Analysis of Surface coverage in regards
to surface functionalization

-A microscopic approach

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Abstract

The understanding of how white blood cells react when coming into contact with various surfaces is of major importance for a wide range of biomaterials and biosensor applications. In this study it is investigated if it is possible to determine how neutrophils react to a certain type of sensor chip called cell clinic being developed. This study investigates the cell surface coverage on the sensor chip and how it correlates to the signal response of the sensor at hand. Neutrophils, as other white blood cells, are cells that quickly adhere to surfaces and during the adhesion process they activate at different levels depending on i.e. type of surface or surface functionalization, this activation can be visualized by the change in morphology.

While measuring the change of capacitance with the cell clinic sensor during cell adhesion, the cell surface coverage is of main importance. The main focus of this diploma work has been to develop an image analysis script capable of conducting automated analysis on a large body of images estimating the surface coverage. Input data for this modeling is taken from fluorescent microscopy images. The experiments conducted during this project have indicated that white blood cells adhered to the sensor surface shows signs of being activated also without external activation. This clearly shows that knowledge of how neutrophils react to surface modifications is of great importance as well as the awareness that any surface may trigger a response from the immune system i.e. neutrophil activation, so also in the cell clinic. It is a fact that it might be difficult to evaluate the effect of a foreign substance on the neutrophils while a significant amount is activated from being in contact with the surface.

Regarding different surfaces the white blood cells does not display any preference of adhering to any specific surface. The surfaces used in this project was silicon oxide wafers, silicon oxide wafers with a nitride surface functionalization and the intended sensor chip; however the addition of PMA clearly shows an effect on how many cells that adheres to the surface as well as the average area of each cell.
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Aims & Goals
The focus of this project has been to investigate the cell to surface interactions for neutrophils adhered to a variety of surfaces. The aim of the work has been to develop an automated image analysis script capable of estimating cell coverage of surfaces in multiple images simultaneously. The input data used for the analysis is fluorescent microscopy images.

The correlation between cell concentration and surface coverage is investigated and in a second step it will be possible to use that information to investigate the correlation between the change in electrical signal measured by the cell clinic and the surface coverage of the sensor. The cell clinic application is described further in the background section.

Ultimately, the long term goal is to determine whether or not it is possible to use the electrical signal from the cell clinic to determine how the cells on the sensor react in the presence of a pathogen.
Background

**Neutrophil granulocytes**, or neutrophils for short, make up roughly 40-70% of blood leukocytes which are an important part of the human body’s defense against foreign substances and particles, they have a short survival time and function through tracking and attacking said threats by chemotaxis and phagocytosis. Chemotaxis is the process of a cell or organism tracking food or prey by the presence of certain chemical stimuli, and phagocytosis is the process of neutrophils ingesting the targeted substance or particle.

The destruction of a pathogen during phagocytosis involves several different chemicals, such as the enzyme called lysozyme and several oxygen radicals, e.g. hydrogen peroxide (H₂O₂) and the superoxide anion (O₂⁻) (Gerard J. Tortora, 2011), these molecules may be possible to detect in electrical measurements.

Furthermore there are two additional mechanisms in which neutrophils eliminate foreign microbes, Neutrophil extracellular traps (NETs) and degranulation.

Neutrophil extracellular traps (NETs) has been shown to represent an important part of immobilizing and killing invading microorganisms. The mechanics behind it is that neutrophils extrude chromatin fibers, covered in antimicrobial peptides and enzymes, which creates a netlike meshwork that will trap microbes. The composition of these NETs might vary depending on the stimuli. (Radic, 2012)

Degranulation involves a series of complex biochemical reactions and activation of effector molecules within the cells. The result of degranulation within neutrophils is a reorganization of the actin cytoskeleton which forms a mesh around the cell which works as a shield against granule docking and fusion, which will prevent invading microbes to enter the cells. (Lacy, 2006)

**Fluorescent microscopy** is used for detecting and analyzing fluorophores. The technique is achieved by staining target cells with a fluorescent molecule designed to bind to, and mark, specific molecules or structures within the cells to enable analysis of the system or a specific reaction. The phenomenon of fluorescent molecules Examples of structures commonly stained is proteins and/or membranes.

