Single Molecule imaging
of the Lck lipid-anchor in live fibroblast cells

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Figure on front page:

Impression of diffusion in the cell membrane. Diffusion in domains, in-between domains and in and out of domains is depicted.
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1. Introduction

The cell membrane has been an object of study for a long time. The general view about structure and dynamics of the cell membrane has changed a lot over the last couple of years. It has been known for a long time that the cell membrane consists of a lipid bilayer that contains different types of protein, which is represented in the fluid mosaic model (19). But only recently scientists think about other, more complex, structures within this global model. The general opinion now is that there are rigid structures inside this lipid bilayer, called rafts or liquid-ordered domains, that assemble and disassemble dynamically (18,2). These liquid-ordered domains or rafts are rich in cholesterol and saturated lipids, like sphingolipids, which is thought to cause a decrease in fluidity (18). The structures are believed to play a role in signal transduction by serving as a ‘meeting point’ for proteins involved in (different) signaltransduction pathways. The measured diameter of these structures ranges from 30 nm up to 700 nm (15,17)

Because these domains are thought to be small it is difficult to see them with conventional microscopes on living cells. Therefore techniques like Single Molecule (SM) measurements are helpful in learning more about these structures. We used this technique, and other biophysical techniques, to learn more about diffusion in the inner leaflet of the membrane and the size of these domains.

By tagging the N-terminal end of the membrane kinase Lck with a fluorescent protein we are able to analyse its diffusional behaviour, using Fluorescent Recovery After Photobleaching (FRAP) and Single Molecule (SM) measurements. Raft localisation can be determined by cholesterol extraction. The combination of the above methods was used to determine if the fluorescent proteins localise in lipid rafts, as was found by biochemical assays (10). Further, information about the size of the rafts is obtained.

1.1 The function and characteristics of Lck

Lck is a protein tyrosine kinase (PTK) (9). It catalyses the transfer of the γ-phosphate of ATP to tyrosine residues on the protein itself or on other proteins functioning as substrates. This phosphorylation modulates enzymatic activity or creates binding sites for recruitment of downstream signalling proteins. The protein tyrosine kinases are ordered in two groups: the transmembrane receptor kinases (RTKs) that are activated by binding of a ligand, and nonreceptor kinases (NRTKs) that are triggered in signalling pathways. Lck is a NRTK of the Src family; this family is involved in processes like mitogenesis, T-and B-cell activation and cytoskeleton restructuring (9).

Most NRTKs are cytosolic, but Lck is anchored to the plasma membrane. The localisation to the membrane is mediated through amino-terminal modifications on Lck. The aminoterminus is myristoylated and palmitoylated (14). These are specific saturated lipids that are coupled to the protein.

Lck itself is constitutively associated with receptors on T-lymphocytes. Initialised by binding of the receptor with antigen, Lck becomes autophosphorylated and in turn it also phosphorylates the receptor. This phosphorylation (chemical binding of a phosphate group) is the way to move a signal detected outside, the ligand binding to the receptor, to a signal on the inside of the cell. The binding of a new NRTK activates the signalling pathway and
thereby leads to the activation of the transcription of involved genes. The binding of this second NRTK is only possible after phosphorylation.

In our construct the last 11 aminoacids of the original Lck construct* (fig. 1 Appendix) were coupled to the enhanced Yellow Fluorescent Protein (eYFP). In this way a membrane localised fluorescent protein was obtained (fig. 2).

Figure 2: Schematical drawing of the Lck construct (A) used for the SM and FRAP measurements. The one letters codes of the amino acids are represented in blue. eYFP is the enhanced Yellow Fluorescent Protein. Comparing white light image (B) and fluorescence image (with scale) (C) shows membrane localization of the construct in 3T3-A14 cells.

By using this construct instead of the full Lck construct we hope to be able to indicate whether there are rafts present in the cytoplasmic leaflet of 3T3-A14 cells and if acylation, which is the coupling of the fatty acids to the protein, of the N-terminus alone is sufficient to induce localisation to lipid rafts (14,3) or interaction with a receptor is required.

