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Optimization and clinical validation of CTC analysis for use in diagnostic applications through microfluidic capture and sorting

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Dear Madam:

This report, entitled "Optimization and clinical validation of CTC analysis for use in diagnostic applications through microfluidic capture and sorting", was prepared as my 2B Work Report for the University of Waterloo. This report is in fulfillment of the course WKRPT 300, as required by my BASc Nanotechnology Engineering degree. The purpose of this report is to present my findings for optimizing and validating the analysis of rare cells such as Circulating Tumour Cells (CTCs).

The University of Toronto is one of the most renowned public research universities in the world, and the Kelley lab is one of the bigger labs within it. The Principal Investigator is Dr. Shana Kelley, who is a biochemistry professor at the University of Toronto, and also has her own company named 'Xagenic'. The overarching theme of the research group is the development of novel molecules and devices enabling biological activities to be measured and manipulated in new ways. The projects underway involve aspects of many different disciplines, such as material science, biomedical engineering and nanotechnology.

I would like to thank Dr. Shana Kelley as well as the University of Toronto for providing me with this great opportunity wherein I have gained valuable experience in the biomedical and nanotechnology fields, as well as experimental resources, including academic papers and instructional guidance for learning new skills, and further developing old ones.

I would also like to thank Dr. Reza Mohamadi, Dr. Gordon Chan, and Dr. Mahmoud Labib who were directly responsible for supervising my work in the lab, and helped me get past any obstacles that I encountered along the way. Additionally, I would like to thank the several PhD students that I worked with for allowing me to help with their projects and vastly grow my knowledge. Specifically, I would like to thank Brenda Green for helping me with all the clinical data and image analysis, and also Mahla Poudineh, with whom I worked a lot on her MagRC chips. Finally, I would like to thank Dr. Sam Chang for teaching me how to fabricate some of the microfluidic devices we use. I hereby confirm that I have received no further help other than what is mentioned above in writing this report. I also confirm this report has not been previously submitted for academic credit at this or any other academic institution.

Sincerely,

Dhilan Bekah
ID 20512356

Contributions

Dr. Shana Kelley's Research Group is part of the faculty of Pharmacy at the University of Toronto, and consisted of ~30 people. While I did interact with the majority of the lab group, the team that I most prominently worked with was the Biomolecular Detection group, which accounted for fourteen people. There were four research associates, one postdoctoral fellow, five graduate students, and four other co-op students.

Professor Kelley taught at Boston College before coming to the University of Toronto, where she progressed to her current position as a Distinguished Professor of Biochemistry, Pharmaceutical Sciences, Chemistry, and Biomedical Engineering. She has founded two molecular diagnostic companies called GeneOhm Sciences and Xagenic Inc. In line with this is her desired area of focus for the research group, which lies in developing novel molecules and devices enabling biological activities to be measured and manipulated in new ways. The four larger themes of the group were Mitochondrial Chemical Biology, Peptide Vectors for Intracellular Targeting, Biotemplated Materials, and Ultrasensitive Biomolecular Detection. As previously mentioned, I was a part of the Biomolecular Detection group, whose primary objective is to generate detection systems applicable to the diagnosis of cancer, infectious diseases, and other disease states.

As a co-op student, my primary task was to validate the microfluidic devices developed by the group and obtain data for the different cancer clinical trials. This included running the blood samples from cancer patients (obtained in collaboration with hospitals) through the chips, using fluorescent microscopy to image the captured cells, and correlate data with clinical trial information. Secondary tasks involved aiding graduate students with their projects, which pertained to CTCs, but were not intended for clinical trial purposes (e.g. Studying Epithelial-to-Mesenchymal Transition (EMT)). Finally, basic lab management and upkeep, such as ensuring an up-to-date inventory of antibodies or picking up and allocating orders placed by the lab, became routine tasks.

This report focuses on my primary task of validating the microfluidic chips and acquiring data in the aims of drawing significant conclusions for the types of cancers analyzed in the trials. More specifically, in doing so, this report serves to demonstrate the improvements that were made in order to ameliorate the entire process. During this time, I have gained a tremendous amount of knowledge, most of which is directly related to my academic experience as a nanotechnology engineer, but also as someone who is

looking into biomedicine as a future career path. Moreover, being exposed to such a large group of great researchers that are far more knowledgeable and experienced than me has given me a lot of insight into what is most commonly used in nanotechnology and biomedical research, and what they are used for. Weekly group meetings were scheduled where two people would present their work, and this was a great way to learn about a variety of diagnostic applications, stemming from electrochemical assays, DNA hydrogels, or mitochondrial function, to name a few.

The purpose of this report in relation to my work term is to provide a brief summary as to the validation process of the novel microfluidic devices developed by the Kelley Lab, and the major improvements that were made. In doing so, I hope to also highlight the comprehensive knowledge I gained in several facets of research, including fabrication, project development through iteration and analysis, as well as group problem-solving. More specifically, I learned a lot about cancer and diagnostics. Working with collaborators on the clinical side also gave me new perspective, which you do not get while working from the lab research perspective.

In the broader scheme of things, my work helped the group continue their existing studies, start brand new ones, and ameliorate the foundational processes common to all. My work primarily helped acquire data to further push the clinical validation of the primary Velocity Valley Chip along, getting it closer to the commercialization stage. In terms of the entire Kelley Lab, I was able to tag onto a variety of different side projects and help PhD students with theirs, contributing to the overall work done by the Lab. In the long term, this helps with grants, and future collaborator prospects if the work is successful.

Summary

The purpose of this report is to demonstrate the significant improvements that were made to the sample-to-answer process involving the detection, capture, and analysis of rare circulating tumour cells (CTCs) in cancer patients. Since cancer is one of the leading causes of death in today's world, and CTCs are a major factor involved in that, it is imperative to be able to detect and analyze them. This has been made possible through clinical study collaborations with hospitals, as well as novel microfluidic devices. The scope of this report encompasses the studies and microfluidic devices developed by the Kelley lab, but more importantly, the optimization of the overall process.

