SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
(Ph.D.)

Optimizing Fluorescence Resonance Energy Transfer Measurements

By: Fábián Ákos István, MD

Supervisor: Professor Dr. Szöllősi János, Ph.D., DSC

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN, 2013

I. Introduction

The theoretical background of resonance energy transfer was published in 1948 by Förster who described the non-radiative short distance transfer of energy from an excited donor to an acceptor molecule. This process involves simultaneous de-excitation of the donor and excitation of the acceptor molecule. When both donor and acceptor are fluorescent molecules the process is referred to as fluorescence resonance energy transfer (FRET). The rate of energy transfer is inversely proportional to the sixth power of the distance between the donor and acceptor, therefore the efficiency of energy transfer (E) strongly depends on the intermolecular distance of the donor to the acceptor:

$$E = \frac{1}{1 + \frac{r^6}{R_0^6}}$$

where r is the separation of the donor and acceptor and R_0 is the so called Förster distance characteristic for the donor-acceptor pair. The Förster distance corresponds to the distance at which the transfer efficiency between donor and acceptor is 50%.

This distance dependence was exploited by Stryer and Haugland who first utilized FRET as a "spectroscopic ruler" to measure the distance between two molecules. With the development of imaging modalities, FRET was later used to study distance relationships in biological samples. FRET experiments have become widespread, with diverse methods for microscopic or flow cytometric setups. The newer methods are able to harness the potential of fluorescent proteins and high through-put methods are also emerging, making FRET measurements a diverse tool to tackle scientific questions of the nanometer distance range.

1. Effect of multiple fluorophores on transfer efficiency

FRET theory describes the interaction of one donor with one acceptor, whereas most imaging modalities involve ensemble acquisition of the signal of several to hundreds of fluorophores simultaneously. Additionally, in biological samples interaction of one donor with one acceptor is not guaranteed. This can be a result of preexistent uneven protein patterns and distributions or dynamic rearrangements arising from complex formation, protein translocations or simple lateral diffusion in the membrane. Additionally, when fluorescently tagged antibodies are used, usually multiple fluorophores are coupled to an antibody to achieve better signal-to-noise ratios. Multiple acceptors interacting simultaneously with the same donor will increase the rate constant of energy transfer. This results in increased transfer efficiency without closer proximity between donor and acceptor. This effect is taken into consideration when measuring transfer efficiency of freely moving fluorophores in a two dimensional plane or energy transfer between a plane of acceptors and a donor above the plane. In these cases special modeling and calibration schemes have to be applied to accurately determine inter-dye distances. Similarly, it has been shown, that an increase in number of acceptor fluorophores increases transfer efficiency measured for fluorescent protein constructs. Since the effect of multiple acceptors interacting with a single donor is realized at the level of rate constants, all methods for measuring FRET will report increased apparent transfer efficiency as compared to the case of interaction between one donor and one acceptor. We therefore sought to investigate how measured transfer efficiency is influenced by multiple interacting fluorophores. This was achieved by varying the fluorophore-to-protein (F/P) ratio of fluorescently labeled antibodies and observing the changes in FRET efficiency in an intramolecular model system.

2. Transfer efficiency in three-dye systems

Originally, FRET was limited to viewing the interaction between one donor and one acceptor species. However, in the early 2000's it was realized that the addition of a third dye could expand the capabilities of traditional FRET measurements. First of all, functioning as a relay point, the third dye increased the interaction range that could be viewed with FRET. Secondly, the third dye allowed FRET to be viewed between three distinct molecular species, so that the relative orientation of three molecules could be assessed at the same time. This has significant potential for biological investigations, where higher order multimers and multi-component signaling complexes play important roles in governing biological function. While providing significant gains, the addition of a third dye also presents several problems. Instrumentation requirements increase, as the instrument of choice has to be able to detect and excite three different fluorophores while at the same time allowing separation of the individual signals.

The theoretical groundwork of three-dye FRET systems was published by Watrob *et al* in 2003 and describes the different energy transfer routes that occur in such a system. Briefly, if the three dyes are designated with increasing excitation wavelength as dye A, B and C, the following behavior can be observed: the dye with the shortest excitation wavelength – dye A – is the global energy donor for dyes B and C; the dye with the intermediate excitation wavelength – dye B – is an acceptor for dye A and a donor for dye C; the dye with the longest excitation wavelength – dye C – is a global acceptor. Regarding the total transfer of energy from dye A to C, two main cases are distinguished: in the first a direct, one-step FRET occurs between A and C (E_{AC}); in the second an indirect, two-step FRET occurs, where energy is first transferred from A to B (E_{AB}) and then from B to C (E_{BC}). The latter

sequence is also called relay-FRET. Since relay-FRET arises from two independent excitation─de-excitation processes, it can be written as the product of E_{AB} and E_{BC}. When direct transfer can occur from dye A to B and from dye A to C as well, the two acceptors compete for the same donor. This has the consequence that instead of the original non-competitive energy transfers, apparent competitive energy transfers are measured. The theory of FRET in a three-dye system is exceedingly more complex than in a two-dye system, resulting in more extensive calculation requirements. Several methods have been developed to measure FRET in a three-dye system. However, the complexity of the three dye system required simplification, either through extensive sample preparation, restricted sample selection or neglecting of transfer routes. We therefore developed a new method – tripleFRET – that can be implemented with a broad range of biological samples and does not require specific sample preparation beyond fluorescent labeling.

II. Scientific goals of the thesis

To determine the effect multiple fluorophores interacting simultaneously have on transfer efficiency, we used an intramolecular FRET system and varied the fluorophore-to-protein (F/P) ratio of the antibodies used. We had three main goals:

- Determine the behavior of different F/P ratio variants of antibodies with respect to intensity and cellular affinity.
- Determine how the interacting number of acceptors influences transfer efficiency.
- Determine how the interacting number of donors influences transfer efficiency.

