**SHORT THESIS DOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)**

# The effect of excitation light intensity and the degree of labeling of antibody on the quantitative fluorescent measurements

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# 1. Intorduction

Fluorescence techniques are widely used to determine the molecular parameters of various biochemical and biological processes due to their sensitivity, specificity, and temporal resolution. There are numerous methods for the fluorescent labeling of the observed biological molecules, but fluorescently labeled antibodies are still the most often used for this purpose. The parameters of fluorescence (fluorescence lifetime and fluorescence quantum efficiency) are influenced by the environment of the fluorophore. One of the manifestations of this phenomenon is Förster resonance energy transfer (FRET), which is unique in generating fluorescence signals that are sensitive to conformational changes, associations, and distances of molecules. Thus, it is able to resolve molecular interactions and conformations with a spatial resolution that exceeds the diffraction limit ($\sim\lambda / 2$) of standard optical microscopy and is compatible with super-resolution techniques. In my work, I sought to find the effect of two techniques, which are often used to increase the signal-to-noise ratio of fluorescence measurements (increasing the labeling ratio of antibodies and strong excitation intensity), on the quantitative reliability of measurements.

## 1.1. FRET

FRET is a distance-dependent process in which energy is transferred in a non-radiative way by a long-range dipole-dipole interaction from a fluorophore in the excited state (the donor) to a suitable acceptor molecule, which, in most cases, is also fluorescent. As a result of this process the donor molecule is de-excited and an electron of a suitable acceptor molecule is transferred to a higher energy level. Since FRET occurs only between molecules that are close to each other, within a range of 1 to 10 nm, it is also commonly referred to as a spectroscopic ruler. Conventional hetero-FRET can occur when the emission spectrum of a donor chromophore overlaps with the absorption spectrum of a chemically and spectroscipically different acceptor chromophore. This phenomenon has several photophysical manifestations, all of which result in an appropriate experimental technique to detect the FRET efficiency: (i) sensitzed emission of the acceptor; (ii) quenching of donor fluorescence emission; (iii) decreased fluorescence lifetime of the donor, a mechanism, which is similar to donor quenching; (iv) reduced rate of donor photobleaching and (v) changes of donor polarization.

It is often believed that FRET can only occur between two spectroscopically different fluorophores. However, FRET can also take place between spectroscopically identical molecules provided that they have a small Stokes-shift (small shift between the excitation and absorption peaks). The energy transfer between like fluorophores is called homo-FRET. The only applicable method to detect homo-FRET is the measurement of anisotropy. The energy transfer efficiency ($E$) is used to characterize the FRET efficiency, which shows the ratio of donors relaxing due to FRET to the total number of excited donor molecules. For FRET, the following conditions have to be fulfilled: 1.) the donor should have high quantum efficiency, 2.) the emission spectrum of the donor should overlap with the absorption spectrum of the acceptor, 3.) the electric field of the donor and the acceptor absorption dipole vector should be close to parallel. This orientation is characterised by the orientation factor. 4.) The distance between the donor and acceptor should be 1-10 nm. FRET can be examined with a number of measurement techniques, of which microscopic imaging and flow cytometry should be highlighted from a biological point of view. While the advantage of flow cytometric FRET (FCET) measurements is that we can examine a large cell population in a short time, the microscopic approach allows us to observe intracellular detail and correlate FRET values with other fluorescently labeled biological parameters pixelwise.

### 1.1.1. Methods of FRET measurement

Fluorescence has a number of spectroscopic characteristics that can be detected with good sensitivity and, fortunately these spectroscopic properties change under the influence of FRET. Therefore, several methods can be used to measure FRET. The most remarkable FRET measurements are based on the following three approaches: 1.) fluorescence intensity-based approach; 2.) fluorescence lifetime-based approach, 3.) fluorescence anisotropy-based approach.

### 1.1.2. Fluorescence intensity-based measurements

Fluorescence intensity-based FRET measurements include measurements of donor quenching, acceptor photobleaching, donor photobleaching, and sensitized acceptor emission. The determination of FRET based on the sensitized emission of the acceptor is the most reliable among the intensity-based methods. Among these approaches the three-channel FRET measurements are the most commonly used FRET technique, which is based on the measurement of three different combinations of the excitation wavelenghts and emission filters. In the case of these measurements the fluorescence intensities of samples singly labeld

with a donor or an acceptor are recorded in three channels: 1.) donor channel ($I_1$): the donor is excited and detected in the range of its own absorption and emission wavelengths; 2.) FRET channel ($I_2$): the sample is excited at the absorption wavelength of the donor and the fluorescence is detected at the emission wavelength of the acceptor, which characterizes the sensitized emission of the acceptor; 3.) the acceptor channel ($I_3$), where the absorption and emission wavelengths correspond to the spectral properties of the particular acceptor that characterize the direct emission of the acceptor. In this method it is necessary to determine different correction factors, known as overspill factors, from single-labeled samples. In the case of the donor only-labeled sample we determine how much the donor sample fluoresces in the FRET or acceptor channel ($S_1$, $S_3$), while in the case of an acceptor-labelled sample the overspill of acceptor fluorescence into the FRET or donor channel ($S_2$, $S_4$) are calculated. The correction factor that greatly influences the value of FRET is the $\alpha$ factor, the value of which shows the intensity of an excited acceptor molecule in the FRET channel compared to an excited donor detected in a donor channel. The detection efficiency of an excited acceptor in the FRET channel can be compared to the detection efficiency of an excited donor photon in the donor channel by labeling a sample with a donor-conjugated antibody specific for a particular epitope and another sample with an acceptor-labeled antibody specific for the same epitope. The molar absorption coefficient and the labeling ratio, i.e. the average number of fluorophores on an antibody in an antibody stock solution, must be taken into consideration for the calculation of $\alpha$. The FRET efficiency can be determined from the measured intensitities ($I_1$-$I_3$), the spectral overspill factors ($S_1$-$S_4$) and $\alpha$.

### 1.1.3. Fluorescence lifetime measurements

Fluorescence lifetime characterizes the time that fluorescent molecules spend on average in the excited state before they return to the ground state by various radiative and non-radiative mechanisms. Fluorescence lifetime is equal to the time during which the population of excited molecules decreases *e*-fold. Fluorescence lifetime is inversely proportional to the sum of all relaxation rate constants. If the appropiate acceptor is near the potential donor, the donor fluorescence life is reduced by FRET. Fluorescence lifetime microscopic FRET (FLIM-FRET) measurements, as opposed to the measurement of the sensitized emission of the acceptor, do not require correction. Furthermore, they are not sensitive to changes in fluorophore concentration and illuminating light intensity, light scattering, or photobleaching.

