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Homo-FRET detection in wide-field, steady-state microscopy, with mCherry as labeling fluorophore

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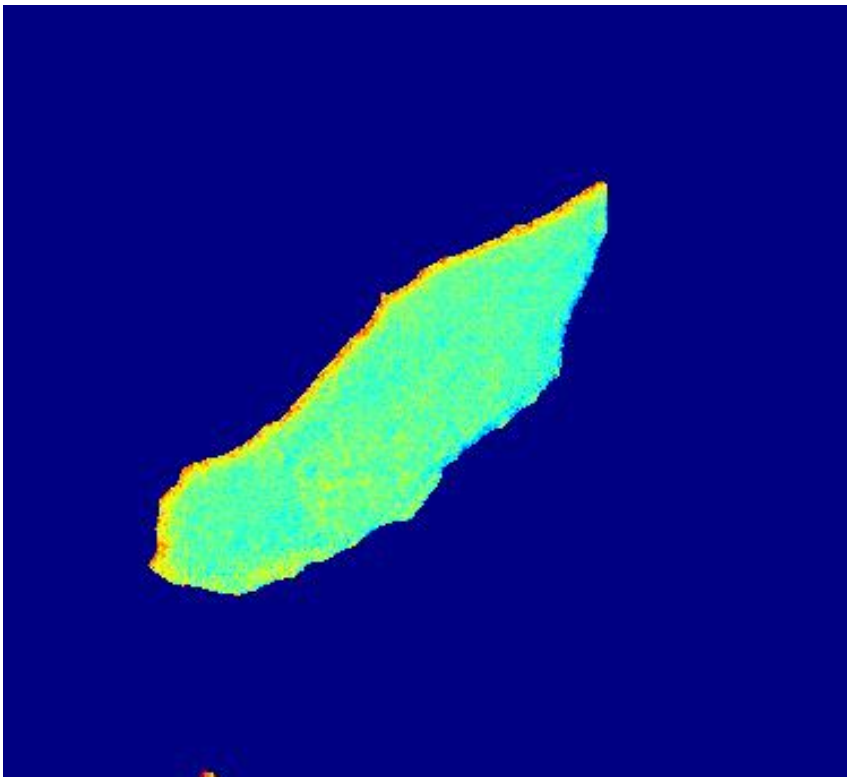


Abstract

HomoFRET is a method in fluorescence microscopy to measure protein clustering in cells by calculating the anisotropy. In this research Homo-FRET is done with steadystate wide-field microscopy. The mCherry fluorophore is used as labeling fluorophore instead of the common mGFP, because it follows from literature that autofluorescence of cells in red spectrum is lower then in the green part of the spectrum. The method obtained anisotropy values with an error of 5%. The main problem of measuring HomoFRET with steady state microscopy turns out to be the camera registration. A depth dependence in registration is possible.

Contents

1 Introduction

Fluorescence microscopy is a widely used tool in cell biology. Fluorescence microscopy is able to visualize cell processes, which are too small to detect with regular microscopy. Since fluorescence microscopy visualizes cell processes by labeling proteins, biological processes are not interrupted or destructed by the measurements. Recent advances in molecular labeling technique has made fluorescence samples widely available. One kind of fluorescence microscopy is FRET, Föster Resonance Energy Transfer. FRET occurs when the distance between fluorophores is in the order of $1 - 10nm$. So imaging FRET tells something about the distances between fluorophores. This could be used to detect clustering. Clustering is an important phenomenon in cell biology, because cell processes as proliferation, migration and growth involves clustering.

A method to detect the rate of FRET is FLIM, Fluorescence Lifetime Imaging Microscopy. When FRET occurs between different fluorophores, this called hetero-FRET, the fluorescence lifetime is affected. Measuring changes in fluorescence lifetime the rate of FRET could be determined. FRET could also occur between similar fluorophores, in this case it is called homo-FRET. When FRET occurs the polarization of the emitted light is affected. Measurements of the polarization of the emitted light enables the detection of homo-FRET. An advantage of homo-FRET above hetero-FRET is that just one fluorophore is used. This makes biological sample preparation far more easier for homo-FRET than for hetero-FRET. It is already possible to do accurate measurements with steady-state as well as time-resolved confocal microscopy. However this method is very time consuming.

This thesis investigates of Homo-FRET could also be measured by steady-state wide-field microscopy. This research is an continuation of the research done by Nivard Kagie and Kiefer van Teutem. Nivard investigates measuring Homo-FRET in steady-state wide-field microscopy with the labeling fluorophore mGFP. He concluded that clustering could be detected, but the result was less significantly then using confocal microscopy. Nivard also mentioned that the autofluoresence is of great importance on the anisotropy values. Therefore he recommended to use red labeling fluorophores in stead of green, because from literature it follows that autofluorescence in the red part of the spectrum is less then autofluorescence in the green part of the spectrum. Kiefer optimized the set-up Nivard had used in his experiment. Measuring autofluorescence cells he confirmed that autofluorescence in the red part of the spectrum was less then in the green part. Kiefer could not measure cells of biological samples, because these where not available.

As well as Nivards and Kiefers research this research is part of a project that is taken place in collaboration with the research group of cell biology at Utrecht university. The ultimate goal of the project is to find a cheap and fast method to image the behavior of EGFR, the epidermical growth factor receiver. Activating EGFR could cause cellproliferation, migration and growth. In cancer cells an over expression of EGFR is detected. Information about the regulation of EGFR is very important for medical purposes. In this project research of EGFR is done with measuring Homo-FRET by steady-state, wide-field microscopy.

This research investigates of measuring Homo-FRET with mCherry as labeling fluorophore improves the results measured with mGFP and of the results are accurate enough to use for biological purposes. The setup used is the setup Kiefer optimized to detect Homo-FRET with steady-state wide-field microscopy.

In this thesis first the theory of fluorescence microscopy is treated. Secondly there is a section devoted to methods and materials, which will discusses the set-up, measurement method and data analysis thoroughly. Thirdly the experimental results obtained will be given and finally there will be a section with conclusions and recommendations.

2 Theory

2.1 Fluorescence

A molecule or atom that absorbs light will become excited. In that case the molecule or atom has an electron in an excited state $S1$. When the electron relaxes to its ground state,$S0$ it can emit light. At each electronic state there can exist a number of vibrational energy levels. After excitation an electron will drop to the lowest vibrational energy level of the $S1$ state through a non-radiative process. Subsequently the electron will relax to one of the higher vibrational energy levels of the ground state under the emission of light. Finally the electron drops to the lowest vibrational level of the ground state, again through a non-radiative process The relaxations to the lowest vibrational levels of the $S1$ and $S0$ state will occur rapidly, in the order of $10^{-12}s$ [4]. The decay from the lowest $S1$ level to one of the vibrational levels of the $S0$ state will take longer, $1 - 10ns$ [1, 4]

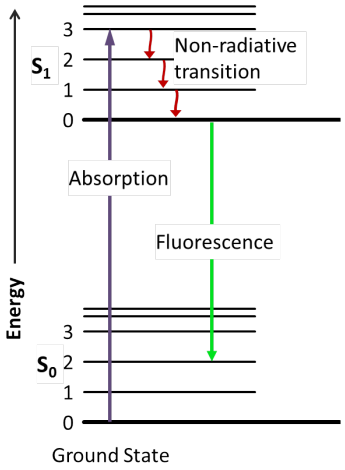


Figure 1: Jablonsky diagram, shows the vibrational states and the stokes shift.[1]

The process of an electron returning to the ground state is usually shown in a Jablonsky diagram, see figure 1. From the Jablonsky diagram it is clear that the energy emitted in the form of light is less than the energy received from the absorbed light. This phenomenon is known as the Stokes-shift. As a consequence the wavelength of the absorbed light is smaller than the wavelength of the emitted light. This difference in wavelength gives us an opportunity to separate the emission beam from the excitation beam, which will be important for our FRET-experiments.

An important variable in fluorescence theory is the transition moment. Each fluorophore has a transition moment. This transition moment consist of two axis, an excitation axis and an emission axis. A fluorophore emits light along its emission axis and the fluorescence process is most effective if the fluorophore is excited with a beam polarized along its excitation axis. Exciting a sample of isotropic fluorophores with a polarized excitation beam, only fluorophores whose excitation axis are aligned approximately[2] along the electric field vector will become excited.

2.2 HomoFRET

When fluorophores are located close to each other an excited fluorophore can transfer energy to a neighboring fluorophore. This process is called Föster Resonance Energy Transfer (FRET). The fluorophore that gets initially excited is called the donor fluorophore, the fluorophore that receives its energy through FRET is called the acceptor fluorophore. If the donor and acceptor fluorophore are of a single type, the

[1] Image 1 is token from *http://en.wikipedia.org/wiki/Fluorescence#mediaviewer/File:Jablonski_Diagram_of_Fluorescence_Only.png*

[2] The absorbtion of light goes with a factor $\cos\theta$, were θ denotes the angle between the electric field vector and the transition moment, this is discussed in 8

energy transfer is called homo-FRET, if the fluorophores are of a different type it is called hetero-FRET. It is important to realize that the energy transfer between donor and acceptor is not a radiative process but a dipole-dipole interaction [4], [9]. There are a few conditions to be met for FRET to occur. First the fluorophores must be located close together, in the order of $10nm$. Second the emission spectrum of the donor has to overlap with the excitation spectrum of the acceptor. Since Homo-FRET is about two fluorophores of a single type, the excitation spectrum and the emission spectrum of the used fluorophore have to overlap. Finally the orientation of transition moments of the involved fluorophores is important, a "wrong" orientation obstructs the energy transfer.

To measure the rate of FRET we introduce the Föster distance R_0. A direct formula for R_0 is [9]:

$$R_0^6 = \frac{9000 \cdot \ln 10 \cdot \kappa^2 \cdot \phi_D}{128 \cdot \pi^5 \cdot n^4 \cdot N} \int_0^\infty \frac{f_D(\nu) \cdot \epsilon_A(\nu)}{\nu^4} \cdot d\nu \tag{1}$$

Here ν is the energy in wave numbers, ϵ_A is the molar extinction coëfficient of the acceptor,$f_D(\nu)$ is the spectral distribution of donor fluorescence,(normalized to unity on wavenumber), N is Avogadro's number, n is the index of refraction of the medium, and ϕ_D is the donor's quantum yield in the absence of an accepter. The factor κ^2 describes the orientation of the dipole moment of the acceptor with respect to the dipole moment of the donor.

The rate at which FRET occurs F is given by [9]:

$$F = \frac{1}{\tau_D} \cdot (\frac{R_0}{R})^6 \tag{2}$$

In this formula denotes τ_D the average lifetime of the excited donor fluorophore, R_0 the Föster distance and R the distance between the donor and acceptor fluorophore. Given a sample of one kind of fluorophores, it follows from formulas **??** that the rate of FRET only depends on the distance R between the fluorophores and κ^2, the mutual orientation of the fluorophores. Note that as the orientation factor $\kappa^2 = 0$, then $R_0 = 0$ and therefore $F = 0$, so no FRET occurs. From formula 2 it follows also that at the distance R_0 the decay rate of the acceptor is the same as the decay rate of the donor in absence of a acceptor molecule.

The efficiency E of the energy transfer is calculated using the following formula [4]

$$E = \frac{{R_0}^6}{{R_0}^6 + R^6} \tag{3}$$

Here R_0 is the Föster distance and R the distance between the donor and accepter fluorophore. From the formula it follows that the Foster distance is the distance at which the energy transfer is 50% efficient.