Previously, three-dye FRET methods were restricted to either semi-quantitative efficiency determination or neglecting of transfer processes to facilitate interpretation of FRET signals. Mostly some permutation of donor quenching was used, necessitating an external reference sample to determine unquenched donor emission. To circumvent these shortcomings, we had the following objectives:

- Lay down the mathematical background for a three-dye intensity-based method that allows computation of direct individual FRET between dyes A, B and C as well as relay- and total-FRET without a reference sample.
- Verify the method with a three-dye labeling scheme of cell-surface proteins and compare results with those obtained with conventional two-dye intensity-based FRET.
- Evaluate the method on molecular systems with variable interacting schemes.

III. Materials and Methods

1. Cell lines

Human gastric cell line NCI-N87 with high ErbB2 (member of the epidermal growth factor receptor family) and major histocompability complex (MHC) class I expression level was obtained from the American Type Culture Collection (Rockville, MD, USA) and grown according to the manufacturer's specification (in RPMI containing 10% fetal bovine serum, 2 mM L-glutamine, and 0.25% gentamicin in 5% CO_2 atmosphere) to confluency. For flow cytometry, cells were harvested by treatment with 0.05% trypsin and 0.02% ethylenediamine-tetraacetic acid before antibody labeling.

2. Conjugation of antibodies with fluorescent dyes

In our experiments we used the following anti-ErbB2 monoclonal antibodies (mAbs): pertuzumab (a gift from Hoffman-La Roche, Grenzach-Wyhlen, Germany); trastuzumab (purchased from Hoffman-La Roche, Grenzach-Wyhlen, Germany); and H76.5 antibody (prepared from the hybridoma cell line, a kind gift of Yosef Yarden). Covalent binding of the monofunctional succinimidyl ester derivates of amine-reactive dyes (Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 555 and Alexa Fluor 647; Molecular Probes/Invitrogen, Eugene, OR, USA) to the lysyl-ε amino groups of antibodies was carried out in 0.1 M sodium bicarbonate buffer at pH 8.3. Dyes dissolved in sodium bicarbonate buffer were added to antibody solutions, and the reaction mixture was incubated. Unreacted dye molecules were removed by gel filtration through a Sephadex G-50 column. To achieve different F/P ratio of the antibodies we changed antibody concentration, pH and/or labeling time.

The F/P labeling ratio was determined from absorption at 280 nm and the maximum absorption wavelength of the dye used by spectrophotometry

(Nanodrop, Wilmington, DE) and was in the range of 1–10 for whole mAbs. In order to prevent artifact production and remove aggregates, dye-conjugated mAbs were centrifuged in the cold (4°C) at 110,000 × *g*, for 20 min in an Airfuge ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) before cell labeling.

3. Labeling cells with fluorescent antibodies

For flow cytometry, freshly harvested cells were washed twice in ice-cold phosphate buffered saline (PBS; pH 7.4). The cell pellet was suspended in PBS at a concentration of 2×10^7 cells/ml. Then 25 µl of conjugated antibodies were added to 25 µl of cell suspension and cells were incubated for 30 min on ice. The excess of antibody was at least fivefold above saturating concentration (final labeling concentration of 100 μg/mL) during the incubation. Thereafter cells were washed twice in PBS and fixed in 500 μL of 1% formaldehyde-PBS. During labeling special care was taken to keep the cells at ice-cold temperature to avoid induced aggregation of cell surface molecules.

4. Instrumentation and sample measurement

For experiments to determine the effects of multiple FRET partners interacting simultaneously, measurements were carried out on a FACSArray bioanalyzer (Becton Dickinson, Franklin Lakes, NJ, USA). The flow cytometer is equipped with a 532-nm solid-state laser and a 635-nm diode laser, and for FRET measurements the detectors with 585/42 band pass (donor channel; I_1), 685 long pass (energy transfer channel; I_2), and 661/16 band pass (acceptor channel; I_3) filters were used. For every sample 20,000 events were acquired.

For tripleFRET measurements, we used a FACSVantage SE with DiVa option flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with a

488-nm water-cooled Argon-ion laser, a 532-nm diode pumped solid-state laser and a 633-nm air-cooled HeNe laser. The fluorescence detection channels for tripleFRET measurements were: 488-nm excitation with 530/30 band pass filter (I_1), 488-nm excitation with 585/42 band pass filter (I_2), 488-nm excitation with 675/20 band pass filter (I_3), 532-nm excitation with 585/42 band pass filter (I_4), 532-nm excitation with 650 long pass filter (I_5), 633-nm excitation with 650 long pass filter (I_6).

5. Evaluation of transfer efficiency

For all FRET experiments, manual gating was performed on the FSC – SSC plot to exclude debris and doublets. Samples labeled with one dye only were used to determine non-specific background corrected intensities in native dye channels. In two-dye systems double-positive populations were gated and used for FRET calculations. For tripleFRET measurements double-positive populations were gated in the triple-labeled sample. Populations either positive for dye A and B or positive for dye A and C were gated. The intersection of the two populations gave a population that is positive for dyes A, B and C. This population was used for tripleFRET analysis. Transfer efficiency histograms were generated for all possible FRET processes, and after manual gating the value of median transfer efficiency was determined.

To evaluate FRET data obtained with flow cytometry, ReFlex software (free-ware, available at http://www.freewebs.com/cytoflex) was used with the equations entered in the equation editor of the program. Intensity-based FRET for two-dye systems was calculated according to the equation:

$$A = \frac{E}{1-E} = \frac{1}{\alpha} \cdot \frac{I_2 - I_1 S_1 - I_3 S_2}{I_1}$$

For tripleFRET measurements the equation set introduced in the Results section of this manuscript was used. Transfer efficiency values are given as median values of transfer efficiency histograms. Flow cytometric dotplots and histograms were generated with ReFlex, three-dimensional transfer efficiency scatter plots were created with Wolfram Mathematica 7 (Wolfram Research, Champaign, IL, USA).

