Effect of SLAMF6 on T cell calcium signalling in response to antigen stimulation

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Degree Project in Physical Chemistry, 2026

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BSc, 30 hp

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Supervisor:
Peter Jönsson
Div. of Physical Chemistry

Examiner:
Felix Roosen-Runge
Div. of Physical Chemistry

Lund University
Department of Chemistry
Division of Physical Chemistry
P.O. Box 124
SE-221 00 Lund, Sweden

Popular Science Abstract

Our immune system protects us from things that can make us sick, such as viruses, bacteria, and other germs. One important part of the immune system is T cells which are a type of white blood cells. They act like a control system, checking whether cells in the body look normal. If they detect abnormal cells, they can destroy them before they become a problem and make us sick.

Our body is constantly renewing cells by making copies of them. Sometimes this cell replication goes wrong and abnormal cells start to grow and multiply uncontrollably. If the immune system detects these abnormal cells early, it can help prevent cancer. However, if it doesn't, cancer may develop and the body might need medical help to fight it.

Acute myeloid leukaemia (AML) is a type of cancer where the bone marrow starts producing abnormal white blood cells. This leads to a weaker immune system, which has a harder time fighting infections. Many cancer treatments can be stressful for the body, and there is ongoing research to improve them. One common treatment is immunotherapy, which works by strengthening the immune system so it can better recognize and attack cancer cells. A potential target for immunotherapy that has been researched is signalling lymphocytic activation molecule family member 6 (SLAMF6). How it affects the immune system and contributes to the elimination of cancer cells is still not fully understood and is therefore being actively studied. In this thesis an experimental system was developed and optimised to study on a physiochemical level how SLAMF6 affects signalling in T cells. To reach this goal, several subgoals were defined:

1) Creating a supported lipid bilayer (SLB) with proteins of interest to mimic a cell membrane. SLBs are models of cell membranes consisting of a lipid bilayer deposited on a glass slide, that have a controlled composition and can be favourably studied with fluorescence microscopy.
2) Binding T cells to the SLB using different relevant receptor-ligand pairs, including CD58-CD2, SLAMF6-SLAMF6 and peptide loaded major histocompatibility complexes (pMHC) binding to T cell receptors (TCRs). CD58 and CD2 are adhesion molecules whereas TCR is a receptor on T cells that initiates signalling upon binding pMHC molecules displaying parts of a pathogen.

3) Inducing and studying calcium signalling in the T cells. The release of calcium ions can be measured, by adding a fluorescent dye that blinks once it detects calcium in the cell. This was done both with TCR binding pMHC in the SLB as well as via antibodies targeting the TCR.
4) The concentration of TCR-pMHC pairs was varied and a dose-response curve for signalling was obtained.
5) The final step was to investigate how SLAMF6 affects the dose-response curve. Will it make the T cell more or less sensitive? This was investigated by comparing how many T cells signalled at a fixed number of pMHC molecules, with and without SLAMF6. The results indicate that the concentration of trigger molecules influences cell signalling and that SLAMF6 lowers T cell activation. Which suggests that it can reduce T cell activation in this system.

Abstract

SLAMF6 is an immunoregulatory molecule studied in cancer research as a possible target for immunotherapy in acute myeloid leukaemia (AML). The absence of SLAMF6 has been shown to elevate T cell activation and improve anti-tumour responses (1). The regulatory mechanism of SLAMF6 is not fully understood. The aim of this thesis was to investigate the effect of SLAMF6 on T cell signalling using fluorescence microscopy. The experimental system was developed stepwise. First a mobile SLB containing relevant proteins (including CD58, SLAMF6, and peptide loaded major histocompatibility complex (pMHC)) was created. Cells were then added to the SLB to analyse binding patterns. The T cells were subsequently activated by pMHC binding T cell preceptors (TCRs) on the T cells, and a dose-response curve of the number of activated cells vs pMHC density was generated. Finally, SLAMF6 was added to the system to investigate how it affects T cell signalling. Fluorescence recovery after photobleaching (FRAP) analysis confirmed the formation of a mobile SLB with proteins successfully incorporated in the bilayer. T cells adhered to the SLB through CD58 mediated interactions and showed accumulation of SLAMF6, UCHT1-Fab and pMHC in the cell-SLB contact at the cell surface. T cell activation was observed through calcium signalling, measured using the calcium sensitive dye Fluo-4am. The fraction of signalling cells increased with increasing concentration pMHC, enabling the creation of a dose-response curve. Lastly the addition of SLAMF6 reduced the fraction of signalling cells, suggesting that SLAMF6 regulates T cell activation in this system.

Keywords: Cell signalling, Fluorescence microscopy, SLAMF6, T cell activation.

Acknowledgement

I would like to thank Peter Jönsson for supervision, feedback, and support throughout this project. I also thank David Campana for continuous support and for always taking the time to help when needed. Finally, I would like to thank everyone who supported me during this project, as well as my family for their encouragement throughout my studies.

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1 List of abbreviations

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt)	POPC:DGS-NTA(Ni)
Acetoxymethyl	AM
Acute Myeloid Leukaemia	AML
Acute Myeloid Leukaemia	AML
Antigen Presenting Cells	APCs
Cancer Thesis Antigen	CTA
Cluster of Differentiation 58	CD58
Fluorescence Recovery After Photobleaching	FRAP
HEPES Buffered Saline	HBS
Major Histocompability Complex	MHC
Peptide- Major Histocompatibility Complex	pMHC
Region Of Interest	ROI
Signalling Lymphocytic Activation Molecule Family Member 6	SLAMF6
Supported Lipid Bilayer	SLB
T Cell Receptor	TCR
Total Internal Reflection Fluorescence	TIRF

2 Introduction

T cells are an important part of the immune system, responsible for fighting foreign pathogens. T cell receptors (TCRs) recognize antigen derived peptides when they are presented by the major histocompability complex (MHC) on antigen presenting cells (APCs). Precise regulation of T cells are important for eliminating foreign pathogens and cancer cells while preventing autoimmune diseases caused by an overactive immune system (2). Signalling lymphocytic activation molecule family member 6 (SLAMF6) is a recently studied protein in cancer research. It's an immunoregulatory molecule expressed on T cells, NK cells and B cells and is involved in regulating the immune system. It is being investigated as a potential target for immunotherapy in acute myeloid leukaemia (AML), as it may contribute to reduced anti-tumour immune responses. The deletion of SLAMF6 has been shown to enhance T cell activation and improve anti-tumour responses (1). However, the underlying mechanisms are not yet fully understood and are currently being studied. In this thesis the aim was to investigate how the presence of SLAMF6 affect the signalling of T cells. To achieve this, different parts of the experimental system had to be developed and optimized. Establishing each part was considered a sub-goal and the work was divided into five different steps. All experimental results were quantified using fluorescence microscopy. The first goal was to create a mobile supported lipid bilayer (SLB) containing proteins such as cluster of differentiation (CD58), SLAMF6, the anti-TCR antibody UCHT1 and the NY-ESO-1 peptide loaded major histocompatibility complex (pMHC). The second step was to achieve binding of the T cells to the bilayer, mediated by the proteins present on the SLB. This binding was quantified through colocalization analysis. The third step was to measure calcium signalling in the T cells interacting with SLBs containing different proteins. The fourth step was to generate a dose-response curve showing the fraction of signalling T cells at different concentrations pMHC. The final goal was to investigate how SLAMF6 affects cell signalling in the established experimental system.

2.1 Fluorescence Microscopy

The process of fluorescence involves the excitation of light followed by the emission of the light at a longer wavelength. This process includes a loss of energy, where the emitted light has less energy than the excited. The loss of energy is shown by the Stokes shift, which explains the change in wavelength from excited to emitted light. Fluorescence indicators with large Stokes shifts are used in fluorescence microscopy to efficiently separate emitted light

from excitation light. A large Stoke shift is advantageous as it reduces background signal and improves the signal to noise ratio by minimizing spectral overlap between excitation and emission wavelengths (3).

Total internal reflection fluorescence (TIRF) is a specific microscope setting, where it produces high resolution imaging in a single plane. The technique works by directing excitation light through a glass surface, such as a coverslip, at a shallow angle so that it totally reflects at the glass slide-sample interface. The reflection creates a very thin (≈100nm) evanescent light field, only exciting molecules close to the surface, as a result only molecules near the glass slide are seen (3). This technique is useful for studying protein movement at the cell membrane, as well as single molecules, cell contacts and cell adhesion.

The fluorescence microscope can also be used when studying activation of T cells, which is shown by the release of Ca^{2+}. This imaging includes a different technique. Instead of using high resolution (100x) and TIRF, using low resolution (10x) and epifluorescence at 488nm. In epifluorescence a parallel beam of light is passed via an objective directly upwards through the sample, focused at a certain distance. Both the illuminated and emitted light travel through the same objective lens. The technique is particularly useful when imaging thick samples. The backside is the higher amount of background compared to TIRF.

2.2 Supported lipid bilayers (SLBs)

Cell membranes are thin, sheetlike structures that form closed boundaries between different compartments. They consist mainly of lipids and proteins. The lipids are primarily arranged in a bilayer, where each lipid are amphipathic molecules meaning they have a hydrophilic head and hydrophobic tail. Phospholipids are the major class of membrane lipids, consisting of one or more fatty acid, attached to a backbone (commonly glycerol), and a polar headgroup formed by a phosphate group linked to an alcohol. The fatty acid components provide a hydrophobic barrier while the remainder of the molecule has hydrophilic properties, resulting in the formation of a lipid bilayer with the hydrophobic tails stacking together. This arrangement creates a semi-permeable barrier, between the inside and outside of the cell, that restricts the passage of most polar and charged molecules while allowing small nonpolar molecules to diffuse more easily. The membrane is fluid, allowing lipids and many proteins to move in the plane of the membrane, described by the fluid mosaic model (4).

Supported lipid bilayers (SLBs) are a mimic of the cell membranes consisting of a lipid bilayer formed on a solid surface. It's often used to understand fundamental functions such as protein interactions and membrane organization. SLBs can be made by the vesicle fusion technique which involves adding small unilamellar vesicles to a target surface. Hydrophobic interactions are the major driving force in the formation of lipid bilayers (4). Electrostatic forces together with other surface forces drive the interaction of vesicles with the solid surface, facilitating vesicle fusion and rupture. Vesicle rupture can trigger the rupture of neighboring vesicles, ultimately leading to the formation of a continuous planar bilayer across the surface (5).

