A SIMPLE MICROFLUIDIC DEVICE FOR
AUTOMATED, HIGH-THROUGHPUT MEASUREMENT
OF MORPHOLOGY OF STORED RED BLOOD CELLS

AN ABSTRACT
SUBMITTED ON THE 15TH DAY OF APRIL, 2013
TO THE DEPARTMENT OF BIOMEDICAL ENGINEERING
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FOR THE DEGREE OF
MASTER OF SCIENCE
BY

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ABSTRACT

Stored red blood cell (sRBC) morphology is currently scored manually by technicians in a slow labor intensive process prone to error. This project proposes a way to simplify, automate, and expedite the morphology scoring process by using a novel microfluidic device that I designed to facilitate the flow of a single layer of red blood cells (RBCs). The appearance of this flow allows for the capture of a series of high clarity images captured via digital camera coupled to a microscope that are ideally suited for image analysis algorithm-based morphological scoring. During storage, RBCs heterogeneously shift from the form of discocyte to the reversibly altered form of discoechinocyte as storage lesion progresses. Beyond this level of degradation, the cell assumes the form of a spheroechinocyte or spherocyte and becomes irreparably damaged. The microfluidic device and image analysis algorithm developed in this research classified the individual morphology of 5000 RBCs taken from storage into the physiologically relevant category of either “discocyte,” “reversibly changed,” or “irreversibly changed.” This process took only 15 minutes. The accuracy in classification was verified as 92.6% in a separate trial when compared against classification of the same sample images via manual inspection. The morphological distribution of the RBC population remained consistent in both cases. The findings of this project suggest that microfluidic device assisted automated image analysis can provide a quick and effective way to quantitatively estimate the viability of a sRBC population and the extent of storage lesion endured. This technology could provide augmented RBC storage and transfusion research capabilities and have clinical applications, such as the ability to conveniently differentiate between the transfusion qualities of two sRBC units of the same age.
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Dedicated to Nano
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Erythrocyte (red blood cell) transfusions are one of the most critical and common medical procedures performed in hospitals today. They are called for when a patient requires improved oxygen transport through the circulatory system. The availability of units of packed RBCs ready for human transfusion is an incredible luxury and a scientific achievement. However, even with modern storage methods, red blood cells in storage do deteriorate in viability to the point where they can no longer provide the patient with a safe and efficient replenishment of \( \text{O}_2/\text{CO}_2 \) transport capabilities for life supporting cellular metabolism throughout the body (Fergusson, et al., 2004). There is much available evidence that blood stored for longer periods of time is more likely to lead to adverse clinical outcomes for the patients (Berezina, et al., 2002); (Alfano and Michael, 2011); (Bessis, 1972); (Henkelman, et al., 2010); (Daryl, et al., 2009).

Since most RBCs are collected and stored using the same standards, the de-facto way of knowing if an individual unit of packed red blood cells is fresh enough to transfuse into a patient who needs an immediate boost in oxygen carrying capabilities is a check of the expiration date on the bag assigned when the blood was collected from the donor. As such, a given unit of packed RBCs is conventionally given a binary rating of transfusion quality; expired or unexpired (D'Alessandro and Liumbruno, 2010). This is a gross oversimplification of the biological complexities that establish the true RBC unit “quality,” or expected positive post transfusion efficacy in terms of improved oxygen delivery, cell survival, and lack of post-operative complications of a unit of red blood cells after a period of storage (Chinyee, et al., 1997). The exact quality of red blood cells has been proven to be at an apex of importance when the patient either requires a massive blood transfusion or has significant pre-existing circulatory
complications (Lelubre and Vincent, 2011) so there is a purpose for measuring the quality of stored RBCs directly.

It is known that individual RBCs heterogeneously change their shape as their biological properties change in deleterous hypothermic storage conditions (Bessis, 1972); (Hovav, et al., 1999). This change in shape may likely provide a metric with which to associate the predictable viability of each cell. As an aggregate score, this could indicate the viability of an entire sRBC unit (Berezina, et al., 2002); (Meiselman, 1981); (Longster and Buckley, 1972). In this project, the concept of associating morphology score with overall sRBC unit quality is utilized in the design of a device that can be easily used to provide images ideally suited in terms of individual cell appearance for digital analysis to reveal a proportional morphological distribution of a sample population representing a quantifiable measure of the “quality” of a unit of sRBCs.

A microfluidic device was specifically designed and created using the relatively recent technique of soft lithography developed at the turn of the millennium (Whitesides, et al., 2001) to create a single flat non-overlapping layer of flowing RBC’s from a small inserted population. This flat flowing layer of cells visible in the device was then observed at a high objective with a camera-equipped microscope and images were taken of the cells in regular intervals so as to reveal an unbiased and sufficiently large sample population. Because of the orientation of cells as they appeared in the device, the morphology of each cell was simple enough to detect and categorize using an automatic segmentation and classification algorithm that was able to quickly report the number of cells observed in an image series that appeared as discocyte, echinocyte (reversibly changed), and spherocyte (irreversibly changed) (Berezina, et al., 2002); (Bessis, 1972).

The results of this project indicate that morphology score detection using images obtained from a device that I designed in conjunction with an image analysis algorithm are
comparable and possibly superior to current methods, verified by comparison with visual
detection and scoring. These results are also easily repeatable since the devices can be made
relatively cheaply and quickly and require a small sample size and short time period of image
capture for proper implementation. The efficiency offered by this system make it a potentially
useful method to enhance the depth of research in the field of RBC storage and transfusion
when used in conjunction with other forms of mechanical/biochemical analysis or manipulation
of sRBCs, and it may feasibly lead to a clinical application for testing sRBC units prior to
transfusion. In this thesis, these ideas are explored as our method is tested and compared to the
current state of automated morphometric analysis technology in the scope of RBC storage and
transfusion.
CH 2: BACKGROUND

The importance of investigating the implementation of a microfluidic device and algorithm to determine sRBC morphology is based on what is currently known about RBC storage and transfusion. In order to interpret a novel way to assess the quality of sRBCs based on imaging, there is a basis of knowledge that must be understood behind the method in terms of the physiology of the RBC itself, how RBC storage and transfusion works, how RBCs react to their storage environment chemically and physically, how these changes can be detected/observed, and what they mean in terms of clinical outcomes. All of these points are critical in supporting the conceptual viability of this research as well as exhibiting the usefulness and potential implications of the method described. The scientific basis described herein will also place this research more precisely within the scope of other subjects in the RBC storage and transfusion field.

2.1 Properties of Human RBC

Human RBCs (erythrocytes) are cells that originate from pluripotent haemopoietic stem cells within bone marrow and are introduced to the blood stream as enucleated biconcave discs containing hemoglobin at a rate of 2.4 million cells/second in adults and they remain alive in the blood stream for an average of 120 days, at which point they are broken down by macrophages (Lipowsky, et al., 1995). At any given time, there are 20-30 trillion RBCs in ~4.7L of circulating blood in an adult human male, comprising approximately ¼ of the total cells in the body (Elert, 2012).

The main function of RBCs in the body is to transport O2 from pulmonary capillaries to tissue capillaries where it is exchanged for CO2 and transported back to the pulmonary
capillaries for gas exchange. This gas transport is facilitated by the some 270 million hemoglobin biomolecules which account for one third of the volume of RBC cytoplasm (Bridges, 2001), allowing a 50-fold oxygen transport capacity compared to that of water (Klinken, 2002) and giving the cells their characteristic red color. This gas exchange in tissues is what allows for metabolic respiration in the cells of these tissues.

The biconcave disk shape of RBCs along with the naturally viscoelastic properties of their phospholipid bilayer membranes allow them to deform by way of bending, folding, and squeezing. This deformability facilitates both passage through narrow capillaries such as those in the spleen (Deplaine, et al., 2011); (Gifford, et al., 2006) and proper contribution to bulk flow viscous properties in larger vessels allowing optimal physiological performance (Higgins, et al., 2009). The average diameter of a healthy human RBC varies between 6.2-8.2µm and the thickness ranges from 0.8µm to 2.5µm (Turgeon, 2005). These cells typically exhibit a 90fL volume and a surface area of about 136µm² (Mclaren, 1987). These geometric dimensions maximize the surface area/volume ratio of the discocyte giving it 50% more membrane than it needs to contain its volume (Hess, 2011) and in doing so, facilitate the cell’s ability to deform and maximize gas exchange by providing it with a loose fitting membrane and a relatively large membrane surface area, respectively.

The survival of the human body relies heavily on the successful functionality of RBCs since they allow cellular metabolism to persist. The functionality of RBCs relies on a number of sensitive variables that can be compromised in many ways. The viscous and chemical properties of blood plasma can change, a large amount of RBCs can be removed from circulation too suddenly, or the physical/chemical properties of the individual RBCs can be irreparably altered rendering them incapable of proper gas transport or microcapillary perfusion (D’Alessandro and Liumbruno, 2010). People commonly suffer from pathological conditions that cause these
effects and the RBC transfusion is the most heavily relied on reparative technique (McLellan and Walsh, 2002).

2.2 RBC Transfusion

It is important to address the RBC transfusion itself to put in perspective the importance of sRBC analysis and why this research was done. The concept of blood transfusion has existed since the discovery of the circulatory system, but the performance of the procedure is thought to originate from Europe, where first known blood transfusion attempt occurred in 1656 when a man transfused blood from one dog to another (Gould, et al., 2007). Only a decade later, an attempt was made to transfer blood from an animal to a man, but due to an edict protesting further experiments with the procedure; it wasn’t until 1818 when an obstetrician named James Blundell conducted the first successful man-to-man blood transfusion using a syringe and defibrinated blood (Edmond, 1940). Transfusions would not become a common clinical procedure until after 1900 when a man named Landsteiner discovered the ABO histocompatibility system which described the role of agglutinins, blood type variation, and other factors of incompatibility that made it possible to select a proper donor for a particular recipient (Spiess, 2007).

Today blood is understood to a much further extent and transfusion has become a mainstay of clinical practice, with millions of units of blood being collected annually worldwide to facilitate the demand for the procedure (Klein, et al., 2007). Now when blood is donated, it is screened for infections and then often fractionated into its components including RBCs, white blood cells, platelets, clotting factors, plasma, and antibodies so that patients can receive a transfusion specifically corresponding to their needs, but this was not always the case. Separating blood by its components began in the 1940’s because blood plasma could be collected and shipped overseas for war-related transfusions but due to the need for constant
refrigeration, whole blood could not. In the 1960’s, RBC transfusion took over the whole blood transfusion as the most utilized type. Thanks to the development of mobile refrigeration, the Vietnam War was the first time Americans were able to donate RBCs that could be transported to field hospitals for soldiers to receive. Albeit lifesaving in many situations, the likely less than ideal condition of the packed RBCs by the time they were transfused in Vietnam has been linked to spikes in lung and respiratory illnesses at the time. This was also when it was realized that an infection screening process should be mandatory for all donated blood (Spiess, 2007).

The RBC transfusion is now vastly the most common type with roughly 80 million units transfused annually worldwide (Hess, et al., 2009). 14 million sRBC units were transfused in the United States alone in 2001 (Gould, et al., 2007). They are used for patients who are experiencing traumatic hemorrhaging or a condition preventing circulatory oxygen transport such as anemia, which is associated with some 95% of critically ill ICU patients by the second day of hospitalization (Kaplan, 2007) (Gettingier, et al., 2004). RBC transfusions are also used by hemophilic, thrombocytopenic, and some cancer patients (Alfano and Michael, 2011).