### 1.1.4. Fluorescence anisotropy measurements

Fluorophores are randomly orientated in space and time. If they are illuminated with polarized light, only those fluorophores became excited from the whole fluorophore population whose absorption dipole is sufficiently parallel to the polarization plane of the excitation photons. This process is photoselection. Furthermore, the orientation of excited fluorophores changes randomly before they lose energy with or without radiation, e.g. with FRET. In conclusion, the light emitted is depolarized relative to the excitation light. Fluorescence anisotropy shows how polarized the fluorescence emitted after polarized excitation. The fluorophore is illuminated with vertically polarized light and then both vertical and horizontal emissions are collected. The processes leading to the depolarization of the emission can be characterized on the basis of these two intensities, and the anisotropy can be determined using these intensities and a correction factor (*G* - different detection efficiency of vertically and horizontally polarized light). Anisotropy is sensitive to the size and shape of molecules, the rigidity and fluidity of the molecular environment, rotational motion, and molecular association events. FRET also influences the anisotropy of fluorophores. Due to hetero-FRET, the lifetime of the donor is reduced, thus giving the donor less time to rotate in the excited state. It can be concluded that donor emission is hyperpolarized (compared to the case where there is no hetero-FRET), which will result increased anisotropy. In the case of homo-FRET, acceptors with an absorption dipole parallel to the emission dipole of the donor are preferentially excited during the FRET interaction, yet each homo-FRET step depolarizes the excited molecular population. Since the donor and acceptor molecules are spectroscopically identical, the emission is less polarized, resulting in a decrease in anisotropy.

## 1.2. Methods of antibody labeling, the effect of labeling on antibodies

Antibodies are proteins of the adaptive immune system that perform effector functions and are able to specifically find target molecules and disease-causing organisms. They are widely used in diagnostics and research, and have become an important therapeutic tool in the clinic for the treatment of various diseases. Since antibodies are amino acid polymers that have a myriad of side chains, these side chains are dominant in conjugation processes. Proteins generally have three functional groups: amino (-$NH_2$), thiol (-SH), and carbohydrate groups for covalent modifications. The most popular reactive group is the amino group, which is present in large amount on the surface of the antibodies. The most widely used conjugation

method is the conjugation of amino groups to N-hydroxysuccinimide ester, resulting in a stable carboxamide bond. In addition to the conjugation method through amino groups, targeted labeling of the sulfhydryl group is also popular due to its specificity for maleimide and its high reactivity. Most thiol groups of cysteines are present in disulfide bonds, which, upon reduction, can readily bind maleimides to thiolated fragments of antibodies by thioester bonding. Beside these methods, carbohydrate moieties located on the Fc portion of antibodies are also used for conjugation processes, which can be covalently labeled with reagents containing hydrazide or hydroxylamine after reduction. Although the labeled functional group is located away from the antigen binding site, it still has a significant effect on the affinity of the antibodies, which can be attributed to the conformational and dynamic changes resulting from the labeling. From among the antibody labeling techniques discussed above, reaction with amino groups is the most preferred conjugation method due to its ease of operation, faster reaction kinetics, low cost, and high dye-antibody conjugate yield.

The average number of fluorescent dye molecules on the antibodies is given by the Degree Of Labeling (DOL) or F/p (fluorophore / protein). These values are often necessary for the quantitative evaluation of fluorescence measurements. Since the F/p value is usually determined for an antibody stock solution, this can be misleading because some antibodies in the antibody stock solution have more and others have fewer conjugated dyes. Thus, in the population of labeled antibodies formed during conjugation procedures, each antibody has a different number of dye molecules, the distribution of which can be characterized by a Poisson distribution.

## 1.3. Effect of antibody labeling on fluorophores

Although fluorescent labeling is very popular nowadays, it still has limitations. Fluorescence self-quenching has been described for biomolecules multiply labeled with the same fluorophore, as a result of which the brightness of the labeled molecule is not proportional to the degree of labeling. This is attributed to the clustering of fluorophores, which form on the biomolecule during labeling. The formation of aggregates is an important reason for quenching the fluorescence of most aromatic π-conjugated hydrocarbons. For certain fluorophores, there is little Stokes shift between the absorption and emission spectra, so when multiple dye molecules bind to a protein, energy transfer can occur between them if their distance is within the Förster distance. Homo-FRET can lead to fluorescence quenching when energy is transfered to non-fluorescent fluorophores.

In addition, a number of other factors play a role in the decrease in fluorescence intensity in the case of over-labeling, such as e.g. dimerization of dye molecules and collision quenching between dye monomers. The mechanism of quenching can be static or dynamic. The two types differ in whether a decrease in fluorescence intensity results in a shortening of fluorescence lifetime. In the case of dynamic or collisional quenching, the fluorophore in its excited state collides with the quencher. In this case another relaxation process appears, and this process shortens the fluorescence lifetime. In contrast, in the case of static quenching, the quencher already forms a complex with the ground state fluorophore, and the complex formed loses its fluorescent property. The rate of quenching measured in the total fluorophore population is influenced by the dissociation equilibrium of the fluorophore and the quencher. In this case the fluorophores not complexed with the quencher are "intact" and only they are responsible for the remainder of the fluorescence. Therefore, static quenching does not result in a decreased fluorescence lifetime.

# 2. Problems

During microscopic measurements, an increased excitation intensity is often used for a better signal-to-noise ratio in order to obtain brighter images. One of the advantages of bright images is that the labeled components can be well distinguished from the background. However, this approach does not always give a scientifically evaluable result. Imaging is based on the general assumption that the location of the fluorescent signal is the same as the appropriate, biologically relevant location of the labeled molecule and that its intensity is proportional to the concentration of labeled antibodies used in the experiment and to the intensity of light illuminating the sample. Unfortunately, due to the phenomenon of saturation, these assumptions are often incorrect.

The sources of saturation can be different. 1.) Biological saturation: this phenomenon is caused by protein overexpression. Due to the extreme protein expression, the protein synthesizing and sorting mechanisms of the transfected cell become saturated, therefore protein aggregates, modified cell constituents appear on the microscopic images, and the transfected proteins are not in the location corresponding to their physiological role. 2.) Detector saturation: in this case we increase the detector voltage or the intensity of the excitation light too much. Fluorescence intensities above the detection limit are indistinguishable. 3.) Fluorophore saturation: in this case most fluorophores are in the excited state. Due to the high intensity of excitation light, the fluorophores are re-excited immediately after relaxation. 4.) Overlabeling of antibodies: by increasing the labeling ratio, the antibodies used for labeling contain more fluorescent molecules. Indirect evidence suggests that the quantum efficiency of the dye and the affinity of the antibody decrease with increasing labeling ratio.

# 3. Aims

The two sources of saturation phenomena mentioned in the first two points of the “Problems” section can cause an easily recognizable, visible problem, which can be easily corrected. However, in the latter two cases, these problems remain hidden, which can lead to incorrect calculations and erroneous results during our measurements. Therefore, in my dissertation I will study the problem of antibody overlabeling and the negative effect of fluorophore saturation on hetero-FRET.

<u>In the case of labeling antibodies with fluorescent dyes, we would have liked to determine:</u>

a) the affinity of antibodies with different DOLs,

b) the labeling ratio of the cell bound fraction of antibodies,

c) the effect of DOL on the fluorescence lifetime of the dyes,

d) distribution of fluorescence intensity of individual antibody molecules in the stock solution and in the bound antibody fraction.