The major points covered in this report concern the improvements made with regards to protocol run time, staining efficiency, scanning and automation, data processing, as well as design development. Microfluidic devices are rapidly growing in the field of diagnostics, and have led to novel ways of studying cancer, and more specifically, CTCs. Methods for capturing and analyzing these CTCs have been developed by the Kelley lab, and a brief description of the general process, including the Velocity Valley chip, is initially given. First, the major issues concerning the staining and protocol run specifics are given. These issues are addressed, and changes regarding the reagents used, as well as the sequential steps involved, are explained. Afterwards, the major issues / areas for improvement concerning the scanning and data processing system are given, and changes regarding the program code, acquisition parameters, and enumeration system are explained. Finally, an alternative microfluidic chip design for improving CTC capture, called the MagRC chip, is introduced, and the optimization of this design is reported.

The major conclusions in this report concern the improvements successfully made to the target objectives outlined in the report body. Firstly, the changes implemented in sample run protocols clearly demonstrate how the run time and amount of resources were effectively reduced. Secondly, the changes implemented in the scanning and data processing stages demonstrate how the consistency of high quality images was increased, as well as how the total data processing time was significantly reduced, while improving the reliability of the results. Finally, the refinement process for the new MagRC chip shows a promising potential for improving CTC analysis, and is close to the established Velocity Valley chip in overall performance after a much shorter time. All of the conclusions that were drawn effectively translate to both short-term and long-term reduction in costs through time and resource savings. Moreover, these

improvements have provided more conclusive results, indicating that the entire process has been rendered more systematic and reproducible, as well as more reliable.

The major recommendations in this report are to adhere to the new systematic implementations so as to create consistent results, further optimize the protocol runs by testing new reagents, explore the scanning software's integrated capability of counting cells, and continue to refine both the VV and MagRC microfluidic chips by exploring new materials and methods. In addition, developing new designs should also be done in order to maximize the possibilities of capture and profiling. Developing the magnetic guidance chip shows great promise and should be pursued.

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1 Introduction

Cancer has been, and still is today, one of the greatest public health problems worldwide. According to the Canadian Cancer Society, it is expected that about 40% of Canadians will develop cancer in their lifetime, while 25% will die from it [1]. The importance of circulating tumour cells (CTCs) was first proposed in the mid 19th century, and today, they are widely recognized as having several potential applications with regards to cancer therapy [2]. CTCs are cells that have shed from a primary tumour into the vasculature, and are carried throughout the body via blood circulation [3]. Once these CTCs settle in another part of the body, they may begin to proliferate, resulting in a new tumour. This process is called metastasis, and accounts for over 90% of cancer-related mortality [2]. As a result, the identification and molecular characterization of CTCs provides crucial insight into the metastatic process [2]. Firstly, CTC enumeration has been used to guide prognosis of the disease, in order to modify the course of treatment as required [2]. In addition, the genotypic and phenotypic characterization of CTCs, taken from a sample of blood (which is significantly less invasive than other methods such as surgery), may be able to serve as a "real-time tumour biopsy" [2]. These are only a couple of the numerous advantages that CTCs have to offer for cancer treatment, and it is therefore important to be able to develop technology that can accommodate these applications.

1.1 Microfluidic Devices

The major technology used today to study CTCs is microfluidics. The field of microfluidics involves the study and manipulation of fluids that are constrained to a sub-millimetre scale [4]. At this scale, the phenomena that govern liquids are very different from those at the macroscale. For example, gravitational forces are less dominating at the microscale, while surface tension and capillary forces are more dominating at the microscale. As a result, there is an increased potential to tackle problems that are encountered at the macroscale. More specifically, microfluidics allows for the development of technologies that enhance the capabilities of those working with macroscale problems in biology and medical research [4]. Applications can include continuous-flow, droplet-based and digital microfluidics, as well as DNA chips, cellular biophysics and optics. This report concerns work that is continuous-flow based, as CTCs are found in blood, which is liquid that continuously flows. More specifically, it pertains to lab-on-chip applications, which is a rapidly growing area of research in the world of microfluidics. Lab-on-chip (LOC) devices are broadly used in life sciences and medical research, and of particularly

interest is diagnostics. These miniaturized systems are able to support precise control of liquids (flowing usually under laminar regime), minimize consumption of reagents and samples due to smaller volumes, favour short reaction times, require little to no power to operate, are portable, and more often than not have lower cost of production [5].

1.2 Current Detection, Capture, and Sorting

The Kelley Lab group created one such device, which they termed the Velocity Valley (VV) Chip. The basic premise of the chip is to have a sample fluid (i.e. blood) run through it, and via biochemical, magnetic and microfluidic properties, capture and sort CTCs in the chip itself. Using diagnostics, isolating cells from blood can lead to analysis of bloodborne cancer cells, infectious pathogens, and prenatal testing [6]. In order to capture CTCs from complex matrices such as blood, a method for marking the cells must be used, and generally, antibodies are used to identify the CTCs based on their phenotypic profiles. In the case of the FDA standard, this includes the expression of various Cytokeratins, lack of CD45 expression, and presence of a nucleus and cell-like morphology. With the VV chip, antibody-coated magnetic nanoparticles are initially used due to their high colloidal stability and surface-to-volume ratio [6]. At practical flow rates, however, capture of nanoparticle-bound cells is difficult due to the small volumes (and thus low magnetic susceptibility). Therefore, these velocity valley microstructures were implemented, as they create pockets of low linear velocity, enabling cells that are passing through to slow down, which in turn allows the applied magnetic force to overcome the reduced drag force and trap the cells [6]. By analyzing the magnetic and drag forces acting on the cells, a threshold linear velocity for capture was obtained and translated to fluid flow in units of Volume / Time, which is what is used to run the sample volumes via an automated pump.

Figure 1 illustrates the integration of the velocity valley concept, along with X-shaped microstructures that further enhance the capture efficiency, into the chip design. Examining label A, CTCs are tagged via magnetic nanoparticles that are functionalized with an antibody against the surface marker EpCAM, which is an epithelial tissue marker. Tagged cells are magnetically captured in velocity valley (VV) regions (low flow regions in blue, gradient towards high flow regions in red). Label B demonstrates a multizone velocity valley design featuring four different regions with varying linear velocities (1x, 0.5x, 0.25x, 0.125x). Cells with high EpCAM expression are captured in zone 1, and cells with decreasing expression are captured in later zones accordingly. Finally, label C provides the flow profiles for zones 1-4 [8].

