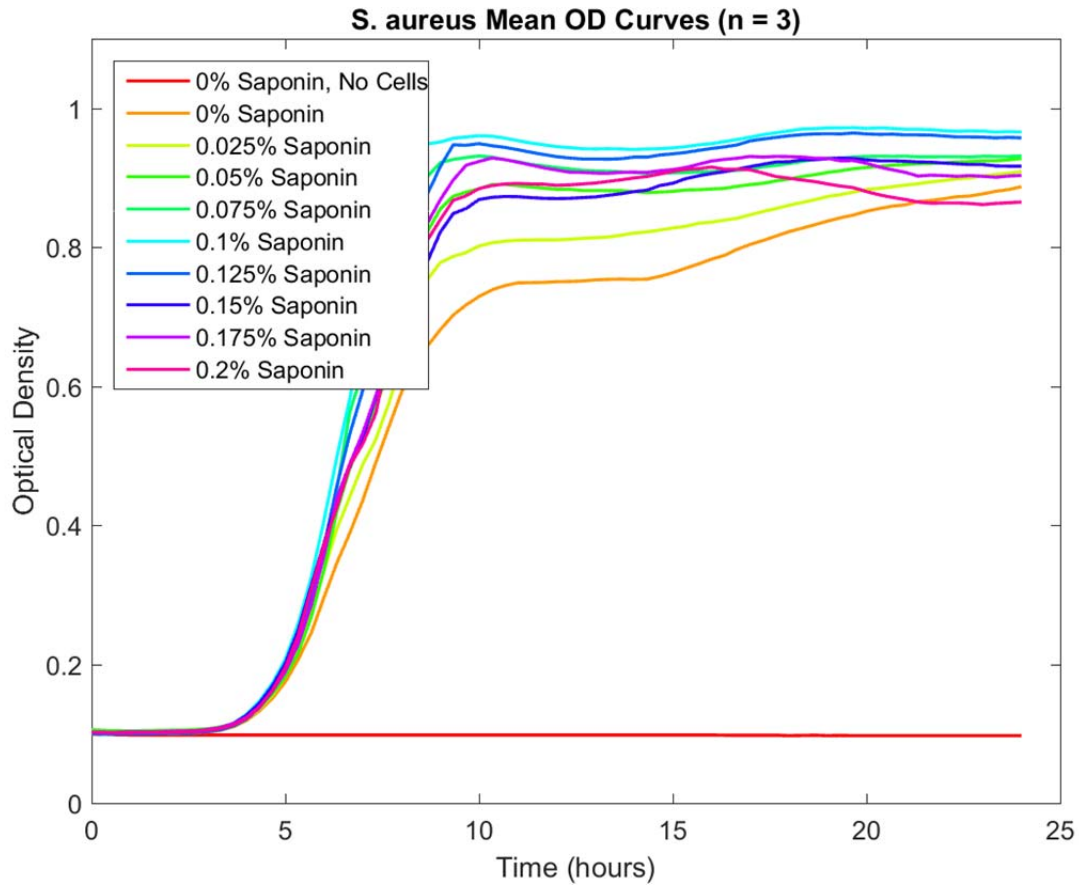


Figure 3: Measuring the Effect of Saponin on the Growth Rate of *S. aureus*. The rate of growth of *S. aureus* allowed to incubate with varying concentrations of saponin is displayed. A similar rate of growth is seen for each concentration of saponin that *S. aureus* is exposed to.

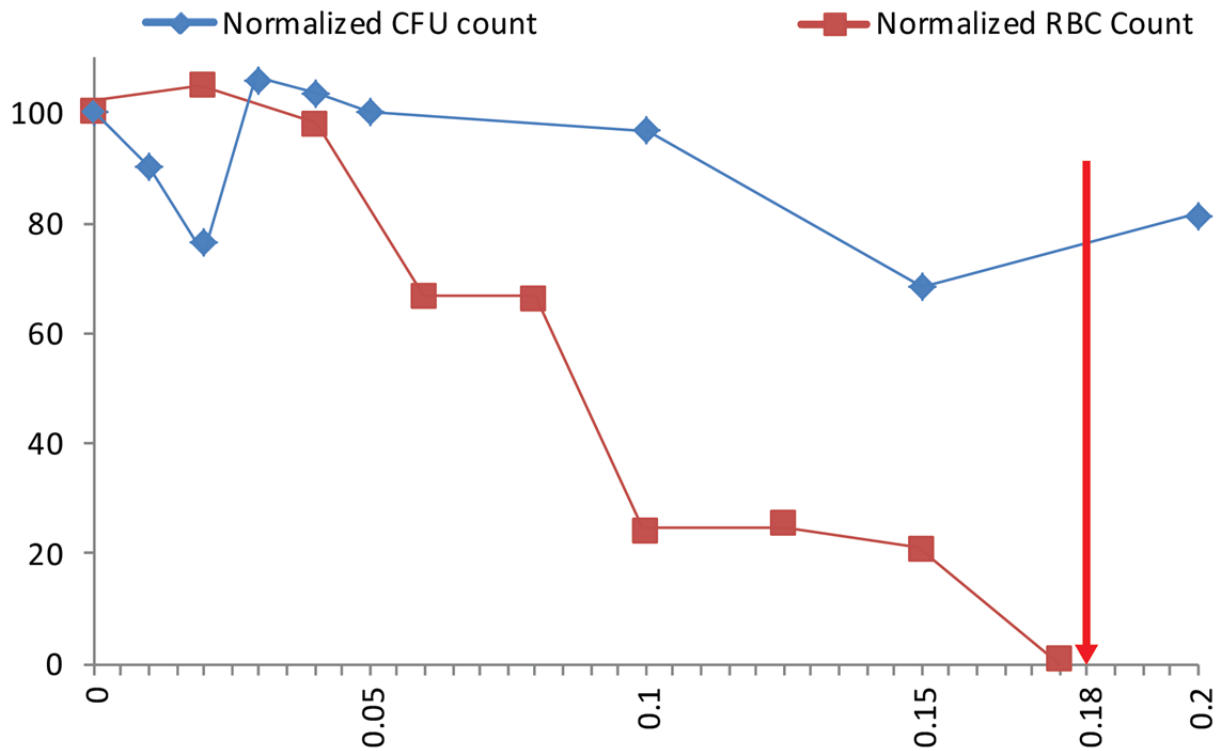


Figure 4: Optimal Saponin Amount for Lysing Solution. The optimal saponin concentration selected allows for optimal lysis of erythrocytes (normalized RBC count) and minimal lysis of bacteria.

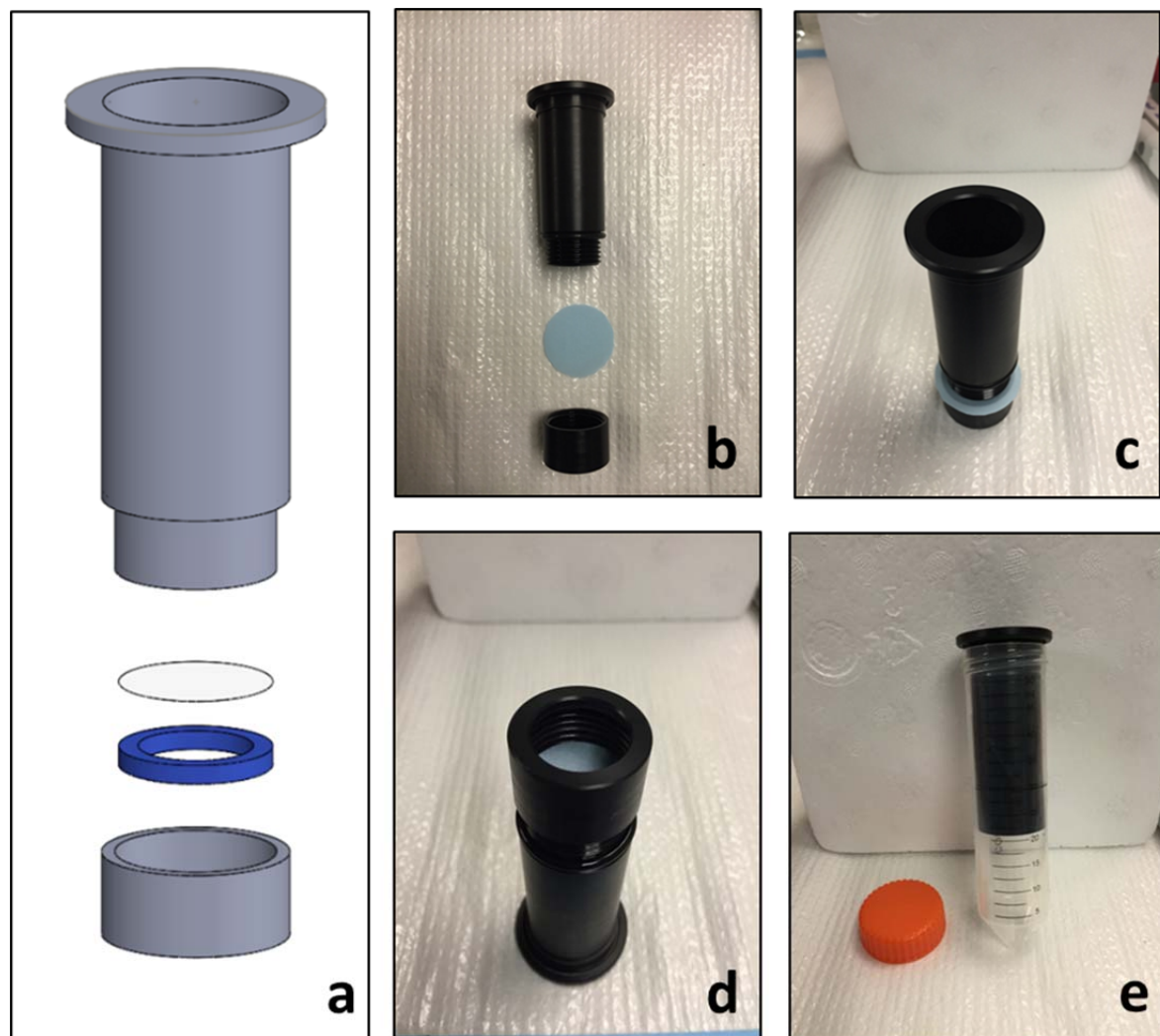


Figure 5: Final Centrifuge Tube Insert Design. a) Computerized drawing of Centrifuge Tube Insert design, with membrane and O-ring insert. b) – e) Photographs of centrifuge insert design with the assembly components, including 50 milliliter conical tubes. The final piece was fashioned using Delrin[®] as the material.

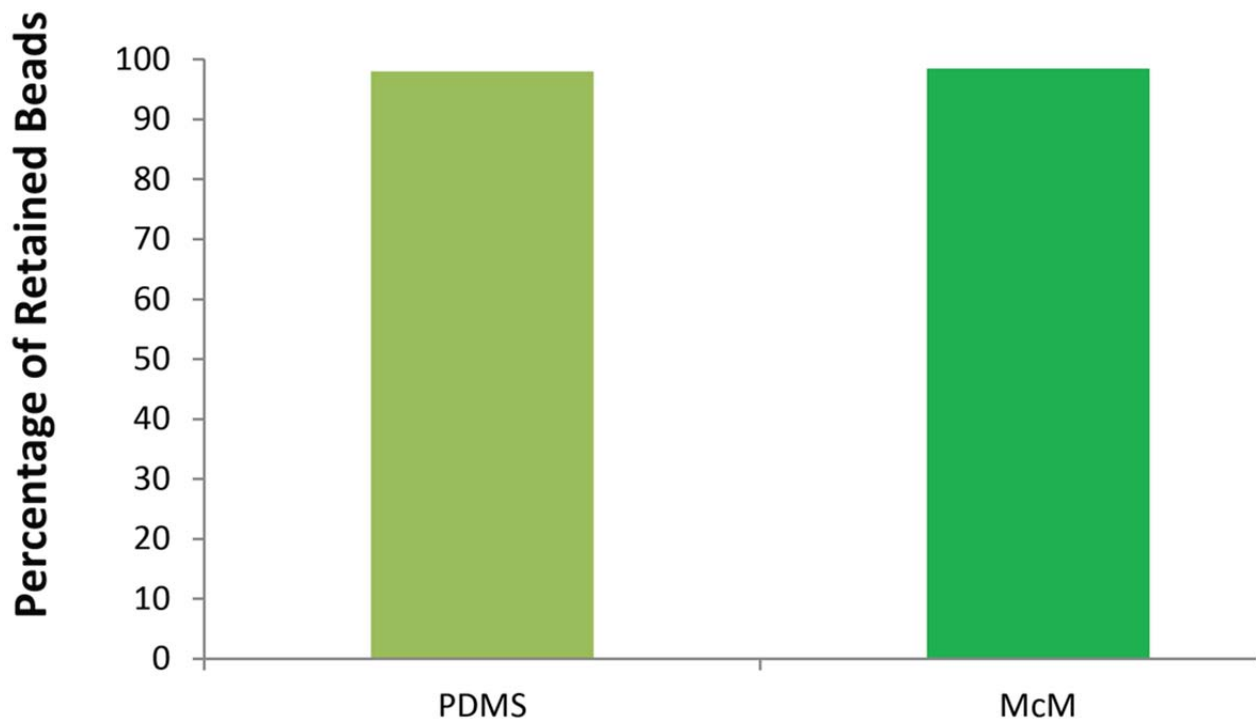


Figure 6: Preliminary Centrifuge Insert Experiments with O-Ring. For these experiments, the ability of the centrifuge insert to perform size exclusion filtration was confirmed. The membrane used for this assembly is a nylon membrane with a 5 micron pore size. Two types of O-rings were tested. Each type of O-ring had three trials conducted. PDMS = polydimethylsiloxane; McM = McMaster-Carr.

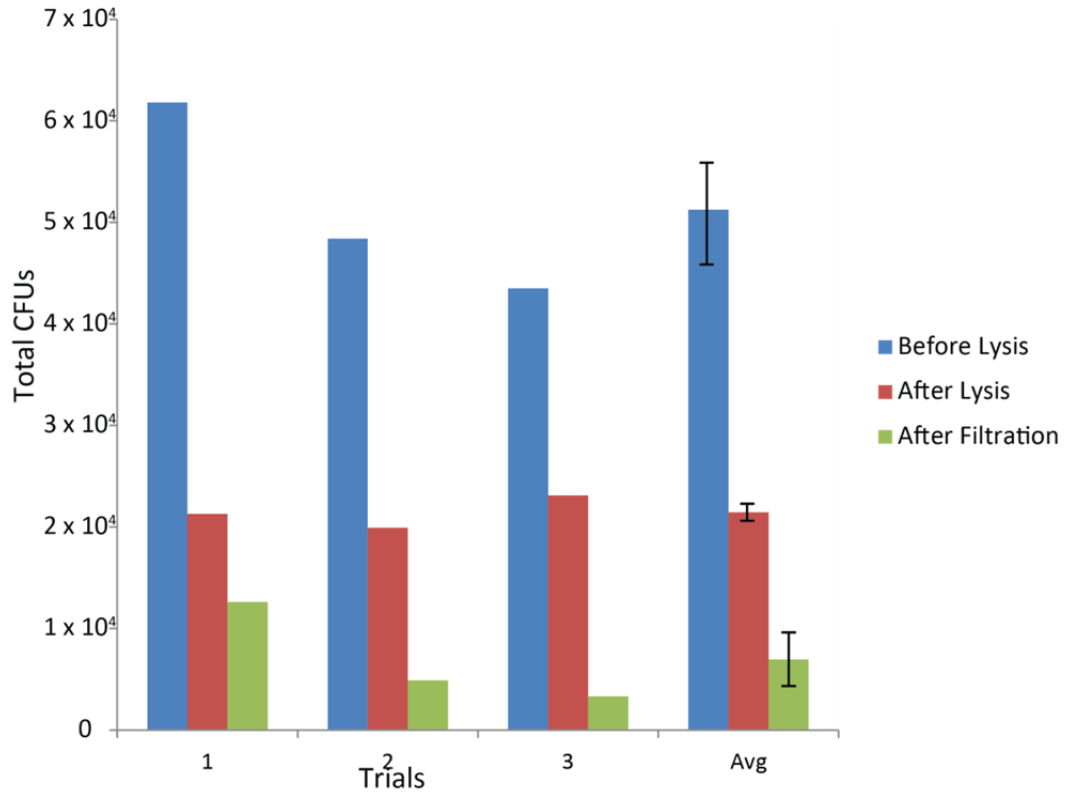


Figure 7: Centrifuge Insert Experiments with Whole Blood. For these experiments, recovery of *S. aureus* was determined at each step of the process. There was a significant loss in the amount of bacteria recovered by the end of the total process. A total of 3 trials were conducted.

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Chapter 3

Rapid Concentration of Bacteria Using Microfluidic Magnetic Ratcheting

3.1 Chapter Summary

Blood stream infections are life threatening, requiring immediate and ideally personalized care, however, currently, the identification of the causative micro-organisms often requires days, and often results in false negative results. Concentrating microorganisms prior to analysis from blood could reduce time and enhance culture positivity. In the presented work, a magnetic ratcheting-based microfluidic chip is used to enrich microorganisms present in suspension bound to functionalized nanoparticles. This technique could be used to prepare clinical microbiology samples for further analysis, potentially with a more rapid turn-around-time. In addition, the microchip used for this work has been further characterized in order to determine the optimal conditions for chip usage.

3.2 Introduction

Rapid identification and treatment of infectious agents has been an essential part of treatment and care that is provided in health care. In addition, the rapid identification of resistant bacterial species has been identified by the President of the United States as a national priority. Indeed, President Obama issued Executive Order 13676: Combating Antibiotic-Resistant Bacteria on September 18, 2014, a document which states that the increase in antibiotic-resistant bacteria is a serious public health threat(Obama 2014). The rapid identification and treatment of infectious

agents in the care of patients with infectious diseases leads to the reduction in the length of stay, cost of care, and ultimately results in better care given to patients in the hospital.

