

Detection of changes in white blood cells after activation using a density gradient inside microcapillaries

Possibilities for detection of malignancy in patients

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Sammanfattning

Infektioner och autoimmuna sjukdomar påverkar idag 1.7 miljarder av världens befolkning [1]. Idag används det flera olika metoder för att försöka att diagnosticera infektioner och autoimmuna sjukdomar, men dessa metoder kan tyvärr inte skilja mellan infektioner på grund av en autoimmun sjukdom eller på grund av patogener. En metod baserad på att skapa en densitetsgradient inuti kapillärer skulle göra det möjligt att skilja mellan infektioner orsakade av patogener och autoimmuna sjukdomar. Metoden går ut på att en kapillär, fylld med droppar med olika densiteter och en droppe av antingen isolerade neutrofiler eller helblod, centrifugeras i 2-25 minuter. Neutrofiler som aktiverats på grund av en infektion, ett tecken på att immunförsvaret är aktiverat, kommer hamna på olika positioner längs med kapillären beroende på deras aktiveringsgrad. Neutrofiler och alla andra immunceller förändras beroende på deras aktiveringsgrad men även beroende på hur de aktiveras, d.v.s. beror aktivering på invaderande patogener eller autoimmunitet: De förändringar som immunceller genomgår vid aktivering kommer leda densitetsförändringar och detta faktum kan användas för att skapa en metod baserad på densitet för att upptäcka aktiveringsgrad. Metoden visade att neutrofiler hamnade på olika platser beroende på deras aktiveringsgrad. Vidare studier behöver göras med blod från patienter med olika sjukdomar, som påverkar immunceller, för att bestämma den kliniska relevansen. Projektet har öppnat upp för fortsatta studier av neutrofilers aktivering men även av metoder baserade på densitetsgradienter inuti mikrokapillärer. Metoden har visat sig vara lovande i kvantifieringen av aktiveringsgrad av neutrofiler men även som ett komplement till tillgängliga diagnostiska metoder för detektering av inflammation.

Abstract

Infections and autoimmune diseases are today affecting at least 1.7 billion of the world population [1]. There are today several methods used to try and diagnose infections and autoimmune diseases, but these cannot differentiate between infection due to different pathogens or autoimmune disease. A method based on a density gradient inside a capillary would aid in the differentiation between the different pathogens causing the disease and autoimmune diseases. The method amounts to a capillary, filled with drops of different densities and either isolated neutrophils or whole blood, inserted into a centrifuge for 2-25 minutes. The neutrophils, activated during infection and a sign for the immune system being activated, will end up in different locations along the capillary depending on their state of activation. The neutrophils, or any other immune cell, changes depending on their activation state but also on how they were activated, i.e. depending on pathogen but also if it is autoimmune, and this can be used to create a method based on density to detect these changes. The method was able to show that neutrophils ended up in different places based on their activation state. The project has opened up for further studies on neutrophil activation but also on methods based on density gradient inside of capillaries. The method has showed to be promising in quantifying the activation state of neutrophils but also as a complement to the available diagnostic tools for infection used today.

Keywords

Neutrophils, activation, density gradient, capillary, centrifuge, infection, autoimmune disease

Detection of changes in white blood cells after activation using density gradient

Populärvetenskaplig sammanfattning

Creation of density gradients in capillaries can make it possible to diagnose viral and bacterial infections but also autoimmune diseases.

Viral and bacterial infections are amongst the most common diseases worldwide. Autoimmune diseases, i.e. conditions where one's own body is attacking itself, are also becoming more common. Viral and bacterial infections and different autoimmune diseases may seem to be separate from each other but have in common that they lead to an activation of the immune system. There is today no fast and easy diagnostic method to evaluate if a disease is due to viral or bacterial causes or self-activation of the immune system. The most common way to evaluate if an infection, i.e. immune response due to virus or bacteria, is by measuring CRP (C-reactive protein). CRP-test will show elevated levels of CRP during infection, but it takes 8 hours after onset of illness to see a results and some medications can also affect the detection of CRP even during a serious infection. Also, a CRP-test does not show the cause of the infection [2] and because of that a blood culture, which takes over 24 hours, will be done to see if bacteria are present. Again, this method does not show virus and a separate test will be done to know if viruses are involved [3]. For conditions such as sepsis, i.e. uncontrolled activation of the immune system systemically and hence the immune cells, every second counts and false negatives can result in a patient's death.

Creating of a density gradient inside of a microcapillary, which will be put inside of a centrifuge for 2-25 minutes, may make it possible to diagnose cases such as sepsis but also other diseases affecting the immune

system. The density gradient inside a microcapillary works due to differences in density depending on the cells activation levels, ways of activation but also due to intracellular content. By using a method based on density gradient it can be possible to determine if a patient's symptoms are due to a viral or bacterial infection or if they are due to an autoimmune disease since these diseases will activate the immune system differently and therefore lead to different spreading of immune cells along the capillary.

Methods available today such as CRP-test, are mostly dependent on detecting changes in levels of substances present during inflammation, e.g. C-reactive protein. For cases such as sepsis but also for patients taking some specific medications this dependency on detecting substances in the body can result in false negative readout even if the body's immune cells are fully activated. The false negative is due to sepsis and the medication affecting the production of the inflammatory substances in the body and the false negative will in turn lead to more tests being done, which take longer time, or going around with a undetected infection which can lead to severe consequences. The proposed method can make it possible to in a fast and easy way determine if an infection is present and what it is caused by depending on the cells distribution along the density gradient created inside of the microcapillary, which is a direct quantification of their activation state. The method will also make it possible to detect the cases that otherwise would have been overlooked by the methods available today and potentially saving lives.

1. Introduction and background

The human body has always fascinated researches because of its complexity but also due to the lack of knowledge regarding many reactions/processes in our bodies. There has been countless research done to understand the body and there is still more needed to be done to grasp the human body's full complexity.

Blood has always been of interest both historically but also scientifically. Blood contains a vast number of cells that all have specialized functions and are of great importance. Blood contains red blood cells, white blood cells, thrombocytes, and many different proteins etc. which all are circulating in our body and most in their non-active state. The red blood cells are the carriers of oxygen, thrombocytes are important during injuries to clog wounds, the proteins have many different roles and can be involved in the immune response and last but not least we have the white blood cells which are our defenders against invaders. White blood cells are crucial for our survival and protects us from harmful substances, microbes, viruses etc. This defense is tightly controlled since the cells that are meant to protect us can, if the inherent control is faulty, start attacking healthy tissues and organs and harm us. The control of the defense is crucial, i.e. that the white blood cells are activated correctly, but so is also the number of cells since a low number can result in a poorer defense against intruders but also a weak immune system. An increased number of white blood cells on the other hand can signal some kind of cancer/leukemia and is a result of the precise control system that the body is faulty.

Detecting the changes on either the number of white blood cells or their activation can give a greater understanding for how they are regulated but also an easier and faster way to detect these abnormalities. White blood cells are involved in many diseases ranging from autoimmune disease to leukemia and deficit immune system, which are affecting a great number of people around the world [4]. There are today no easy and fast way to detect abnormalities in white blood cells since most diagnostic tools need

a specialist to look at the white blood cells to determine what the problem is or a manual count is needed, computerized counting has been developed and is used more and more, but trained personnel still has to do a lot of manual work.

There are today several methods developed to help the healthcare personnel to evaluate if a patient is having an infection or not directly instead of cell counts, by using quick tests like CRP and ESR. CRP, C-reactive protein, shows changes in concentration of a protein involved during infection and an elevation will indicate an infection. CRP only takes 5 minutes but has a small detection window [2]. ESR, erythrocyte sedimentation rate, on the other hand takes one hour but has a longer detection window than CRP. ESR measures how fast red blood cells are clumping together, which is dependent on levels of fibrinogen, and an elevated ESR indicates infection, i.e. elevated levels of fibrinogen [5].

In the case of sepsis, an uncontrolled activation of immune cells in response to invading pathogens, blood culture is the mostly used diagnostic tool to find pathogens in the blood since both CRP-test and ESR are too unspecific and not always able to detect infections. CRP-test and ESR that normally are used are indirectly showing if immune cells are activated or not by measuring proteins released during an inflammation. Unfortunately blood cultures, which specifically show the presence of bacteria, takes a long time, approximately 48 hours or more depending on the pathogen, and for patients with suspected sepsis every second counts since the survival chances decrease the longer it takes to confirm the disease [3]. Not only does it take a long time to get results but the results can also give false negatives which do not exclude the chances of a patient having sepsis. The need of a fast and easy method is therefore of great interest and in this master thesis a method using density gradient to be able to separate cells depending on their activation state is evaluated.

With the development of technology the tools used by health care is also developing and becoming more computerized but regardless of those changes there are still some problems needed to be solved, for example trying to in an easy and fast way separate the different blood components form each other and being able to separate activated from non-activated cells independent of e.g. detection of CRP, since the methods available today are not able to do so.

In this work I have evaluated to what extent blood cells' biophysical characteristics can be used to diagnose infections. In the future this can be extended to a multidimensional infection-signature through simultaneous measurement of several biophysical characteristics on single cell level. I have specifically in this work examined the changes in density of neutrophils as a result of activation.

1.1 Concept

The main purpose of this project is to see if it is possible to separate activated from non-activated immune cells by separation in a density gradient inside microcapillaries and visualizing the immune cells inside with a microscope. The neutrophils undergo changes during activation and depending on what stage they are their properties will change, e.g. granule production will increase, their size will change and they will become bigger and in the end after releasing their granule they will go into apoptosis. These changes that the neutrophil undergoes will not only result in changes in granule and size but also density. Normally the density of a neutrophil is approximately 1.1- 1.06 g/cm³, where the higher density is normal for non-activated cells meanwhile the lower density is most often seen in activated neutrophils. The changes in density should be big enough to be able to be detected using a method based on density gradient [6]. Since the density changes are present, a method based on creating a density gradient inside a capillary can possibly be used and neutrophils could be separated based on their activation stage.

The first step of creating a density gradient inside of a microcapillary is having a solution, e.g. Iodixanol, with different densities. Drops from the different solutions and either enriched neutrophils or whole blood will be placed on a hydrophobic plate, see fig.1. The drops will later be sucked into a capillary by angling it 45° and putting the tip on the drop, see fig.2. The capillary force will result in the drop being sucked into the capillary. Repeat the previous step for all the drops in the plate and after the last drop add some kind of plug to the capillary to keep the liquids inside of the capillary, see fig.3 and fig.4. The capillary, with the density gradient and cells inside will later be placed inside of a centrifuge for 2-25 minutes on 700-13550 RCF (relative centrifugal force). After the centrifugation neutrophils of different activation stages, e.g. activated and non-activated, should end up at different places along the capillary, see fig.4.

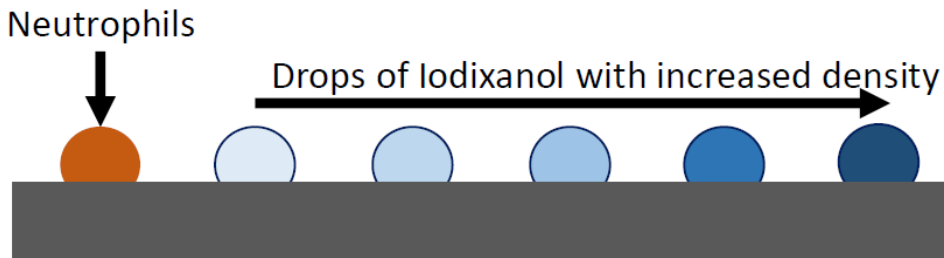


Figure 1: The drops of cells, i.e. neutrophils, and Iodixanol with different densities were placed on a plate with a hydrophobic surface.

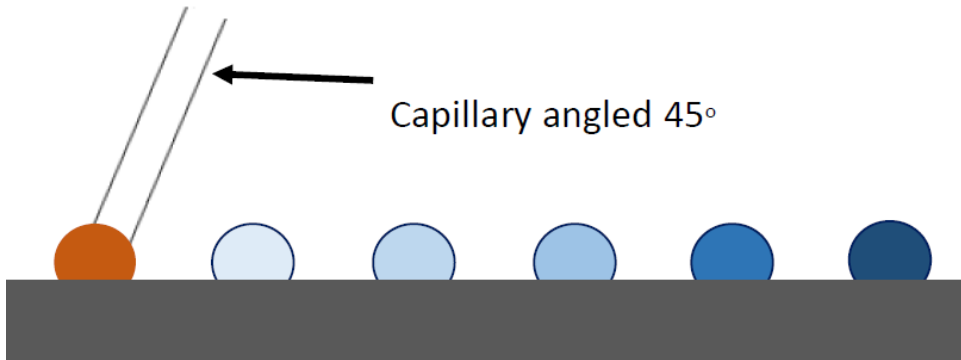


Figure 2: Angle the chosen capillary 45° to achieve best suction of the whole droplet into the capillary and decrease the amount of residue left on the plate. The more the surface is hydrophobic the better will the result be.

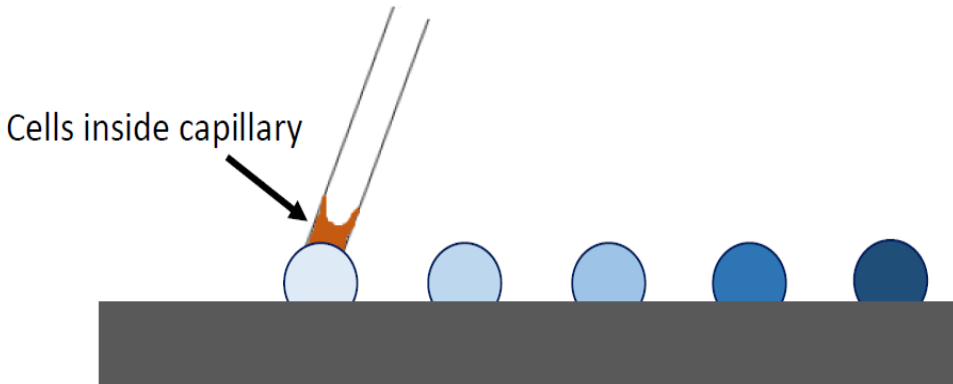


Figure 3: After placing the capillary on the first drop, capillary action will pull the drop will get inside of the capillary. Move to the second drop and repeat the same procedure as with the first drop, i.e. angle the chosen capillary 45°.

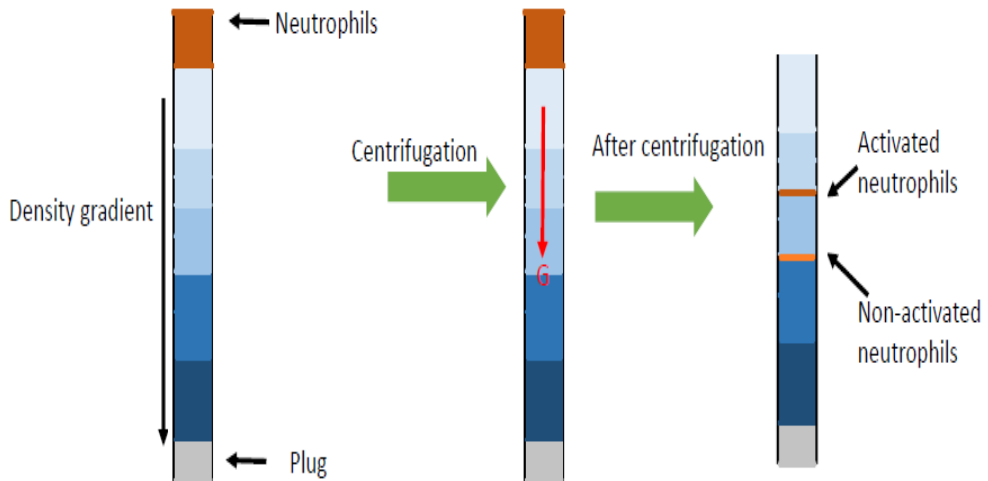


Figure 4: After all the drops are inside the capillary, plug it using adhesive dabs after sucking in the last drop. Take the prepared capillary and insert it inside a centrifuge for 2-25 minutes on 700-13550 RCF. After the centrifugation the activated and non-activated neutrophils will be separated and end up in different locations along the density gradient created with different concentrations of Iodixanol.