6. Determining alpha-, cross-excitation- and spillover-factors

Since alpha factors are scaling factors correcting for the difference in the fluorescence quantum yields and detection efficiencies of donor and acceptor fluorophores, the intensity of the same number of excited donor and acceptor fluorophores has to be measured at given wavelengths. This is most easily done by labeling a cell-surface protein with donor- and acceptor-tagged antibodies in separate samples. For our experiments, the average intensity of several thousand cells singularly labeled with Alexa Fluor 488, Alexa Fluor 546, Alexa Fluor 555 or Alexa Fluor 647 was used for the calculation of alpha factors. Cross-excitation and spillover factors were measured on single-labeled samples.

7. Anisotropy measurements

Fluorescence anisotropy measurements were performed on a Fluorolog-3 spectrofluorimeter (Horiba Jobin Yvon, Longjumeau, France). The excitation light was provided by a 450-W Xe-arc lamp. Anisotropy of Alexa Fluor 546, -555 and -647 conjugated trastuzumab, free dye solutions and PBS solution were measured with FL-1044 polarizers in L-format configuration. The concentration of the fluorescent conjugated antibodies and free dyes were in the range of 10^{-7}–10^{-6} M, where absorption of the sample was below 0.05 to ensure negligible inner filter effects. A 1 cm optical pathlength quartz cuvette

(Hellma, Müllheim, Germany) was used. Excitation and emission monochromator wavelengths were set according to emission and excitation maxima of the dyes applied. Slit width and acquisition time were chosen so that all polarizer-mode intensities (I_{VV}, I_{VH}, I_{HH} and I_{HV}) for all concentrations remained below 1,000,000 counts per second. Data were analyzed with DataMax for Windows v2.1 software.

IV. Results

1. Determining the effect of multiple FRET partners interacting simultaneously

a) Comparison of different F/P ratio variants of antibodies

For our experiments, ErbB2 proteins on NCI-N87 cells were labeled with trastuzumab and pertuzumab monoclonal antibodies conjugated with Alexa Fluor 488, Alexa Fluor 555 or Alexa Fluor 647. The F/P ratio of antibodies used was determined by spectrophotometry. To verify the labeling ratios, cells were labeled with different F/P ratio versions of the same antibody and the mean intensities of the cells were measured. The mean intensity of cells increased with an increase in antibody F/P ratio, however the increase was not linearly proportional, with a drop off of the intensity signal especially towards the upper limit of the used F/P range. Alexa Fluor 546 displayed the largest F/P dependent intensity saturation, while the effect was least prominent with Alexa Fluor 555. The intensity curve plotted as a function of antibody F/P ratio was similar for trastuzumab and pertuzumab conjugated with the same dye, whereas different dye variants of the same antibody yielded dissimilar curves. Intensity saturation curves as a function of labeling antibody concentration were the same for different F/P ratio variants of the same antibody. The saturation of intensity as a function of antibody F/P was also detected with the spectrofluorimeter for free antibodies in solution and was very similar to the intensity curve of the same antibody bound to the cell surface.

Dye conjugated antibodies were further characterized with anisotropy measurements of the antibodies in solution. Anisotropy was measured at several concentrations and then plotted as a function of concentration. The

individual measurement points of antibodies were fit with a line and the y-intercept, designated by us as intrinsic anisotropy (which corresponds to the anisotropy of an infinitely dilute antibody solution and is equal to the anisotropy of a single antibody without the perturbing concentration effects), was used to compare the antibodies. We found that as the F/P ratio increased, intrinsic anisotropy decreased. This change of anisotropy as a function of F/P ratio was true for all antibodies used; however the curves were characteristic for the fluorophores investigated. The anisotropy of antibody conjugated dyes was substantially higher than the anisotropy of the free dye.

b) Energy transfer measurements with different F/P ratio antibody variants

Increasing the number of acceptors interacting with a given donor increases the probability of an excited donor to find an acceptor partner before de-excitation and leads to an increase in the rate of transfer and subsequently in transfer efficiency. If all n acceptors interacting with the donor are identical in terms of FRET interaction probability, then the system's rate of transfer will be n times the rate of transfer for one acceptor. In this case the relationship between E_0 (the original energy transfer efficiency with one acceptor) and E_n (transfer efficiency after n-fold increase of the rate of transfer) is the following:

$$E_n = \frac{n \cdot k_{transfer}}{n \cdot k_{transfer} + k_{other}} = \frac{n \cdot k_{transfer}}{k_{transfer} + k_{other}} \Bigg/ \frac{k_{transfer} + k_{other} + (n-1) \cdot k_{transfer}}{k_{transfer} + k_{other}} = \frac{nE_0}{1 + (n-1)E_0}$$

The seemingly complicated term can be linearized if the term $A = E/(1 - E)$ is used instead of E:

$$A_0 = \frac{E_0}{1 - E_0}$$

$$A_n = \frac{E_n}{1 - E_n} = n \cdot \frac{E_0}{1 - E_0} = n \cdot A_0$$

Therefore plotting A as a function of n yields a straight line, with A_0 as the y-intercept. The value of E_0 can in turn be calculated from A_0. Since E_0 is the characteristic transfer efficiency for the interaction of one donor with one acceptor, it can be used for distance calculations according to the original Förster equations.