In the past, there have been various mechanisms that have been used to achieve this objective of identifying and treating infections. These mechanisms include performing serial culture steps coupled with using biochemical phenotypic methods to detect organisms that preferentially metabolize different substrates. The length of time for recovering microorganisms from patient clinical samples by culturing is often lengthened by various factors, including the presence of fastidious organisms, the presence of antimicrobials which are frequently given empirically, and also the fact that these organisms may be present in rare/low amounts(Shenep, Flynn et al. 1988). Modern technology and improvement or recent advances in science and technology and genetic engineering has allowed us to improve ways to rapidly identify these infectious agents. These advances have allowed improvement in the care provided to patients.

Bacterial and fungal bloodstream infections are an important cause of patient hospital morbidity and mortality. Whether these bloodstream infections are central-line associated, mucosal barrier injury related, or primary in origin, bloodstream infections are a significant burden in health care(Epstein, See et al. 2016). Approximately 200 000 cases of bacteria have been reported to occur annually in the United States(Coburn, Morris et al. 2012, LeBude and Diemer 2014). Bloodstream infections require immediate and, ideally, personalized care, in terms of antimicrobial therapy. While immediate, empirical antimicrobial therapy is often given when the onset of bloodstream infection is suspected, the type of specific personalized therapy that can

impact patient outcomes cannot be instituted until the exact causative agent of the bloodstream infection has determined. Given the life threatening nature of bloodstream infection, rapidly diagnosing bloodstream infection can improve patient care by reducing patient hospital length of stay, cost of care, morbidity, and ultimately patient outcome(Beekmann, Diekema et al. 2003, Kumar, Roberts et al. 2006). Using the current standard methodology, the identification of the exact causative agent in a bloodstream infection usually requires 48 – 72 hours, and often longer (see Chapter 1, Table 1). This can certainly be improved upon.

In developing a diagnostic that can rapidly diagnose bloodstream infection, it is important to consider the characteristics of the fluid sample in question, and the findings present in this fluid that correspond with the presence of bloodstream infection. The physical characteristics of whole blood are as follows: whole blood is regarded as a “non-Newtonian, shear thinning fluid,” with viscosity that has been measured to have values between $1 - 10^3$ mPa·s or centipoise(cP). Blood viscosity is strongly dependent on the volume concentration of erythrocytes present, temperature, plasma protein concentration, shear forces, and the diameter of the tube through which it flows(Pries, Neuhaus et al. 1992, Simmonds, Meiselman et al. 2013). Whole blood has a relative density that has been measured to have a value of 1.0506 at 37 degrees C, a characteristic that is also dependent on temperature and volume concentration of erythrocytes(Trudnowski and Rico 1974). Additional physical properties to consider include it’s electrical parameters, such as dielectric permittivity, conductivity, capacitance, resistance, and magnetic properties, particularly with respect to hemoglobin, which is abundantly present within whole blood and is also responsible for blood iron transport(Sosa, Bernal-Alvarado et al. 2005). The chemical composition of blood includes water, ions, proteins, carbohydrates, and lipids.

The whole cells present within whole blood include erythrocytes, leukocytes, and platelets, which are present with blood of healthy adults in the following quantities: $4.5 - 5.9 \times 10^6$ erythrocytes per cubic mm, $4.5 - 11 \times 10^3$ leukocytes $\cdot \text{mm}^{-3}$, and $150 - 350 \times 10^3$ platelets per cubic mm (Kratz, Ferraro et al. 2004). Erythrocytes have a reported density of 1.086 – 1.122 grams per milliliter, leukocytes have a reported density of 1.057 – 1.092 grams per milliliter, and platelets have a reported density of 1.072 – 1.077 grams per milliliter (Pitt, Alizadeh et al. 2016). Bacteria in the sample of a patient with suspected bloodstream infection has been described to be present in varying amounts, ranging from non-detectable amounts, to 10^7 colony forming units (CFU) per milliliter of blood (Shenep, Flynn et al. 1988). It is unclear which fraction of blood that microorganisms are usually present, but very likely they are associated with platelets. Given the sheer abundance of whole cells within blood, it is important to consider that whole blood cells can obscure this signal, the presence of bacteria within whole blood, which corresponds with the presence of bloodstream infection. Thus, a methodology to separate bacteria from whole blood cells, such as red cells, can be very useful in the development of a diagnostic for bloodstream infection.

Furthermore, in terms of clinical diagnostic test development, there are particular issues that need to be addressed. To start with, what is the clinical syndrome of interest for which the new test is being developed, as defined by the medical community? With respect to this clinical syndrome, are there quantifiable metrics already known to correlate with the presence or absence of this clinical syndrome based on the detailed observations of the scientific and research

community at large? These quantifiable metrics, whether single or multiple, can be thought of as signals, which are to be detected or measured by the proposed test being developed for diagnosing the clinical syndrome of interest. What are the signals that have been described and how can these signals best be clearly detected? What other signals present within the system can affect the detection of the signal of interest, either by obscuring it, mimicking it, reducing it, or even amplifying it? Importantly, the gold standard test, or the generally accepted reference used to determine if the clinical syndrome in question is present, should be identified during the test development process. This standard will need to be incorporated into test development and implementation plans, so that the final accuracy of the developed test can be verified (Schulzer 1994, Greenberg, Daniels et al. 1996, Lalkhen and McCluskey 2008). Ultimately, the new technology will be subject to review and approval by the FDA (Shawar and Weissfeld 2011).

With regard to bloodstream infection, the current gold standard for detecting the clinical condition of bloodstream infection is blood culture. In this case, the signal that is detected is the presence of bacterial colony forming units (CFUs) within whole blood. It is widely known, however, that blood culture does not always detect the presence of bloodstream infection (Schifman, Meier et al. 2015). As has been previously stated, the number of bacterial CFUs isolated from a person's bloodstream can vary widely. In fact, there is evidence that during daily activities such as brushing teeth or defecation, in humans, the bacteria that constitute the normal flora inhabitants of surfaces where bacteria are known to reside, can escape into a person's blood stream (LeFrock, Ellis et al. 1973, 1975, Everett and Hirschmann 1977). These bacteria are thought to be rapidly eliminated by the immune system, so that these bacteremias spontaneously resolve (Aronson and Bor 1987), and are not allowed to persist within the blood

stream to cause bloodstream infection. Certainly, the manipulation of infected tissues can cause leakage of microorganisms into the bloodstream(Bennett Jr and Beeson 1954). However, there are clearly circumstances where bacteria that have entered a patient's bloodstream do persist and cause blood stream infection. In order to diagnose bloodstream infection, amplifying the signal of interest will allow the proposed diagnostic method to be a successful one, and an improvement over the ability of the current methodology to isolate a pathogen of interest.

Several authors have looked methods to increase the yield of the isolation of pathogens from patient blood cultures(Baron, Scott et al. 2005, Lee, Mirrett et al. 2007, Baron 2011). The introduction of continuous-monitoring blood culture systems (CMBCS) has had a significant impact on the detection of bloodstream infection(Murdoch, Koerner et al. 1995, Weinstein 1996). Previously, a standard of practice was established, whereby several collections of patient blood samples are obtained and cultured to aid in the isolation of microbial blood pathogens and the diagnosis of suspected bloodstream infection. This practice was established based on several literature reports that indicated an increase in sensitivity in blood culture results, with an increase in the number of collected samples(Washington and Ilstrup 1986). More recently, however, it has been reported that as many as 90% of blood cultures fail to reveal a pathogen(Baron 2011, Weinstein and Doern 2011). Several techniques have been proposed for the rapid detection of bloodstream infection(Cartwright, Rottman et al. 2016, Sheldon 2016). An approach that incorporates the use of microfluidics could make an important contribution in solving this problem. Microfluidics offers the possibility of creating a device with many repeatable (or parallelized) components, allowing high volume processing in a rapid amount of time, which would be helpful in large volume whole blood sample preparation. In addition, prototypes can

be quickly formed, revised, and updated, leading to a quick and low-cost solution. Importantly, the device to be used can be easily designed to be user-friendly.

Within microfluidics, several techniques have been described which could potentially be used to address the problem of rapidly diagnosing bloodstream infection. For example, Yung and colleagues reported the creation of a micromagnetic-microfluidic blood cleansing device, where a functionalized super-paramagnetic 1 micron beads along with a magnet were used to remove *Candida albicans* from whole blood(Yung, Fiering et al. 2009). Also, authors have used inertial effects within microfluidic channels, rather than a magnetic based approach, to separate bacteria from whole blood. Using inertial microfluidics, authors designed a massively parallel microfluidic device to separate *Escherichia coli* from whole blood(Mach and Di Carlo 2010). In another example, authors present a microfluidic system that allows selective lysis of blood cells with recovery viable bacteria(Zelenin, Hansson et al. 2015). Using saponin as the primary lysing agent for red blood cells, along with deionized water for an osmotic shock step to lyse white blood cells, authors demonstrate the ability to recover bacteria such as *Escherichia coli* and *Micrococcus luteus* from whole blood on chip with a flow rate of 48 microliters per minute. In this work, a blood cleansing technique was developed, which makes use of functionalized magnetic nanobeads functionalized with a genetically engineering mannose-binding lectin (FcMBL)(Kang, Super et al. 2014). These functionalized magnetic nanobeads are allowed to incubate in whole blood containing bacteria, after which this suspension is introduced into a microfluidic channel with the use of a peristaltic pump, and bacteria bound to functionalized magnetic nanobeads are drawn into a parallel microfluidic channel where saline is also flowing because of magnets located adjacent to this channel. With this technique, authors were able to

demonstrate clearance of over 90% of *Staphylococcus aureus* or *Escherichia coli* present within tested blood.

As seen with these prior approaches, in the development of new technology for the diagnosis of bloodstream infection, the focus has been on performing the separation of microorganisms from other cells present within whole blood. The prior described techniques are able to separate microorganisms from whole blood with significant recovery. However, these techniques will require integration with a technique that allows detection and identification of the recovered microorganisms. While separation of microorganisms is achievable, an important next step would be to amplify the signal that correlates with the presence of bacteria in blood. The signal, in this case, would be the actual number bacteria in the blood of a patient with bloodstream infection. Blood culture of a patient's sample is one method by which this signal can be amplified. Another technique would involve concentrating bacteria so that the actual number of bacteria within a specific area of to be analyzed for the presence of bacteria is increased. In this regard, the rapid diagnosis of bloodstream infection can likely be achieved with the use of nanomedicine(Sanvicens, Pastells et al. 2009, Tallury, Malhotra et al. 2010, Zhu, Radovic-Moreno et al. 2014), where newer nanotechnologies are applied for the purpose of solving this important clinical problem.

The concept of a molecular motor, where molecular complexes are able to transfer energy, whether chemical or potential energy, onto on an object in order to perform work on it, has been

described by several authors (Jülicher, Ajdari et al. 1997, Reimann 2002, Hänggi and Marchesoni 2009). The physical term “work” is defined by equation (1):

$$W = F \cdot s \quad (1)$$

where the work (W) performed on an object is equal to the product of the magnitude (F) of a constant force acting on the object to produce a net displacement (s). With regard to a model of the molecular motor example, and, in particular, a protein molecular motor model, the Brownian fluctuations present within an elastic element are captured for the purpose of generating a unidirectional force. A molecular ratchet has been described as a molecular motor where chemical reactions allow the motion generated by thermal fluctuations to be biased (Wang and Oster 2002). In other words, the molecular movement occurring due to thermal fluctuations can, with the assistance of chemical reactions, occur in a specific constant direction causing net displacement. This concept has further applied to study the movement of paramagnetic particles in the presence of a magnetic flux, in the use of magnetic ratcheting (de Souza Silva, Van de Vondel et al. 2006, Tierno, Reimann et al. 2010). More recently, Murray et. al. developed a microfluidic chip that leverages this physical phenomenon of magnetic ratcheting to develop a quantitative magnetic separation technology (Murray, Pao et al. 2016). The same physical phenomena of magnetic ratcheting can be potentially applied for the purpose of concentrating magnetic particles, which could be further used to concentrate bacteria. A concentration step is important so that an abundance of material that has been concentrated into a small area is available for analysis, where this enhances the yield of analytic technique to be used.

The work addressed in this chapter focuses on Aim 2 of the dissertation. The goals of Aim 2 are to: 1) Rapidly concentrate bacteria present within a liquid suspension using microfluidics and 2) Characterize the performance of the microfluidic chip designed to rapidly concentrate bacterial cells of interest. I achieve these goals by using a microfluidic magnetic ratcheting microchip, with *Staphylococcus aureus* as a prototype organism. Here, I present a system that can be used to rapidly enrich organisms for the purpose of rapidly identifying infection. *S. aureus* is indigenous to skin and can be a cause of serious infections, including necrotizing fasciitis infection and bloodstream infection. As discussed, Murray et. al. previously reported on a quantitative magnetic separation technology used to extract rare eukaryotic target cells from biological samples that made use of magnetic field amplification near large electroplated magnetic microelements(Murray, Pao et al. 2016), for the purpose of magnetic ratcheting. Here, I focus on using this platform for enriching microorganisms, present in blood in rare amounts, that cause bloodstream infections(Washington and Ilstrup 1986). The ability to enrich microorganisms will significantly aid in diagnosing bloodstream infection, by allowing signal amplification for further downstream identification and analysis of the enriched microorganisms. As compared to previously described platforms, this platform has unique advantages in allowing high-throughput processing and extreme concentration of the sample that can be integrated with other downstream analytical techniques such as mass spectrometry, in order to rapidly identify microorganisms causing bloodstream infections, with the goal of little to no culture steps.

3.3 Methods

3.3.1 Description of the Platform.

The platform used in this study (Figure 1) is a microfluidic magnetic ratcheting chip that has been designed to perform high-throughput enrichment of magnetically-tagged entities of interest from a background liquid suspension using a cycling magnetic field. This cycling magnetic field is generated using a Halbach wheel arrangement of permanent magnets to create dynamic potential energy wells on the surface of the chip that leads to the unidirectional ratcheting of magnetically-tagged entities (Figure 2). The magnetizable elements that comprise this microchip, which are permalloy micropillar arrays, allow for the creation of dynamic potential energy wells on the surface of the chip that, again, lead to the unidirectional ratcheting of magnetically-tagged entities. In this case magnetically-tagged entities are super-paramagnetic microparticles bound to bacteria. They are ratcheted with a frequency of 10 Hz for 30 min for the purpose of enrichment. Super-paramagnetic particles in solution are ratcheted over a surface area of 9.92 cm² into a surface area of 0.10 cm², a 100-fold area concentration (Figure 1).

3.3.2 Microfluidic Magnetic Ratcheting.

To perform bacteria ratcheting experiments, 500 nm particles were functionalized, the ratcheting platform was assembled, and then the ratcheting platform was set up to run on the ratcheting system. A cycling magnetic field was created by a Halbach arrangement of rare earth magnets within a circle that rotates. This specialized arrangement and circling motion creates a cycling magnetic field. The permalloy coated pillars on the microfluidic chip allow for the surface of each pillar to become magnetized. Magnetized elements are extracted from the bulk fluid, and move in direction opposite to the rotating field. These elements are compelled to move forward as the surface potential energy landscape is transformed to allow magnetic ratcheting to occur. In this case, particles find it to be energetically favorable to move in a direction opposite to the

cycling magnetic field (Figure 3). Each part of the experimental process is described in each section below.

3.3.3 Particle Production.

500 nm particles were functionalized with a biotinylated Anti-*Staphylococcus aureus* IgG3 mouse monoclonal antibody (ab21028, abcam[®]) that is specifically targeted against peptidoglycan within the cell wall of *S. aureus* (Figure 4). Particles were functionalized such that this antibody has been attached to coat the surface of these particles. Particles were functionalized using the streptavidin-biotin interaction (Figure 4)(Hsing, Xu et al. 2007). To perform this functionalization process, 5 microliters of a biotinylated anti-*S. aureus* antibody was added to 50 microliters of a stock solution of 500 nm streptavidin-coated dextran iron oxide composite nanoparticles (nanomag[®]-D, Micromod GmbH, Germany) in a solution of 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS). After incubation at 4 degrees for 60 minutes, particles were collected using an Eppendorf based magnetic bead collector. Collections steps were performed at 4 degrees C. After the initial incubation, 3 wash steps were performed.

3.3.4 Microfluidic Magnetic Ratcheting Microchip.

The magnetic ratcheting chip was designed and prepared based on a previously reported technique using electroplated magnetic micro-elements(Murray, Pao et al. 2016). Within the microchip, there are 3 regions: the large surface area region, where magnetized entities are extracted from the bulk fluid, an area over which particles become concentrated, using magnetic ratcheting, into a smaller and smaller area, until reaching the collection patch area. In comparing

the larger surface area of the chip with the collection patch, this represents a 100 fold reduction in surface area, allowing for enrichment of magnetically tagged entities (Figure 1).

To prepare the microchip for use, each microchip was coated with polystyrene in order to reduce the effect of friction on particle movement. In order to do this, a 5% (w/v) solution of polystyrene in toluene was prepared. After this, in a cleanroom setting, each chip was immersed in SU8 developer for 5 minutes; the chip surface was then rinsed with acetone, methanol, isopropanol, and then deionized water. Chips were then dried with nitrogen gas, and then placed on a 100 degree Celsius hotplate to complete the drying process. Dried chips were placed in hexamethyldisilazane (HMDS) chamber for 15 minutes, to ensure adhesion of the polystyrene to be spun coated onto the chip surface. After this 15 minute incubation step, the polystyrene solution was spun coated onto chip surface with use of a spin coater using the following recipe: spin 1; 500 rpm, 100 rpm, 5 seconds, spin 2; 1500 rpm, 1000 rpm, 30 seconds. After this procedure, each microchip was soaked in a 2% pluronic (w/v) solution in deionized water for at least 30 minutes, washed with deionized water, and then air dried prior to use. The use of pluronic solution was to further reduce the effect of friction on particle movement along the surface of the microchip.