If coupled with image analysis, fluorescent microscopy opens up for fast screening of samples, over time tracking of stained cells, quantitative analysis and comparison between objects.
Cell Clinic. The Cell clinic referred to in this document is a device under research in collaboration between Linköping University in Sweden, the University of Oulu in Finland and the University of Maryland in USA. In the cell clinic project it is investigated whether it is possible to utilize an electrical sensor device molded from LTCC (low temperature co-fired ceramics) plates (Kilpijärvi, 2015), to make electrical measurements on live cells whilst exposing the cells to various chemicals and/or molecules to evaluate the response in signal in regards to response of the cells adhered to the surface. The change in signal strength is related to the change in the dielectric constant of the medium, this means that adding cells and/or chemicals to the surface may either increase or decrease the signal depending on the electric properties of the added media. A schematic drawing of the measuring procedure is shown in Figure 1, with “substance” being cells, fluorescent dyes, PMA or any other molecule that is being evaluated.

![Figure 1. Schematic drawing of how measurements with the Cell clinic sensor device is conducted](image-url)
Laboratory work
To enable the acquisition of, for this project, necessary information regarding neutrophils and surface coverage the neutrophils used first needs to be separated from donor blood.

Separation of Neutrophils from donor blood
Separation of neutrophils from donor blood is being done by in a process following the step by step procedural explanation written in whole in this section.

- Take 50ml heparinized newly tapped, room tempered blood.
- Place 25ml polymorphprep (Nycomed (Axis-Shield PoC AS)) in 2 separate 50ml Falcon tubes and let 25ml of blood run slowly down the tube wall making the blood become layered on top of the polymorphprep.
- Centrifuge 480 x g (1350rpm for Farm), for 40 minutes, in RT (room temperature) in a swingout rotor centrifuge.
- Remove the top three layers (the light yellow one on top, the unclear layer below the yellow layer on top and the clear layer below that). Collect the next unclear layer and the slightly pink one below it into a new Falcon tube (50ml) for further centrifuging.
- Mix with equal parts RT 0.45% NaCl solution and add approximately 20ml RT PBS.
- Centrifuge at 400 x g (1323 rpm for Farm), 10min in RT in a similar centrifuge as above.

Following steps should be performed on ice
- Lysate remaining erythrocytes by adding 4.5ml of cold dH20 (or milliQ water), wait for 35 seconds and add then 1.5ml PBS containing 3.4% NaCl and 5ml of physiologic solution (KRG or Hapes) without calcium.
- Centrifuge 400 x g, 5min, +4 degrees C. Dispose of the supernatant (the liquid lying above the solid residue on cells in the bottom of the falcon tube) and resuspend the pellet (solid residue of cells). Lysate once more as above.
- Centrifuge 400 x g, 5 min, +4 degrees C
- Dispose of the supernatant and carefully dissolve the pellet. Add 1 ml ice cold physiological solution i.e. KRG with calcium or Hapes-buffer. Remove 10 microlitre for cell counting in a Burker chamber.
- Dilute to desired concentration with physiological saline solution. 
  \[ V_1 \times C_1 = V_2 \times C_2 \] (1)

By utilizing equation 1 above it is easy to dilute your cells to a desired concentration. In the equation \( C_1 \) is the calculated concentration (cells per ml) acquired from counting cells in a Bürker chamber, explained below, \( V_1 \) is the original volume (in this case 1ml, following the procedure above), \( C_2 \) is the desired concentration and \( V_2 \) is the final volume reached to balance the equation.

The reason for using heparinized blood is because of the fact that heparin is a naturally occurring anticoagulant, preventing the blood from coagulating during transport, storage or laboratory procedures. The heparin needed to prevent the blood from coagulating is generally preexistent on the inside of the test tubes into which the blood is tapped from the donor meaning no further treatment of the blood is needed once you extract it.

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Bürker Chamber
The bürker chamber is, as mentioned above, used to calculate cells. Figure 2 below shows a Bürker chamber and its 3x3 square grid, each 1mm$^2$ in size, used to count leucocytes.

By utilizing capillary force it is possible to force the leucocytes to spread across the surface. After this has been done the chamber is placed in a bright field microscope for counting the cells. To get an accurate value of cell concentration, cells are counted in 10 (at least) squares and then averaged to get a statistically more actual value. The cell concentration is then expressed in $X \times 10^6$ cells/ml, where X is the calculated cell average across the squares of the chamber. (BRAND, 2005)

Preparations for Imaging
Depending on whether or not the imaging needs to be conducted on live cells, the preparation procedure looks slightly different, for example, some fluorophores are not usable for staining live cells. Simultaneously if the application requires imaging of live cells fixation is not viable.