2.3 Anisotropy

In this section anisotropy is discussed as a measure to detect Homo-FRET. Assume a polarized excitation beam, an isotropic sample of fluorophores and fluorophores with parallel excitation and emission axes. As a consequence of the random orientation of the fluorophores, the transition moments of the fluorophores may have different orientation. When FRET occurs the emitted light could, compared to the excitation beam, be polarized in a different direction or become depolarized, because the transition moment of the donor molecule could be oriented differently then the transition moment of the acceptor molecule. By measuring the polarization of the emitted light with respect to the polarization of the excitation beam we can measure the rate of FRET. A quantity to indicate the depolarization is the anisotropy r. The anisotropy is given by the following formula:

$$r = \frac{I_{||} - GI_\perp}{I_{||} + 2GI_\perp} \tag{4}$$

Here $I_{||}$ is the intensity of the emission light that is parallel polarized with respect to the excitation beam. $I_\perp$ is the intensity of the emission light that is perpendicular polarized with respect to the excitation beam. Note that $I_{||} + 2I_\perp$ is the total intensity of the fluorescence. G is a factor that corrects for the polarization preference of the set up, this will be explained in section 4.2.1 . From formula 4 it follows

that depolarization of light decreases the anisotropy, so FRET decreases the anisotropy. Notice that the formula 4 has nice properties. Given two fluorophores a and b, then the total anisotropy is given by:

$$r_{a+b} = f_a r_a + f_b r_b \tag{5}$$

Where r_{a+b} is the total anisotropy, r_a is the anisotropy of fluorophore a, r_b is the anisotropy of fluorophore b and $f_a = \frac{I_a}{I_{a+b}}$ and $f_b = \frac{I_b}{I_{a+b}}$ are the relative intensities of the fluorophores a and b.

- **Anisotropy of 1 fluorophore**

In order to quantify the polarization decrease when FRET occurs, the fluorescence process if one fluorophore gets excited is discussed first. An important notion is the lifetime of a fluorophore τ, which denotes the expected time the fluorophore will stay excited. The natural lifetime τ_0 denotes the expected time a fluorophore is involved in only radiative processes. An other important notion is the quantum yield ϕ, which denotes the ratio between radiative decay and total decay. Let now $\rho(t)$ denote the function that gives the probability that a fluorophore is excited at a time t. Then the following differential equation describes the exponential decay of the fluorophore [9]:

$$\frac{d\rho}{dt} = -\frac{1}{\tau} \cdot \rho(t) \qquad [\rho(0) = 1], \quad [\rho(\infty) = 0] \tag{6}$$

Solving this differential equation analytical the following decay probability function $\rho(t)$ is found:

$$\rho(t) = e^{-\frac{t}{\tau}} \tag{7}$$

And the quantum yield is given by:

$$\phi = \int_0^\infty \frac{1}{\tau_0} e^{-\tau t} = \frac{\tau}{\tau_0} \tag{8}$$

So the expectation value of the intensity of the emitted light equals the quantum yield ϕ.

- **Anisotropy of 2 fluorophores**

In a cluster of N-fluorophores a decrease in anisotropy is expected because FRET occurs. In order to understand the process with a cluster of N fluorophores, a cluster of two fluorophores a and b is considered first. Suppose that fluorophore a is excited. There is a change that fluorophore a will relax under emission of a photon and no FRET occurs. An other possibility is that fluorophore a will transfer its energy to fluorophore b. If the fluorophores are located close together and the FRET efficiency is high it can occur also that energy is transferred back and forth between the two fluorophores. Now the derivatives of the decay-probability functions of fluorophore a and b, ρ_a and ρ_b could be investigated. These decay-probability functions are coupled, since they involve energy transfer between a and b. The rate of change in the decay probability functions is simply a sum of the factors that gives the probability for a fluorophore to decays, transfers its energy to another fluorophore, or receives energy from another fluorophore. This is written down in the following coupled system of differential equations.

$$\begin{aligned} \frac{d\,\rho_a(t)}{d\,t} &= -\frac{\rho_a(t)}{\tau} - F_{ab}\rho_a(t) + F_{ba}\rho_b(t) \qquad [\rho_a(0) = 1] \quad [\rho_a(\infty) = 0] \\ \frac{d\,\rho_b(t)}{d\,t} &= -\frac{\rho_b(t)}{\tau} - F_{ba}\rho_a(t) + F_{ab}\rho_a(t) \qquad [\rho_b(0) = 0] \quad [\rho_b(\infty) = 0] \end{aligned} \tag{9}$$

Here τ is the average lifetime of this particular type of fluorophore, $\rho_a(t)$ and $\rho_b(t)$ denote the function which give the probabilities that fluorophore a resp. b are in an excited state at time t. F_{ab} gives the rate of energy transfer from a to b, F_{ba} gives the rate of energy transfer from b to a. Since the fluorophores a and b are of a single type $F_{ba} = F_{ab} = F$.

The system of differential equation 9 can again be analytical solved, which give functions ρ_a and ρ_b [9]:

$$\begin{aligned} \rho_a(t) &= \frac{1}{2} \cdot (1 + e^{-2 \cdot F \cdot t}) e^{\frac{-t}{\tau}} \\ \rho_b(t) &= \frac{1}{2} \cdot (1 - e^{-2 \cdot F \cdot t}) e^{\frac{-t}{\tau}} \end{aligned} \tag{10}$$

It follows that the sum ρ_a and ρ_b gives the same probability decay function as in the case of a single fluorophore. So the total quantum yield stays the same. With these formulas the quantum yields of the single fluorophores could be calculated. Since the quantum yield gives the expected intensity, the anisotropy can be calculated with formula 4 and 5. It follows

$$\begin{aligned} r_{tot} &= r_1 \cdot \frac{I_1}{I_{tot}} + r_{et} \cdot \frac{I_2}{I_{tot}} \\ \Rightarrow r_{tot} &= r_1 \cdot (\frac{1+\tau \cdot F}{1+2 \cdot \tau \cdot F}) + r_{et} \cdot (\frac{\tau \cdot F}{1+2 \cdot \tau \cdot F}) \end{aligned} \tag{11}$$

Here r_{tot} and I_{tot} are the total anisotropy and intensity, r_1 and I_1 are the anisotropy and intensity of the light emitted by initially excited fluorophore,r_et and I_2 is the anisotropy, resp intensity of the light emitted after energy transfer from the initially excited fluorophore to his neighbor. Recall that $F = \frac{1}{\tau_D} \cdot (\frac{R_0}{R})^6$, see formula 2,section 2.2. Here r_1 is the anisotropy of a random oriented fluorophore which gives r_1=0.4. A complicated calculation gives us $r_{et} = 0.16$ [1]. Remark that $0.16 = 0.4^2$. Recall that $F = \frac{1}{\tau_D} \cdot (\frac{R_0}{R})^6$. Substituting this in the formula above gives:

$$r_{tot} = r_1 \cdot \frac{1+(\frac{R_0}{R})^6}{1+2 \cdot (\frac{R_0}{R})^6} + r_{et} \cdot \frac{(\frac{R_0}{R})^6}{1+2 \cdot (\frac{R_0}{R})^6} \tag{12}$$

From the formula above it follows that if $\frac{R_0}{R} \ll 1$, the total anisotropy r_{tot} is almost equal to the anisotropy of one fluorophore. This is easy to see, because in that case $R \gg R_0$ and FRET will hardly occur. Inversely if $\frac{R_0}{R} \gg 1$ then $R \ll R_0$ and FRET will occur frequently. In this case the total anisotropy will come equally from the donor and acceptor molecule and will be $0.4 \cdot \frac{1}{2} + 0.16 \cdot \frac{1}{2} \approx 0.2$.

- **Anisotropy of N fluorophores**

The case of N fluorophores is a generalization of the case of two fluorophores. It turns out the anisotropy is then given by [9]:

$$r_{N,tot} = r_1 \cdot \frac{1+F \cdot \tau}{1+N \cdot F \cdot \tau} + r_{et} \cdot \frac{(N-1) \cdot F \cdot \tau}{1+N \cdot F \cdot \tau} \tag{13}$$

Here r_{et} denotes the average value of all individual emission of fluorophores excited trough energy transfer. From literature it follows that $r_{et} = r_1^N$. Notice that formula for the case of N fluorophores is analogous to the case of 2 fluorophores. Recalling $F = \frac{1}{\tau_D} \cdot (\frac{R_0}{R})^6$, it follows that if $\frac{R_0}{R} \gg 1$ then each fluorophore contributes a equal part of the anisotropy. In this case $r \to r_{et}$ and so for large values of N $r \to 0$.

2.4 Dimerization

The idea of Homo-FRET is to measure protein clusters in cells. From section **??** it follows that FRET decreases the anisotropy. In order to let FRET occur it is important that fluorophores are located close to each other. Because cell processes involve protein clustering, measuring FRET can be used to study cell processes, as mentioned in the introduction. Actually the clustering of fluorophores can take place in absence of cell processes. In that case two monomer fluorophores stuck together and form a dimer. This process is called dimerzation. The rate of dimerization is specified by the dissociation constant K_d. This constant denotes the concentration of fluorophores at which the number of dimers is equal to the number of monomers. Consequently, for studying cell process by measuring homo-FRET it is important that the concentration of the used fluorophore is much smaller than the dissociation constant.

2.5 Polarization affecting factors

Apart from FRET there are other factors that influences the polarization of the emitted light and in this way the anisotropy.In this section I will discuss several factors which affects the polarization of the light.

[1] For a derivation of r_1 see appendix A 8, the value of r_{et}, is taken from [9].

2.5.1 Rotational diffusion time

Due to thermal heat, molecules and atoms rotate. When a molecule or atom rotates, the transition moment of the fluorophore rotates also. As a consequence the polarization of the emitted light is affected and the anisotropy decreases. In order to reduce this rotational diffusion it is important to measure a sample of fluorophores which rotate as less as possible during the decay process. The decrease of the anisotropy due to rotational diffusion for steady state anisotropy measurements with a single-exponential intensity decay, as in this experiment, can be calculated easily by the following form of the Perrin equation [4, p 367].

$$r = \frac{r_0}{1 + \frac{\tau}{\theta}} \tag{14}$$

Here r is the actual anisotropy, r_0 is the anisotropy in absence of rotational diffusion ,τ is the average lifetime and θ is the rotational correlation time,a measure for the rate at which a molecule or atom rotates due thermal heat. From the formula it follows directly that if $\tau >> \theta$ the anisotropy will drop to zero, $r \to 0$ and if $\tau << \theta$ the anisotropy is almost equal to the fundamental anisotropy, $r \approx r_0$.

2.5.2 Non-parallel emission and excitation axis

Most of the fluorophores do not have parallel emission and excitation axis. Consequently the polarization of the emitted light isn't parallel to the polarization of the absorbed light. Thereby the anisotropy decreases without FRET occurs. A same derivation as in the case of a 2 fluorophore cluster see section **??** is done for a fluorophore whose angle between the excitation and emission axis is β. In this case the maximal anisotropy$r$ is given by:

$$r = 0.4\frac{3\cos^2(\beta) - 1}{2} \tag{15}$$

Fortunately for small angles we can use the approximation $\cos(\beta) = 1$. Up to a misalignment of 7.4 degrees, the maximum anisotropy $r = 0.39$. A greater problem is that the misalignment is dependent of the excitation wavelength. How shorter the excitation wavelength, the more energy contained in the light, and more different oriented transition moments that are able absorb light.