3.3.5 Bacterial Ratcheting Experiments.

An assembly was completed with a metal base, the microfluidic magnetic ratcheting chip, a polydimethylsiloxane (PDMS) gasket with inlet and outlet portions cut, and a Plexiglas cover (Figure 5). The use of this assembly allows the creation of a water tight seal. The PDMS gasket

was created using a laser cut mold onto acrylic, and PDMS was cured after conventional a degassing procedure performed on PDMS mixed at a 1:10 ratio for cross-linker:base. After completing this assembly, this chip was first “primed” using a 1% BSA in PBS solution: 1000 microliters of a 1% BSA in PBS solution was introduced into the inlet, followed by removal of excess fluid. The chip was then loaded with test liquid suspension for each experimental run. 1 milliliter of fluid was introduced at the inlet, and 100 microliters of fluid was removed from the outlet (Figure 6). Experiments were performed by spiking *S. aureus* into each test liquid suspension, in this case Tryptic Soy Broth liquid media. The *S. aureus* used strain was generously donated from a collaborator. In order to perform these experiments, 3 types of suspensions were processed on chip for comparison: a suspension containing *S. aureus* only, a suspension containing non-functionalized 500 nm super-paramagnetic particles with *S. aureus*, and a suspension containing functionalized 500 nm super-paramagnetic particles with *S. aureus*.

After spiking *S. aureus* into Tryptic Soy Broth liquid media, then, 40 microliters of either a suspension of 500 nm functionalized particles, a suspension of 500 nm non-functionalized particles, or phosphate buffered saline (PBS), was added. This was followed by incubating this entire suspension for 60 min. To determine the ability of this microchip to concentrate bacteria, aliquots of the suspension from the inlet and outlet were cultured in order to compare colony counts of cultured bacteria. Colony counts of bacteria were completed from an aliquot (1 microliter) of the liquid suspension introduced at the inlet and an aliquot (1 microliter) of fluid taken from the outlet after 30 min of ratcheting. This experiment was performed with functionalized particles, non-functionalized particles, and a control suspension without particles, with 3 trials for each condition, where each suspension was processed on chip for 30 minutes.

3.3.6 Microchip Characterization Experiments.

In order to further understand and characterize magnetic ratcheting particle movement on chip, experiments were performed using superparamagnetic 2.8 micron beads (Dynabeads® M-280 Streptavidin, ThermoFisher Scientific). Each chip was prepared using the previously prescribed procedure. The PDMS gasket was prepared using a 1:15 cross-linker:base ratio, in contrast to the 1:10 cross-linker:base ratio used for bacteria ratcheting experiments. Particle movement was documented with serial image capture. 980 microliters of fluid was introduced at the inlet, and 294 microliters of fluid was removed from the outlet. Particle suspension concentration was determined by performing counts of beads present in suspension with the use of a hemocytometer; 20 microliters was removed in order to perform bead counts.

3.4 Results

3.4.1 Bacterial Ratcheting Experiment Results.

The results from experiments performed using this platform are shown here (Figure 7). The enrichment factor for each trial is calculated as a ratio defined in equation 2 as follows:

$$\text{Enrichment Factor (EF)} = \frac{(\text{CFUs})_{\text{outlet}}}{(\text{CFUs})_{\text{inlet}}} \cdot 10 \quad (2)$$

Here, CFUs outlet would be *S. aureus* colony forming units cultured from the outlet, and CFUs inlet would be *S. aureus* colony forming units cultured from the inlet. In each trial, there is increase in *S. aureus* colony CFUs cultured from the outlet as compared to the inlet (Figure 7).

In all, there is increased mean enrichment factor of *S. aureus* colony forming units (CFU), 18 fold vs. 4 fold for control solutions (Figure 7).

3.4.2 Microchip Characterization Experiment Results.

The results from experiments performed to characterize the functionality of the microchip are summarized in Figures 8 – 14. Figure 8 displays the benchtop ratcheting system. The system has several configuration options, in order to perform several functions such as separation of magnetically-tagged entities and concentration of magnetically-tagged entities. Initial characterization experiment results indicate that in addition to beads concentrating at the chip outlet, beads also concentrate at 2 additional locations on the microchip. Based on these findings, additional ratcheting system orientations were evaluated (Figure 10). These experiments demonstrate that orienting the rotating magnet-containing wheel at an angle of 30 degrees relative to the microchip biases on-chip particle movement such that the majority of particles concentrate at one location. Using this optimized configuration, experiments using different rotation frequencies for the rotating magnet-containing wheel were performed. An efficiency factor was calculated as defined in equation 3:

$$EnFc = \frac{[Beads]_{outlet}}{[Beads]_{inlet}} \cdot 100\% \quad (3)$$

Figures 12-14 display the measured EnFc for each experimental condition, where time and frequency were varied to determine microchip functionality. As displayed, EnFc is shown to increase with time, and also with ratcheting frequency.

3.5 Conclusions and Future Direction

This platform can be used to successfully separate and enrich magnetically tagged entities, within fluid suspensions, without performing additional steps. In this case, the use of this platform was applied for separating and enriching bacteria present within a liquid suspension, without performing bacterial culture step. In contrast to other reported techniques, which primarily include electrodynamic methods to concentrate bacteria(Zhang, Do et al. 2010), and the use of magnetic forces to separate bacteria(Kang, Super et al. 2014), both the separation and enrichment of bacteria on this microchip platform was achieved by using magnetic forces. Potential applications include concentration of bacteria for diagnostic purposes, or enrichment for further downstream analysis.

There are several advantages with the use of this technique. Notably, the platform is easy to use and reduces the need for an additional culture step. Integration with a downstream analysis technique also optimized to identify microbes present in low quantities would increase the ability of this technique to aid in microbial identification without performing culture steps at all. This platform has the potential for integration with downstream techniques for the purpose of performing further analysis of separated and enriched microorganisms, such as mass spectrometry. In terms of potential drawbacks, the increased cost of the nanoparticles and the microchip production should be considered. However, the expected benefit to be gained by reducing the time to diagnosis for bloodstream infection would likely make an increase in cost for this new technique an allowable one. In addition, the use of a specific antibody as the surface functionalization ligand for these particles will limit the applicability of these particles

to the organism for which the antibody is specific to. This can be improved by using a ligand that can interact with a larger number of organisms. This system can certainly be modified so that the entire process is completely contained. In this way, bacterial separation and concentration can occur in a confined fashion on-chip, reducing the likelihood of sample contamination or laboratory technician environmental exposure. These undesirable events could happen in a sample processing flow that, for example, would include performing magnetic pull down within an Eppendorf tube, followed by centrifugation/wash steps needed to purify and concentrate bacteria within a sample.

In conclusion, by using this microfluidic magnetic ratcheting microchip, with the assistance of functionalized super-paramagnetic particles, the separation and enrichment of *S. aureus* present in a liquid suspension is achieved, which is Aim 2 of this dissertation. Furthermore, the microchip used for this process has been further characterized so that chip performance can be enhanced. For future work, this approach can potentially be modified so as to allow the separation and enrichment of *S. aureus* present within patient blood samples for those patients with bloodstream infection.

3.6 Acknowledgements

This chapter contains a modified figure with permission from John Wiley and Sons from the publication: Murray, C., E. Pao, P. Tseng, S. Aftab, R. Kulkarni, M. Rettig and D. Di Carlo (2016). "Quantitative Magnetic Separation of Particles and Cells Using Gradient Magnetic

Ratcheting." Small 12(14): 1891-1899. I would like to acknowledge Dr. Coleman Murray, allowing me to use a version of Figure 3c, which I modified.

3.7 Figures

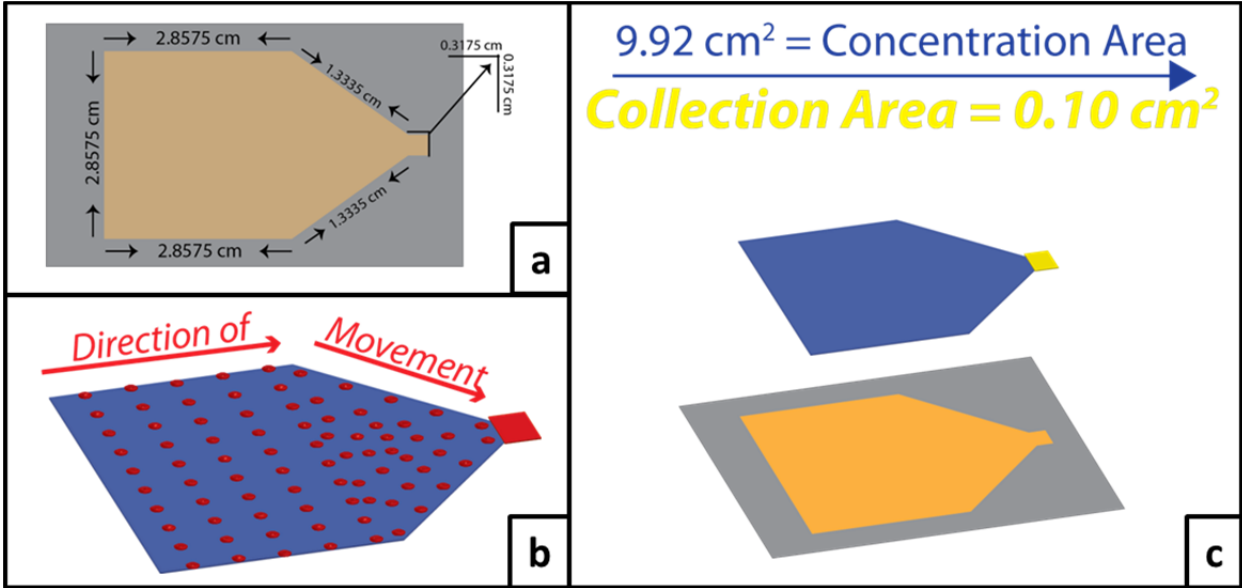


Figure 1: Microfluidic Magnetic Ratcheting Microchip Overview. a) Microchip dimensions. b) Schematic showing the movement of magnetically-tagged entities on the microchip surface. c) Schematic showing microchip surface area for particle concentration and collection.

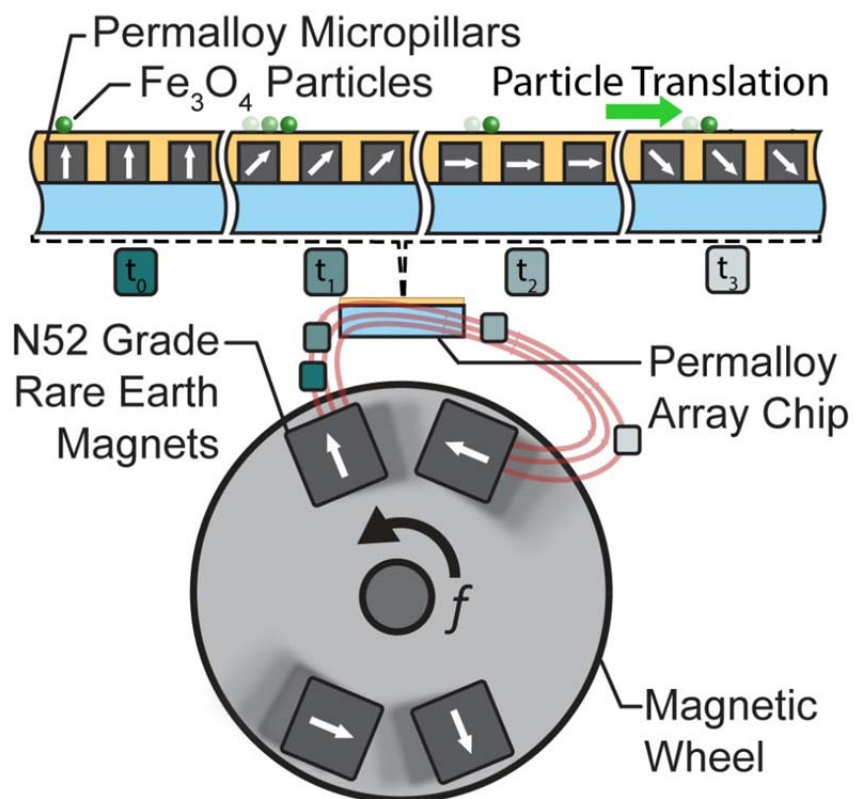


Figure 2: Schematic of Microfluidic Magnetic Ratcheting Chip Surface. A cycling magnetic field is generated using a Halbach wheel arrangement of rare Earth magnets. Particle translation occurs on the microchip surface in response to the generated magnetic flux.

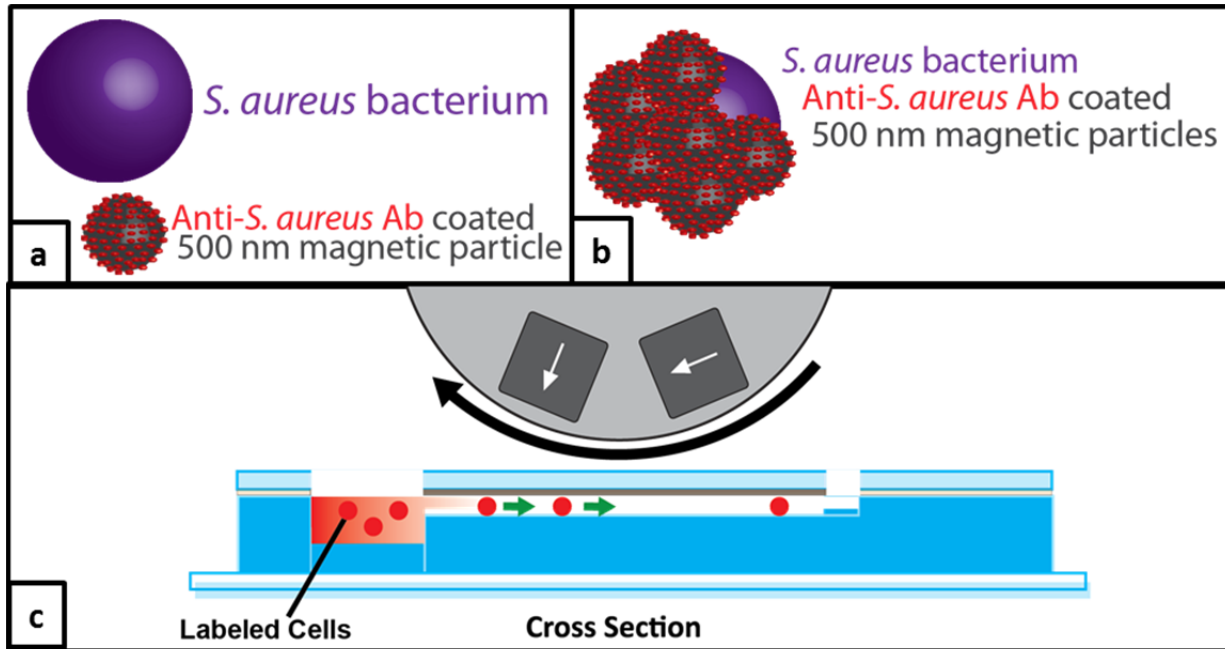


Figure 3: Particle Movement on Microfluidic Magnetic Microchip Surface. a) *S. aureus* bacterium alongside a functionalized 500 nm super-paramagnetic particle. b) *S. aureus* bacterium that has been covered with functionalized particles. c) Schematic displaying particles being extracted from within a liquid suspension which are then translated along the microchip surface.

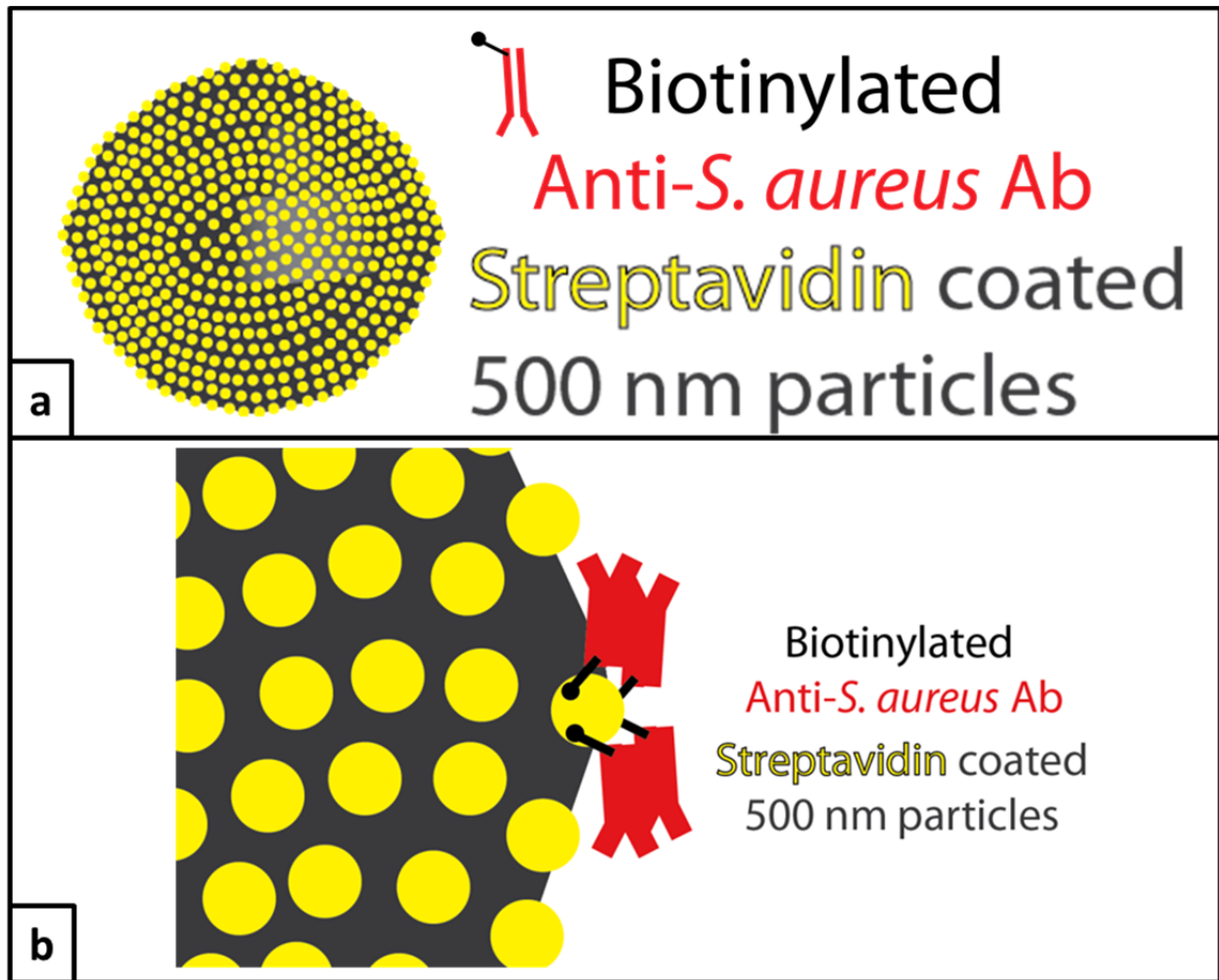


Figure 4: Particle Functionalization Process. a) Schematic of streptavidin coated 500 nm particles with a biotinylated *Anti-S. aureus* antibody. b) Schematic of streptavidin coated particles that have been functionalized with biotinylated *Anti-S. aureus* antibodies.