*The Lck construct was a gift of the group of A.I. Magee from the division of Membrane Biology at the National Institute for Medical Research in London.
2. **Theory**

Two methods to study the diffusional behaviour of Lck were applied, namely Single Molecule (SM) measurements and Fluorescence Recovery After Photobleaching (FRAP). In the following sections the two methods are explained and a theoretical description of diffusional properties of particles in a membrane is outlined.

### 2.1 A general description of Diffusion

Diffusion is described as the free movement of particles inside a solvent, which is a random walk characterized by a diffusion constant \( D \). The diffusion constant is determined by the viscosity \( \eta \) of the solvent and the size of the particle \( R \). The relationship between these parameters is given by:

\[
D = \frac{kT}{f} \tag{1}
\]

Where \( f \) is the friction coefficient \((12,16)\). This friction coefficient depends on the type of diffusion in question. In the simple case of three-dimensional diffusion of a sphere imbedded in a fluid the Stokes-Einstein relation holds and \( f \) is given by eq.(2).

\[
f = 6\pi\eta R \tag{2}
\]

In the case of protein diffusion in a bilayer the movement is only two-dimensional and the friction coefficient changes. The bilayer is treated as a viscous fluid sheet with a very small thickness \( h \) and a viscosity \( \mu \), bound on both sides by a three-dimensional fluid of much smaller viscosity \( \mu' \). The diffusing molecule is represented as a cylinder of height \( h \) and radius \( R \). (fig. 3)

![Figure 3: Schematical representation of two-dimensional diffusion in a sheet. In a sheet of thickness \( h \) and viscosity \( \mu \) diffuses a cylinder with height \( h \) and radius \( R \). The medium surrounding the sheet has a smaller viscosity \( \mu' \).](image)

\[\text{Figure 3: Schematical representation of two-dimensional diffusion in a sheet. In a sheet of thickness } h \text{ and viscosity } \mu \text{ diffuses a cylinder with height } h \text{ and radius } R. \text{ The medium surrounding the sheet has a smaller viscosity } \mu'.\]
The friction coefficient for this form of diffusion was derived by Saffman and Delbrück (16).

\[ f = \frac{4\pi\eta h}{(\ln \frac{\mu h}{\mu' R} - \gamma)} \]  

(3)

Where \( \gamma \) is Euler’s constant (0.5772).

Because we measure the displacement of a particle in time we need a relation between the diffusion coefficient and this displacement. For a two-dimensional homogeneous system the diffusion coefficient is related to the mean square displacement \( \langle r^2 \rangle \) by.

\[ \langle r^2 \rangle = 4Dt \]  

(4)

This relation is derived from the diffusion equation and fully describes data obtained by all ensemble-type measurements.

Given that for the SM measurements individual molecules are followed over time we are able to determine the full distribution function of the square displacements (SD), which will be developed in the following.

The probability \( p(r,t) \) to find a molecule at position \( r \) at time \( t \) is given by Fick’s 2\textsuperscript{nd} law.

\[ \frac{\partial p(\mathbf{r},t)}{\partial t} = -D \frac{\partial^2 p(\mathbf{r},t)}{\partial r^2} \]  

(5)

Using the initial condition that the molecule was at the origin at time \( t=0 \), this differential equation has a solution of the form (12) (using eq.(4)):

\[ p(r,t)dr = \frac{1}{\pi \langle r^2 \rangle} \exp\left(-\frac{r^2}{\langle r^2 \rangle}\right) dr \]  

(6)

For analysis the distribution of the squared displacements, \( r^2 \), is of particular interest. For this we transform eq.(6) into polar coordinates and integrate over the angular distribution, which leads to eq.(7).

\[ p(r^2,t)dr^2 = \frac{1}{\langle r^2 \rangle} \exp\left(-\frac{r^2}{\langle r^2 \rangle}\right) dr^2 \]  

(7)

Finally, we integrate eq.(7) to find the cumulative distribution function for the square displacement (20).

\[ P(r^2,t) = \int_0^{r^2} p(r^2,t)dr^2 = 1 - \exp\left(-\frac{r^2}{\langle r^2 \rangle}\right) \]  

(8)

Eq.(8) gives the probability that the particle stays within a circle of radius \( r \) during a timelag \( t \).