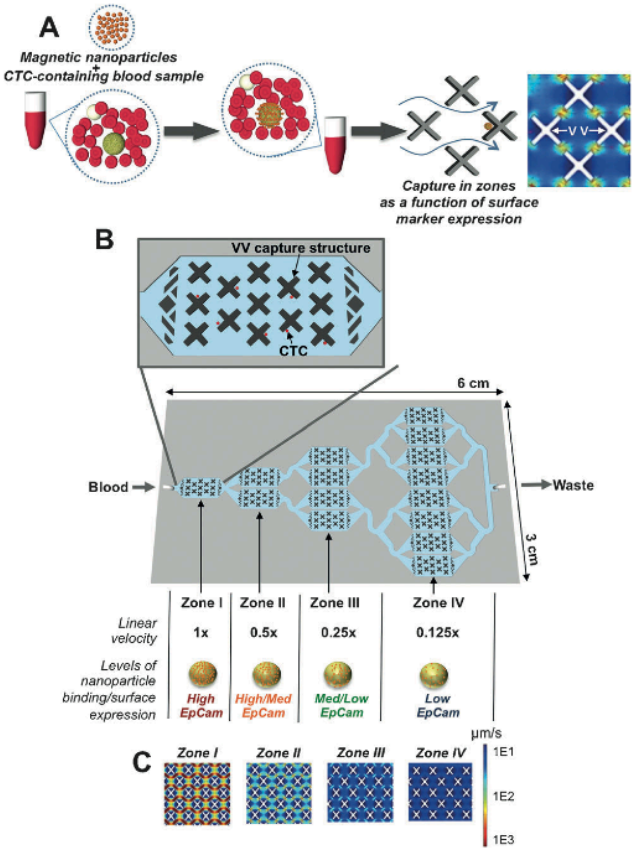

Figure 1. Velocity Valley chip for surface-expression-guided binning of heterogeneous CTCs.

This chip has presented a large potential for highly sensitive clinical capture and sorting of CTCs as compared to CellSearch, the only FDA-approved testing method for capturing and enumerating CTCs. The VV chip, on average, can capture CTCs with both high and low levels of EpCAM, using just 1 mL of blood per chip, while CellSearch can only capture CTCs with high and not low EpCAM expression, using 7.5 mL of blood. More evidence through cell line validation, as well as clinical sample data, however, is required in order to definitively clear the method under FDA standards, and complete the commercialization process.

In addition to using a microfluidic device, a protocol with specific reagents was implemented for capturing and staining the CTCs. Moreover, a scanning system for viewing and counting the CTCs was also already established. These will be further elaborated on in sections 2 and 3, respectively, when addressing the improvements that were made.

1.3 Objectives for Improvements

The CTC research process that has been described above gives a relative indication of the stage that it was at, prior to any of the work discussed in this report. Henceforth, this report will describe the improvements made to the overall process, as well as the results of those implementations. More specifically, the following three major objectives were targeted for improvement:

1. Minimize Protocol Run Time & Resources
2. Minimize Results & Analysis Time (includes scanning, counting, and automation)
3. Increase Overall Chip Performance

Each of these three requirements will be explored and discussed in more detail in sections 2, 3, and 4, respectively.

2 Protocol Run

In order to demonstrate how the standard operating procedure (SOP) was improved upon, a baseline description is necessary. Different clinical studies have slightly different protocols depending on the desired outcomes that are being tested, as well as the type of cancer being investigated, but they all have very similar sequential steps. Referring to Appendix A as a guiding example, firstly, after adding the magnetic nanoparticles conjugated to anti-EpCAM (Epithelial Cell Adhesion Molecule), the sample blood is added to the VV chip. Once the blood has passed through fully, the cells expressing EpCAM (including the CTCs) have already been captured by the applied magnetic field. Afterwards, PBS-EDTA is run through as a wash, and the EDTA thins and keeps the blood from clogging in the chip. Then, the PFA (formaldehyde) solution is used to fix the cells. Then, a detergent is used to permeabilize the cells – this is a crucial step in the process, as it will allow for immunofluorescent staining in the next step. Different antibodies, tagged for different surface markers, and to different fluorescent colours, are chosen in order to later distinguish between the different cells in the blood matrix (e.g. white & red blood cells, CTCs). Finally, a few washes are performed with a mild detergent solution, and the final stain for the nucleus is added.

As previously mentioned, different clinical studies have varying protocols. This is primarily due to the fact that CTCs from different cancers (e.g. bladder, prostate, breast, renal) are marked more effectively

with different antibodies. The following sections will serve as a guide for the entire sample-to-answer process from the sample pickup to the image analysis for certain clinical studies, in an effort to amass enough conclusive data to validate the VV chip.

2.1 Minimizing Run Time and Resources

To address the optimization of the protocol runs, two major studies will be discussed: Prostate and Breast cancer. Patient samples tested with the VV chips are collected via pickup from nearby hospitals, which the clinical studies are in partnership with.

2.1.1 Prostate Cancer Study

This particular Prostate Cancer study was carried out in collaboration with the Princess Margaret Hospital, and is the longest ongoing single study for cancer at the Kelley Lab. The Kelley Lab's major objective was to examine how CTCs change over disease progression using different CTC markers: Cytokeratin, N-Cadherin, Androgen Receptor (AR-555), and Androgen Receptor Variant 7 (ARV7). For this study, four VV chips per patient sample were initially used in parallel, and a fifth was added later on, while the original fourth was discontinued. The combinations are presented in Table 1 below.