Cell surface FRET measurements were carried out with the donor (Alexa Fluor 546 or Alexa Fluor 555) conjugated to trastuzumab and the acceptor (Alexa Fluor 647) conjugated to pertuzumab. Transfer efficiency for a given donor increased non-linearly with the increase of acceptor F/P ratio and followed the FRET saturation curve predicted by our theoretical calculations. All donors had similar saturation curves, however with a shift of the curve towards higher transfer efficiency values with the increase of donor labeling ratios in the case of Alexa Fluor 546. The correlation between the labeling ratio of the donor and transfer efficiency was weak, with a calculated Pearson's correlation coefficient of 0.14 for Alexa Fluor 546 and 0.038 for Alexa Fluor 555. The measured transfer efficiency achieved with a given acceptor showed only slight increase from increasing the donor F/P ratio. On the other hand transfer efficiency was strongly correlated with the labeling ratio of the acceptor, with a Pearson's correlation coefficient of 0.95 calculated for all acceptor-donor combinations. Increasing acceptor F/P ratio drastically increased transfer efficiency with any given donor. Energy transfer also showed correlation with the acceptor/donor (A/D) ratio. This is however a byproduct from calculating A/D ratio as F/P ratio of the acceptor divided by F/P ratio of the donor. The non-causal relationship between A/D ratio and transfer efficiency is supported by the results showing that the same A/D ratio can result in very different transfer efficiency values depending on the individual F/P ratio of the acceptor and very similar transfer efficiencies can

be measured with different A/D ratios if the F/P ratio of the acceptor is the same. The term A plotted as a function of acceptor F/P ratio showed a linear relationship as predicted by our theoretical model. The slope of the fitted lines was used to determine A_0 and then calculate characteristic transfer efficiency for the dyes. The characteristic FRET efficiency was then plotted as a function of donor F/P ratio and we saw a dye-specific linear increase with an increase in donor F/P ratio.

2. *TripleFRET: a method to measure transfer efficiency in three-dye systems*

a) TripleFRET calculations

An initial equation set to calculate both direct and relay-transfer from dye A to C is presented below. The calculations require detection of six independent emission intensities, I_1-I_6. The six intensities can be interpreted as follows: I_1 – quenched emission of donor A (by acceptors B and C), native intensity channel to detect dye A; I_2 – sensitized emission (from donor A) and quenched emission of acceptor B (by acceptor C), I_3 – sensitized emission of acceptor C (from donor A and donor B excited through donor A), I_4 – quenched emission of donor B (by acceptor C), native intensity channel to detect dye B; I_5 – sensitized emission of acceptor C (from donor B) and I_6 – native intensity channel to detect dye C. The equation set assumes competitive FRET efficiencies E'_{AB} and E'_{AC} for FRET from A to B and from A to C, respectively. There are altogether seven unknowns: three unperturbed intensities I_A, I_B and I_C from the three dyes and four transfer efficiencies E'_{AB}, E'_{AC}, E_{BC} and E_{relay}.

$$I_1(488nm \rightarrow 530/30BP) = I_A(1 - E'_{AB} - E'_{AC})$$

$$I_2(488nm \rightarrow 585/42BP) = S_1 I_A(1 - E'_{AB} - E'_{AC}) + \alpha_{AB} I_A E'_{AB}(1 - E_{BC}) + S_4 I_B(1 - E_{BC})$$

$$I_3(488nm \rightarrow 675/20BP) = \alpha_{AC} I_A(E_{relay} + E'_{AC}) + S_{12}\alpha_{AB} I_A E'_{AB}(1 - E_{BC}) + S_9 I_A(1 - E'_{AB} - E'_{AC})$$

$$+S_{10} I_B(1 - E_{BC}) + S_7 I_C$$

$$I_4(532nm \rightarrow 585/42BP) = I_B(1 - E_{BC})$$

$$I_5(532nm \rightarrow 650LP) = S_2 I_B(1 - E_{BC}) + \alpha_{BC} I_B E_{BC} + S_3 I_C$$

$$I_6(633nm \rightarrow 650LP) = I_C$$

Since in its present form the equation system is underdetermined (with only six independent equations for seven variables), a further equation is required for a solution:

$$E_{relay} = E'_{AB} \cdot E_{BC}$$

The equation set can be solved, however yields complicated expression for most variables. These can be simplified with a set of factorization terms:

$$I_{X1} = I_1$$

$$I_{X2} = I_2 - S_1 I_1 - S_4 I_4$$

$$I_{X3} = I_3 - S_{12} I_2 - (S_9 - S_1 S_{12}) I_1 - S_7 I_6 - S_4 I_{X5}$$

$$I_{X4} = I_4$$

$$I_{X5} = I_5 - S_2 I_4 - S_3 I_6$$

The terms correspond to bleed-through corrected sensitized emission (I_{X2}, I_{X3} and I_{X5}) and quenched donor (I_{X1} and I_{X4}) intensities. The solutions for the variables can be given as:

$$I_A = I_{X1} + \frac{I_{X2}}{\alpha_{AB}} + \frac{I_{X3}}{\alpha_{AC}}$$

$$I_B = \frac{I_{X5} + \alpha_{BC} I_{X4}}{\alpha_{BC}}$$

$$I_C = I_6$$

$$E'_{AB} = \frac{\alpha_{AC} I_{X2} (I_{X5} + \alpha_{BC} I_{X4})}{\alpha_{BC} I_{X4} \left[\alpha_{AC} I_{X2} + \alpha_{AB} (I_{X3} + \alpha_{AC} I_{X1})\right]}$$

$$E'_{AC} = \frac{\alpha_{AB} \alpha_{BC} I_{X3} I_{X4} - \alpha_{AC} I_{X2} I_{X5}}{\alpha_{BC} I_{X4} \left[\alpha_{AC} I_{X2} + \alpha_{AB} (I_{X3} + \alpha_{AC} I_{X1})\right]}$$

$$E_{BC} = \frac{I_{X5}}{I_{X5} + \alpha_{BC} I_{X4}}$$

The non-competitive FRET efficiencies can also be calculated:

$$E_{AB} = \frac{I_{X2} (I_{X5} + \alpha_{BC} I_{X4})}{I_{X2} (I_{X5} + \alpha_{BC} I_{X4}) + \alpha_{AB} \alpha_{BC} I_{X1} I_{X4}}$$

$$E_{AC} = \frac{\alpha_{AB} \alpha_{BC} I_{X3} I_{X4} - \alpha_{AC} I_{X2} I_{X5}}{\alpha_{AB} \alpha_{BC} I_{X4} (I_{X3} + \alpha_{AC} I_{X1}) - \alpha_{AC} I_{X2} I_{X5}}$$