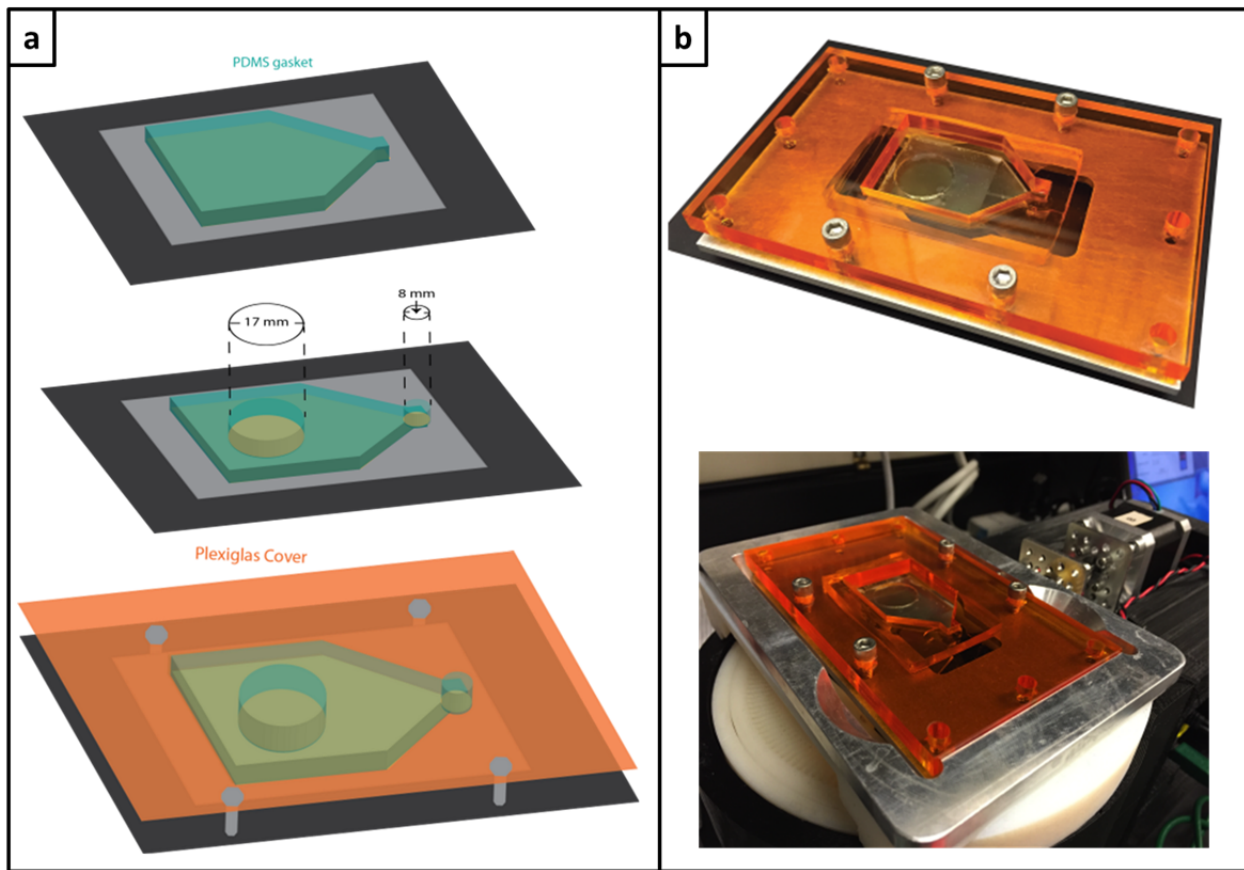


Figure 5: Ratcheting Experiment Assembly. a) Schematic of microfluidic magnetic ratcheting chip assembly with PDMS gasket and Plexiglas cover. b) Photo of ratcheting experiment assembly with assembly placed on benchtop ratcheting unit.

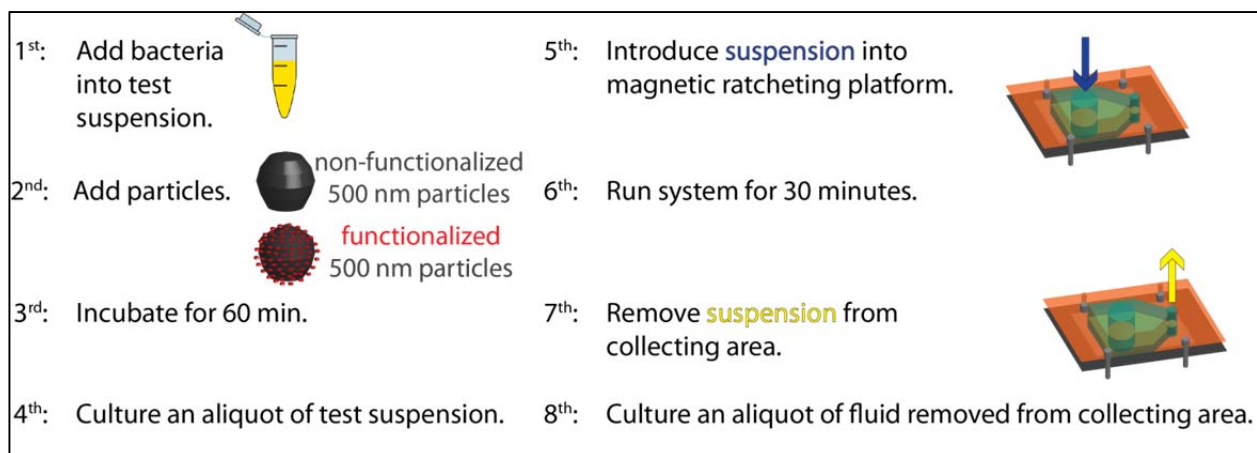


Figure 6: Flow Diagram of Experimental Set up. Each experiment was conducted with the series of steps described in this flow diagram.

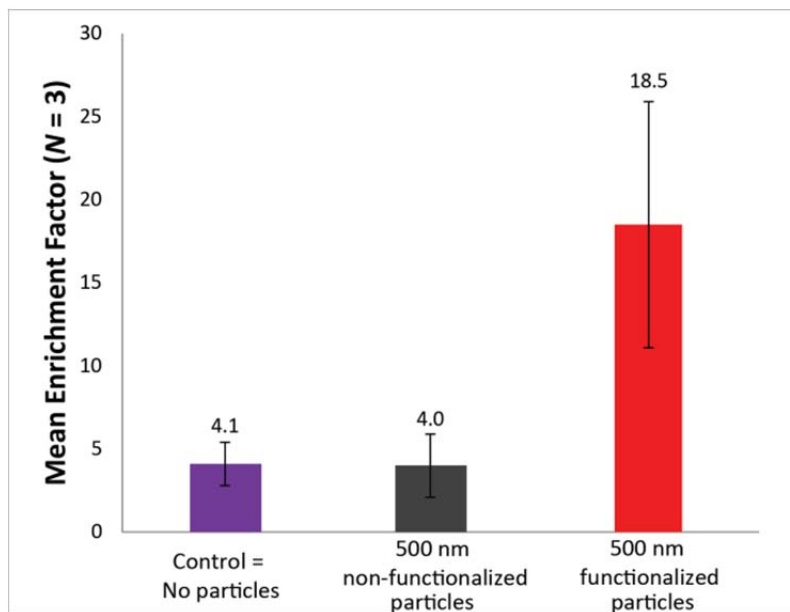
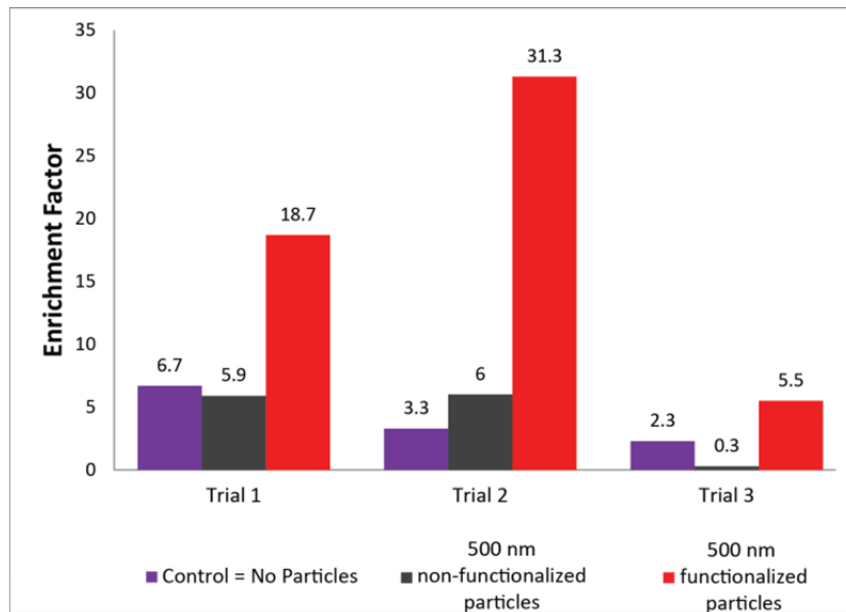


Figure 7: Bacterial Ratcheting Experimental Results. In three trials, an enrichment effect can be seen, where functionalized particles were able to enrich bacteria at a level above the control condition. The mean enrichment factor for functionalized particles was 18.5, as compared with a mean enrichment factor of 4.1 for the control condition and 4.0 for non-functionalized particles.

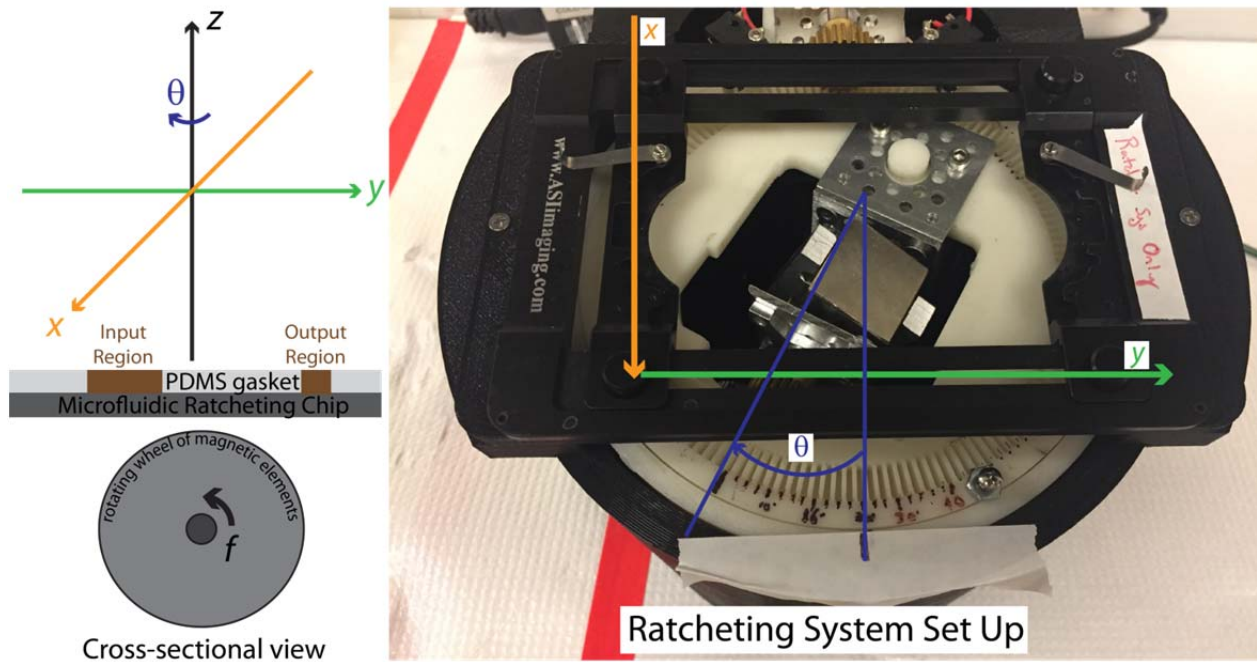


Figure 8: Ratcheting System Orientation and Set Up. The benchtop ratcheting unit has been designed to allow several configurations for use. Depending on the desired function, the rotating magnet-containing wheel can be aligned at different angles with respect to the magnetic microfluidic chip. In the shown configuration, the rotating magnet-containing wheel has been rotated in the x-y plane 30 degrees relative to the microfluidic chip loading station.

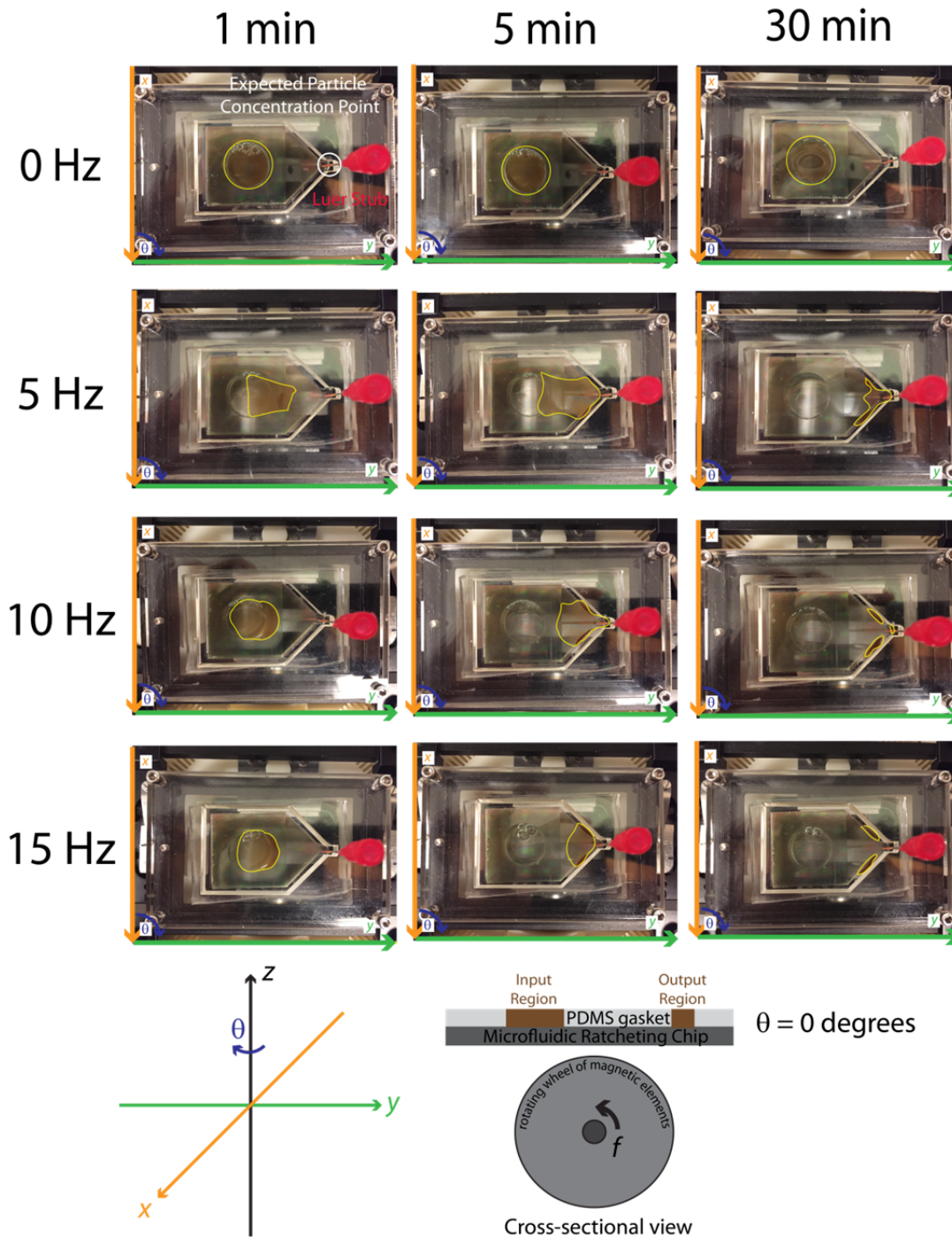


Figure 9: Initial Magnetic Microfluidic Chip Characterization Experimental

Results. 2.8 micron superparamagnetic beads concentrate at 3 discrete locations on the microchip, using the stated orientation for the rotating magnet-containing wheel.