<u>In fluorophore saturation studies, our objectives were:</u>

a) to examine how the intensity of the illuminating light affects the FRET values calculated by the conventional formula.

b) If it affects, we would have liked to derive a formula that takes fluorophore saturation and FRET frustration into consideration.

# 4. Theory

## 4.1. Fluorophore conjugation and antibody affinity studies

### 4.1.1. Distribution of antibodies with different labeling ratios in the cell-bound fraction

The distribution of antibody species with different labeling ratios in an antibody stock solution with a given DOL is assumed to follow Poisson distribution:

$$f_k = c_{tot} \frac{DOL^k}{k!} e^{-DOL} \tag{1}$$

where $f_k$ is the concentration of antibody species with a a labeling ratio of $k$, $DOL$ and $c_{tot}$ are the average labeling ratio and concentration of an antibody stock solution, respectively. The concentration of each bound antibody species with a labeling ratio of $k$ can be determined by writing the following binding equations, provided that no ligand depletion is present, i.e. $f_k = f_{k,tot}$.

$$\begin{aligned}
&K_{d0}\ fb_0 = f_0\ s_{unbound}\\
&K_{d1}\ fb_1 = f_1\ s_{unbound}\\
&K_{d2}\ fb_2 = f_2\ s_{unbound}\\
&\ldots\\
&K_{dn}\ fb_n = f_n\ s_{unbound}\\
&fb_0 + fb_1 + fb_2 + \ldots + fb_n + s_{unbound} = s_{tot}
\end{aligned} \tag{2}$$

where $K_{d0}$, $K_{d1}$, $K_{d2}$, … are the dissociation constants of the unlabeled and labeled antibody species with a labeling ratio of 1, 2,…, respectively, $s_{tot}$ and $s_{unbound}$ are the total concentrations of the binding site (epitope) and the concentration of epitope not bound to the antibody, respectively. The analytical solution of the equation system gave the concentrations of the bound antibody species with different labeling ratios ($fb_n$).

The total fluorescence intensity of the antibody stock solution is given by the total fluorescence intensity of the different species:

$$F_{free} = \sum_{k=1}^{highest\ DOL} f_k\ k\ Q_k \tag{3}$$

where $Q_k$ is the quantum efficiency of the fluorophores for an antibody labeled with $k$ number of fluorophores.

Using these principles the total fluorescence intensity of the bound antibody fraction can be determined as follows.

$$F_{bound} = \sum_{k=1}^{highest\ DOL} fb_k\ k\ Q_k \tag{4}$$

### 4.1.2. Dependence of the anisotropy of antibody solutions on the labeling ratio of individual antibody species for the free and bound fractions

The anisotropy of a mixture of antibodies with different labeling ratios can be determined by averaging the intensity-weighted anisotropy of the different species within it. The anisotropy of the antibody stock solution can be given by the following equation:

$$r_{free} = \frac{\sum_{k=1}^{highest\ DOL} f_k\, k\, Q_k\, r_k}{\sum_{k=1}^{highest\ DOL} f_k\, k\, Q_k} \quad (5)$$

where $Q_k$ is the fluorescence quantum yield of dyes present in an antibody with a DOL of k.

The anisotropy of the bound antibody fraction can be determined by the following equation.

$$r_{bound} = \frac{\sum_{k=1}^{highest\ DOL} fb_k\, k\, Q_k\, r_k}{\sum_{k=1}^{highest\ DOL} fb_k\, k\, Q_k} \quad (6)$$

## 4.2. Examination of fluorophore saturation

### 4.2.1. Effect of donor saturation on apparent FRET efficiency

Let us investigate the phenomenon of fluorophore saturation in the absence of FRET. For a moment, it is assumed that a fluorophore has only ground or excited singlet state. In equilibrium the number of ground state donors getting excited is equal to the number of excited donors relaxing. This condition is summarized by the following matrix equation:

$$\begin{pmatrix} 0 \\ 0 \\ D_{all} \end{pmatrix} = \begin{pmatrix} -\Phi_D\, \sigma_D & \frac{1}{\tau_D} \\ \Phi_D\, \sigma_D & -\frac{1}{\tau_D} \\ 1 & 1 \end{pmatrix} \begin{pmatrix} D \\ D^* \end{pmatrix} \quad (7)$$

where $\Phi_D$ is the photon-flux of the donor exciting laser, $\sigma_D$ and $\tau_D$ are the absorption cross-section and fluorescence lifetime of the donor, respectively. $D$ and $D^*$ are the concentration of

donors in the ground and excited states, respectively, and $D_{all}$ is the total concentration of donors. The solution of the matrix equation for $D$ and $D^*$ are shown below.

$$D = \frac{D_{all}}{1+\sigma_D\, \tau_D\, \Phi_D}, D^* = \frac{D_{all}\, \sigma_D\, \tau_D\, \Phi_D}{1+\sigma_D\, \tau_D\, \Phi_D} \tag{8}$$

Fluorophore sturation is a phenomenon, where the fluorescence intensity of excited fluorophores is not directly proportional to the intensity of illumination light. In this case, most fluorophores are in the excited state.

The extent of fluorophore saturation ($D_{sat}$) can be determined by the following formula:

$$D_{sat} = \frac{\sigma_D\, \tau_D\, \Phi_D}{1+\sigma_D\, \tau_D\, \Phi_D} \Rightarrow D^* = D_{all}\, D_{sat} \tag{9}$$

Since the fluorescence intensity of the donor is proportional to the intensity of the donors in the excited state, the apparent FRET efficiency calculated from donor quenching can be determined as follows:

$$E_{apparent} = 1 - \frac{D_A^*}{D_{no\,A}^*} = \frac{E}{1+(1-E)\sigma_D\, \tau_D\, \Phi_D} = \frac{(1-D_{sat})E}{1-D_{sat}E} \tag{10}$$

where $E$ is the theoretical FRET efficiency measured at low excitation intensity. The above equation provides an obvious opportunity to correct the calculated, apparent FRET efficiency for donor saturation:

$$E = \frac{E_{apparent}}{1+D_{sat}\left(E_{apparent} - 1\right)} \tag{11}$$

### 4.2.2. Determination of FRET efficiency considering fluorophore saturation during intensity-based measurements

We created a model system to investigate the effect of fluorophore saturation taking both the donor and the acceptor into consideration. The resulting equations also do not correct for FRET frustration. The equations are too long to be presented in the thesis.

### 4.2.3. Determination of FRET efficiency considering fluorophore saturation and FRET frustration

FRET frustration is a phenomenon when an excited acceptor cannot serve as an acceptor for an excited donor. FRET frustration was taken into account in a system that includes a complex of a donor and an acceptor. In order to use this model for a donor-acceptor system in which their ratio is different from 1:1, free acceptors were assumed in the system. The numerical solution of the equations describing the system was calculated in Matlab.