2.2.1 Occurrence of Morbidity and Mortality Linked to RBC Transfusion

After receiving a blood transfusion, a patient will experience some physiological reaction as the previously stored blood as it is assimilated as part of their body. Assuming the proper storage and transfusion procedures are followed correctly, these reactions are usually minor and temporary, but severe and possibly fatal physiological effects can occur from transfusion in certain cases (Gould, et al., 2007). There have been studies seeking the relation between RBC storage duration (within 42 days) and clinical outcomes of transfusions both that indicate a strong relation between the two (Koch, et al., 2008) and that indicate that a relation is too difficult to determine (D’Alessandro and Liumbruno, 2010); (Lelubre and Vincent, 2011);
(McLellan and Walsh, 2002). Some of this discrepancy may be due to the use or lack of leukoreduction in separate studies (McLellan and Walsh, 2002) or donor variation between individual stored units (Alfano and Michael, 2011). The current common consensus is that when using modern techniques including liquid hypothermic storage, component separation and pathogen screening, a typical RBC transfusion with an unexpired (<42 days old) unit does not pose enough of a safety risk to be of concern in most individual cases (Zimrin and Hess, 2009). Although there is room for improvement, most healthy, screened, and properly sRBC units that fall within the 42 day expiration date are safe to transfuse (Klein, et al., 2007). However, there are a number of postoperative clinical complications following transusions that have been attributed to the condition of the RBCs following storage (Fergusson, et al., 2004); (Frenzel, et al., 2009); (Vamvakas and Carven, 1999). In these cases, the associated adverse clinical outcomes are fairly well recognized, while the mechanisms that cause them after transfusion that stem from the effects storage deterioration are very complicated and less understood.

In a RBC transfusion, the primary goal for any patient is to improve oxygen delivery by providing them with a new population of RBCs with hemoglobin capable of facilitating this. Any so called adverse clinical effect can be defined as any unintended result of the transfusion that inhibits the effectiveness of the therapy in improving oxygen transport or causes harm to the patient’s body. Some examples of adverse clinical outcomes resulting from RBC transfusion include bleeding complications, renal insufficiency, longer postoperative hospitalization period (Koch, et al., 2008), recurrence of postoperative infection (Nielsen and Mynster, 2000), time between recurrent transfusions, multiple organ failure, immunoparalysis, and transfusion related acute lung injury (TRALI) (Zimrin and Hess, 2009) or circulatory overload (TACO) (Anon., 2013), among others. All of these outcomes are either directly or indirectly related to patient morbidity or mortality rates (Hod and Sptalnik, 2011); (Gettinger, et al., 2004).
2.3 RBC Storage

The true science of blood storage began in 1904 when Peyton Rous discovered that citrate could be used as an anticoagulant for blood, and glucose could help keep it metabolically stable in storage. In 1915, glucose-citrate solutions were used to store rabbit RBCs for their use in a heterophile agglutination syphilis test (D'Alessandro and Liumbruno, 2010). The same sort of solution was used to preserve blood for up to 26 days that was transfused to some soldiers during WW1, but this technique was criticized due to bacterial growth in the blood catalyzed by the presence of glucose. Citrate itself could preserve blood for about 5 days, which was rarely enough time for the blood to go from the donor to the recipient. This was especially the case for soldiers on the battlefront (Zimrin and Hess, 2009). As blood transfusion became a more popular practice, scientists began to realize the potential benefit and relative challenge of storing and maintaining blood to facilitate them. The techniques have become more advanced since the early 20th century, but the principle of using a citrate-glucose storage solution and refrigeration remains.

2.3.1 Modern RBC Storage Standards

Since it was first put into practice, the common method for blood storage has been improved largely based on trial and error. In the case of RBCs, it is now the most common practice at blood banks to remove plasma from whole blood via light centrifugation and leukocyte reduce or leukoreduced the sample through a centrifugation and filtration procedure. Leukoreduction/depletion is the practice of removing leukocytes from a RBC unit prior to storage. It is thought to minimize the risk of virus transmission, alloimmunization, and immunosuppression, though its usefulness has been disputed (McLellan and Walsh, 2002).
Leukoreduction is not a standard part of sRBC unit collection in all nations, but its effect in improving the durability of RBCs during storage has been demonstrated (Antonelou, et al., 2012). In the research explained in this thesis, leukoreduced blood was used. The separated RBCs are then added to a citrate-based anticoagulant solution that, with the addition of phosphate, supports osmotic balance. This solution is combined with a hypertonic additive solution to support prolonged cellular metabolism and reduced hemolysis, commonly SAGM (sodium, adenine, glucose, mannitol 370 Osm/L) and stored at 4 ± 2°C (Liumbruno, et al., 2009) in polyvinyl chloride (PVC) blood bags, which have proven to minimize hemolysis compared to alternate bag materials and glass (Hess, et al., 2009). By convention, this technique and its slight variants are considered to keep blood in a transfusion safe state for 42 days. This is a widely accepted shelf life, since the average age of a stored unit when it is transfused in the US is 21 days (Gettinger, et al., 2004). It is important to understand how this expiration date was determined for this method of storage.

2.3.2 Expiration Date System

In the United States, the FDA currently mandates that blood is considered safe to transfuse if the selected storage method (eg. new additive solution patent applicant) has been proven via chromium-51 labeling to allow that at least 75% of transfused cells remain in the recipient circulation 24 hours after the transfusion and total hemolysis remains below 1% (Raat and Ince, 2007) (Fergusson, et al., 2004) (Daryl, et al., 2009). For this test, the recipient is the same person as the donor (an allogeneic transfusion) so as to eliminate donor/recipient incompatibility effects from the results. This method of establishing a standard safe storage period for different methods was developed through trial and error and has been criticized for its susceptibility to variability based on the test patient used, among other things (Reid, et al., 1999).
2.4 Storage Lesion and Associated Effects In Vivo

The time dependent degradation of the quality of sRBCs in terms of expected transfusion efficacy is due to a series of gradual physical and biochemical changes to the cells that are collectively referred to as storage lesion (Daryl, et al., 2009); (Chinyee, et al., 1997). Most of the changes that occur within RBCs during storage lesion are interconnected and stem from related causes. These changes can be categorized as biochemical, physical, or immunologic (Daryl, et al., 2009). Since we are dealing with leukoreduced RBCs, we will only consider the biochemical and physical changes. Storage lesion is a very complex phenomenon that is not fully understood, but extensive research on the subject has revealed the most significant aspects relating to transfusion success or failure (Daryl, et al., 2009).

2.4.1 Reversible Biochemical Damage

While in storage, RBCs will experience diminished metabolic activity leading to lowered levels of 2,3 DPG (diphosphoglycerate), ATP, a lowered pH, increased extracellular potassium concentration, and eventually oxidative damage (Hess, 2010). The 2-4C refrigeration temperature causes the sodium/potassium ion pumps on the cell membrane to become inactive, leading to high extracellular potassium concentration. The additive solution allows glycolysis to continue within the cells, resulting in a buildup of lactic acid and protons; the products of the glycolytic pathway. With no way to dispose of these products, the acidity of the cells and their environment drops and glycolysis slows down as the excess of protons begin to inhibit enzymes within the glycolytic pathway. This acidosis first rapidly diminishes levels of 2,3 DPG, an allosteric hemoglobin modifier necessary for O2 delivery, to almost zero after 2 weeks of storage (Chinyee, et al., 1997). The breakdown of 2,3 DPG allows a short boost of ATP production early on in storage followed by slowed production once 2,3 DPG is diminished and
eventually depletion of 20-25% of the initial ATP concentration (Alfano and Michael, 2011) after 5 weeks of storage as metabolism continues to slow down (Zimrin and Hess, 2009). Low 2,3 DPG, ATP, and pH levels do not independently cause cell death and with the aid of pre-transfusion rejuvenation solution, can all be recovered by cells once they are reintroduced to circulation in as little as 48-72 hours (Heaton, et al., 1989). These effects on their own are not of much concern to RBC quality (Hogman, 1985), but they create conditions conducive to harmful oxidative damage while the blood remains in storage.

2.4.2 Oxidative Stress

The biochemical effect of oxidative damage that occurs among RBCs after prolonged storage can cause irreversible harm to the cell and lead to storage induced apoptosis. The hemoglobin within stored cells is usually 75% saturated with oxygen when drawn from venous blood (Hess, 2010), and in storage conditions, these oxygen molecules constantly bind and detach between different iron atoms of hemoglobin molecules resulting in oxidative stress. On occasion, one such oxygen molecule will pull off an electron with it forming ferric radical methemoglobin or superoxide. Methemoglobin is usually reduced by cytochrome b5 reductase to superoxide, but the availability of this enzyme decreases as glycolysis slows down over the duration of storage. The significant presence of unstable methemoglobin leads to free iron atoms, allowing superoxide molecules to go through a Fenton reaction whereby they react with iron atoms and water to form hydroxyl radicals. These free radicals gradually damage proteins and lipids within the cell including the critical ion exchanger band 3 proteins which can cause the formation of immunoclearance antigens (Chinyee, et al., 1997). Band 3 oxidative damage diminishes the lifespan of cells in circulation and the destruction of certain intercellular lipids causes the release of lysophospholipids, which contribute to additional hemolysis through
membrane loss (Zimrin and Hess, 2009). The rapid release of lysophospholipids and similar cellular waste has been associated with suppressed patient immunity (S. Gould).

### 2.4.3 Physical Characteristics

The physical changes experienced by RBCs as a result of storage lesion include a gradual reduction in cellular membrane surface area, a change in cellular shape and size, and altered rheological properties. These changes occur as a direct result of both reversible and irreversible biochemical effects of storage lesion, and their manifestation serves as a direct indicator of how the physical properties of the cell have changed and how well the cell will function in vivo (Park, et al., 2010); (Nagaprasad and Singh, 1998); (Meiselman, 1981). RBCs begin as healthy discocyte shaped cells, but alter in shape through a linear series of morphological stages named by Bessis et al. that can be summarized as echinocyte (deformed discocyte with abnormalities in texture and protrusions known as spicules, slightly lesser membrane surface area), spheroechinocyte (echinocyte with many protruding spicules assuming more of a sphere shape than disc as membrane surface area decreases), and spherocyte (a small sphere with no spicules) (Bessis, 1972). This morphological
shift can be largely attributed to the effects of oxidative damage and lowered ATP levels. It is important to note that among a population of sRBCs, the morphological shift occurs heterogeneously. At any given point, there will be a mixture of well-preserved discocytes, reversibly damaged echinocytes, and irreversibly damaged spherocytes. Blood that has been recently placed in storage will contain some spherocytes due to the variance of the age of the cells when they were collected from the donor; some cells are naturally senescent when they are collected (Cohen, et al., 2008).

This disc-to-sphere shape transition is due to the continuously decreasing ratio of cellular membrane surface area to volume. It is estimated that a RBC will lose 25% of its total phospholipid membrane surface area after 42 days of storage (McLellan and Walsh, 2002). The mechanism by which this occurs is the same that creates the appearance of spicules during the medial stages of the morphological shift; the micro vesiculation of cellular components damaged from oxidative stress being expelled from within the cell (Hess, 2010). The size of these vesicles and the concentration of their damaged contents continuously increase as the concentration and variability of proteins and lipids within the cell decrease (D’Alessandro and Liumbruno, 2010). This rapid vesiculation is also promoted by the absence of high cytosolic ATP concentration. High levels of ATP are necessary for controlling the activity of aminophospholipid translocase, a flippase that helps to keep phosphatidylserine (PS) on the cytosolic side of the protein membrane. When ATP levels drop as storage persists, PS becomes exposed on the outside of the membrane. This process, which is accelerated by the effects of oxidative stress, is a natural part of apoptosis (Yoshida and Shevkopylas, 2010). This changing of shape can be reversed with the aid of rejuvenation treatment, which entails warming the cells and treating them with a pH neutral solution. However, rejuvenation is impossible for cells that have progressed in lesion beyond the intermediate echinocyte stage (Berezina, et al., 2002) and the
amount of membrane lost will permanently prevent the cell from returning to its discoid shape. At this point of irreversible change, the cell morphology will continue shift to adopt the shape of a sphere (Nagaprasad and Singh, 1998).