2.5.3 Objective

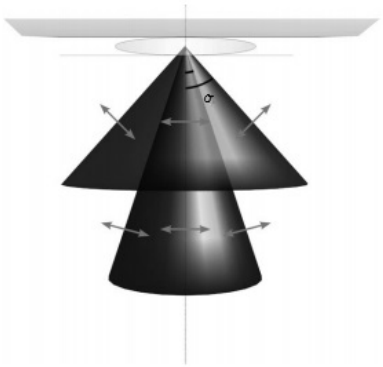


Figure 2: The effect of the objective on the polarization of light for a small and large NA [1]

The microscope objective affects the polarization of the emitted light substantially, because light that passes through a objective is a divergent cone, see figure 2. An objective is characterized by it's numerical aperture NA given by the formula:

$$NA = n \sin \sigma \tag{16}$$

Here n is the refraction index of the substance the objective is immersed in and σ is the maximum angle under which the objective can collect light, see figure 2. Therefore the NA increases with increased

[1] Image is token from D. Axelrod, *Biophys. J* **1979**,26,557

refraction index and with σ. If σ increases the intensity of the light collected by the objective will increase, simply because the objective collects more light. So the larger σ is, the brighter your image. Since the resolution of an objective is proportional to $\frac{\lambda}{NA}$, it follows that the resolution is better if the NA is higher. As can be easily seen from the picture, also the depth of focus DOF, decreases with increasing NA. Therefore a larger NA yields a better depth resolution.

For this reasons it seems that a higher NA will improve our measurements, but this is not the case. An other very important consequence of the increasing NA is that light becomes depolarized, because the light cone of the objective induces the mixing of horizontal an vertical polarization, as shown in figure 2. The graph 3 shows the relation between σ and the anisotropy r. So a balance have to be made between the lowering anisotropy effect of the NA and the brightness, DOF and resolution. In [2] is recommended to use a maximal NA of 0.75. In this experiment an microscope objective with a NA of 0.6 is used. This will reduce the maximum anisotropy by 6%.

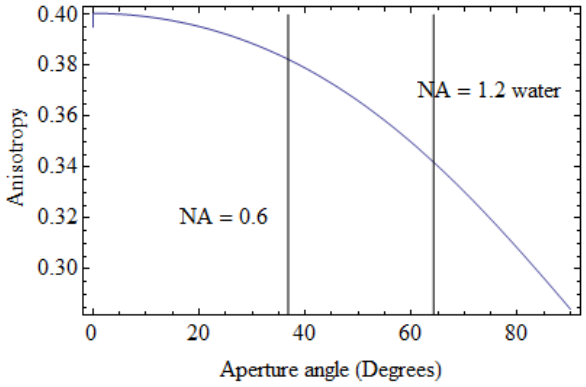


Figure 3: The effect of the NA on the maximum anisotorpy [1]

2.5.4 Background

When measuring the intensity of the emitted light, a part of the received signal is caused by background signal. This background signal consist of camera background, environmental background and autofluorescence. Components of the camera background are random noise, a fixed pattern and read-out noise. The environmental background consists of environmental light and scattering of the objective. Autofluorescence is the fluorescence of unlabeled cells. In section 2.6 the background will be discusses thoroughly. Nevertheless it is important that these background signals can be polarized differently than the light emitted by the fluorophores. Since the anisotropy is calculated by the intensities of parallel and perpendicular polarized light, with respect to the polarization of the excitation beam, background will definitely affect the anisotropy.

2.5.5 Bleaching

Measuring a time at one point in your sample, the intensity of the fluorescence light will decrease. In fact when the sample is illuminated the number of active fluorophores will decrease. This phenomenon is called photo bleaching. Photo bleaching means that the average distance between to fluorophores increases and FRET will occur less frequently. As a consequence the anisotropy will decrease. For this reason it's important to minimize photo bleaching. This is done by shortening the exposure time of your sample. However shortening your exposure time will increase the signal to noise ratio, as explained in the next section. It is the challenge of this experiment to find a balance between uncertainty and photo bleaching.

[1] This image is token from [5].

2.6 Uncertainty

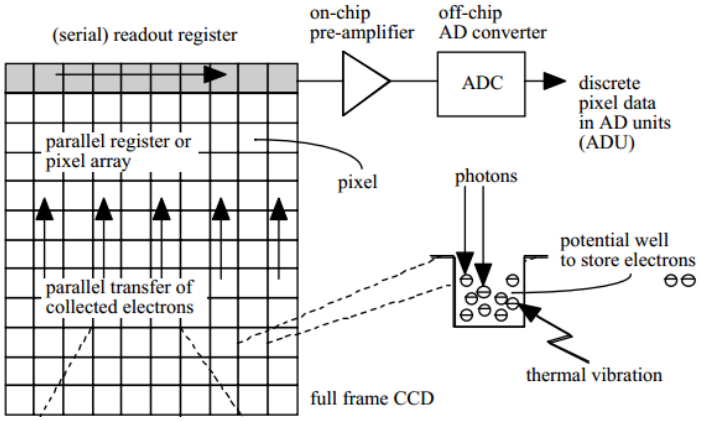


Figure 4: Schematical representation of working of the cameras.[1]

This section will discuss the uncertainties involved in anisotropy measurements. Therefore the functioning of the cameras, which is schematically drawn in figure 4 has to be discussed. The camera consists of a light-sensitive chip with a semiconducting layer. When a photon hits the chip, an electron can be released from the valence-band to the semiconducting band and creates thereby an electron-hole pair. Such an electron is called a photo-electron. During a given exposure time photo-electrons are stored in a potential wells. After the exposure time the photoelectrons are transferred to a serial readout register. Then the pixels are read out one by one and the charge is amplified. Subsequently the charge is send to an ADC (Analog Digital Converter), where it is converted to ADU (Analog digital units)

2.6.1 Photon statistics

The photons that are detected by the camera obey photon statistics. Therefore the uncertainty in the measured intensity is equal to $\sqrt{N}$, where N is the number of counted photons. The relative uncertainty decreases if N increases, so in order to reduce the fundamental uncertainty as many photons as possible must be measured. Calculating the uncertainty in the anisotropy propagation of uncertainties is used on formula 4. This gives for the variance in the anisotropy $v(r)$ [6]:

$$v(r) = \frac{9G^2 I_{\perp}^2}{(2GI\perp + I_{||})^4} v(I_{||}) + \frac{9G^2 I_{||}^2}{(2GI\perp + I_{||})^4} v(I_{||}) + \frac{9I_{\perp}^2 I_{||}^2}{(2GI\perp + I_{||})^4} v(G) \tag{17}$$

Here $I_{||}$ and $I_{\perp}$ are the parallel and perpendicular intensities and $v(I_{||})$ and $v(I_{\perp})$ are the variances in the parallel and perpendicular intensities. G is the G-factor and $v(G)$ the variance in the G-factor. Substituting the anisotropy formula 4 in formula 17 gives [6]:

$$v(r) = \frac{(1-r)(1+2r)(1-r+G(1+2r))}{3I_{tot}} \tag{18}$$

Here r is the anisotropy.Recall that the standard deviation is given by the square root of the variance so $\sigma(r) = \sqrt{v(r)}$. We see that the standard deviation of the anisotropy not only depends on the total intensity, but also on the anisotropy itself. In figure 5 for $r = 0$, totally unpolarized light and $r = 0.4$, totally polarized light. It follows that measuring anisotropy there is a final limit in reducing the uncertainty.

When calculating uncertainties in the parallel and perpendicular intensities, it is important to realize

[1]The figure is taken from [8]
[2]This image is taken from [5]

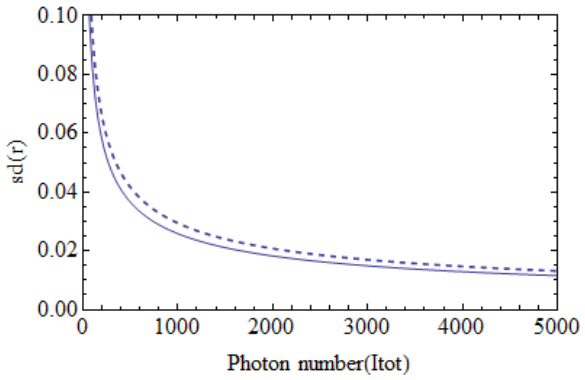


Figure 5: Dependence of the standard deviation on the number of counted photons I_{tot}. The dashed line denotes the maximum anisotropy $r = 0.4$, the straight line denotes $r = 0.$[2]

that the signal of the A/D-converter is amplified and therefore not equal to the number of detected photons. So if the signal is equal to αN, where α denotes the gain factor and N denotes the number of detected photons, then the measured standard deviation is $\alpha\sqrt{N}$ and not $\sqrt{\alpha N}$, the square root of the obtained signal.

2.6.2 Thermal noise

Besides photon excitation also thermal vibration can cause electron-hole pairs. This phenomenon of thermal excited electrons is called dark current. Because of impurities in the chip-layer, some pixels are far more sensible to dark current then others. These pixels can become hot pixels, even after a few moments of exposure time. The amount of dark-current is directly related to the temperature. Cooling the chip for 6 degrees, will diminish dark current with a factor 2 [8] In our experiment the chips are cooled to 30 °C.

2.6.3 Read-out noise

A third kind of noise is the read-out noise. This noise that originates from the read-our process and is almost completely caused by the amplifier. The read-out noise depends only on the read-out rate and increases speeding up the read-our rate. For high read-out rates,e.g.$560MHz$ as used in the experiment the read-our noise is significant.

2.6.4 Autofluorescence

Autofluorescence denotes the fluorescence of cells in absence of labeling fluorophores. A cell contains many different proteins and some of them are fluorescent. The fluorescence of the cells can be polarized differently than the fluorescence of the fluorophores. In this way autofluorescence will effect the anisotropy. The effect of autofluorescence can be corrected by subtracting the autofluorescence signal from the total intensity:

$$r = \frac{(I_{||} - A_{||}) - (I_{\perp} - A_{\perp})}{(I_{||} - A_{||}) + 2(I_{\perp} - A_{\perp})} \tag{19}$$

Here $A_{||}$ denotes the part of autofluorescence that is parallel polarized and $A_{\perp}$ denotes the part of autofluorescence of the perpendicular polarized. In figure 6 the effect of autofluorescence is shown for $I_{||} = 23000$ and $I_{\perp} = 10000$, so $r = 0.3$ in absence of autofluorescence.

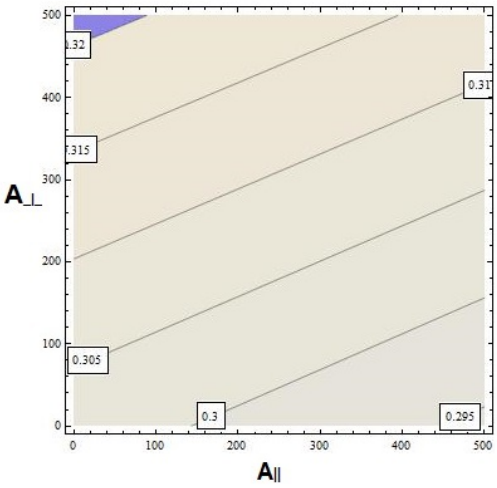


Figure 6: Dependence of the anisotropy on different values of the autofluorescence. In this figure $I_{||} = 23000$ and $I_{\perp} = 10000$

3 Microscopy Techniques measuring fluorescence anisotropy

Measuring fluorescence anisotropy could be done with a wide range of microscopy techniques. In this sections a few important notions will be discussed. Measuring fluorescence can be done with wide-field as well as confocal microscopy. In wide-field microscopy a 3-D object is projected onto a 2-D detector. Thereby the whole field is imaged. In confocal microscopy a pinhole filters out the out of focus light and one point at a time is measured. In this way thin layers are measured, from which a 3-D images can be constructed. As a consequence wide-field microscopy is a fast method and gives bright images, but has no depth resolution and out-of-focus blur. Confocal microscopy has a better resolution, but is time consuming.

Two other important notions in fluorescence microscopy are stead-state and time resolved microscopy. In time-resolved microscopy a sample is illuminated with a pulse as short as possible. After the excitation the fluorophore is not excited again before it's totally decayed. In this way fluorescence lifetime could be measured but also the anisotropy as a function of time could be measured. In steady-state microscopy the sample is constantly illuminated for during a given exposure time. The rate of FRET can be detected by calculating the anisotropy and comparing this values with anisotropy values of reference samples.