The SLBs made in this project consisted of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (POPC:DGS-NTA(Ni)). POPC is a common phospholipid used in synthetic membranes. DGS-NTA(Ni) is a functionalized lipid containing a nickel-nitrilotriacetic acid (Ni-NTA) group that has high affinity for histidine-tagged (His-tagged) proteins, allowing them to bind specifically to the bilayer. When embedded in a POPC bilayer, it enables controlled anchoring of His-tagged proteins to the membrane.

2.3 T cells

T lymphocytes (T cells) are an important part of the adaptive immune system. Their main tasks are to respond quickly to foreign antigens and eliminate them, generate memory T cells for long-term protection and cooperate with B cells to ensure an effective immune system. T cells recognize foreign pathogens presented by major histocompatibility complex (MHC) molecules on antigen presenting cells (APCs). When the T cells receptor (TCR) on the T cell surface discover a matching antigen accompanied by costimulatory signals and cytokines from APCs T cells can become activated. The T cells then proliferate and differentiate to kill infected cells, produce cytokines, and regulate immune responses (2).

Precise regulation of the T cell activation process is crucial for maintaining the balance between generating specific T cell subtypes to fight various infections and cancer while preventing autoimmunity and inflammatory disease caused by dysregulated T cell responses (6). One of the molecules involved in regulating the T cell is SLAMF6.

2.3.1 Signalling Lymphocytic Activation Molecule Family Member 6 (SLAMF6)

SLAMF6 is an immunoregulatory molecule expressed on T cells, NK cells and B cells. It functions as a self-ligand receptor and plays an important role in regulating both innate and adaptive immune responses. It can either act activating or inhibiting on immune responses depending on the expression of intracellular adaptor protein SAP, which SLAMF6 signals through. In the presence of SAP, it has been observed that SLAMF6 signalling can promote T cell activation, whereas in the absence of SAP, it recruits inhibitory phosphatases and transmits inhibitory signals (7). SLAMF6 has been shown to function as an immune inhibitory mechanism in acute myeloid leukaemia (AML) cells, protecting the cancer cells from recognition and elimination by the immune system. Genetic deletion of SLAMF6 in AML cells enhances T cell activation and promotes cytotoxic elimination of leukemic cells in coculture systems. Similarly, targeting SLAMF6 with an antibody against its dimerization site disrupts homophilic interactions, leading to T cell activation and killing of AML cells. Research is currently exploring whether SLAMF6 can serve as a targetable immune checkpoint for AML immunotherapy (1), but the molecular mechanism of how SLAMF6 influences T cell sensitivity is currently under debate.

2.3.2 Calcium signalling and Fluo4-am

T cell activation through the TCR triggers a signalling pathway that leads to an increase in intracellular calcium levels. Activation of an enzyme results in the production of 1,4,5-triosphosphate (IP3). IP3 binds to receptors on the endoplasmic reticulum and causes the release of calcium into the cytosol. In response to the loss of calcium from the endoplasmic reticulum calcium channels in the plasma membrane open, allowing extracellular calcium to enter the cell. This results in a sustained increase in cytosolic calcium concentration (6).

Fluo-4AM is a calcium indicator that increases its fluorescence intensity when it binds to calcium ions. Fluo4-AM can diffuse across the cell membrane because of the attached acetoxymethyl (AM) group which makes the molecule hydrophobic. Inside the cell, in the cytosol, intracellular esterase's cleave the AM groups, converting it to Fluo-4, which is trapped inside the cell (8).

2.3.3 NY-ESO-1 and MHC

NY-ESO-1 is a cancer thesis antigen (CTA), expressed in various cancer types. Its highly immunogenic, inducing strong T cell and antibody responses. Normal somatic tissue does not express it, making it a good tumour antigen for immunotherapy. In cancer cells, the NY-ESO-1 gene is aberrantly activated, and the protein is processed into peptides that are presented on MHC class I molecules, making them recognizable to T cells. The antigen presentation leads to the activation of CD8+ cytotoxic T cells which can recognize and kill NY-ESO-1 expressing cells (9). The experiment will include both wild-type Jurkat T cells, which do not interact with NY-ESO-1, and Jurkat cells transduced with TCR against NY-ESO-1.

3 Materials and Methods

3.1 Sample preparation

3.1.1 Cleaning glass slides

Piranha solution was used to clean glass slides from organic residues and ensure proper SLB formation. Piranha is a mixture of 3:1 sulfuric acid (4.5ml, 95-97% purity, Sigma Aldrich) and hydrogen peroxide (1.5ml, 30%, Sigma Aldrich). Due to the strongly exothermic nature of the reaction, the hydrogen peroxide was added dropwise to the sulfuric acid. A microscope glass slide was added to the solution and the solution was heated for 30 min using a hot plate (∼80°C). The slide was stored in deionized water and, prior to use, rinsed with deionized water and dried using a nitrogen gun. A silicone well was cut and attached to the glass slide.

3.1.2 Forming Bilayers

HEPES Buffered Saline (HBS) (25μl) was added to the silicone well to wet the surface. A solution containing HBS (45μl) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (95 wt%) and 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (5 wt%) (POPC:DGS-NTA(Ni) = 95:5 wt%) (5μl, 1mg/ml) was prepared and added to the well to facilitate vesicle fusion and rupture. The SLB was incubated for 30 min, followed by washing with HBS (5x50μl).

3.1.3 Addition of SLAMF6 and CD58

The Ni^{2+} ions in POPC:DGS-NTA(Ni) coordinate with Histidine-tags, enabling the specific binding of recombinant, fluorescently tagged proteins such as CD58 (Alexa Fluor 488 tagged) and SLAMF6 (Alexa Fluor 647 tagged) that had been engineered with multi-histidine tags at the protein C-terminal. Recombinant, his-tagged proteins (SLAMF6 and CD58) were added at various concentrations (2-5μl, 0.1-0.001mg/ml, R&D Systems), incubated for 30 minutes and washed with HBS (3x30μl).

3.1.4 Addition of UCHT1-Fab and pMHC

UCHT1-Fab, an antibody fragment targeting the CD3 part of the TCR complex and pMHC providing antigen specific stimulation were used in separate experiments for T cell activation. Both proteins were fluorescently tagged with Alexa Fluor 647 and were added at various

concentrations (3-5 μl, 0.1-0.001mg/ml, obtained from R&D Systems and KACTUS Bio respectively), incubated (1-2h) and washed with cell media (3x50μl).

3.2 Cell culturing

The T cells used in the experiments were from the Jurkat cell line, including both transduced Jurkat cells expressing TCR against NY-ESO-1 and non-transduced (wild-type) Jurkat cells. The Jurkat cells were grown in RPMI 1640 medium with 2mM L-glutamine and sodium bicarbonate (Sigma Aldrich), 10% Fetal bovine serum, 1% sodium pyruvate, 1% HEPES, 1% Penicillin-Streptomycin. The cells were incubated in 37°C with 5% CO_2. The cells were split and given fresh media every two days, or whenever they reached a density of $1*10^6$cells/ml, to ensure proper nutrition.

3.3 Binding of T cells to the SLB

The bilayer was observed under the fluorescence microscope to ensure a proper, mobile bilayer. The T cells (15μl) were added to the bilayer containing the proteins of interest and allowed to settle for approximately 20 minutes prior to analysis. The optimal incubation time for the interaction between the cell and SLB mediated through CD58 was studied, along with cell binding to SLAMF6, UCHT1-Fab and pMHC. Protein distribution was also investigated.

3.4 Calcium signalling

3.4.1 Fluo-4am sample preparation

Fluo4-am kit from Thermo Fisher Scientific (F10489) was used. Probenecid solution (1000μl, 1x) was made by mixing Probenecid (10μl, 100x) and HBS (990μl). Approximately $1*10^5$cells were centrifuged (2rpm*1000, 2min), depending on the experimental condition either Jurkat cells or Jurkat cells transduced with anti NY-ESO-1 TCR were used. The centrifuged cell pellet was resuspended in supplement free RPMI 1640 (100μl), Probenecid (100μl, 1x) and Fluo4-am dye (1μl), incubated (37°C, 10min) followed by incubation (20°C, 20min). The cells were centrifuged (2rpm*1000, 2min), resuspended in Probenecid (900μl, 1x) and centrifuged (2rpm*1000, 2min). The cells were then resuspended in HBS, ready for analysis.

3.4.2 Calcium triggering: UCHT1 on glass

As a standard system for T cell activation anti-CD3 (UCHT1R, 30μl, 0.01mg/ml, 1-2h, R&D Systems) on glass was used, which was incubated (≈1h) and washed with cell media (3x50μl). The focus was adjusted by addition of unlabelled T cells (3μl). The Fluo-4am labelled cells (15μl) were added to the well and a video with 5s intervals was taken meanwhile, to observe the landing and triggering of the T cells.

3.4.3 Calcium triggering: UCHT1-Fab

As a second test system for signalling, a glass slide was cleaned according to section 3.1.1 and a bilayer according to section 3.1.2 and CD58 (3-5μl, 0.01mg/ml) and his-tagged UCHT1-Fab (Fab2, 30μl, 0.01mg/ml, 1-2h, R&D Systems) was incubated (≈1h) and washed with cell media (3x50μl). The focus was adjusted by addition of unlabelled T cells (3μl). The Fluo-4am labelled cells (15μl) were added to the well and a video with 5s intervals was taken meanwhile, to observe the landing and triggering of the T cells.

3.4.4 Calcium triggering: pMHC

A glass slide was cleaned according to section 3.1.1 and a bilayer according to section 3.1.2, CD58 (3-5μl, 0.01mg/ml) and pMHC was incubated in differing concentrations (HLA-A*02:01/B2M/NY-ESO-1 monomer protein, 3-5μl, 0.1mg/ml-0.002mg/ml, 1-2h, KACTUS Bio) and washed with cell media (3x50μl). The focus was adjusted by addition of unlabelled T cells (3μl). The Fluo-4am labelled cells (15μl) were added to the well and a video with 15s intervals was taken meanwhile, to observe the landing and triggering of the T cells.