If the system is not homogeneous but consist of a homogenous membrane with two populations distinguishable by their mobility, the solution will be a superposition of eq.(8) with \( R \) (indicating mobility (section 2.2)) the relative contribution of the two systems to the distribution.

\[ P(r^2,t) = 1 - \left[R \exp\left(-\frac{r^2}{\langle r_1^2 \rangle}\right) + (1-R) \exp\left(-\frac{r^2}{\langle r_2^2 \rangle}\right)\right] \]  

(9)

Where \( r_i^2 = 4Dt \).
2.2 Fluorescence Recovery After Photobleaching (FRAP)

In this technique the intrinsic property of a fluorescent molecule to lose its fluorescent abilities on intense exposure to light is used. Measurements are done using a highly intense laser beam with a sharp focus (FWHM ~ 1.14 µm) to induce irreversible photobleaching in a small area (relative to the surface area of the whole membrane). A second weak beam (~ 1% intensity) with a larger width (FWHM ~ 6.5 µm) is used to scan the sample and record the data. The data are sampled in a sequence of illuminations to follow the recovery of the fluorescence due to diffusion (fig. 4).

![Figure 4: The general layout of a FRAP experiment. Fluorescence on the cell membrane is homogeneous before bleaching (A). A small spot is bleached using a laser (B). Fluorescent molecules from other parts of the cell membrane diffuse into this spot. After a certain time the fluorescence is homogeneous again if the molecules are fully mobile (C).](image)

In this way FRAP data are used to determine the diffusion coefficient and the fraction of mobile protein in a heterogeneous mobile/immobile sample. Fluorescence recovery will be less than 100% if there is an immobile fraction. The reason for an immobile fraction of proteins can be ‘real’ immobility due to e.g. anchoring to the cytoskeleton structures, or due to restrictions in the diffusional space, which are smaller than the size of the bleach beam and therefore cannot be replaced by new fluorescent molecules.

Assuming the diffusion to be two-dimensional and free, the fluorescence recovery is determined by the characteristic halftime of recovery \(t_{1/2}\), the initial fluorescence level before imaging \(F^0\), the fluorescence level directly after photo bleaching \(F(0)\) and the mobile fraction \(R\) \((1,21,4)\).

\[
F(t) = \frac{F(0) + (R(F^0 - F(0)) + F(0)) \left(\frac{t}{t_{1/2}}\right)}{1 + \left(\frac{t}{t_{1/2}}\right)}
\]  
(10)

The diffusion constant, \(D\), is determined by the characteristic halftime of the recovery and the beam radius \(w\).

\[
D = \frac{\beta w^2}{4t_{1/2}}
\]  
(11)
In this equation $\beta$ is a constant that is determined by the bleach depth, which is the amount of fluorescence that is lost by photo bleaching, and is 1 in the case of no more than 70% bleaching (1).

With this method bulk diffusion is studied, assuming a population of fluorescent proteins divided in a mobile and immobile fraction. These fluorescent proteins are localised at the cell membrane.
2.3 Single Molecule measurements (SM)

Another way to determine the diffusion behaviour is by the analysis of the trajectories of individual molecules for this we measured the movement of these individual fluorescent molecules on the apical membrane of the cell.

For this type of experiment a very low concentration of fluorophores (<0.1/µm²) is required, therefore the transient transfection protocol was optimised to achieve this level of fluorophore expression. This low concentration of fluorescence makes sure that the detected signal is from a single fluorescent molecule. Several checks are performed to ensure this assumption (7).