Relay-transfer and total transfer are:

$$E_{relay} = \frac{\alpha_{AC} I_{X2} I_{X5}}{\alpha_{BC} I_{X4} \left[\alpha_{AC} I_{X2} + \alpha_{AB} (I_{X3} + \alpha_{AC} I_{X1})\right]}$$

$$E_{total} = \frac{\alpha_{AB} I_{X3}}{\alpha_{AC} I_{X2} + \alpha_{AB} (I_{X3} + \alpha_{AC} I_{X1})} = \frac{I_{X3}}{\alpha_{AC} I_A}$$

Similar equation sets were also developed for the cases of relay-FRET without direct FRET from dye A to C, direct FRET from dye A to C without relay-FRET and no direct or relay-FRET.

b) Transfer efficiencies of two- and three-dye systems

We chose the dyes Alexa Fluor 488 (dye A), Alexa Fluor 546 (dye B) and Alexa Fluor 647 (dye C) for our experiments. NCI-N87 cells were labeled with trastuzumab, pertuzumab and H76.5 antibodies against the ErbB2 protein. Samples were prepared as a three-dye system as well as a corresponding set of two-dye systems. For validation purposes and to demonstrate the applicability of our method in three-dye systems, all samples were evaluated (in addition to our own equations) using the intensity-based method for two dye systems. All permutations of a two-dye system with the three fluorophores resulted in measurable transfer efficiencies (E_{AB} = 13.5%, E_{AC} = 4.9%, E_{BC} = 45.1%). Analyzing FRET in two-dye systems according to the tripleFRET method produced identical transfer efficiency values as conventional two-dye intensity-based FRET analysis. FRET analysis of the three-dye system with tripleFRET resulted in transfer efficiency values very similar to the ones obtained in two-dye systems (E_{AB} = 12.9%, E_{AC} = 4.0%, E_{BC} = 44.4%). Correction for competition between the two acceptors further increased the agreement with the values from two-dye systems (E_{AB} = 13.4%, E_{AC} = 4.6%,). At the same time, traditional intensity-based FRET failed to reproduce the FRET values of the two-dye systems in the triple-labeled sample. Specifically, E_{AB} was underestimated (7.9% instead of 13.5%) and E_{AC} overestimated (11.4% instead of 4.9%). The addition of dye B to the labeling scheme consisting of only dyes A and C substantially increased the total energy transferred from A to C (E_{total} = 4,9% →10.4%), providing evidence for a relay transfer process in our intramolecular model system.

To demonstrate the sensitivity of tripleFRET calculations and its power to dissect populations with different protein association patterns in biological systems, we mixed together 7 different samples in the same tube. Then energy

transfer was measured by flow cytometry for the mixed sample. The evaluation of 3 FRET efficiencies allowed us to discriminate between all seven differently labeled specimens in the same sample. The transfer efficiency values measured in such a fashion were in good agreement with FRET efficiencies obtained from the specimens measured individually.

c) TripleFRET in three-dye systems with different spatial distributions of dyes

Lastly, we altered the labeling scheme so that the three dyes could not co-localize on the same protein due to competition between antibodies. This way we either achieved a dye configuration where the transfer process from A to B is intermolecular or dye B excited by energy transfer from dye A was not on the same ErbB2 protein as dye C, causing relay-FRET to become minimal. Transfer efficiency was calculated with different initial equation sets considering four scenarios: simultaneous relay and direct transfer from A to C; only relay transfer without direct transfer; only direct transfer without relay transfer; no relay or direct transfer. In the case of intramolecular localization of all three dyes, the scheme supposing direct and relay transfer to dye C gave the best approximation of energy transfer values from two-dye systems without neglecting any transfer processes. The same was true for the case of intermolecular energy transfer from dye A to B, where assuming only relay transfer neglected the substantial direct transfer process from dye A to C and supposing only direct-FRET underestimated energy transfer from A to B. However, in the case when dye B excited by energy transfer from dye A was not in close proximity of dye C, analysis involving simultaneous direct and relay transfer failed to give results with a physical meaning, as A to C transfer was found to be negative. Calculations with only relay transfer produced a relay-FRET value that was higher than the total energy transfer from A to C. Therefore a scheme involving only direct transfer gave the best results, with

physically plausible results obtained for all calculated transfer efficiencies. However, in this case a small but relevant amount of relay transfer was neglected, since total transfer was higher in the three-dye system than in a system with only dyes A and C.

V. Discussion

1. Effect of multiple FRET partners interacting simultaneously

a) Intensity quenching and anisotropy of antibodies

First, we compared the properties of the different F/P ratio variants of the antibodies. We encountered a non-linear increase in fluorescent signal with increasing antibody F/P ratio, implying loss of signal through quenching or some other effect. Labeling antibody concentration dependent intensity saturation curves were nearly identical (after normalization to maximum intensity) for small and large F/P ratio antibodies, so F/P ratio did not influence antibody binding. Free antibodies in solution displayed the same F/P ratio dependent intensity saturation as our cellular experiments, which further ruled out an influence of antibody-antigen interaction. We also investigated whether different F/P ratio variants of the same antibody differ in absorption, excitation or emission spectra. Our data did not show any significant differences in the spectra of the different F/P ratio variants, therefore this was also ruled out as a cause of intensity saturation. The F/P ratio dependent saturation curves were different for different dyes bound to the same type of antibody, whereas different antibodies conjugated with the same dye had similar curves. Therefore the saturation effect was characteristic for the dye used.