# 5. Materials and methods

## 5.1. Cell lines and antibodies

Our experiments were performed on SKBR-3, A431 and JY cell lines. SKBR-3 is a trastuzumab-sensitive human breast tumor cell line overexpressing ErbB2, A431 is a human epithelial carcinoma cell line overexpressing ErbB1, and JY is an Epstein-Barr virus transformed B-lymphoma cell line. The cells were grown according to their specifications. For microscopic measurements, cells were grown in an 8-well chamber to 80% confluence. For measurements in suspension, cells were removed from the bottom of the flask using trypsin-EDTA-PBS solution. ErbB2 proteins were labeled with trastuzumab and pertuzumab, which are humanized monoclonal antibodies specific for two different, non-overlapping epitopes of ErbB2. ErbB1 receptors were labeled with Mab528 antibody obtained from the supernatant of HB-8509 mouse hybridoma cells. W6/32, a monoclonal antibody specific for the MHC-I heavy chain, L368 binding to β-2-microglobulin, and L243, which recognize MHC-II, were also made by using mouse hybridoma cells. N-hydroxysuccinimide esters of AlexaFluor488, AlexaFluor546 and AlexaFluor647 dyes were conjugated to the amino group of purified monoclonal antibodies according to the manufacturer's specifications. The dye/protein labeling ratio (DOL, number of dye molecules bound to an antibody) was determined by spectrophotometry, and it ranged from 0.5 to 6.

## 5.2. Fluorescence labeling of cells on coverslips and in suspension

In order to perform fluorophore saturation measurements, SKBR-3 cells were grown to 80% confluence in an 8-well chamber slide. After washing twice with PBS, cells were labeled with fluorescent antibodies on ice at a concentration of 20 μg/ml in 150 μl of PBS containing 1% (w/v) BSA, protected from light for 30 minutes. For FRET measurements, samples were labeled with a mixture of antibody solutions containing antibodies labeled with donor and acceptor dye. Samples singly labeled with the donor or the acceptor were required to calculate $\alpha$ and the overspills factors. Samples were washed twice with PBS and then fixed with 1% formaldehyde. The process was performed on ice.

For fluorophore conjugation measurements, A431 and SKBR-3 cells were used to label ErbB1 and ErbB2 by Mab528 and trastuzumab, respectively. MHC-I, MHC-II, and β-2-microglobulin were labeled by W6/32, L243, and L368 monoclonal antibodies, respectively, on JY cells. In order to determine the equilibrium binding of fluorophore-conjugated

antibodies, freshly harvested and trypsin-treated cells were washed twice with cold PBS, and $10^5$ cells were labeled on ice for 30 minutes in the dark with concentration series of AlexaFluor546 or AlexaFluor647-labeled antibodies in 100µl of 1mg/ml BSA-PBS. Unbound antibodies were removed by washing twice with PBS and the cells were fixed with 1% formaldehyde. In order to determine the binding affinity of unlabeled antibodies, cells were washed twice with PBS, then $10^5$ cells were labeled with a series of concentrations of unlabeled antibody in the presence of a constant concentration of labeled antibody specific for the same epitope in 100 µl of 1 mg/ml BSA-PBS on ice for 30 minutes and protected from light. Then the cells were washed twice and fixed with 1% formaldehyde. Fluorescence intensities were measured with a flow cytometer. A binding curve for labeled antibodies and a competitive binding curve for unlabeled antibodies were fitted to the measured data to determine the $K_d$ of labeled and unlabeled antibodies.

## 5.3. Transient trasfection and plasmids

In order to measure FRET between two fluorescent proteins, SKBR-3 cells were transiently transfected with a fusion plasmid coding for EGFP (donor) and mCherry (acceptor) linked by a sequence of five amino acids (RDPPV). Cells transfected with pEGFP-C3 and pmCherry-C3 were used to determine the overspill factors required for FRET calculations. SKBR-3 cells were grown in an eight-well chamber and 0.5 µg of plasmid was added per well. Transfected cells were prepared with a 2:1 (v/w) lipid/DNA ratio, using Lipofectamine 2000 according to the manufacturer's specifications.

## 5.4. Confocal microscopy

A Zeiss LSM 880 confocal laser scanning microscope was used to image the fluorescently labelled samples. During FRET measurements between AlexaFluor488-trastuzumab and AlexaFluor546-pertuzumab, the donor molecule was excited with an argon ion laser line at 488 nm in the donor and FRET channels, and then emission was detected between 500-530 nm and 550-610 nm in the donor and transfer channels, respectively. The acceptor was excited with a 543 nm HeNe laser, its emission was detected in the range of 550-610 nm.

FRET measurements were also performed between AlexaFluor546-trastuzumab and AlexaFluor647-pertuzumab, where the AlexaFluor546 dye molecule was excited with a 543 nm laser beam, its emission was detected between 550 and 610 nm in the donor channel and

the sensitized acceptor emission was recorded at 635–755 nm. AlexaFluor647-pertuzumab was excited at 633 nm and the fluorescence emitted by it was detected between 635 nm and 755 nm. In order to determine FRET efficiency on transfected cells, the donor (EGFP) was excited with a 488 nm laser and its emission in the donor channel was measured between 495-575 nm, and FRET-sensitized acceptor emission was detected between 580-670 nm. The acceptor (mCherry) was excited by a 543 nm laser line, the emission of which was detected in the wavelength range of 575-695 nm. Fluorescence images were taken with a 63X (NA = 1.4) oil immersion objective. Images from a single field of view were recorded with increasing laser intensity (1-5-10-15%). Subsequently, images were taken from another area with decreasing (15-10-5-1%) excitation intensity.

## 5.5. Measurement of Saturation of Mobile Fluorophores

In order to measure the saturation of mobile fluorophores, antibody stock solutions were diluted to a concentration of 200 nM in PBS. Because fluorophores are mobile in solution, photobleaching is negligible. A relatively larger volume (200 μl) of this diluted antibody solution was added to the 8-well chambered coverslip to avoid undesirable reflection. During the measurement, the fluorescence intensity was measured as close to the bottom of the chamber as possible with the excitation and emission parameters mentioned in the previous section. The excitation laser intensity was first gradually increased (1-5-10-15%) and then gradually decreased (15-10-5-1%). The two measurements were performed under the same conditions with the same measurement error. The measured fluorescence intensity was normalized to the fluorescence intensities at the lowest excitation intensity.

## 5.6. Measurements of laser intensity

Laser power measurements were carried out with a Thorlabs optical power meter equipped with a sensor with a spectral sensitivity range of 350-1100 nm. On the microscope, the different laser intensities (488, 543, and 633 nm) were varied on a percentage scale, and the adjusted laser power was measured with an optical power meter. The measured values were converted to photon flux taking into account the size of the focal point and the energy of each photon.

## 5.7. Image analysis

Fluorescent image analysis was carried out in Matlab supplemented with the DipImage toolbox. Membrane pixels were identified with a custom-written implementation of the

manually-seeded watershed segmentation algorithm. In order to determine FRET and all of correction factors the rFRET program was used in Matlab.