The microscope used for this project is an inverted confocal microscope, meaning that incident light will hit the observed sample from below. As a result of this any non-transparent substrate used with the neutrophils will require them to be suspended “upside-down” in the microscope. This will render ordinary bright field microscopy unusable in this project because of the fact that all of the used substrates are non-transparent.

In the case of imaging live cells, as needed for any means of correlation to the signal acquired in the cell clinic application,

All of the procedures described below are conducted in a tissue culture plate (VWR 24 wells).

Activation
After the neutrophils are separated from the donor blood, since the majority of steps in the separation process are done in low temperature (4°C), it is necessary to let them regain some temperature as well as recalcifying them.

The recalcification of the neutrophils is achieved by adding CaCl$_2$ to each well after the neutrophils are added to the surface at a final solution concentration of 1mM.
**Triggering**

It is possible to add PMA (Phorbol 12-myristate 13-acetate, $C_{36}H_{56}O_{8}$) a substance which can be used to resemble the effect similar to that of an invasive molecule (substance or bacteria), to the wells in an attempt to force a reaction from the neutrophils, this will be done to evaluate how/if the neutrophils change their appearance as they get triggered. PMA works as a specific activator for protein kinase C (PKC) and hence also nuclear factor NF-κB. (Phorbol Myristate Acetate - PMA) It has been shown that the nuclear factor NF-κB does indeed play parts in leukocyte activation as well as proinflammatory cytokine production which both are important contributors to the inflammatory response, making it a good external activator for investigating cell response in white blood cells. (Lawrence, 2009)

**Fixation**

The process of fixating the neutrophils onto the surfaces that are present in the wells are accomplished by adding PFA (paraformaldehyde) to the wells in a final concentration of 4% for a period of time larger than 30 minutes. After the incubation of PFA, to fixate the neutrophils, are completed it is important to clean the surfaces before adding any fluorescent dyes to the wells, this washing is being done by the repeated addition/subtraction of buffer solution (in this case Hepes) in a large volume (~300-500 µl) 5-6 times, this to be sure that any PFA that still remains in the wells are of very low concentration.

The commonly used fixation agent constitutes approximately 10% formalin, which is a 3.7% solution of formaldehyde in water with 1% ethanol present. This solution does not cause excessive tissue shrinkage or distortions of the cellular structures. The commercially available formaldehyde solution in its undiluted state generally contains approximately 10% ethanol to prevent spontaneous condensation reactions, and because of this the commercial formalin fixation agents is a 2-phase fixative, initially dehydrating the cell causing hardening of tissues and membranes followed by cross-linking mediated by aldehyde. (Fox CH, 1985)

**The dye**

In this project BodiPY FL phallacidin, B607, is the fluorophore used to stain the fixated samples. Before staining the cells however it is necessary to dilute the fluorophore, this is achieved by mixing 1/20 stock BodiPY solution (~6.6µM) with 2/20 LPC (lysophosphatidylcholine) and 17/20 PBS (Phosphate buffered saline).

The B607 fluorophore utilized in this project is a fluorescent phallotoxin which stains F-actin within cells at nanomolar concentrations. Phallotoxins are extremely water soluble fluorophores which makes it a reasonable candidate for labeling, identifying or quantitating F-actin in the type of cellular experiments used in this project. Phallotoxins in general has a similar affinity in regards to actin regardless of size, binding in an approximate ratio of one molecule per subunit of actin, allowing for visual separation between cells of different thickness. (Invitrogen, 2006)
**Staining the Neutrophils**
The process of staining the neutrophils is a lot like the process of fixing them onto the surface. Start with adding a big enough volume of the fluorophore solution, enough to cover the entire surface, to ensure that as many neutrophils as possible gets stained. After adding the fluorophore onto the surface, incubate for 30-40 minutes. When the incubation is complete the final step is to wash the surfaces with the same procedure as when washing after fixation.

**Shaking**
During the process of fixating as well as staining the cells, during some of the experiments, it was investigated if it is possible to get a consistent distribution of cells across the surface as well as better staining of the cells. This was done by using a Heidolph Inkubator 1000, shaking the cells throughout the whole process, with a speed of 35 revolutions per minute.