3.5 Calcium triggering: pMHC and SLAMF6

A glass slide was cleaned according to section 3.1.1 and a bilayer according to section 3.1.2, CD58 (5μl, 0.01mg/ml), SLAMF6 (5μl, 0.01mg/ml) and pMHC (5μl, 0.003mg/ml) was incubated (1-2h) and washed with cell media (3x50μl). The focus was adjusted by addition of unlabelled T cells (3μl). The Fluo-4am labelled cells (15μl) were added to the well and a video with 15s intervals was taken meanwhile, to observe the landing and triggering of the T cells.

3.6 Measurements on TIRF

The imaging of SLBs and cell contacts were done using a high resolution (CFI Apo TIRF 100XC oil, Nikon) oil immersion objective and TIRF. The microscope used is Nikon's Eclipse Ti. TIRF microscopy excites only molecules closest to the surface, which are visible due to their tagging or staining with a fluorophore. The imaging was controlled with the program MicroManager.

3.6.1 Diffusion measurements

To ensure a proper bilayer formation diffusion measurement was done. This was done either by just observing mobility in the bilayer, or by analysing the fluorescence recovery after photobleaching (FRAP). FRAP provides diffusion rates and the mobile and immobile fractions by bleaching a defined region and tracking how quickly unbleached fluorescent molecules move back into the bleached area (10).

3.6.2 Cell signalling measurements

The calcium triggering was performed using a low resolution objective (CFI Plan Fluor 10x, Nikon) and epifluorescence setting, exciting fluorophores throughout the entire thickness of the sample, not just near the surface. The imaging was controlled using the MicroManager program.

3.7 Analysis

The data obtained from the fluorescence microscope was analysed using Fiji and Matlab along with relevant plugins within these programs. Cell signalling measurements were analysed using a custom Fiji script built on the TrackMate plugin, presented in appendix A. The script provides both the number of detected T cells and the proportion of cells that are signalling. The FRAP data was analysed in Fiji, to assess fluorescence recovery and a MatLab plugin was used to determine diffusion coefficients, as well as mobile and immobile fractions (10). Intensity measurements were analysed in Fiji, by selecting a region of interest (ROI) and plotting time on the x-axis against intensity on the y-axis. Colour merges of images from the same area were generated in Fiji to visualise the distribution of proteins within the same ROI.

4 Results and discussions

4.1 Model cell surface characterization

The first step in the development of the experimental system was to create a functional mobile SLB with proteins. An illustration of the system is shown in figure 1.

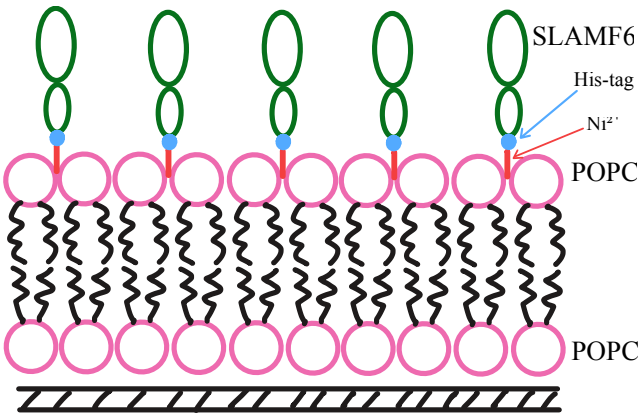

Figure 1. A schematic representation of a SLB presenting SLAMF6. SLAMF6 is anchored to the SLB via His-tag and Ni-NTA interactions. Both the SLB and the anchored proteins remain laterally mobile within the membrane, allowing diffusion in the plane of the bilayer.

To ensure a proper bilayer with mobile proteins, diffusion measurements were done in form of FRAPS, shown in figure 2 and 3. The results were then analysed in Fiji, to ensure recovery of the bleached area.

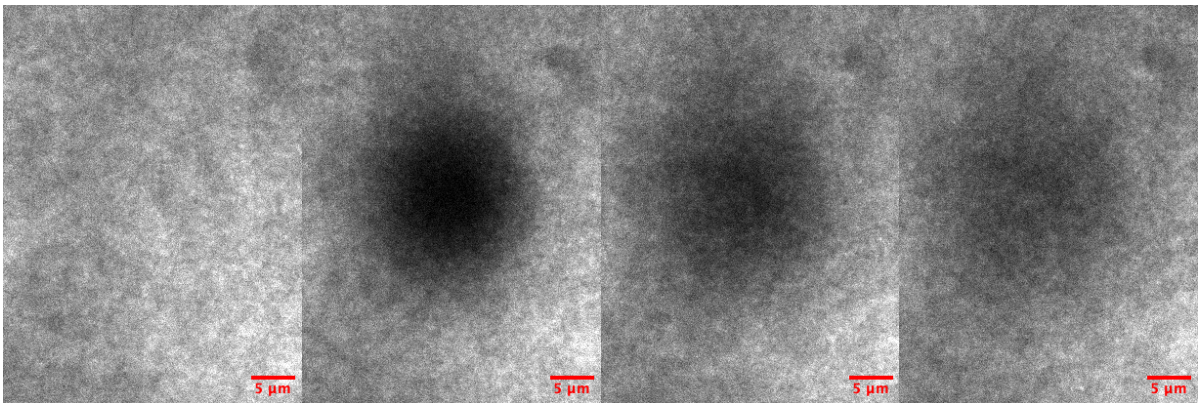

Figure 2. FRAP of fluorescently labelled SLAMF6. First picture from the left shows the bilayer before bleaching, second directly after bleaching, third 10 seconds after bleaching and fourth 20 second after bleaching.

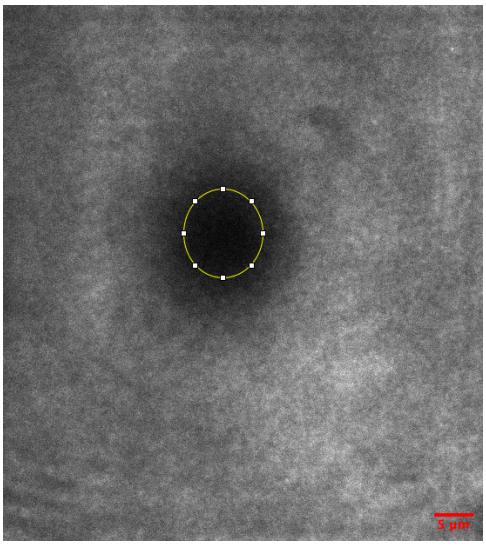

Figure 3. Shows the analysed bleaching spot of the FRAP (yellow circle), using ROI.

The recovery of the FRAP was high as shown in figure 4, indicating that most of the proteins are freely mobile within the bilayer and can diffuse back into the bleached area. The concentration of the proteins affects the diffusion coefficient. A lower protein density leads to less crowding and an increased diffusion rate, while a higher concentration may cause dimerization and crowding of the proteins, acting as a barrier for diffusion. The fluorescence intensity is directly correlated with protein concentration, a higher concentration results in higher intensity due to the presence of more fluorescent tags. Finding an optimal concentration was therefore crucial. This was tested by evaluating a range of different concentrations, and the optimal concentration, shown in figures 2,3 and 4, was determined to be 0.01mg/ml.

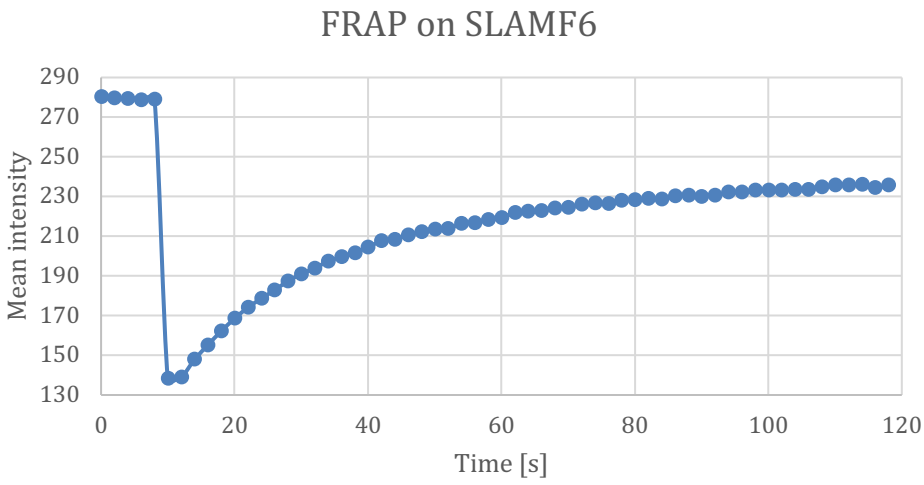

Figure 4. Recovery of FRAP, analysed in Fiji by plotting the mean intensity in the ROI shown in figure 3.

The laser used for imaging also photobleaches the whole frame when imaging the recovery. This causes a gradual decrease in fluorescence intensity across the whole frame, shown in figure 5, which also needs to be considered when analysing the recovery of the fluorescently labelled molecules.

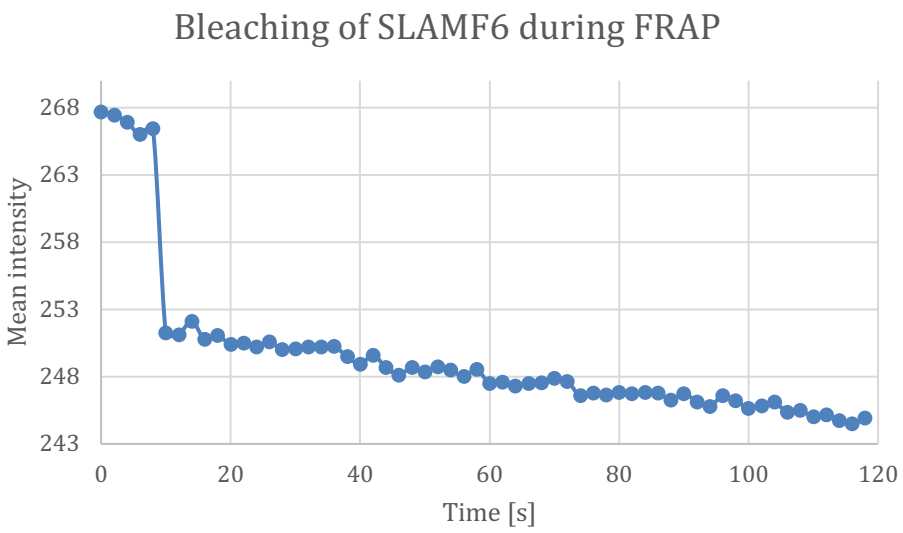

Figure 5. Shows the bleaching of the whole frame (not just ROI) when doing a FRAP.