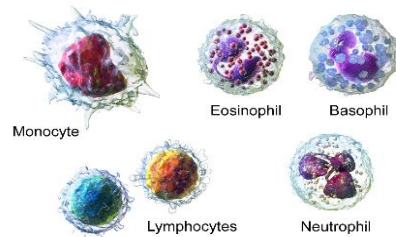
The proposed method can also be applied to other cells than neutrophils, but in this work focus has been put on the different activation states of neutrophils. The changes that immune cells undergo may be different and triggered by different stimuli but they all lead to changes in density, which is a prerequisite to be able to use a method based on density gradient inside of microcapillary.

1.2. White blood cells

White blood cells are of great importance for our immune system and are vital for our ability to resist viral and bacterial infections. White blood cells are divided into five main types, due to their difference in structure but also cell division lineage;

monocytes, neutrophils, lymphocytes, eosinophils and basophils [7]. The function and morphology of these cells are different but they are all involved in protection of the body against the unknown, even though the cells have their own way of protecting us. At times this protection against the unknown and unwanted can backfire

and start attacking our own cells, i.e. as in the case of autoimmune diseases, or the body will start producing defect white blood cells, as seen with infectious mononucleosis. The activation of white blood cells itself will result in changes of the cells morphology [8]. The fact that activation of the white blood cells result in changes in their appearance has been known for a while and has been studied. Not only does the white blood cells have changes in their morphology but also in the number of cells present in the blood due to e.g. bacterial infection or viral infection but also different diseases, such as leukemia and different autoimmune diseases etc. The changes that can be seen in the morphology of the white blood cells can, as mentioned, be due to a disease but it can also be due to vitamin deficiency, i.e. hypersegmented neutrophils can be seen when a patient has a deficiency in either folic acid or vitamin B₁₂, and to be able to detect these deficiencies a microscope and highly trained health care personnel is needed [9].



White Blood Cells

Figure 5: The different kinds of white blood cells present in human blood [10].

The activation of neutrophils will differ depending on of the activation is due to pathogens or autoimmune disease, since neutrophils in autoimmune diseases have shown to have abnormalities in phenotype but also function in different autoimmune disease. Since neutrophils are having abnormalities in their function meanwhile neutrophils involved in

infection caused by pathogens have their functions intact it should make it possible to differentiate an infection caused by an autoimmune disorder or due to invading pathogens [10]. Not only are there changes in the neutrophil function but also depending on what is causing the infection different cells will be activated, i.e. during a parasitic infection eosinophils are the primary immune cells being activated etc., and this can also be used to be able to differentiate between the different causes for an infection [9].

Being able to detect these changes seen in white blood cells using a density gradient would make it possible to in an easy way determine if an infection is present in the body, without expensive devices or highly trained personnel. The separation of white blood cells due to their activation state will also make it possible to examine samples easier, since the personnel will not need to go through every blood cell and will only need to look at the abnormal cells, since they will be separated. Not only will it possibly make it feasible to find differences in cells due to activation but also due to their maturity and changes in their internal composition. This information can later be used for diagnostics of diseases.

1.2.1. Monocytes

Monocytes are the largest among the white blood cells (i.e. WBC or leukocytes) and is part of the innate immune system, i.e. the system that provides an immediate defense against infection but is not specific.

Monocytes also influences the adaptive immune system, i.e. which has a more specific response to an infections and can eliminate or prevent pathogen growth. Monocytes, constituting ~5% of the total WBC, will migrate from the blood into the peripheral tissue and differentiate to

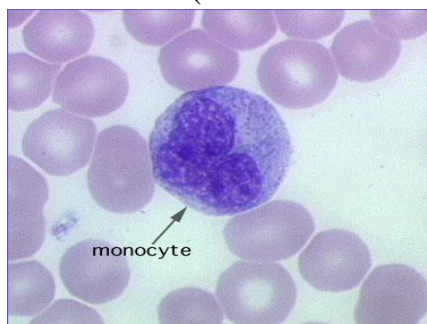


Figure 6: A blood smear showing the monocyte and red blood cells, the pink/red circles [12].

macrophages or dendritic cells, which are tissue resident. Macrophages are “all eaters” that protect tissue from foreign substances but also gets rid of and digests dead cells and other debris from the body. Dendritic cells and macrophages are also a bridge between the innate and adaptive immune system. The macrophages and the dendritic cells will process the antigen material and present it on their cell surface to be able to present it to T-cells, i.e. T-lymphocytes. The function of monocytes and the cells it can differentiate into can be summarized into three parts:

1. Phagocytosis: Process of the uptake of particles or microbes that later will be digested and destroyed.
2. Antigen presentation: Antigens are presented on its surface, which is a fragment of the digested microbe etc., to be able to activate the adaptive immune system.
3. Cytokine production: The production of substances that can regulate the functions of the monocytes or the chemotaxis, i.e. the process in which the monocytes finds its target etc. by moving towards a concentration gradient of a certain substance e.g. secreted by a microbe.

1.2.2. Lymphocytes

Lymphocytes are compared to the other white blood cells are more common in the lymphatic system than in blood. There are three major divisions of lymphocytes, B-cells (or B-lymphocytes), T-cells (or T-lymphocytes) and natural killer cells (NK-cells), and these in turn can have their own subdivisions. B-cells are involved in making antibodies that in turn can bind to pathogens, block any invasion by pathogens, activate the complement system (a system that is part of the innate immune system and enhances the ability of antibodies and phagocytic cells to get rid of microbes and damaged cells) and enhance destruction of pathogens. Natural killer cells on the other hand are able to kill cells that do not display MHC class I molecules (located

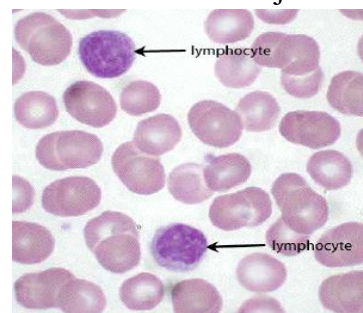


Figure 7: A blood smear showing the lymphocytes and the red blood cells, the pink/red circles [12].

on the surface of cells and has the function of displaying fragments of non-self-proteins within the cells to cytotoxic T-cells) or that displays stress markers, such as MIC-A. The importance of getting rid of cells that do not display MHC class I molecules, i.e. decreased expression, or have upregulated MIC-A is that those are signs that cells are infected by a virus or intracellular pathogen but it is also a sign that those cells can become cancerous. T-cells are more complex than both B-cells and natural killer cells and are divided into several subdivisions depending on their main task.

T-cell subdivision:

- **Helper T-cells:** Bind to antigen peptides presented on MHC II complex, showing non-self-protein fragments, on the surface of antigen presenting cells, such as dendritic cells, B-cells, phagocytes and some endothelial cells. Helper T-cells synthesize cytokines (cell signaling molecules that affect behavior of other cells) and other substances which coordinate the immune response.
- **Cytotoxic T-cells:** These cells bind to antigens presented by MHC I complex of virus-infected cells or tumor cells and kills them.

1.2.3. Eosinophils

Eosinophils constitute about 2-4% of the total white blood cells and the number of these cells fluctuate during the day/season but also due to hormonal changes, such as during menstruation. Eosinophil numbers rise as a response to allergenic substances, parasitic infections, collagen diseases but also during diseases in the spleen and the central nervous system. Eosinophils are rare in the blood and are instead more common in the mucous membranes of the respiratory, digestive and lower urinary tract. The main function of the eosinophils is to deal with parasitic infections but also has a predominant role in allergic reactions.

Eosinophils secrete chemicals that will destroy the parasites that otherwise are too big for the other white blood cells to phagocyte.

1.2.4. Basophils

Basophils are chiefly responsible for allergenic and antigen responses and has a role when tissue is injured. Basophils releases histamine as a response to those stimuli mentioned. Histamine has many roles in the body but the main role is to dilate blood vessels, which increases the blood flow to the injured area. Basophils are the rarest of all the white blood cells and constitute 0.5% of the total WBC. The histamine will, as mentioned, dilate blood vessels and this will not only increase the blood flow to the affected area but also make blood vessels more permeable so that neutrophils and clotting proteins easily can reach the injured area. Basophils also secretes heparin, which is an anticoagulant that inhibits blood clotting but also promotes recruitment of white blood cells to the affected area. Basophils can also secrete chemical signals to specifically recruit eosinophils and neutrophils to an infection site.

1.2.4. Neutrophils

Neutrophils are the most abundant white blood cell, constituting 60-70% of the total circulating white blood cells.

These have the same phagocytic functions as macrophages, i.e. they digest unknown substances and microbes. They mostly protect us from bacterial and fungal infection and are the first to respond to a microbial infection. In comparison to the monocytes, which can survive after digesting a few pathogens, neutrophils die after digesting pathogens. Neutrophils are the first to be recruited during the healing of a wound but also the most common cell type seen in the early stages of

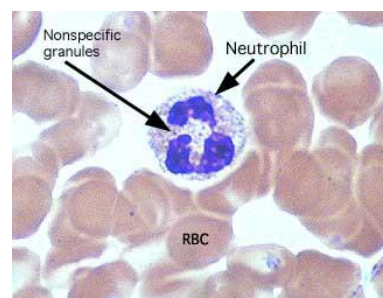


Figure 8: A blood smear showing a neutrophil and its granules, but also red blood cells (marked as RBC) [12].

an acute inflammation. Neutrophils are responsible for forming pus, pus is dead neutrophils that have died after being activated and digesting potential microbes at the wound site and other debris. Neutrophils can phagocytose cells, release a cocktail of chemicals that will damage cells and tissue and activate and recruit other neutrophils. Neutrophils contain a variety of degradative enzymes, such as proteases, hydrolases, nucleases etc., in their granule but also have the ability to generate reactive oxygen species (ROS), cytokines, chemokines and other regulatory molecules. The enzymes and ROS can act together to kill a large variety of microbes, due to their ability to damage microbes but also cell membranes which in turn will lead to cell death, and this is one of the reasons for why neutrophils have the highest cytotoxic potential amongst all the immune cells.

Normally neutrophils are circulating in the blood in their non-active state and are activated by regulatory or chemotactic signals, i.e. cytokines, chemokines and host- or pathogen-derived factors, which in turn will make them move from the blood to the infection site. During its transition from the circulation to tissue neutrophils undergo a process called priming. The priming will result in changes in the neutrophil, such as changes in their surface properties of the cell via movement of the cytoplasmic granule to the cell surface but also activation of de novo gene expression, i.e. expression of a certain gene in a greater extent than before due to the activation. Priming is resulting in neutrophils having a greater cytotoxic capacity, an extended lifespan but also enhanced functions to be able to be as effective as possible during an acute inflammation.

Neutrophils have a key role in the host defense and these cells contribute to the tissue damaged seen during an inflammation but also the tissue damage associated with inflammatory diseases. Both human and animal studies have implicated that the products, i.e. proteases and ROS, from neutrophils that are released during their active state result in tissue and organ damage associated with human disease, such as rheumatoid arthritis, vasculitis, chronic obstructive pulmonary disease and inflammatory bowel disease, such as IBS. Neutrophils can in these diseases infiltrate tissue and become wrongly activated, as a result of an infection or via immune complexes, and start to secrete molecules that

normally should be retained in vesicles. These vesicles contain molecules that can attack healthy tissue, if they are of higher concentration than endogenous tissue levels of anti-proteases or anti-oxidants. Neutrophils don't only have a direct effect in initiating tissue damage in inflammatory diseases but are also involved in regulating inflammatory functions of other immune cells, i.e. by releasing regulatory molecules, chemokines and cytokines. Neutrophils are important during the response of inflammatory signals by generating a cytotoxic response but are also involved in the control of hematopoiesis, i.e. formation of blood components, and modulating the function of B- and T-cells.

1.2.5. Immune cell abundance

As mentioned previously the abundance of immune cells differ and table 1 shows the percentage of each of the white cells present in the whole blood.

Table 1: Summary of the relative abundancies of different leukocytes in whole blood [9].

Neutrophils	60–70%
Basophils	0.5–1%
Eosinophils	2–4%
Monocytes	3–8%
Lymphocytes	20–25%

1.2.6. Tissue resident leukocytes

Some of the white blood cells will migrate to tissue and stay there permanently instead of circulating in the blood. These cells will often have a special name depending on which tissue they have settled in, i.e. fixed macrophages in the liver are called Kupffer cells. The most common resident leukocytes are:

- **Dendritic cells:** Present in tissues that are in contact with the external environment, such as skin, lining of nose, stomach, lungs and intestines. Important to note that dendritic cells will migrate to lymph nodes after ingesting antigens to show them to lymphocytes.
- **Mast cells:** Contains granules rich in histamine but also heparin. Have the same function as a basophil but are resident and develop from another hematopoietic lineage than basophils.
- **Microglia:** Resident macrophages in the central nervous system, i.e. the brain and spinal cord. Microglia act as the first and main immune defense in the central nervous system.

The presence of these resident immune cells in the peripheral blood is in most cases a symptom of a pathogenic disease, such as rheumatic disease etc. These cells would be possible to differentiate and detect using the proposed method, i.e. a density gradient inside of a microcapillary, which in turn would make it possible to easily detect resident immune cells that should not be present in blood normally.

1.2.7. Density of immune cells

As has been mentioned throughout the Introduction, white blood cells and red blood cells in the blood have different densities. The inherent differences in density amongst blood cells, see fig.9, makes it possible to use a method based on density gradient to separate the blood cells.

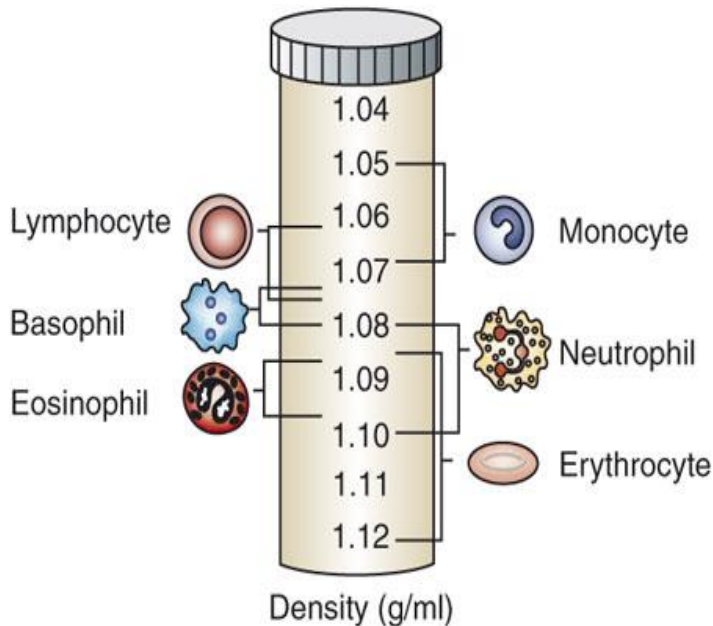


Figure 9: The different blood cells have different densities and will therefore end up in different locations along a density gradient [10].