Individual molecules are followed in time by taking images at regular time intervals, ranging from 4 up to 55 ms. Illumination was in all cases set to 3 ms. On average molecules can be followed in three subsequent images. This number is limited by photo bleaching therefore the intensity of the used laser is kept low to minimize this.

The fluorescence peaks in the images are fitted to a Gaussian profile to determine their position, width and intensity. In this way the displacement, from picture to picture, of Single Molecules can be determined. The accuracy in peak positioning is determined by the signal to noise ratio and is 50 nm on average in our experiments. Ensembles or time averaging of the displacement data yield the mean square displacement (MSD) as a function of time (t).

Plotting the cumulative probability density function of the SD shows an exponential behaviour. Using eq. (9), it is possible to determine a parameter set \( \{r^2_1, r^2_2, R\} \) from which diffusion is calculated. If the value of \( R \) is zero (or 1) the population of the fluorophores is homogeneous, resulting in a monoexponential distribution. Otherwise the population is heterogeneous, the optimal function is a biexponential.

If the lipid-anchored eYFP is raft localised, diffusion of at least one of the populations should be restricted. In that case displacement would be confined to an area and the mean square displacement would no longer be linear in time (11). Assuming that diffusion within this domain is free and the size of this domain is \( L \), the mean square displacement would depend on the timelag \( t \) and the initial diffusion \( (D_0) \) in the following way (11).

\[
\langle r^2(t) \rangle = \frac{L^2}{3} \left( 1 - \exp \left( -\frac{12D_0 t}{L^2} \right) \right) \quad (12)
\]

Fitting the mean square displacement as a function of time with this function, if restriction is apparent in the data, will yield the size of the domain the fluorescent molecule is localised in. Comparison with cholesterol extraction data will indicate if these domains are detergent resistant.
2.4 Cholesterol extraction

In trying to determine whether diffusion is influenced, as suspected, by raft like structures we used a drug called methyl-β-cyclodextrin (MβCD) (Sigma-Aldrich chemie). MβCD (fig. 5) is structured like a spherical shell in which cholesterol can be caught. Incubation of cells with this chemical does significantly (~ 30%) extract cholesterol from the cell membrane (8).

Figure 5: Structural formula (A) and schematic representation (B) of MβCD. (The β in image B indicates that this is the structure of MβCD, and not for MαCD for instance).
3. **Experimental set-up**

3.1 **Cell growth**

Cells were transfected using Fugene (Fugene 6 Roche diagnostics corporation (~ 50% efficiency)) to transiently express the Lck construct (fig. 1) in 3T3-A14 cells (mouse fibroblast cell line, expressing the insulin receptor#).

Cells are cultured in DMEM medium with streptomycin and penicillin (100 µg/ml) supplemented with 10% bovine serum. Growth conditions are 37°C, 5% CO₂. Measurements were conducted at 37°C while cells were kept in Phosphate Buffered Saline (PBS), 2-3 days after transfection for FRAP and 6-7 days for the SM experiments.

For the cholesterol extraction experiments cells were converted to just DMEM medium with pen/strep 24 h before measurements and incubated in DMEM medium with 5mM MβCD for 70 minutes before measuring. Otherwise measurements were conducted as described above.

3.2 **Single Molecule imaging and FRAP set-up**

Cells adherent to glass slides were mounted on an inverted microscope equipped with a 100x oil immersion objective. The samples were illuminated using Ar⁺-laser at a wavelength of 514 nm. For the FRAP experiments we split the laserline in to a weak beam (4%) and a strong beam (96%). In the weak beam we used a divergence lens to be able to scan a ‘large’ sample area. For the SM experiments we only used the strong beam containing a divergence lens at an intensity of ~2kW/cm². Using an appropriate filter combination permitted the detection of the signal by a liquid nitrogen-cooled CCD camera (fig. 6 Appendix). The set-up has a detection efficiency of ~ 8 %.

To analyse this data different Matlab programs were used which were previously written for this purpose. Origin was used for subsequent fitting of the data to eq.(10).