A large amount of spherocytes introduced to the blood stream can obstruct small capillaries such as the 3µm diameter splenic capillaries and cause ischemia (Chinyee, et al., 1997); (Reinhart and Chien, 1986). Vascular occlusions can also occur in larger capillaries where local thrombosis creates a smaller route of passage for RBCs (Chien, 1987). The cytoplasm viscosity of irreversibly damaged cells increases (Hess, 2010) and the mechanical deformability of the cell is diminished as a result of the increased membrane surface tension (Relevy, et al., 2007) in addition to oxidative damage sustained by the cytoskeletal structure (Yoshida and Shevkoplyas, 2010). When transfused, these rigid dysfunctional cells will die or be culled from circulation by phagocytic cells without delivering oxygen (Hess, 2010) (Cooper and Jandl, 1969). The rapid destruction of a large population of spherocytes in a patient’s blood stream can contribute to iron overload in chronically transfused patients (Bosman and Werre, 2008) or cause inflammation and infection from massive extravascular lysis (Hod and Sptalnik, 2011). RBCs also increase in aggregability, or tendency to adhere to each other due to a reduced cell surface charge caused by a reduction of sialic acid content that occurs during storage (Hovav, et al., 1999).

2.5 Microfabrication Using Soft Lithography

To design and fabricate a device that can meet the functional requirements defined in section 3.2, the microfabrication technique of soft lithography was selected because of its success in similar in vitro studies involving high speed cellular imaging (Shevkoplyas, et al.,
2003). Soft lithography is a newer form of photolithography invented by researchers at Harvard University in 1998 (Whitesides, et al., 2001). The essence of this technique is to use a master mold to emboss a pattern on an elastomer substrate. This method was selected for a number of reasons that make it very convenient for this research. Using SL, one can create a master mold with geometric features specified using a CAD program with one micrometer level resolution that can be used to cast multiple replicas of PDMS (polydimethylsiloxane) structures that adopt the geometric patterns present on the master mold to their surfaces. All this can be achieved at an efficient pace and a reasonable cost (Duffy, et al., 1998), making SL an ideal technique for the production of experimental microfluidic devices. It is the use of an elastomer such as PDMS that differentiates this process from conventional photolithography.

The chemical and physical properties of PDMS are well suited for the intended functionality of this device. PDMS is permeable to gas, which means that fluids such as blood can fill confined microchannels as the air they replace is permeated out of the channel through the material. PDMS is chemically stable and has a low interfacial free energy (~21.6 dyn/cm) meaning that its surface is less likely to cause adverse aggregation or reactions while in contact with other polymers or molecules and will allow non-destructive separation of the material from the mold (Xia, et al., 1996). PDMS is an optically transparent material making it convenient for microscope imaging. It is also durable, non-

Figure 3: Illustrated process of negative photoresist. The photoresist coated on the silicon wafer is selectively exposed to UV light beneath the photomask. Exposed portions of photoresist crosslink and the developer solution washes away the non-exposed photoresist.
hygroscopic (will not swell with humidity), and thermally stable below 200°C (Whitesides, et al., 2001). The only drawbacks of PDMS in the scope of this research are that it is hydrophobic and deformable enough to limit the dimensional aspect ratio of channels due to the tendency of collapse, a form of a phenomenon known as pairing (Toepke and Beebe, 2006). These issues are both addressed in the design and preparation of the devices.

2.5.1 General Process of SL for Negative Photoresist

Preparing for SL is a multiple step process, starting with design and ending with a master mold that can be used to make replica PDMS microdevices (refer to appendix for details). The microfluidic design is first drawn to exact scale using a CAD program. This file is then sent to a facility that uses a high resolution printing system to create a film photomask with the pattern etched in. The substrate, which is a silicon wafer, is then covered with a light-sensitive chemical called photoresist which reacts to UV radiation. This wafer is placed under the photomask on a mask aligner which exposes the photomask and wafer below it to UV light. A treatment with a developing solution after exposure isolates either the exposed or unexposed photoresist depending on whether the photoresist is classified as “negative” or “positive”, respectively. In a negative photoresist, the type used in this study, exposure to UV light initiates molecular cross linking that allows exposed photoresist to “resist” the developer solution while the rest of the photoresist is washed away. This process allows for the transparent patterns etched in the photomask to appear as a precise negative or relief of the pattern desired to be embossed to the elastomer (Munn and Jain, 2009). The final product is the master mold.
CH 3: METHODS AND MATERIALS

3.1 Overview

In order to achieve the goals of this project by creating a system whereby the morphologically specific proportions of a population of sRBCs can be determined, a visual approach was selected. The central concept for this approach was to use an inverted microscope to record a series of images that depict a population of sRBCs with high enough resolution for the morphology of each cell to be detected and compiled for an accurate profile of the proportion of each morphological classification of sRBCs that comprise the population. This entailed microfabrication of a suitable device, an experimental procedure with sRBC samples for imaging using this device, and a way to interpret this data with an algorithm. The mutually compatible designs of the microfluidic device and algorithm are why this can be considered a “hybrid” approach.

3.2 Microfabrication Using Soft Lithography

The design of the microfluidic device used to provide suitable images for morphological analysis was essential in ensuring that the images collected from the microscope could be rapidly and accurately analyzed using a MATLAB algorithm created by lab members Xiaoxi Yang, Nathaniel Piety, and Sergey Shevkoplyas. As such, the microfluidic device was designed and fabricated to not only preserve the morphological identity of the sRBCs that it would display, but also to provide images that conform to the requirements of the algorithm.

The primary functional requirements of the device are such:

1. Injectable with sample of sRBC via handheld pipette
2. Can be mounted on an inverted microscope platform
3. Can function while stationary with no external pressure to drive flow
4. Does not alter the morphologic condition of sRBCs

The image yield requirements of the device are such:

1. Cells are individually discernible
2. Adequate through flow so no cell appears in more than one image
3. Minimal cell overlap, adhesion
4. Sufficient cell/background contrast
5. Minimal flipping of cells

3.2.1 Device Design
The device was designed using CleWin, an inexpensive 2-dimensional geometric layout editor created by WieWeb Software (Netherlands) for mask design. This interface allowed for micrometer sized feature drawing on separate layers and the output file type “.cif” can be sent to Photo Sciences Inc. (Torrance, CA) for the production of a high quality laser imaged photomask with greater feature definition or, when some precision in feature definition can be spared, the “.cif” file can be sent to Fineline Imaging (Colorado Springs, CO) for a less expensive mylar photomask.

Early Experimental Designs: Issues
I changed the design of the device over the course of multiple iterations before arriving at the pattern that met the design criteria stated in section 3.2 and was able to thus provide images that allowed the MATLAB algorithm to most effectively obtain a morphology score. Initially, it was presumed that the view field of the microscope would be adjusted to capture populations of cells rather than allowing the field to stay stationary while the cells passed through it in intervals. For this approach, two designs were tested using the less expensive mylar
transparency photomask [Fineline Imaging, Colorado Springs, CO]. The first design included arrays of “U” shaped brackets within a wide channel for the “capturing” of cells and as structural support. The second design consisted of arrays of 8×8µm sink pits with 10µm depths meant for “trapping” the cells with gravity as they passed over the pits. These designs provided unsatisfactory results for a number of reasons and the latter was much more difficult to fabricate since it involved multilayer fabrication. This made it apparent that the approach should not be to trap cells but should rather be to allow them to pass through a single stationary view field with minimal interference and with the simplest geometric features possible to accommodate the analysis algorithm.

*Final Iteration:*

The continuous flow-based imaging approach was selected and the functional criteria of the intended role of the device governed the specific design. The spatial dimensions were chosen based on the microscopic field view size we would be using. For the algorithm to be able to detect the morphology of each cell, the optical zoom of 40 × 1.6 is required. The rectangular dimensions of this field of view were measured to be 280 × 225µm. We also took into account the expected effect of fluid shear stress at the lateral walls of the channel and ignored the viscous drag caused by the top and bottom walls of the channel since this effect would be uniform across the width of the channel and would therefore have a negligible effect on sample bias. The design needed to accommodate for a symmetrical velocity

![Figure 5: Overhead general view of final microfluidic design showing inlet/outlet ports with scale bars in microns.](image)
profile so that cells appearing in images were moving through at a constant rate to minimize the potential sampling bias of having a lateral variation in flow velocity (see figure (7)). The natural deformability of PDMS was taken into account as the material has a very low approximate Young’s modulus of 1.2 MPa (Eng, 2005), although the Young’s modulus of PDMS can vary depending on the mass ratio of base and curing agent used to create it. This deformability causes a channel of any considerable width with the intended shallow depth of 5 µm to be susceptible to collapse (pairing). Such a collapse would compromise the entire device. This was addressed by incorporating an array of simple columns through the channel that would act as supports and prevent collapse with minimal interference to the cells, including at the inlet and outlet of the device as seen in figure (5). The functionality of the algorithm depended on simple geometry detection within captured images to calibrate the intended area of cell recognition in each image. The shape of a square was chosen for its straight edges. These squares could also be used as the needed supports in the channel.
Taking all of these considerations into account, the optimal design was chosen as an array of 20x20µm squares as seen in figure (6). The shape and spacing of these squares allows for an adequate flow rate for rapid imaging purposes and helped to create a dispersed and uniform distribution of cells as they moved through. The orientation of the squares is such that 5 columns of enclosed perimeters are formed, and each perimeter and the 8 squares that surround it fit within the field of view of the microscope with a 10 µm tolerance in all directions as visible in figure (8). The appearance of these squares in images allows for spatial calibration with the algorithm and helps the operator ensure that the optical settings on the microscope are the same for each device used. Since there are 5 columns of these matrices, the central column can be assumed to be the least susceptible to selection bias caused by the fluid shear stress on the lateral sides of the channel, assuming incompressible flow and no slip boundary conditions. The total length of the channel was 7.5mm and the width from edge to edge was 1.5mm. In addition, the support column dimensions and alignment of this design provide enough support to prevent the collapse of the thin channel (Huang, 2005). The height of the intended 50µL sample volume injected at the circular inlet \( h = \frac{0.05cm^3}{\pi \cdot 2cm^2} \approx 4mm \) would
generate a sufficient pressure gradient against the atmospheric pressure at the outlet of the
device capable of driving adequate flow (measured as \( \sim 0.54 \text{nL/s} \)) at a relatively steady rate. At
the given flow rate, 0.162\( \mu \text{L} \) (0.03%) of sample would be displaced during a typical 5 minute
experiment so it is assumed 0.03\% of the inserted sample height would be lost. This is a
negligibly small change in sample column height over the course of an experiment, so we
assume a negligible change in flow rate through the device for our purposes.

![Diagram](image)

Figure 10: A schematic of how an assembled microfluidic device appears: A PDMS block, glass slide, and column of
sample creating a pressure gradient to drive flow at the inlet.

### 3.2.2 Microfabrication Process

The microfabrication process results in a PDMS block with a negative relief of the microfluidic
design adhered to a PDMS coated glass slide with an accessible inlet and outlet on either end of
the channel.

**Photomask Acquisition and Wafer Spin Coat**

The “.cif” file of the final design was sent to Fineline Imaging (Colorado Springs, CO) for
photomask production and a negative photomask made of mylar film was received with the
design etched in. The mylar film photomask was selected instead of the more expensive
alternative sodalime glass with low reflective chrome photomask (1+/-.25µm feature resolution) because this design did not require feature resolution <5µm (Munn and Jain, 2009). 3” silicon test wafers (Wafer, South Boston, MA) were spin-coated (PWM32-PS-R790, Headway Research, Inc., Garland, TX) for uniform coverage of a layer of SU-8 2010 negative photoresist (MicroChem Corp, Newton, MA) using the manufacturer-provided protocol of ramp/speed/duration for intended height of mold features. This was a critical step in the fabrication process, as explained further in section 4.1. This protocol accounts for the viscosity of this particular SU-8 photoresist and corresponds sharply with the resulting photoresist layer thickness (within an approximate 0.5µm tolerance) to enable fabrication of channel depth with the precision needed to minimize cell overlap and discocyte flipping without impeding the flow of the cells through the channel.