1.3 Diagnostic tools for infection in health care

In primary health care there are many diagnostic tools used to determine if an infection is present but also the severity of the infection. As with any methods used there are some inaccuracies that may be present, e.g. false negatives or false positives. There are several methods used today in the different departments in healthcare/health services, e.g. care centers and infection unit in the hospital etc.

In care centers the most used diagnostic tools are: CRP-test, erythrocyte sedimentation rate (ESR) and blood cultures. In the infection unit the most common tests are more concentrated on finding antigens from certain viral/bacterial/parasitic infections or antibodies against these pathogens, detection of immunosuppression (immune system is deficient) and detection of autoimmune diseases and different proteins involved in the immune response, such as cytokines and other markers, The infection unit is also interested in doing white blood cell differential/count, i.e. counting either the number of a certain white blood cell or determining the percentage of a certain white blood cell in the blood. These tests will give the healthcare provider different information but they also have different accuracies.

Blood cultures are used to determine if there is an infection, and this takes 2 hours to 7 days to get results depending on the pathogen cultured. Blood tests can also be done and are much faster, e.g. a CRP-test (C reactive protein) or erythrocyte sedimentation rate (ESR). CRP-tests and ESR are very common in care centers and are most often the first tests done when a doctor is suspecting some kind of infection [9]. CRP-test takes 5 minutes to get a result and the main drawback is that there is a window for when CRP is detectable in the blood. For many patients CRP-tests may show that they have normal levels, even though their white blood cells have been activated and they have a big inflammation [2]. ESR is an old method that takes 1 hour to do and will show the changes that otherwise would have been missed by CRP, though it is important to note that again not all changes can be detected and that false negatives are common [5]. There are also today different tests to determine if the most common pathogens are present, e.g. pathogens causing common cold such as bacterial infection by group A Streptococcus, which takes 5 minutes to do. For other pathogens, blood cultures are done to determine which specific pathogen is causing the disease, and the time to get a result can range from 2 hours to several days depending on the pathogen that is of interest [3].

In the infection unit there are more advanced tests being conducted since their patients have more serious diseases than a common cold, e.g. severe autoimmune diseases or severe infections such as sepsis, or are undergoing chemotherapy and are immunosuppressed. In the cases for patients in contact with the infection unit the tests are more concentrated on the number of white blood cells and different markers for infection, such as different proteins involved in the immune response and activation

of white blood cells. Based on the specific protein being evaluated or a white blood cell count takes between 1 to 2 hours or several days to get a result. Not only are certain infection markers of interest but some cases receptor expression is of interest and in those cases specific antibodies labelled with a fluorophore, which will bind specifically to a certain receptor, can be used and evaluated with flow cytometry [9].

Regardless if it is the primary health care or infection unit, an easy and fast test based on cells biophysical properties, which is giving blood cells infection-signature, which give clinically useful information in addition to the currently available methods it sought after. The infection-signature would be able to detect activation of immune cells in the cases in which the available methods today are insufficient, e.g. in cases of sepsis.

1.4. Central point of concept

In this work I have examined cells density, more specifically neutrophils density. By creating a density gradient inside of microcapillaries cells of different densities can be divided into different sections along the capillary. The separation of the different components of the blood can be done by density since the subpopulations of blood cells but also the red blood cells have different densities. The granulocytes are generally denser than agranulocytes but less dense than red blood cells. A separation based on the cells activation state should also be possible since they undergo different changes in their morphology that in turn will result in changes in density [6], a prerequisite for separation to be possible using a density gradient.

2. Theoretical background

2.1 Sedimentation

Sedimentation is the tendency in which particles in a suspension settles in the fluid in the location in which the density of the particle is equal to the density of the suspension. When creating a density gradient with a medium and adding a mixture of particles of different densities it is possible to separate them using a centrifuge. The separation is possible due to the motion of the particles through the fluid in response to the forces acting on them, these forces can be everything from gravitational to centrifugal acceleration to magnetic, electric, di-electric and acoustic forces. In this experiment a centrifuge will be used to create the force acting on the particles respectively the cells. As mentioned the forces acting on the particles/cells can be either due to gravity or as a result from the test tube being put in a centrifuge. The forces acting on the particles will be from gravity or centrifugal, as in this experiments case, and will act to drag the particles down meanwhile at the same time there will be a counteracting force acting on the opposite direction, i.e. the drag force, see fig. 10.

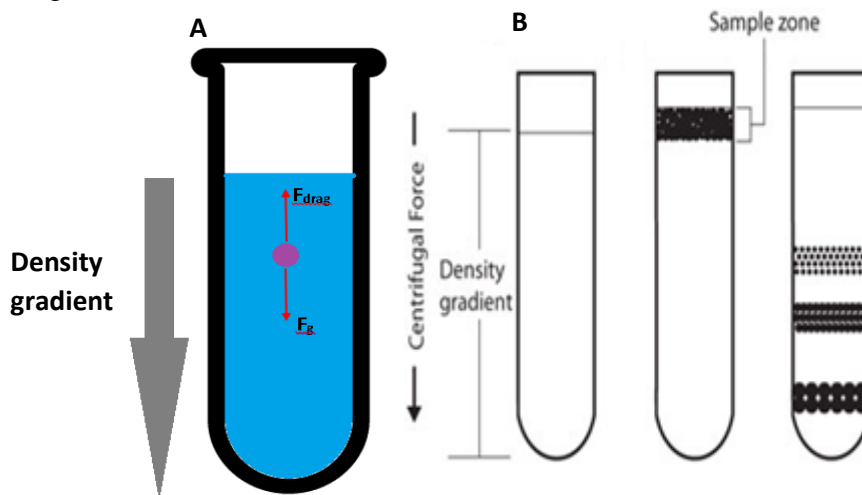


Figure 10: The forces acting on the purple particle suspended in a density medium. The forces are F_{drag} , i.e. frictional force also known as Stokes drag, and F_g which can be gravitational or in this case centrifugal acceleration, see A. The particle will after being centrifuged end up in the position that best matches its own density, i.e. in the place where the density of the medium is either equal to or close to the density of the particle, see B [13].

The drag force, F_{drag} , or as it is better known as the Stokes drag, is a force acting on the interface between the fluid and the particles suspended in the fluid. It is a frictional force that is exerted on particles with very low Reynolds number, i.e. on very small particles, suspended in a viscous fluid. To be able to use the formula for Stokes drag some assumptions have been made, i.e. [14]:

- ✓ Assume laminar flow
- ✓ Have spherical particles
- ✓ The particles are made out of homogenous material
- ✓ The particles have a smooth surface
- ✓ The particles do not interfere with each other

The drag force (F_{drag}), see eq. 2.1, is as mentioned a force acting on particles suspended in a medium and is dependent on the viscosity of the medium (η) the radius of the particle (a) and the terminal velocity of the particle (v_{sed}).

$$F_{\text{drag}} = 6\pi\eta av_{\text{sed}} \quad (2.1)$$

As mentioned previously the applied force which will cause the particles to be accelerated to their terminal velocity in this experiment will be the centrifugal force. The centrifugal force (F_g), eq. 2.2, is dependent on the centrifugal acceleration (G), see eq. 2.4, the difference in density between the particle, denoted p , and medium, denoted m , it is suspended in ($\Delta\rho = \rho_p - \rho_m$) and the volume of the particle (V_p), see eq. 2.3.

$$F_g = \Delta\rho V_p G, \text{ where} \quad (2.2)$$

$$V_p = \frac{4\pi a^3}{3} \quad (2.3)$$

The centrifugal acceleration is the constant that can be changed by the operator easily and fast directly on the centrifuge used, and this just by changing the RPM, rotation per minute, see eq. 2.5. The centrifugal

acceleration if dependent the angular velocity (ω) the distance from a chosen position on the tube to the rotor (R) and the buoyant mass (m).

$$G = Rm\omega^2 \quad (2.4)$$

The centrifugal acceleration, i.e. G-force, could not be measured directly and was instead calculated by converting the RPM, i.e. the rotation of the centrifuge per minute, to the G-force, see eq. 2.5. The centrifugal acceleration can, as mentioned, easily be changed by changing the RPM on the centrifuge. The conversion from G to RPM is dependent on the radius center of the rotor to a point in the sample/capillary (r_c), see eq. 2.5.

$$RPM = \sqrt{\frac{G}{r_c}} \quad (2.5)$$

During the sedimentation experiment the applied force accelerates the particles in the fluid to a terminal velocity at which the applied force is exactly cancelled by the opposing drag force, i.e. $F_{drag}=F_g$, see eq. 2.6. Because these two opposing forces will be equal when the particle has reached its terminal velocity it is possible to calculate the terminal velocity, v_{sed} and see eq. 2.7, but also the time it takes for the particle to reach its equilibrium position, i.e. where $\rho_p=\rho_m, t_{sed}$ [14].

$$F_{drag} = F_g \quad \leftrightarrow \quad 6\pi\eta a v_{sed} = \Delta\rho V_p G \rightarrow \quad (2.6)$$

$$v_{sed} = \frac{\Delta\rho V_p G}{6\pi\eta a} = \frac{\Delta\rho \frac{4\pi a^3}{3} G}{6\pi\eta a} = \frac{2\Delta\rho a^2 G}{9\eta} = \frac{2\Delta\rho a^2 \omega^2 R}{9\eta} \quad (2.7)$$

The time for the particles to reach its equilibrium state can be deduced from eq. 2.7 by integrating the velocity, see eq. 2.8 to 2.9.

$$\frac{dR}{dt} = v_{sed} \rightarrow \frac{dR}{dt} = \frac{2\Delta\rho a^2 \omega^2 R}{9\eta} \rightarrow \frac{dR}{R} = \frac{2\Delta\rho a^2 \omega^2}{9\eta} dt \rightarrow \quad (2.8)$$

$$\rightarrow \ln(R_2 - R_1) = \frac{2\Delta\rho a^2 \omega^2}{9\eta} t_{sed} \quad (2.9)$$

The final step done to get the time for the particle to reach equilibrium is to take eq. 2.9 and move the different constants until t_{sed} is alone, see eq. 2.10.

$$t_{sed} = \frac{9\eta \ln(R_2 - R_1)}{2\Delta\rho a^2 \omega^2} \quad (2.10)$$

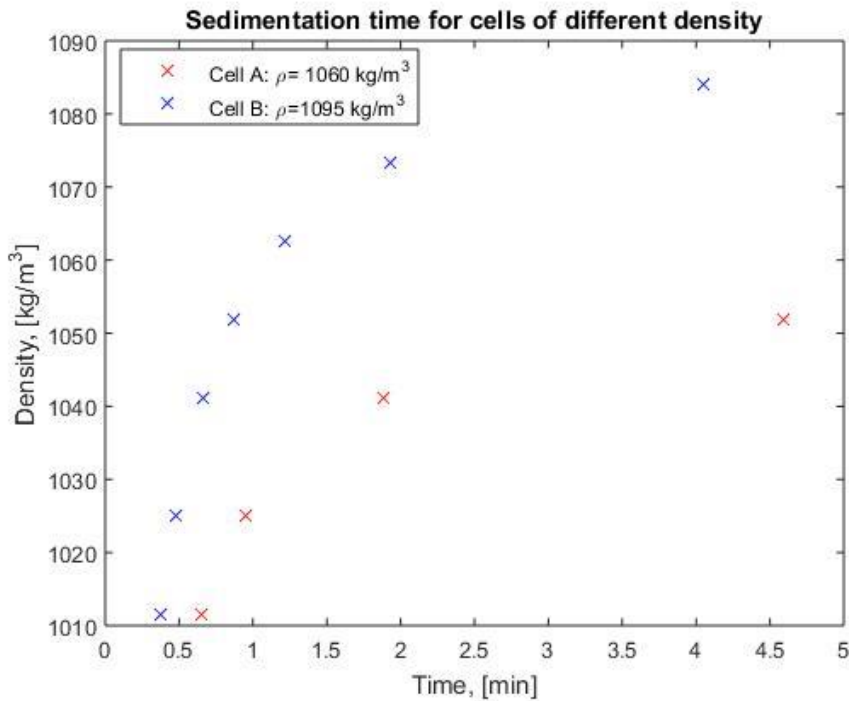


Figure 11: The plot is showing the time it takes for a cell to reach its equilibrium point, i.e. the point in the density gradient that matches the cells inherent density.

In fig. 11 it can be seen that depending on the density of the cell inside of the capillary it will take a different amount of time for it to reach its equilibrium point, i.e. the point that matches its inherent density, depending on the properties of the cell. In the purpose of highlighting the change in time depending on the density better the size and other parameters concerning the morphology, e.g. the size, of the cells were put constant. In fig.11 it is assumed that every step in the gradient is of equal height and the number of steps are equivalent to density changes, going from 1012 kg/m^3 to 1115 kg/m^3 Cell A which has a lower density reaches its equilibrium point after four steps and comparing to Cell B it reaches each step slower than Cell B, this is explained by that eq. 2.10, i.e. t_{sed} , will be shorter since the difference between the density of the particle and the medium will be greater for large densities. Cell B, with a higher density, reaches its equilibrium point after seven steps, see fig.11. The time it takes for cells to reach equilibrium is not only dependent on the density of the cells and medium but also on the RCF used, i.e. the higher the RCF is the faster will the cells reach equilibrium. Though, it should be noted that there is a trade-off between how fast the RCF can be and the end result, since immune cells, especially neutrophils, are more likely to be activated by the high RCF and even go into apoptosis. The time it takes to induce activation of immune cells by mechanical activation can be as short as a few seconds, which is one of the problematics when working with immune cells and especially neutrophils for health care.

3. Aims

The main purpose of this project is to see if it is possible to create a density gradient in a capillary and thereby be able to separate activated from non-activated neutrophils but also to image single cells inside of the capillary with a microscope.

Aims and specific actions:

1. Generating a density gradient in a capillary (Aim I)
 - a. Elaborate a method to create a density gradient along a capillary, in which particles can be separated based on their density.
 - b. Visualization the gradient: Is it possible to visualize the density gradient by adding colors or fluorophores to the density medium.
 - c. Demonstrate density-based separation of particles inside of a capillary based on differences in density.

2. Demonstrate separation of activated neutrophils (Aim II)
 - a. Find the safety regulations for handling of blood and protocols.
 - b. Separate neutrophils from whole blood.
 - c. See if it is possible to detect neutrophils inside of the capillary without a microscope
 - i. If no, can the cells membrane or cytoplasm of the cell be colored to achieve better detection?

3. Visualize separated single cells and particles inside the capillary (Aim III)
 - a. Identify suitable methods for visualization of single cells.
 - b. Compare capillary geometries to improve visualization of cells.

4. Method

The project's experimental part was divided into several parts, in which the first parts were method development and proof of concept and the later parts were the actual work with cells, i.e. white blood cells, and exploration of different visualization techniques. To be able to create a gradient for density-based separation and visualization of cells inside of a capillary the method described briefly in the section Concept was evaluated. The method also consists of interviews conducted to get a better understanding of the clinical relevance for the proposed method but also to identify which information clinicians and researches find relevant and important for their work.

4.1. Experimental method

This part is a more detailed description of how a density gradient is created inside of a capillary and a complement to Concept.

4.1.1. Sample preparation

The main objective with the experiment is to create a density gradient inside of a capillary and to do so some preparations are needed to be done. Iodixanol, density gradient medium used for cell and subcellular organelle isolation, of different concentrations were created by diluting Iodixanol with different amounts of PBS. The different liquids got different concentrations depending on the ratio of Iodixanol and PBS. The mixtures of Iodixanol and PBS were later placed on a hydrophobic plate, i.e. a plate created with glass slides covered in tape, with a pipette. The drops placed on the plate were not only different concentration of Iodixanol, but also particles, such as PMMA, Silica and polystyrene, cells and whole blood, see Appendix IV for handling of blood and enrichment of neutrophils from whole blood. For some of the experiments different colors/dyes were added to the Iodixanol mixtures, the colors were store-bought food coloring but also FITC-labelled Ficoll, i.e. Ficoll labelled

with a fluorophore. For a more detailed list regarding the materials see Appendix VI.