#The 3T3-A14 cells were a gift from J.A. Maassen from the department of Medical Biochemistry of the Sylvius Laboratory at Leiden University.
4. Results

4.1 Fluorescence Recovery After Photobleaching:

![Images of the bleach spot and a typical FRAP curve. Top right is the image just after bleaching which is used to determine the area of bleaching (top left). Calculation of the fluorescence used in the graph is done for this area. The fluorescent intensity at the bleach spot is plotted for all images in the graph (bottom). There are three distinct regions in a FRAP curve: 1. prebleach region $t < 0$, 2. bleaching $t=0$, and 3. Recovery $t > 0$ (see text below for details).](image)

An example of a typical FRAP curve is given in fig. 7. There are 3 distinct regions visible in this curve. The first part is the prebleaching; here we see the average level of fluorescence on the cell membrane. At time $t=0$ we bleach and a clear drop in fluorescence is visible. This bleaching should be less than 70% of the initial fluorescence, for reasons explained in section 2.3. The last section in the curve is the recovery. The flow of new fluorophores from other parts of the membrane into the bleached area is observed here.

The parameters $t_{1/2}$ and $R$ come from this part of the curve. $R$ is determined from the level of fluorescence long after bleaching compared to the level before bleaching and $t_{1/2}$ is the time it takes to get to 50% of the recoverable fluorescence.

The parameters are:

- $D = 0.04 \pm 0.02$
- $R = 0.89 \pm 0.06$
- $t_{12} = 36.3 \pm 13.0$
- $\alpha = 1.00 \pm 0.00$
Fitting this type of curve with eq.(10) yields parameters $R$, representing the mobile fraction, and the characteristic halftime of recovery ($t_{1/2}$).

$D$ is calculated from $t_{1/2}$ using eq.(11)

Averaging over 20 of these measurements we find a diffusion coefficient $D$ ($w^2=1.3 \mu m^2$) of $D=0.79 \pm 0.44 \mu m^2/s$ and a mobile fraction $R=0.92 \pm 0.11 \%$ ($N=20$).

The large error in the diffusion coefficient is due to variations in $t_{1/2}$ from cell to cell. This indicates that a large number of measurements should be done to get an acceptable error in the data. Because of this problem we decided to use the FRAP data only as an indication of the diffusion constant necessary for the analysis of the SM measurements.

![Graph showing diffusion constant (D) and mobile fraction (R) for different constructs](image)

**Figure 8: The diffusion constant and the mobile fraction for different constructs.**

The measurements at 20°C in HEK 293 cells were done by J. Butter. The measurements on Lck at 37°C in 3T3-A14 cells were done for this project. There is no significant difference between the three constructs. Temperature and/or cell type seems to have some influence on both the diffusion constant and the size of the mobile fraction.

The results of this measurement are compared to the measurements done by J. Butter. (fig. 8) She did FRAP measurements, on the same and similar constructs, on HEK 293 cells at 20°C. The diffusion in the 3T3-A14 cells is much higher. This is probably influenced by the higher measurement temperature. The mobile fraction is similar for all constructs in all cell types at both temperatures.
4.2 Single Molecule measurements

We accumulated SM data by focussing on top of the cell to measure movement of individual molecules in the cell membrane.

After construction of the cumulative probability density functions (will be called just probability density function or pdf in the rest of the report) from the data we fitted them with a biexponential (eq.(9)) to see if the population of fluorophores was homogeneous or heterogeneous. As (fig. 9) indicates this mono- or biexponential form is only apparent in the two shortest timelags. At longer time lags a large fraction of the population seems not to be moving (within our measuring accuracy), for this reason we refitted all the data with a triexponential function assuming that part of the population is not moving. Due to an error in the positioning of peaks a population that is not moving will show up in the data as a population that is moving with a MSD of $2.5\times10^{-3} \text{m}^2$. The equation used in fitting was:

$$p(r^2, t) = 1 - \left( \beta \exp\left(-\frac{r^2}{0.0025}\right) + (R)\exp\left(-\frac{r^2}{r_1^2}\right) + (1-R)\exp\left(-\frac{r^2}{r_2^2}\right) \right) \quad (13)$$

Fitting the data with eq.(13) clearly gives better fitting results (as can be seen in fig. 10). It is not clear whether the improvement of the quality is due to the introduction of one extra parameter or that this triexponential is indeed a better description of the different populations of the protein. After looking at the behaviour in time of the parameters we got from fitting with eq.(13) we could not see any sensible pattern.