To obtain the diffusion coefficient and mobile/immobile fractions the data were analysed in MATLAB using the frap_analysis package. The package provides several fitting models to best describe the data. As shown in figure 6, fit no.2 for example includes an additional parameter, gamma0, which accounts for the immobile fraction.

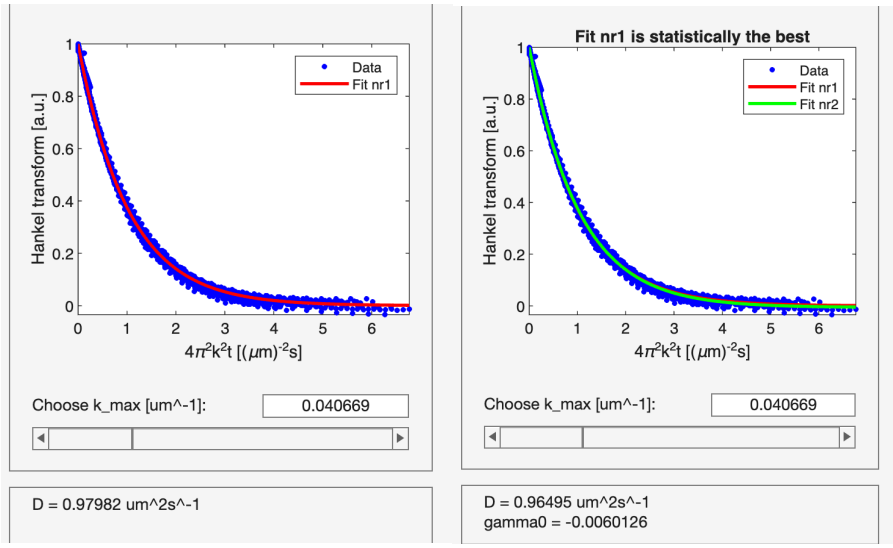

Figure 6. Diffusion coefficients of SLAMF6. The figures show two different fitting models, no immobile fraction (left) and with immobile fraction (right). The data shows almost no immobile fractions of SLAMF6 and therefore the fit to the left is statistically the best.

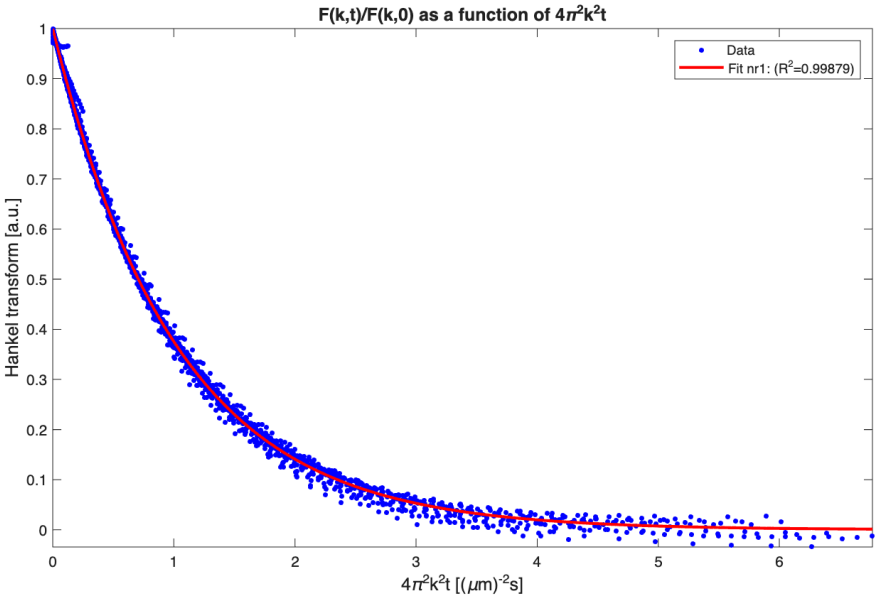

Figure 7. Shows fit no.1 from figure 6.

The diffusion coefficient from figure 7 was calculated to 0.98$\mu m^2/s$ which is within the reported range for membrane proteins in SLBs (0.2-2.6 $\mu m^2/s$). SLAMF6 is a transmembrane protein, which typically has a lower mobility compared to lipids. For comparison lipid diffusion in fluid bilayers is generally higher (1-4 $\mu m^2/s$) (11).

4.2 Binding of T cells to the SLB

The accumulation of protein at the cell surface indicates a receptor-ligand interaction and specificity of binding and is therefore investigated in the sections below.

4.2.1 Binding of T cells mediated by CD58

Once a functional and mobile SLB had been validated, T cells were added to examine accumulation at the interface between the bilayer and the cells. Redistribution of cell bound CD58 was observed within the cell contact area, see figure 8.

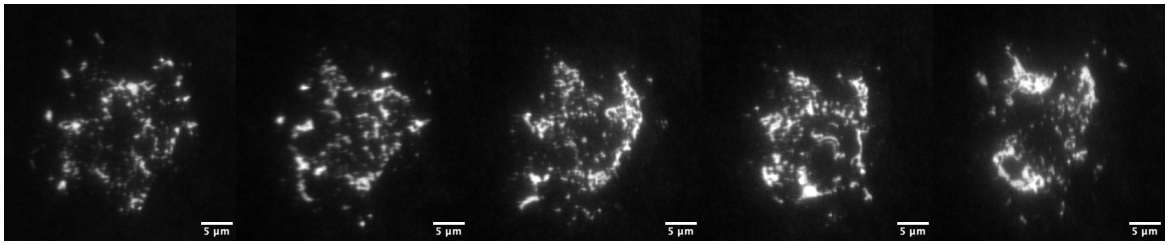

Figure 8. Shows the cell contact at different times after landing between the cell and the bilayer mediated by CD58. The white areas correspond to accumulation of fluorescent CD58 in the SLB in the contact. The different times are, from left to right [min]: 12, 17, 21, 28, 40.

Figure 8 shows how CD58 redistributes within the contact. The mean intensity of CD58 within the cell contact area was measured over different time points and is shown in figure 9. The mean intensity in general only increase moderately in the range 8 to 72 minutes.

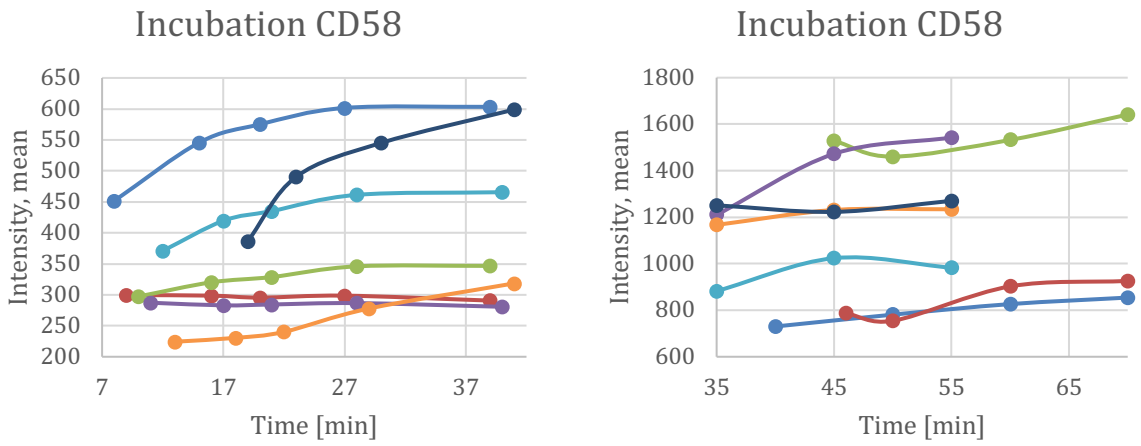

Figure 9. Change in mean intensity in different cell contacts at increasing time (left: 8 to 40 minutes; right: 35 to 72 minutes) after adding the cells. Each color is a separate contact. Left and right data are from two different experiments.

CD58 is a protein that mediates adhesion between the cell and the bilayer and is therefore present in all the measurements described below.

4.2.2 Binding of T cells mediated by SLAMF6

The ability of SLAMF6 to accumulate at the SLB-cell contacts was next investigated and confirmed, as shown in figure 10.

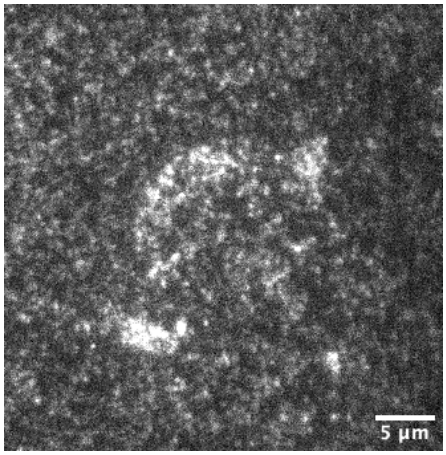

Figure 10. Shows the cell contact between the cell and the bilayer mediated by SLAMF6.

4.2.3 Binding of T cells mediated by UCHT1-Fab

The accumulation of antibody fragment UCHT1-Fab around the T cells was studied and shown to bind to the cells. A distribution analysis was also made to show localization patterns, shown in figure 11.