Table 1. Prostate Cancer Sample Chip Combinations

Chip	Capture	Staining
1	EpCAM	• Ncad-Biotin • Androgen Receptor (AR-555) • CD45-APC
2	EpCAM	• Ck-Biotin • Androgen Receptor (AR-555) • CD45-APC
3	EpCAM	• Ck-Biotin • Androgen Receptor Variant (ARV7) • CD45-APC
4 (discontinued)	EpCAM	• Ck-Biotin • M30 Apoptotic Dye • CD45-APC
5	CDH11, anti-Biotin beads	• Ck-Biotin • Androgen Receptor (AR-555) • CD45-APC

Briefly, N-Cadherin conjugated to Biotin (Ncad-Biotin), as well as Cytokeratin conjugated to Biotin (Ck-Biotin), are the two antibodies that were used to tag and stain the CTCs. Two different antibodies were used to cover a broader range of protein binding. In addition, this study followed patients undergoing treatment that used Androgen Deprivation Therapy. Thus, two reagents, AR-555 and ARV7, were used to stain the Androgen Receptors and examine drug resistance over the course of treatment. Figure 2 demonstrates the positive stain for both reagents using cell lines, in order to validate the staining in patient samples. Image A shows an ARV7 stain for a VCaP Prostate Cancer Cell Line, while image B shows an AR-555 stain for a VCaP Prostate Cancer Cell Line. Image C shows an ARV7 stain for a Prostate Cancer Patient Sample, while image D shows an AR-555 stain for a Prostate Cancer Patient Sample. Over the time scope of the work done for this report, no significant conclusions were found as to whether ARV7 is more or less effective than AR-555. This information, however, is needed in order to explain the improvements made to this study. As illustrated in Table 1, Chip 5 was introduced as a different method of capture, as opposed to simply changing the staining. This was done in an effort to compare profiles, as a means to double check the average CTC counts, which proved to be conclusive. In addition, Chip 4, which used an apoptotic dye to determine the percentage of cells that would be living, was discontinued. This is because no further significant analysis could be achieved with the results concerning this chip, as the percentage was consistently around ~67%. This, in turn, reduced the time of each run as well as the number of chips per run, back to its original amount, while improving the reliability and reproducibility per sample.

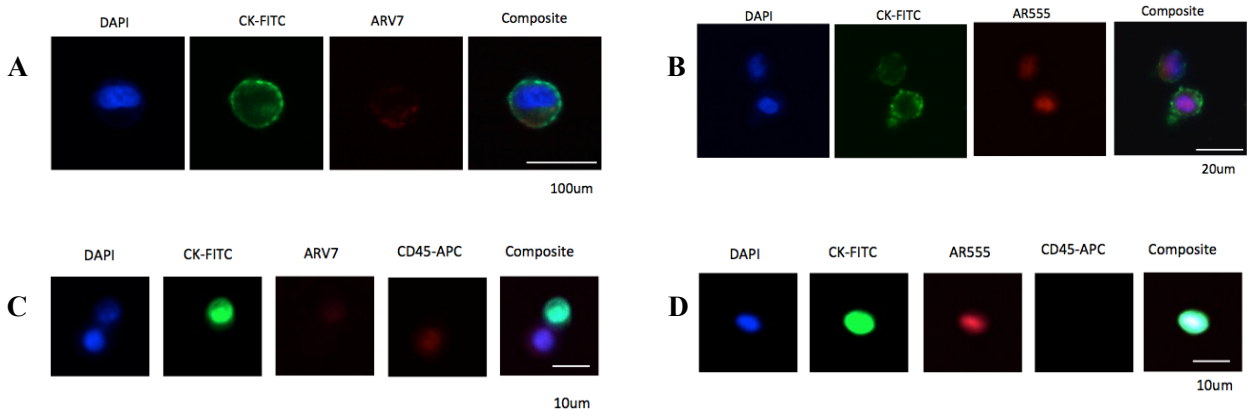

Figure 2. Prostate Cell Line and Patient Sample Staining

Moreover, the results from incorporating Chip 5 into this study lead to the belief that a change in the number of CTCs for a patient has virtually no correlation with the patient's Prostate-specific antigen (PSA) levels, which is often used as a measure of cancer progression on the clinical side. This is an important observation as it raises questions as to whether PSA is actually a good indicative marker for prostate cancer. As a result, more direct research has been invested in determining whether current treatment based on PSA is correct, which will greatly reduce the amount of PSA-related work on both the research and clinical sides in the future, should it be incorrect.

2.1.2 Breast Cancer Study

This study, in collaboration with Sunnybrook Hospital, began after the onset of the work encompassed in this report. As a result, many changes were implemented in the protocol at the very start of the study, in order to improve efficiency but also address certain challenges that arose in previous studies. Firstly, using the Yellow Avidin Nanobeads (refer to Appendix A, highlighted in blue) for fluorescing the CTCs proved to be inconsistent. These beads were initially used in an animal study to enhance the fluorescence of the Ck-18 antibody tagging of the CTCs, and they had worked well. However, over a prolonged period of time, they appeared to lose their reliability. It appeared to be quite arbitrary as to when the beads would lose their viability and induce lots of non-specific binding, making it difficult to count CTCs accurately. This could have been due to a variety of factors, such as how long the operator kept the avidin beads out of the fridge, whether they are mixing the stock solution each time before removing aliquots, and so on. Moreover, ordering new stocks would come from different batches as it turned out, since some seemed to be no good from the beginning, and for a full month, no working stock could be obtained. This inconsistency led to problems when running patient samples during that time. As a result, an alternative antibody was found which did not require the use of Avidin beads. The working mechanism of the beads was such that, once bound to the standard Ck-18 labeled CTCs, they would appear very bright under the Fluorescein isothiocyanate (FITC) channel of the microscope. Instead, an alternative Ck-18 antibody was conjugated directly (albeit dimmer) to the FITC channel. This greatly improved reproducibility and, indirectly, the time and number of resources used in the study. This is due to the fact that, with the previous inconsistencies, results would appear unreliable after processing, which meant that more patient samples would have to be collected in order to gather a conclusive data set. By reducing the number of patient samples, fewer chips and reagents were used.

This study also introduced the crossing of multiple antibodies for a certain marker. The aim of this was to increase the CTC detection range by accounting for cells that may lack a certain antibody binding ability. Cell lines were used to ensure that there was no surplus coverage (i.e. non CTCs), and the known number of cells input validated the staining. The new antibody combination consisted of the new Ck-18 conjugated to FITC, C-11 (a pan-Ck comprising of multiple cytokeratin antibodies), a CD45 antibody conjugated to APC (for staining the white blood cells), and a DAPI nuclear stain. Thus, through this selective staining, the different cell types could be distinguished. Looking at label D of Figure 2 for reference (omitting the Androgen Receptor stain), all the Ck antibodies conjugated to FITC bind to the CTCs and appear green through fluorescent microscopy, while the CD45-APC binds to White Blood Cells (WBCs) and appears red, and the DAPI nuclear stain binds to all cell nuclei and appears blue. Moreover, the total time for every protocol (since every protocol used DAPI to stain every cell in order to distinguish from debris), was cut down by 30 minutes. Alongside a Research Associate, Gordon Chan, a realization was made after noticing that the cells were already permeabilized by the antibody step. Thus, the addition of the DAPI nuclear stain at the same time as the antibody step was hypothesized to introduce no functional difference. This way, both wash steps as well as the DAPI step, which were each 10 minutes long (refer to Appendix A, highlighted in red), could be removed. This is a significant change as a 30-minute shorter protocol saves a lot of time after numerous runs, especially when there are samples to be run, scanned and counted everyday.