Soft Bake

Manufacturer-provided soft bake protocol for 5µm thickness was followed to ensure mechanical integrity of the photoresist by evaporating SU-8 solvents still present on the wafer. The wafer was baked for two minutes at 95°C flat on a hot plate (Torrey Pines Scientific, Inc., Carlsbad, CA). The soft bake was an important step in the process, since resist tends to peel off during development on under baked wafers and it tends to crack or deform on over baked wafers (Munn and Jain, 2009). The wafers were then allowed to cool for approximately one minute.

Exposure

The wafers were then exposed to a UV light on an ABM mask aligner (ETI/6/350/NUV/DCCD/M, Evergreen Technology Inc, San Jose, CA) through the mylar transparency photomask placed between the wafer and the light source (FineLine Imaging, Colorado Springs, CO). The exposure time of eight seconds was sufficient to propagate cross-linking throughout the entire depth of
the photoresist without causing exposure to unintended areas of the photoresist (and
subsequent loss of resolution) due to diffraction, refraction, and reflection effects associated
with excessive exposure (Zhang and Hong, 2001).

Post Bake
Following exposure, a post bake was conducted according to manufacturer protocol, and the
wafers were placed on the hotplate for three minutes at 95°C. This part of the procedure
accelerated the cross linking reaction initiated by the UV exposure and required the same
precision as the soft bake. Following the post bake, the wafers were allowed to cool for one
minute.

Development
Wafers were then treated with SU-8 developer (PGMEA, Propylene Glycol Monomethyl Ether
Acetate) for one minute following manufacturer-provided protocol. After development, the
wafers were rinsed with isopropyl alcohol and dried with pressurized air. Isopropyl alcohol
effectively removes the developer and can reveal portions of unexposed photoresist that were
not removed by the developer (Bogdanov and Peredkov, 2000). The surface features on the
wafers were visually clear following the alcohol rinse. Any wafers that had obvious flaws in their
negatives were discarded while wafers that appeared to have successful negatives were
inspected on a microscope (BX51, Olympus, Center Valley, PA) for microscopic flaws.

Silanization
The final treatment for the wafer was an overnight surface exposure to CF3(CF2) 6(CH2)2SiCl3
(tridecafluoro-1,1,2,2-tetrahydrooctyl) trichlorosilane (CAS #78560-45-9, Gelest Inc, Morrisville,
PA) in a vacuum desiccator via vapor deposition. 10 small drops through a glass pipette
(approximately 0.2ml) of trichlorosilane per wafer were added to an open vial and the vial was
placed in the desiccator along with the wafers. This step did not require much precision since vapor deposition is a self-limiting application method (Munn and Jain, 2009). The trichlorosilane acts as an augmenting low surface-energy release agent in conjunction with the naturally low surface energy properties of PDMS and helps prevent cured PDMS from damaging or removing the photoresist negative when it is peeled off of the master.

**Doming**

Once the silinization of the wafers was complete, three-dimensional dome shaped structures were added to the inlet and outlet sections of the negatives for each device so as to increase the effectiveness of the inlets and outlets on the devices they cast and minimize the chance of channel collapse at entry/exit (Burns, et al., 2011); (Forouzan, et al., 2011). The masters were removed from the vacuum desiccator and placed on a hot plate while 23µl of the high viscosity SU-8 2007 negative photoresist were placed on each inlet/outlet section so as to form a free standing hemisphere encompassing the 4mm diameter of each. The hot plate was set to 95°C overnight using a heating ramp of 180 °C/hour to avoid stress, cracking, and peeling of the dome structures that could occur if they were to heat or cool non-homogenously. The wafers were then exposed to near-UV light in two three minutes intervals and were then post baked at 95°C for five hours. The master wafers were then exposed to another overnight vapor deposition treatment of trichlorosilane using the same procedure. The next day, the master wafers were removed from the vacuum desiccator, ready and prepared for PDMS molding. (Hulme, et al., 2010); (Forouzan, et al., 2011); (Burns, et al., 2011).

**PDMS Preparation**

After the masters are complete, polydimethylsiloxane (PDMS) is used to create many replica devices with them. PDMS was prepared by manually combining a liquid silicon rubber
base with a curing agent (SYLGARD 184, Dow Corning Corporation, Midland, MI) in a 10:1 mass ratio prior to use. The two liquids were weighed on an electronic balance (PM4600, Mettler Electronics Corp., Anaheim, CA) in a clean room to ensure the correct mass ratio, and were then mixed in a plastic cup using a plastic knife for five minutes. The mix was then placed in a vacuum desiccator to draw out all of the air pockets within the solution that formed from the mixing process so that the resulting block would be homogeneous and air-free. After this, the PDMS mixture, still a viscous liquid was poured over the silicon wafer stamp in a 3” diameter petri dish. This dish was then placed in an oven (Isotemp 500 series, Fisher Scientific, Hampton, NH) at ~67°C for at least 4 hours, after which the PDMS was solidified via hydrosilylation reaction between vinyl groups and hydrosilane groups in a process known as curing (Whitesides, et al., 2001) and the channel imprint was established. Using a sharp edged scalpel, the PDMS was cut around the perimeter of the petri dish and then excised by individual blocks with interfaces separating each microchannel imprint. The blocks would then be removed, each with a relief of the channel designed with CleWin on the “bottom”. Scotch tape was used to cover these channels once exposed to air (Munn and Jain, 2009). Once the PDMS was removed, the master could be used again. The first PDMS blocks created using a new master were inspected to ensure the correct channel depths were created. This inspection involved using a scalpel to cut a narrow (<1mm) cross section of the PDMS block where the channel depth was critical and viewing it under a microscope (BX51, Olympus, Center Valley, PA) and measuring it based on the size of the view field.
4mm diameter tubular biopsy punches (Acu Punch, Acuderm Inc., Fort Lauderdale, FL)) were then used to gently punch holes in each device at either end of visible channel so as to create an inlet and outlet that can be accessed from the “top” side of each device.

Glass Slide Preparation

Regular glass microscope slides (VWR, West Chester, PA) were used to form the bottom surfaces for the micro channels and render the devices mountable on the microscope stage. The preparation of these slides included an initial cleansing with antimicrobial soap and warm water followed by a removal of surface particles using kim-wipes and pressurized air. The devices were then placed in a spin-coater (Spin-Coater KW-4A 1000rpm, Chemat Technology, Northridge, CA) and were treated with a small amount of liquid PDMS (prepared the same way, although a lower cross-linking agent ratio of 20:1 as opposed to 10:1 creates a stronger seal (Munn and Jain, 2009). The spin coater was then activated with a nine second ramp and 30 second full 1000rpm cycle, which was sufficient to uniformly spread the PDMS across the top surface of each slide. The slides were then removed from the spin coater and placed in large petri dishes. The petri dishes were then put in the oven at ~67°C for at least four hours to allow the PDMS to cure.

Air Plasma Treatment

The PDMS treated glass slides and PDMS blocks with channel imprints were cleaned of most surface particles by using scotch tape and placed with their intended active sides facing up inside the plasma cleaner (PDC 3-xG, Harrick Plasma, Ithaca, NY). Air is evacuated from the plasma chamber and the device is then activated with the “high” setting for 100 seconds. This oxygen plasma treatment renders the naturally hydrophobic surface of PDMS to become temporarily hydrophilic by disrupting organic bonds on the surface and removing absorbed
water, gases, and low molecular weight molecules. Specifically, the native methyl groups are converted to silanol groups, leaving the surface functionalized (Munn and Jain, 2009). The exposed channel side of the PDMS block and the PDMS treated side of the glass slide were then immediately very gently pressed together. This treatment causes the surfaces to adhere tightly due to oxidized attraction without added pressure that could cause the wide and narrow channels to collapse (Munn and Jain, 2009).

### 3.2.3 Device Preparation

Prior to experiments and immediately following plasma treatment, the channels of the devices were chemically treated in a two-step process to minimize RBC and protein adhesion with the walls of the channel and ensure smooth flow (Burns, et al., 2011). The first step of the treatment was to pipette (Finnipipette, Thermo Fisher Scientific, Waltham, MA) device channels with 50µl of a 1% w/v solution of mPEG-silane (MPEG-SIL-5000-1g, Laysan Bio Inc, Arab, AL) and deionized water and allow the filled devices to remain in a humid chamber for four hours, after which the solution is removed. During this step, the mPEG-silane molecules adhere to the walls of the channels, which cause them to act as a “lubricant” for passing cells (Shevkoplyas, et al., 2003).

The second step of the treatment was to fill each channel with 50µL of GASP buffer (9 mmol/L Na2HPO4, 1.3mmol/L NaH2PO4, 140 mmol/L NaCl, 5.5 mmol/L glucose, 1% bovine serum albumin [BSA], 290 mmol/kg, pH 7.4) and allow devices to spend two hours in a humid
chamber. After this, the GASP buffer was removed. 4mm diameter plastic tubing attached to a 50ml syringe (BD, Waltham, MA) was attached to the outlet of each device and decompressed to ensure that GASP buffer and any debris were fully removed.

3.3 Experimental Procedure

All experiments conducted for results in this project followed the same protocol for consistent results that best showcase the abilities of the device, including blood sample preparation, device loading, blood sample loading, and image acquisition and analysis.

3.3.1 Blood Sample Preparation and Loading

Units of packed red blood cells (CPD>AS1, leukoreduced, blood type: O+, A+, AB+, hematocrit: 67.5 +/- 2.5%, MCV: 95 +/- 3 fl, MCHC: 30.7 +/- .6 g/dL) were acquired from a blood bank (The Blood Center, New Orleans, LA) and stored in our lab’s blood bank refrigerator (Jewett BBR6-1B18, Thermo Fisher Scientific, Asheville, NC). Prior to sample extraction, the unit of blood was removed from the refrigerator and placed on a rocking platform (model 100, VWR, West Chester, PA) for five minutes and then manually massaged and turned for approximately one minute. The blood bag was visually inspected for dark spots as obvious signs of premature coagulation or bacterial infection. A sampling site coupler (Fenwal Inc, Lake Zurich, IL) was then placed into one of the bag’s sampling ports. A syringe (BD, Franklin Lakes, NJ) sterilized with isopropyl alcohol was used to extract 5ml from the bag for sampling. A stock solution of glutaraldehyde (8%, Ted Pella Inc. Redding, CA) was diluted 50x with PBS to a concentration of 0.16%, and 750µL of this solution was added to the 750 µL of extracted RBC sample, (resulting in a 0.08% glutaraldehyde, 32.5-35%hct solution) mixed, and incubated at room temperature (22°C) for 10 minutes. 1ml of this blood was then diluted 25x to a functionally ideal ≤2% hematocrit with GASP buffer in an aliquot to quench the cross-linking reaction (Shevkoplyas, et
A hematology analyzer (Medtronic M-Series, Boule Medical AB, Stockholm, Sweden) was used to verify cell count and hematocrit. This hematology analyzer cannot determine hematocrits below 2%, so unregistered readings were assumed to be close to, but less than 2% based on the consistency of the dilution scheme.