The volume (V_{cap}^x), see eq. 4.1 and eq. 4.2, of the capillary of length L (square (width x) and round (radius r)) was calculated and depending on the number of steps wanted in the gradient different volumes of the drops were used, i.e. divided the total volume of the capillary with the number of steps wanted. The droplets were placed on a plate, which was of glass but covered in duct tape to make the surface hydrophobic, to easily be able to suck up the drops into the capillary.

$$V_{cap}^{round} = \pi r^2 L \quad (4.1)$$

$$V_{cap}^{square} = x^2 L \quad (4.2)$$

4.1.2. Microcapillary

The drops created on the plate are later sucked into the capillary, following the method previously described in concept, by holding the capillary at a 45° against the top of the drop and repeating it for all of the drops. After the last drop place a finger at the top of the capillary to hinder the density gradient inside the capillary to spill out before the plug/adhesive is added to the bottom of the capillary. Later on, the capillary is placed inside a centrifuge at 2-25 minutes at 700-13550 RCF depending on the specific experiment conducted.

4.1.3. Visualization of the microcapillary

After the centrifugation the microcapillary is taken out and later placed on a setup created to hold the capillary and then imaged with the combination microscope with florescence and bright-field setting and the phase-contrast microscope vertically or horizontally, depending on the experiment. For the phase-microscope a ruler was placed in the close proximity to the capillary, i.e. both the scale of the ruler and the capillary could be visualized, and used to keep track of the position of the cells along the capillary. Depending on what is examined inside of the capillary the zooming and other properties will be changed accordingly.

4.2. Interviews

Achieving better knowledge about the immune cells in the body but also the potential clinical relevance of the project interviews were conducted. To gather different views a medical doctor working at the department of hematology and transfusion-medicine in Skåne University Hospital and a researcher and docent in among others rheumatology, inflammation and immunology. The different point of views but also the different expectations that they have on machines for detection of activated and non-activated neutrophils.

5. Results and discussion

The differences in density between the different blood cells should make it possible to do density measurements in a capillary, see fig.9. The method in which a capillary is used, in which a density gradient has been created, could make it possible to in an easy and fast way determine the density of cells and/or particles suspended in the density gradient, since the particles will stop at the point in the density gradient that matches their inherent density. To be able to test the accuracy of such a density gradient but also if it is possible to separate particles with different densities, particles of known density were used to be able to have a constant when examining the precision of the system at hand. The density gradient is created by sucking up drops of different concentrations of Iodixanol but also sodium-polytungstate (NPTS) into the capillary, which occurs due to the capillary forces. Particles and/or cells are sucked up and later the whole capillary, with the particles suspended in the density gradient, will be centrifuged for 2-25 minutes with the speed of 700-13550 RCF.

5.1. Capillary filling

The importance of having a hydrophobic surface of the plate was great to be able to achieve the best results when sucking up the drops into the capillary. The more hydrophobic the surface was the more insignificant was the residue left on the plate after sucking up the droplets. As can be seen in fig. 12 the five droplets to the left were laid on a more hydrophobic surface meanwhile the last drop to the right, which is more flat and has a smaller contact angle with the surface, was laid on a less hydrophobic section of the plate. More was left on the plate after trying to suck in the droplet to the right into the capillary than when sucking up the drops to the left, and this due to the hydrophobicity of the surface.

Besides the role of hydrophobic surface of the plate the angle of the capillary against the drop also played a role in achieving the best result. Using an angle of approximately 45° when holding the capillary towards the plate showed to result in the best results and less residue left on the plate (see fig. 13), though the angle had to be increased the more drops were sucked into the capillary and in the end an angle of 160° was needed to suck up the last drop, which has to do with gravity.

An issue that was present was air bubbles being created inside of the capillary, see fig.14, and affecting the gradient since it created a discontinuity of the gradient and resulted in the capillary not being used. There were times were the air bubble vanished after centrifugation but most of the times it never disappeared.

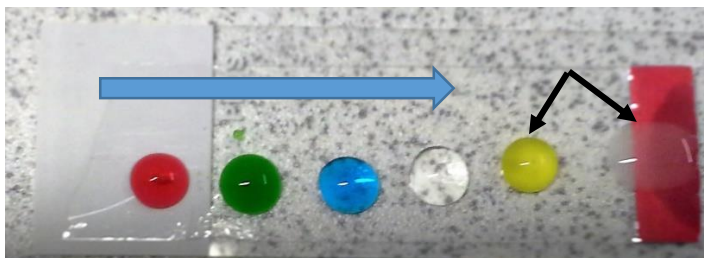


Figure 12: The droplets with different concentrations of Iodixanol but also sodium-polytungstate (NPTS, 75%w) and particles. The blue arrow shows how the density gradient goes from low, i.e. red droplet, to high density, i.e. white droplet. The black arrows point out the particles, i.e. polystyrene particles in the white droplet and Silica particles in the yellow droplet.

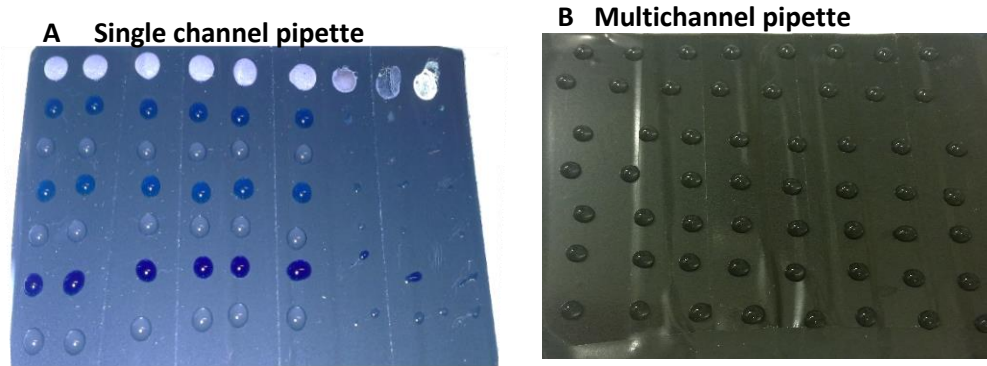


Figure 13: As can be seen in A some residue is left from the drops after being sucked into the capillary. Some of the drops in A were fully internalized by the capillary, see the column furthest to the right, meanwhile some left a tiny drop on the plate. In B a multichannel pipette was used to achieve better reproducibility, i.e. the droplets drawn into the capillary showed more uniform volume.

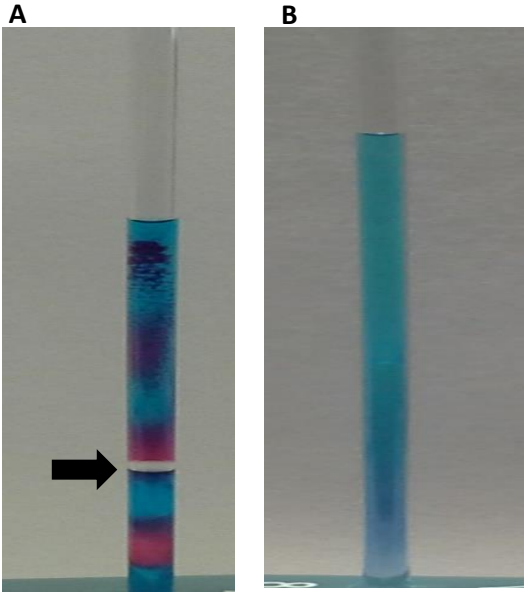


Figure 14: The problem with air bubbles, see arrow, getting created inside of the capillary can be seen in A and this bubble didn't disappear even after centrifugation. B shows a capillary with no air bubble. The blue and red are food coloring added to each density solution of Iodixanol to easier be able to see the density gradient.

For the primary experiment a pipette with a single channel was used, but later on a multichannel pipette was used. The multichannel pipette made it possible to achieve both better reproducibility but also better results when sucking in the drops, since the drops were more uniform and less residue was left on the plate, see fig.13 (A and B) for comparison.

5.2. Visualization of the gradient

Using a clear liquid to create the density gradients made it difficult to visualize the actual density gradient and not only the locations of the particles. To make it easier to visualize the gradient the different droplets with different densities of Iodixanol were colored using food coloring. The droplets of Iodixanol were colored blue, green and red and had the concentrations of Iodixanol 40%, 30% and 20% respectively, see fig. 12.

After putting the capillary into the centrifugation the red color was seen shedding its color and that the shedding after a while ended up at the same position as the polystyrene particles, i.e. at the place that has the density 1.05 g/cm^3 , see fig. 15. The blue food coloring didn't show the same phenomenon and it can be due to the difference in what type of substance was used to achieve a certain color. The red food coloring had the substance carmine in it meanwhile the blue had E133 and the difference in substance can explain why the red started shedding and why the blue did not.

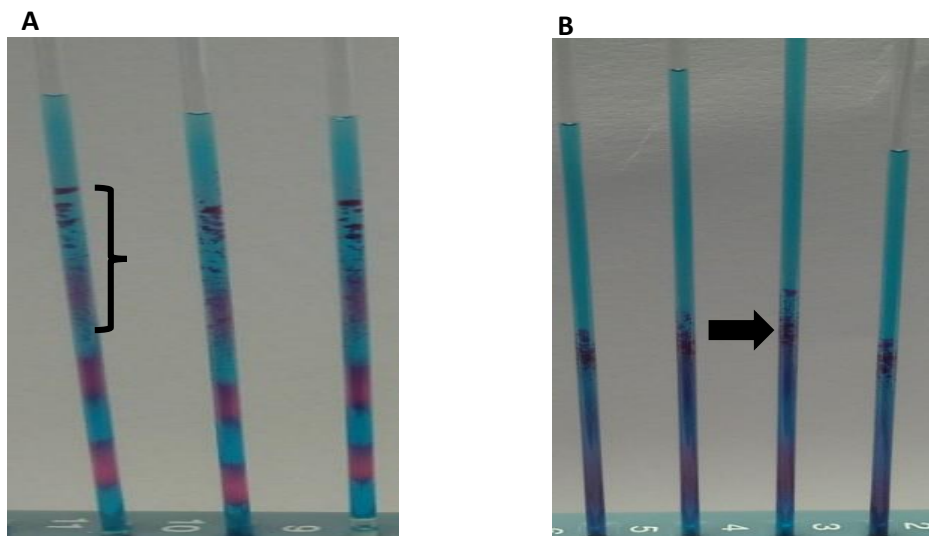


Figure 15: The shedding of the red food coloring can be seen in both A and B. In the beginning shedding could be seen at the places with lowest density, see bracket in A. After some time, the shedding from the red food coloring ended up at the same place as the polystyrene particles, i.e. at the location where the density is 1.05 g/cm^3 see arrow in B. The red food coloring did not shed when added to higher densities than 1.05 g/cm^3 .

The shedding of the color was unexpected and the final position of the shedding at the same location as the polystyrene seemed to be coincidental. The usage of food coloring to be able to visualize the density gradient was later left and new experiments were conducted using FITC-labelled Ficoll, i.e. Ficoll labelled with a fluorophore. Ficoll, i.e. FITC-labelled Ficoll + Ficoll, can be used to create a density gradient and to read out the density depending on the intensity of the fluorescence, due to it being labelled with the fluorophore FITC. Though, in the experiments conducted the density gradient was created with Iodixanol and the visualization of the gradient was done with FITC-labelled Ficoll, i.e. gradient where intensity of the fluorescence changes along the capillary.

To be able to enable quantitative measurement of the density tracing of fluorescence, by adding FITC-labelled Ficoll molecules instead of Iodixanol, was done. A good understanding of how the gradient behaves, i.e. if it is linear and does it have steps etc., will make it easier to evaluate the accuracy of the method but also makes it easier to be able to draw

conclusions based on where the cells end up along the capillary. FITC-labelled Ficoll of different concentrations were used to create a gradient based on how strong the fluorescence is, the higher concentration of FITC-labelled Ficoll the stronger will it shine, as can be seen in fig.16. The preliminary data, see fig.17 and fig.18, shows that the fluorescent is behaving as expected, i.e. a capillary filled with Ficoll should have high intensity along the capillary and a capillary that is empty or filled with water should have minimum intensity.

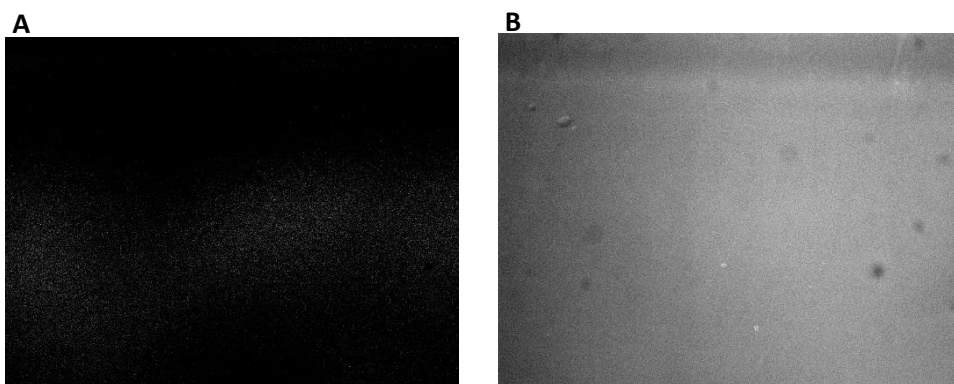


Figure 16: Images taken with a fluorescence microscope, and in (A) the capillary filled with water and in (B) the capillary had PBS with Ficoll labelled with a fluorophore added in high concentration.

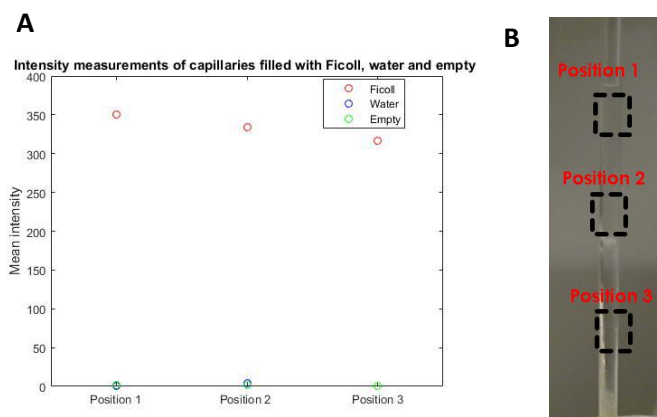


Figure 17: Measurements done along the width of the channel (A) shows differences in intensity along the height of the channel for the three different capillaries. The capillary with Ficoll, i.e. the fluorescently labelled molecule, is showing some fluctuations along the capillary. (B) shows the three different locations measured along the capillary's height.

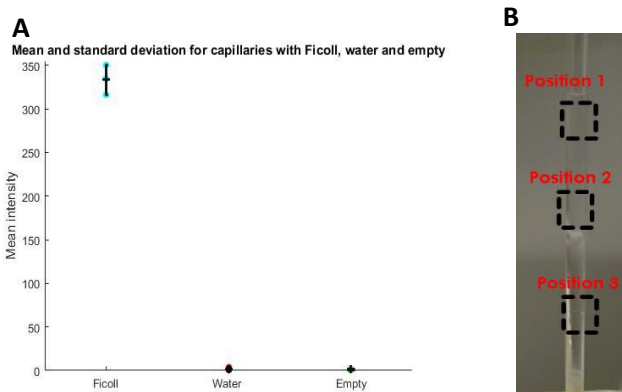


Figure 18: (A) shows the mean and standard deviation of the different measurements conducted for the capillaries filled with Ficoll, water respectively empty for three different positions. (B) shows the three different locations measured along the capillary's height.