3 Sample Analysis

3.1 Scanning and Automation

After running the patient samples through the VV chips in accordance with the protocols, the chips would be stored in petri dishes containing some water (to prevent drying out of the inside of the chips), wrapped tightly with parafilm, and stored in the 4^0C fridge in order to later analyze the results, using fluorescent microscopy. Since the cells were permeabilized, however, scanning would have to be performed within a few days of the run in order to avoid leakage of the fluorescent dyes out of the cells.

The Nikon Eclipse Ti-E was the microscopic instrument used to scan the chips, in conjunction with an integrated software application entitled NIS-Elements Advanced Research. After prepping the chips to be scanned, they were loaded onto the x-y stage, and more specifically, onto the piezo stage, which was on top of the main x-y stage. The piezo stage allows for much finer control (down to the subnanometer

range), which increases not only the scanning precision but also the image acquisition capabilities. More importantly, however, the piezo stage allows for automatic focusing, which is a key feature in allowing for automation, and for multiple sequential images to be taken. Figure 3 illustrates the basic interface and capabilities of the software. Label A shows the controls for shutter and image format settings, but namely exposure time, while label B shows LED power sliders for different fluorophores. Label C shows the automated scanning program interface, allow for chip and stage alignment, as well as choice of objective lens and filter. Finally, label D shows the list of customizable scanning programs. The Kelley Lab Group has created several microfluidic devices, and each has several potential applications. Advantageously, the NIS software allows for the creation of a customized program with set parameters (i.e. size of scanning area, aperture, acquisition speed, number of filters etc…) within the NIS software to suit the experiment's requirements.

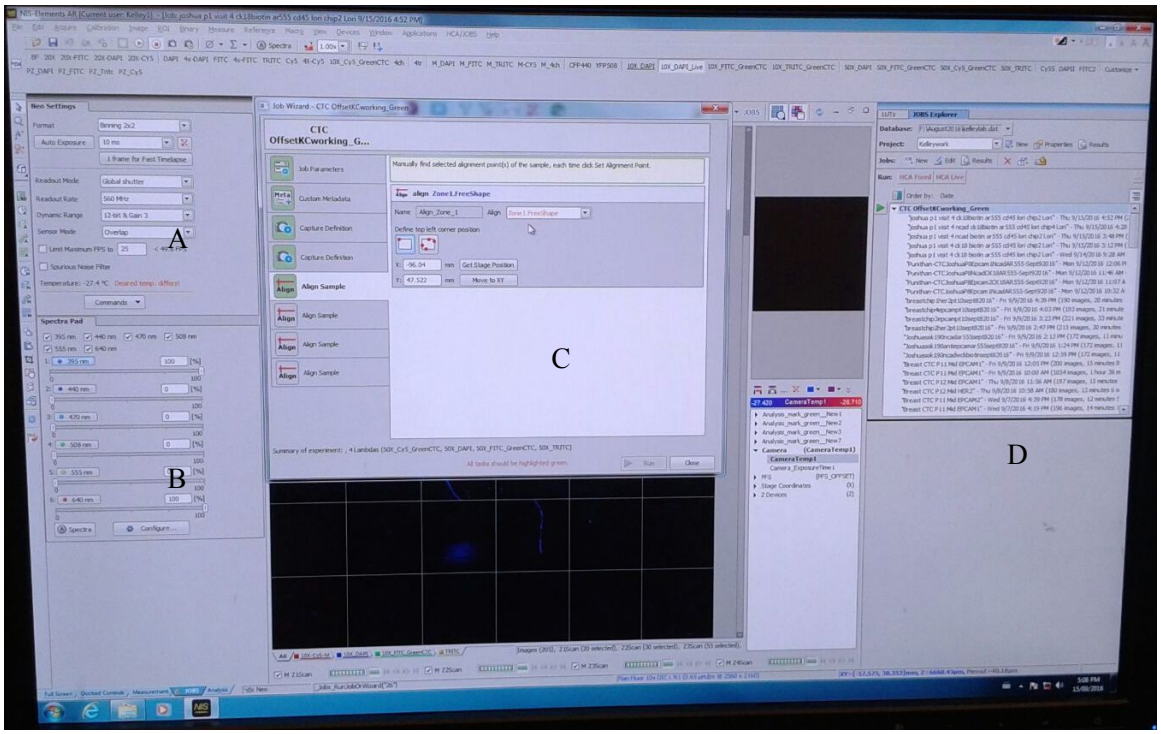

Figure 1. NIS-Elements Advanced Research Interface.

Prior to the improvements made, the scanning process had loose definitions of the imaging parameters, and also did not take full advantage of the automation capabilities. As previously mentioned, the premise of CTC detection was based on fluorescent microscopy. More specifically, three major filters were used for differentiation: FITC (green), APC (red), and DAPI (blue). Through cell line and patient sample runs,

image acquisition settings were well worked out so that each scan would use the same exposure times and LED power settings for any similar reagent. Ultimately, the following were chosen:

- Nuclear stain DAPI: 10 milliseconds, at 100% power
- Anti-Ck-FITC: 150 milliseconds, at 50% power
- Anti-CD45-APC: 500 milliseconds, at 100% power
- AR-555 TRITC: 70 milliseconds, at 100% power
- ARV7 TRITC: 30 milliseconds, at 100% power

These settings appeared to be the most effective through breast cancer and prostate cancer cell line validation, and were consequently used for the Breast Cancer study, as well as every other study wherein staining was identical and applicable, such as the Bladder Cancer study. They were also used with the Prostate Cancer study as additional scan data to the original settings, and it was found to be more accurate based on the more conclusive appearance of cells, with well-defined staining for each component of the cell (i.e. nucleus and surface). Figure 4 compares two images of captured CTCs, where the left and right pictures are before and after image acquisition optimization, respectively. It is clear that the picture on the right provides a much clearer distinction between the nuclear stain (blue) and the Cytokeratin stain (green).