The device was placed on the mechanical microscope stage with the fresh GASP buffer still inside of it. The microscope system was an inverted IX71 (Olympus America Inc, Center Valley, PA) equipped with a high-speed CMOS digital camera (MC1362, Mikrotron GmbH, Germany) connected to an HP Pavilion Elite e9180f (Core i7 920 / 2.66GHz / 9 GB / 1 TB Hewlett-Packard Company, Palo Alto, CA) desktop via camera link analog video frame grabber (PIXCI E4, EPIX Inc, Buffalo Grove, IL). To improve the contrast of images by darkening the RBCs in blue light, a narrow band-pass blue filter (394 ± 50nm, B-390, Hoya Corp USA, Fremont, CA) was implemented on the microscope. A 2mm diameter elbow reduction tube fitting (L420/410-6, Value Plastics, Fort Collins, CO) attached by tubing to an open topped syringe containing GASP buffer mounted to an adjustable height water column device was inserted tightly into the outlet port of the device. Lowering the syringe on the column would create a pressure gradient between the device and the level of GASP in the syringe that would cause a controllable flow of GASP buffer from the device into the plastic tubing. A kim wipe was then used to absorb most of the standing GASP buffer in the inlet channel. 50µl of the prepared blood sample was then aspirated from the aforementioned aliquot and ejected into the inlet channel of the device using a pipette. The red blood cells in the channel were manually brought into focus on the microscope and the field of view was fixed between one selected matrix of supports in the device channel.

3.3.2 Image Acquisition
The camera was then set to acquire short image sequences of one image per second for up to five minutes using 1fps capture rate, 5ms exposure and global shutter. The blood flow could be seen on the computer monitor. Images and automatically timed series of images could be recorded using image analysis software designed to operate with the video frame grabber (XCAP-Viewer, EPIX, Buffalo Grove, IL).

### 3.4 Computational Data Analysis

An algorithm written with MATLAB R2009 (MATLAB, Mathworks, Natick MA) as a collaborative effort of Sergey Shevkoplyas, Xiaoxi Yang, and Nathaniel Piety was used to analyze the series of images captured by the video frame grabber software. The goal of the algorithm was to classify each cell as either discocyte, reversibly changed, or irreversibly changed. This morphological classification technique has been employed before because of its apparent physiological relevance (Berezina, et al., 2002); (D’Alessandro, et al., 2012) and is addressed further in section 5.4.2. Although the images were recorded at 1fps (4ms exposure, global shutter), the algorithm was used to analyze every 3rd image so as to further reduce the occurrence of slow individual cells being counted twice (this is discussed further in section 5.7). The parameters used in classifying the morphology of each cell are based on the standards described by (Bessis, 1972) and the standards used by (Albertini, 2003); (Wagner, et al., 2005) and the quality of the images obtained using our microfluidic device increased the efficiency of the code and minimized the necessity to overcomplicate the filters. The operation of this algorithm can be summarized as three stages:

#### 3.4.1 Pre-Processing
The algorithm detects the perimeter columns in an image to calibrate the designated area of cell detection. The image is then converted to gray-scale and the contrast is polarized so as to help cells stand out from their surroundings and optimize edge detection.

### 3.4.2 Cell Segmentation

A black and white binary mask of the image is then applied to remove variability caused by minutely varying focus during image acquisition, the perimeter columns themselves, and cells that do not fit fully within the designated detection perimeter using a size based closing procedure with high and low limits based on cell size. Pairs or groups of cells that overlap are then differentiated using a watershed splitting method that separates cells based on pixel features apparent in the binary mask based on the topography (Xiong, et al., 2010). The original grayscale intensity image and binary mask are then combined in a composite image which allows detection of the size, color, and texture of each cell. Cell segmentation is highly prone to error if the cells are overlapping or aggregating, so this part of the algorithm depends on the success of the microfluidic device in minimizing this occurrence.

### 3.4.3 Feature Extraction/Classification

The 3 sets of data describing each cell are numerically compiled in a three dimensional dataset that determines which of the three classifications (1-discocyte, 2-reversibly...
changed/stomatocyte or echinocyte, 3-irreversibly changed/spheroechinocyte or spherocyte) of morphology each cell occupies. This process is applied to a series of the desired number of images (more images theoretically would yield greater accuracy) and the total number of cells that meet each of the three criteria are presented as totals, providing an easily comprehensible morphology score of the sample population. This part of the image analysis can be compromised by abnormal appearance of cells, namely sideways orientation of discocytes, and therefore relies on the success of the microfluidic device in mitigating this phenomena as well.
CH 4: RESULTS

The novel microfluidic device that I designed was tested to determine if it adequately met the design criteria that were decided upon based on the capabilities of a relatively simple MATLAB algorithm and the capabilities of soft lithography. If successful, the images captured using the microfluidic device I designed would allow accurate, fast, and high throughput morphological assessment via the MATLAB algorithm. This required experimental determination of the most effective microfluidic design and image capture method to use. Once this was determined, the optimal channel depth needed to be experimentally determined. Once it was confirmed that we had images that would allow morphology detection with our algorithm, we proceeded to compare the performance of the algorithm with a morphology score obtained using manual counting both with images obtained using our device and using a blood smear. We were able to compare the algorithm’s classification of each cell to manual classification from the same set of images obtained from the microfluidic device. We also verified our microfluidic device + algorithm scoring method by determining a high throughput morphology score and comparing it to a morphology score obtained using the standard blood smear and manual counting method, showing that our system can obtain similar results based on a much higher sample size and all within a matter of minutes instead of hours.

4.1 Channel Depth Determination

The depth of the channel on the microfluidic device was a critical factor in ensuring it provided the desired type of cell orientation for ideal images. In order to prevent cell overlap or discocyte flipping, (two phenomena that when captured in an image, disrupt the classification process of the algorithm) the channel needed to be as shallow as possible without inhibiting the
movement of the cells or causing cells to become stuck as this would skew the results of the algorithm. The length of a discocyte is 7-8µm, so the height needed to be less than 8µm to minimize flipping, so 7µm was tested as the maximum height. The diameter of a spherocyte is usually 3µm so this was tested as the minimum height. 5µm height was tested as an intermediate. The higher and lower channel heights were achieved by fabricating master wafers with slower and faster photoresist spin coating angular velocities, as per instructions provided by the photoresist manufacturer.

Figure 14 - Cross section of channel depth drawn to relative scale with spherocyte (left) and discocyte (right) with spherocyte diameter and discocyte width and height reported in microns. The spherocyte is free to move and the discocyte is somewhat restricted from flipping. Overlapping is less likely.

Figure 15: A comparison of device depths of 3µm (a/d), 5µm (b/e), and 7µm (c/f) reveals that a 5µm depth minimizes unwanted overlapping and discocyte flipping (as seen in excess with 7µm depth) without hindering cells from entering and traversing the channel (as seen in excess with 3µm depth). Velocities, as per instructions provided by the photoresist manufacturer. There was an
approximate half μm tolerance in the channel height using this method. The depths were verified as explained in section 3.2.4.

Although the depth of channels could be controlled with high precision, we anticipated unpredicted effects and decided to test all three of our possible depths to find which would be optimal. We found that at a 3μm depth, overlapping and cell flipping was reduced to satisfaction, but the entryway to the channel became impinged (likely due to the pressure applied with biopsy punch) such that some cells could not enter the channel, which would cause a morphology sampling bias. Also, too many cells would become stuck between the floor and ceiling of the channel. At the 7μm depth, there was no apparent bias of cells able to enter the channel and discocyte flipping was minimal, but overlapping was much too excessive and variation of cell height within the channel made it more difficult to find a single focus that made all cells identifiable by the code. The 5μm depth was clearly optimal, as it kept cell flipping and overlapping to a minimum without causing impingement at the entryway or significant impedance of the flow of individual cells. It was also noted that structural collapse along the channel, which occurred more frequently with the 3μm depth, was minimal at the 5μm depth.

4.2 Image Appearance

The channel depth of 5μm had been established as being the most effective at minimizing the occurrence of cell overlap, discocyte flipping, and morphologically biased flow inhibition as compared to the closest taller and shorter depths attainable using the approximate +/-0.5μm resolution of our photoresist spin coating procedure. Therefore the rest of our devices were fabricated with this depth and we used them to collect a series of images that were analyzed with the MATLAB algorithm. The results were visually inspected on an individual cell
basis by dissecting the algorithm (illustrated in figure 16) and the images were deemed suitable for the code because the cells that appeared in the images were sufficiently spaced apart, not overlapping, not flipping, and were generally properly identified based on the parameters that were selected to differentiate them. This preliminary result indicated that the system was ready for a high volume precision test and a low volume accuracy test where data would be collected.

Figure 16: Algorithm is able to discern between discocytes (red diamond), reversibly changed (yellow circle), and irreversibly changed (blue asterisk) cells as they move through the narrow microfluidic channel

4.3 Accuracy Assessment

In order to determine the accuracy of our system in terms of automated morphological categorization facilitated by the microfluidic device, we acquired five images with a device using six week old blood sample and compared the classification ascribed to each cell by the algorithm to that ascribed by myself and two lab members. The five images contained a total of 205 different cells that fit within the selected perimeter of columns. The three of us each individually inspected the images and classified each cell as belonging to one of the three morphological classes. In the case where there was a discrepancy in classification of a cell between observers, the classification chosen by two of the three observers was used for that cell.

Dissecting the algorithm as mentioned in section 4.2, we were able to visually review the morphological category that each cell was automatically assigned after analysis in each image. We compared, on a cell by cell basis, the classifications found using manual counts and
those found with the algorithm. This data was compiled in a confusion matrix (Table 1) that indicates more precisely how the code and manual observations differed in classification of each cell. The confusion matrix is a common method for evaluating the accuracy of automatic image element classification (Hu and Osuna-Highley, 2010). To interpret the data in the confusion matrix, consider the case of discocyte classification. The sum of the numbers composing the discocyte “column” represent the total number of algorithmically identified discocytes (AIDs) and each number making up this column represents how many AIDs were manually identified to belong to each classification, indicated by the labels to the left. In this case, 82 AIDs were also counted as discocytes by the manual observers, but six AIDs were categorized as reversibly changed RBCs by manual counters, and one AID was classified as an irreversibly changed RBC by manual observers. The inverse can be applied to the rows.

<table>
<thead>
<tr>
<th>True classes</th>
<th>Prediction by classification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Discocyte</td>
</tr>
<tr>
<td>Discocyte</td>
<td>82</td>
</tr>
<tr>
<td>Reversibly changed RBC</td>
<td>6</td>
</tr>
<tr>
<td>Irreversibly changed RBC</td>
<td>1</td>
</tr>
</tbody>
</table>

Features used are 3D static, size, color intensity, temporal texture. The overall accuracy is 92%.

Table 1: Confusion matrix comprehensively displays all discrepancy and concurrence between the manual count and the automated count with our system on an individual cell inspection basis.

This confusion matrix provides a way to measure the overall “accuracy” of the algorithm as the percentage of the total number of cells that were similarly classified by both the algorithm and the observers (indicated by the sum of the diagonal numbers), found to be 92.6%:

\[
\frac{190 \text{ agreed cells}}{205 \text{ total cells}}
\] Since there is no way to determine the absolutely accurate morphological classification of each cell, the percentage of cells that with matched classification between the
algorithm and by the trained manual observers serves as the best feasible measurement of the algorithms accuracy. The implications of this accuracy determination are discussed further in section 5.1.

The most common discrepancy between the code and manual observer was in discerning between a discocyte and a reversibly changed cell. The code accurately identified all of the irreversibly changed RBCs but four, and did not designate any cell as irreversibly changed that the observers did not. Of the four irreversibly changed cells that it misidentified, the algorithm assigned one as a discocyte, and three as reversibly changed RBCs. These results are encouraging in that they correlate well with what a manual counter might determine, since there is always some subjectivity in deciding between a discocyte/reversibly changed cell or between a reversibly changed/irreversibly changed cell. This means that the microfluidic device can provide images that allow for this sort of comparative accuracy since it was used to capture these images.