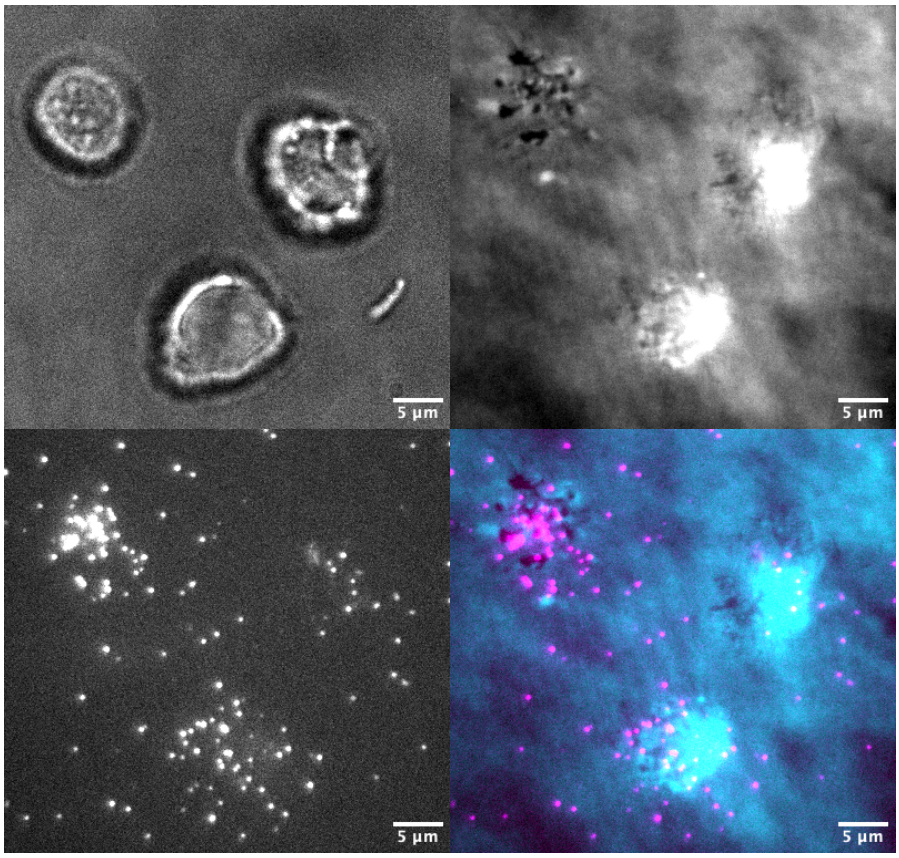

Figure 11. From left to right: cells pictured on the bilayer, the cell contacts between the cells and the bilayer mediated by CD58 and the accumulation of UCHT1-Fab around the cells. Merge of CD58 (blue) and UCHT1-Fab (pink) shows the distribution of proteins.

The merged images shown in figure 11 visualises the distribution of CD58 and UCHT1-Fab. The UCHT1-Fab which was at very low density in the SLB strongly binds to the cell interface observed as the accumulation of white dots in figure 11. Although not completely clear from figure 11, CD58 and UCHT1-Fab tend to occupy different regions of the contact, thus preferentially not colocalizing. This might be due to an effect of height difference between the two protein pairs.

4.2.4 Binding of T cells mediated by pMHC

Binding of pMHC to the NY-ESO-1 transduced T cells was next studied and the results are shown in figure 12 and 13.

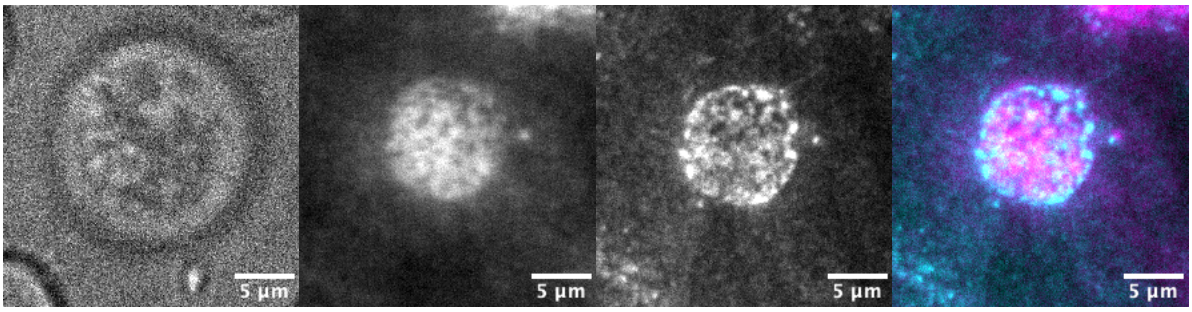

Figure 12. From left to right: a cell pictured on the bilayer, the cell contact between the cell and the bilayer mediated by CD58, the accumulation of pMHC around the cell and merge of CD58 (pink) and pMHC (blue) images.

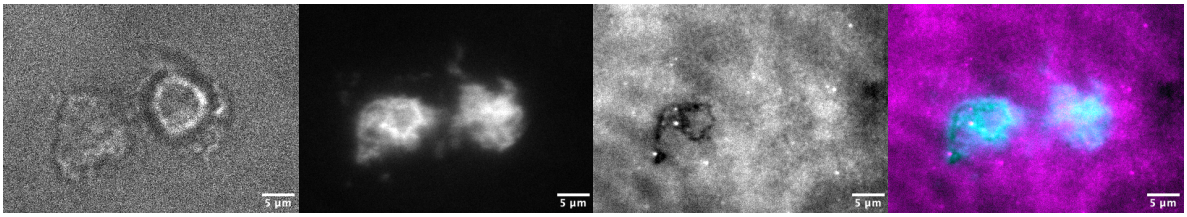

Figure 13. From left to right: cells pictured on the bilayer, the cell contacts between the cells and the bilayer mediated by CD58, the accumulation of pMHC around the cells, and image merge of CD58 (blue) and pMHC (pink).

The cells shown in figure 13 illustrate two adhered cells, one of which appears strongly activated (left cell). This is indicated by its shape, showing a spread and irregular morphology. Cell activation typically induces changes in the cytoskeleton, resulting in a flatter cell with increased surface area (12). Compared to figure 12, both cells in figure 13 show no clear pMHC accumulation. An explanation to this is that the pMHC density on the SLB is much higher in figure 13 than for the case shown in figure 12. Since the cells only have a limited number of TCR the contacts in figure 13 are likely already saturated with bound pMHC and the accumulation becomes less clear due to the high density of free pMHC.

As shown in figure 12 the pMHC and CD58 appear to colocalize. To examine this further it was also studied whether pMHC undergo lateral movement during contact formation/activation. A video of a pMHC mediated cell contact was acquired and showed redistribution of pMHC around and within the contact area, shown in figure 14. The mean fluorescence intensity within the cell contact was quantified and analyzed in Fiji, revealing a fluctuating behavior, which further indicates protein redistribution, shown in figure 15. However, this could also be due to fluctuations in illumination since the curves closely follow

each other. The maximum fluorescence intensity within the cell contact was also analyzed in Fiji and showed transient increases, suggesting the formation of local regions with elevated protein concentrations, visualized in figure 16.

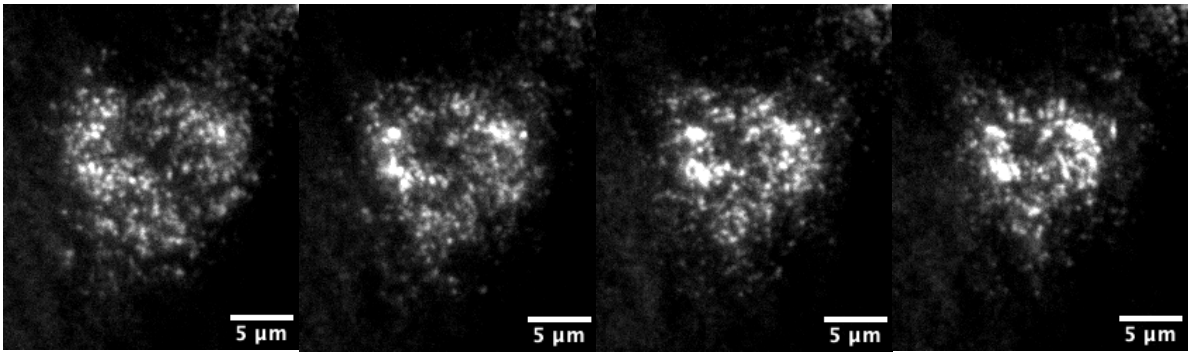

Figure 14. Fluorescently labelled pMHC in a cell contact at different times. Visualizes that the proteins remain cell associated while exhibiting dynamic redistribution around and within the T cells. From left to right: frame 1; 20; 40; 60 [15s intervals].

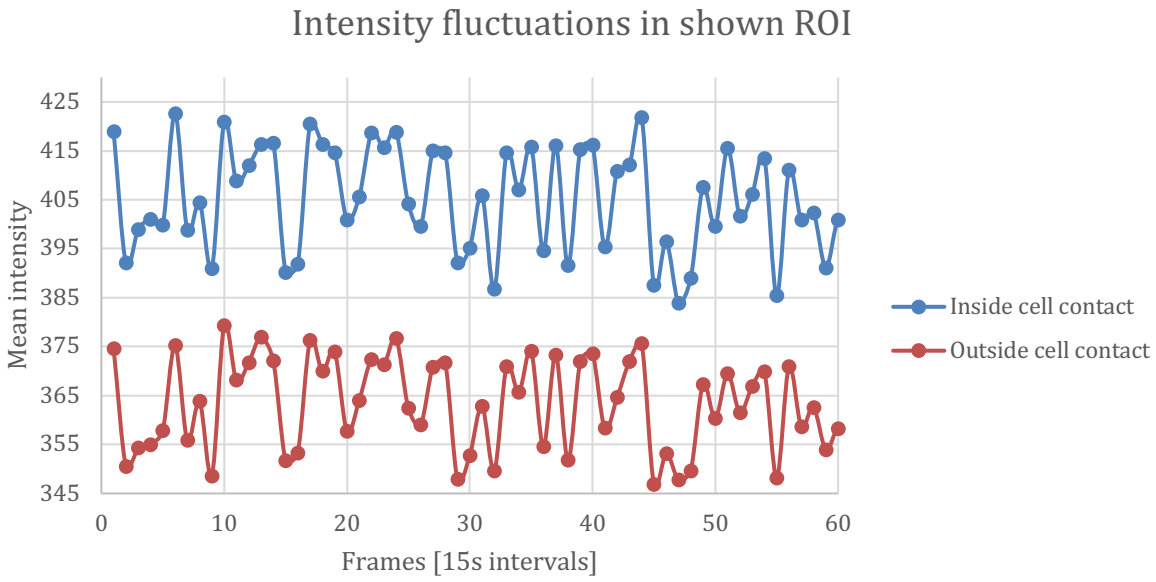

Figure 15. Shows how the fluorescence intensity within and outside of the cell contact fluctuates over time, indicating dynamic redistribution of the protein. Frame 1-60, later frames showed similar behavior.