## 5.8. Flow cytometry

Flow cytometric measurements were performed on a FACS Aria III flow cytometer. AlexaFluor546 dye was excited with a laser beam at 561 nm and its emission was detected through a 595 ± 25nm bandpass filter, while AlexaFluor647 was excited at 633 nm and the light emitted by it was recorded using a 635 nm high-pass filter. The mean fluorescence intensity of cells was analyzed with a software tool, ReFlex. The average fluorescence intensity of cells was analyzed after gating the population of living cells on dot-plots containing forward and side scattering data. Before further analysis the average fluorescence intensities were background corrected.

## 5.9. Fluorimetry, measurement of fluorescence anisotropy

A Fluorolog-3 spectrofluorimeter was used for fluorescence intensity and anisotropy measurements. AlexaFluor546 was excited at 550 nm and fluorescence intensity was detected at 590 nm, while AlexaFluor647 was excited at 650 nm and its emitted light was recorded at 675 nm. 20 nM antibody stock solutions were prepared and the intensity and anisotropy of the stock solutions were measured. Equations (3). and (4) were fitted to the dependence of the fluorescence intensity of the antibody solutions and the cell-bound antibody fractions on the labeling ratio.

## 5.10. Isolation of cell-bound antibodies by immunoprecipitation

Five million cells were labeled in suspension with AlexaFluor546- and AlexaFluor647-labeled IgG, and the cells were washed twice with PBS. The cells were then lysed with lysis buffer on ice for 10 minutes, followed by centrifugation at 600 g for 5 minutes, and the resulting supernatant was immunoprecipitated with protein G at 4 °C for one hour. Samples were washed 3 times with lysis buffer, and then antibodies bound to protein G were removed with 100 mM glycine-HCl. The eluted antibodies were obtained from the supernatant after centrifugation followed by phosphate buffer neutralization. As a control, a diluted, 100 nM antibody stock solution was immunoprecipitated and subjected to all of the above steps. Fluorescence anisotropy of the immunoprecipitated samples was measured in parallel with non-immunoprecipitated samples on a fluorimeter.

## 5.11. Fluorescence lifetime measurements

For fluorescence lifetime measurements, an inverted IX81 fluorescence microscope equipped with a Lambert Instruments LIFA fluorescence lifetime imaging module and a modulated LED light source in the wavelength range of 405–640 nm was used. AlexaFluor546 was excited by using a 527 nm LED light and a 510-552nm bandpass filter, and then a 570 nm dichroic mirror and a 590 nm high-pass filter were used to separate the emitted light. AlexaFluor647 dye was excited with a 639 nm LED through a ZET642/20 bandpass filter, and its emission was recorded using a ZT647rdc-UF2 dichroic mirror, an ET665 high-pass filter, and an ET700/75m bandpass filter. The fluorescence lifetimes of antibody stock solutions with different labeling ratios and a concentration of 20 μg/ml (~133 nM) were determined in the frequency domain. Unconjugated dye solutions were used for calibration, and the lifetimes of AlexaFluor546 and Alexaluor647 were adjusted to 4.1 and 1 ns, respectively, known from the literature.

## 5.12. Single molecule fluorescence measurements

In order to measure single molecule fluorescence of cell-bound antibodies SKBR-3 cells, grown in eight-well chambers, were labeled by 10 μ??g/ml AlexaFluor546- or AlexaFluor647-tagged trastuzumab in the presence of a 500-fold or 1000-fold molar excess of unlabeled trastuzumab on ice for 30 min. Unbound antibodies were removed by washing twice with PBS and then fixed with 1% formaldehyde. In order to analyze antibody stock solutions at the single-molecule level the same kind of antibodies were immobilized on the surface of epoxy-functionalized coverslips at a concentration of 0.1 and 0.05 mg/ml. The images were taken as close as to the coverslip bottom as it possible by a Zeiss LSM880 confocal microscope in photon counting mode. AlexaFluor546 and AlexaFluor647 were excited with laser beams at 546 nm and 633 nm, respectively, and their emissions were detected at 575-680 and 638-755 nm, respectively. Matlab was used for image analysis.

# 6. Results

## 6.1. Effect of antibody labeling on antibody affinity and the photophysical properties of dyes

### 6.1.1. Effect of labeling on the affinity of antibodies

The effect of fluorescence labeling on the affinity of antibodies was examined for two different fluorescent dyes (AlexaFluor546 and AlexaFluor647) and five different IgGs (trastuzumab, Mab528, W6/32, L368 and L243). Antibody stock solutions with relatively high DOL were selected to label the cells and their saturation binding was determined by flow cytometry. Competitive binding experiments were also performed to shed light on the binding affinity of unlabeled antibodies. One of the examined antibodies, the Mab528 antibody, showed no particular change when conjugated with AlexaFluor dye, while in case of the other 4 antibodies dissociation constant was significantly increased with labeling. Our data confirm previous evidence showing that conjugation of fluorescent dyes decreases antibody affinity in most cases. In addition, it is even revealed that conjugation with AlexaFluor647 affects antibody affinity more significantly than labeling with AlexaFluor546.

### 6.1.2. The fluorescence intensity of free and bound antibody differs depending on the labeling ratio

An antibody stock solution characterized by a certain average labeling ratio contains antibody species with different numbers of fluorophores, whose distribution in the solution approximately follows a Poisson distribution.

Regarding that fluorescence labeling negatively affects the affinity of antibodies, it is expected that the dissociation constant of individual antibody species will increase as a function of DOL in the same stock solution. Thus, antibodies with low affinity will be underrepresented in the cell-bound fraction. This phenomenon could be measured by comparing the fluorescence intensity of the antibody stock and that of the bound fraction. When plotting the fluorescence intensities of unbound antibodies according to DOL, the results showed that these increases in intensity fell short of the linear increase that would be expected in the absence of self-quenching. The antibodies differed significantly in this respect. In addition, the fluorescence intensities of the bound fraction were determined using labeled cells by flow cytometry. Cells were labeled with antibody stock solutions whose

fluorescence intensity was determined previously. Not only did the intensity increase of cell-bound antibodies as a function of the DOL lagged behind the linear increase, it also differed from the curves of the stock solutions in most cases. A systematic difference was also observed for the two fluorophores tested. While the lowest DOL-normalized fluorescence intensity of cell-bound AlexaFluor546-labeled antibodies was higher than that of antibody stock solutions labeled with the same dye, the opposite relationship was obtained for AlexaFluor647-conjugated antibodies. Equations (3) and (4) were fitted to the measured data. The fit reproduced the main trend of the measured values and also showed that antibody labeling greatly influenced the quantum efficiency of AlexaFluor546, while AlexaFluor647 primarily affects the affinity of the antibody. We could conclude that the fluorescence of bound and free antibodies depends differently on the labeling ratio. This result supports our hypothesis that antibody species with different DOLs in the same stock solution have different affinities. Furthermore, it has became obvious that the mean DOL of the bound and unbound fractions must be different.