There is a difference in intensity between the capillary filled with water, empty and Ficoll, which is expected since water/air should not have any fluorescence properties, which of course Ficoll has as a fluorescent, see fig.16. The preliminary results, see fig.17 and fig.18, show that Ficoll is behaving in an expected way, i.e. the fluorescence intensity is fairly constant, i.e. it shows some fluctuations since the intensity decreases from top to bottom, along the capillary when the concentration is constant, which made it a good candidate for further studies. The next experiments conducted where using Ficoll in different concentrations and using it to create a density gradient and later evaluating how the gradient behaves, see fig.19 and fig.20.

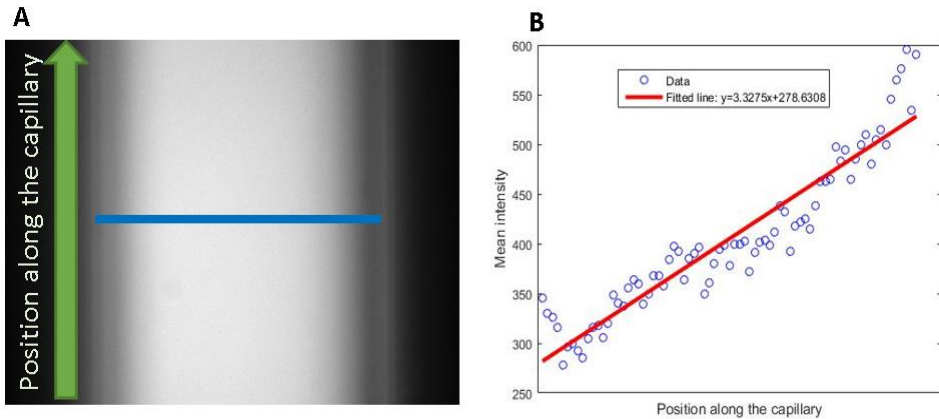


Figure 19: In A the mean intensity was obtained by taking the mean intensity along the width of the capillary. In B the mean intensity along the whole capillary was later used to fit a line. The concentrations used were 10%, 12.5%, 15%, 20%, 25%, 33% and 50%.

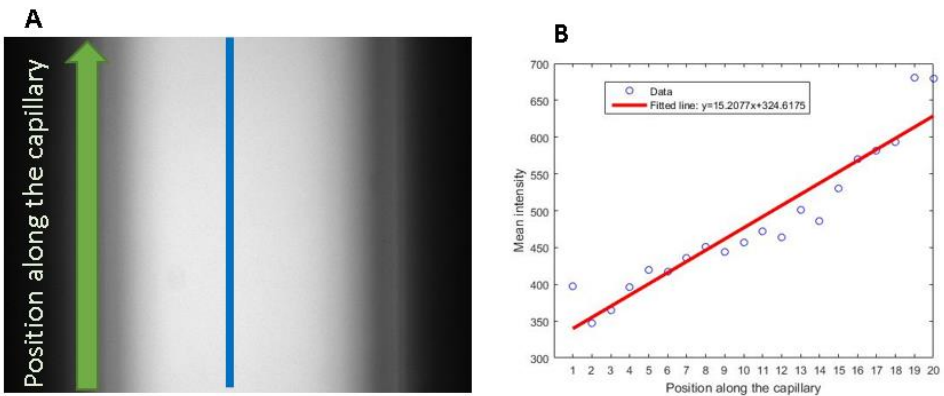


Figure 20: In A the same method was used as in fig. 17 but instead of taking the mean along the width of the capillary it was obtained for along the length. In B the mean intensity along the whole capillary was later used to fit a line. The concentrations used were 10%, 12.5%, 15%, 20%, 25%, 33% and 50%.

As can be seen in both fig.19 and fig.20 the intensity increases with increased concentration and behaves in a linear way. The linear behavior is not expected since the figures 19 and 20 should have showed a step like behavior since the gradient is created with drops in which the density increases in a step like manner. The reason the gradient is behaving in a linear way seems to be due to diffusion/mixing occurring at the intersection between every step, i.e. molecules diffuse and mix and create a smooth continuation of every step instead of a jump. As can be seen in

both the fig.19 and fig.20, there are some outliers, especially in the beginning and in the end, corresponding to position 1 respectively 20 in fig.20. These outliers can be explained by the fact that at the end of the capillary is a plug, to hinder the liquid to run out of the capillary, and this plug has been used several times and has been contaminated with fluorescent from previous uses. The explanation for the last parts is that there is a big jump between the higher concentrations, i.e. from 33% to 50%, which why these are seen as outliers, i.e. positions 18 to 20 in fig.20.

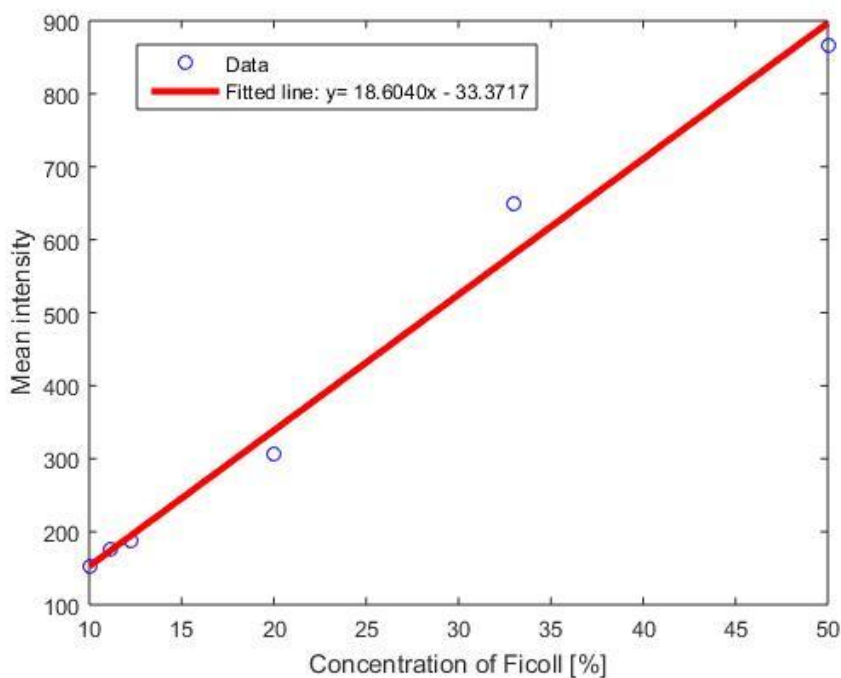


Figure 21: In this part of the experiment capillaries where filled with single concentrations of Ficoll, ranging from 10% to 50%. These measurements show that the concentration changes of Ficoll behaves in a linear way.

If one adds fluorescent molecules in proportion to the amount of density-modifying molecules it will be possible to calculate the density gradient from the fluorescence gradient. Figure 21 showed to behave in a linear way made it possible to try and write an equation that takes the mean

intensity and converts it to density. There will be need for two different equations where the first will give the concentration depending on the intensity of the fluorescence, see eq. 5.1, and the other equation will take use the concentration from the previous equation and give the density of the Iodixanol step, see eq. 5.2, based on density information from the data sheet.

First and foremost pictures of the fluorescence and the background will be taken. The mean intensity can be calculated by choosing an area in the picture of following the same procedure as in fig. 19 or fig.20 in the previous experiment with the density gradient. The mean intensity of the fluorescence from the chosen area needs to be converted to a concentration, but before that can be done the background (I_B) needs to be removed from the picture taken of the fluorescence ($I - I_B$) but also from the maximum intensity (I_o), previously measured. To convert the intensity to a concentration ranging maximum (C_{max}) and minimum (C_{min}) the following equation can be used:

$$C(I) = \left(\frac{I - I_B}{I_o - I_B} \right) (C_{max} - C_{min}) + C_{min} \quad (5.1)$$

The concentration will later be added in an equation that will give the density (ρ) of Iodixanol, since the concentration can be converted to density using eq. 5.2, [16].

$$\rho = 5.245 * C(I) + 1005 \quad (5.2)$$

The density of Iodixanol can be given by eq. 5.2, though it should be noted that this shows that, theoretically, the conversion of fluorescence intensity can be converted into density. No experiments were conducted with cells and the density gradient with a fluorophore added to confirm that it works, but it should be possible.

5.3. Separation of beads

As mentioned in earlier parts, i.e. Introduction and Concept, the differences between the different white blood cells in the blood should make it possible to separate them by using a density gradient. Before live cells can be used some things needs to be examined and some concepts should be proven to be able to accurately determine that the separation that is seen in cells is due to the density gradient and not just a phenomenon that cannot be explained. Before tests are done with cells the accuracy of the density gradient in separating particles of known density will be determined by conducting further tests, i.e. Test A and B.

The different experiments conducted:

- I. Test A: Create a density gradient and add a particle with a known density and calculate the accuracy of the experiment. By replicating the experiment a number of times the accuracy of the experiment can be evaluated and potential problems can be understood.
- II. Test B: Create a density gradient and add two particles of different density and see if it is possible to separate them, and again evaluate the accuracy of the experiment by doing a number of replications.

5.3.1. Method for Test A and Test B:

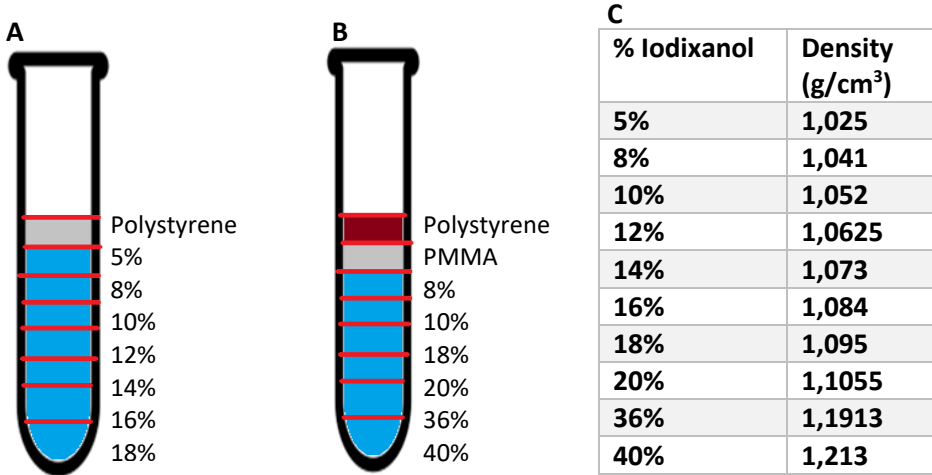


Figure 22: A showing the density gradient created for Test A and B showing for Test B. The reason for the big difference in the steps for Test B is due to the fact that there was a big difference in density between PMMA and polystyrene, i.e. 1.05 g/cm³ respectively 1.19 g/cm³, which made it hard to create small steps. (C) shows the density for the different steps in the gradient.

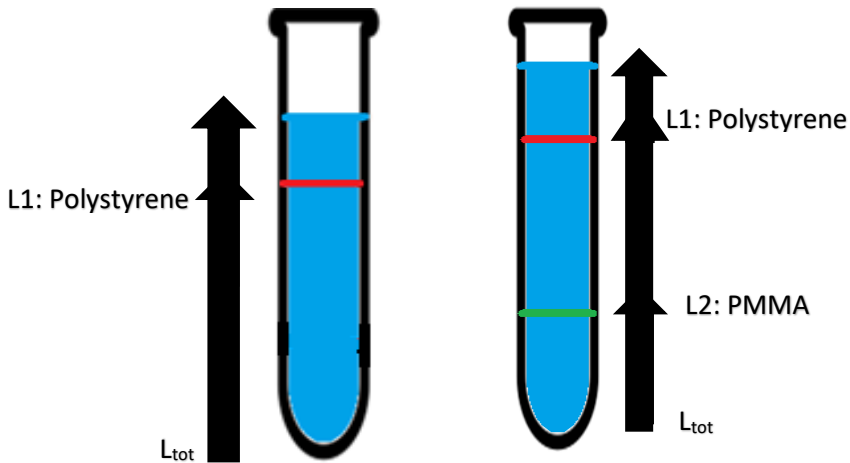


Figure 23: For Test A only polystyrene particles was added, see A. The location of the particle strip, i.e. polystyrene, from the top was measured and later a quota was calculated, i.e. L_x / L_{tot} , and a mean and standard deviation for each location was calculated. The same quota and the same quota was calculated for Test B, which only had two different particles, i.e. polystyrene and PMMA, in it, see B.

5.3.2 Test A

The purpose of this part was to evaluate the accuracy of the density gradient created inside the capillary. The evaluation of the accuracy of the experiment needs to be done since some interesting things were seen during the course of the experiment. The first thing that was noticed is the fact that air bubbles could be created when sucking in the drop of either the Iodixanol or the particle, which in some cases could disappear after centrifugation but which in other cases stayed its place in the capillary. Beside the air bubble inside the capillary it is not possible to suck in exactly the whole drop and there is always some residue left on the plate, which affects how accurate the method is.

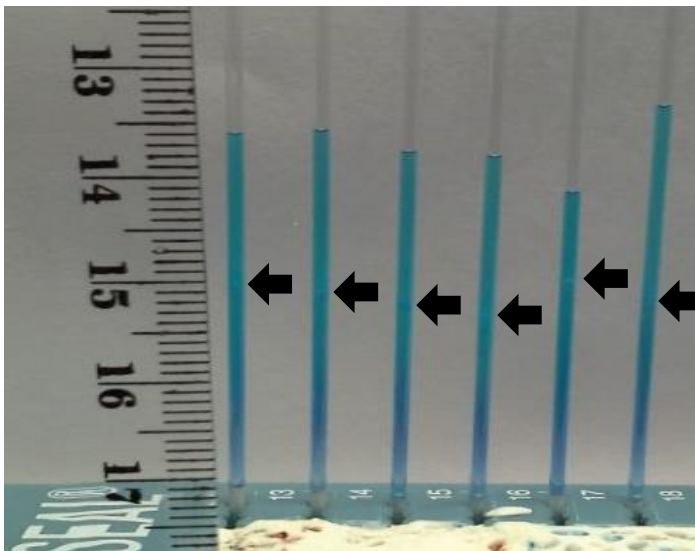


Figure 24: Polystyrene particles (with a density of 1.05 g/cm^3) were added to a density gradient ranging from 5-18% Iodixanol ($1.025\text{-}1.095 \text{ g/cm}^3$), with a 2 % step starting from 8 %, but the first step from 5 % to 8 % had a 3% difference. Black arrows are pointing at where the polystyrene particles have ended up along the capillary.

As can be seen in fig.24 there is a difference in height between the capillaries but also a difference in where the polystyrene particles end up along the length of the capillary.

5.3.3. Test B

In this part in the experiment the main purpose was to see if it is possible to separate particles of different density from each other. The possibility to separate particles from each other indicates that it should be possible to also do so for white blood cells. In this part polystyrene and PMMA particles were used, which had densities of 1.05 g/cm^3 respectively 1.19 g/cm^3 . It would have been ideal to use particles whose density were closer to each other to be able to evaluate the accuracy of the density gradient and would also have proven that it is possible to separate cells even with small differences in density. The test primarily showed that it is possible to create bands of particles with different densities that ended up in different locations.