Using steady-state microscopy for measuring anisotropy, there are two methods of detecting polarization. One method uses a polarization beam splitter and two cameras, one for measuring the intensities of the parallel beam, one for measuring the intensity of the perpendicular beam. In this way both polarizations could be detected at the same time, but the acquired parallel and perpendicular images has to be aligned. The other method uses no polarization beam splitter, but changes the orientation of the polarizer. In this way the just one camera is needed, so the images do not have to be aligned. However in this case the images are not acquired at the same time. Therefore if there is photo bleaching, this method wouldn't be usable.

In this experiment our goal is, to find a quick and accurate method to detect homo-FRET. for this reason a steady-state wide-field microscopy is used. Because of the significant bleaching that occurs during the exposure time, there are two cameras used.

4 Methods and Materials

In this section the setup, calibration of the setup, and data-analysis will be discussed thoroughly. Apart from this a short measurement protocol and a protocol for the analysis is given.

4.1 Optical Set-Up

The aim of this experiment is to measure protein-clustering in cells. For biological and medical purposes the measurement method has to balance between a fast and accurate process. In order to calculate anisotropy, a polarized excitation beam is needed and a detector that measures the polarization of the emitted light. This experiment makes use of steady-state, wide field microscopy with a T-setup. A Nikon Ti epsilon microscope body is used. This common used microscope body is suitable for a wide range of purposes. In this section the set-up is thoroughly discussed. The first part describes the excitation beam, the second part the emission beam, the third gives the characteristics of the used dichroic cubes and the fourth will describe the characteristics of the used cameras. An illustration of the set-up is shown in figure 7.

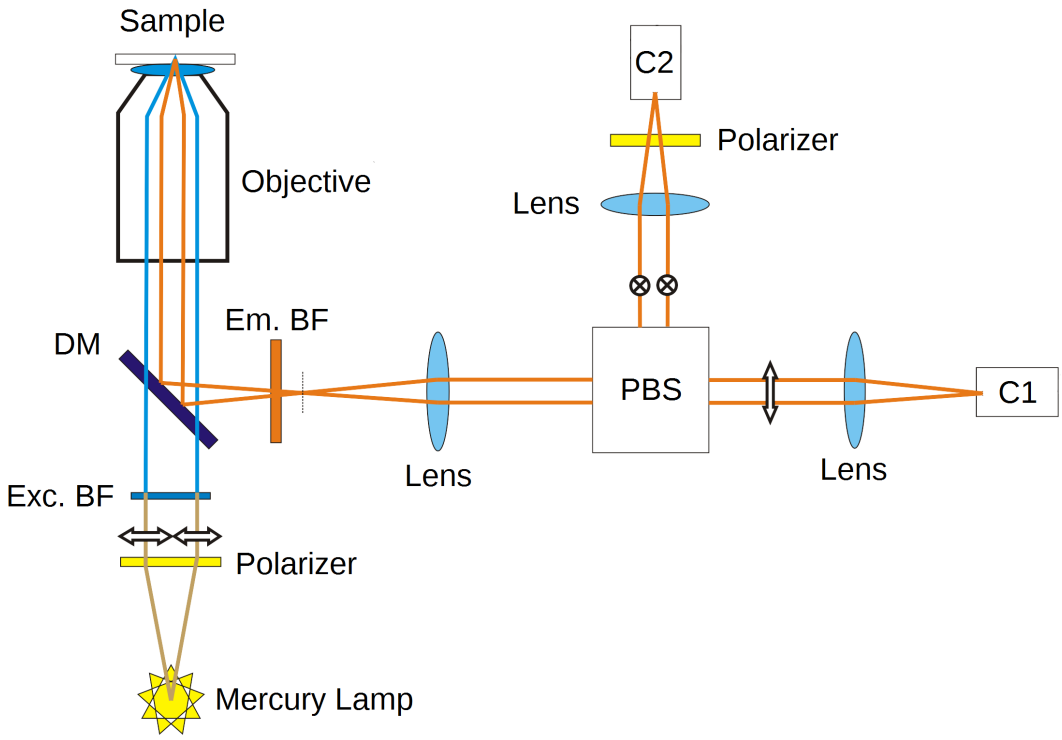


Figure 7: Schematic reproduction of the setup, the blue rays represents the excitation beam, the orange rays denote the emission beam. Note that the functioning of the dichroic mirror for the emission beam is actually reversed.[1]

Excitation beam

A short-arc Mercury Lamp is used to create an excitation beam. Mercury Lamps are commonly used in microscopy, because of their broad emission spectrum. The light from the Mercury lamp travels trough a polarization filter. The polarization filter is a ultra broadband wire grid polarizer,a *Thorlabs, WP25L-UB*. The transmission of the polarizer is 70% and the extinction ratio is over 5000 : 1. This polarizer is important for creating a well-defined polarization beam. After passing through the polarizer, the excitation light hits the dichroic cube. A dichroic cube consists of two bandpass filters and a dichroic mirror. The first bandpass filter selects a specific range of wavelengths, after which the selected beam hits the

[1] This picture is token [5]

dichroic mirror. The dichroic mirror has a characteristic wavelength. Light with a wavelength lower then the characteristic wavelength is reflected, light with a wavelength higher than the characteristic wavelength passes through, in the figure this beam is shown as reflected in order to gain a clear view of the setup. The reflected wavelengths are absorbed by an absorption wall in the microscope-body. In this way the dichroic cube selects wavelengths suitable for the experiment. Subsequently the light travels through the objective, which focusses the light on the sample. The objective used in this experiment is a Nikon S Plan Fluor air-objective with NA=0.6 and a magnification of 40×.

Emission beam

A part of the fluorophores gets now excited and will emit light. The emitted light passes through the objective and hits the dichroic mirror of the dichroic cube. Now the light with a wavelength higher than the characteristic wavelength passes through to the second bandpass filter of the dichroic cube. This ensures that only fluorescence light will pass. The emission beam will be collimated by a lens with focal point of $80nm$. Subsequently the collimated light travels through a polarization beam splitter (PBS), in this case a $Thorlabs, CM1 - PBS - 251$. Light with a parallel polarization continues and light with a perpendicular polarization is reflected. After exiting the PBS, each light beam is focussed on an Andor sCOMS camera. In section 4.1 will be explained how the cameras function. The extinction ratio of the PBS for the parallel beam is 1000 and of the perpendicular beam is 40. Because of the poor perpendicular extinction ratio of the polarization beam splitter, an extra polarizer is placed before the sCMOS camera that detects the perpendicular beam. This extra polarizer is a $ThorlabsWP25M - VIS450 - 700nm$ wiregrid polarizer, with a tranmission of 90% and a extinction ratio of about 300.

Dichroic cubes

The beads measured in this experiment are yellow-green fluorescent beads. For this purpose a dichroic cube is used with an excitation filter of $460 - 500nm$, a dichroic mirror of $505nm$ and a emission filter of $516 - 556nm$. For measuring the other samples a dichroic cube is used with an excitation filter of $540 - 580nm$, a dichroic mirror of $585nm$ and a emission filter of $590 - 670nm$.

Cameras

The cameras used in the set-up are Andor sCMOS Neo 5.5 cameras. The cameras are sensible to NIR-light. The chips of the cameras have 2560×2160 pixels. Each pixel gets a 16 bits signal after the ADC converts the signal. This means each pixel has one of 65536 gray-values. The cameras have two read-out modes, a Global and a Rolling Shutter. The Global shutter read out all the pixels at the same time, the rolling shutter read out sequentially in rows. Kiefer[] has optimized the camera settings and find that the best results are obtained by using the rolling shutter with a readout rate of $560MHz$ and just using the $500x500$ pixels in the center of the chip. The rolling shutter is preferred above the global shutter, because it has a greater amplifier. In this way the lowest signal to noise ratio is obtained, because the noise isn't time-dependent. Kiefer also investigated the gain factor of the camera. Measuring a bright sample he found that the gain factor is actually around 1.7 or 1.8, so the $\alpha = 1.7$. In order to reduce thermal noise the camera chip can be cooled down to $-30\,^{\circ}\mathrm{C}$

4.2 Calibration of the Setup

4.2.1 G-factor

Optical elements in the setup affect the polarization of the lightbeam by letting through one polarization more efficiently than another. Especially the objective, see section 2.5.3 influences the polarization of the light, but also mirrors and lenses can effect the polarization of the light. Calculating the anisotropy the intensities have to be corrected for the polarization preference of the setup. This is done by measuring a sample of the dye Rhodamine-B in water. Since Rhodamine-B has a short rotatianol correlation time and water is not viscous, this will result in mostly isotropic fluorescence, $r = 0$. Now the G-factor can be calculated with the following formula.

$$G = \frac{I_{||}}{I_{\perp}} \tag{20}$$

Here $I_{||}$ is the intensity of the light polarized parallel with respect to the excitation beam and $I_{\perp}$ is the part of the light polarized perpendicular with respect to the excitation beam. It is important that the G-factor is measured on a pixel per pixel bases. For example an objective is a cone, therefore light on different places of the cone will have a different distortion of the polarization.

4.2.2 Maximal Anisotropy

In section 2.5 it was mentioned that, rotational diffusion, not parallel excitation, emission axis, the objective and the background could have a lowering effect on the anisotropy. Especially the effect of the objective is quite large. In order to determine the reliability of the measurements, the maximum anisotropy available by the set-up need to be determined. This is done with a sample of MCherry in glycerol. MCherry is a fluorescein, a fluorescent protein. MCherry is a large protein and so it's rotational correlation time is long and glycerol is a very viscous fluid. Therefore the rotational diffusion is reduced to a minimum.

4.3 Biological Samples

In the experiment the following biological constructs are measured:

- EGFR-FKBP-mCherry not stimulated
- EGFR-FKBP-mCherry stimulated with AP
- EGFR-FKBP-mCherry stimulated with EGF
- EGFR-2FKBP-mCherry not stimulated
- EGFR-2FKBP-mCherry stimulated with AP
- EGFR-2FKBP-mCherry stimulated with EGF

Here EGFR is the epidermal growth factor receptor, FKBP stands for the FK 506 binding proteïn and mCherry is the labeling fluorophore. When an EGFR is exposed to EGF a cell get signals to start processes like cell proliferation, migration and growth. Enduring these process the cells cluster. When EGFR is exposed to AP clustering takes also place, but AP do not induce cell processes.

4.4 Measurement Protocol

- **General settings**

Before doing a measurement the environmental light should be diminished to a minimum. Switching on the cameras make sure that the chips are cooled down to 30 °C. For the measurements the rolling shutter is used. The parallel camera has to have an internal start and the perpendicular camera an external, in order to ensure that the cameras measure exactly at the same time. In order to minimize bleaching the shutter has to be closed in between measurements. For simplifying the data analysis it is recommend to save parallel and perpendicular images in distinct folders. Saturated pixels have to be avoided by measuring with shorter exposure times. Finally be aware that the mercury lamp needs to warm up for $30min$.

- **Instrumental background**

A measurement without mercury lamp should be done in order to determine dark current and hot pixels. Subsequently a measurement with mercury lamp should be done in order to detect scattering effects and environmental background.

- **Beads**

For camera registration bead samples are measured. This beads are $200nm$ yellow green beads. The dichroic used has a excitation filter $460-500nm$, dichroic mirror $505nm$ and emission filter $516-556nm$. In order to obtain as bright images as possible the polarization filter is let out. Because of diffraction phenomena the beads appear as blobs of around 10 pixels on the screen. Focussing the microscope the airy disk around the beads has to minimize as much as possible. For the data analysis is important that beads are located not to close together as will be explained in section **??**

- **G-factor**

The G-factor is measured by a sample containing $5\mu m$ Rhodamine B in an aqueous solution. The sample is treated with BSA in order to prevent that rhodamine gets stuck on the coverslip or the sample glass. The exposure time has to be long enough to get a high signal, in this experiment at least 10000 out of 65536 counts. The number of pictures that could be taken on one place in the sample depends on the amount of bleaching. To determine the background a sample containing only water is measured.