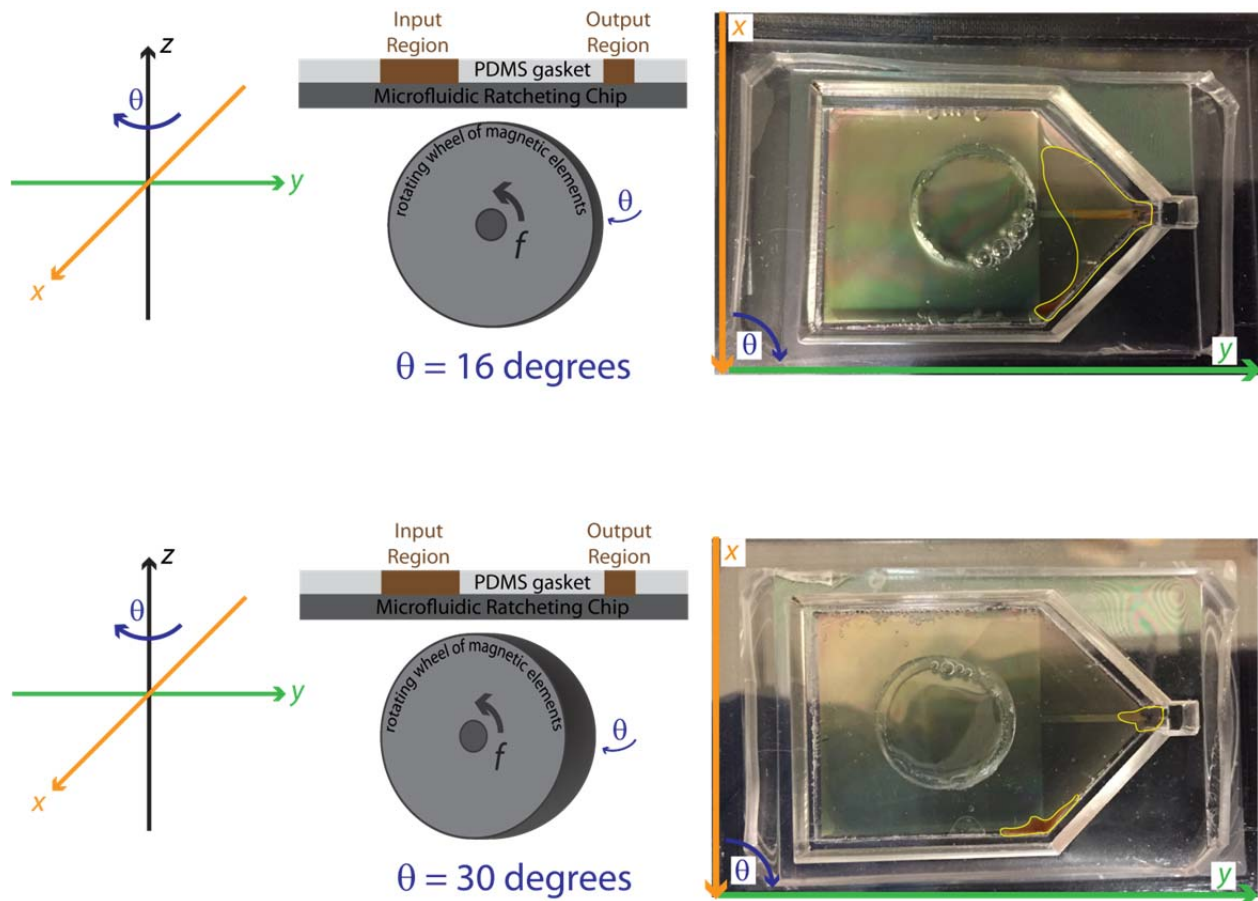


Figure 10: On Chip Particle Movement Using Different Ratcheting System Orientations.

By changing the angle of orientation for the rotating magnet-containing wheel with respect to the microchip, the majority of 2.8 micron superparamagnetic beads present within a liquid suspension move to one discrete location. Particles can be collected at this location and quantified. Experiments were performed using a rotating wheel frequency of 15 Hz for 10 minutes.

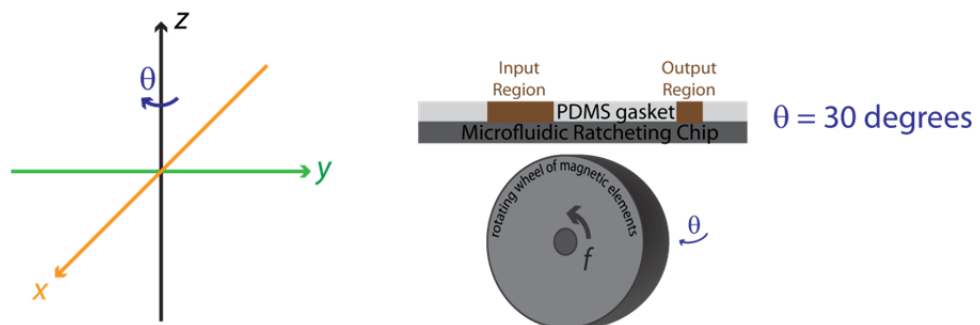
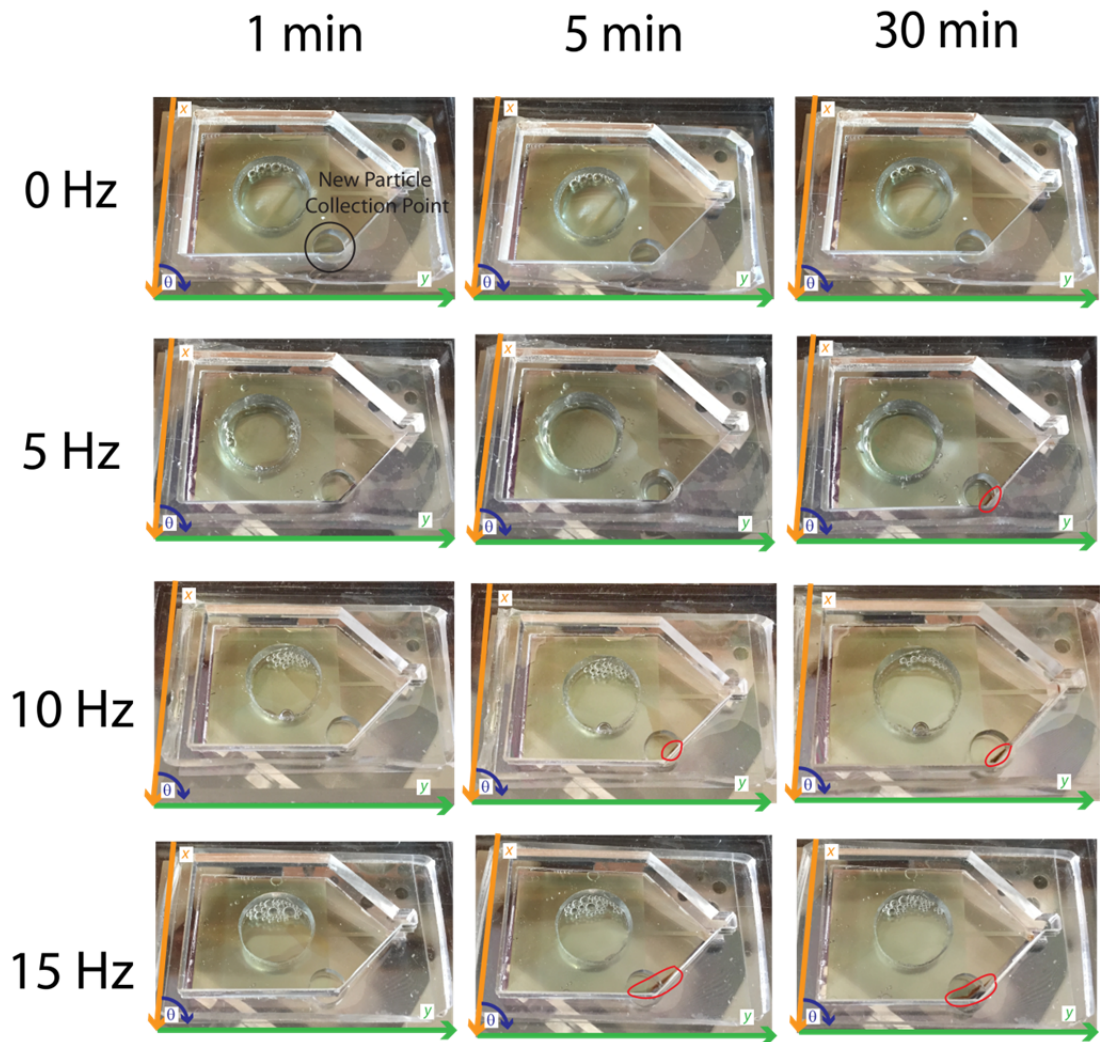


Figure 11: Magnetic Microfluidic Chip Experimental Results Using Optimal

System Configuration. 2.8 micron superparamagnetic beads concentrate at 1 discrete location on the microchip, using the updated optimal orientation for the rotating magnet-containing wheel, and updated collection point on-chip.

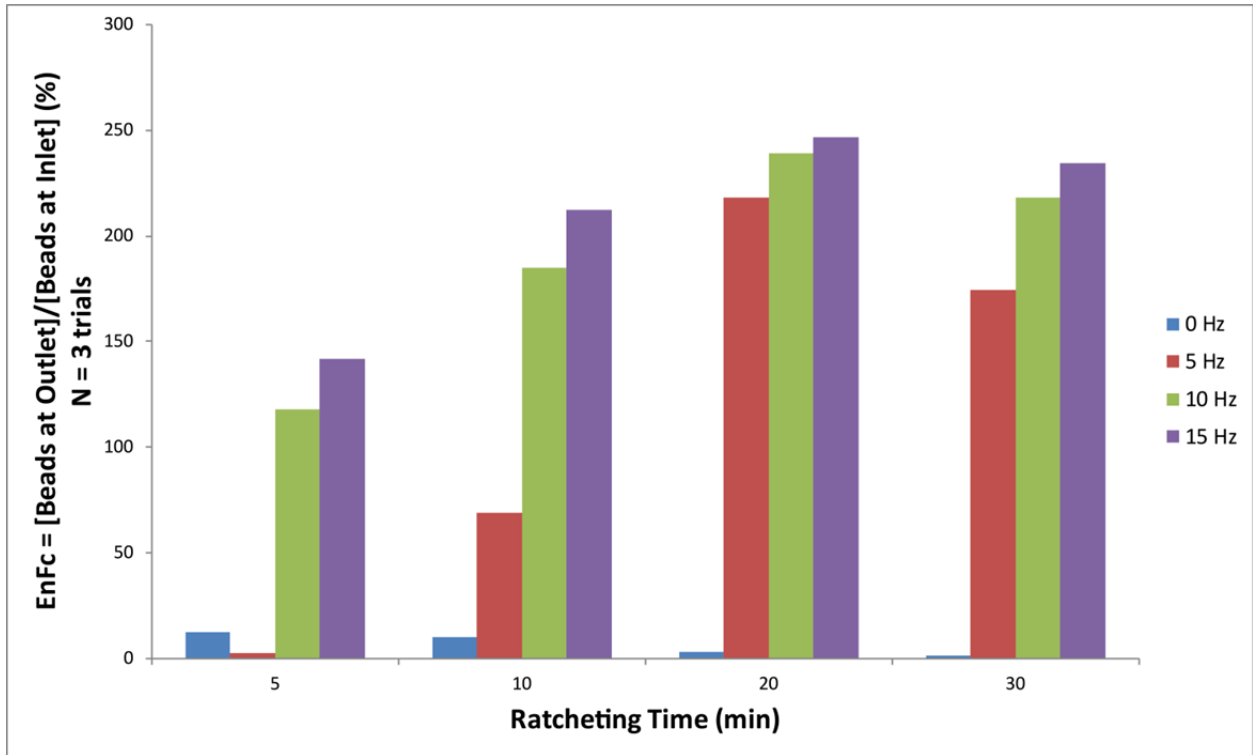


Figure 12: Quantification of Particle Concentration Using Optimized Ratcheting System

Orientation. Concentration of 2.8 micron superparamagnetic beads present in a fluid suspension was performed using the optimized ratcheting system orientation. The concentration of the bead suspension introduced into the system and the concentration of bead suspension withdrawn from the system was determined using a hemocytometer. Each result is the average EnFc determined from 3 trials for each tested condition.

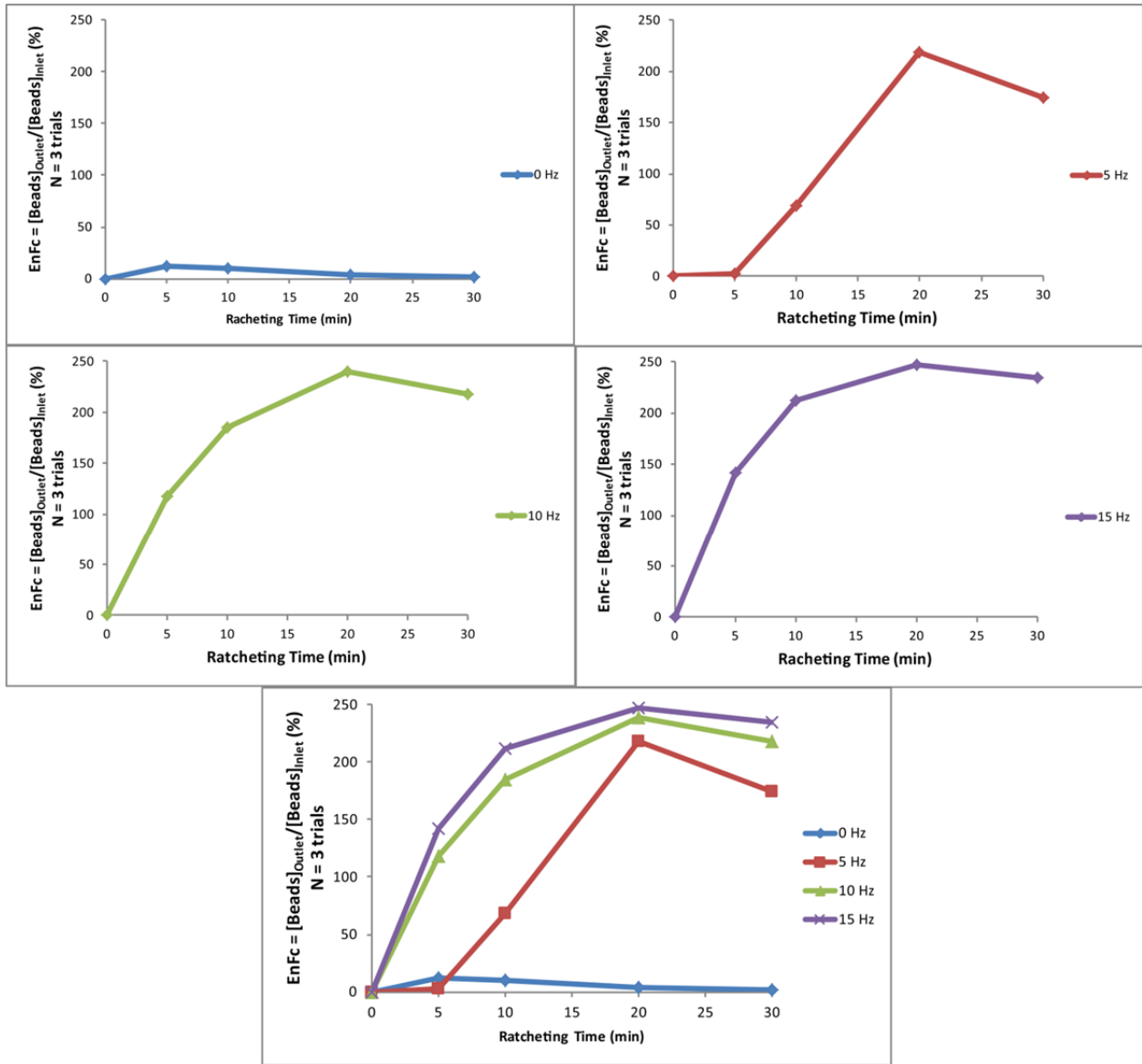


Figure 13: Particle Concentration on Chip at Tested Ratcheting Frequencies as a Function of Time. EnFc is shown to increase with time, and also with ratcheting frequency, using the optimized ratcheting system orientation.