### 6.1.3. Simulation of fluorescence intensities of free and bound antibodies

The interpretation of our results presented in the previous section suggests that fluorescence labeling induces changes in the quantum efficiency and the affinity of antibodies. Our goal was to evaluate quantitatively the different behaviors of the two AlexaFluor dyes. An antibody stock solution was assumed to contain antibody species with different DOLs competing for the same epitope and different affinities and quantum efficiencies were assigned to these fluorescently labeled species. The predicted fluorescence intensities of the stock solutions and the bound fractions were determined according to equations (3) and (4) in four different cases. If the fluorescence labeling does not affect the affinity of the antibody, the normalized fluorescence intensities of the stock solution and the bound fraction would completely overlap, despite the decrease in quantum efficiency as a function of increasing DOL. If the quantum efficiency is constant but the affinity of the antibodies decreases with high DOL, the fluorescence of the stock solution would increase linearly, while the normalized fluorescence intensity of bound antibodies would increase less steeply. Furthermore, two complex models were analyzed in which labeling affects both the affinity of the antibody and the quantum efficiency of the dye. In Model 1, the deterioration in antibody affinity is more pronounced, while in Model 2, quantum efficiency is more sensitive to labeling. Models 1 and 2 reproduce the different behavior of the two AlexaFluor dyes. In Model 2 the normalized fluorescence intensity of bound antibodies was higher, while in

Model 1 the intensity of antibody stock solutions was higher. If fluorescence labeling affects quantum efficiency more than antibody affinity, the normalized intensity of the bound fraction is expected to be higher, which was the case for AlexaFluor546. On the other hand, if the antibody affinity is more sensitive to fluorescent labeling than the quantum efficiency, the normalized intensity of the stock solutions will be higher, corresponding to AlexaFluor647. Although the DOL of the stock soluion was increased up to 5, the labeling ratio of bound antibodies remained around ~3-4. This suggests that the predicted mean fluorescence intensity of the bound fraction is lower than that of the stock solutions for both models.

### 6.1.4. Determination of the labeling ratio of the bound antibody fraction by fluorescence anisotropy measurements

Although our experimental results and modeling presented in the previous chapters pointed out the difference in labeling ratios between bound and unbound antibody fractions, we tried to find an independent method to confirm these results. Therefore, fluorescence anisotropy measurements were performed. Cells were labeled with antibody stock solutions with different DOL, and after lysis and immunoprecipitation, the cell-bound antibody fraction was recovered. The anisotropy of both the isolated antibodies and the stock solutions was then measured. In order to examine whether immunoprecipitation has any effect on the antibodies or our measurements, the used antibody solutions were immunoprecipitated in the same way as the cell samples. The fluorescence anisotropy of the stock solution decreases as a function of the DOL. The anisotropy of untreated and immunoprecipitated antibody stock solutions does not differ, suggesting that immunoprecipitation has no significant effect on anisotropy. On the other hand, the anisotropy of cell-bound antibodies was typically higher than that of stock solutions for both AlexaFluor dyes. This suggests that the DOL of cell-bound antibodies is lower.

In some cases, the anisotropy of cell-bound antibodies was barely reduced, despite the fact that the cells were labeled with antibody solutions with increasingly higher DOL. This observation suggests that individual antibody molecules with a low labeling ratio almost exclusively bound to the cells. Although conjugation with AlexaFluor546 and AlexaFluor647 appears to affect antibody affinity differently, anisotropy measurements show the same trend in the case of both dyes. In our further investigations, the model presented in the previous chapter was used and extended to take into account the homo-FRET-dependent anisotropy between fluorophores for the stock solutions and the bound fraction. The results of the

calculations predict that the bound antibody fraction can be characterized by a higher fluorescence anisotropy regardless of how dye conjugation affects antibody affinity. Our experimental results and model calculations suggested that the mean labeling ratio of cell-bound antibody was typically lower than that of the stock solutions.

### 6.1.5. The effect of antibody labeling on the fluorescence lifetime and spectra of fluorophores

The results presented in the previous sections suggest that a multi-labeled fluorescent antibody has reduced brightness. We were interested in whether static or dynamic quenching underlies the phenomenon, so fluorescence lifetime measurements were performed in the frequency domain with a dilute solution of antibody stock solutions. The apparent phase and modulation lifetimes of the antibodies labeled with AlexaFluor546 and Alexafluor647 were determined, and then the results were normalized to the lifetime measured for the antibody with the lowest DOL. The fluorescence intensity of the same antibody stock solutions was determined separetly by fluorimetry, and it was normalized with the lowest DOL. Both the normalized lifetimes and fluorescence intensities were plotted against the DOL. Although normalized fluorescence lifetime decreased with increasing DOL, the magnitude of this change was still much smaller than the change in normalized fluorescence intensity, which implies that the decreased quantum yield was attributed to static quenching. In order to reveal whether non-fluorescent dimers, also known as H-aggregates, contribute to the quenching process, the absorption and excitation spectra of fluorophore-conjugated antibodies were recorded. The appearance of a blue-shifted peak and DOL-dependent increase in the absorption spectra compared to the main absorption band, and the absence of this band in the excitation spectra, means that non-fluorescent dye aggregates are present. The absorption spectrum of free, non-conjugated dyes is the spectrum of monomeric species. The excitation spectra of unconjugated, free AlexaFluor647 differs from that of the antibody-bound dye, while these spectra overlap each other in the case of AlexaFluor546. Cyanine dyes are known to form cis and trans isomers that are slightly spectroscopically different, which may have caused a difference in conjugation with AlexaFluor647 dye compared to free dyes. In summary, lifetime measurements and spectral data are consistent: dye aggregates contribute significantly to fluorescence quenching.

### 6.1.6. Distribution of the fluorescence intensity of single antibody molecules in the stock solution and in the cell-bound fraction

All our previous experimental and theoretical results suggest that the labeling ratio of cell-bound antibodies is lower than that of the antibody solutions used for labeling, since the multiply-labeled antibodies are underrepresented in the bound fraction due to their low affinity. In order to further verify that the above conclusions are correct, single-molecule measurements were performed with AlexaFluor546- and AlexaFluor647-labeled antibody stock solutions and their cell-bound fractions. Sufficiently diluted antibody solutions were immobilized on an epoxy-treated cover slip so that a detected fluorescent spot is indeed derived from a single antibody. For the same reason cells were also labeled with sufficiently diluted fluorescent antibodies. In the case of both samples images were recorded close to the bottom of the coverslip. Samples labeled with AlexaFluor488-conjugated antibodies were used for background determination since the fluorescence of AlexaFluor488 helped us find the appropiate plane. Samples labeled with AlexaFluor488 can be used as a negative control because the spillover of AlexaFluor488 into the AlexaFluor546 and AlexaFluor647 channels is negligible. Furthermore, these measurements proved that the signal of AlexaFluor546- and AlexaFluor647-conjugated antibodies are stronger than the autofluorescence. The intensity distributions of the fluorescent spots were also examined for the stock solutions and the cell-bound fraction using AlexaFluor647-trastuzumab antibody with a high labeling ratio (DOL = 3.8). Antibody solutions with high DOL are expected to contain a number of antibodies conjugated to 3-4 fluorophores, and the distribution of antibody species therein follows a Poisson distribution. Surprisingly, when examining the stock solution, low-intensity spots were the most abundant, which is in contrast to the Poisson distribution. This phenomenon has been attributed to the inverse relationship between high labeling ratio and quantum efficiency. The average intensity of the antibodies was calculated from the photon number of each spot, which was only 25% higher for the antibody with a DOL of 3.8 than the intensity of the antibody with a DOL of 0.9. This observation was in accordance with our previous results. When comparing the intensity distribution of stocks and cell-bound antibodies molecular species with a higher single molecule DOL were clearly underrepresented in the bound fraction for the AlexaFluor647-trastuzumab with a high degree of labeling (DOL = 3.8). Examination of a solution of AlexaFluor647-trastuzumab with a lower labeling ratio (DOL = 0.9) revealed no significant difference in the intensity distribution of bound antibodies and stock solution. When the intensity distribution of the bound fraction of