<table>
<thead>
<tr>
<th>Code Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Falsey Identified</td>
</tr>
<tr>
<td>Irreversibly Changed RBC</td>
</tr>
<tr>
<td>Reversibly changed RBC</td>
</tr>
<tr>
<td>Discocyte</td>
</tr>
</tbody>
</table>

*Figure 17: An alternative visual interpretation of the confusion matrix. In the case of discocyte, it is to be read as 7 cells were identified as discocyte by the algorithm that were identified differently by manual count, 5 cells were identified as discocytes by manual count that were identified as otherwise by the algorithm, and 82 cells were identified as discocyte by both the algorithm and the manual count.*

4.4 Method Comparison

In a separate experiment we wanted to test the sensitivity of our method with a larger sample size to detect any possible flow based morphology selection bias we may have not accounted for. We manually counted approximately 600 cells found in 3 images in randomly
chosen fields of view from a blood smear with glass slides. Using a sample from a 6 week old (RBC at this age using our storage technique can be expected to exhibit a large variability in morphology (Berezina, et al., 2002); (Bessis, 1972) leukoreduced (CPD>AS-1) unit of packed RBC, we used our microfluidic device (3 separate replicate devices were used to reach this total) and algorithm to count approximately 5000 cells found in 100 images from a centrally located view field designated by a perimeter of square columns. The image acquisition rate used was 1fps and the algorithm counted one image for every three that were taken.

The morphological distribution of cells roughly matched between both the manual glass slide observation and automatic scoring with the microfluidic device, indicating that the device and algorithm do not have an extreme selection bias. Also, the fact that the microfluidic device and algorithm were used to count many more cells would imply that the morphology score it yields is a more statistically significant representation of the blood bag population as a whole. The standard deviation of morphological distribution among images was significantly higher for the manually counted images, indicating the need for more images to be counted for a more accurate representation of the population, which would take much more time. The standard deviations also indicate the advantage of consistency in categorizing cells of a certain appearance as the same morphological class. A person who counts and must make guesses on cells that are difficult to discern may accidentally skew the data whereas the
automated system would remain consistent in its judgment. The only way to test the accuracy of the device and code here would be for operators to manually count approximately 200 cells in 25 blood smears. The concentration and endurance required for this is sort of labor would obviously be difficult to achieve.

4.5 **Timing Assessment**

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>RUNNING TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Device Pre-loading</td>
<td>2 min</td>
</tr>
<tr>
<td>Image Capturing / 300 Images</td>
<td>5 min</td>
</tr>
<tr>
<td>Morphology Classification / 5,000 Cells</td>
<td>5 min</td>
</tr>
</tbody>
</table>

Table 2: Task time breakdown for 5,000 cell automated morphology score

To determine the efficiency of this morphology scoring method, we measured the time it took to complete each step of the process. Assuming the operator is familiar with the procedure and experienced implementing it, it would take approximately 2 minutes to load a device with a sample, mount it on the microscope, and find an appropriate view field to use for image capture. The image capture process takes 5 minutes, as the blood needs time to flow between each image capture so that the numbers of cells that appear in more than one image are kept to a minimum. A total of 300 images are taken in five minutes, and one out of three is used as iterated before. Since there are 50 cells per image on average as evidenced in section 4.4, this would result in 5000 cells. The morphology classification algorithm takes 5 minutes to analyze the image data and provide a morphology score. There is some time between when the image capture is completed and when the analysis begins, but this is entirely dependent on the operator. With an optimal length of time being 12 minutes for one sample accounting for 300 images and 5,000 cells, this method is indisputably faster than the manual counting method,
which usually takes hours between blood smear preparation and manually inspecting images (Kamath, 2011).

CH 5: DISCUSSION

5.1 General Results Summary

In this project, I developed a microfluidic device that can facilitate a single layer self-propelled flow of RBCs suitable for microscope-obtained image sequences that can be more accurately and efficiently analyzed with a relatively simple image analysis algorithm designed for scoring the morphological composition of a RBC population. We have shown that the design of the device facilitates a continuous flow of a single layer of RBCs without a morphology based flow bias that would alter the represented proportions defining a morphology score. We have shown that the appearance of RBCs in images obtained with this device can be analyzed with an algorithm that uses image based parameters for classification by comparing the automated classification of 205 cells to a manually determined classification of each cell in the same set of images obtained from the microfluidic device. This allowed us to find a reasonable estimate of the accuracy of the code in conjunction with the microfluidic device. This thesis has explained and demonstrated that the microfluidic device that I experimentally developed following certain design criteria can provide images that yield a morphology profile of a population of sRBCs comparable to one obtained using the traditional blood smear and manual analysis method. Furthermore, this research has definitively shown that our automated hybrid approach is much faster and easier to implement than the traditional approach, allowing for a much higher cellular throughput (and therefore higher statistical significance in data) and reduced contingency for human error.
The experimentally estimated 92.6% accuracy of our algorithm is an encouraging result, but this percentage cannot be considered the true intrinsic accuracy of the device since there is no way to perfectly determine the morphology score for comparison. Rather, it indicates the expected correlation between results from an average manual inspector and the algorithm when inspecting images from our device. This correlation is still an important indication of the success of the device and the same correlation based on a confusion matrix has been used to measure the accuracy of other automatic microscopy image classification algorithms (Boland, et al., 1998); (Wheeless, et al., 2005) and an accuracy of 92% determined in this way has been considered favorable in similar automated morphometric analysis research (Hu and Osuna-Highley, 2010); (Huh, et al., 2009). The method comparison (section 4.4) also revealed that our algorithm did not falsely identify any cells as irreversibly altered, though it did identify some discocytes as reversibly altered cells and vice-versa. This tendency is likened to the trouble that a manual observer might encounter while trying to discern between the two classifications. The difference between a healthy discocyte and an early stage echinocyte can be difficult to determine, but an irreversibly changed spheroechinocyte or spherocyte is more distinct from the other two groups. These results would indicate that the current accuracy deficiencies of the code are similar to those of a human observer. The difference, as indicated by the standard deviation measurement, is that the code will misidentify cells with consistency since automated determination is based on strict image based parameters while a manual inspector may be more inclined to make a guess.

5.2 Popularity of Automated Microscope Image Based Detection

The use of automated microscopy in morphological measurement is an emerging trend being applied to improve research in various fields of cellular biology, and it is becoming an
increasingly popular interest to those studying microscopic phenomena due to recent technological advancements (Taylor and Giuliano, 2005). Those that are utilizing this technology praise it for the common advantages it offers in various biological applications related to diagnosis or research; speed, accuracy, user objectivity, simplicity, and convenience. RBC morphology detection is only one of many different biological applications of automated microscopy. For example, technology similar to what we used in our study has been applied to improve the efficiency of location proteomics with an identification algorithm that detects subcellular protein locations by automatically classifying patterns with images gathered using fluorescence microscopy (Hu and Osuna-Highley, 2010); (Huh, et al., 2009). Digital image analysis has also been applied to blood smears to identify, count and classify leukocytes by the separate (segmented) morphological appearance of their nuclei and cytoplasm in order to diagnose leukocyte-symptomatic diseases like acute leukemia. Applications of a method like this would be applied to rapid disease screening using patient whole blood samples (Sadeghian, et al., 2009); (Huang, 2012); (Angulo, et al., 2006).

5.3 Various Methods of RBC Morphology Detection

Even within the scope of RBC morphology detection, there are variations in the goal of the detection in different applications, meaning different morphological features are sought to be identified and classified. This means different morphology detection applications require a different appearance of images, which changes how the sample is prepared and loaded.

5.3.1 Automated Microscopy for Disease Diagnosis

Automated microscopy has been utilized successfully to rapidly diagnose malaria by using digital images obtained from a Giemsa-stained blood smear with similar segmentation algorithms designed to count the total number of RBCs pictured and identify how many of them
are infected based on specific morphological abnormalities associated with malaria that are identified with a threshold check (Purwar, et al., Dec. 2011); (Di Ruberto and Dempster, 2002).

Even more similar to our research, automated detection of RBC morphology has been used before to classify cells as either “normal”, “abnormal”, or “sickle -cell” by using an algorithm that makes classifications based on 42 parameters (as opposed to our three: area, average color, and surface noise) that can be deduced from the microscope obtained images of each cell. In this study, the accuracy of the method was also determined by comparison between automatically calculated and manually determined results, and was found to range from 73-92%. However, in this study, samples were put on glass slides and images were taken from random view fields. Cells that appeared to overlap or were not in focus in each image were omitted from analysis (Wheeless, et al., 2005). The microfluidic device used in our method provided images that didn’t require omissions and our algorithm was far less complicated and was able to achieve an equivalent, if not greater identification accuracy with the same number of possible morphology classifications. A separate study used a blood smear and a seven tier classification scheme along with advanced image analysis software (Albertini, 2003).

5.3.2 Manual Electron Microscope for the Three Classes of Storage Degradation

Scoring the morphology of a population of RBCs and classifying them according to the investigation conducted by Bessis (Bessis, 1972) has been done in prior research on multiple occasions. Notably, Berezina (Berezina, et al., 2002) developed the concept of simplifying the classification of cells into the three groups that

<table>
<thead>
<tr>
<th>Day of storage</th>
<th>Discocyte (%)</th>
<th>Reversibly changed RBC (%)</th>
<th>Irreversibly changed RBC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5th</td>
<td>79.0 ± 2.7</td>
<td>14.0 ± 1.7</td>
<td>7.0 ± 1.6</td>
</tr>
<tr>
<td>7th</td>
<td>78.0 ± 3.0</td>
<td>13.6 ± 1.7</td>
<td>8.4 ± 1.6</td>
</tr>
<tr>
<td>14th</td>
<td>57.4 ± 3.8†</td>
<td>27.9 ± 1.9†</td>
<td>14.7 ± 2.6†</td>
</tr>
<tr>
<td>21st</td>
<td>53.7 ± 3.5*</td>
<td>30.8 ± 3.0*</td>
<td>15.7 ± 3.3*</td>
</tr>
<tr>
<td>28th</td>
<td>47.0 ± 3.6*</td>
<td>35.2 ± 1.8*</td>
<td>17.2 ± 4.1†</td>
</tr>
<tr>
<td>35th</td>
<td>37.5 ± 5.0†</td>
<td>40.6 ± 3.4†</td>
<td>21.0 ± 5.0</td>
</tr>
<tr>
<td>42nd</td>
<td>23.3 ± 4.3†</td>
<td>46.8 ± 6.7*</td>
<td>29.0 ± 4.0†</td>
</tr>
</tbody>
</table>

* P < 0.01 vs 5th day.
† P < 0.01 vs previous point.

Figure 19: In this table, it clear that as the length of storage continues, the % discocyte drops and the % irreversibly changed RBC increases (Berezina, et al., 2002)
were used in this study; discocytes, reversibly changed cells, and irreversibly changed cells. This is a convenient way to measure RBC morphology in terms of expected clinical efficacy and it has been proven to corroborate the shift from healthy discocyte to irreversibly changed RBC during the length of storage. This three tier classification of morphology has been applied to observe changes in the morphology of RBCs of people who recently experienced severe shock (Berezina, et al., 2004), and in time course studies measuring storage lesion (D’Alessandro, et al., 2012); (Blasi, et al., 2012); (Antonelou, et al., 2012). In all of these studies, the researchers came to conclusions similar to those found by Berezina in 2002 (Berezina, et al., 2002). It was hypothesized that the proportion of RBC’s taken from storage occupying each of the three morphological conditions would shift from a high majority of discocytes toward the irreversibly changed condition as the sRBCs approached their expiration date. Berezina et al. confirmed their hypothesis by manually inspecting SEM images and categorizing each cell and finding that between the 5th and 42nd day of storage, the proportion of discocytes dropped while the proportion of reversibly and irreversibly changed RBCs increased, as seen in figure (19). This data supports our assertion that a morphology score can be used clinically to measure the condition of RBCs as they degrade in storage, but the methods that were used in each of these studies to obtain this data are highly inefficient.