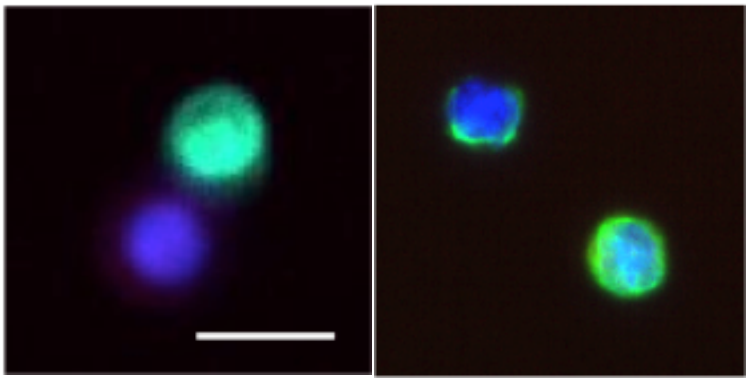

Figure 2. Comparison of CTC imaging for a Prostate Cancer Patient Sample

Another important aspect of the scanning that was optimized was the high-magnification image capture. Initially, the programs would only function reliably and consistently with the low 10x magnification lens. In brief terms, the microscope would first scan the entire chip and take autofocused images with the regular 10x lens. If any CTCs were detected by the program specifications (in simple terms, green and

blue but no red), then the program would tell the microscope to go back to those coordinates, and an autofocused image would be taken with the 50x objective lens. A major obstacle that was encountered during this period was an offset issue that occurred whenever the objective lenses would switch from the 10x to the 50x. As a result, no CTC image would be captured. This was due to the fact that people using the microscope would often forget to turn off the piezo stage before manually adjusting the chip on the main stage, which created position offsets. Upon this discovery, the group members were notified of the situation so that this would cease. To address the offset already established, however, a manual position offset was added to the programming after several iterations and adjustments. An ROI (region of interest) adjustment was also made to ensure that the cell of interest was actually found within the image. With the aid of the Nikon instrument expert, all of the offset problems were addressed. Finally, after adjusting the necessary criteria for inclusion and selectivity in the program code regarding the colours, through trial and error, the high 50x magnification was consistently and successfully implemented by creating a new custom program for not only the VV chip (or what was colloquially termed the 4-zone chip), but also the 10-Zone MagRC chip (which will be elaborated on in Section 4).

3.2 Data Processing

After the scanning process had been optimized, the counting of the CTCs was addressed due to the subjectivity of the process. Multiple people ran patient samples, and while it was consistent in that one person was primarily responsible for one study (unless they were unable to run a sample for that particular instance), the nature of counting CTCs was still subject to personal differences across the studies. As a result, a system for validating whether a cell was a CTC or not was implemented, using a research paper that was published in Molecular Oncology in 2016, entitled ‘Challenges in circulating tumour cell detection by the CellSearch system’, as a guide. They created a flow chart, depicted below in Figure 5, to help process the CTC on a yes or no basis.

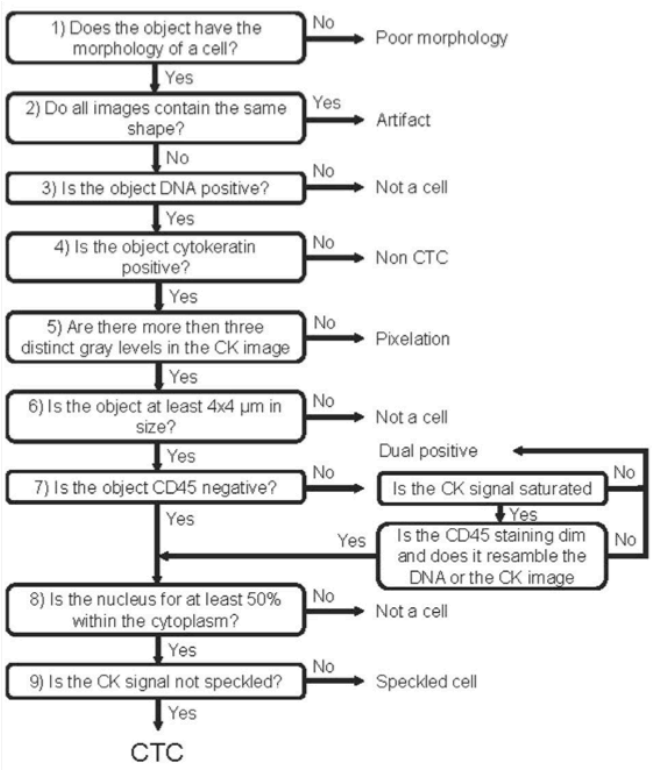

Figure 3. Criteria for a CTC [9].

This system, in combination with the high-magnification image capture and more robust acquisition settings, greatly improved the quality of analysis, since it provided clarity as to what was a considered a CTC. There was no longer a need to zoom into every frame that potentially had a CTC – looking through the 50x images and using the flow chart above made distinguishing between a CTC and a non-CTC much easier. Having a more robust imaging system allowed for a better SOP for each clinical study, improving reliability and reproducibility, which indirectly reduced the number of resources used from inconclusive results. Furthermore, having better 50x scans as opposed to the 10x scans greatly reduced the enumeration time of CTCs per sample, as there were less scans to examine, and they were easier to analyze in conjunction with the established CTC criteria flow chart in Figure 5. More importantly, however, it further refined the results that were being obtained, making extrapolations for disease prognosis more reliable.

4 Improving Chip Performance

4.1 New Designs

While VV chip was the primary microfluidic device used to capture and sort CTCs in clinical studies, another chip was newly developed by a graduate student in an effort to improve capturing and sorting, and required validation. It was termed the 'MagRC' chip, because it used Magnetic Ranking Cytometry to capture and sort the cells. The detailed mechanism for this design is beyond the scope of this report, however, and so it will only be discussed as a variation to the VV chip as a means for improvement.