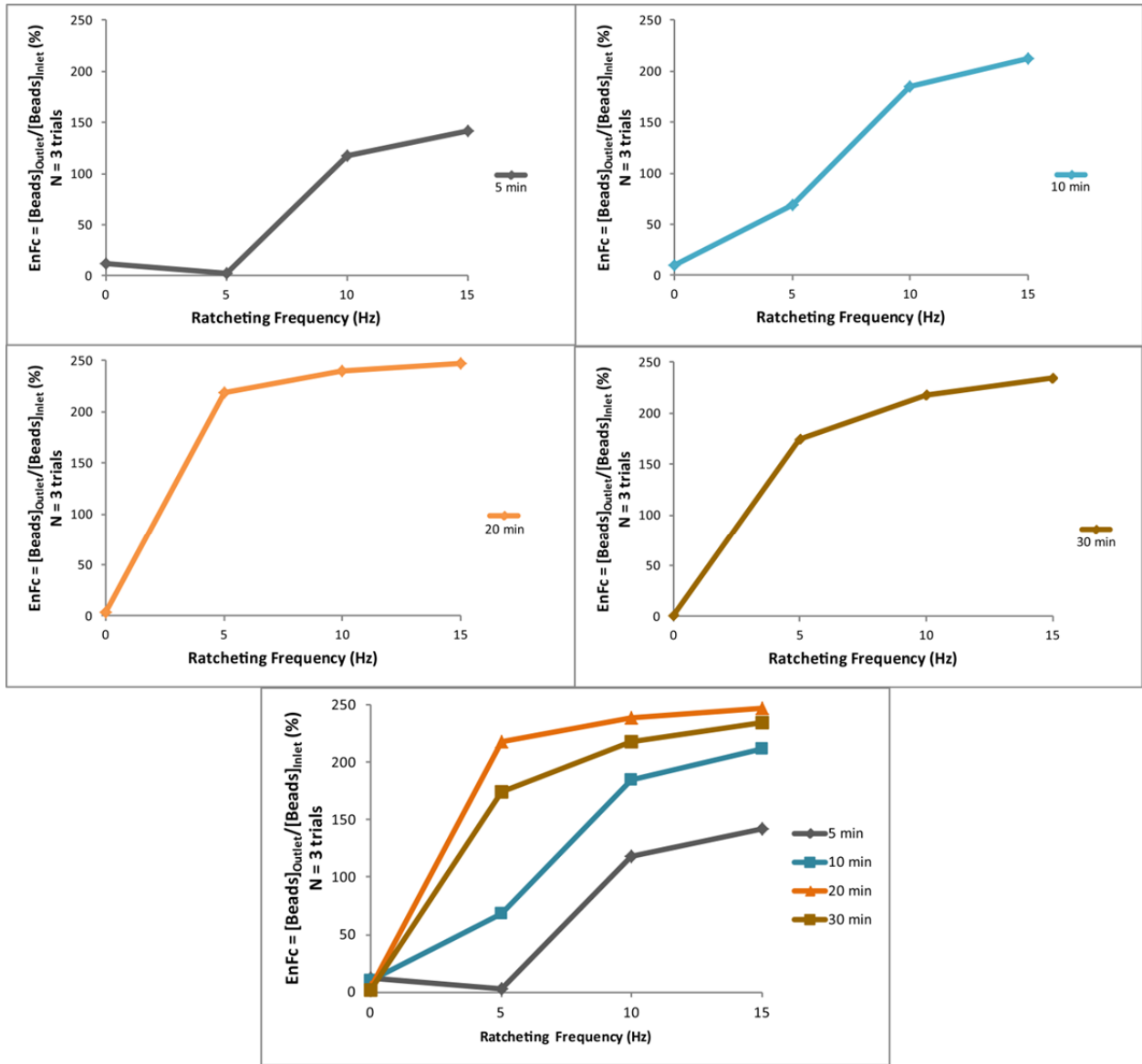


Figure 14: Particle Concentration on Chip at a Given Time as a Function of Ratcheting Frequency. EnFc is shown to increase with ratcheting frequency. A different ratcheting chip was used for a set of experiments where a particle concentration was measured at a constant time point across a series of tested frequencies.

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Appendix 1

Current status of microfluidics-assisted cytology: the application in molecular cytology

Chapter summary:

Recent advances in miniaturization and fluid handling enabled by microfluidics are poised to have an impact in the preparation of clinical samples and analysis of cells. In this chapter, I introduce the fundamental technological advances and microfluidic technologies that are addressing current challenges in cytopathology, and illustrate how these technologies can expand the opportunities for analysis and samples made available for use by the cytopathologist. For example, by isolating rare sub-populations of cells from blood or expeditiously purifying body fluid samples, these technologies can improve cytomorphological, molecular, and cytogenetic testing by the cytopathologist. Contrary to previous smear-based conventional cytology and current liquid-based cytology preparation methods, microfluidics-assisted cytology techniques may provide more suitable cell preparations for molecular analysis. Many of these technologies are currently in development in academic laboratories, however, commercialization is in progress, and microfluidics-assisted cytology promises to reach the clinical lab in the near future.

[A] Introduction: What is microfluidics?

Born out of several fields, including the fields of molecular analysis and microelectronics, microfluidics emerged as a discipline in the early 1990s. Since that time, developers have been

designing and testing small-scale devices to perform primarily sample analysis, using techniques that have their origin in the principles of microfabrication used in the semiconductor industry. Microfluidics is the science and technology of platforms containing elements constructed within the millimeter scale, that operate on and process fluid, where the fluid amount is often also within the microliter scale. This essentially allows a miniaturized laboratory platform. There are several advantages associated with using miniaturized laboratory analysis that is offered with microfluidic techniques. Two rather obvious advantages are the potential for developing low cost tests that are portable and require little maintenance. As well, consider that, in many clinical instances, only a small volume of a patient's specimen is available for multiple needed analyses; here, techniques that can achieve the same level of accuracy using a fraction of the sample in question would be extremely valuable. An emerging field, nanofluidics, focuses on platforms with features constructed on the nm scale! In the microfluidic field's infancy, the focus had been on chemical analyte analysis. However, developers are quickly realizing the potential for platforms in this area to address additional needs within medical diagnostics and clinical pathology in particular. With regard to cellular diagnostics, microfluidics has much to offer. Given the similar length scales to human cells (tens of micrometers) it is not surprising that these systems have been widely used to manipulate, separate, and interface with cells.

In this chapter I will discuss the steps involving device formation, how microfluidics is poised to meet the needs of molecular cytopathology, example technologies that are near prime time for clinical use, and what future advances may be required in this field.

[B] Device Formation

An attractive feature of the field of microfluidics is the ease with which devices may be formed using simple fabrication techniques that were adopted from the field of microfabrication and the semiconductor industry. Microfluidic devices are frequently manufactured using a combination of photolithography and soft lithography. Lithography, or, the formation of an object by etching shapes into a substrate, is the mainstay by which many microfluidic devices are created. Though much of the work of device creation is done bench top in an academic setting, commercial grade microfluidic devices have been developed and are positioned to make a remarkable impact in medical science. As an introduction to their use, it is instructive to consider the steps by which such devices are commonly manufactured.

B.2 Process flow

A common process flow for microfluidic device development includes these series of steps: a) creation of a device mold using photolithography, b) formation of an elastomer from the device mold using soft lithography, and c) final device fabrication. Figure 1 illustrates this process conceptually. Note that for clarity, the features displayed here are not drawn necessarily to scale. Typical device features are on the micrometer scale, whereas the silicon wafers used are usually 4" in diameter.

To start with, to make a device mold, a developer will first deposit a thin film of a chemical called photoresist (PR) onto a silicon wafer. Photoresist chemicals are usually polymer

compounds that are photoactive; that is, the compound will undergo physical property changes once exposed to light. In this example, the photoresist depicted is a negative photoresist: one that will harden when exposed to light. After this thin film of photoresist is deposited, the developer will place a mask over the wafer, and then expose the mask-covered wafer to UV light. Here, the area corresponding to the planned device features is translucent, so that light can shine through and cause the photoresist in the exposed area on the wafer to react. The rest of the mask is opaque, preventing the unexposed photoresist from reacting. After this, the mask is removed from the exposed wafer, heated to further harden the photoresist layer, and then placed in photoresist developer, which will dissolve unexposed photoresist. (Figure 1a)

After creation of a device mold, at a prototyping stage, we usually use an elastomer compound for device formation. Very commonly, the compound used is poly(dimethylsiloxane) or PDMS. PDMS allows for rapid prototyping, is optically translucent, which allows for easy microscopic evaluation of the device and cells within and, is biocompatible, and gas permeable (thus compatible with living cells) (Bélanger and Marois 2001). In this case, the developer pours PDMS (the example elastomer compound) over the device mold, to form one part of the final device.

Finally, the elastomer compound mold is bonded to a glass slide to fashion the final device. Note, both surfaces are usually activated, so that they will bond to one another. The act of bonding the molded elastomer to a glass slide, in this case, allows for the formation of microchannels for sample fluid flow. Fluid can be introduced into the device via tubing

connected through holes punched through the elastomer mold prior to bonding onto a glass slide. Mold creation using photolithography in general occurs in a clean room (Figure 2); after which fabrication of device prototypes can be done on the bench top in a laboratory setting. The entire process can be performed over the course of 1-2 days.

B.3 Device design

Device designs are usually created using a software package used for drawing, such as AutoCAD. Once created, designs are then transferred onto a mask. The created mask is then used for the process described above.

B.4 Device elements

Typical device components include channels, valves, filters, mixers, pumps, and reservoirs for reagents (Whitesides 2006). These components are designed using a drawing software package as described above. The final device drawing is converted to a photomask, which is used for photolithography (Figure 1), to make a device mold. The device mold is then used for soft lithography to create the final device, as described above.

[C] Microfluidics and Molecular Cytopathology – Addressing the Need to Generate Pure Populations of Specific Cell Types for Molecular Analysis

As it relates to medical diagnostics, thus far much of the work in the microfluidics community has focused on miniaturization in the area of chemical analyte analysis, flow cytometry, and differential blood cell counts (Cheung, Gawad et al. 2005, Golden, Kim et al. 2009, Holmes, Pettigrew et al. 2009, Hur, Tse et al. 2010). However, the community has now recognized that additional needs are present, and that these needs are ideally addressed with microfluidic tools. Particularly in the area of the analysis of cytology-based diagnostics, the focus within the field is shifting into one where developers are working to create microfluidic chips that can simplify and enhance existing tools. For example, in cytopathology, there is a pressing need for improved methods to enrich and purify cells from cytological samples, after which, these samples may be then reviewed by a pathologist for evidence of malignancy. Current sample preparation methods are most often completed on a laboratory bench top manually with macroscale instruments (Rivet, Lee et al. 2011). This practice likely reduces the consistency between operators and labs. One can imagine several clinical scenarios where a diagnosis may be potentially missed due to sampling error or inadequate sample preparation.

Many microfluidic approaches employed to address sample preparation have aimed to scale down macroscale techniques. For example, with one macroscale technique, cells can be isolated in a conical tube by incubating cells with specific immunomagnetic beads, followed by placing a strong magnet in proximity to the tube, and then performing multiple rinse steps to wash isolated cells. Microscale technologies followed suit, by integrating with on-chip and off-chip magnets for separating cancer cells, bacteria, and fungi from blood (Yung, Fiering et al. 2009). Other concepts were borrowed from analytical chemistry, like affinity and size-based chromatography, using instead “columns” sized for cells and affinity approaches specific to cell biomarkers like

size or surface proteins. Approaches that make use of unique physics accessible in microfluidic systems are also poised to make an impact (Chun and Ladd 2006, Seo, Lean et al. 2007, Bhagat, Kuntaegowdanahalli et al. 2008, Di Carlo 2009). The reader is referred to several comprehensive reviews that discuss the physical operating mechanisms of various microfluidic cell separation and concentration approaches (Nilsson, Evander et al. 2009, Dharmasiri, Witek et al. 2010, Gossett, Weaver et al. 2010).

Pathologists continue to refine the art and science of accurately identifying abnormal morphological characteristics that denote malignancy. Microfluidics can be an important partner in this endeavor by engineering devices whereby quality samples are prepared in a high throughput, robust and durable manner, then delivered to the pathologist for review, and more importantly for molecular analysis. Retrieving target cell populations in solutions containing mixed cell populations and preparing them for analysis with minimal perturbation is a complex sample preparation task. In this regard, there are some challenges facing the microfluidics community; I will review these challenges here.

C.2 Concentrating rare cells from patient specimens with large volumes

Such patient specimens, where a large volume may be submitted for analysis, include but are not limited to blood, urine, pleural, peritoneal, and broncho-alveolar lavage fluid samples. For example, 50 mL of pleural fluid is adequate to gather enough material for a cell smear in malignant pleural fluid analysis. In another case, the presence of >5 cancer cells within 7.5 mL

of blood was shown to be an independent predictor of overall patient survival in metastatic breast cancer (Cristofanilli, Budd et al. 2004). A sample with a low cellularity combined with inadequate sampling and the presence of rare cells can lead to unsatisfactory results, prompting further diagnostic testing. Concentrating in the presence of a large number of background cells is also challenging and requires combined separation and concentration approaches.

In order to achieve large volume processing, the goal is to process samples at flow rates in the millimeter•min⁻¹ scale instead of the microliter•min⁻¹ scale to satisfy the workload requirements of a clinic with a single machine or achieve rapid turnaround to aid in quicker clinical decisions. Scaling up from microliter•min⁻¹ to millimeter•min⁻¹ represents a 10³ order of magnitude increase. An increase such as this raises concerns that cells in a device using these higher flow rates can be exposed to high shear stress in the microchannels, leading to cell damage. To mitigate this concern, developers can scale up flow rates by creating massively parallel arrays of devices, or by increasing the channel dimensions of the device, enabling more volume throughput while maintaining acceptable forces on cells.

To that end, it is important to design systems that can be expanded in a parallel fashion and still retain the fundamental fluid flow mechanics (Davis, Inglis et al. 2006, Di Carlo 2009, Mach, Kim et al. 2011). These mechanics typically take advantage of particle, or, in this case, cell, size, shape, deformability and density using hydrodynamic forces induced by microchannel features. For example, Deterministic Lateral Displacement (DLD) and inertial microfluidics uses the physical phenomena unique to these systems in various channel shapes and orientations to guide

particles above certain size cutoffs into specified outlets for collection. Since these channels can easily be arrayed, such that multiple channels are patterned onto the chip surface, there is no limit to the amount of parallelization that can take place. By performing parallelization, numerous arrays of channel are made available for fluid processing, offering macroscale regime flow rates in a compact microfluidic device. In another technique, acoustic waves maneuver cells to positions of defined pressure nodes within a standing acoustic energy field (Petersson, Åberg et al. 2007, Lin, Mao et al. 2012, Nordin and Laurell 2012). From these defined positions, maneuvered particles can be collected. While able to process at high flow rates, the technology requires an external piezo or patterned interdigitated transducer (IDT) that will convert electrical signals into surface acoustic waves in order to initiate fluid flow, which may present a challenge for parallelization. Still, forces from these systems are large compared to other types of forces, such as dielectric and magnetic forces. Therefore device systems that incorporate these physical phenomena can operate with appreciable flow rates ($0.05\text{--}0.5\text{ mL}\cdot\text{min}^{-1}$) with only a single channel. The ideal technology would be one where microchannels can be stacked indefinitely to obtain rapid processing times.

C.3 Efficiently preparing small volume samples for multiple assays

For the majority of clinical circumstances where the sample of volume available for testing is limited, efficient sample preparation is paramount. Factors that may reduce the sample available for further analysis include dead volume in a chip or external tubing, parallel assays, and loss of cells due to an inefficient on-chip sample preparation process. Innovative approaches to load and pump fluids without significant dead volume will lead to a reduction in the effective volume

of sample needed for analysis, while sequential assays can make the most of a sample volume. Traditionally, the microfluidic field has touted the small volume processing capabilities as an inherent advantage of the small scales of operation. For the purpose of small volume processing, various methods have been developed that take advantage of microchannel dimensions as well as the materials used to create the microchannels. Example methods include capillary-driven and vacuum-driven fluid flow.

In capillary-driven flow, fluid flow is initiated by surface tension, in which the flow can be driven by differences in surface energy upon the wetting of a channel, network of channels, or porous structure such as paper (Juncker, Schmid et al. 2002, Martinez, Phillips et al. 2009, Osborn, Lutz et al. 2010). Contrast this with a technique using vacuum-driven flow, where fluid flow is initiated by using the porous structure of normal polydimethylsiloxane (PDMS) as a negative pressure source after the device is taken out of a vacuum chamber (Hosokawa, Sato et al. 2004, Dimov, Basabe-Desmonts et al. 2011) While both techniques offer a standalone system for working with small liquid volumes and concentrating cells with no moving parts and external components, these approaches potentially have long processing times, which may be prohibitive for certain point-of-care diagnostic applications.

Another technique that requires additional external components makes use of centrifugation of a small volume of biofluid on a microfluidic chip to achieve pumping. As with traditional centrifugation – but with much smaller volumes – this approach allows for separation of plasma from whole blood and the subsequent mixing of the plasma with lyophilized reagents for

detection (Amasia and Madou 2010). A recent innovation, which works most effectively with microscale volumes, separates cells specifically bound to immunogenic magnetic micro beads by, instead of removing background fluid, pulling the bead-bound cells themselves through an immiscible fluid phase into a second wash volume (Berry, Strotman et al. 2011).

The above-described techniques in general apply for continuous-flow microfluidic systems, where fluid flow occurs in one continuous fluid stream. However, there is significant interest in developing platforms on which fluid samples are analyzed after conversion into a series of droplets. This is the main focus of the area in microfluidics called digital microfluidics. There are some advantages gained by using the approach of converting fluid samples into droplets for further downstream testing, such as reduced risk for contamination and improved mixing efficiency. In this example, investigators combine two methodologies, electrowetting-on-dielectric (EWOD) and optoelectronic tweezers (OET), into a single microfluidic device for the purpose of converting a suspension of HeLa cells into droplets containing HeLa cells. The droplets can then be manipulated using electrical signals (Shah, Ohta et al. 2009). The EWOD technique is attractive in that samples are converted to droplets with electrical energy, and not active mechanical chip components, such as pumps and valves (Shen, Fan et al. 2014). It will be important to see how this technology can be further adapted for use in cytological analysis.

C.4 Preparing samples with high cellularity

Patient specimens that are highly cellular, such as blood or bone marrow aspirates will contain diverse and large populations of cells. These high cellularity samples can be challenging for the cytopathologist and for microfluidic processing for many reasons, given a large background of healthy cells. To start with, cells within these samples can interact within fluid flow to prevent accurate separation. Also, cells have a higher likelihood of aggregating, and clusters of cells can clog flow within microfluidic channels. Of course, there is the need to achieve a high purity of a specific population of cells as they are separated from a diverse background population of cells. Finally, it can be difficult to retrieve specific cells from within a highly cellular solution, particularly if these specific cells are present in rare numbers. Despite these challenges, there has been much progress in processing samples that have high cellularity, especially for isolating specific cell populations from complex fluids like blood. There are several mechanisms that can be used for the purposes of cell separation and many approaches that can be used to address these challenges, some of which we will now discuss.