Going back to the original biexponential fit we decided to take a closer look on the data from the two shortest time lags because this data was clearly biexponential (assuming the quality of the model increases using less parameters) both before and after cholesterol extraction.

To get an idea of the changes in diffusion in time we not only looked at one step trajectories but also analysed longer, up to 4 steps, trajectories (meaning a peak can be followed in 5 subsequent images).

Even for the 4 step trajectories we still see biexponential behaviour as can be seen in fig. 11. If we analyse the data from the parameters of this biexponential fit we see a clear pattern in the data for both the slow (immobile) and fast (mobile) fraction (fig.(12)). These results are discussed in the next sections.
Figure 9: An overview of the probability density functions for the MSD at different timelags. The pdf’s on the right are before cholesterol extraction, on the left are the pdf’s after cholesterol extraction. Eq. (9) is used to fit the data from all timelags to a biexponential. The quality of the fit clearly decreases as the timelag becomes longer.
Figure 10: An overview of the probability density functions for the MSD at different timelags. The pdf’s on the right are before cholesterol extraction, on the left are the pdf’s after cholesterol extraction. Eq. (13) is used to fit the data from all timelags to a triexponential. The fits are significantly better than the biexponential fits (fig. 9).
Figure 11: The probability density function (pdf) for the 40 ms timelag data (241 datapoints (A)) and the 4 step trajectories of the 10 ms timelag (302 datapoints (B)). The first pdf cannot be fitted with a biexponential function, the second can. This indicates that the data becomes less accurate if the timelag increases. An increase in timelag means a larger area has to be scanned to find peaks in subsequent images which increases the change to mix up different proteins.
Figure 12: Overview of the biexponential fit results. MSD as a function of time for the slow (or immobile (A)) fraction and the fast (or mobile (B)) fraction. The blue line indicates the measurement error in the MSD. And the percentage of protein in the fast fraction as a function of time $R$ (C).
4.2.1 Diffusion behaviour of the Slow (immobile) fraction

Looking at fig. 13 we can see that the mean square displacement of the slow fraction is not linear in time but constant. We see that the MSD is limited to $3 \cdot 10^{-3} \, \mu m^2$ and that this behaviour is not influenced by cholesterol extraction. Our error in the positioning of the peaks results in an overestimation of our MSD by a factor $2\sigma^2$ (where $\sigma^2$ is $(\Delta y)^2 + (\Delta x)^2$ and $\Delta y = \Delta x = 50 \, nm$) which is in our case $\sim 1 \cdot 10^{-2} \, \mu m^2$ (13). Due to this error we cannot say anything about MSD’s smaller than $\sim 1 \cdot 10^{-2} \, \mu m^2$. The MSD of $3 \cdot 10^{-3} \, \mu m^2$ that we found is much smaller than our accuracy. It is not clear why we get such a small value for the MSD, according to the theory (13) this is not possible. One explanation is that we overestimated the error in our peakpositioning. With our set-up we are not able to tell if this fraction is moving in domains with a size smaller than our error of $1 \cdot 10^{-2} \, \mu m^2$, or is actually completely immobile. For the same reason we are not able to rule out any influence of cholesterol extraction. If there is an influence it would have to be very large to be significant. We could only observe an increase in mobility leading to a MSD $> 1 \cdot 10^{-2} \, \mu m^2$.