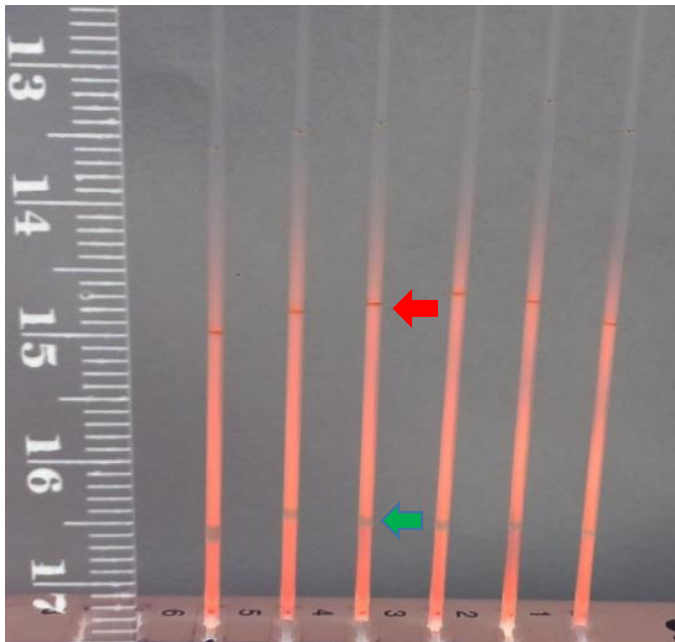


Figure 25: Polystyrene particles (with a density of 1.05 g/cm^3) and PMMA particles (with a density of 1.19 g/cm^3) where added to a density gradient ranging from 8-40% ($1.041\text{-}1.213 \text{ g/cm}^3$) Iodixanol. Red arrow pointing at where the polystyrene particles are located and green arrow at where PMMA particles are located

As can be seen in fig.25 there is again a difference in height between the capillaries but also a difference in where the polystyrene particles and PMMA particles end up along the length of the capillary. The difference that can be seen compared to Test A is that it seems like the difference in the location of the particles is smaller. To be noted fig.25 should look like fig.24 regarding the color of the gradient and the only difference is that fig.25 is taken with the setting negative to better show the location of the particles.

The bigger the difference between the steps in the density gradient the more accurate will the outcome be but at the same time the resolution in the density separation will be lost since the difference between the steps is greater. The accuracy of Test A showed to be in question since there was a big difference in the quota (L_1/L_{tot}). This could in turn lead to problems when trying to separate activated from non-activated neutrophils since the density difference will be very small and a density gradient like Test A will be necessary.

5.3.4. Results from Test A and Test B

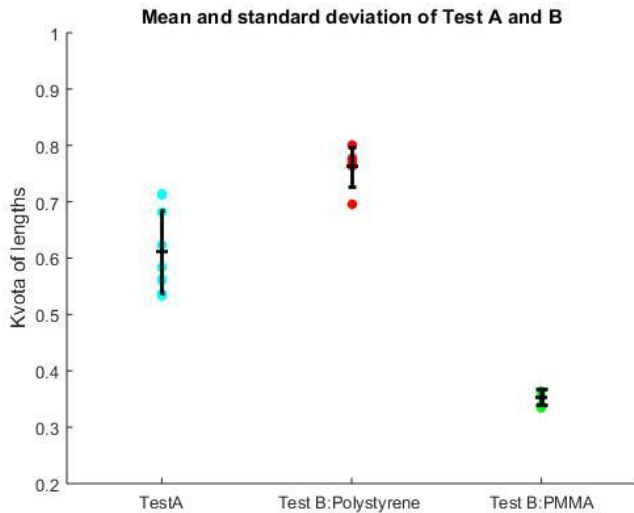


Figure 26: The mean and standard deviation for the different quotas from Test A and Test B. As can be seen the standard deviation is smallest Test B, which had a bigger difference in density from one step to another, meanwhile Test A had much smaller differences in density.

As fig.26 shows there is a difference between Test A and Test B and the accuracy of Test B is many times better than Test A. The standard deviation for Test A is 0.0735 meanwhile for Test B for polystyrene particles is 0.0355 and for PMMA particles 0.0142. The reason for this difference in standard deviation can be explained by a number of factors, starting with human error since the procedure of filling up the capillaries is done by hand and the reproducibility is very difficult but also since there is some residue left on the plate after sucking up a droplet. Another factor is the fact that for Test A the density gradient steps were more narrow and the droplets were much closer in density than for Test B and this can result in mixing of the liquids during centrifugation which in turn could mean that the density gradient is not as fine as thought, hence resulting in a bigger difference between capillaries. Compared to Test B there were some outliers that affected the standard deviation and could be the reasons for the high standard deviation. More capillaries need to be examined and during a longer time to be able to separate differences due to occurrences and get a standard deviation closer to reality.

5.4. Separation of cells

A density gradient was created inside of a microcapillary and enriched cells or whole blood was added. The capillary was later placed inside of a centrifuge and was afterwards examined both with a microscope and directly with the eye.

The separation and activation of neutrophils followed Appendix IV respectively Appendix I.

The possibility to see the location of cells inside of the capillary, regardless if the density gradient was colored or not, was difficult. The volume needed of the enriched neutrophils had to exceed at least half the total volume of the whole capillary, see eq. 4.1 and eq. 4.2, which in turn resulted in a decrease in the number of steps possible in the capillary, to be able to be seen with one's own eyes, see fig.27. Single cells were not possible to see with the eye but could possibly be seen with a microscope. The possibility to be able to visualize cells would make it easier to determine their spreading, if we can detect single cells, and therefore possibly be able to show that cells either have an inherent spreading of non-activated or activated neutrophils or that the differences in the spreading is due to the neutrophils being in different states. If single cells can be detected any outliers would be possible to detect.

To try and improve the visualization of the neutrophils some of the enriched cells had Evans Blue, a dye, added to see if it would make it easier to visualize the cells from the liquid, see C in fig.27. What could be seen in this experiment is that something, possibly neutrophils, could be seen at the intersection between the gradient and the enriched cells with Evans Blue.

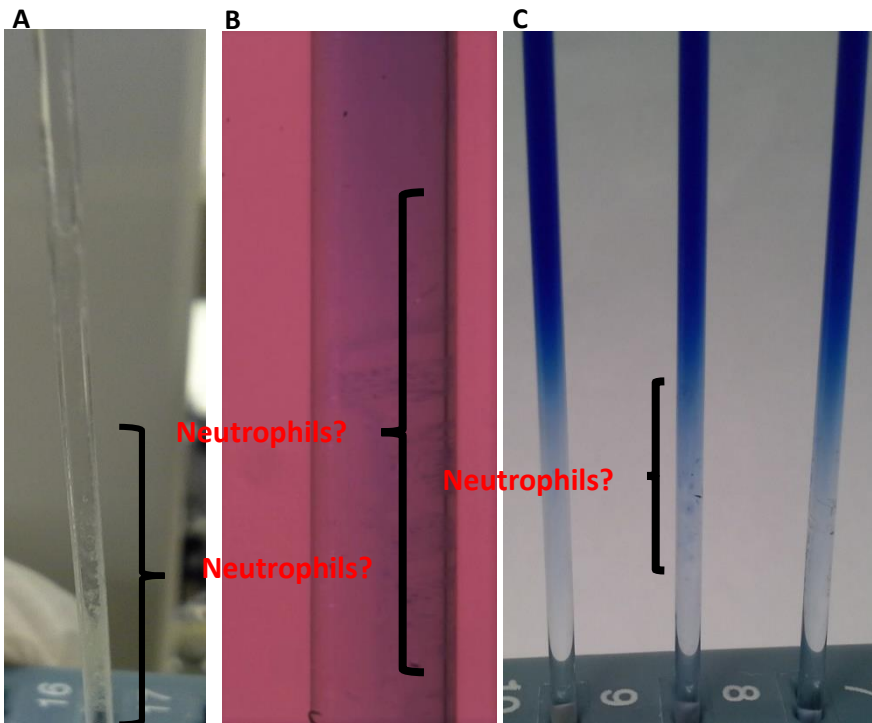


Figure 27: As can be seen in A, B and C it is difficult to see cells, even though the A and B show something closely similar to neutrophils. In A, it is more or less impossible to see anything, which is due to that the volume of the enriched neutrophil solution was much smaller than in the other two pictures. The brackets show where the neutrophils are possibly located.

5.5. Microscopy of single cells

5.5.1. Imaging of cells in whole blood

Cells could be visualized using two types of microscopes, in which one was a phase-contrast microscope, in a capillary with no gradient, see fig. 28, as well as in a density gradient, see fig.29 and fig.30. As can be seen in fig.29 and fig.30 there is a difference in size and morphology between the cells. These changes can be due to differences in activation or that they may be different cells, i.e. whole blood was used. Depending on the location of the cells along the capillary their appearance changed, see

fig.29 and fig.30. Neutrophils and monocytes differ in size and a monocyte may look like an activated neutrophil, which resulted in the conclusion that it is hard to say if the cells seen are monocytes or neutrophils.

Red blood cells in the whole blood used, see B in fig.29, will not create any problems since their density is higher than that for white blood cells, ranging from 1.077 g/cm^3 to 1.19 g/cm^3 [17]. The red blood cells will after centrifugation end up at the bottom normally, but since I added Iodixanol with a higher density than the red blood cells they ended up a small distance further up from the bottom of the capillary, see B in fig.30.

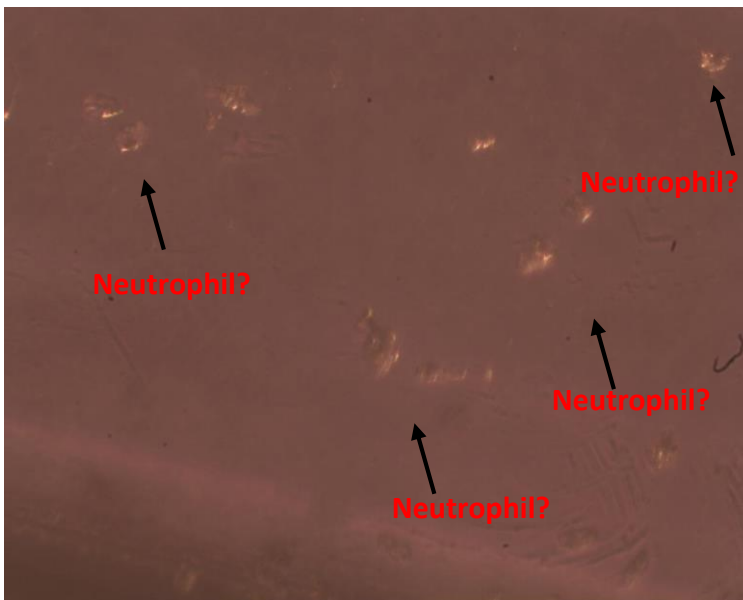


Figure 28: Possible neutrophils are shown with black arrows, taken with phase contrast microscope.

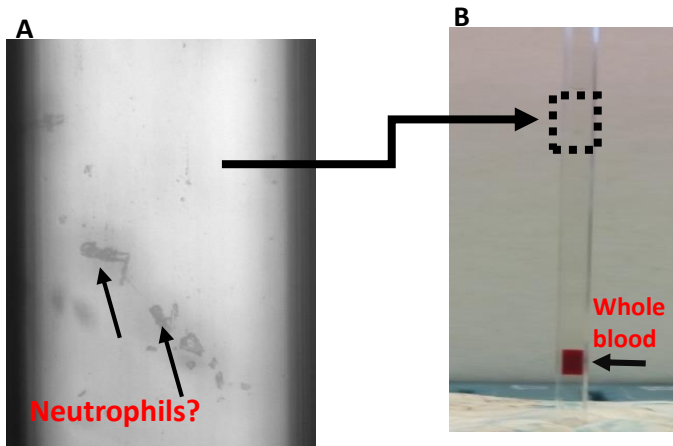


Figure 29: In A possible neutrophils can be seen, in their activated state or monocytes. The location of where A is taken is marked with a box and arrow in B.

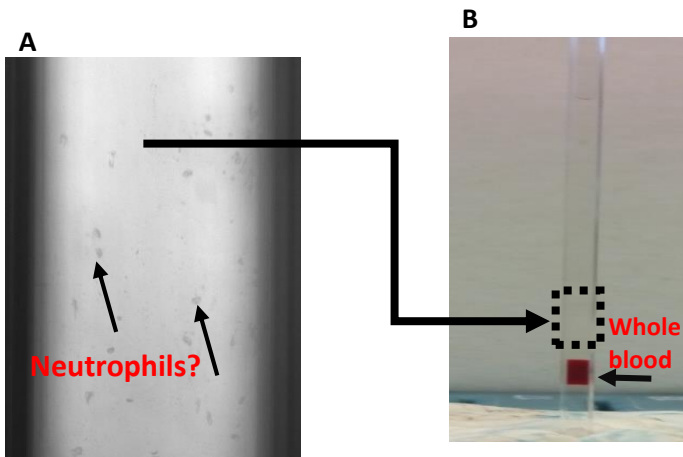


Figure 30: In A possible neutrophils can be seen, in their non-activated state. B shows whole blood inside the capillary and a density gradient. The location of where A is taken is marked with a box and arrow in B.

5.5.2. Analysis of enriched neutrophils

To be sure of what is detected enrichment was done and used as a control, since they mostly contain one cell type (i.e. neutrophils), up to 99% of the cells in the enrichment will be neutrophils according to the manufacturer of the enrichment kit used [19]. Again the results showed a spreading along the capillary and there were places that had no cells, which means that the density at this location did not fit any of the cells inherent density. The majority of the cells ended up close to $1.04\text{-}1.1\text{ g/cm}^3$, see fig.30, and this is not far off from the truth since neutrophils have shown to span between $1.06\text{-}1.1\text{ g/cm}^3$. The reason for the detection of cells of lower density than normal can be due to either the presence of monocytes (have a density close to 1.04 g/cm^3), that the neutrophils are so called low-density neutrophils [9] or that these cells that had low density had gone through apoptosis, i.e. where dead, see fig.29 [6].

5.5.3. Visualization of labeled neutrophils

To really validate the results obtained from previous experiments a control was done using fluorescent labeling specific for live cells. Since the enrichment resulted in the liquid containing 99% neutrophils it is possible to say that the cells being labeled are most likely neutrophils and not any other cells. Same tests were conducted as before, i.e. enriched neutrophils that were labeled were added into a capillary filled with a density gradient. The capillary was later visualized using a microscope with a combination of fluorescent microscope and a bright-field microscope, see fig. 31. The results here showed similar to those seen in fig.29 and fig.30, i.e. cells looked different and ended up in different locations. The cells in fig.31 ended up at the same location as fig.29 meanwhile cells in fig.32 ended up at the same location as fig.30 in the capillary. This shows that cells are separated from each other due to differences in their density along the capillary.

There were differences in shape but also size when looking at the neutrophils labeled, see fig. 31 and fig.32, and this can be due to their activation state. During the labeling of the neutrophils they were centrifuged to remove the excess dye and this can be one of the reasons that some have changed their form, probably were they activated by the force from the centrifugation. Neutrophils are very sensitive cells and can become activated by many different things, for example if the person was smoking or stressed before the blood test, the enrichment process itself can activate neutrophils but also the centrifugation of the cells inside a capillary can result in them becoming activated.