AlexaFluor647-trastuzumab was compared for DOL 3.8 and DOL 0.9, there were no significant differences between the curves. We also examined AlexaFluor546-labeled trastuzumab (DOL = 1.9), where we also found that individual molecules with high DOL were less present in the cell-bound fraction, although this effect was less pronounced than in the case of AlexaFluor647-trastuzumab with a high DOL. All of these findings are in agreement with the previous conclusions. It can be concluded that single-molecule measurements confirm the results of flow cytometric and fluorimetric experiments, as well as anisotropy measurements, according to which antibodies with a lower labeling ratio within an antibody stock solution bind to their antigens in most cases.

## 6.2. Reducing the detrimental effects of saturation phenomena in FRET microscopy

In the case of high excitation photon flux, the FRET-induced quenching of donor fluorescence is reduced according to theoretical considerations, because the donor relaxed by energy transfer enters the excited state almost immediately in this case. A quantitative reflection of the phenomenon was described in the "Theory" chapter of the dissertation, which predicts that the FRET efficiency calculated from donor quenching decreases as a function of saturated donors. The rearrangement of the formula describing this phenomenon provides an opportunity to correct the FRET efficiency calculation for donor saturation. Since the excitation photon flux commonly used in confocal microscopy is often in the range where the phenomenon of saturation is present, we considered that this problem is worth further investigation. Fluorophore saturation is significantly affected by intersystem crossing, as up to 50-80% of the dye can accumulate in the triplet state, which may also affect the apparent FRET efficiency. As described in the theoretical part of the dissertation, the apparent FRET efficiency calculated from donor quenching decreases according to the fraction of saturated donors, even when the fluoreophores accumulate in the triplet state. We can conclude that: (i) it is not the fraction of donors in the excited state, but the normalized fractional saturation of the S state, which determines the apparent decline of the FRET efficiency evaluated from donor quenching; and (ii) if the triplet state is populated, the apparent decrease of the FRET efficiency is even higher. The observations mentioned above allow us to correct the apparent FRET efficiency for donor saturation. The equations discussed so far have not taken into consideration the phenomenon of FRET frustration, which occurs when the acceptor is in the excited state and therefore the donor is unable to relax by FRET. We also derived another

system of equations that takes both fluorophore saturation and FRET frustration into account. For the AlexaFluor488-AlexaFluor546 donor-acceptor pair, the principles mentioned above were used to determine the FRET values. As expected, both FRET efficiency and $\alpha$ decrease sharply with increasing donor excitation laser intensity. After correction of the $\alpha$ factor for fluorophore saturation, it became independent of photon flux. The FRET efficiency was corrected in three different ways: (i) the conventional equation was corrected according to equation 11; (ii) considering fluorophore saturation but disregarding frustrated FRET; (iii) considering fluorophore saturation and frustrated FRET as well. The first two approaches provided identical results and reduced the photon flux-dependence of the FRET efficiency, while the third one almost completely eliminated it.

By comparing the experiments in which we changed either the intensity of the donor-exciting laser, the intensity of the acceptor-exciting laser, or both, we concluded that the photon flux exciting the donor matters the most.

We also tested our methods for the donor-acceptor (GFP + mCherry) pair formed by fluorescent proteins. The system of equations was slightly modified so that the $\alpha$ and FRET values could be determined from the same measurement. These measurements also confirmed that the application of the equation taking fluorophore saturation into account successfully eliminates the dependence of the FRET efficiency on excitation photon flux.

The validity and applicability of the above-mentioned methods is confirmed by the fact that the calculated FRET values were successfully made independent of the illuminating photon flux. There are several deexcitation pathways for a fluorophore, of which photobleaching and singlet-singlet annihilation can affect FRET calculations. We neglected both of them for the following reasons. The occurrence of singlet-singlet annihilation is unlikely in the experimental system we presented since: (i) the model disregarind singlet-singlet annihilation was shown to eliminate the excitation power-dependence of the apparent FRET efficiency. This phenomenon already implies that singlet−singlet annihilation does not take place in our systems. (ii) In a complex of one donor and one acceptor singlet-singlet annihilation occurs when both fluorophores are in the excited state (D*A*). Based on our calculations, it can be concluded that D*A* complexes are present in significant amounts only at high FRET values. Since FRET values for cellular FRET measurements are rarely higher than 0.3–0.4, ignoring singlet-singlet annihilation is a reasonable assumption in such experiments.

Photobleaching is also a phenomenon that can influence FRET measurements by decreasing the amount and density of donors and acceptors and by changing the ratio of

donors/acceptors. Although in the three experimental systems we examined, the intensity of the dyes decreased by ~30% during the experiment due to photobleaching, this phenomenon had no significant effect on the calculated FRET efficiency, as the FRET values were not affected by the duration of previous illumination with excitation light. If FRET values calculated in the conventional way or by any of the methods proposed in the current manuscript turn out to be sensitive to bleaching, they should be corrected for bleaching after careful consideration of the applicability of any correction formula.

# 7. Discussion

## 7.1. Effect of fluorescence labeling on the properties of antibodies and fluorophores

Previous results have reported that conjugation of antibodies to fluorophores negatively affects their affinity and the quantum efficiency of antibody-bound fluorophores. The main findings of our experimental results on the affinity and quantum efficiency of antibodies are as follows: (i) the fluorescence quantum yield of antibody-conjugated fluorophores decreases as a result of both dynamic and static quenching, with the latter being the dominant factor, according to fluorescence lifetime measurements; (ii) the affinity of fluorescent antibodies decreases as a function of the labeling ratio leading to a lower mean DOL of the bound fraction than that of the stock; (iii) according to our model calculations and experimental results labeling with different dyes has different effects on the affinity and quantum efficiency of the antibodies, as demonstrated by our studies with AlexaFluor647 and AlexaFluor546.

The quenching of multiply-labeled antibodies is mainly dominated by static processes, but dynamic effects also contribute. The formation of dye aggregates and FRET processes in which energy is transferred to non-fluorescent clusters or dimers play an important role in self-quenching. Comparison of the absorption and excitation spectra revealed that these non-fluorescent aggregates were present in both AlexaFluor dyes.