- **Maximal Anisotropy**

The maximal anisotropy is measured by a sample containing $0.5microM$ Mcherry in a 50/50 PBS Glycerol solution. The Mcherry sample is also treated with BSA. Again the exposure time chosen on bases of the obtained number of counts. Also a background sample containing 50/50 PBS Glycerol has to be measured. Because mCherry is a protein it is important to store the sample in the freezer.

- **Autofluorescence**

In this experiment autofluorescence cells in mowiol are measured. Because the autofluorescence cells could have a very low expression, it could be helpful to search with the upper microscope light. After this the cells are measured for $1s$ and subsequently $3s$ with the light from the mercury-lamp. The measurements of $3s$ are needed to define a good region of interest, that is to define were the borders of the cell in the image are.

- **Biological Samples**

In order to get a good anisotropy results images of cells needed at least 10000 out of 53000 counts. Measuring cells they have to be selected on the amount of counts they give. Very bright cells could cause saturated pixels by low exposure times, $1s$ or $0.5s$, these are not suitable for measurements. In order to obtain at least 10000 counts and not more than 53000 counts the exposure time could be varied between $1s$, $2s$ and$3s$. The cells needed to be imaged as most in the center of the image as possible.

4.5 Data Analysis

The most of the data analysis is done with Mathlab. Besides Mathlab the programme Image J in used, mostly for analysing image stacks. In this section the data-analysis is outlined. A summary of the Matlab scripts/function could be found in the APPENDIX. An extended discussion of the transformation matrix could be found in APPENDIX

4.5.1 Transformation Matrix

In order to calculate the anisotropy the parallel and perpendicular image have to overlap perfectly. So for each pixel in a parallel image the corresponding pixel in a perpendicular image has to be determined. This can be done by finding a transformation matrix. Applying this matrix on the perpendicular image will align the parallel and perpendicular images. A transformation matrix is calculated of the following form:

$$T = \begin{bmatrix} a & b & 0 \\ c & d & 0 \\ t_x & t_y & 1 \end{bmatrix}$$

Here the entries a, b, c, d are the geometric parameters that determines together the rotation, shearing and scaling of the perpendicular image, t_x determines the translation in the x-direction, t_y determines the translation in the y-direction.[1]

[1] The real case is somewhat more complex for this see 8

4.5.2 Analysing different regions within one cell

Analyzing the cells, there is a opportunity to distinguish between different regions of the cell, e.g. the membrane, the cytoplasm and the nucleus. This can give interesting information of the place in the cell where clustering occurs or where the autofluorescence is large. Actually for treating the parts of the cell differently then others, there is a need to distinguish these parts. In wide field microscopy this is quite difficult because the resolution is limited and one pixel on a image of a cell will could show a part of the membrane and cytoplasm or cytoplasm and kernel at once. For this reason the analysis of the experiment do not use different regions of interest inside one cell.

4.5.3 Uncertainty in the anisotropy

As mentioned in 2.6,the uncertainty in the anisotropy is caused by photon statistics, instrumental background and autofluorescence. Actually also the ojective has a lowering effect of the anisotropy. Because in this experiment measurements are only compared to each order and each images is influenced in the same way by the objective, this uncertainty is not taken in account. Uncertainty is also caused by registration of the images. Theoretically this uncertainty has to be less then one pixel. For this reason the uncertainty in the registration is not taking in account by calculating the anisotropy.

In the anisotropy the uncertainty could be divided in tree parts. First each pixel in an anisotropy images has a certain amount of uncertainty. Secondly to each cell a average value is attached, which has a certain uncertainty. Thirdly a value of anisotropy is obtained for each group of cells of a same biological sample. The first uncertainty is calculated by propagation of uncertainties for each pixel. So the uncertainty is given by:

$$\sigma_r = \sqrt{(\frac{\partial r}{\partial I_{||}}\sigma_{I_{||}})^2 + (\frac{\partial r}{\partial I_{\perp}}\sigma_{I_{\perp}})^2 + (\frac{\partial r}{\partial A_{||}}\sigma_{A_{||}})^2(\frac{\partial r}{\partial A_{\perp}}\sigma_{A_{\perp}})^2 + (\frac{\partial r}{\partial G}\sigma_G)^2 + (\frac{\partial r}{\partial B_{||}}\sigma_{B_{||}})^2(\frac{\partial r}{\partial B\ \perp}\sigma_{B\perp})^2}$$

Where r denotes the anisotropy, $I_{||}$ and $I_{\perp}$ denotes the parallel and perpendicular intensities, $\sigma_{I_{||}}$ and$sigma_{I_{\perp}}$ denotes the uncertainties in the parallel and perpendicular intensities,$A_{||}$ and $A_{\perp}$ denotes the parallel and perpendicular autofluorescence intensities, $\sigma_{A_{||}}$ and$sigma_{A_{\perp}}$ denotes the uncertainties in the parallel and perpendicular autofluorescence, G is the G factor and $B_{||}$ and $B_{\perp}$ denotes the parallel and perpendicular instrumental background intensities, $\sigma_{B_{||}}$ and$sigma_{B_{\perp}}$ denotes the uncertainties in the parallel and perpendicular background. The uncertainties in the intensities, autofluorescence and background are all determined by applying photon statistics to each single pixel, the uncertainty in the G-factor is determined by propagation of uncertainties. In this way an error for each pixel in the anisotropy image is obtained.
For the weighted average of a whole cell the uncertainty is based on the deviation on the average of the pixel values. In this case it would make no sense to work with propagation of the uncertainties, because for each region a different anisotropy is expected. Especially in the case of clusters, the difference in anisotropy values between regions inside a cell is expected very large. So the uncertainty of the anisotropy of a whole cell the standard deviation in the anisotropy values of the pixels inside the cells is calculated. The way of calculating the error of a group a cells is done in the same way as calculating the error for a whole cell. In this case all the anisotropy values of pixels of cells belonging to one group are put together. Again the standard deviation of the whole bunch of pixels is calculated to obtain the uncertainty in the average anisotropy value of a group of cells.

4.6 Analyse protocol

4.6.1 Determining the Transformation matrix

As described in 4.5.1the transformation matrix is determined in Matlab. There are two ways of doing this. The first method discussed here is the script `alim` that Kiefer has written for determining the transformation matrix. The`alim` needs to imput arguments, a parallel and corresponding perpendicular image. The script uses the mathlabfunction `imregtform`. `Imregtform` is a function that estimates a transformation matrix to align one image to another. The function uses the optimizer *registration.optimizer.RegularStepGradientDescent* which rely on the number of overlapping pixels. The

optimizer detects the relative intensity of each pixel and counts the number of pixel which parallel and perpendicular relative intensity are similar. Then the optimizer tries a transformation matrix. If there are not more overlapping pixels after the transformation, the moving image is replaced by the image resulting from applying the transformation matrix to the original moving image. If not the original image is maintained. Now the process starts over again. In this way the to images are aligned and a transformation matrix is defined. For this analysis the optimizer has a maximum of 500 iterations. The result of the alignment is directly showed in a figure where the parallel image is colored green upon the distorted perpendicular image which is colored pink. Where two images exactly overlap, the pixels has a same relative intensity, the picture is gray. The obtained transformation matrix is stored as a affine 2d matrix in the workspace. Using this optimizer in combination with bead images a few problems could occur. In the first place if there are beads on the edge of the parallel image, these beads could fall outside the 500×500 pixels of the perpendicular camera. In this case the optimizer wouldn't work. Secondly the optimizer could stuck in a local maximum or minimum. This registration method seems to work quite well with bright and large images as a whole or a group of cells. On bead images the method works poorly most of the time. This can be explained because there are to less intense pixels which the optimizer can compare.
A second method for the registration is obtained by comparing positions of beads. This script is called `alimpointsmeerdere`. The script starts with reading in all bead images measured. This is done by the function `imread`. The Matlab function `imread` is used to load a series of images into the workspace. Data the is read in this way is stored in a cell. This cell is not a cell of the biological sample but denotes a special array in Matlab, each element of the cell denotes a single image.This method determines the position of a bead according to the average intensity of pixels of the bead. This is done for all the parallel and perpendicular images. Then the positions of the parallel and perpendicular beads are compared. If a bead on one image hasn't a corresponding position on the other image the bead is thrown away. After this a optimizer find a transformation matrix, which transforms the perpendicular positions such that they became the same as the parallel positions. In the end the transformation matrix is given and the two images are shown in figure. Again the parallel image is shown in purple and the perpendicular image is shown green, if they overlap perfectly the total images is gray.
The second method works very fast, but is only usable for a registration on bead images. This method has also the advantage that a lot of bead images can be combined in one optimizing process, just by listing the position of all the bead images. Actually in the experiment the registration is also done on cells. For cells only the first method is usable. Luckily the first registration method works quite well for cells in contrast with the bead images.

4.6.2 G-factor

The G-factor is calculated by the Matlab function `calgaangepast`.The function `calgaangepast` needs five input arguments, a parallel image of the rhodamine sample, a perpendicular image of the rhodamine sample, a parallel image of the background sample, a perpendicular image of the background sample and the transformation matrix. If there is a stack of rhodamine-images or rhodamine background images measured, first the average intensity of these images is calculated on a pixel per pixel basis in image J. In this way an average parallel and an average perpendicular image are obtained. The function `calgaangepast` first applies the T-matrix to the (average) perpendicular images. After this the function calculates for each pixel the G-factor by first subtracting the background on pixel per pixel bases and then dividing each pixel of the parallel image through the corresponding pixel of the perpendicular image. Some pixels in the parallel image may have no corresponding pixel in the perpendicular image, because some pixels of the perpendicular image will lost after applying the transformation matrix. To compensate for this phenomenon all pixels with a value greater than 1.45 are set to 0. In this way a 500×500 figure of the G-factor is obtained. The image is directly shown and average value of the image is given in the output of the function. The uncertainty in G is calculated in Mathlab with the script `sigma-G-factor`. This script needs the same input elements as the function `calgaangepast` and an input argument that denotes the amount of images in a images stack. The script applies propagation of uncertainties on a pixel per pixel basis. Again pixels in the parallel image that haven't a corresponding value in the perpendicular image are set to zero. The script produces immediately a picture of the uncertainty in the G-factor.

4.6.3 Maximum Anisotropy

The maximum anisotropy is calculated by the function `anisaangepast`. This function needs six input arguments, a parallel images of the mCherry sample, a perpendicular image of the mCherry sample, the transformation matrix, the image of the G-factor, a image of the parallel mCherry background and a image of the perpendicular mCherry background. If there are more images in one image stack, again first the average of these images has to be calculated with Image J. First the background intensities are subtracted from the measured intensities then the function calculates the anisotropy on pixel per pixel bases by 4. The result is shown directly in a figure. The uncertainty of the mCherry measurements is calculated with the function `sig_ani`. These function needs the same input arguments as the function *anisaangepast* and the number of images of the image stack. The uncertainty is calculated on pixel basis according propagation of uncertainties. A figure of the uncertainty in the maximal anisotropy is directly shown on the screen.