Antibody variants were further characterized with anisotropy measurements. Anisotropy quantifies the degree of polarity lost between fluorescent emission and excitation by a polarized excitation light. To compare the anisotropy of different F/P ratio variants, we compared the intrinsic anisotropies introduced in the Results chapter. As expected, the free dye had substantially lower anisotropy than the antibody conjugated variants, a consequence of slowed movement from the added bulk of the antibody. Interestingly, intrinsic

anisotropy decreased with an increase in antibody F/P ratio. This cannot be explained with just the increased complex size of higher F/P ratio variants (further decrease in movement speed from the increase in size of the dye-antibody conjugate from additional dyes should actually increase anisotropy). Several processes can take place between the dyes conjugated to the same antibody that alter anisotropy. Homo-FRET is a FRET process where both donor and acceptor belong to the same dye species and is possible because of the overlap between emission and excitation spectra of any given dye. Homo-FRET allows excitation energy to be passed from one dye to the other without fluorescent emission. While the individual excited dye state is shortened just as with hetero-FRET, actual fluorescent emission will occur later than without homo-FRET. Also, since FRET does not require emission and excitation dipoles to be perfectly aligned, the emission polarity can be changed in leaps. The combined effect is diversification of emitted light directions by homo-FRET, which reduces anisotropy. On the other hand, collision quenching for instance shortens the fluorescence lifetime and causes an increase in measured anisotropy. Our measurements show that increasing F/P ratio reduces the anisotropy, which suggests that homo-FRET is the dominant underlying process. The minimal value of intrinsic anisotropy was correlated with the F/P ratio dependent intensity quenching exhibited by an antibody, i.e., the larger the intensity saturation of an antibody, the higher the plateau value of intrinsic anisotropy. This is in line with the assumption that intensity saturation is a consequence of collision quenching, therefore larger saturation means more quenching which can counteract the effects of homo-FRET.

b) Effects of acceptor abundance

Our FRET experiments showed the labeling ratio of the acceptor to be highly correlated with the measured transfer efficiency. Transfer efficiency measured

with the same donor and different F/P ratio acceptor variants increased non-linearly with the labeling ratio. The experimental measurement points closely matched the theoretical curves predicted by our calculations. The $A = E/(1-E)$ plots also displayed the linear relationship that our theory postulated. The fact that plotting $E/(1-E)$ as a function of the acceptor labeling ratio yields a line demonstrates that each acceptor dye behaves similarly, increasing the probability of FRET interaction to the same extent. Also, our transfer efficiency curves as a function of acceptor F/P ratio closely resemble previously published curves for varying concentrations of dyes randomly distributed in solution, further supporting our theory that acceptors bound to a single antibody have a non-preferential, equal chance to interact with the same donor. Our measurements prove acceptor availability as a limiting factor for measured FRET efficiency. Measured transfer efficiency was increased nearly two-fold just by increasing the acceptor F/P ratio.

c) Effects of donor abundance

Theoretically, increasing the number of donor dyes does not increase the probability of an individual donor to interact with an acceptor and so the fraction of donor molecules losing the absorbed energy through FRET does not change and transfer efficiency stays unchanged. Multiple donors interacting with an acceptor should not affect transfer efficiency negatively, since donor de-excitation is such a fast process, that the chance of two simultaneously excited donors competing for the same acceptor is minimal. Therefore under conventional circumstances systems with multiple donors within interaction distance of the same acceptor (such as antibodies labeled with multiple dyes) are regarded as a single donor system with respect to transfer probability.

In our experiments we saw a slight increase of transfer efficiency from the increase of donor F/P ratio, which was especially evident with the Alexa Fluor 546 dye as donor. The same was true for characteristic transfer efficiency, with a linear increase with increasing donor labeling ratio. This effect is most likely caused by the increasing homo-FRET between the donor dyes upon increasing the number of dyes bound to the antibody. The effect of homo-FRET can be explained as follows. As the donor and acceptor move through their possible spatial positions, the relative orientation of the donor emission and acceptor excitation dipoles also constantly change, cycling from relative orientations favoring FRET transition to ones that essentially preclude it. From the donor's stand point this means in certain positions FRET is likely and therefore dominates other de-excitation processes, such as fluorescence. In other positions FRET transitions are not likely (small rate of transfer), therefore other de-excitation processes determine the fate of the excited state. In our proposed model homo-FRET acts as a lifeline for the excited state in positions where de-excitation would take place without a contribution from FRET. Instead of non-FRET de-excitation, the donor's excited state can be conserved and transferred by homo-FRET to positions that favor FRET. By transferring energy from positions where FRET has negligible probability larger FRET efficiency is detected without a decrease in acceptor-donor distance.

d) Implications for FRET measurements

Our results show that manipulating antibody labeling ratios can be a simple tool for increasing measurement sensitivity beyond a better signal/noise ratio of the measured intensities. Based on our results the R_0 of the acceptor-donor dye system can be manipulated by changing the labeling ratios. If we denote

R_n as the Förster distance for a FRET system with n acceptors, then the relationship between R_n and R_0 is:

$$E_n = \frac{n \cdot R_0^6}{n \cdot R_0^6 + R^6} = \frac{R_n^6}{R_n^6 + R^6}$$

$$R_n = n^{\frac{1}{6}} \cdot R_0$$

Simply by changing the R_0 of the system we can shift the intermolecular distance-FRET response curve. Therefore by increasing acceptor labeling ratio the value of FRET efficiency can be increased. This can be useful in low-FRET systems, where FRET levels can be elevated to detectable levels above background. Further, the curve can be shifted so that all possible distance changes in a given system are followed by a significant change in FRET. For instance reducing acceptor F/P ratio can be beneficial, if FRET values are near saturation, since a smaller F/P acceptor reduces system R_0 and increases FRET change over that particular distance range.