**Storage**
After the cells are fixated and stained onto surfaces they aren’t very sensitive in regards to storage, the fluorophores however might degrade a bit over time. This means that imaging and analysis of the attached cells might become more difficult over time.

Storage of samples with live cells is not possible, in this project not even overnight, because of the short lifespan of the neutrophils, less than 24 hours in the bloodstream due to programmed cell death, apoptosis, to maintain homeostasis. (Allen, 2014) Another thing that needs to be considered is that if the surfaces are let to dry manually there might occur spots as residue from drying which might complicate image acquisition, for this reason surfaces are generally blow dried using nitrogen gas due to its inert nature.

**Microscopy**
The microscope used for the fluorescent microscopy is an inverted confocal microscope with two different Nikon cameras attached for different capture modes, one Nikon Eclipse Ti and one Nikon DS Ti12, the later one being used for image acquisition in this project. A multitude of images was taken for every substrate used to calculate values, of e.g. surface coverage, more likely to represent the whole surface rather than every single image for itself.
Image Processing

To enable extraction of the desired data from the images, after they have been acquired in the microscope, they need to be processed in a way that enables the acquisition of desired data. In this project the image analysis has been conducted using Matrix Laboratories (MatLab).

The processing is automated to make the procedure faster as well as to reduce the influence of biased analysis possible to occur if images are to be manually handled. In this context, the words automated means that with a single click the script will be run on every single file of a predetermined type of image, analyze them, and save them individually as a new image with the same image name, only with the addition of an indicator as to that the image has indeed been analyzed.

During the project a variety of different algorithms for image analysis were tested, with varying quality, each algorithm and approach described in sections below. Each approach has the same initial steps shown in Figure 3 below.

![Figure 3. Schematic drawing of the working procedure of analysis scripts developed](image)

First approach

The first approach was to investigate each pixel under a threshold condition, which was set manually trying to get reasonable conformity between the analyzed image and its counterpart, to determine whether or not it was bright enough to be considered a cell, or if it was shared intensity from nearby pixels. As a first approach this gave a decent estimate, however further improvements were needed, as shown in Figure 4.

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Figure 4. Analyzed image to the left, microscopic image to the right. Individual pixel analysis, displayed value is surface coverage.

One issue with this approach, in addition to not being able to clearly distinguish the edges of the cells in the image, is that in an image with loads of cells, and possibly overlapping, the image gets very cluttered and chaotic, see Figure 5.

Figure 5. Microscopic image to the right, analyzed image to the left. Clear indication that a bright image with lots of cells is an issue.

**Gradients Analysis**

The second algorithm that was tested were developed based on gradients analysis, in an attempt to determine where in the image cells are by analyzing the changes in intensities over the image. This approach used linear filters, one for x- and one for y-direction, and calculated the gradient in each pixel by taking the square root of the sum of the roots of these linear filters individually. (Gradient = sqrt(Xfilter^2 + Yfilter^2)) For an image with low coverage and cells reasonably spread this approach works better than the first algorithm, see Figure 6. If a comparison between the first approach and gradients analysis is made it clearly shows the difference in individual cell detection, see Figure 7.
Figure 6. Microscopic image to the right, Analyzed image to the left. Shows better individual cell detection which can be clearly seen with the separation of the two cells in the center of the image.

Figure 7. Difference between first approach (top) and gradients analysis (bottom). Showing that gradients analysis works better.
The gradients analysis approach, as shown in the images above, yielded better results with respect to individual cell distinction in an image with a low cell count. However, this approach induces problems in a cluttered image where lots of cells are clumped together and/or overlapping since the edges are less distinguishable, see Figure 8.

Figure 8. Gradients analysis in steps showing a cluttered image, as seen in the top right (and bottom left) image, gradients are difficult to detect.

In a comparison between Figures 4 and 6, shown in Figure 7, it can be shown that the gradients analysis approach yields better, yet not sufficiently good, results in the case of cluttered images.
Final Approach
After numerous iterations of scripts, each with a different approach with the aim to find and determine which areas of the images should be considered a cell, a final version (final in the terms of the timeframe of this project) has been developed. This final version utilizes parts of previous iterations, in terms of image conversion, eroding and closing connected areas as well as the creation of a new image of the same size onto which the detected cell areas would be written, as well as new functions, mainly algorithms for perimeter detection, that together works significantly better, as shown in Figure 9 below.