4.6.4 Autofluorescence

The analysis of autofluorescence data is a delicate subject, because the autofluorescence varies strongly between different cells and in different parts of the cells. In this analysis an average perpendicular and parallel value of the whole cell is calculated. This analysis made no distinction between different parts of the cell, because in wide-field microscopy it is very difficult to point out the borders between these regions. In order to calculate the average value of the autofluorescence, the area the cell occupies has to be made into a region of interest.A region of interest is a image that is 1 on each pixel occupied by the cell and 0 on the other pixels. The region of interest is determined on the image of the parallel cell, because the parallel images is generally brighter than the perpendicular image. If the intensity values of the parallel images are significantly higher than the background, intensities of at least 250 counts, a threshold function in mathlab can determine a region of interest and calculating the mean value. If the intensities of the parallel images are two low, the region of interest, ROI, should be made by hand in Image J. When using the threshold function in mathlab the script `autofluor` is used for determining the ROI. The script need four input arguments, the link where autofluorescence cells are stored, the number of cells and a cut off level. After this the intensity of the images is compared with the background and on this ratio a threshold is determined. Pixels with an intensity above the threshold are set to one, pixels with an intensity below the threshold are set to zero. In this way the region of interest is created. The script `autofluorinlezen` needs two input arguments, the link containing the parallel and perpendicular images of the autofluorescence cells and a link containing the parallel and perpendicular masks of each cell. Since for the average value of the intensity of the parallel and perpendicular autofluorescence no overlap between the images is needed, it is possible to work with to masks. It is also possible to work only with a mask bases on the parallel image. The second mask will be obtained by applying the transformation matrix of the first mask. However the measurements of the autofluorescence didn't has a good transformation matrix, two masks are used. script will multiplier the region of interest with the original image. After determining the ROI's the scripts will go on in the same manner. First the ROI-image is multiplied by the corresponding parallel and perpendicular images. Now all pixels which are not in the ROI are set to zero. Subsequently the mean value of the cell is calculated. It is difficult to give a reasonable uncertainty in the autofluorescence. The difference between cells are so large, almost nothing can be said about the uncertainty. In this case taking simply the squirt of the measured counts is meaningless. For this reason the uncertainty the standard deviation of the mean values is used.

4.6.5 Cells

The images of the cells are analyzed with a Matlab function called `cellen alles in een`. This function has seven input variables the link where the cells are stored, an array n, the transformation matrix T, the G-factor, the uncertainty in the G-factor, the maximal anisotropy, and an other array called skip. The first array is used to define groups of cells of an identical construct. The first element in the array is denoted zero, the last the number of cells. The number of a cell which is the first of a new construct should be denoted in this array. The second array contains the numbers of the cells which aren't take along in the analysis. Reasons could be a very low intensity or the transformation matrix isn't working. The output of the function consist of an absolute histogram giving the anisotropy per construct and a histogram giving the anisotropy relative to the maximum anisotropy. Apart from this for each cell five

figures are obtained, the first shows the effect of the transformation matrix, the second shows the region of interest of the cell, the third shows the anisotropy, the fourth shows the anisotropy throwing away values smaller then 0 or greater then 0.4, the fifth shows the uncertainty per pixel of the anisotropy. The script start reading the cells in in the workspace. Subsequently on basis of the ration between average intensity and background a ROI is determined. Then the anisotropy per cell per pixel is calculated according to 4. Also the weighted average per cell is calculated. The uncertainty per pixel is calculated according to the propagation of uncertainties. The uncertainty of the averaged anisotropy is calculated on the deviation of the anisotropy values of the pixels. The anisotropy isn't expected to be the same all parts of the cell, recall in the parts where clustering is expected the values of anisotropy should be lower. Calculating the deviation of the anisotropy values gives a good representation of the differences inside one cell. Then all the anisotropy values of pixels of cells of one construct are listed together. In this way a weighted average of the anisotropy values

5 Results

5.1 Background Measurements

The camera background was measured with and without mercury lamp. Without mercury lamp there were taken 30 images with exposure time $0.1s$ and 30 images with exposure time $1s$. The results are shown in table 1. Lengthening the exposure time from $0.1s$ to $1s$ have little effect on the background noise of the cameras, when averaging 30 pictures. In order to detect dark current and hot pixels, the highest measured pixel values out of the 30 images for both exposure times are compared . The highest signal in one pixel measured with exposure time $1s$ was for the parallel signal 119 counts and perpendicular signal 122 counts, for $0.1s$ the parallel signal returns 118 counts and perpendicular signal 117counts. So the 1s measurements turned out slightly higher values.

The results of imaging 30 pictures of background noise with mercury lamp are also given in tabel1. The parallel values turns out to be larger than the perpendicular values and also lengthening the exposure time has a increasing effect on the background signal measured with mercury lamp.

tijd	par	σpar signal	per	σper
0.1s without mercury lamp	100	6	100.4	2.3
1s without mercury lamp	100.3	2.3	101	3
1s with mercury lamp	119	6	111	5
2s with mercury lamp	140	9	123	7
3s with mercury lamp	161	11	135	8

Table 1: Results of the instrumental background, the values in the entries are the averaged signals over 30 images given in counts

5.2 Camera registration

Both registration methods explained in section 4.6.1 were applied to the bead images. The registration method based on the position of beads turned out best, looking only to beads images. The `alim` function worked sometimes, but many times the algorithm got stuck. Applying a transformation matrix obtained from bead images to a cell image gives mostly no satisfactory result. Therefore registration matrices were obtained by applying the *alim* on cell images. It was remarkable that the `alim` didn't get stuck using cell images. This is probably because the amount of data usable for the optimizer is much larger for cell images then for bead images. The transformation matrices obtained this way were almost all better then the ones obtained from the bead images. However, registration on different cells gives sometimes quite different transformation matrices and not all transformation matrices worked equally well. For this reason a depth dependence in the registration is suspected. A measurement with a water objective was done, because the DOF of a water objective is smaller than the DOF of an air objective. Therefore measuring with a water objective should be less sensible for an eventually depth dependence in the

camera registration. The obtained result seems slightly better on first sight. Also measuring beads on different heights with the usual air objective gives different transformation matrices.

Because there are no quantified results of the camera registration, figures comparing between different transformation matrices were made. The figure below,8 give the anisotropy of a cell twice, in the left image a transformation matrix bases on bead images is used, in the right a transformation matrix based on cells is used. In the figure the left edge of the cell has a strange red line with anisotropy values higher or equal to the theoretical maximum when the transformation matrix of the beads is used. On the right edge quite low values visible. This extreme high values aren't present in the image were the bead transformation matrix is used and also the right edge the values are higher. In this way the figure shows the effect of a wrong transformation matrix in the final anisotropy values.

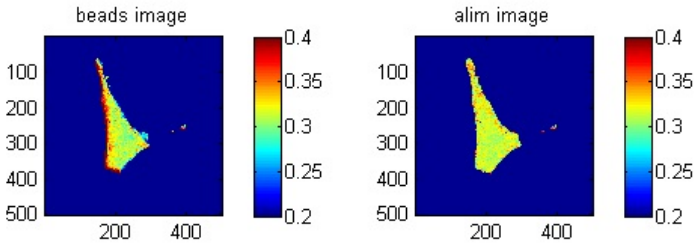


Figure 8: The consequence of a bad alignment, the left image shows anisotropy using a transformation based on beads, the right image shows the anisotropy, using a transformation matrix based on cells.

5.3 G-factor

The G-factor varied strongly between different measurements. Values of the G-factor were obtained between 1.28 and 1.39 were obtained. In the measurements is tried to acquire around 30000 counts by a maximum 53000 counts. The parallel and perpendicular background intensities were measured around 165 and 135 counts. So the noise band is about an 0.5% of the total intensity. To ensure the uncertainty is small, more images were taken in quick succession on a predetermined position in the sample. Deriving the G-factor from one image gives a uncertainty in signal of around 0.017 counts, calculating it from 10 images gives an error of 0.008 counts and calculating it out of 20 images gives an error of around 0.003 counts. This is in agreement with the $\sqrt{N}$ rule. When taking more images at once bleach occurs. Bleaching occurs almost equally for the perpendicular and parallel intensities, for both around 5% in 20 measurements of $4.5s$ at one place. Because of the G-factor defines the ratio between the parallel and perpendicular intensities, bleaching of 2000 counts wouldn't influence the G-factor significantly. This way the best measurements gives an relative error of 0.5%.
Measuring the G-factor it occurred that for a few weeks the G-factor was around 1.28 and for another few weeks the G-factor was around 1.39. For this difference, that amounts nearly 10%, no reasonable explanation could be found.

5.4 Maximum anisotropy

For the maximum anisotropy values around $r = 0.31$ were found. A typical measurement returns $r = 0.3104 \pm 0.0014$. In the measurements is tried to acquire around 30000 counts by a maximum of 53000 counts. The m-Cherry background turned out 160/130 counts. So the signal to noise ratio was around 0.5%. Actually this measurement gives a much lower maximum anisotropy then theoretically expected, $r = 0.37$. For this reason the maximum anisotropy was measured with samples of $0.5 microM$ and $0.05 microM$. It was expected that a sample of $0.05 microM$ should give a higher value than a $0.5 microM$ sample, because less dimerization would take place in a lower concentration. It turns out that the $0.5 microM$ sample gives a higher maximum anisotropy than the $0.05 microM$ sample. Because the sample of $0.5 microM$ gives more counts, it was chosen to measure only this sample. In order to detect of mCherry was stuck on the cover slip, new mCherry $0.5 microM$ samples were made. The new samples turned out to be much more intense then older once. However the new samples didn't give a higher anisotropy. Because no reason could be found for the low values of the mCherry sample, the maximum anisotropy values are not taken in account for the measurement of the cells. For this reason only absolute anisotropy values are reported in stead of relative values with respect to the maximal anisotropy.

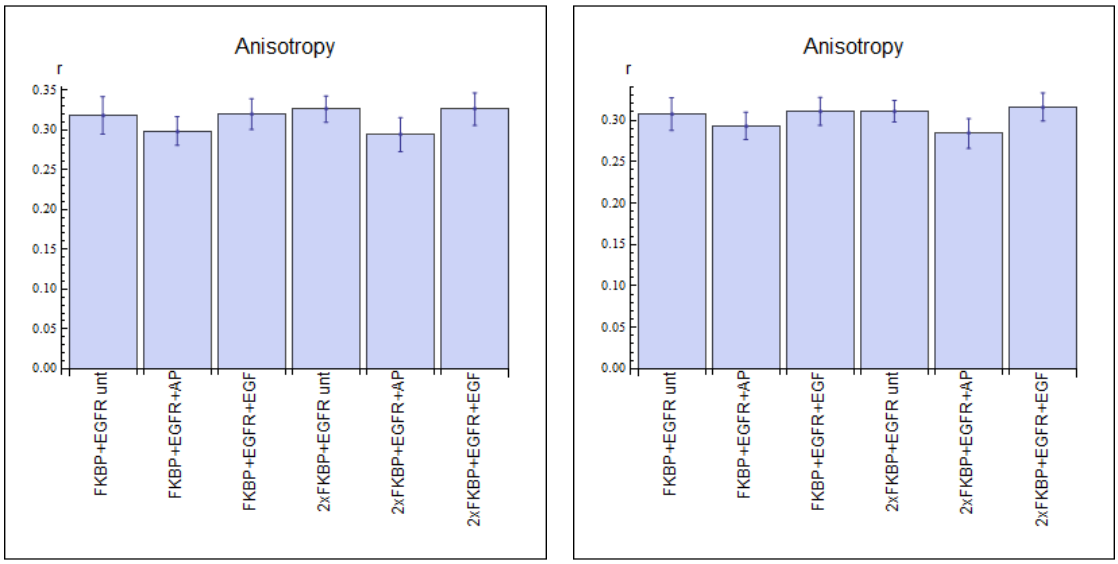
5.5 Autofluorescence

The measurements of the autofluorescence gives two very different results. Using an exposure time of $1s$ three measurements turned out a weighted average of 402 ± 277 counts for the parallel intensity and 269 ± 151 for the perpendicular intensity. For these measurements no sample of the autofluorescence background was available. Examining the background by hand in imageJ returns a background intensity of 190 counts for parallel and a background intensity of 130 counts for perpendicular. A fourth measurement, were also an exposure time of $1s$ was used returned a weighted average of 170 ± 56 counts and 128 ± 42 counts for perpendicular and parallel respectively. This measurements included the instrumental and environmental background. However measuring a background sample with only mowiol, the averaged background of autofluorescence turns out 191 ± 5 counts for parallel and 152 ± 4counts for perpendicular. This is higher than the autofluorescence values. Examining the background of the autofluorescence cell images by hand in imageJ gives around 150 counts for parallel and 125 counts for perpendicular. Therefore new autofluorescence measurements have to be done. However the contrast of the autofluorescence cells and their background is much less in the fourth measurement then in the first three measurements.