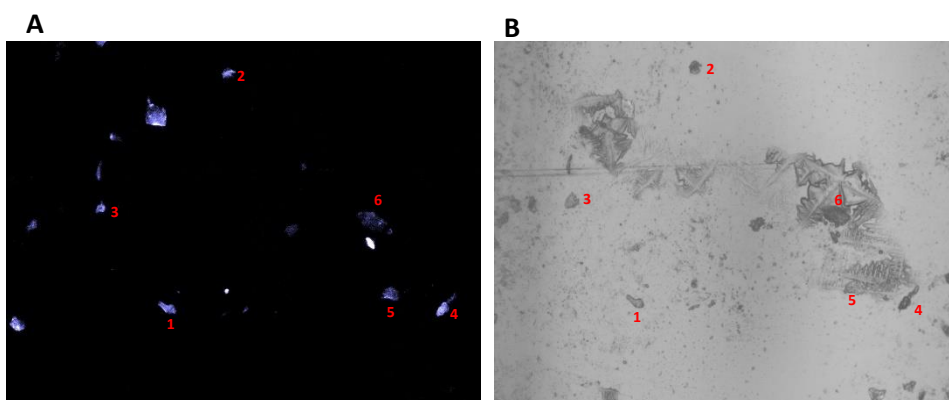


Figure 32: Visualizing neutrophils by marking them with fluorescence, A, and comparing it with a bright field image, B. Size differences could be detected. The numbers on the two pictures corresponds the same neutrophil, i.e. number 1 on A is the same cell as number 1 on B.

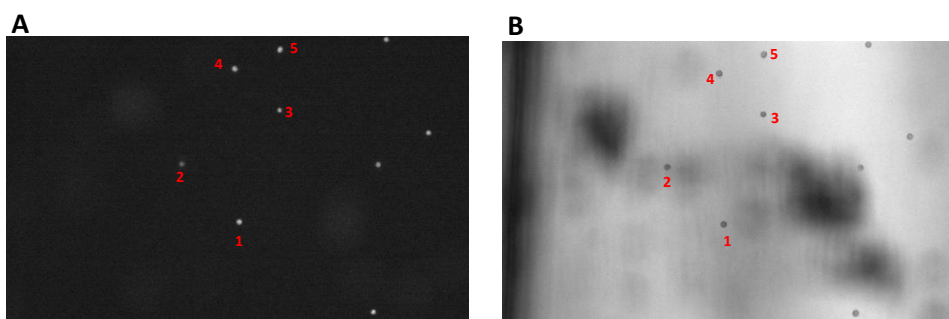


Figure 31: Visualizing neutrophils by marking them with fluorescence, A, and comparing it with a bright field image, B. A homogenous collection of neutrophils can be seen. The numbers on the two pictures corresponds the same neutrophil, i.e. number 1 on A is the same cell as number 1 on B.

5.5.2. Fixed vs non-fixed neutrophils

To validate that the difference in size is truly due to changes in their activation neutrophils after enrichment were divided into two fractions, i.e. one fraction got fixed meanwhile the other were cells kept alive, and both fractions were labeled with fluorescence. One of the batches were fixed with PFA by following protocol in Appendix V. The fixation kills/stops the neutrophil and inhibits it from changing meanwhile the cells only labeled can go through activation and in the end lysis. By having fixed and non-fixed cells it should be possible to compare activated from non-activated cells. Though, strictly speaking there are other ways in which would be better to be able to evaluate the cells activation, the fixation does not insure that the cells that are seen have not gone through some activation, since they can be activated and be kept in that state by fixation. The primary results did on the other hand show differences in size and morphology depending on if they were alive or fixed.

The differences seen in fig.33 can be due to the setting of the centrifuge, i.e. RCF, but also the time. The RCF used for the centrifugation is important since the higher speed can result in activation of neutrophils but also in their destruction since they would not manage to hold against the forces from the centrifuge. Speed is not the only factor but also the time neutrophils are being spun, the longer they are inside the centrifuge the more likely it is that they become activated or even die.

5.5.2.1. Comparison of different geometries of capillaries

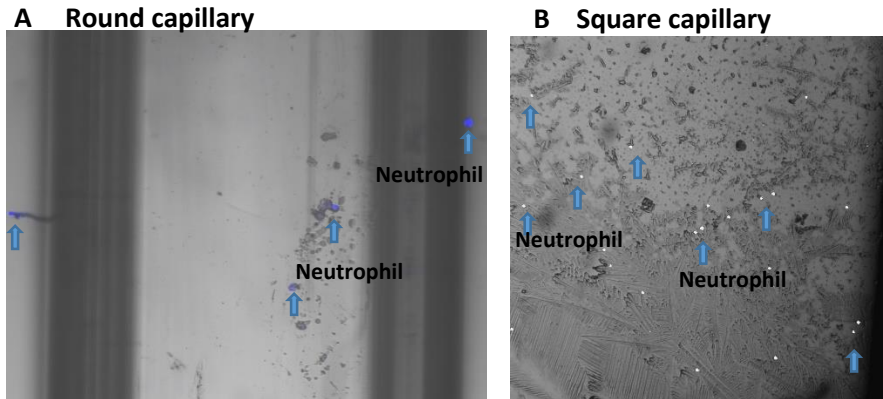


Figure 33: A shows neutrophils inside a capillary with a density gradient. The blue markings in A shows where the cells are and there are some cells not being marked, which can be dead or some cells not being labeled. B shows cells inside of a capillary with a density gradient and as can be seen the white dots, i.e. labeled cells, are hard to visualize if there was only a bright field picture.

An issue that was discovered was a problem with coating on the capillary walls but also difficulties in imaging certain types of capillaries. To visualize the problem the best pictures taken with the fluorescence microscope and pictures taken with the bright-field microscope were overlapped, to show problematics regarding the coating etc., see fig.33 and fig.34.

The square capillaries were prone to contamination or having some kind of coating on the capillary wall, making it hard to visualize cells using the bright field microscope. The capillaries used were in the beginning square but after some tests a round capillary, which is normally used for hematocrit tests, was used. The round capillaries didn't result in the problem with a coating forming, since they most likely have some coating on the inside which prevents attachment of cells on the capillary wall. The round capillaries resulted on the other hand in difficulties when trying to visualize them with the microscope, the round shape became problematic and only the center of the capillary could be visualized with certainty. The round capillary made it hard to determine where the capillary walls ended and started and created some kind of mirroring effect, seen in fig.33 (A)

and it looks like the capillary has several walls, but gave a much cleaner capillary. The contamination could also have come from the plate that the capillaries were placed on when looking at them with the microscope or from inside the centrifuge.

There are also size differences between A and B in fig. 33, which can be explained by the fact that (B) only had fixated and labeled neutrophils meanwhile the capillary in (A) had non-fixed neutrophils that were only labeled. The fixation puts the neutrophil in a state that inhibits it from undergoing any changes, hence the name fixation, and this results in neutrophils not being able to activate regardless of stimuli, e.g. the force from the centrifugation etc. The neutrophils only being labeled are still alive and can undergo changes depending on the stimuli, e.g. being activated or even dying through apoptosis.

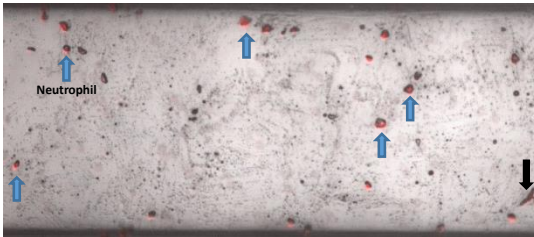


Figure 34: The neutrophils labeled with fluorescent, colored red and with blue arrows. There is a size difference amongst the neutrophils, which is a sign of different activation states but also can be due to inherent size differences. Without the red color showing where the neutrophils are it would be difficult to with certainty know which ones of the dots are actual cells.

The neutrophils used to obtain fig.34 were alive, hence the size difference between the cells. In the same figure it can also be seen that there are some dots, looking similar to neutrophils, amongst the labeled neutrophils. These can either be other immune cells, since the enrichment separated granulocytes and not neutrophils specifically, dead neutrophils that have gone through apoptosis or neutrophils not being labeled. In fig.34 one of the labeled neutrophils, marked with a black arrow, had a strange shape, i.e. was more shaped like a worm than like a dot. This can

be due to different reasons, either that the cell is going into apoptosis, is trying to phagocytose some other dead cells in the enrichment or that it has clumped together with platelets.

The visualization is of great importance since it gives us the chance to directly determine at which stage the cells are but also what density they have. This will make it possible to create a diagnostic method based on where the neutrophils will end up along the capillary, since different diseases will result in different activation levels of the neutrophils.

5.6. Outtakes from the interviews

A researcher and a medical doctor were interviewed to identify the issues they have in their everyday work and get a greater understanding of the importance of the detection of different activation states of neutrophils. During the literature study a subject started to be repeated over and over again amongst many writers, i.e. the fact that neutrophils are very sensitive cells and that it is today very difficult and time consuming to examine immune cells in general. The researcher and medical doctor gave two different perspectives to the same subject, i.e. identification of neutrophils and the possibility to differentiate them based on their activations state.

The researcher mentioned that there is a difference between health care and research concerning time and aim with the information at hand gained from a certain method. Health care personnel are more interested in using the method to achieve a certain diagnosis, i.e. is the patient sick or not, meanwhile in research the main purpose is to understand the mechanism for a disease. The time aspect is also different since it in research can take the time it needs to get a result as long as it is of clinical importance, e.g. today it can take 2-3 days to get results from a certain method, on the other hand time is of great importance in health care and the faster it is to achieve a diagnosis the better it will be. In research it would also be of interest to look at and examine single cells and the method should

therefore be integrated in a way that allows separation of the neutrophils based on their activation state after being separated in the capillary. According to the researcher it would be interesting for them to be able to know how different cells are in regard to their activation, not only morphologically but also on a molecular basis and looking at the receptors that these cells express etc. The researcher noted that they today have problems handling neutrophils since they are so sensitive and can rupture easily and be activated by the slightest changes. The condition of the neutrophils can change if e.g. the patient have been running, been stressed before the blood test or if the person was smoking before.

The medical doctor, working at the department of hematology and transfusion-medicine, expressed that there are today not enough methods that are giving doctors all the information they would like to have concerning the state of patient. There is today very difficult for doctors to determine when a patient can stop with their medications, doctors have no idea what the chances are for that patient to worsen during the time they are not in their medication. The patients in the infection unit have diseases that results in them having relapses/symptoms in periods. A method that would make it possible to quantify the neutrophils based on their activation would be an interesting tool since it could be used to determine the chances of a patient to have recurrence of their symptoms. The doctor pointed out that the importance lays on if the quantification would show any clinical importance but also if it would show that the distribution of activation states differentiates between sick and healthy individuals. The doctor also noted that they themselves have problems with neutrophils since they are so sensitive and the slightest changes can result in their activation and in the end apoptosis, e.g. the slightest changes in temperature during the transportation of test tubes with blood. In this part of the medical service time is not as important as long as the information is of clinical relevance, i.e. would help in stopping relapses of disease, help in determining if a patient should continue with a certain medication or what medication should be given to a patient based on the activation states of neutrophils. The importance here is the functionality of the examined cell, i.e. what role does it have in the disease, what properties does the cell have that makes it responsible for a certain condition etc., and if separating neutrophils based on their activation state could help in

achieving an understanding of diseases affecting the immune system it would be of great interest for clinicians today.

The conclusions from the interviews conducted showed that there is a need for new methods for evaluating infections. Time is not always important for e.g. chronic diseases, such as autoimmune diseases, since the importance of the information can be of such importance that time can be overlooked. Neutrophils are cells that are very sensitive and can be problematic in their handling, i.e. can be activated easily, go into apoptosis fast and start aggregating. A method being able to quantify the activation states of neutrophils is interesting if it shows any clinical relevance, i.e. can show a difference between sick and healthy individuals but also a difference depending on the disease.

6. Conclusion

The method of creating a density gradient has been used for a long time and for different purposes, ranging from separation of cells to calculation of density for unknown objects. The usage of density gradient for diagnostics has yet to be used, but people are today working to develop methods based on density. The most recent work being published is a method based on density gradient used to detect iron deficiency, a drop of whole blood is sucked into a capillary with three density steps and then put inside a capillary. Depending on if the patient has an iron deficiency or not their red blood cells will end up in different locations along the capillary [18]. In my work I have instead chosen to focus on white blood cells, and especially neutrophils, since there are today many different diseases and factors that may affect the white blood cells. My proposed method is using a density gradient with many steps and by adding neutrophils that have either been enriched or whole blood to look at how they spread along a capillary. Based on their activation state it could be possible in an easy and fast way determine if this change is due to a bacterial or viral infection or inflammation due to an autoimmune disease. The activation of the white blood cells differs depending on what resulted in their activation and there is today no fast way to determine the cause of an infection in a fast way.

By looking at the activation of the white blood cells, more specifically neutrophils, diseases will be detected since these are the cells that first and foremost are activated by invaders but also are responsible for different autoimmune diseases, i.e. when the immune system starts attacking one's own cells like they were intruders. Understanding the way neutrophils are activated but also the differences between the different ways of activation will not only be used as a diagnostic tool but also to better understand diseases and hopefully find new ways to treat them.

The preliminary results showed that it is possible to detect cells along a capillary and that there is a spread being detected of neutrophils along the capillary. Neutrophils were stained with a fluorescent to be able to with

certainty say that the finding done were really cells and not artefacts. It was possible to detect neutrophils of different sizes and shapes along the capillary, which is a sign of neutrophils being in different activation states. There is tough a trade-off needed to be done depending on the capillaries used in the experiments, i.e. usage of round capillaries resulted in less coating of the capillary wall but difficulties when trying to visualize it using a microscope meanwhile square capillaries made it easy to visualize but had problems with coating. The problem with the capillaries made it more difficult to determine which type of capillary is best fitted for the experiment since they had different drawbacks, though it should be possible to coat the inside of the square capillary also and this should have made the square capillary the best choice in experiments.

The blood being used in this experiment were from healthy individuals and there is still work needed to be done with sick blood regarding the speed needed and the time, since neutrophils from sick individuals are more fragile than from healthy individuals and adjustments will be done accordingly. A better understanding of the different ways to activate neutrophils, i.e. RCF setting in the centrifuge and the time the samples are being centrifuged, is of great importance since the main goal is to be able to quantify the activation state of neutrophils and minimal activation should occur during the preparation of the sample and the handling, as mentioned in Discussion. The understanding of how neutrophils are activated is a big area and a lot of work has been done. The work that has been done is mostly concentrated on finding differences in receptor expression that can be correlated to their activation state, most common experiments have been conducted by activating cells and adding a cocktail of fluorophore-conjugated antibodies that bind to receptor only present when the cell is activated and then analyzed with flow cytometer [9].

The proposed method where a density gradient is created inside of a microcapillary is not dependent on any immune substances created by the body but is showing the activation states if neutrophils and showing how the neutrophils are dividing up along the capillary, which makes the

method useful for also the cases where available methods today are not able to set a diagnosis. The method is making it possible to quantify the neutrophils according to their activation states but also making it possible to take a closer look at single cells using a microscope. The method is not explicitly used for neutrophils but can be used for any other immune cells since they all go through some kind of activation that in turn would lead to changes in their density. The method is fast, easy and uses materials and devices already available in most hospitals etc.

The human body is complex and there is still a lot more research that needs to be done to better understand how the white blood cells in the human body activates but also reacts to different stimuli. The method where density gradient is used has shown to have potential, not only in my work but also by others for different applications, to be an easy and fast method to be used in the healthcare sector or in research as a tool to better understand the changes white blood cells can undergo.

6.1. Outlook

6.1.1. Labeling of cells

To be able to correlate the cells density to their phenotype specific fluorescence labels will be needed and it would also be a good idea to put some of the samples through a flow cytometer to determine the precision of the method used for separation but also labeling. The optimal option will then be to detect the labeled cells directly in the gradient, i.e. do specific labelling of neutrophils and combine with density measurements. The flow cytometer would also help to determine the amount of live and dead cells for each experiment would have been interesting to know since it would help to determine if the cells not being labeled are dead or not. It would have been interesting to have done some tests on sick individuals and see how their neutrophils would have managed all the preparation and finally the centrifugation.