In each case, a blood smear and SEM microscope was used. There are two main weaknesses to this system. First, it is known that blood smears require a level of expertise to use properly and allow for operator subjectivity. They can cause a non-uniform cell distribution with varying thickness, streaking, and clumping, leaving it to the operator to decide on a “good working area,” (Xiong, et al., 2010) which leaves room for subjectivity and inconsistency between operators and smears (Houwen, 2002); (Kamath, 2011). Furthermore, the limitation in total cell count in each of these studies inhibits the statistical significance of their results. The
only way to increase the cell count would be to have the cell counter labor over more images and likely grow tired and become more prone to make mistakes (Kamath, 2011). A manual counter may have experience, but he or she will never be able to count as quickly or as consistently as a computer algorithm (Xiong, et al., 2010). In the aforementioned research in this section, researchers monitored their cells manually by counting “500-1500 cells” (Berezina, et al., 2002), “1000-1500 cells” (D’Alessandro, et al., 2012), “600 cells” (Blasi, et al., 2012), and “2000 cells” (Antonelou, et al., 2012) at each time point. The disadvantages of manually counting RBCs in SEM images to obtain a morphology score as performed in these studies are clear. For a sample size of classified RBCs to be considered statistically significant enough to be representative of an entire sRBC unit (as was the goal in these studies), technicians had to spend an enormous amount of time and effort counting hundreds of cells and leaving room for mistakes brought on by fatigue or distraction.

5.3.3 Automated Morphology Detection Machines

The practice of applying automated microscopy to blood morphology scoring has become commonplace to the point of commercialization, with devices such as the Diff Master Octavia and CellaVision DM96 (Cellavision AB, Sweden) that are designed to be used in labs to find the leukocyte class distribution of a population of whole blood or identify the proportion of red blood cells that appear to exhibit abnormalities based on the morphology of each cell apparent in images from a blood smear. These machines can perform these tasks within 5 minutes. These devices were both evaluated and found to provide accuracy (defined the same as it is in our study) ranging from 71-94% for high throughput screening in the case of RBC morphology classification (Ceelie, et al., 2007). These devices are much more clinically applicable than manual SEM cell counting, but they do come with their own setbacks. These devices are relatively expensive and are often only affordable in large hospitals, making them
less viable as research tools and less accessible for small clinics. Also, with extremely high throughputs, slight miscalibrations or unexpected particle morphology/clumped particles may lead to high counts of misidentified cells that an operator may not be aware of since he or she cannot directly monitor the images or classifications being processed by these machines in real time (Kamath, 2011). While these instruments rely on blood smears for images, some newer devices such as PixCell Medical’s “CBC Tester” (PixCell Medical Technologies, South Industrial Zone, Israel) use a rheological phenomenon known as viscoelastic focusing (Leshansky, et al., 2007) whereby the elasticity of a polymer solution used as a medium with a patient whole blood sample can be adjusted to control the lateral “focusing” of particles/cells within the blood that exhibit certain size or rheological properties (such as deformability) as they flow through the microchannel of a disposable microfluidic device. The resulting flat plane of particles can then be imaged without an optical zoom adjustment, providing images that are easy to morphologically analyze in the same sense that our images are. This is relatively new technology and there are limited reviews available for incite on further advantages or disadvantages involved in this approach. Our device accomplishes the same goal in providing a plane of particles, but instead of relying on the elastic properties of our buffer, we rely on the mechanical constrictions of our channel. There is no need for our device to be tunable since it is only intended to analyze one type of particle (RBCs). The commercial concept of the “CBC Tester” device as being an all-in-one microscope and image analysis package with disposable microfluidic sample cartridges could serve as an example of what a clinical device that implements the method described in our project could possibly look like.

5.4 Different Methods of Stored RBC Quality Detection

Morphological assessment, as used in our research, is not the only approach that has been taken to quantify the quality of a population of sRBCs. RBC quality, as it pertains to
expected post transfusion efficacy, has also been measured in terms of deformability (Burns, et al., 2011), membrane fragility (Alfano and Michael, 2011), aggregability (Nagaprasad and Singh, 1998), and shear viscosity/stability (Alexy, et al., 2005);(Uyuklu, et al., 2009). All of these measurable properties of RBCs theoretically indicate the extent of storage lesion and predict post transfusion survivability and proper performance of cells in-vivo, so there is some physiological significance in the measurement of each.

Deformability has been the focus of much research in the field of RBC storage and transfusion because of its direct importance in transfusion efficiency in terms of capillary bed perfusion (Chien, 1987). Deformability is a general term used to describe a complex characteristic. Since deformability lacks a single intrinsic descriptor of measurement (Evans, 1989), researchers have resorted to a variety of techniques to quantify it including flow cytometry, image cytometry, ektacytometry, laser-assisted optical rotational cell analysis (Hardeman, et al., 1994), optical tweezers, adhesion assay, micropipette aspiration (Musielak, 2009), and artificial microvascular network perfusion (Burns, et al., 2011), among others. Each of these techniques accounts for some, but not all of the parameters that define deformability, and some are more physiologically relevant than others (Musielak, 2009).

The aggregability and shear viscosity of a sRBC population, which are directly related are not difficult to measure and are considered an important indicator of the physiological condition of a population of RBCs, but these characteristics are not considered to intrinsically cause physiological complications and are thus considered less critical metrics than altered cellular deformability (Baskurt and Meiselman, 2013). However, they are easier to describe since they are defined by less parameters, and a common method used to quantify these characteristics involves the use of image sequence analysis (Forsyth, et al., 2010). Measurements of aggregability and shear viscosity can be regarded as parameters of deformability so they are
often studied in conjunction with deformability (Forsyth, et al., 2010); (Nagaprasad and Singh, 1998).

It is arguably counterproductive to consider each of these RBC quality metrics individually. The visual morphology of sRBCs, along with their deformability, membrane fragility, aggregability and shear viscosity are all interrelated in how they indicate the extent of storage lesion. For example, as a cell loses its membrane surface area, the cortical tension of the membrane will increase, simultaneously lessening the deformability of the cell (Guo, et al., 2011). The reasons why one characteristic would be studied in lieu of another or why one parameter would be measured to describe a characteristic in lieu of another is because the ultimate goal of all of this research is to maximize the precision of a single physiologically relevant metric that can serve as an aggregate of sRBC quality.

It is conceivable then, that in order to maximize the precision of a sRBC quality test, all of these factors should be measured together. This may eventually be possible, but it would be difficult to implement properly, would require extensive resources, and may not be worth the effort. In the scope of this paper, we argue that a visually detectable morphology score can and should be used as an aggregate indicator of all of these characteristics. The biological explanation of storage lesion and its effects on RBCs in relation to their expected in vivo function support this assertion.

5.5 Strengths of Our Approach

By using a microfluidic device and an algorithm that were designed to work in unison, our method adds a unique simplicity to an otherwise overcomplicated landscape of parallel research. The drawbacks of using a blood smear to obtain images for automated analysis in terms of their interpretability with a morphology based algorithm are largely eliminated with
the use of our microfluidic device, and the drawbacks of manual counting, which are obvious in some of the research discussed previously are also minimized.

The operator dependent subjectivity of the process is decreased in multiple ways, including the manual preparation required to prepare the sample. With a blood smear, there is some level of finesse in preparation that will vary between operators and affect the appearance or distribution of the cells based on subtleties such as the angle of film application, and it is important to have an experienced operator to handle the task of preparing smears (Kamath, 2011). With our microfluidic device, the operator only needs to follow the protocol of preparing the sample and injecting it into the device with a pipette. Among experienced lab technicians, there is little room for variability in the performance of this task. Also, the subjectivity and time consumption of manually deciding the multiple view fields of a blood smear to sample is reduced by allowing a morphologically unbiased flow of cells to naturally provide a fairly varied profile (as indicated by the results in section 4.4) of the population instead. The single height of the plane at which all cells appear relative to the microscope lens means the image analysis errors associated with refocusing are mitigated as well, since the focus can remain the same for all cells within the device and the similar cells will have a relatively uniform color and texture. Our device prevents the depth variability that can cause some cells to appear out of focus, which also allows for a more reliably constant level of feature detail in every image.

Once the images are gathered, the morphology analysis algorithm, which is not very robust compared to those used in other studies (Vromen and McCane, 2010); (Di Ruberto and Dempster, 2002); (Purwar, et al., Dec. 2011) can be relied on to classify the small variance in appearance of RBCs as they pass. The user friendly simplicity of the algorithm makes it easy to adjust and troubleshoot in case there is an apparent issue with identification.
Finally, the time saving high potential throughput of our device (>1000 cells/minute) allows for greater precision (as demonstrated in section 4.5), consistency of digital parameter based distinguishing between discocyte/reversibly changed cell (further reduction of manual count subjectivity allowing inaccuracy in scoring to be recognized and accounted for), and relative accuracy make our method vastly superior to manual counting. The reproducibility of our results also contribute to lower error, since many PDMS devices can be fabricated from a single master wafer to run multiple tests on a single sample without requiring time, effort, or funds. Our method is cost effective, labor-light, does not require a skilled operator, and our high throughput of cell counting allows us to make statistically significant conclusions about cell populations where a statistically significant manual count would take too long (Kamath, 2011). The aforementioned rapid automated RBC morphology scoring devices (described allow for high throughput, but still suffer from the disadvantages of relying on blood smear images in addition to the aforementioned issues mentioned in section 5.3.3.

### 5.6 Limitations of our Approach

The research presented in this project, while encouraging, is only a preliminary trial of an uncommonly used approach of coupling a simple microfluidic device with a simple image analysis algorithm. The simplicity of this approach simultaneously accounts for its novelty and vulnerability to error.

We have shown that the general quality of images we can obtain using a PDMS device is substantial in terms of compatibility with a simple morphology detection algorithm such as the one we have developed when compared to a blood smear, but the system is still far from perfect. Although the RBCs are significantly restricted

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Figure 20: A discocyte moves with a slanted orientation causing an awkward appearance
from flipping by the 5µm depth of the channel, some discocytes are still able to partially flip, fold, or move along with a slanted orientation as seen in figure (19). This means that in a series of images, there may be some that contain a sideways oriented discocyte. This often results in a misidentified cell, as the algorithm is not designed to account for this phenomenon. The optimal functioning of this system also heavily relies on the freshness of the GASP buffer being used. Fresh GASP buffer ensures that there will be minimum cellular adhesion to the walls of the channel, but after the purity of the buffer has diminished beyond a certain point due to factors such as exposure to bacteria, undesirable cellular adhesion to channel walls is inevitable. In this study, wall adhesion likely skewed our results to some extent by causing the morphological classification of slower moving cells to have a proportionately higher score than what would be indicative of the true population (figure 21, table 3). This is because when a slow moving cell enters the view field while image capture is initiated, that single cell will appear in multiple, if not all of the images as it is impeded from flowing out of the view field with the other cells. This is a very rare phenomenon with fresh GASP buffer but it did happen on occasion. Cell to cell aggregation was also a problem, as the code was not very efficient at discerning between tightly aggregated cells with no space between them. However, cell to cell aggregation can be mitigated in future experiments by excluding glutaraldehyde, which is known to increase aggregability, from the sample dilutions (Burns, et al., 2011). Glutaraldehyde treatment is more essential in preserving cell shape and structural integrity in blood smear preparation and may be omitted in preparation of samples for our approach.