For example a simple solution to address aggregation would be to include a dilution step, which would decrease the potential for cell aggregation and microchannel clogging. However, adding a dilution step increases sample volume and will then increase the time required for sample processing. Another approach to reduce cell aggregation involves designing assays where cell separation occurs at the microchannel surface, rather than within the crowded fluid phase. An example that demonstrates this technique would be an affinity capture-based approach (Nagrath, Sequist et al. 2007, Gleghorn, Pratt et al. 2010). Here, the microchannels are coated with antibodies that are specific to receptors of target cell populations so that cells are captured on-chip. These affinity based-platforms have been developed for isolating circulating tumor cells

from blood (Nagrath, Sequist et al. 2007, Gleghorn, Pratt et al. 2010, Stott, Hsu et al. 2010, Wang, Liu et al. 2011).

Alternatively the crowded fluid phase may be used to aid in separation through the margination of particular cell populations to preferred locations within a fluid flow stream (Shevkoplyas, Yoshida et al. 2005, Hou, Bhagat et al. 2010). This can be done using a mechanism that exploits cell size to assist in separating cells within a sample. Consider DLD, where the physical phenomenon at work directs particles to move at an angle with respect to flow through an array of microposts. Larger cells undergo a bumping mode whereby cells are deflected from the normal trajectory, while smaller particles follow streamlines in a zigzag mode. This approach operates effectively even with samples that have high cellularity. Tuning the shape of microstructures in the flow or creating mixing flows to allow surface contact even in highly cellular solutions is a challenge that is being addressed (Gleghorn, Pratt et al. 2010, Stott, Hsu et al. 2010, Zhao, Cui et al. 2012).

Downstream sample analysis may also be facilitated by segmenting cells within a highly cellular solution into more easily analyzed single cells. This would be beneficial in many clinical scenarios. One such scenario involves developing diagnostics for mutation-targeted drug therapies. Here, it would be important to isolate and analyze single cells with particular genetic mutations so that these specific mutations can be accurately identified. A possible solution is to segment highly cellular solutions into droplets containing single cells using droplet generators

(Chabert and Viovy 2008, Edd, Di Carlo et al. 2008). One main advantage is that single cells can be detected using fluorescent imaging and separated into different zones for further analysis.

While these systems offer rapid throughput and high efficiency of cell capture, a successful technology should be able to balance these aforementioned criteria, in addition to achieving high purity and having the ability to retrieve cells in solution.

C.5 Automating multi-step sample preparation

To standardize the sample preparation process the steps involved in sample preparation should be automated. These steps include centrifugation, pipetting, and cell staining, all operations usually manually performed in clinical labs. For example, when working with a large volume patient specimen (e.g. peritoneal fluid obtained after paracentesis), technicians must aliquot, centrifuge and manually pipette the mixed sample to make cell smears. However, mishandling and user error in processing, such as neglecting a step to homogenize the sample before performing a decanting step, may lead to misleading results. A standardized fluid plumbing system in which an entire volume is mixed and processed through a device would address this issue by removing user-sampling bias; it can also allow for processing large liquid volumes. Automation of these steps to minimize sample handling will preserve sample integrity and lead to less error overall, ultimately leading to cost reduction and increase in the diagnostic accuracy.

Some microfluidic technologies already offer multiple steps that are integrated onto a single platform. Centrifugal microfluidics is a technology that can prepare blood samples through a series of pumping, valving, volume metering and mixing (Gorkin, Park et al. 2010). Abaxis currently sells this sample-to-answer technology in the Piccolo clinical blood analyzer system for both medical and veterinary diagnostics. Other technologies like the Vortex (or Centrifuge) Chip, a method that recreates the functions of a benchtop centrifuge in a microfluidic format, combines cell concentration, separation, and staining. This technology is particularly useful for samples with low cellularity (Mach, Kim et al. 2011). An ideal technology would automate cell-staining techniques in a microfluidic platform to enable efficient uniform labeling using traditional and immunocytochemical stains as well as to enhance the performance of cytogenetic analysis, e.g. fluorescence in situ hybridization (FISH).

C.6 Obtaining high purity for molecular assays (Table 1, Figures 3 & 4)

Achieving high purity of specific cell populations from heterogeneous solutions presents a critical challenge in clinical sample preparation. Purity is important in preparing cellular samples for nucleic acid analysis, for cell counting of specific selected sub-populations, and for reducing the presence of background cells and other non-cellular particulates that can mask the evaluation of cells of interest. For example, bloody specimens containing leukocytes can contaminate molecular analysis results when attempting to detect gene mutations or perform nucleic acid sequencing in a target cell population (Billah, Stewart et al. 2011). Strategies directed toward the removal of contaminating cells can also aid in the development of platforms for cell counting and sorting of specific subpopulations. The removal of blood cellular components and the

concentration of target cells into a small field of view may expedite and increase the accuracy of cytology examinations (Dharmasiri, Witek et al. 2010, Mach, Kim et al. 2011, Pratt, Huang et al. 2011).

A few microfluidic approaches address these issues and are capable of extracting cells at high purity (Stott, Hsu et al. 2010, Wang, Liu et al. 2011, Plouffe, Mahalanabis et al. 2012). However, it is worth noting that some methods lack the ability to make cells readily available in solution after sample preparation, limiting the ability to integrate these techniques with downstream cytological or flow cytometry. Some technologies have been successful in recovering cells after on-chip processing (Mach, Kim et al. 2011, Shah, Yu et al. 2012). In the “Centrifuge-on-a-Chip,” target cancer cells are selected by size from a bloody sample, collected in a concentrated solution, and imaged in a small field of view (Mach, Kim et al. 2011). Specifically, this technology has been shown to improve the detection of KRAS gene mutations in lung cancer cells processed on this chip, as compared with lung cancer cells processed with standard bench top centrifugation (Che, Mach et al. 2013). This device has been further optimized for high-purity extraction of circulating tumor cells (CTCs), which can be performed on high liquid volumes with a rapidity that highlights the capability of Vortex technology use in clinical sample preparation (Sollier, Go et al. 2014). In another technique, viable cells are recovered from an affinity-based cell isolation chip using a hydrogel coating layer which can be subsequently degraded to release cells for downstream molecular assays (Shah, Yu et al. 2012). The microfluidic methods discussed here are critically addressing this challenge of obtaining high purity cell separation. Methods such as these are poised to open up new opportunities for the

cytopathologist to analyze blood and other body fluids for rare cells of diagnostic importance that were previously not accessible given the background of healthy cells.

[D] Case Studies of Microfluidic-Assisted Cytopathology

The introduction of new technology and tools into a discipline is sometimes met with inherent skepticism from those within the discipline regarding whether these tools are as helpful as they claim to be. In some instances, by proposing to altogether eliminate current methodologies upon which standards of practice have been built, the introduction of new tools can be met with frank hostility. However, the very nature of work within the field of microfluidics should hopefully lessen the likelihood of this negative response. Successful work within the field requires an interdisciplinary approach. This means that all stake holders need to be involved, including microengineers, industry, laboratory technicians, research assistants, and physicians, even at the early inception stage. Within this framework innovators can more likely develop effective tools that will meet the needs of pathologists who practice medicine in the 21st century. Thus far we have endeavored to give context regarding how microfluidics and pathology can be partners in addressing unmet needs in medical diagnostics. In this section are highlighted specific examples where microfluidic technology has been shown to assist in making important pathologic diagnoses, and where this multidisciplinary framework has proven to be successful.

D.2 Purification of body fluids with Vortex Technology

Previously, in the “Centrifuge-on-a-Chip” system, designers fashioned a microfluidic chip that performs the functions of a bench top centrifuge, such as concentration of rare cells and solution exchange, with high throughput volume processing (Mach, Kim et al. 2011). This device concentrates and removes rare cells of interest (such as circulating tumor cells) from a larger background of cells in diluted blood by using physical phenomena unique to fluid flowing through microchannels and additional microstructures. This technology has been shown to rapidly enrich cells of interest based on their size from body fluids like pleural effusions and blood. Using this technology, malignant and mesothelial cells of interest to a cytopathologist were purified and concentrated from background erythrocytes and leukocytes from clinical samples. Besides potentially improving the accuracy of cytomorphological reads, such an approach was shown to improve molecular detection accuracy of clinically relevant gene mutations (see Figures 3 & 4) (Che, Mach et al. 2013). The Vortex Technology has also been shown to have the capability of performing high purity separation of circulating tumor cells from whole blood with a short amount of processing time (20 minutes for 7.5 mL of blood) (Sollier, Go et al. 2014). This high purity separation of cells from within a small volume enables cytopathologists to have access to rare cells from highly cellular samples like blood and conduct any desired downstream staining or molecular testing. In this way, it also facilitates molecular analysis by cytogenetics, given that there are fewer non-target cells to screen. In addition, cells purified in this manner are now available for gene mutation analysis for personalized medicine.

D.3 Automated and quantitative analysis of pleural effusions

In this example, the authors developed and used a microfluidic based technology to aid cancer diagnosis by using the platform in a pre-screening role to identify malignant cells in pleural

fluids. The technique, called deformability cytometry (DC), allows one to robustly perform rapid and label-free measurements of the mechanical properties of cells, properties which appear to have origins in traditionally analyzed cytomorphological features, such as nuclear to cytoplasmic ratio, chromatin structure, and cytoskeletal arrangement (Gossett, Tse et al. 2012). This automated technology is capable of evaluating the mechanical properties of cells with significantly higher throughput than other platforms, approximately 2000 cells per second in a flow-through format (see Figures 5-7). Using this platform, investigators analyzed pleural effusions from 119 patients (Tse, Gossett et al. 2013) to identify mechanical markers associated with malignancy and inflammation. An advantage of the approach is that only red blood cell lysis is required, followed by a 5 minute quick processing, and automated analysis to yield a result. In the analysis, the authors used an algorithmic diagnostic scoring system that incorporates information on cell size, deformability, and the distribution of these characteristics in the cell population. With this technique, 63% of the samples could be correctly classified as malignant or benign, with 100% positive predictive value and 100% negative predictive value. In this way, the platform could potentially be used to pre-screen and classify a large number of samples that are clearly negative or positive for malignancy. This pre-screening step would save time and effort for cytopathologists, who could then focus on the diagnosis of more challenging samples. In addition, in the same study, using this technology improved the diagnosis of challenging samples (approximately half of the samples identified as containing atypical cells were definitively diagnosed using the DC approach). Deformability cytometry can then provide assistance with the analysis of atypical cells, and could potentially be combined with other technologies to analyze cells and perform downstream purification. Future work using such technologies include exploring the ability to detect specific sites of origin for cells disseminated

into pleural or other body fluids using deformability and other label-free physical markers of cells.

[E] The Future

Looking ahead, there are many opportunities for collaboration between microfluidic developers and molecular cytopathologists. This chapter has largely focused on microfluidic platforms designed to efficiently prepare body fluid samples for cytological tests and molecular analysis. Note, however, that there has also been progress in developing microfluidic systems to allow for tissue staining and preparation to be done *on chip* (Lovchik, Kaigala et al. 2012). One such system evaluates the presence of four important breast cancer cell biomarkers: estrogen receptor (ER), human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR), and Ki67 on human breast needle biopsy samples (Kim, Kim et al. 2010). We have a grand challenge before us; to create devices that allow high purity isolation of clinically relevant cells that can also be done in a high throughput manner (Figure 8), and provide these cells obtained not only for morphological but also, more importantly, for molecular-based analysis. We are very nearly realizing this, as evidenced by devices currently nearing the final stages of development (Figure 9). To further address this challenge, we need intense collaboration between developers and end users, so that we correctly address the problems at hand. This area allows for interdisciplinary work, and a team approach, with all partners contributing their expertise for the common goal of improving our methodology for not only diagnosing illness but also defining the correct targeted therapy.

[F] Acknowledgements

This chapter contains a modified version of the publication: Adeyiga, O., A. J. Mach, J. Rao and D. Di Carlo (2016). Current Status of Microfluidics-Assisted Cytology: The Application in Molecular Cytology. *Molecular Cytopathology*, Springer: 261-283(Adeyiga, Mach et al. 2016) with permission from Springer.

[G] Figures

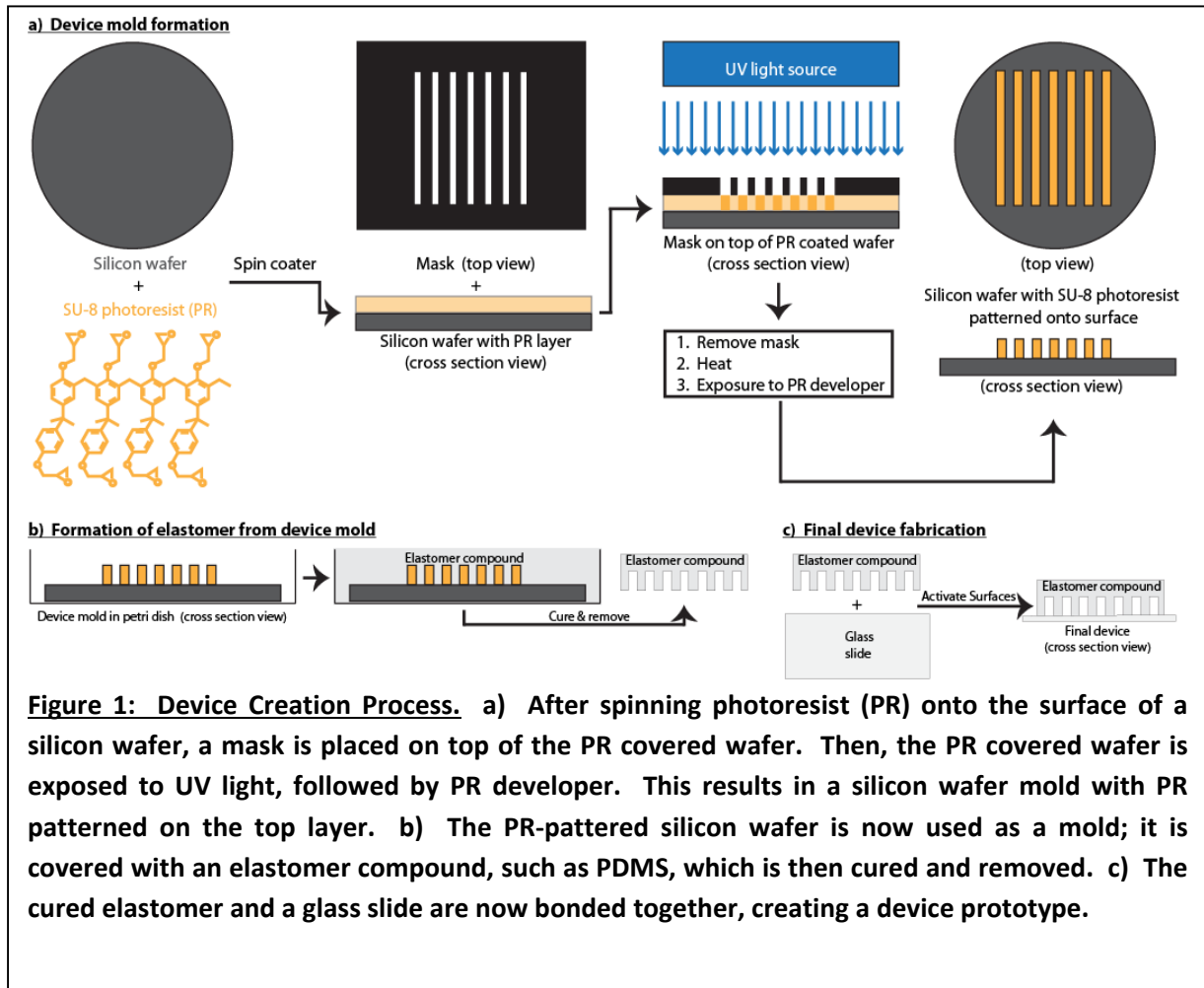
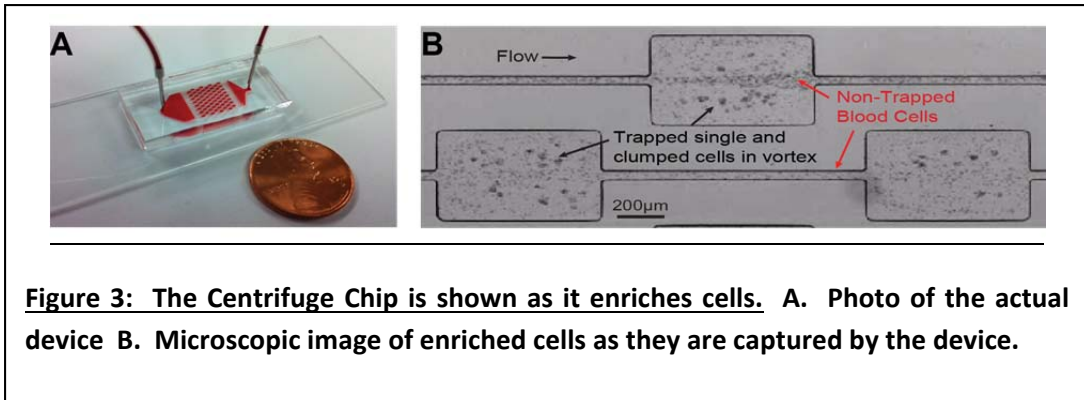


Figure 1: Device Creation Process. a) After spinning photoresist (PR) onto the surface of a silicon wafer, a mask is placed on top of the PR covered wafer. Then, the PR covered wafer is exposed to UV light, followed by PR developer. This results in a silicon wafer mold with PR patterned on the top layer. b) The PR-patterned silicon wafer is now used as a mold; it is covered with an elastomer compound, such as PDMS, which is then cured and removed. c) The cured elastomer and a glass slide are now bonded together, creating a device prototype.



Figure 2: Clean room microengineering. Left: An example silicon wafer. Right: Substrates undergoing surface activation using clean room instrumentation.