Our three different experiments demonstrate that multiply-labeled antibodies are underrepresented in the cell-bound antibody fraction: (i) we compared the fluorescence intensities of the antibody stock solution and the cell-bound antibodies as a function of the labeling ratio. (ii) We compared the anisotropy of antibody stock solutions and their bound fractions. (iii) The intensity distributions of the individual antibody molecules in the bound fraction and the antibody stock solution were measured directly. In order to predict the anisotropy of the bound fraction, model calculations were performed that found that the curve fitted to the anisotropy of the antibody stock solutions as a function of DOL could be used as a calibration curve to determine the labeling ratio of the bound fraction. The decreased affinity of the antibody may be explained by the altered or inhibited intrinsic flexibility of the molecule due to conjugation. Although antibodies and fluorophores show significant differences in their sensitivity to fluorescent labeling, the average labeling ratio of the bound fraction is almost always lower than the DOL of the antibody stock solutions used to label the cells. The fluorescence intensity distributions of single molecules in the stock solutions were

not always in accordance with our expectations, e.g., for the AlexaFluor647-conjugated trastuzumab antibody with a high labeling ratio, the peak with the lowest intensity values contained the highest number of molecules. This phenomenon was attributed to self-quenching and the resulting non-linear intensity change in the case of antibodies with high DOL. On the other hand, it should be considered that the distribution of antibody molecules with different labeling ratios in a stock solution does not follow a Poisson distribution, which would also explain this phenomenon.

For the Poisson distribution to be valid for fluorophore-labeled antibodies the following contitions have to be fulfilled: (i) each lysine residue must be characterized by the same probability of being labeled and (ii) subsequent labeling steps must be independent of each other. Since none of these conditions is likely to be fulfilled for fluorescent labeling of antibodies, the Poisson distribution can only be used as a simplifying, practical approach.

In summary, we have developed an experimental approach that characterizes the effect of fluorescence labeling on the affinity of antibodies and the quantum efficiency of fluorophores. We have successfully shown that the labeling process negatively affects the properties of both the antibody and the fluorophore. Due to the formation of dye aggregates and the energy transfer to non-fluorescent dimers, the quantum efficiency of dyes decreases. On the other hand, the affinity of multiply-labeled antibody species in a stock solution decreases, so antibodies with a high labeling ratio in the antigen-bound fraction will be underrepresented. The significance of the results presented is important in experiments where it is necessary to determine the fluorescence of antigen-bound antibodies. For quantitative, intensity-based or ratiometric FRET measurements, certain constants ($\alpha$ factor) are required in the calculations, which characterize the sensitivity of the excited donor or acceptor detection system. The fluorescence intensity of the fluorescent antibodies used to determine the constants should be normalized to the labeling ratio of the antibody using the DOL of the antibody stock solution. As our results showed that the labeling ratios of bound antibodies and antibody solutions are significantly different, these calculations will be incorrect and will result in misestimation of the correction factors. Antibody labeling ratios are also important in super-resolution localization imaging, where statistical calculations are used and corrected for antibody labeling ratios. If corrections are made to the labeling ratio of the antibody stock solution during the calculations, the number of localizations will be erroneously determined. In conclusion antibodies with a low DOL are typically preferred in quantitative fluorescence studies.

## 7.2. Fluorophore saturation measurements

Förster-type resonance energy transfer (FRET) provides a popular opportunity to study protein-protein interactions under physiological and pathological conditions due to its flexibility and relative ease of use. Although many forms of measurement exist, the most widespread is the intensity-based or ratiometric approach, which examines donor quenching and measures the fluorescence of the sensitized and directly excited acceptor. In order to achieve a better signal-to-noise ratio in our measurements, we often use a strong excitation intensity, which leads to fluorophore saturation. Under these conditions, the emitted fluorescence is no longer directly proportional to the illuminating photon flux, which can be lead to incorrect calculations. This problem is the most common in microscopic measurements, but it is absent from fluorimetric and flow cytometric measurements due to the low excitation intensity and the low numerical aperture objective (or lack of objective).

Although the phenomenon of saturation is present at laser intensities often used in confocal microscopy, it is not taken into consideration when performing intensity-based FRET calculations.

Based on our experiments and model system, the FRET efficiency and the α factor required for its determination are highly dependent on the intensity of the illuminating light. The higher the excitation photon flux, the greater the decrease in FRET efficiency calculated by the conventional formula is. The phenomenon was examined assuming only a singlet state and both a singlet and a triplet state. With the model where only the S1→S0 transition was assumed, an error is made because the triplet state is ignored. Therefore, we also considered a system where fluorophores accumulate in the triplet state, but for the practical application of this model, the rate constants of the S1→T1 and T1→S0 transitions should be known. Since these parameters are generally unavailable and difficult to determine experimentally, we evaluated FRET measurements using a simpler model that neglects the triplet state. The mistake resulting from neglecting the triplet state was compensated by the use of an apparent photon flux that overestimated the real photon flux in the evaluation. Our studies also demonstrated that donor saturation is the most significant factor causing the decreased FRET efficiency at high illumination intensity. In our calculations, we created three different formulas for determining the FRET efficiency: (i) conventional formula corrected for donor saturation; (ii) new intensity-based formula considering donor saturation; (iii) new formalism considering both donor saturation and FRET frustration. Based on the measurements of three different donor-acceptor pairs, it can be seen that the methods we used largely eliminated the

dependence of the FRET efficiency on excitation photon flux and provided much more reliable results. The above-mentioned methods were implemented in rFRET, a program running in Matlab. During the application of these formulas the dependence of the energy transfer on the intensity of the excitation light can be significantly reduced. It should be emphasized that saturation is a real problem in microscopic measurements, which should be taken into consideration for more quantitative and device-independent measurements.

# 8. Summary

In order to achieve the best possible signal-to-noise ratio during fluorescence measurements, the two most commonly used methods are to increase the labeling ratio of antibodies and to increase the intensity of the excitation light. Nonetheless, they affect our measurements undesirably, resulting in erroneous calculations. In our experiments, it was found that labeling antibodies with fluorescent dyes negatively affects the affinity of the antibodies and the quantum efficiency of the fluorescent dyes. Antibodies with high DOL will be underrepresenteted in the cell-bound fraction due to their decreased affinity. Furthermore, the energy transfer and quenching processes between the fluorophores will result in reduced brightness. In addition, during microscopic measurements, the intensity of the illumination light at the focal point of a large numerical aperture objective is so high that the fluorophores saturate. With model calculations and experimental measurements, we confirmed that the FRET efficiency calculated by the conventional formula greatly decreases as a function of the excitation photon flux. We have successfully created equations that take both donor saturation and FRET frustration into consideration. By applying these equations, the dependence on the illumating laser intensity of the FRET efficiency and the $\alpha$ factor required FRET calculations can be almost completely eliminated. In summary, all these measurements, models and calculations significantly contribute to the implementation of more quantitative, accurate and device-independent fluorescence measurements.

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