![Figure 9. Montage of original microscopic images on the top, and corresponding images with overlays of the same image, and its analyzed counterpart on the bottom.](image)

As presented in Figure 9 above the script is showing some slight problems in precision (mostly visible in the low coverage images) with circles clearly being larger than the corresponding cells in the original image. At the same time in the higher coverage images to the left some small parts are not being detected properly, this due to difficulties in detecting edges. In Figure 10 below a step-by-step series of images is displayed describing how the analysis script works.
Figure 10. Step-by-step visualization of the working procedure of the final approach

As Figure 10 indicates, the image analysis script initially loads the image (A), converts it into a HSV (hue, saturation, value) color map, in a step which is not displayed. The next step is to extract information of detected perimeters and write this information in a new black image (B). After perimeter detection has been made, the perimeter is filled to create a representation of the detected cell area (C). The last step is to create an overlay of the original image that enables simple ocular examination of the conformity between the original image and the analysis (D).
Cell Size Estimation
Multiple approaches were investigated, to be able to estimate the number of cells detected in the images, before settling for doing it manually. In this case doing it manually means that cells were measured in the original microscopic images using the MatLab function “imdistline” which creates a manipulative line with which it is possible to measure the diameter of cells. In this case 80 cells with/without PMA were measured and an average was calculated. This average value was then used to calculate a higher and a lower cell area average. The calculated lower cell size average, if converted to µm (~9µm) is a reasonable fit to what is stated in the literature (Gerard J. Tortora, 2011).

Cleaning Surfaces
Each surface is cleaned by using Piranha Solution (Piranha Etch) prior to the procedure of applying cells to the substrate surfaces. The cleaning procedure was done to remove organic residue from the surfaces, e.g. photoresist which is a commonly used material in the microelectronics industry. Regarding the piranha solution, there are many different mixing ratios commonly used. The piranha solution utilized in this project is sometimes called “base piranha” which is a mixture of ammonium hydroxide (NH₄OH) and hydrogen peroxide (H₂O₂). Cleaning surfaces with base piranha solution is commonly referred to as TL1 cleaning. The ratios in base piranha is 3:1, however in this project where cleaning is supposed to remove atmospheric residue and eventual fat/hair/dead cells that may have come into contact with the surface during handling. Due to this the mixture is much less concentrated, 1/7 ammonium hydroxide, 1/7 hydrogen peroxide & 5/7 ultra clean water (MilliQ). This mixture is then heated on a heating pad for roughly 15 minutes to an approximate temperature of 80°C whilst the surfaces that are being cleaned are placed in the solution.

Due to the fact that the solution contains ammonium hydroxide it is desirable to keep the solution in a chemical fume hood, both during mixing and during cleaning because of the fact that ammonium exerts a very distinct and unpleasant smell.

A concentrated base piranha solution is extremely exothermic and may cause sudden boiling and/or splashing. Because of this protective equipment are important.

Safety
Due to the acidic and oxidizing nature of the constituents of the piranha solution direct contact with the skin may cause long lasting cellular damage. Hence a variety of safety equipment such as glasses and gloves should be used. (Piranha Solution, Standard Operating Procedure Template, 2015)

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User Interface
To simplify usage of the developed scripts a User Interface was created so that an inexperienced user easily will be able to analyze images using the script without making changes to the code, potentially rendering it unusable. This was achieved by using a built-in MatLab function for creating applications.

Results & Discussion
In this chapter on top of displaying results and discussing ideas of how to move the project forward will be added.

Surface coverage to Surface Functionalization
The surface coverage analysis has been conducted on three different types of surfaces, SiO$_2$, SiO$_2$ with a nitride surface functionalization as well as an actual sensor chip developed for use in the Cell clinic sensor application. The surface coverage values, as well as number of cells, for these surfaces are displayed in Chart 1 and Table 1, below.

![Chart 1](Image)

Chart 1. Staple chart showing max-, min- and average surface coverage for the different surfaces, with and without PMA

As can be seen in chart 1 above, there can be no real conclusion made about if white blood cells are more likely to attach to any of these three used surfaces. Even though the original SiO$_2$ surface display the highest value of surface coverage, it also displays the lowest. Regardless of this the average across all surface types lies within a few percentages (12.0 – 15.3%).
In Table 1 below, each noted value is a calculated substrate average calculated from five images taken on each substrate representing a more statistically probable value for the number of cells on an area of the substrate corresponding to the images. However there is no record of the calculated cell number for each image because of the stated fact that the analysis script runs for multiple images calculating an average across. Because of this the standard deviation value needs to be taken lightly since it is calculated from averages instead of individual images. To get values for each individual image the script needs to be tweaked to save the value from each image instead of calculating the average, were this to be done the values in Table 1 might change significantly.