The concept has been recently utilized to extend the Förster-distance of protein systems, so that transfer efficiency is detectable at intermolecular distances of 15 nm. Labeling the protein of interest with multiple acceptors randomly distributed on the protein surface elevated transfer efficiency above the level measured when just a single acceptor is used. Although with this approach the acceptor can no longer be treated as having a well defined, point-like distance from the donor, it allows detection of protein interaction at distances exceeding the conventional range of FRET experiments. This should allow a much broader mapping of molecular networks to encompass interaction partners that were missed because of the distance restrictions that apply to energy transfer measurements.

2. *TripleFRET measurements*

a) *TripleFRET: a novel method to measure energy transfer in three-dye systems*

We identified and broke down to quantifiable components six different emission intensities in total, which, in a system of equations allow the individual FRET between each member of the system to be assessed, which in turn carries information about the relative spatial organization of the studied molecules or epitopes. Both uncorrected and competition-corrected transfer efficiencies were calculated to determine the apparent FRET of the dye system, while still obtaining the competition-free FRET values of a two-dye system.

In our experiments we used Alexa Fluor 488, Alexa Fluor 546 and Alexa Fluor 647 fluorophores as dyes A, B and C, respectively. There is sufficient spectral overlap between the excitation and emission spectra of these fluorophores to allow for all theoretically possible energy transfer routes. When measuring two-dye systems, evaluation with the classical intensity-based FRET and with the tripleFRET method gave comparable results. Also, FRET efficiencies obtained by the tripleFRET approach in three-dye systems for any dye-pair were in good agreement with the values measured and calculated for the corresponding two-dye system. However, when using the two-dye intensity-based method in a three-dye system, we measured significantly lower E_{AB} and significantly higher E_{AC} values compared to the corresponding two-dye systems. This can be attributed to the quenching of fluorophore intensity and augmentation of sensitized emission by the third fluorophore, so that distorted values are used as acceptor and donor fluorophore intensities during energy transfer calculation.

b) Transfer efficiency in systems with multiple transfer schemes

The results show that while our method is accurate, it fails to distinguish between different spatial distributions that produce near-identical transfer efficiency profiles. In these cases an accurate model can only be constructed with knowledge of antibody binding stochiometry (i.e. just one recognized epitope per protein). Theoretically, a slight increase in individual transfer efficiencies from the presence of additional transfer routes distinguishes the cases from one another; however, the contribution of these routes is mostly small and can be masked by measurement noise and biological variability.

In most cases, various distinct molecular interaction schemes allow physically plausible E_{AB}, E_{AC} and E_{BC} values to be calculated from the same quenched donor and sensitized acceptor emission intensities. This in turn means that being able to calculate a given transfer efficiency does not guarantee that the FRET process is actually taking place at the molecular distance deduced. For instance, sensitized emission of dye C can be attributed to direct FRET between A and C, relay excitation through B or both. Based solely on intensity data we cannot distinguish between these cases or tell which one of them apply to a given situation. Even if multiple orientations are considered in FRET calculations, as long as the relative contribution of each to the ensemble FRET signal is not known, precise efficiency values cannot be calculated. The same effect is achieved when not all fluorophores participate in the transfer process, for instance, when three different proteins are labeled. The presence of single-dye species without transfer partners under such conditions is a problem even in traditional ensemble measurement types. Theoretically, an initial equation set can be developed to take multiple simultaneous distributions into account; however, the number of variables does not allow the equation set to be solved with the six measurable intensities. Therefore,

accurate intensity-based calculations require prior knowledge about possible transfer routes, either from measurements in two-dye systems or known and/or limited spatial distribution of the imaged dyes.

This limitation was not addressed in previous papers because the model system used to test the method ensured co-localization of all three dyes and no variation in the interaction scheme. This is an inherent property of single-molecule imaging methods, since only one fluorophore triplet and as a consequence one interaction scheme is detected at a time. Further, all measurements with DNA strands, fixed distance three-fluorophore constructs or multimers, where FRET is only possible in a given relative conformation of the imaged molecules ensured transfer processes were restricted to individual trimers of dye A, B and C. This corresponds to the scheme represented by Sample 1 in Figure 10. The key restrictions of this scheme are: FRET only takes place within the dye-trimer; a shared dye B participates in E_{AB} and E_{BC}, so if E_{AB} and E_{BC} are detected relay-FRET also occurs and E_{AB} is quenched by E_{BC}; only dominant E_{AB} and E_{BC} contribute to relay-FRET; if E_{AC} is measured it places dyes A, B and C in the corners of a virtual triangle. In these restricted systems tripleFRET equals the efficacy of previously published three-dye methods, however without the need for an external reference sample. Whether other methods fare better when multiple interaction schemes are present is not known, since the model systems used to demonstrate these methods did not allow such diverse interaction schemes to occur. In such a fashion, either by chance or design, the restricted applicability of three-dye FRET measurements was not unmasked. It should also be noted that these considerations are only vital when precise absolute transfer efficiency values are needed and can be partially neglected when FRET is only used as a semi-quantitative indicator or interaction scheme changes during the experiment can be ruled out.

VI. Summary

We set out to characterize different F/P ratio variants of fluorophore conjugated antibodies and then utilize them to determine the effects of dye abundance on transfer efficiency. Our major results are:

- Fluorescence intensity of dye conjugated antibodies does not increase linearly with F/P ratio and dye-specific intensity saturation is present.
- Acceptor and donor F/P ratio directly influence measured transfer efficiency.
- Acceptor abundance has the greatest effect on FRET efficiency, with a non-linear increase in transfer efficiency from increasing the interacting number of acceptors.
- We were able to predict dye influence with our theoretical model, which facilitates manipulation of the FRET system in a purposeful way to yield better results.

We wanted to contribute to the growing field of three-dye FRET measurements in two key areas, which are also part of the appeal of two-dye FRET: ease of use and applicability in cellular systems. Our novel three-dye method, tripleFRET has the following characteristics:

- Can be performed on regular flow cytometers.
- Allows calculation of all individual transfer efficiencies in a three-dye system without the need for an external reference sample.
- Matches the sensitivity of previous three-dye methods.
- Equals the performance of traditional two-dye FRET in two-dye systems and delivers more reliable results in three-dye systems.
- Allows direct comparison of FRET data from two- and three-dye systems when prior knowledge about the spatial localization is also available.