To some extent, the skew of results caused by occurrence may be mitigated by the fact that if some RBCs are slowed down due to aggregation or wall adhesion while within the view field, it can be assumed that similar cells experiencing the same effect upstream from the view field will not move fast enough to enter the view field during image acquisition and will
therefore not be counted. In this sense, the multiple capture effect may in some cases compensate for a lower total number of morphologically equivalent cells passing through. The system also allows for some operator subjectivity as the operator chooses among the central column of over 40 possible view fields enclosed by support columns in order to ensure there are no permanently adhered cells or structural imperfections within the specific field being captured at the time that the capture sequence is initiated. For our purposes, this was an acceptable compromise because the possible skewed results that could result from thickly adhered cells or structural imperfections that likely occurred during fabrication surpass the magnitude of the effect on the results that interoperator subjectivity could cause.

![Image of cells tracked](image)

Figure 21: Seven RBCs of varying morphology and location are identified (left) and spatially tracked using background removal and projection over the span of 6 consecutive images taken at 1fps (right) to reveal varying speeds and paths. Cell 6 is an echinocyte and aggregates with another cell along its path through the view field and takes 6 seconds to pass through, meaning it would be counted twice by the algorithm (1 image is counted for every 3)

<table>
<thead>
<tr>
<th>Cell</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Type</td>
<td>Discocyte</td>
<td>Echinocyte</td>
<td>D</td>
<td>D</td>
<td>DE</td>
<td>E</td>
<td>D</td>
</tr>
<tr>
<td>Avg. Speed (µm/s)</td>
<td>35.33</td>
<td>45.78</td>
<td>27.86</td>
<td>40.98</td>
<td>39.13</td>
<td>31.98</td>
<td>37.08</td>
</tr>
<tr>
<td>SD (µm/s)</td>
<td>4.415646</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3 The average speed of each cell tracked in figure 21 is displayed with the standard deviation. Largely due to aggregation and adhesion, the cells vary in speed significantly.
Another concern we have about our device is the fluctuation in flow rate we observed among the devices tested. This variation cannot be easily accounted for because each of the devices were fabricated and prepared following the exact same protocol and the consistent 50µl sample injection volume theoretically should result in the same flow rate. This variation in flow rate could be caused by seemingly insignificant differences in the individual fabrication of each device. The deformability of PDMS makes perfect device replication difficult to achieve. For example, the pressure applied with the biopsy punch to create the inlet and outlet could have some small effect on the rate of sample flow allowed to enter the channel or air allowed to exit.

As mentioned previously, the algorithm used in this research is not designed to account for significant variation in the appearance of cells in that it cannot identify undefined morphological abnormalities. The algorithm itself can be augmented to account for some of these more common abnormalities, but in order to showcase the effectiveness of the device in diminishing the frequency and severity of such abnormalities, the relative simplicity of the algorithm was an intended feature of the system.

5.7 Future Directions

The research presented here is an encouraging indication that our hybrid approach of using a microfluidic device to facilitate improved image capture in conjunction with a fast and simple image analysis algorithm may be a more effective way to analyze the morphology of RBCs in order to evaluate the expected efficacy of a stored unit or investigate the relationship between sRBC morphology and another related phenomena.

5.7.1 Strengthen Proof of Concept
There are opportunities for further research to confirm the validity of this method with a more stringent proof of concept. A time trial study where the morphology score of a unit of stored blood would be monitored over the course of its 42-day storage lifespan would provide crucial insight on how the morphology score detected with our system correlates with the expected decline in RBC quality which would be reflected by a population shift towards the irreversibly changed morphology. This has been observed in other sRBC morphology time course studies (Berezina, et al., 2002); (Antonelou, et al., 2012). This study could be conducted with different sRBC units for a higher sampling size and with different operators to measure the effect of operator induced variability.

5.7.2 Potential for Research Applications

Our system could be applied in other research involving RBC storage lesion as a useful tool for comparing different storage/transfusion related phenomena. The notable correlation between RBC morphology and deformability has been a subject of interest in previous research (Meiselman, 1981); (Nagaprasad and Singh, 1998); (Hogman, 1985); (Park, et al., 2010); (Burns, et al., 2011). The associated phenomena of sRBC aggregability and endothelial cell interaction are also highly researched (Henkelman, et al., 2010). The mechanical changes that are reflected by the morphological shift from discocyte to spherocyte are a major factor in the decline of cellular deformability and increase in aggregability (Blasi, et al., 2012); (Longster and Buckley, 1972). Although many morphology scoring methods already exist as described in section 5.3, the method described in this thesis that uses a microfluidic device to change and debatably enhance the process of image capture to facilitate automated morphometric analysis could help these researchers quickly, easily, and accurately determine the morphological profile of the cells using the advantages of our approach to enhance the depth of their particular study (eg. testing
improved storage solutions, blood bag materials, effectiveness of leukoreduction (Antonelou, et al., 2012) etc.) with less effort. The high cellular throughput of our system makes it ideally suited for deformability studies where metrics are obtained from the behavior of a large population of cells (Burns, et al., 2011). Likewise, any research with the intent of studying a particular morphological sub-population of cells (e.g. spherocytes) could use our method to associate the results of a deformability/aggregability analysis with the presence or absence of that sub-population.

5.7.3 Clinical Potential

As described in Ch.2, the degradation of a sRBC unit population is complex and heterogeneous among individual cells. The expiration date system described in section 2.3.2, while sufficient for most clinical cases, fails to account for the multitude of variables that can change the shelf life of sRBCs, ranging from infection preventative gamma irradiation (Relevy, et al., 2007); (Ran, et al., 2011) to donor dependent factors (Hess, 2010); (Hess, et al., 2009), to the position of the bag while it was being transported (Liumbruno and AuBuchon, 2010). In one study, inter-donor variability was demonstrated to be the leading cause of in-bag hemolysis in sRBCs during storage (Hess, et al., 2009). Accepting storage duration as the sole indicator of expected transfusion efficacy makes an unnecessary assumption that could be circumnavigated with a device such as the one described in this project. Two separate 30 day old blood bags from different donors will never be identical in quality, even if they were stored the same way (Hess, 2012). There would be an advantage to being able to discern between the qualities of the two blood bags, especially at times when there are blood donor shortages.

If investigated further, the method used in this research could lead to the discovery of a consistent correlation between a morphology score obtained using our microfluidic device and
image analysis algorithm approach and the physiologically relevant mechanical/biochemical properties of the RBCs being analyzed. This information could lead to an inferable correlation between morphology score and the expected post transfusion efficacy of a stored blood unit, providing a quantitative quality assessment of individual units of sRBCs (De Korte, et al., 2008); (Longster and Buckley, 1972); (Meiselman, 1981). This type of quality assessment could provide hospitals and health clinics with a way to differentiate between the quality of two units of RBCs that are the same age for critical care patient prioritization and allow them to transfuse units that are beyond 42 days old (some 42+ day old units are still adequate for transfusion but are discarded by default). A U.S. Army Institute of Surgical Research study found that a combat support hospital in Baghdad, Iraq transfused 5,294 RBC units over the course of a year that averaged 33(+/- 6) days in age at the time of transfusion (Spinella, 2007). In difficult to access places such as combat zones where sRBC units are in great demand but are less accessible, the ability to assess the quality of older units to avoid wasting transfusion adequate units could be highly useful. Likewise, if there were ever an international blood shortage in the future (eg. due to the spread of an infectious disease), this clinical ability would become equally, if not more important.
CH 6: CONCLUSION

I have designed a novel microfluidic device suited to facilitate automated high throughput stored RBC morphology scoring. In this research, this device was incorporated with a microscope equipped with a digital camera and a MATLAB image analysis algorithm designed by other members of the lab. This unique system has been tested in terms of its accuracy, speed, and ease of use, and it was compared to similar methods in the same regards. Specifically, this microfluidic device was designed and fabricated using soft lithography and can accept a small, easily prepared sample from a sRBC unit and, while mounted on a microscope with proper digital image capture capabilities, has shown that it can provide an even and unbiased flow of a single cell thick plane of non-overlapping/fliping RBCs. We have demonstrated that this continuous single layer flow of RBCs imparted by the microfluidic device, when digitally captured in a flow rate dependent time interval, can be quickly (section 4.5) analyzed with a customized MATLAB algorithm (section 3.4), providing an accurate (section 4.3) and physiologically relevant (section 2.4) morphology profile of the RBCs that reflects the extent of their heterogeneous progression of storage lesion indicative of the population comprising the unit that the sample was taken from.

Microfabrication is a step dependent process and even the slightest error in design drafting, master wafer fabrication, PDMS molding, device fabrication, device preparation, or experimental procedure will result in images that are insufficient to use with our algorithm as this is an extremely sensitive application where 1µm can be the difference between success and failure, as shown in the channel depth determination section and explained in the discussion. However, the reward for meticulous attention to detail in microfabrication and experimental
preparation is a powerful tool that allows for less dependency on the algorithm for determining an accurate morphology score indicative of storage lesion in an efficient manner comparable, if not superior to other methods that have been used in relevant applications that depend on either painstaking manual image analysis or highly complex image analysis models that account for some but not all of the inevitable abnormalities that appear in images obtained from conventional blood smears.

Based on the relationship supported by previous research (Gettinger, et al., 2004); (Ho, et al., 2003); (Koch, et al., 2008); (Steiner, 2009) between known biological mechanisms of storage lesion and the clinical outcome of RBC transfusions in patients, it is proposed in this thesis that the comprehensive morphology profile obtained with our hybrid approach compiled based on an automated determination of thousands of individual RBCs abilities to survive in circulation post transfusion could provide the clinical advantage of differentiation between the quality of two sRBC units of the same age that, according to current convention, would be considered to have no discernible difference in transfusion efficacy, even though such a difference can and in many cases does exist (Hess, 2012); (Alfano and Michael, 2011).
APPENDIX

1. Abbreviations

AIDs.............Algorithmically Identified Discocytes
mPEG.............Methoxy- Polyethylene Glycol
PDMS..........Polydimethylsiloxane
PS...............Phosphatidylserine
RBC...............Red Blood Cell/Erythrocyte
sRBC.............Stored Red Blood Cell
TACO...............Transfusion Associated Circulatory Overload
TRALI............Transfusion Related Acute Lung Injury
II. Visually Detailed Explanation of Microfabrication Progress and Soft Lithography

1) The design is drawn on Clewin
2) The .cif file of the Clewin drawing is sent to Fine Line Imaging
3) The mylar photomask containing a negative stamp of the design is received
4) A 3" silicon wafer is selected
5) The wafer is spin coated with photoresist
6) The wafer is pre-baked to solidify and remove solvents from PR
7) The wafer and photomask are placed on the mask aligner
8) The mask aligner emits high intensity UV light through the photomask and onto the wafer
9) The wafer is postbaked
10) The wafer is treated with developer solution to dissolve unexposed photoresist
11) The wafer is rinsed in isopropyl alcohol
12) The wafer is left overnight in a vacuum desiccator exposed to trichlorosilane
13) Dome droplets of photoresist are added to inlet and outlet of each channel
14) The wafer is baked overnight
15) The wafer is given to 2 consecutive 3 second direct UV exposures.
16) The wafer is baked for an additional 5 hours
17) The wafer is left in the vacuum dessicator overnight for a second trichlorosilane treatment
18) The completed master wafer is stored in a petri dish where it will be used to mold PDMS blocks with microfluidic channel imprints.
LIST OF REFERENCES


Mr. Mathew I. Triscott earned his Masters of Science and Bachelors of Science and Engineering in Biomedical Engineering at Tulane University in May 2013. Both degrees were conferred simultaneously upon completion of the 5 year “4+1” program that he began in 2008. He worked as an intern at an in-vitro diagnostic company called Instrumentation Laboratory during the summers of 2010 and 2011. He is currently seeking employment as an engineer in Boston, MA, Silver Spring, MA, Austin, TX, Raleigh, NC, and New Orleans, LA. His career interests include design, marketing, and regulation of biomedical devices.