Table 1. Calculated number of cells in the analyzed images as well as Max-, Min- and Average values.

<table>
<thead>
<tr>
<th>Cells\Surface</th>
<th>Chip</th>
<th>Chip PMA</th>
<th>Nitride</th>
<th>Nitride PMA</th>
<th>SiO&lt;sub&gt;2&lt;/sub&gt;</th>
<th>SiO&lt;sub&gt;2&lt;/sub&gt; PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>62</td>
<td>26</td>
<td>87</td>
<td>28</td>
<td>52</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>87</td>
<td>30</td>
<td>87</td>
<td>28</td>
<td>68</td>
<td>16</td>
</tr>
<tr>
<td></td>
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<td>26</td>
<td>76</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>76</td>
<td>28</td>
<td>119</td>
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<td>Min</td>
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<td>26</td>
<td>76</td>
<td>28</td>
<td>51</td>
<td>15</td>
</tr>
<tr>
<td>Average</td>
<td>70,3</td>
<td>27,3</td>
<td>81,5</td>
<td>28</td>
<td>69,5</td>
<td>22,5</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>14,4</td>
<td>2,3</td>
<td>6,4</td>
<td>0</td>
<td>23,0</td>
<td>8,2</td>
</tr>
</tbody>
</table>
As the calculated values in Table 1 indicates together with the surface coverage areas displayed in Chart 1, it looks like the addition of PMA to the surfaces reduces the likelihood that cells will adhere properly to the surface. This statement seems rather clear since the average cell count on surfaces with PMA is half (or less) than on surfaces without PMA, even if the average surface coverage is approximately the same. However, without taking before/after images for adding PMA to the surface this is only speculations since without first hand evidence the statement could be based on circumstantial events regarding each individual surface.

Addition of PMA

One of the main goals with the cell clinic sensor application is to distinguish if it is possible to measure the effect of external stimuli on neutrophils. If there is to be any possibility for such measurements, investigations needs to be conducted to investigate whether or not the substrate in itself has any effect on the morphology of the neutrophils.

The experiments conducted to investigate this is to externally activate the neutrophils by adding PMA, as described in the “Triggering” section under methods, and compare the images to determine the difference between the substrates with/without PMA.

A comparison between the different substrates, as well as with/without PMA, is displayed in the Figures 11, 12 and 13 below.

![Cell images](image)

*Figure 11. SiO₂ substrate without PMA to the left, with PMA to the right.*
As can be seen in the left part of Figures 11 and 12 there are barely any neutrophils deviating from the standard circular shape with a distinct cell structure. Meanwhile if we investigate the difference between the left and right parts of Figure 13, it is easy to see that a significant part of the cells, even without added PMA, is showing signs of being activated.

This has the potential of becoming a significant problem when trying to measure the effect of an external response in the actual cell clinic application for this image analysis script.
Cell Concentration

The experiments conducted in this project have all been with a stock solution of $2 \times 10^6$ cells/ml after separating the neutrophils from whole blood. In this regard, it would have been desirable to use a variety of different concentrations and investigate whether or not the correlation cell concentration to surface coverage is a linear function. However to better utilize the time at hand, as well as to get more data for the experiments conducted, it was decided to only use one concentration. The reason behind not conducting even more experiments is that fresh blood could only be acquired once every two weeks, or perhaps once a week.

Shaking

After manually analyzing the images and investigating whether or not the process of shaking the plates during cell adhesion and fixation has any influence in the way cells distribute across the surfaces and the quality of the staining, the results were inconclusive. Nothing in the experiments that were conducted and the images that were taken can say with certainty that shaking the culturing plate during cell adhesion and fixation has any impact on the heterogeneity of the surface coverage, this is shown in Figures 14 and 15 below.

![Figure 14. Comparison between cell adhesion whilst shaking the tissue cultivating plate (left) and cell adhesion without shaking the plate (right) whilst simultaneously triggering them with PMA.](image-url)
As Figures 14 and 15 indicates there doesn’t seem to be any evidence at hand suggesting that shaking the culturing plates during adhesion has any effect on the distribution of cells over the surface. However in a larger scale experiment with a different aim and a more sophisticated analysis method, one might get a different result. In this project however, a few tests were conducted to investigate whether or not the process of shaking gave a conclusive result where cells tended not to clump together allowing for the image analysis script to have an easier time distinguishing between individual cells, yielding significantly better cell counts and more reliable average surface coverage, and this does not seem to be the case.