4.1.1 100-Zone Design

Figure 6 depicts the schematic for the "100-Zone" MagRC Chip, wherein varying sized Nickel Post Microstructures change the capture profile along the chip, effectively allowing for '100-Zones' through the length of the chip.

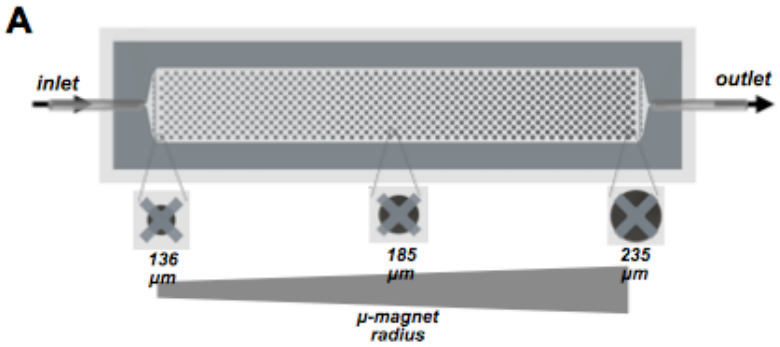

Figure 1. 100-Zone MagRC Chip Schematic.

This term was coined because there were effectively 100 mini-zones as the size of the nickel posts increase through the length of the chip. Due to the design of the chip, there was increased method specificity, and the sensitivity of capture was supposed to have greatly increased, consequently increasing the profiling ability of CTCs. The initial results with cell lines were great, and in line with the flow cytometry data. As a result, validation with preliminary patient samples (not following a specific study) began, in order to determine any problems and potential improvements. This chip, however, proved quite problematic at this point.

Firstly, the fabrication took longer as compared to the VV chip, as it needed to be made in the clean room, since etching was involved in the process (as opposed to the 4-zone which was done purely with soft lithography). Additionally, there was a low throughput as only one chip could come from a single mask due to its length, which was half the throughput of the VV chip.

Secondly, once the chips were fabricated, they rarely made it to an actual sample run, as they would get damaged from the degassing process. Before every run, the chips that are to be used are degassed with some fluid, usually Pluronic. This is to prevent air bubbles in the chip during the run, as this would impede the fluid flow, affecting the expected flow properties, thereby lowering the efficacy of the capture. The 100-Zone Chip however, is designed such that there is a layer of PDMS attached to a layer of SU-8 (photoresist), and this is held together by fairly weak forces. As a result, degassing these chips would often cause the PDMS layer to lift, and leakage would occur as a result. Once this has occurred, the integrity of the chip is lost, and it cannot be fixed. Several things were tried, such as leaving the chip to bake for longer, using a thinner PDMS layer, and even super gluing the sides (as impractical as it was). However, none of these alternatives provided a good, permanent solution.

4.1.2 10-Zone Design

After raising the issues that were present with the current design with the group (primarily the graduate student), a new design was introduced, as depicted by Figure 7.

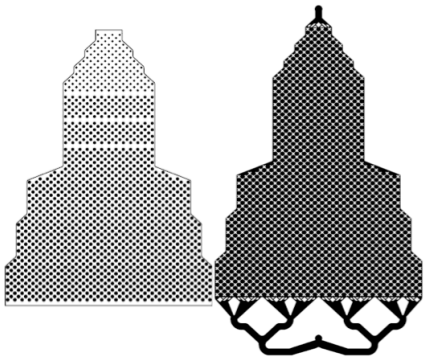

Figure 2. 10-Zone MagRC Chip Schematic

While a 100-zones would have provided a very specific profiling, it was decided to be unnecessary for the moment (especially given the fact that the next highest sorting chip was the velocity valley or 4-zone chip). Much of the adhesion problem between the SU-8 and the PDMS was attributed to the length of the

chip, which made it much more likely for a particular section to have not settled as well, which would in turn affect the rest should it come to separate. As a result, the new design featured 10 zones, dictated by their size and consequently their respective flow rates. The smaller length and refined fabrication process allowed for a much more consistent production of these chips. In addition, the smaller size meant a higher throughput, which made it more practical for clinical studies (which require many chips at a time). After validating the chip with cell lines and flow cytometry data, preliminary clinical samples were used. This time, however, the problems encountered previously did not occur. While this report encompasses only the early stages of clinical validation, the initial results seem promising. Figure 8 illustrates the capture of a CTC form a patient sample in a 10-Zone device. Unfortunately, the 50x image was lost, and so it is at 10x magnification.

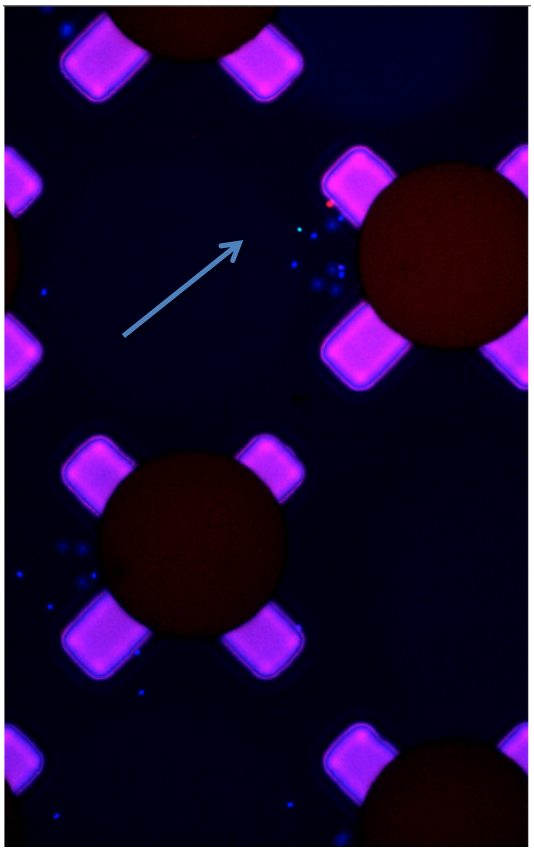

Figure 3. 10-Zone Capture of a CTC, 10x Magnification

In order to gain a better understanding as to which of either the new MagRC chip and the established VV chip is and/ or will be more efficient, a decision matrix was created, as shown in Table 2.