Looking at fig. 13 we see a difference in the behaviour for the datapoints originated from the 10 ms (points at 10, 20, 30, 40 ms) timelag data and the 4.3 ms (points at 4.3, 8.6, 12.9 ms) timelag data (both before extraction). This difference might be due to the change from monoexponential behaviour to biexponential behaviour when going from one step trajectories to more steps in the data from the fastest data acquiring (4.3ms) (fig. 14).

**Figure 13:** The MSD as a function of time for the slow (immobile) fraction is shown in this figure. The blue line indicates the measurement error in the MSD. The MSD is constant in time and has a value of $\sim 3 \cdot 10^{-3} \, \mu m^2$. There is no visible influence of cholesterol extraction.
Figure 14: The pdf of the MSD from the 4.3 ms timelag data. The data from the one step trajectories (A) is a monoexponential distribution. The two-step trajectories (B) on the other hand have a biexponential distribution. This change from monoexponential to biexponential behaviour seems to have an effect on the MSD as a function of time (fig 13).
4.2.2 Diffusion behaviour of the Fast (mobile) fraction

Fig. 12b shows the MSD of the fast (or mobile) fraction as a function of time. The data shows a clear difference in behaviour before and after cholesterol extraction. Before cholesterol extraction the MSD is linear in time. But the diffusion becomes confined after the extraction of cholesterol.

![Graph showing MSD as a function of time](image)

Figure 15: The MSD as a function of time for the fast fraction before cholesterol extraction. The blue line indicates the measurement error in the MSD. The MSD is linear in time. This indicates free diffusion.

In fig. 15 we only plot the data before cholesterol extraction. All datapoints are overestimated because of the error in our peak positioning of \( \sim 1 \times 10^{-2} \ \mu\text{m}^2 \) (13). Therefore, the first point is not \((0,0)\) but \((0,0.01)\). We use a linear fit starting at \((0,0.01)\) and determined the diffusion coefficient with eq.(4).

From this fit we get a diffusion coefficient \(D = 0.55 \pm 0.03 \ \mu\text{m}^2/\text{s}\).
If we look at the behaviour of the MSD in time (fig. 16) after cholesterol extraction we see that the MSD is now confined.

![Graph](image)

**Figure 16:** MSD as a function of time for the fast (mobile) fraction. The blue line indicates the measurement error in the MSD. After cholesterol extraction the MSD is not linear in time, indicating confinement.

In this case the datapoints are also overestimated so we also have a new starting point (0, 0.01).

Using this and fitting the MSD, as a function of time, after cholesterol extraction with the equation for confined diffusion (eq.12) we find a size of the confinement zones of: \( L = 182 \pm 60 \text{ nm} \) and an initial diffusion of: \( D_0 = 1.15 \pm 0.84 \text{ µm}^2/\text{s} \).

We see a clear effect of cholesterol extraction. Before the extraction there is no confinement. The construct is not localised in domains and is able to move freely through the membrane, or is localised in very large domains (larger than ~1 µm in diameter). After cholesterol extraction we see that the diffusion is confined. It is very likely that these confinement zones are rafts because cholesterol has an influence on the diffusional behaviour.
4.2.3 Distribution of the two fractions

Figure 17: The percentage of mobile fraction as a function of time ($R$ is $\alpha$). The proteins are almost evenly distributed between the slow- and fast fraction. Cholesterol has no big influence on this distribution.

The fraction of the population that has a fast diffusion is reduced in time from $55 \pm 5\%$ to $40 \pm 5\%$ (fig. 17). It is not clear what could happen to the proteins that disappear from this fraction. The amount of protein that is resident in rafts indicates that rafts probably make up a large part of the membrane. This is also indicated in recent literature (3,5).
5. **Conclusion**

In this project we combined FRAP and SM measurements. In this way we wanted to learn more about the diffusion of proteins that are anchored in the cell membrane, which could in turn tell us something about the structure of the cell membrane of mammalian cells. But when we looked at the FRAP data we saw that there is a large spread in the data from different cells. We only used FRAP to get an indication of the diffusion because of this problem. This indication is very useful in the analysis of the SM data. FRAP in general gives less information about diffusion than SM measurements.