6.1.2. Capillary geometry

The problem with the capillaries would also have been interesting to evaluate more and see if it would have been a good idea to coat square capillaries with some substance to reduce the coating seen on the wall of the capillary, and if so which coating medium should have been used that resulted in limited/no activation of neutrophils.

6.2.3. Immune cells

Lymphocytes would have been a better choice for the preliminary tests since they are more stable and less sensitive than neutrophils. These cells are not as common as neutrophils but would be great candidates for further research since they would not go into apoptosis as fast and not activate as easily as neutrophils. There are today many diseases affecting lymphocytes and since they are more stable it would have been interesting to have tried several immune cells and see how they behaved.

7. Ethics

Whole blood was used in many of the experiments conducted and as in any experiments using any parts from humans, either it being tissue or blood as in my case, there are protocols needed to be followed but also applications being sent for usage of blood. In my case all the paper work concerning usage of blood was done by the Biomedical department in Lund University, LTH, at which I conducted my master thesis.

Whole blood will be needed for the experiments and can create some discomfort for the individuals donating blood. The amount of blood needed from the patient is not much and it does not take a long time for someone to donate blood. The amount of blood needed is not much and most patients have either gone to the doctor to do some blood tests or donated blood, the procedure is fast, simple and safe for the patient. In the future blood can be taken from the finger, i.e. a prick in the finger, instead from blood vessel in the arm. This will reduce the time for the patient but will also reduce the discomfort. Most patients that have any kind of disease affecting the immune system are used to taking several blood tests and one extra 5 ml test tube of blood would in most cases not be a problem, except form really small children.

8. Acknowledgements

I would like to take this chance and thank everyone that has helped me with my master thesis, everyone from my supervisor to the whole departments of biomedical engineering for their patience every time I have asked for supply etc. I would also like to thank everyone that agreed to be interviewed by me. I cannot forget to thank my family, and especially my mother and brother, for always standing by my side and giving me the strength to keep on going.

I would also like to give my supervisor Per Augustsson a special big thanks for always making me think a step further and making me use the tools I have collected during my time as an engineering student. Without him I would not had the chance to learn what I did but also to learn as much as I did during this project.

Supplementary

The protocols listed in appendices I to III were adapted from Hao-Wei Su's doctoral thesis *Electrical Characterization of Leukocyte Activation for Monitoring Sepsis Progression Using Dielectrophoresis [19]*. Some modifications were made to better suit the setup and chemicals at hand, which are also listed in the Appendix for each protocol.

Appendix I: Protocol for white blood cell separation

Materials and chemicals

1.1 Materials

50mL Conical Tubes

15mL Conical Tubes

Pipette Tips

Sterile 250mL bottles

1.2 Reagents/Buffers

Ficoll-Paque Plus GE Healthcare Cat#17-1440-02 or similar at room temperature

HBSS

Trypan Blue Counting Solution

1.3 Equipment

Centrifuge

Microscope

Hemocytometer

Procedure

Day before blood arrives

1. Make Sure there is a centrifuge at room temperature for blood processing. To ensure this open the centrifuge door and turn it off.

Day of blood arrival

1. Before blood arrives:

- a. Set centrifuge to room temperature (18-20°C).
- b. Allow Ficoll to reach room temperature (store Ficoll at 4°C after using).

2. Transfer whole blood from collection tube to 250mL container.

- a. Alternatively if blood comes in the form of a leukopack/buffy coat: Transfer buffy coat from blood bag to sterile plastic media bottle, carefully clamp off tube, and cut with scissors. These bags may be flimsy and difficult to handle, get an assistant to help stabilize the bag.

3. Estimate blood volume and dilute 1:1 with room temp HBSS. Mix well by inverting several times.

- a. Alternatively if buffy coat: Dilute 1:4 with HBSS
Buffy coat(higher density of WBCs)

4. Aliquot blood into 30mL portions in 50mL tubes.
 5. Slowly underlay 11mL Ficoll in each tube (can also do it overlay).
 6. Centrifuge for 30 min at 400g, 18-20°C with no brake
 7. Remove 5mL of the upper plasma layer containing plasma and most of the platelets.
Transfer to fresh tube. Save sample for freezing. Aliquot into cryovials, label with patient ID
#, date and plasma dilution. Freeze at -80°C.
- 105
8. After taking the plasma aliquot, aspirate the plasma from all 10 tubes – be careful not to disturb the PBMC layer.
 9. Transfer the mononuclear cell layer to another centrifuge tube via a transfer pipette removing the band and some of the layer above and below. Split layer into 4 50mL conical tubes equally and wash cells by adding an excess of HBSS to fill tubes.
 10. Centrifuge at 400g for 10 min at 4°C.
 11. Remove supernatant; re-suspend cells in each 50mL conical tube and combine into 1 50mL conical tube and wash by adding an excess of HBSS.
 12. Centrifuge at 400g for 10 min. at 4°C.
 13. Remove supernatant; re-suspend cells in 20mL and count cells by taking 10uL on Hemacytometer.

a. Equation = Count (average per 1 square)* DF*10⁴

14. Take aliquot of cells for HLA typing.
15. Centrifuge at 400g for 10 min. at 4°C.
16. Cells are now ready for freezing or sorting. Refer to specific protocol.

Appendix II: Neutrophil density isolation and activation protocol

Materials:

- 1) PBS ++ 20mLs (need to be kept in the refrigerator)+ 1% BSA
- 2) PBS - - 8W0mLs + 1% BSA
- 3) Sucrose 270mM with 1%BSA
- 4) Heparin
- 5) Monopoly density based separation media. Keep in room temperature.
- 6) 1.6mM PMA (in the big fridge)

Steps:

- 1) Add in 3mLs of Monopoly density media into 15 mL falcon tubes and then 4mL of blood.
- 2) Use 400g to centrifuge for 40mins, use slow ramp down.
- 3) Prepare PMA solution: 1.5 µL PMA(1.6 mM) into 2.5 mL PBS ++ to make a 1µM PMA in PBS++/1% BSA
- 4) Take out the tubes from centrifuge, suck out the plasma, suck out the upper layer(FR1), the mononuclear layer, and the second later(FR2), PMNs,
- 5) FR2 in to 15 mL falcon tube and fill to 12 mL with PBS - -
- 6) Centrifuge the tubes in 250g for 10 mins.
- 7) Suck out supernatant, resuspend the FR2 in PBS - -
- 8) Mix 0.5 mL of resuspended FR2 with 0.5 mL PMA to activate. Mix another 0.5 mL FR2 with 0.5mL PBS++ as control.
- 9) 20 min incubation under 37°C water bath.
- 10) Count the cells with remaining FR2.

11) 5 min / 2500 rpm in small centrifuge to spin down the two vials and then resuspend in PBS

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Optional

Add in Syto dye 1 μ L to 7e6 cells Incubate for 15 mins

Use 5 mins 2500rpm to spin down the cells.

Appendix III: Human neutrophil activation assay protocol

Reagents

1x PBS buffer (with 1% HSA)

CD66b-FITC (eBioscience), CD11b-PE (eBioscience), CD18-APC (eBioscience)

EasySep™ Direct Human Neutrophil Isolation Kit (Stellcell Technologies)

5 tubes:

1. No stain
2. Stain, no incubation
3. Stain, with incubation
4. Stain, with incubation + 5 μ M fMLP
5. Stain, with incubation + 1 μ M PMA

Neutrophil Isolation Procedures:

1. Put 0.5 mL of whole blood in 15mmx75mm BD FACS tube.
2. Add in 50 μ L magnetic beads and 50 mL cocktail of EasySep Direct Human Neutrophil Isolation Kit.
3. Incubate 5 minutes
4. Add in 3 mL buffer and put the tube in magnets.
5. Incubate 5 minutes
6. Poor the liquid out in a new 15mmx75 FACS tube. Add in 50 mL magnetic beads.

7. Incubate 5 minutes
8. Put the tube in magnets.
9. Incubate another 5 minutes
10. Poor the liquid out in a new 15mmx75 FACS tube. Put the new tube in magnets.
11. Incubate another 5 minutes
12. Poor the liquid out in a new 15mmx75 FACS tube.
13. The cell are now ready to use (3~3.5 mL to use).

Neutropil Activation Procedures:

1. Take 3 tubes for sample 3,4,5, and add 0.5 mL of isolated cells in each.
2. Put another 0.5 mL of buffer/10 μ M fMLP/2 μ M PMA in sample 3,4,5.
3. Incubate sample 3,4,5 in 37 degree incubator for 30 minutes.
4. Add in another 0.5 mL to each to dilute the stimulants.
5. Spin down the cells with 2800 rpm for 4 minutes on 4°C.
6. Remove the supernatant until 0.1 mL
7. Add 100 mL to tube 2
8. Add 50 μ L of premixed stain to tube 2,3,4,5 (1 μ L CD66b, 1 μ L CD11b , 2 μ L CD18 per sample)
9. Place tubes on ice and incubate for 30 minutes.
10. Add another 1.35 mL to each tube for washing the stain.
11. Spin down the cells with 2800 rpm for 4 minutes on 4°C.
12. Remove the excess liquid until 0.1 mL
13. Add 200 μ L buffer to each tube and gently pipette.
14. Add 100 μ L isolated cells to tube 1. Add 200 μ L buffer to tube 1.

15. Now 5 tubes are ready to be measured in flow cytometry.

Flow cytometry setting:

1. Medium speed
2. Each sample runs for 2 minutes.

Appendix IV: Human neutrophil enrichment protocol

Materials

- Blood
- EasySep™ Human Neutrophil Enrichment Cocktail
- EasySep™ Magnetic Nanoparticles
- “The Big Easy” magnet
- 14 mL (17 x 100 mm) polystyrene round-bottom tube, four bottles in total
- Pipette and tips
- Bio-waste bucket/bin
- Gloves
- Duct tape
- Ethanol
- PBS buffer (Phosphate-buffered saline)

Before opening the container with blood and doing any work with it some preparations are needed.

Preparations before handling the container with blood and other chemicals

- Always wear gloves during the handling of blood to reduce contamination of the blood but also any transfer of disease etc. from blood to the person handling the blood.
- Use a duct tape and mark out the area that will be used and which will be in contact with the blood. This step is to have an isolated and specific area that you know have been in contact with blood and therefore making it easy to know exactly where to clean. By having marked out an area it also makes you more conscious of the possible equipment and materials being in contact with blood.
- Clean the area with ethanol to make sure that the area is clean.

- Get all the equipment that will be used during the preparation of the blood in close proximity to the area which was marked with duct tape.
- Use if possible equipment specially used when handling blood otherwise use what is available.
- Have a bio-waste bin near your working area so that you can always get rid of vials and other waste.
- Mark your tubes beforehand with what they will contain, date and initials.

Protocol for neutrophil enrichment

The protocol was taken from the manufacturer of the enrichment kit used in the enrichment of neutrophils with small adjustments [20].

1. Prepare sample at the indicated cell concentration within the volume range, i.e. 5×10^7 cells/mL. Add 0.5 - 6.5 mL of sample into a tube.
2. Add 50 μ L/mL of Selection Cocktail, i.e. EasySep™ Human Neutrophil Enrichment Cocktail, to sample.
3. Mix and incubate for 10 minutes in 2-8°C.
4. Mix Magnetic Particles. Pipette up and down more than 5 times before usage.
5. Add 100 μ L/mL Magnetic Particles to sample.
6. Mix and incubate for 10 minutes in 2-8°C.
7. Add PBS medium to top up the sample to the indicated volume. Mix by gently pipetting up and down 2-3 times.
 - a. Top up to 5 mL for samples < 3 mL
 - b. Top up to 10 mL for samples \geq 3 mL
8. Place the tube (without lid) into the magnet and incubate for 10 minutes.
9. Pick up the magnet, and in one continuous motion invert the magnet and tube,* pouring off the enriched cell suspension into a new 14 mL tube.

10. Remove the tube from the magnet and place the new tube (without lid) into the magnet and incubate for a second separation for 10 minutes.
11. Pick up the magnet, and in one continuous motion invert the magnet and tube,* pouring the enriched cell suspension into a new 14 mL tube.
12. Isolated cells are ready for use
 - a. Note that if the final solution still looks pink, which means that there are still RBC's left, redo steps 5 to 11.

After finishing the enrichment of neutrophils

- Throw away all tubes and pipette tips etc. that are not needed into the bio-waste bin.
- Remove you gloves and put on new ones.
- Take ethanol and soak the paper with it and wipe everything that has been touched, i.e. everything inside the marked area and the things close to it.
- Return everything to its place and again take paper soaked in ethanol and wipe the area that was marked out.
- Remove the gloves and wash your hands with soap and water and wipe with clean paper towels. Use hand sanitizer if available.
- Store your blood and enriched sample in the refrigerator when not in use. Always use gloves when handling blood and the enriched sample.

Appendix V: Labeling and fixation of enriched neutrophils

Materials:

- Enriched neutrophils, see protocol IV
- PFA, 4%
- Fluorescent, Ficoll
- PBS, 0.001 M
- Centrifuge
- Pipette and tips with filter
- Waste tube
- Two test tubes with lids

Method labeling of neutrophils:

Take the enriched neutrophils and divide into two batches and add these into two test tubes. The enriched neutrophils are in a liquid still containing many proteins etc. which can affect the end result of the labeling of neutrophils. Before the fluorescent is added into the two batches take the test tubes and insert them into the centrifuge, 2500 RPM for 5-10 minutes. After the centrifugation the cells will have ended up at the bottom of the tube. Take a pipette and remove as much of the liquid as possible without also removing the bottom layer of cells. After removing the liquid add PBS, amount should match the same volume added into the test tube. Take the pipette and suck up and down the liquid to mix the neutrophils with PBS. Insert the tubes of neutrophils in PBS into the centrifuge again and repeat the previous step.

Add 5 μ l of the fluorescent into each of the tubes, labeling of neutrophils cannot occur if they have been fixated. Let the tubes incubate for 20 minutes. After incubating the cells for 20 minutes insert them into the centrifuge, 2500 RPM for 5-10 minutes, and after the centrifugation take a pipette and remove the access liquid, carefully to not disturb the bottom layer with neutrophils or sucking them up. Add PBS to achieve the same original volume in the tubes, or less depending on the wanted concentration of cells. Repeat the steps with the washing, i.e. putting test

tubes into centrifuge and removing excess liquid and adding PBS, in total two times. After that the neutrophils have been labeled and can be used.

Method fixation of labeled neutrophils:

Take the labeled neutrophils and put them inside of a centrifuge, 2500 RPM for 5-10 minutes, and remove excess liquid. Add 250 μ l of 4% PFA into the test tube and leave for 5-10 minutes. After the incubation perform the washing, i.e. putting test tubes into centrifuge and removing excess liquid and adding PBS, in total one time. After that the neutrophils have been fixated and can be used.

Appendix VI: Materials

Materials:

- Polystyrene particles (10 μ m, 1.05 g/cm³)
- Silica particles (10 μ m, 1.96 g/cm³)
- Iodixanol (60% , 1.319-1.321 g/ cm³)
- Sodium-polytungstate (3.1 g/ cm³)
- Glass capillaries, round, rectangle and squared with an inner diameter of 0.5/1.15/1 mm
- Centrifuge, i.e. Hettich hematocrit centrifuge (700-13550 RCF)
- Pipettes and disposable tip
- PMMA particles (8 μ m, 1.19 g/cm³)
- Whole blood, 5 ml
- Separation kit for human neutrophils, EasySep™ Direct Human Neutrophil Isolation Kit (Stellcell Technologies)
- PBS
- Multichannel pipette

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