The maximum fluorescence intensity, shown in figure 16 initially increases and then levels out. At the same time, figure 14 indicates the formation of clusters and a more compact contact structure, however the reason for this cluster formation was not investigated in this project.

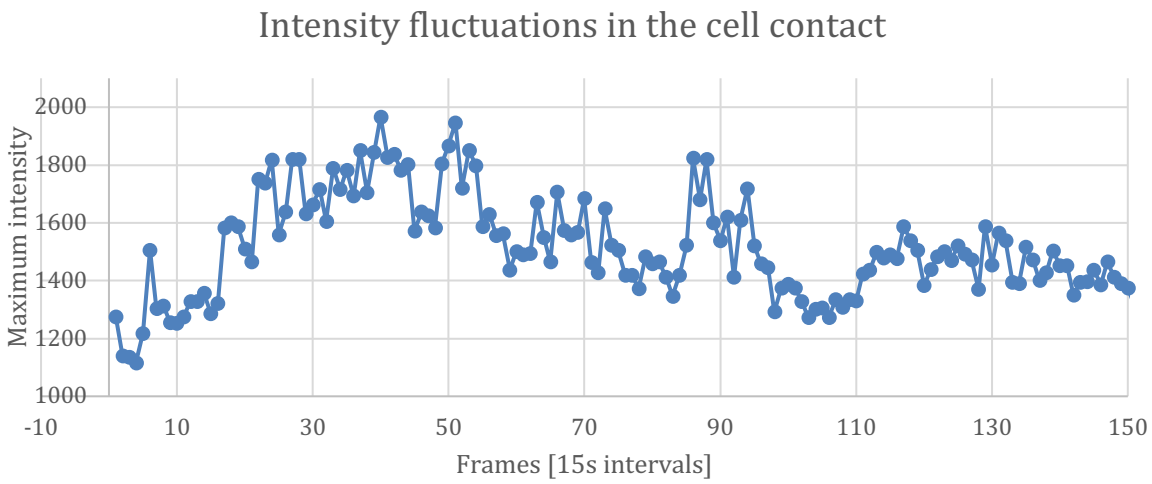

Figure 16. Shows fluctuations in maximum intensity within the cell contact, with transient increases suggesting the formation of localized regions of higher protein concentration. Frame 1-150, later frames showed similar behavior.

The accumulation of protein at the cell surface indicates receptor-ligand interactions and is crucial for T cell activation and signalling.

4.3 Calcium signalling

4.3.1 UCHT1 on glass

To measure T cell activation, the T cells were labelled with Fluo-4am, a fluorescent indicator whose fluorescence intensity increases drastically with rising intracellular calcium levels caused by triggering. The procedure was first established using Jurkat cells stimulated with UCHT1 antibody on glass to confirm that the fluo-4am sample preparation was successful, shown in figure 17 and 18. The data was analysed using a custom script in Fiji built on the TrackMate plugin, which provided the fraction of activated T cells, the script is presented in appendix A.

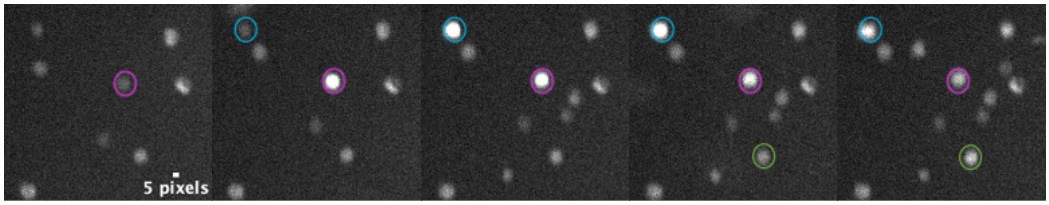

Figure 17. Cell signalling of activated T cells. Frame 40, 50, 70, 80, 90 [5s intervals]. Using UCHT1 as a trigger on glass. The circled cells are the ones activated in the current time interval. Only a few selected frames are shown here for illustration, while all frames were analysed. As signalling occurs at different time points, this may give the impression that only

a small fraction of cells are signalling at any given time. Signalling of 36.2% of the cells (25 of 69).

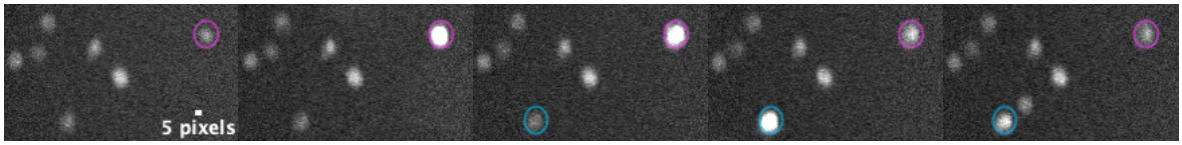

Figure 18. Cell signalling of activated T cells. Frame 40, 50, 70, 90, 100 [5s intervals]. Using UCHT1 to trigger the cells, on glass. The circled cells are the ones activated. Only a few selected frames are shown here for illustration, while all frames were analysed. As signalling occurs at different time points, this may give the impression that only a small fraction of cells are signalling at any given time. Signalling of 36.2% of the cells (25 of 69).

4.3.2 UCHT1-Fab

Once the fluo-4am sample preparation had been established an SLB was incorporated. A schematic illustration of the experimental system is shown in figure 19.

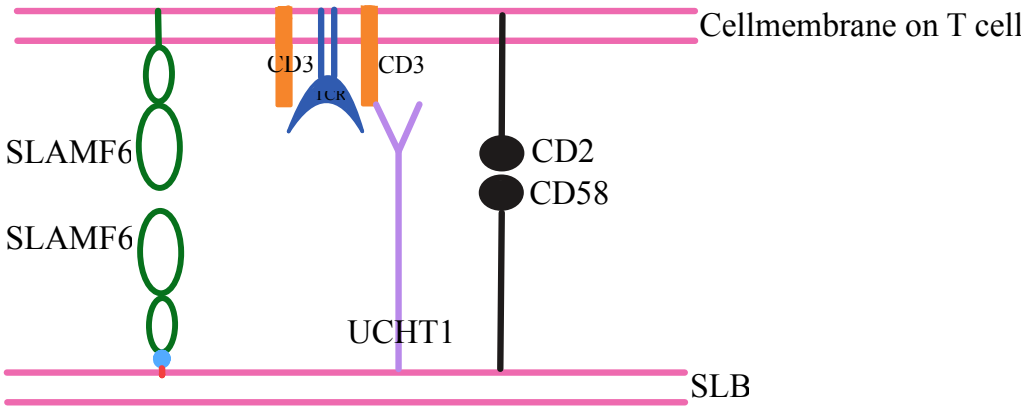

Figure 19. Schematic figure of a SLB presenting SLAMF6, UCHT1-Fab (anti CD3) and CD58. UCHT1-Fab on the SLB binds CD3 on the T cell and activates it without antigen. The binding between CD58 and CD2 promotes adhesion, while SLAMF6 forms homotypic interactions between the SLB and the T cell. Proteins on the SLB are anchored via His-tag and Ni-NTA interactions.

Calcium signalling was then studied with an antibody fragment to activate the T cells, UCHT1-Fab, as shown in figures 20 and 22. Only a few selected frames are shown for illustration, while all frames where analysed. Signalling occurs at different time points, which may give the impression that only a small fraction of T cells are signalling at any given time.

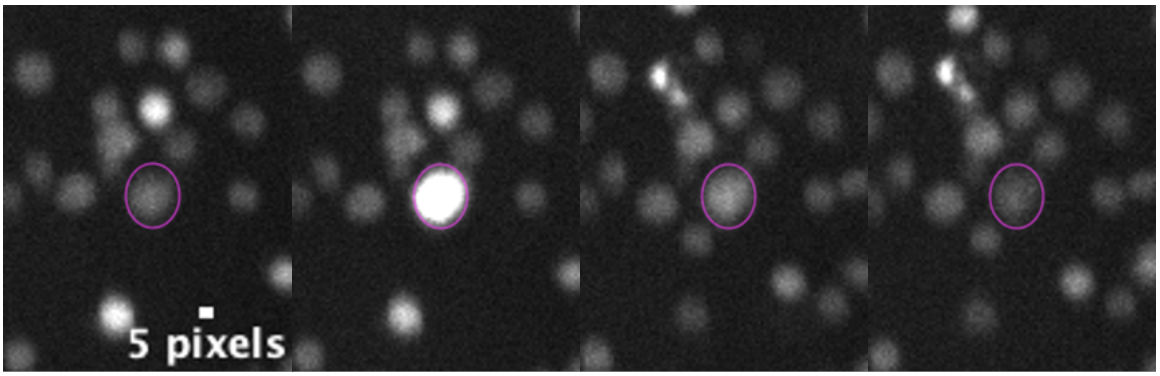

Figure 20. Cell signalling of an activated cell. Frame 115, 120, 160, 170 [5s intervals]. Using UCHT1-Fab as a trigger. The circled cell is the one signalling. Signalling of 51.8% of all cells (133 of 257).