**Exposure**

In the later stage of this project an experiment was conducted to test the reliability of the image analysis script in regards to different exposures. This was accomplished by using a built-in function in the acquisition program for the microscope allowing for the user to change the acquisition time as well as set a multiplicity for each count in the image, creating images with higher contrast. The images to the left in Figure 6 display images taken with higher exposure and the resulting analyzed image showing good results. Simultaneously, whilst yielding satisfactory analysis results, the high exposure image testifies that the analysis script in question is robust when it comes to noise in the image. However, overexposing the images will yield worse results due to the images becoming too bright causing the analysis script to detect faulty areas as well as not indicating actual cells as cells; this is shown below in Figure 16.
Figure 16. Explanation of overexposing images, left image is overexposed making the analysis script fail to detect the center of the large area of cells.

As shown in the image above overexposing the sensor while taking the image will result in a bad analysis result. The difference between the left and the right parts of Figure 16 is a 0.8 times multiplier (1s collection, 2x multiplier vs. 1s collection, 2.8x multiplier) in the acquisition program of the microscope. If instead the exposure is reduced as in Figure 17 below (1s 2x vs. 400ms 1x) there is a clear indication that a high exposure, albeit not overexposure yields the best analysis results for this particular analysis script.
Figure 17. Comparison between high exposure to the left and low exposure to the right

Whilst Figures 16 and 17 above, is used to differentiate between the quality of the analysis script for various rates of exposure, they simultaneously clearly shows that the developed image analysis script shows resilience in regards to noise, as the images with best cell detection (higher exposure) also has a high amount of noise.
Image Analysis Script

In its current state the image analysis script displays resilience towards image noise, as well as showing sufficient, for this application, cell detection abilities. One does not need work hard to find flaws in the analysis even in the images where the script has generated the best results. Regardless of this, in the cell clinic application there is no need for exceptional cell detection as the application is being developed around the ability to measure changes in electrical signal whilst exposing blood to a foreign substance.

At this point there is an issue regarding cell size detection, this due to the fact that the image analysis script utilizes a function that determines areas in regards to brightness in the image. A side effect of this is that in a crammed image where lots of cells are adjacent to each other, possibly even overlapping, the script will detect a larger area than a single cell and indicate that this is in fact one cell. To solve this issue manual size estimations were made as described in the methods area, however in the experiments conducted on electrical sensor chip surfaces, as displayed in Figure 13, a significant number of cells indicates being activated even thou no PMA was added. This is an issue because in images without external activation, to be able to estimate the number of cells on the surface the user would need to estimate the amount of cells displaying activated states after which a new calculated cell area average for that particular image could be calculated. As a direct result of this analysis would be for individual images, so this idea was discarded since the whole idea behind the script, and especially the graphical user interface, was to enable automated analysis of a large body of images with the click of a single button.
Graphical User Interface

To make the produced image analysis script easy to use for personal not familiar with the code or with using MatLab in general, a simple user interface was developed to simplify making changes in the code without risking breaking the script. Figure 17 below shows how the User Interface looks. A description of buttons and functions can be found below the Figure.

![Image Analysis](image)

**Figure 18. Picture of the Graphical User Interface developed to simplify image analysis via the created scripts**

As can be seen in the Figure above some areas has been highlighted for further description. These highlighted areas are numbered and each number has its description below.

1. Choose Folder - Opens the folder in which the UI file is located and lets the user to browse his folder tree in search for the folder in which the images that he/she wants to analyze is stored.
2. Textbox - Once a folder has been chosen via the choose folder button described above, the pathway to that particular folder is displayed in this textbox.
3. Textbox Coverage - After the analysis script has been run on the images the calculated value of average surface coverage is displayed in this box, value displayed in the range 0 - 1.
4. Textbox Cells - After the analysis script has been run on the images the calculated value of average number of cells is displayed in this box.
5. ImageType button group - A group of buttons that is used to change the type of files the image analysis script will look for in the chosen folder once the analysis script is called.
6. Analysis button - Pressing this button will call the analysis script and run it on all detected files of the chosen image type in the predetermined folder.
7. PMA checkbox – Checking this box will change the calculated cell area average to a higher value corresponding to cell activated with PMA