Using Single Molecule measurements we were able to study the diffusional behaviour of our construct in great detail. We have proven the existence of cholesterol detergent domains (rafts) and were able to indicate the size of these structures.

Part of our protein population (~50%) is not moving or moving in very small domains with a size much smaller than ~50 nm in diameter. Cholesterol extraction seems to have no influence on the diffusional behaviour of this fraction. Because we have an error of $\sim 1 \cdot 10^{-2} \mu m^2$ in our measurements we cannot make more definite statements about the behaviour of this fraction. We are not able to distinguish between proteins that are moving in a domain smaller than our resolution and proteins that are not moving at all. There are several possibilities that could explain these measurements. The protein could be anchored to the cytoskeleton or resident in caveolea. To determine what is causing the behaviour of the protein more (biological orientated) research is necessary.

We are able to say a lot more about the fast fraction of the population. The proteins move freely before extraction of cholesterol or are confined to very large domains that we do not resolve in our timescale of measuring. The diffusion constant of this movement is $0.55 \pm 0.03 \mu m^2/s$. The behaviour of the proteins becomes completely different if we extract part of the cholesterol from the cell membrane. The MSD is no longer linear in time but is confined. The size of these confinement zones is $182 \pm 60$ nm. The diffusion in these zones seems to be faster $D_0 = 1.15 \pm 0.84 \mu m^2/s$ but this result is not significant. The large error on the diffusion constant indicates that the first part of the fit is very sensitive to errors. There are not enough points to determine this part of the graph. Because cholesterol has a big influence on these confinement zones it is very likely that they are rafts.

Movement of the construct is strongly influenced by cholesterol. It is hard to say if cholesterol extraction induces raft formation or is just influencing the size of the rafts (6). We would expect that the rafts get smaller (or disappear) if cholesterol is essential for the structural integrity of the raft. The raft needs to be smaller in order to keep the concentration of cholesterol inside constant. If cholesterol extraction would make the rafts disappear we would not see confinement of the MSD after cholesterol extraction. Another explanation could be that cholesterol influences the barriers of the rafts. Extraction could make the barriers between the raft and the rest of the membrane less permeable. In that case the rafts do not change in size but get (more) ‘visible’ after cholesterol extraction. The two fits (before and after extraction) give very different numbers for the initial diffusion. Because we don’t have any points in the first part of the fit used after extraction it is not clear...
if this result is a good description of the MSD at very small timescales. For this reason it is hard to draw any conclusions from the difference in diffusion constant.

The lipid anchor of the Lck protein seems to be sufficient to induce raft localization. We cannot say if this is a good description of the raft localization of the full length Lck protein. To address this matter further measurements should also be done on the intact, functioning protein in the celltype in which it is naturally found in. The size of the rafts we found (after cholesterol extraction) is roughly the same as was found with Single Particle Tracking (STP) measurements (3).

In analysing the data we found that fitting the peaks and finding the traces becomes more and more subject to failure as time between images is increased. This is most likely due to the increase in area that has to be investigated to find the diffusing peak found in a previous image and wrong traces due to photo bleaching or photo blinking. Data analyses becomes more accurate if only timelags shorter than 20 ms are used and from this timelags trajectories with more than one step are used to look over a broader time spectrum.
6. References


*The Lck construct was a gift of the group of A.I. Magee from the division of Membrane Biology at the National Institute for Medical Research in London.
#The 3T3-A14 cells were a gift from J.A. Maassen from the department of Medical Biochemistry of the Sylvius Laboratory at Leiden University.
7. **Appendix**
Figure 1: Representation of the vector that is used to transfect cells with to let them express the Lck construct. The important restriction sites and the part of the vector that is unique to this construct are shown.
Figure 6: Representation of the set-up used in both the FRAP and SM measurements. The set-up is essentially the same for both experiments. The weak beam is not used in the SM measurements. The strong beam is adjusted to an intensity of 2 kW/cm² for the SM measurements.