The difference in the autofluorescence values is probably a consequence of the biological preparation. Because of the very different autofluorescence intensities, the analysis is done once with the high autofluorescence values and once with the lower autofluorescence values. The result is shown in figure 9(a) and 9(b). It turns out that average decrease in anisotropy amounts 3%. The standard deviation of anisotropy calculated with the high autofluorescence values is 0.020 and for the low values 0.017. So with the lower autofluorescence the uncertainty reduces with 15%

5.6 Biological Samples

The results of a typical measurement is shown in figure 9(a) and 9(b). Measuring the biological samples a decrease in anisotropy is found between the untreated cells and cells with AP. Using high autofluorescence values the difference was 6% using 2× FKBP and 10% using FKBP. For the low autofluorescence values the result was a difference of 5% for $2 \times FKBP$ and 9% of $FKBP$. Cells stimulated with EGF did not seem to cluster, because the anisotropy is not lower, see figure 9(a) and 9(b). However the uncertainty for anisotropy calculated by the high autofluorescence was 6% and for the low autofluorescence 5% with respect to the unlabeled FKBP cell.



(a) revs1 (b) revs2

Figure 9: Anisotropy, **a**) shows the anisotropy results calculated with autofluorescence of 402 ± 277 $/269 \pm 151$, **b**)shows the anisotropy results calculated with autofluorescence 170 ± 56 / 128 ± 42

6 Conclusions and Recommendations

The result that lengthening the exposure time from $0.1s$ to $1s$ didn't have an effect on the background noise of the cameras, when averaging 30 pictures, is in agreement with the fact that the readout noise is time independent, see section2.6.3. Dark current is expected to increase by longer exposure times and comparing the highest measured pixel value out of the 30 images for both exposure times, the $1s$ measurements indeed turned out slightly higher values. However averaging over a stack of 30 images measured with exposure time $1s$ no significant account of dark current could be detected.

Measuring with the mercury lamp the parallel signal turned out to be larger then the perpendicular signal. The background measured with the mercury lamp includes environmental light and scattering light, who both has to travel through at least a part of the set up. In section 5.3 it turns out that the set-up lets parallel polarized light easier through then perpendicular polarized light. This explains the higher background signal for the parallel camera. As expected the longer you measure, the larger the values. This is of course because more environmental and scattering light will hit the camera chip when the exposure time is lengthened.

The transformation matrix obtained by both registration methods on bead images, didn't work on the cell images. An explanation for this is that the wavelength used for the bead images is different from the wavelength used for the cells. The measurement of beads on different heights in the sample and the measurement with a water objective reveals that there is probably indeed a depth dependence. However to get a reliable result, more measurements with a water objective and measurements with beads on different heights sample are required. So in conclusion the registration of the cameras is probably dept dependence and dependent on the wavelength. The fact that the registration of the cameras was a problem also Nivard [5] encountered, when measuring green beads and green fluorescent cells with the same dichroic, is another strong indication that the camera registration is depth dependent. If it turned out there is really a dept dependence in the registration, measuring HomoFRET with wide field microscopy couldn't obtain accurate results.

Measuring the G-factor the obtained signal to noise ratio was accurate, 0.5%. Problem is the G-factor varied strongly over weeks. The variance in the G-factor is a fact Nivard [5] also encountered in his research. Kiefer [7] found a G-factors around 1.29, which is consistent with some of our measurements. At this moment, no reasonable explanation of this variation could be given. For this reason it is questionable how reliable the measurements are. If the strange variance in our G-Factor could be traced this problem would be solved.

The low value of the maximum anisotropy is an other problem that couldn't be explained. Kiefer [7] encountered the same low maximum anisotropy in his research. In order to explain the low maximum anisotropy values he calculated the rotational diffusion time of mCherry, but it turned that the effect of rotational diffusion on the maximum anisotropy is negligible. Because the $0.5microM$ sample returns a higher maximum anisotropy then the $0.05microM$ sample, also dimerization is not a satisfactory explanation.

The low value of the fourth autofluorescence measurement is very probably caused by a neutral density filter which was inserted in the microscope body. This is also an explanation for the lower background values in autofluorescence images than the values measured from the mowiol image. However the contrast between background and cells was very much smaller than in the first three measurements. A quick investigation of the sample without neutral density filter shows that the contrast between background and cells is indeed much smaller, but there was no time left for a complete measurement. An explanation for the difference with the first three measurements is that the samples were prepared differently. Because the effect of a neutral density filter in our setup is not investigated no conclusion could be drawn. For a reliable result more measurements have to be done.

Measuring the biological samples it was expected that the anisotropy should decrease by AP as well as EGF. However the EGF samples did not show a decrease in anisotropy. This is probably because something is wrong with the used EGF. However FKBP-samples stimulated with AP show an anisotropy decrease. For this reason the measurement method is able to detect clustering by calculating anisotropy. Apart from this it is remarkable that the $2\times$ FKBP shows less anisotropy decrease than FKBP. It is probable that the biological samples are interchanged.

The goal of this research was to investigate of measuring Homo-FRET with steady-state wide-field microscopy is possible and to optimize this method. The decrease in anisotropy in the AP samples shows that it is possible to detect clustering. However even the anisotropy decrease in AP is not significant,

since 2× the standard deviation is still larger than the decrease in anisotropy. Apart from this the camera registration induces a large uncertainty, which couldn't be taken in account reporting the uncertainty. Also for the maximum anisotropy which is a useful reference value is no reliable value found, which is the same for the G-factor and autofluorescence. In conclusion it could be said that in this research it is not managed to obtain accurate results by measuring Homo-FRET with steady-state wide-field microscopy.

For further research on measuring Homo-FRET with steady-state wide-field microscopy there are a few recommendations. In order to detect the dept dependence of the camera registration orange beads has to be measured. If the problem isn't solved adjusting the wavelength, then more measurements with the water objective and of beads on different heights in the sample has to be done. For the G-factor has to be traced out, what causes the strong variances. A literature study of the maximum anisotropy is required in order to figure out what the recorded maximum anisotropy is in a experimental set up and finally more measurements on autofluorescence has to be done.

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8 Appendices

Appendix A

In this appendix the maximum anisotropy is calculated. Assume that the electric field vector of the excitation beam is oriented in the z-direction. Denote the position of the transition moment of a fluorophore with polar co-ordinates $\vec{x} = (\rho, \theta, \phi)$. If the electric field vector is aligned along the transition dipole moment the fluorophore gets excited. It is also possible that the electric field vector is partially aligned along the transition moment of the fluorophore. The electric field vector could be resolved in a part parallel and a part perpendicular to the transition dipole moment. Because luminous intensity is proportional to the squared absolute value the electric field vector, the intensity of the absorbed light by the fluorophore is given by [nikon site]:

$$I_{abs} \propto \langle \vec{x}, \vec{E} \rangle^2 \propto (\vec{E} \cos\theta)^2 \tag{21}$$

Here $\vec{x}$ denotes the position of the transition moment, $\vec{E}$ denotes the electric field vector,θ denotes the angle between the transition moment and the z-axis. When a fluorophore decays, the emitted light is polarized parallel too the transition dipole moment.The emitted light could be resolved in a part polarized parallel with respect to the excitation beam and in a part perpendicular with respect to the excitation beam. In order to calculate the anisotropy, the fractions of the emitted light that are parallel and perpendicular polarized with respect to the excitation beam have to be known. Assume parallel excitation and emission axis, and let again θ denote the angel between z-axis and transition moment, then the parallel and perpendicular fractions of the emitted light could be determined by examining the process. The absorption of the light by the fluorophore occurs with a factor $\cos\theta$, so the intensity of the absorbed light is $\propto \cos^2\theta$. Fluorescence emission goes again along the dipole moment. Therefore the emitted light polarized parallel to the polarization of the excitation beam will have an intensity $\propto \cos^2\theta$ relative to the absorbed light. The emitted light polarized perpendicular to the excitation beam will have an intensity $\propto \sin^2\theta$. the fluorescence process could be summarized in the following formulas:

$$\begin{aligned} I_{em||} &\propto \cos^2\theta \cos^2\theta = \cos^4\theta \\ I_{em\perp} &\propto \cos^2\theta \sin^2\theta \sin^2\phi \end{aligned} \tag{22}$$

In an isotropic solution of fluorophores each position of the transition moment is equally probable. So with 22the averaged emitted intensities will become:

$$\begin{aligned} I_{av,||} &\propto \frac{1}{4\pi} \int_0^{\pi} \int_0^{2\pi} \cos^4\theta \sin\theta \, d\phi \, d\theta \\ I_{av,\perp} &\propto \frac{1}{4\pi} \int_0^{\pi} \int_0^{2\pi} \cos^2\theta \sin^2\theta \sin^2\phi \, d\phi \, d\theta \end{aligned} \tag{23}$$

Evaluating the integrals results in $I_{av,||} = \frac{1}{5}$ and $I_{av,\perp} = \frac{1}{15}$. The average anisotropy of a single fluorophore is now caculated by 4:

$$r = \frac{\frac{1}{5} + \frac{1}{15}}{\frac{1}{5} + 2 \cdot \frac{1}{15}} = 0.4 \tag{24}$$

So measuring a complete isotropic sample of fluorophores where no FRET occurs we expect $r = 0.4$

Appendix B

Transformation matrix

In order to calculate the anisotropy the parallel and perpendicular image have to overlap perfectly. So for each pixel in a parallel image the corresponding pixel in a perpendicular image has to be determined. This can be done by finding a transformation matrix. Applying this matrix on the perpendicular image will align the parallel and perpendicular images. According to [3] the matrix T is build in the following way:

$$T = \underbrace{\begin{bmatrix} 1 & 0 & t_x \\ 0 & 1 & t_y \\ 0 & 0 & 1 \end{bmatrix}}_{\text{translation}} \cdot \underbrace{\begin{bmatrix} \theta_c & -\theta_s & 0 \\ \theta_c & \theta_c & 0 \\ 0 & 0 & 1 \end{bmatrix}}_{\text{rotation}} \cdot \underbrace{\begin{bmatrix} 1 & k & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{bmatrix}}_{\text{shear}} \cdot \underbrace{\begin{bmatrix} s_x & 0 & 0 \\ 0 & s_y & 0 \\ 0 & 0 & 1 \end{bmatrix}}_{\text{scaling}} = \begin{bmatrix} s_x\theta_x & s_y(k\theta_c - \theta_s) & t_x \\ s_x\theta_s & s_y(k\theta_s + \theta_c) & t_y \\ 0 & 0 & 1 \end{bmatrix} = \begin{bmatrix} a_1 & a_2 & a_3 \\ a_4 & a_5 & a_6 \\ 0 & 0 & 1 \end{bmatrix}$$

Here $t_x, t_y, \theta, k, s_x, s_y$ are called the geometric parameters and are defined in the following way:

t_x = translation in positive x-direction

t_y = translation in positive y-direction

θ =counterclockwise rotation angle w.r.t. the x-axis, $\theta_c = \cos\theta$ en $\theta_s = \sin\theta$

k = shear factor along the x-axis, defind as $tan(skew)$, with $skew$ the skew angle w.r.t. the y-axis

s_x = magnification factor in the x-direction

s_y =magnification factor in the y-direction

The entries $a_1, a_2, a_3, a_4, a_5, a_6$ are called geometric transformation parameters. This transformationmatrix is defined in a way it uses the center of each pixel for transformation. Actually the Matlab function `imregtform`, which is used in this experiment, determines a matrix which transforms pixels according to the position of the upper left corner. So the transformation matrix needs to be adjusted, this gives according to [3]:

$$\begin{bmatrix} s_y(\theta_c + k\theta_s) & s_x\theta_s & 0 \\ s_y(k\theta_c - \theta_s) & s_x\theta_c & 0 \\ t_x + x_c - s_y x_c(\theta_c + k\theta_s) + s_y y_c(\theta_s - k\theta_c) & t_y + y_c - s_x\theta_s x_c - s_x\theta_c y_c & 1 \end{bmatrix} = \begin{bmatrix} t_1 & t_4 & 0 \\ t_2 & t_5 & 0 \\ t_3 & t_6 & 1 \end{bmatrix}$$

Now the geometric parameters could be expressed in $t_1, t_2, t_3, t_4, t_5, t_6$ in the following way:

$$\begin{aligned} s_x &= \frac{t_5}{\cos\theta} \\ s_y &= \frac{t_1}{\theta_c + k\theta_s} \\ \theta &= \arctan\frac{t_4}{t_5} \\ k &= \frac{t_2\theta_c + t_1\theta_s}{t_1\theta_c - t_2\theta_s} \\ t_x &= t_3 + \frac{N}{2}(s_y(\theta_c + k\theta_s) - 1) - \frac{M}{2}s_y(\theta_s - k\theta_c \\ t_y &= t_6 + \frac{M}{2}(s_x\theta_c) + \frac{N}{2}s_x\theta_s \end{aligned}$$

Here $[M, N]$ is the new pixel size in the transformed image.