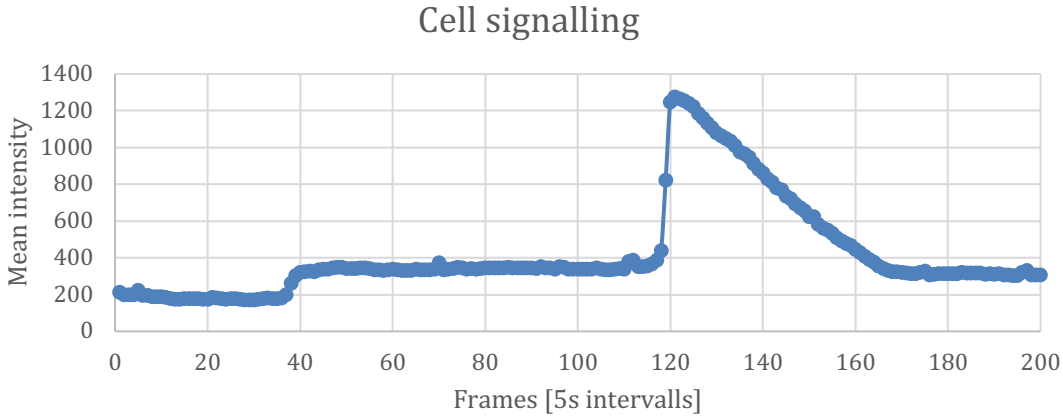

Figure 21. Mean fluorescence intensity of the signalling cell in figure 20. Shows the landing at frame 39, followed by the maximum intensity (signalling) at frame 121.

Figure 21 visualises a calcium burst, characterized by a rapid increase in mean fluorescence intensity followed by a gradual return to the baseline. This response is characteristic of intracellular calcium signalling, where stimulation triggers a transient increase in cytostolic calcium in the T cell before the Ca^{2+} in the cytosol is returned to basal levels.

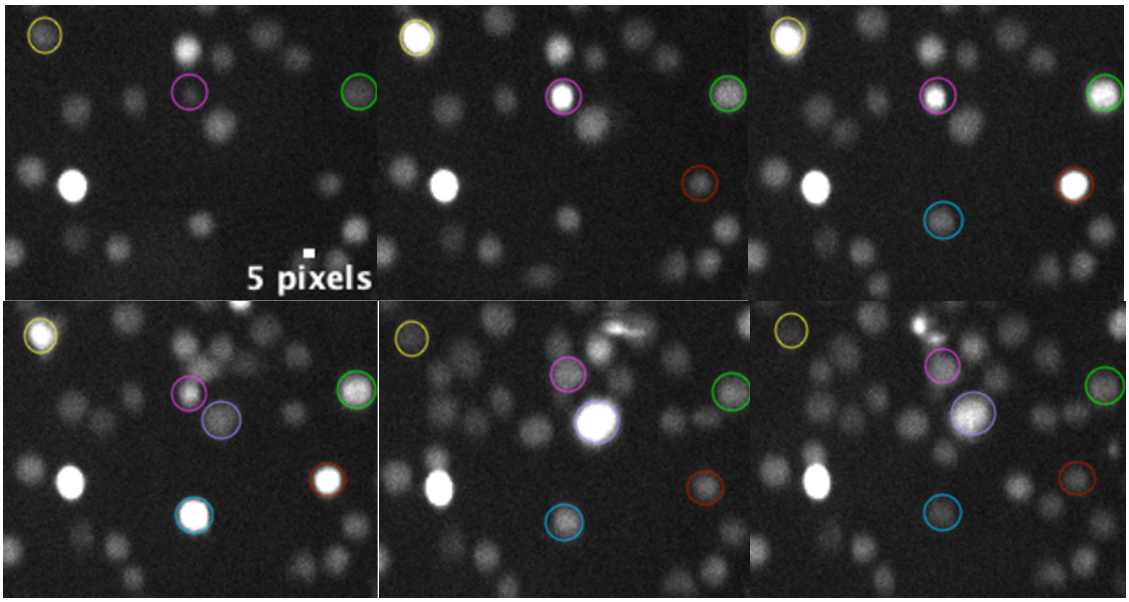

Figure 22. Cell signalling of activated T cells. Frame 60, 70, 80, 90, 130, 150 [5s intervals]. Using UCHT1-Fab to activate the cells. The circled cells are the ones signalling. Signalling of 51.8% of the cells (133 of 257).

The relatively low signalling percentage, 51.8%, is likely due to a low antibody concentration used, although this was not verified in this project.

4.3.3 pMHC

Once the cell signalling procedure was established using Jurkat cells, Jurkat cells transduced with NY-ESO-1 was studied with pMHC as a trigger. The data was analyzed in Fiji using a custom script built on the TrackMate plugin, presented in appendix A, which provided the fraction of activated T cells. A schematic illustration of the experimental system is shown in figure 23.

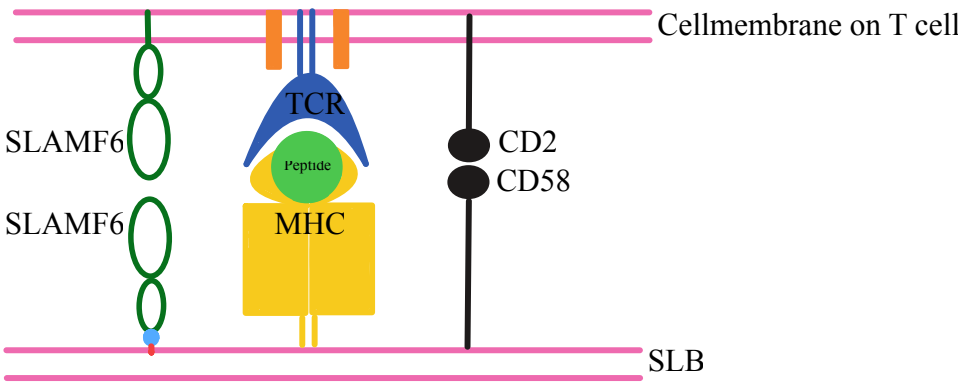

Figure 23. Schematic figure of a SLB presenting SLAMF6, MHC and CD58. MHC presents a peptide that is recognized by the TCR on the T cell, leading to activation. The binding between CD58 and CD2 promotes adhesion, while SLAMF6 forms homotypic interactions

between the SLB and T cell. Proteins on the SLB are anchored via His-tag and Ni-NTA interactions.

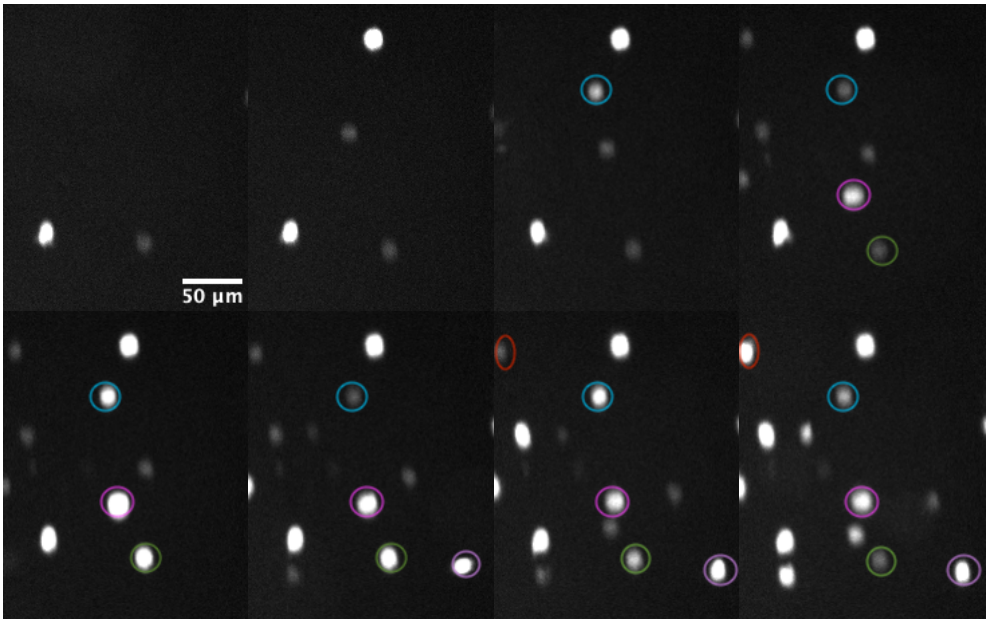

Figure 24. Signalling T cells, frame 10, 20, 30, 40, 50, 60, 70, 80 [15s intervals]. Using pMHC to trigger the cells. The circled cells are the ones signalling. Signalling of 57.6% of the cells, only a few frames are shown for illustration, signalling occurs at different time points.

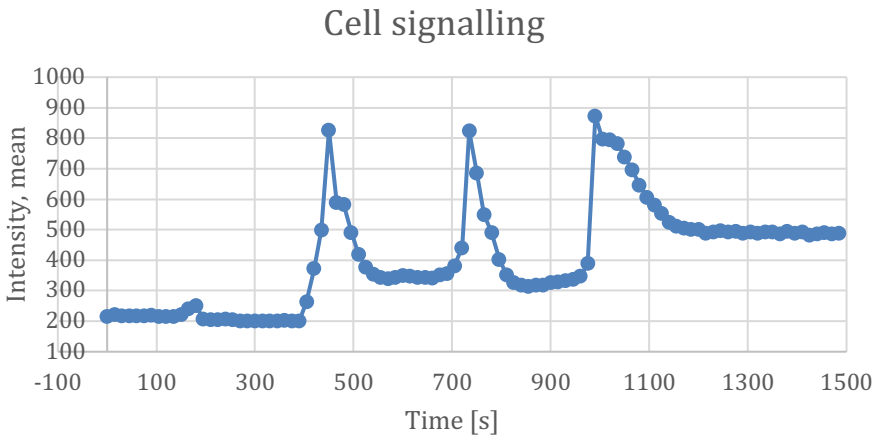

Figure 25. Multiple Ca^{2+} activations from a signalling cell (blue cell in figure 24).

As figure 24 and figure 25 vizualises, some T cells show repetitive calcium bursts, rather than a single spike. The amount of calcium spikes is however dependent on the single cell. Some of them have only one calcium burst (during the observed time), as shown in figure 21, while others have multiple or none. It's not only dependent on the concentration of ligand, pMHC in this experiment, but also on the ability of the cells to repeatedly initiate signalling events (13).

Another possible explanation why only a fraction of the cells signal is variability in TCR expression levels, where some cells may not express a sufficient number of TCRs to initiate signalling.