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As can be seen in the GUI code, if one were to analyze the operating procedure of the code, the analysis button runs two separate controls before actually executing the analysis script. Firstly, it controls what button in the image type button group that is pressed and changes the ImageType argument for the analysis script accordingly. After that, it checks whether the PMA checkbox is checked or not and sets the average cell area, InArea variable, depending on the outcome of this control. The script then moves on with extracting the Average Cell Coverage and the Average Number of Cells from the script and overwrites the existing value in the corresponding textboxes, number 3 and 4 in the description above, accordingly.
Future Work

Moving forward from this point would mean working on two different fronts, first of all as mentioned above the analysis script, as with any kind of programming, could be further developed, and secondly experiments while measuring response in the cell clinic needs to be conducted to see whether or not the application is viable.

An issue that might occur when attempting to measure the cell response in the cell clinic is that under the framework of this project only a solution of white blood cells has been used whilst it is desirable for the cell clinic to be able to measure on whole blood. To determine how whole blood will affect the measurements it might be necessary to conduct control measurements with blood from which white blood cells have been removed, and investigate how the signal changes. However this might not be a valid measurement due to the possibility of extensive cell damage of erythrocytes during centrifuging (L. B. LEVERETT, 1972).

Despite this risk, such an experiment might be vital to investigate the effect of the fluorophore as well as the foreign substance on the other constituents of blood. However should the experiment be successful the effect on white blood cells can easily be calculated by deducting the signal of blood without white blood cells.

As the realization that changing the rate of exposure might change the reliability of the image analysis script came at a very late stage of this project one would want to take new images for all surfaces using a higher exposure rate. This since the scripts shows difficulties detecting cells in a reliable way unless they are bright enough. However, if ocular examination of the images compared to the presented results is made, the results are reasonable.

Nevertheless, if the image analysis script from this project is to be used in the cell clinic application, it would be desirable to go over all the surfaces again, take new images at higher exposure rates and investigate whether or not the yielded results better fit the truth.

By conducting an analysis of histograms there are several potential development aspects of the image analysis script, first and foremost it might be possible to create a noise reduction loop from the histogram by using extracted values when setting thresholds, this could make analysis of images with lower exposure yield better results.

Secondly, doing a histogram analysis might present possibilities to distinguish between cells before and after activation, this due to the fact that it is possible by ocular investigation to distinguish between the two states of cells in regards of not only size and morphology but also in intensity in the image. By using this information it might be possible to extract two different, but distinct, ranges of intensities corresponding to the cells if they are activated or not. Would these ranges be clearly separated it would be possible to calculate the significance of spontaneous activation when the neutrophils come into contact with the used surface.

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In regards to the spontaneous activation of neutrophils adhered to the sensor chips, the newest produced chips have been cleaned in a new way using oxygen plasma cleaning. Due to this it is necessary to conduct new experiments to investigate if the spontaneous activation occurred due to contaminations due to insufficient cleaning or if the cells actually react to the materials on the sensor chip.

If a reliable way to find the actual sensor electrode in the image is developed it would enable the calculation of a more reliable cell area to signal ratio since you would not take into account cells or parts of cells that isn’t actually affecting the sensor. Although seeing as to how the sensor measures the dielectric constant and how it changes when introducing cells or molecules, all cells on the surface might contribute to the change in signal, this needs to be evaluated.
Conclusion
In this project an image analysis script capable of detecting cells in fluorescent microscopy images has been developed as well as a graphical user interface enabling simplified image analysis of a large number of images instead of analyzing single images at a time. The analysis that has been conducted does not indicate that white blood cells indicate any specific adhesion tendencies towards any of the used surfaces; however the white blood cells applied to the electrical sensor chip show signs of being activated in significant amounts. Thus leading to the conclusion that further investigations into how white blood cells react to the sensor surface as well as to how experiment needs to be conducted to enable the cell clinic application to work as desired.
Appendixes
All of the code files regarding the graphical user interface and the image analysis script were submitted separately, this because it was not possible to format the code in a way to make it reasonably presentable in this format.
Bibliography


Image Analysis:
https://se.mathworks.com/help/images/ref/imdistline.html (function description)

https://se.mathworks.com/help/images/ref/.... (function description)

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