Table 2. VV vs. MagRC Chip Decision Matrix

Category	**Score Weight**	**VV Chip (/10)**	**MagRC Chip (/10)**	**VV Weight**	**MagRC Weight**
Cost	5	9	6	45	30
Ease of Fabrication	4	9	6	36	24
Specificity	5	7	9	35	45
Reproducibility	4	8	7	32	28
Staining	3	8	8	24	24
Scanning	3	9	7	27	21
Novelty	5	4	9	20	45
Total				**219**	**217**

The novelty factor was introduced as a representation of the time they have both existed, in order to allocate the potential of the MagRC chip adequately. With that in mind, it comes pretty close to the already established VV chip in performance. If the MagRC chip were to be optimized as the VV chip has been, it would provide a much higher specificity in profiling, allowing for the capture of cell subpopulations that even the VV chip could not. Consequently, this will push detection limits for diagnosis further. While it may be more expensive to produce as compared to the VV chip, it would be used in cases where higher specificity would be required, and thus more sparsely. In addition, as the refining process proceeds, the cost is expected to come down.

5 Conclusions

In conclusion, based on the analysis reported above, it can be concluded that significant improvements were made to the sample-to-answer process with regards to each of the three objectives initially outlined in the introduction.

The clinical studies showed significant improvement throughout the work term. From a protocol perspective, several changes were implemented in order to both, reduce the run time, as well as reduce the number of resources being used, all while improving the quality of the study. In the case of the Prostate Cancer study, the number of chips per sample remained the same, and the reliability and reproducibility increased significantly. Moreover, an important PSA observation was made, which will further improve the focus and allocation of resources with regards to treatment. With the Bladder Cancer study, the Avidin beads were replaced with an alternative Ck-18 antibody conjugated directly to the FITC channel, increasing the consistency of the CTC counts, thereby decreasing the number of patient samples ultimately required for a conclusive dataset. Furthermore, the run time of every protocol was decreased by 30 minutes due to the shift in the DAPI nuclear stain step. All of the above translate directly to cost savings in the form of time and / or resources, with the added bonus of increased study quality.

From a scanning and automation perspective, adjustments to the program code and software parameters greatly increased the consistency of quality images from chip to chip, while also further facilitating the data processing afterwards. Establishing a more rigid set of parameters for identical antibodies reduced subjectivity, and allowed for a more robust SOP for use between individuals. The implementation of a consistent and automated, high 50x magnification system greatly reduced data processing time, as it became easier to classify CTCs based on the clearer image, in addition to reducing the number of image frames to go through. Finally, incorporating the CTC criteria flow chart further decreased processing time, by further facilitating the distinction between correct and incorrect CTCs. Again, all of the above translate directly to time savings, and increased study quality.

Finally, working through and improving the quality of the new MagRC chip design allowed for a parallel way to address the ultimate problem of capturing and sorting CTCs in patient blood in an attempt to aid cancer treatment and prognosis. Based on the decision matrix created, while the VV chip is still better, albeit marginally, the improvements made to the MagRC chip have greatly reduced the gap between the two. Given enough time, it is expected that this new design will out-perform the VV chip overall.

6 Recommendations

Based on the analysis and conclusions drawn in this report, the main recommendations in this report are to further optimize the protocol runs by testing new reagents and minimize run time, further improve the scanning and automation system to increase enumeration speed and consistency, as well as to continue refining / inventing novel chip designs to increase overall performance. More specifically, the key steps to be taken moving forward are:

1. Have every operator / researcher use the flow chart that defines the criteria for what should be classified as a CTC going forward, so as to standardize the counting process.
2. Continue exploring new potential antibodies / reagents in order to bind more specifically to CTCs, as well as increase signal strength. This exploration may also allow for run time reduction in the process.
3. Work on the NIS-Elements program's integrated capability of counting cells by working on the parameters for CTC detection. This would, ideally, eliminate the need for human counting, reducing human error.
4. Further refine the new MagRC chip design by exploring materials and methods to reduce fabrication and time costs.
5. Explore new chip designs to further improve capture and profiling of rare cells. One such potential idea would be a system where capture and release (as in the case currently) is not necessary, but rather implement guiding of the cells to a certain outlet. A similar system was beginning to be implemented and worked on by a graduate student after the scope of this report, but it showed tremendous promise based on preliminary results and presentations. A large advantage that it would present would be a reduction in the loss of cells since they would not longer stop, and risk getting stuck in the chip.
6. Testing Slippery Liquid Infused Porous (SLIP) surfaces for reducing adhesion of cells inside the chip. Very preliminary tests were conducted, with a major obstacle being that the oil reagent used tends to swell the PDMS structures in the chip, degrading the integrity and usability.

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Appendix A

Step	Solution	Volume	Flow	Time
1	1ml blood + 10ul Nano-Beads	1 ml	600 µl/h	100 min
2	PBS-EDTA	200 µl	600 µl/h	20 min
3	PBS-EDTA	200 µl	600 µl/h	20 min
4	4% PFA in PBS 11 µl (36.5% PFA) + 89 µl PBS	100 µl	600 µl/h	10 min
5	Triton X-100, 0.2% in PBS	100 µl	600 µl/h	10 min
6	Anti-CK Biotin 2ul + 93ul PBS 1% BSA, Tween 20 0.1% + 5ul Anti-CD45-APC 3ul (MHCD4505)	100 µl	100 µl/h	60 min
7	PBS- Tween 20, 0.1 % BSA	100 µl	600 µl/h	10 min
8	PBS- Tween 20, 0.1 % BSA	100 µl	600 µl/h	10 min
9	Yellow nanoB Avidin (1ul) + 49ul buffer, then from that, 2ul + 98ul buffer. Buffer is: 1% BSA, Tween 20 0.1% in PBS	100 µl	200 µl/h	30 min
10	PBS- Tween 20, 0.1 % BSA	100 µl	600 µl/h	10 min
11	PBS- Tween 20, 0.1 % BSA	100 µl	600 µl/h	10 min
12	DAPI solution 1 drop + 1ml PBS 1 drop should = 30ul (can measure this)	100 µl	600 µl/h	10 min
13	PBS	100 µl	600 µl/h	10 min