In conclusion, our work delivers new insights into the FRET processes in three-dye and multi-fluorophore systems. This allows us to gain additional information from the investigated system and optimize FRET measurements.

Experimental work was performed with the support of the TÁMOP-4.2.2.A-11/1/KONV-2012-0025 project. The doctoral training program was supported by the TÁMOP-4.2.2/B-10/1-2010-0024 project. The projects are co-financed by the European Union and the European Social Fund.

VII. Publications

UNIVERSITY AND NATIONAL LIBRARY UNIVERSITY OF DEBRECEN
KENÉZY LIFE SCIENCES LIBRARY

Register Number: DEENKÉTK/125/2013.
Item Number:
Subject: Ph.D. List of Publications

Candidate: Ákos Fábián

Neptun ID: V2H8VR

Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Fábián, Á.**, Horváth, G., Vámosi, G., jr. Vereb, G., Szöllősi, J.: TripleFRET measurements in flow cytometry.
   Cytom. Part A. 83 (4), 375-385, 2013.
   DOI: http://dx.doi.org/10.1002/cyto.a.22267
   IF:3.729 (2011)

2. **Fábián, Á.I.**, Rente, T., Szöllősi, J., Mátyus, L., Jenei, A.: Strength in numbers: Effects of Acceptor Abundance on FRET Efficiency.
   Chemphyschem. 11 (17), 3713-3721, 2010.
   DOI: http://dx.doi.org/10.1002/cphc.201000568
   IF:3.339

List of other publications

3. **Fábián, Á.**, jr. Vereb, G., Szöllősi, J.: The hitchhikers guide to cancer stem cell theory: Markers, pathways and therapy.
   Cytom. Part A. 83A (1), 62-71, 2012.
   DOI: http://dx.doi.org/10.1002/cyto.a.22206
   IF:3.729 (2011)

4. **Fábián, Á.**, Barok, M., jr. Vereb, G., Szöllősi, J.: Die Hard: Are cancer stem cells the Bruce Willises of tumor biology?
   Cytometry A. 75A (1), 67-74, 2009.
   DOI: http://dx.doi.org/10.1002/cyto.a.20690
   IF:3.032

H-4032 Debrecen, Egyetem tér 1. e-mail: publikaciok@lib.unideb.hu

5. Varga, Z., Csépány, T., Papp, F., **Fábián, Á.**, Gogolák, P., Tóth, Á., Panyi, G.: Potassium channel expression in human CD4(+) regulatory and naive T cells from healthy subjects and multiple sclerosis patients.
Immunol. Lett. 124 (2), 95-101, 2009.
DOI: http://dx.doi.org/10.1016/j.imlet.2009.04.008
IF:2.906

6. Fazekas, Z., Petrás, M., **Fábián, Á.**, Pályi-Krekk, Z., Nagy, P., Damjanovich, S., jr. Vereb, G., Szöllősi, J.: Two-sided fluorescence resonance energy transfer for assessing molecular interactions of up to three distinct species in confocal microscopy.
Cytom. Part A. 73 (3), 209-219, 2008.
DOI: http://dx.doi.org/10.1002/cyto.a.20489
IF:3.259

7. Kovács, T., Békési, G., **Fábián, Á.**, Rákosy, Z., Horváth, G., Mátyus, L., Balázs, M., Jenei, A.: DNA flow cytometry of human spermatozoa: Consistent stoichiometric staining of sperm DNA using a novel decondensation protocol.
Cytometry A. 73 (10), 965-970, 2008.
DOI: http://dx.doi.org/10.1002/cyto.a.20618
IF:3.259

8. jr. Sipka, S., Bráth, E., Tóth F., F., Aleksza, M., Kulcsár, A., **Fábián, Á.**, Baráth, S., Balogh, P., Sipka, S., Furka, I., Mikó, I.: Cellular and serological changes in the peripheral blood of splenectomized and spleen autotransplanted mice.
Transpl. Immunol. 16 (2), 99-104, 2006.
DOI: http://dx.doi.org/10.1016/j.trim.2006.03.013
IF:2.297

9. jr. Sipka, S., Bráth, E., Tóth F., F., **Fábián, Á.**, Sipka, S., Németh, N., Bálint, A., Furka, I., Mikó, I.: Distribution of peripheral blood cells in mice after splenectomy or autotransplantation.
Microsurgery. 26 (1), 43-49, 2006.
DOI: http://dx.doi.org/10.1002/micr.20209
IF:0.882

10. Horváth, G., Petrás, M., Szentesi, G., **Fábián, Á.**, Park, J.W., jr. Vereb, G., Szöllősi, J.: Selecting the right fluorophores and flow cytometer for fluorescence resonance energy transfer measurements.
Cytometry A. 65A (2), 148-157, 2005.
DOI: http://dx.doi.org/10.1002/cyto.a.20142
IF:2.115

11. ifj Sipka S., Bráth E., Tóth F. F., **Fábián Á.**, Furka I., Mikó I.: Lép autotransplantatio és lépeltávolítás haematologiai és immunologiai hatásainak összehasonlító vizsgálata egerekben. *Magyar Seb. 58*, 84-88, 2005.

12. Szentesi, G., jr. Vereb, G., Horváth, G., Bodnár, A., **Fábián, Á.**, Matkó, J., Gáspár, R., Damjanovich, S., Mátyus, L., Jenei, A.: Computer program for analyzing donor photobleaching FRET image series.
Cytometry A. 67 (2), 119-128, 2005.
DOI: http://dx.doi.org/10.1002/cyto.a.20175
IF:2.115

Total IF: 30.662

Total IF (publications related to the dissertation): 7.068

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

29 March, 2013