Appendix C

This Appendix will give a short summery of the used Mathlab functions and their input arguments.

- camera registration

On basis of the position of the beads:
`[T,R_new,allpPAR,pPERnew,wrong] = alim_points_meerdere(allpPAR, allpPER, maxdistance)`
Here `allpPAR` is the link were the bead image from parallel camera, `allpPER` is the link were the bead images of the perpendicular camera are stored, `maxdistance` is the maximum number of pixels between the bead position on the parallel image and the bead position on the perpendicular image. If the distance is larger than `maxdistance`, the beads are not involved in calculation the transformation matrix.

The output arguments are respectively, the T matrix, the distances between beads after transformation, the positions of the parallel beads and the position of the beads after transformation, and `wrong` are the beads which had now corresponding bead position within the maxdistance

Using `imregtform`
`T=alim(im1,im2)`
Here `im1` is an image of the parallel camera, `im2` is a bead image of the perpendicular camera. The output argument `T` is the transformation matrix.

- G-factor

`G=calgaangepast(rhodPAR,rhodPER,rhodPARbg,rhodPERbg,T`

Here `rhodPAR` is the average parallel image of the rhodamine sample, `rhodPER` is the average perpendicular image of the rhodamine sample, `rhodPARbg` is the average parallel image of the rhodamine background sample, `rhodPERbg` is the average perpendicular image of the rhodamine background and sample `T` is transformation matrix obtained by the camera registration. The output argument `G` is an 500×500 matrix containing the G-factor for each pixel

`[sigmaG]=sigmaGfactor(rhodPAR, rhodPER, T, G, bgpar, bgper, N)`

Here the input arguments are the same, except for `G`, which is the G-factor obtained with `calgaangepast` and `N` which denotes the number of images in an image stack. The output argument is a 500×500 matrix containing the uncertainty of the G-factor for each pixels.

- Maximum anisotropy

`[ani]=anisaangepast(im1,im2,G,T,bg1,bg1)`

Here `im1` denotes the average parallel image of the mCherry sample, `im2` denotes the average perpendicular image of the mCherry sample, `G` is the G-factor matrix obtained with the function `calgaangepast`,`T` is the transformation matrix and `bg1` is the average parallel image of the background sample and `bg2` is the average perpendicular image of the background sample. The output argument `ani` is a 500×500 matrix containing the value of the maximum anisotropy of each pixel.

`[sig_maxani] = sigma_anis(I_par, I_per,bgpar,bgper, T, G, sig_G, N)`

Here `I_par` denotes the average parallel image of the mCherry sample, `I_per` denotes the average perpendicular image of the mCherry sample, `bgpar1` is the average parallel image of the background sample and `bgper2` is the average perpendicular image of the background sample, `T` is the transformation matrix, `G` is the G-factor matrix obtained with the function `calgaangepast`,`T` is the transformation matrix and `sig_G` is the uncertainty in the G-factor, `N` is the number of images in the image stack in imageJ.

- Autofluorescence

Using a mask made by hand in imageJ

```
[auto] = automeerdere(link, map, aantal)
```

Here `link`, denotes the link where the autofluorescence images are stored, `map` is the parallel or perpendicular map, `aantal` denotes the number of autofluorescence cells. The output is a mean autofluorescence value per cell.

Using a threshold.

```
[autopar,autoper] = autFLall(link, TB, cutoff, aantal)
```

Here `link` denotes the folder were the autofluorescence cells are stored, `TB` has two options, `me` for a threshold based on the mean autofluorescence and `ma` for a threshold bases on the maximum autofluorescence. `cutoff` denotes the percentage of the background used to determine the threshold and `aantal` denotes the number of autofluorescence cells. The output gives the mean autofluorescence values for parallel `autopar` and perpendicular `autoper`.

- Cells

Because this is the most important Matlab script, the whole script is included.

```
function [matrix, groepmatrix] = cellenallesin1(link, n, T, G, sig_G, skip)
%alles voor cellen in 1x uitrekenen.
%gem_per_groep          =  rij met gemiddeldes, 1 waarde per groep
%gem_verh_per_groep     =  rij met gemiddelde verhouding per gorep (1
%                       waarde per groep)
%anirow                 = cell: rij met 'plaatjes', ieder plaatje bevat de
%                       anisotropy van 1 cel
%sig_ani                =cell: rij van 'plaatjes', ieder plaatje bevat de
%                       onzekerheid in anisotropy van 1 cel
%groepen_ani            = cell: rij van rijtjes, ieder rijtje bevat een
%                       lijst van anisotropy waardes, per groep 1 lijst.

%WAARDES
%T = affine2d([1.00022678967681,0.00980122082357918;-0.00969827820289732,
% 1.00009144198519;6.14738046347580,1.40502034190811]);
% camera achtergrond
bgpar = double(imread('D:\Margriet&Kyo\DATA\
2014-04-11_G_bleking_mChoud&nieuw\Par\camerabgpar\AVG_camerabg_1s_par.tif'));
bgper = double( imread('D:\Margriet&Kyo\DATA\
2014-04-11_G_bleking_mCh_oud&nieuw\Per\camerabgper\AVG_camerabg_1s_per.tif') );

% autofluorescence
%bepalen we los 1x (zie excel bestand) dus waardes voor autofluorescence
%kunnen we hier inzetten
auto_par = double(402 - mean(bgpar(:)) );
sig_auto_par = double(277);
auto_per = double( 269 - mean(bgper(:)) );
sig_auto_per = double(150);
%auto = {auto_par,auto_per};

aantalcellen = n(end);
%VANAF HIER ANDERE SCHRIPTS NODIG

%cellen inlezen en roi bepalen
[x]=cellen_inlezen_mexpt(link, aantalcellen);
roi = maakroizp(x, aantalcellen);

%HIER BEGINT HET 'REKENEN'
[anirow]=process_exptime_zp(x, G, T, roi, [auto_par, auto_per], bgpar,...
    bgper , aantalcellen); % anisotropy van alle cellen

if (nargin>5)
    [anirow] = cellenskip(anirow, skip);
    % gooit cellen die je onbetrouwbaar vind er uit
end

anirow_cor = anicor(anirow,aantalcellen);
% gooit bij iedere cel waardes onder 0 en boven 0.4 er uit
%
% verhouding = cell(1,aantalcellen);
%
% for i=1:aantalcellen
```

```
%  verhouding{1,i} = anirow_cor{1,i}./maxani;
% geeft de verhouding: anisotropy / maximale anisotropy
% end

sig_ani = cell(1,aantalcellen);
for i=1:aantalcellen
%     if anirow{1,i}==0  HOEFT niet meer door aanpassen skip function
%         sig_ani{1,i}=0;
%     else
   sig_ani{1,i} = sigma_ani_cellen_zp(x{i,2}, x{i,3}, T, G, sig_G,...
    roi{i,1},bgpar, bgper, auto_par, auto_per, sig_auto_par, sig_auto_per);
    % geeft de standaard deviatie van iedere cel (per pixel)
%    end
end

%groepen = cellen_groeperen(anirow, n);

%groep_verhouding = cellen_groeperen(verhouding, n);

for i=1:aantalcellen
    figure;
%     exptime = x{i,1};
%     imPAR = x{i,exptime*2};
%     imPER = x{i,exptime*2+1};
   subplot(2,3,1), imshowpair(x{i,2}, imwarp(x{i,3},T,'OutputView',...
   imref2d(size(x{i,2})))), title(strcat('cell',num2str(i), 'exp time: ',...
   num2str(x{i,1}),': overlap'));

subplot(2,3,2), imshow(roi{i,1}), title('roi');
subplot(2,3,4), imagesc(anirow{1,i}),afgroot, title('anisotropy');
subplot(2,3,5), imagesc(anirow_cor{1,i}), af, title('0 < anisotropy < 0.4');
subplot(2,3,6), imagesc(sig_ani{1,i}), colorbar, caxis([0,0.1]), ...
    axis equal tight, title('sigma in anisotropy, per pixel');
end


[matrix,gemgroep] = sigmacellengem(anirow, sig_ani, aantalcellen, n);
groepen_ani = cellen_groeperen(anirow_cor, n);
% gooit waardes van cellen met zelfde behandeling bi elkaar

sig_per_groep = zeros(1,length(n)-1);
for i=1:length(n)-1
sig_per_groep(1,i) = std(nonzeros(groepen_ani{1,i}));
%spreiding binnen een groep
end
groepmatrix = [gemgroep;sig_per_groep];
```

```
Error: File: D:\Margriet&Kyo\MATLAB\cellenallesin1.m Line: 17 Column: 23
A MATLAB string constant is not terminated properly.
```

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References

[1] Raimond Heukers Paul M.P. van Bergen en Henegouwen Cecilia de Heus, Nivard Kagie and Hanns.C. Gerritsen. Analysis of egf receptor oligomerizatin by homo-fret. *Methods in Cell Biology*, 117:305–321, 2013.

[2] Gabriele S. Kaminski Schierle Fiona T.S. Chan, Clemens F. Kaminski. Homofret fluorescence anisotropy imaging as a tool to studey molecular self-assembly in live cells. 2010.

[3] Amanda Gaudreau-Balderrama. Multi-modal image registration. 2012.

[4] Joseph.R.Lakowicz. *Principles of Fluorescence Spectroscopy*. 3*rd* edition, 2010.

[5] Nivard Kagie. Homo-fret detection by fluorescence ploarization anisotropy in wide-field microscopy. Master's thesis, Utrecht University.

[6] Diane S. Lidke Keith A. Lidke, Bernd Rieger and Thomas M.Jovin. The role of photon statistics in fluorescence anisotropy imaging. *IEEE Transactions on image processing*, 14:1237–1245, september 2005.

[7] Kiefer van Teutem. On using mcherry as a label for use in wide-field homo-fret induced fluorescence anisotroy microscopy. Master's thesis, Utrecht University.

[8] L.J. Van Vliet. Digital fluorescence imaging using cooled ccd array cameras. *Cell Biology*, 1998.

[9] Loren W.Runnels and Suzanne F. Scarlata. Theory and application of fluorescence homotransfer to melittin oligomerization. *Biophysical Journal*, 69:1569–1583, October 1995.