4.4 Threshold concentrations of pMHC

The procedure of activating NY-ESO-1 T cells with pMHC had been established, but the dose-response curve (threshold) of the NY-ESO-1 T cells was unknown. To find the triggering threshold of the NY-ESO-1 T cells, different concentrations of pMHC was examined, see appendix B. The data was then analyzed in Fiji using a custom script built on the TrackMate plugin, presented in appendix A, which provided the fraction of activated cells, shown in figure 26.

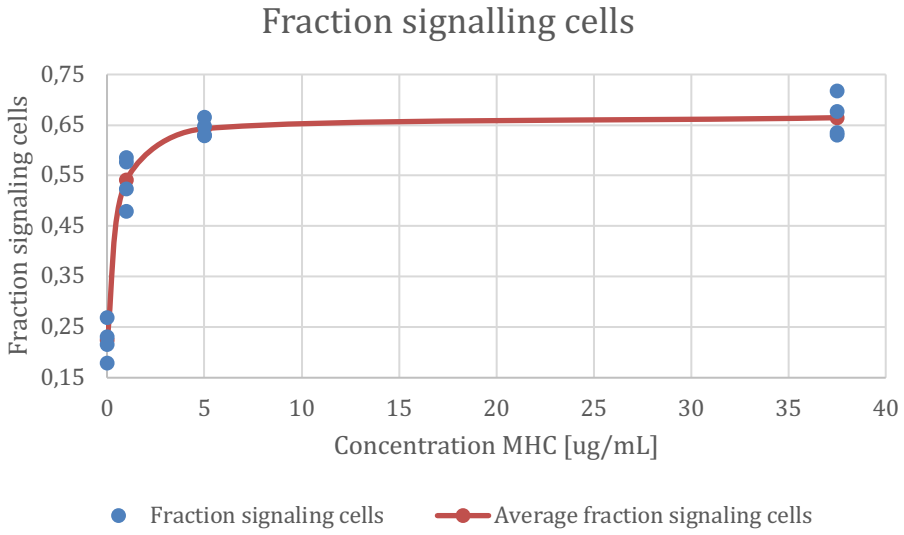

Figure 26. Shows how different concentrations of pMHC affects the fraction of signalling T cells.

As shown in figure 26 the fraction of signalling cells level of at around 65%. The cellular response is typically neither 0% or 100%, as shown in various studies (14). T cell activation is not only dependent on the concentration of pMHC, but also a combination of dynamic immunological synapse formation, cell to cell variability and signalling threshold (13). The sharp increase in the fraction of signalling cells suggests a threshold dependent activation mechanism, where signalling is rapidly initiated once a critical level of pMHC-TCR interactions is reached. The measured concentration of 1ug/ml pMHC corresponded to an intermediate level of cellular response in the dose-response curve.

4.5 How does SLAMF6 affect cell signalling

Once the dose-response curve had been established and a concentration between the maximum and minimum responses had been identified, this concentration was investigated further with the addition of SLAMF6 to determine whether signalling was affected. The signalling was reduced with the presence of SLAMF6, as shown in figure 27. This indicates that SLAMF6 lowers T cell activation. The negative control was added HBS instead of pMHC.

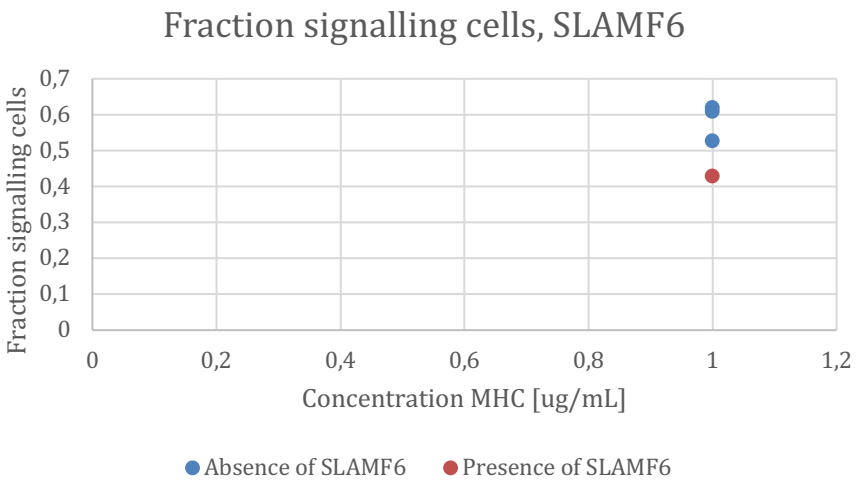

Figure 27. Shows how SLAMF6 affect the fraction of signalling T cells.

This experiment was only done once due to time constraints and the thesis deadline. The measurement in the presence of SLAMF6 was performed approximately 30 minutes after the measurement without SLAMF6. Therefore, the observed decrease in the fraction of signalling cells may be due to cell death over time and should be treated with some caution.

5 Conclusions

In conclusion, several experiments involving proteins on SLBs and T cells were performed to better understand the activation of T cells and the role of SLAMF6. The initial experiments aimed to develop and optimize the experimental procedures, including the formation of a mobile SLB containing proteins. FRAP analysis confirmed the formation of a mobile SLB with recombinant proteins successfully incorporated in the bilayer. T cells were successfully adhered to the bilayer through CD58. Accumulation of SLAMF6, UCHT1-Fab and pMHC at the cell surface indicated specific receptor-ligand interactions. Successful sample preparation using Fluo-4am was achieved to measure calcium signalling in the cells. Different pMHC concentrations were then tested to evaluate their effect on cell triggering, and a dose-response curve was established. The final goal was to investigate how SLAMF6 affects cell signalling in the established experimental system. The results indicate that the concentration of pMHC influences cell signalling and that SLAMF6 lowers T cell activation in this experimental system although further experiments are needed to verify this.

6 Future aspects

It would be interesting for future studies to further investigate the effects of the presence and absence of SLAMF6 in this experimental system. Due to time constrains and the thesis deadline, the experiment investigating whether SLAMF6 reduces T cell signalling was only performed once.

7 References

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8 Appendix

Appendix A. The script below is used for calcium analysis. It provides the number and fraction of signalling cells.

```
from ij import IJ
from java.lang import Double, Integer
from ij.measure import CurveFitter
from ij.gui import Plot
from java.awt import Color
from fiji.plugin.trackmate import Model, Settings, TrackMate
from fiji.plugin.trackmate.detection import LogDetectorFactory
from fiji.plugin.trackmate.tracking.jaqaman import
SimpleSparseLAPTrackerFactory
from java.lang import Math

imp = IJ.getImage()
nFrames = imp.getNFrames()
stack = imp.getStack()

for f in range(1, imp.getNFrames()+1):
    ip = stack.getProcessor(f)
    ip.subtract(ip.getStatistics().median)

imp.updateAndDraw()

model = Model()
settings = Settings(imp)

settings.detectorFactory = LogDetectorFactory()
settings.detectorSettings = settings.detectorFactory.getDefaultSettings()
settings.detectorSettings['DO_SUBPIXEL_LOCALIZATION']    = True
settings.detectorSettings['RADIUS']    = Double(6)
settings.detectorSettings['THRESHOLD'] = Double(3)

settings.trackerFactory = SimpleSparseLAPTrackerFactory()
settings.trackerSettings = settings.trackerFactory.getDefaultSettings()
settings.trackerSettings['LINKING_MAX_DISTANCE']     = Double(12)
settings.trackerSettings['GAP_CLOSING_MAX_DISTANCE'] = Double(12)
settings.trackerSettings['MAX_FRAME_GAP']            = Integer(0)
```

```
settings.addAllAnalyzers()

from fiji.plugin.trackmate.features import FeatureFilter

tm = TrackMate(model, settings)
tm.checkInput()
tm.process()

dt = IJ.getImage().getCalibration().frameInterval

def running_median(x, w):
    half = w // 2
    out = []
    for i in range(len(x)):
        lo = max(0, i - half)
        hi = min(len(x), i + half + 1)
        out.append(sorted(x[lo:hi])[len(x[lo:hi])//2])
    return out

factor = 2

int_all = []
t_all = []
triggered = []
counter = 0
for trackID in model.getTrackModel().trackIDs(True):

    spots = list(model.getTrackModel().trackSpots(trackID))
    spots.sort(key=lambda s: s.getFeature('FRAME'))

    if spots[0].getFeature('FRAME') < 1:
        continue

    if len(spots) < 20:
        continue

    int_track = []
    t_track = []
    for i in range(len(spots)-1):
        dx = spots[i+1].getFeature('POSITION_X') -
spots[i].getFeature('POSITION_X')
```

```
        dy = spots[i+1].getFeature('POSITION_Y') -
spots[i].getFeature('POSITION_Y')
        dr2 = dx*dx + dy*dy
        if dr2 > 3:
            continue

        int_track.append(spots[i].getFeature('MEAN_INTENSITY_CH1'))
        t_track.append(spots[i].getFeature('FRAME') * dt)

    if len(int_track) < 20:
        continue

    int_all.append(int_track)
    t_all.append(t_track)

    int_track_median = running_median(int_track, 5)

    int0 = int_track_median[0]
    flag = any(v > factor * int0 for v in int_track_median)
    triggered.append(flag)

    if counter == 10:
        title = "Intensity vs Time (threshold exceeded = %s)" % flag
        plot = Plot(title, "t", "I")
        plot.setColor(Color.black)
        plot.add("line", t_track, int_track)
        plot.setColor(Color.red)
        plot.add("line", t_track, int_track_median)
        plot.show()
        counter = 0

    counter += 1

x = sum(triggered)
y = len(triggered)
percent = 100.0 * x / y if y > 0 else 0.0
print("%d of %d cells triggered (%.1f%% triggered)" % (x, y, percent))
```

Appendix B. Shows the percentage of signalling cells, with varying concentrations of pMHC. Visualized in figure 26.

Concentration [ug/ml]	Percentage activated cells [%]
0	23.1
0	26.9
0	21.6
0	17.9
1	57.6
1	58.6
1	52.3
1	47.9
5	64.7
5	63.0
5	62.9
5	66.5
37.5	63.0
37.5	67.6
37.5	63.5
37.5	71.7