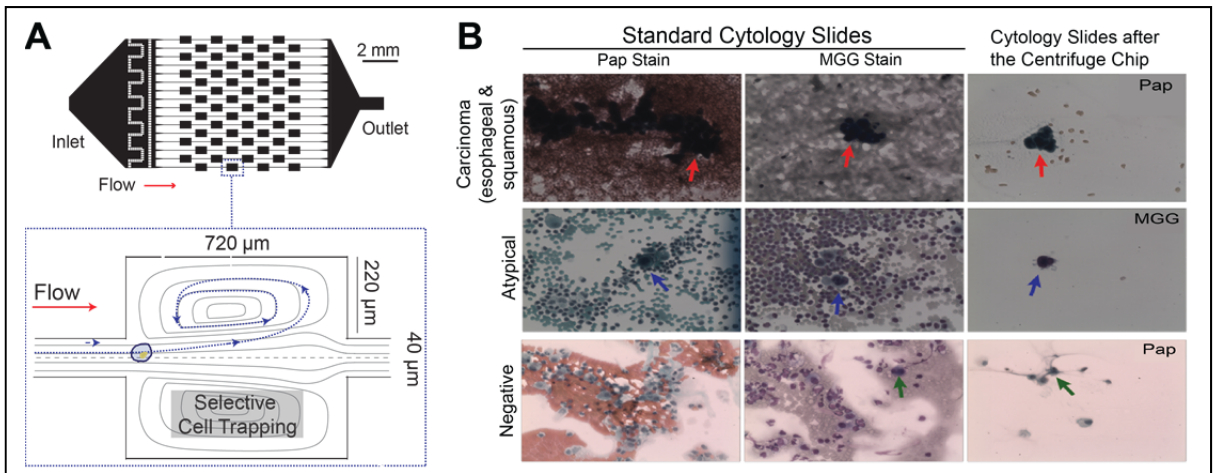


Figure 4: The Centrifuge Chip schematic with slides prepared from cells extracted using this technology. A. Device schematic B. Centrifuge Chip processed sample cytology slides showing reduced cellular background. Patient samples were prepared for cytology review with traditional cytological methods (e.g. centrifugation) and the Centrifuge Chip. Note that slides prepared with the Centrifuge Chip have a reduction in background material, as compared with cytology slides prepared with standard methods. This results in a clearer view of diagnostically important cells. Pap = Papanicolaou. MGG = May-Grunwald-Giemsa.

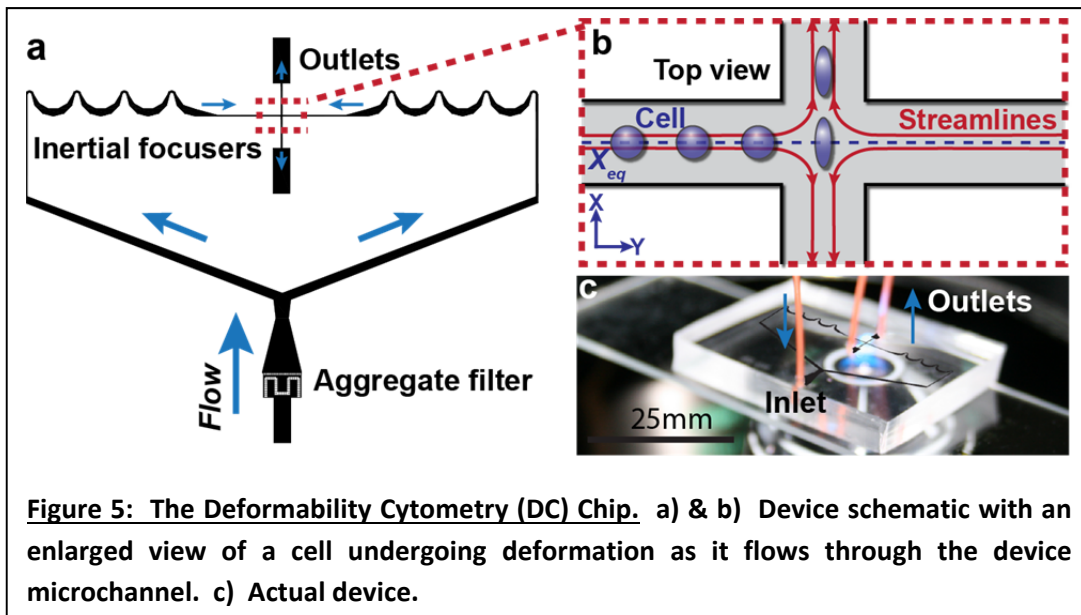


Figure 5: The Deformability Cytometry (DC) Chip. a) & b) Device schematic with an enlarged view of a cell undergoing deformation as it flows through the device microchannel. c) Actual device.

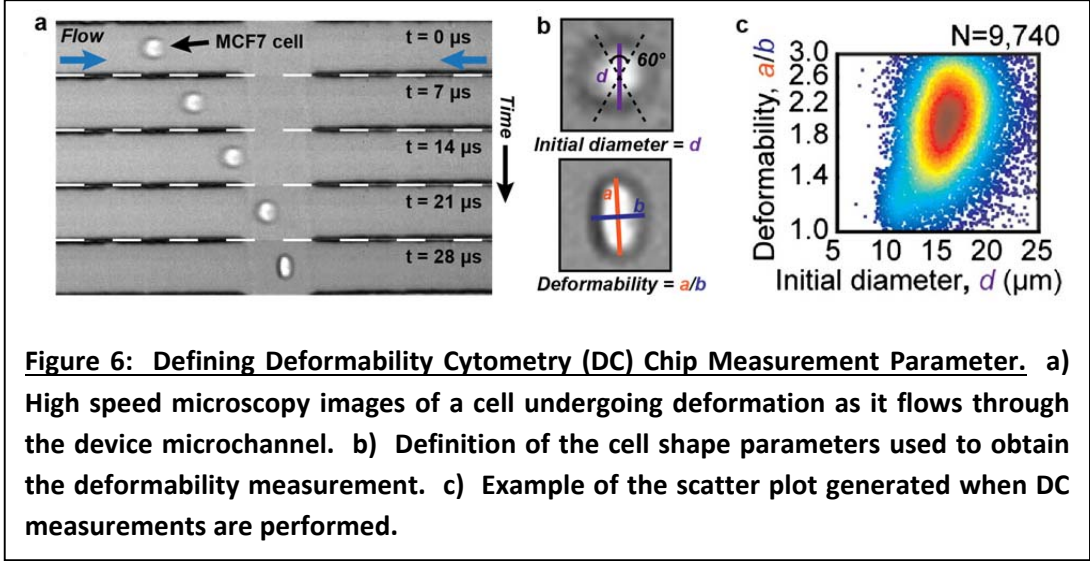


Figure 6: Defining Deformability Cytometry (DC) Chip Measurement Parameter. a) High speed microscopy images of a cell undergoing deformation as it flows through the device microchannel. b) Definition of the cell shape parameters used to obtain the deformability measurement. c) Example of the scatter plot generated when DC measurements are performed.

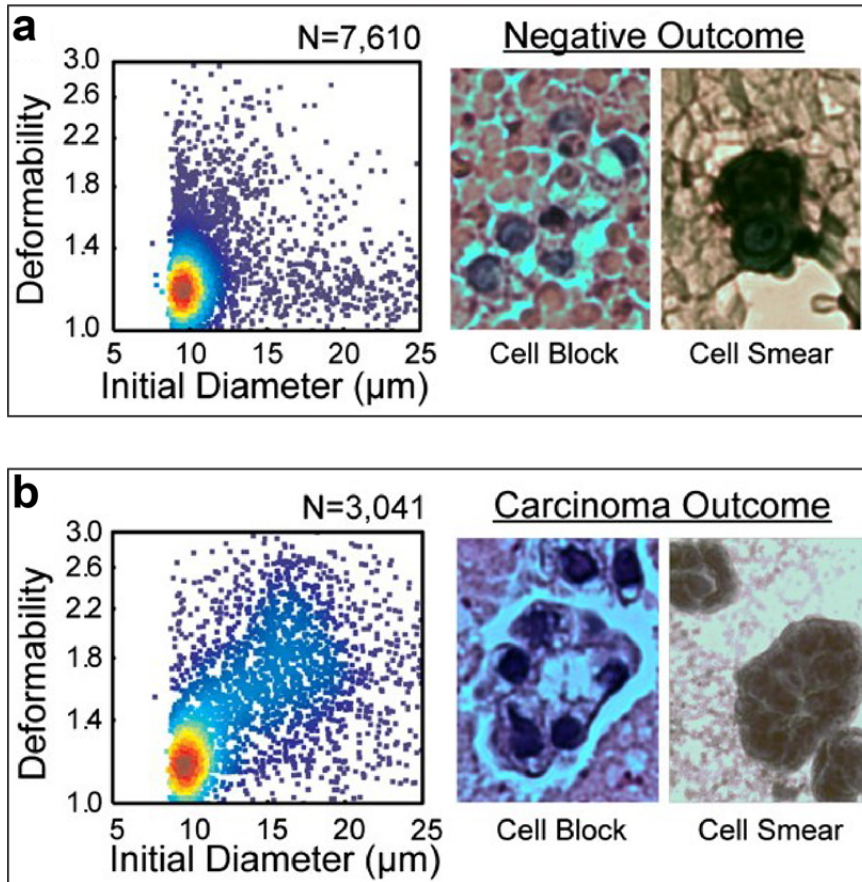


Figure 7: Deformability Cytometry measurements of patient pleural samples displayed with cell block and cell smear preparation of the same samples. DC measurement scatter plots show a different pattern of deformation for each clinical outcome: a) no malignancy as compared with b) carcinoma.

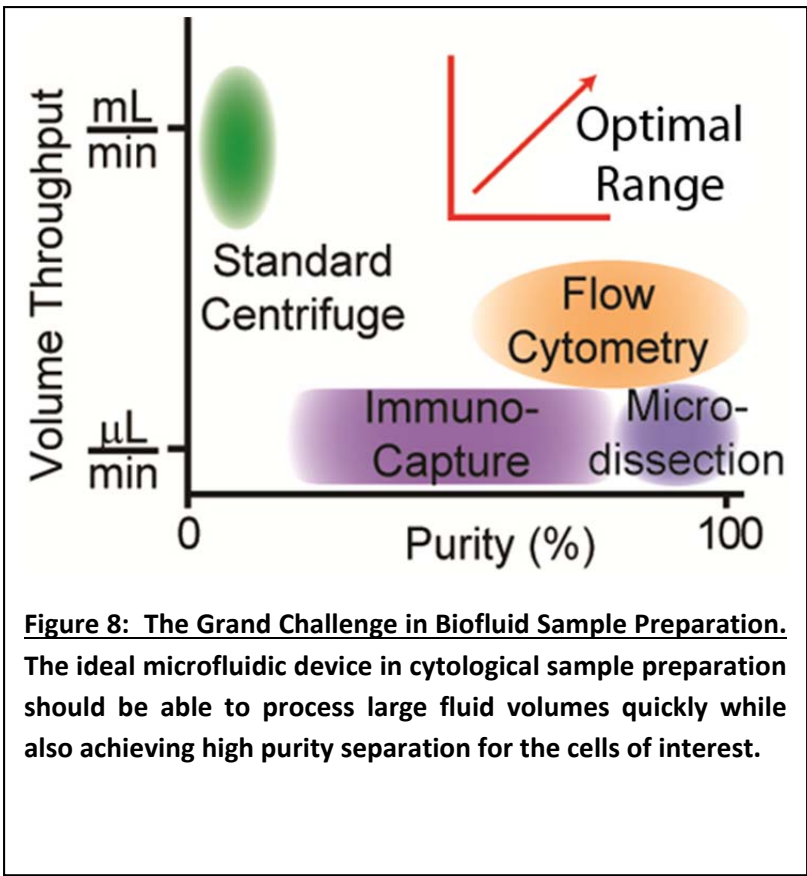


Figure 8: The Grand Challenge in Biofluid Sample Preparation.

The ideal microfluidic device in cytological sample preparation should be able to process large fluid volumes quickly while also achieving high purity separation for the cells of interest.

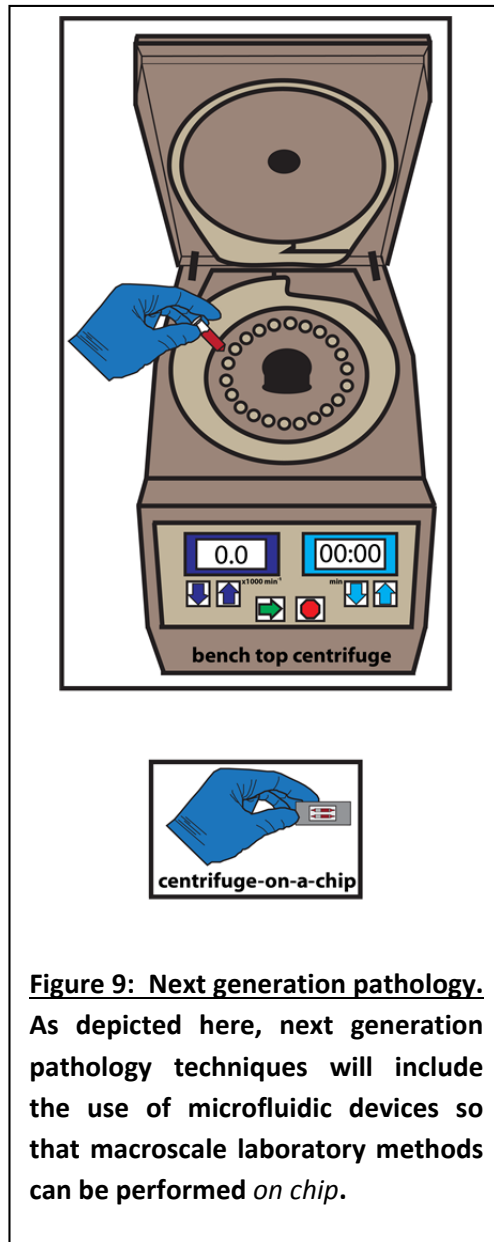


Figure 9: Next generation pathology.
As depicted here, next generation pathology techniques will include the use of microfluidic devices so that macroscale laboratory methods can be performed *on chip*.

[H] Tables

Table 2: The Grand Challenge in Biofluid Sample Preparation

Challenges	Requirements	Methods	Throughput	Max Volume	Operational Cellularity	Integration Ability	Average Purity	References
Concentrating Rare Cells from Large Volumes	1) High volumetric flow rate (mL/min)	DLD	0.35 mL/h, ~10 ⁷ cells/min	None	Diluted blood (50%)	Detection	99%	(Davis, Inglis et al. 2006)
	2) Ability to parallelize channels	Inertial	~10 ⁶ cells/min	None	Diluted blood (~2%)	Detection	80-99.71%	(Bhagat, Kuntaegowdanahalli et al. 2008, Di Carlo 2009, Wu, Willing et al. 2009, Mach, Kim et al. 2011)
	3) Continuous flow	Acoustics	80 µL/min, ~10 ⁸ cells/min	None	Whole blood with sheath	Detection	100%	(Petersson, Åberg et al. 2007, Lin, Mao et al. 2012, Nordin and Laurell 2012)

Preparing Small Volume Samples for Multiple Assays	1) Reduced dead volume 2) Sequential assays	Capillary	0.8-20 nL/s	300 nL	NA	Assays, Detection	NA	(Juncker, Schmid et al. 2002)
		Vacuum	0.5–2 nL/s	5 mL	Whole blood	Assays, Detection	99%	(Hosokawa, Sato et al. 2004, Dimov, Basabe-Desmonts et al. 2011)
		Centrifugal	5 nL/s – 0.1 mL/s	2 mL	Whole blood	Assays, Detection	>99%	(Amasia and Madou 2010)
		Mechanical Filters	0.75 mL/min, ~10 ⁹ cells/min	None	Whole blood	Assays, Detection	90%	(Zheng, Lin et al. 2007)
		Immiscible Phase	1 cm/s	20 mL	Whole blood	Assays, Detection	>80%	(Berry, Strotman et al. 2011)
Preparing Samples with High Cellularity	1) Cell separation at channel surface	Affinity-Based	1 mL/hr	None	Whole blood	Assays, Detection	9.2-68%	(Nagrath, Sequist et al. 2007, Gleghorn, Pratt et al. 2010, Stott,

	2) Segment highly cellular populations into single cells							Hsu et al. 2010, Wang, Liu et al. 2011)
		Cell Margination	NA	15-70 microliters	Whole blood	Assays	NA	(Shevkoplyas, Yoshida et al. 2005, Hou, Bhagat et al. 2010)
		Droplet	8 microliters/hr	NA	Diluted blood (10%)	Assays, Detection	99%	(Chabert and Viovy 2008, Edd, Di Carlo et al. 2008)
		DLD	0.35 mL/h, ~10 ⁷ cells/min	None	Diluted blood (50%)	Detection	99%	(Davis, Inglis et al. 2006)
Automating Multi-Step Sample Preparation	1) Standardize fluid plumbing system	Inertial Solution Exchange	NA	None	Diluted blood (10%)	Assays	NA	(Amini, Sollier et al. 2012)
	2) Minimize sample handling	Centrifugal	5 nL/s – 0.1 mL/s	2 mL	Whole blood	Assays, Detection	>99%	(Amasia and Madou 2010)
	3) Ability to	Droplet	8	NA	Diluted	Assays,	99%	(Chabert

	stain, concentration, and lyse cells		microliters/hr		blood (10%)	Detection		and Viovy 2008, Edd, Di Carlo et al. 2008)
		Digital	NA	>1 mL	NA	Assays, Detection	NA	(Barbulovic-Nad, Yang et al. 2008)
Achieving High Purity Cell Populations	1) Reduced background of cells	Biopolymer System	2 microliters/min	NA	Whole blood	Assays, Detection	NA	(Shah, Yu et al. 2012)
	2) Concentrate cells into small field of view 3) Make cells readily available in solution	Centrifuge Chip	0.1 mL/h	None	Diluted blood (5%)	Assays, Detection	~40%	(Mach, Kim et al. 2011)

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