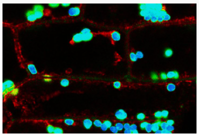

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FLIM FRET and Biosensors: Versatile Tools for Biomedical Research

Fluorescence Lifetime Imaging (FLIM) in combination with Förster Resonance Energy Transfer (FRET) has proven to be very beneficial for investigations in biomedical research for a wide range of structural elements and dynamic changes in cells. FRET allows to monitor molecular interactions, as the FRET signal depends strongly on the distance of the two FRET partners. This allows to investigate interaction of molecules, like ligand-receptor pairs, protein-protein interactions or interactions of effectors with DNA. On the other hand, the FRET principle has been utilized to engineer sensors, that change the FRET efficiency of two fluorophores inside the probe upon binding or release of a ligand. In this case, we have two types of molecular interactions: the FRET partners in the sensor and the interaction of the probe with the ligand. This article explains the component methods (FLIM and FRET) and how the combination of them opens wide fields of applications.

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Measuring specific parameters requires specific sensors. Temperature is measured by a thermometer and speed by a speedometer. Biomedical research is often concerned with measuring parameters in cells or extracellular liquids. Here, there are countless parameters of interest for researchers: for example all sorts of ions, small molecules or other metabolic compounds, and even polarity or electrical potential are key. Researchers want to measure these parameters to unlock the truth behind life, the universe, and everything. These days, most probes for biological parameters are based on fluorescence. A fluorescent probe may increase or decrease in brightness, depending on the concentration of the analyte investigated. Unfortunately, intensity is not only influenced by the concentration of the fluorochrome, but also the illumination intensity, degradation due to bleaching, as well as absorption and shadowing effects. To avoid these problems in part, ratio-dyes are preferred which allow calibration of the fluorochrome emission in comparison to a constant background which falls within the spectrum of that fluorochrome. Still, intensity-based probing is not very reliable, because of limited reproducibility. It also requires elaborate calibration and correction methods. A better approach is offered by fluorescence lifetime imaging (FLIM). The lifetime is independent of the dye concentration, illumination intensity, and absorption and scattering in the sample. Therefore, variations of these during the experiment makes no difference in terms of lifetime. On the other hand, fluorescence lifetime depends very significantly on the molecular environment, which renders it feasible for measuring the effect of environmental parameters. In a special case of a molecular environment, there may be a second dye that can absorb the excitation energy from the first dye – FRET. This process offers a sensitive method for lifetime measurements. For this combined technique, more and more probes (“analyte-meters”) are developed and made available for biomedical research: FLIM FRET biosensors.

What is FLIM?

Excitation of fluorescence occurs with photons of appropriate energy. After absorption of the photon (blue arrow, Figure 1a), the electronic system releases a small amount as heat (dotted line Figure 1a). The emitted photon is therefore of longer wavelength as compared to the absorption (Figure 1b). Instead of spontaneous emission, the energy release can be triggered by interaction with a low energy photon (Figure 1c), which is known as stimulated emission, an essential process for laser radiation and STED-microscopy. Finally, the energy can also be released entirely without emission of a photon, thereby reducing the fluorescence efficiency (quenching, Figure 1d).

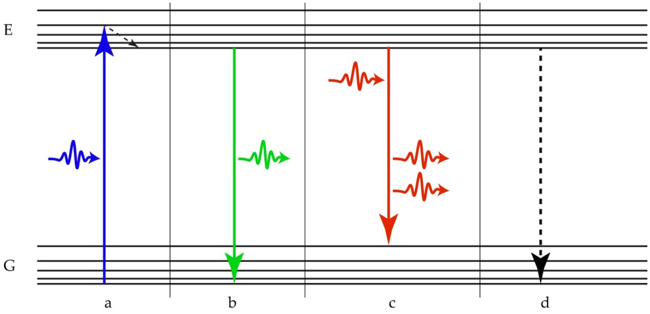

Figure 1: Quantum events in fluorescence: (a) absorption, blue, (b) emission, green,(c) stimulated emission, red and (d) quenching. Events that involve a photon are shown in solid arrows with the corresponding color. Non-radiative events are shown in dotted black lines. G: ground state; E: excited state.

Fluorescence images are commonly thought of as two-dimensional intensity distributions of the emitted light. The intensity can be measured as a function of excitation wavelength or emission wavelength or both. Emission detection is performed either by a series of simultaneously detected spectral bands, incremented spectral bandlets, or a combination of both. The results are used to create colorful images, to separate signal channels, to identify interactions in space and time, and much more. Yet, the fluorescence process offers an additional world of information: the fluorescence lifetime, which is independent of the intensity. The lifetime can be used to identify and separate fluorescent species under controlled conditions and reveal details about the molecular environment.

After excitation, the fluorescent molecules stay for a certain time in the excited state, and then decay back into the ground state. The time they stay in the excited state is not predictable, as it is a quantum-mechanical governed stochastic process. A well-known example for such a behavior is the radioactive decay of instable atom nuclei, identified by the half-life period which is very specific for each nuclide. For fluorescence, the life time is described as the characteristic lifetime, τ, for an excited chemical species. That renders the lifetime as a means to differentiate fluorochromes, in addition to separation via intensity characteristics.

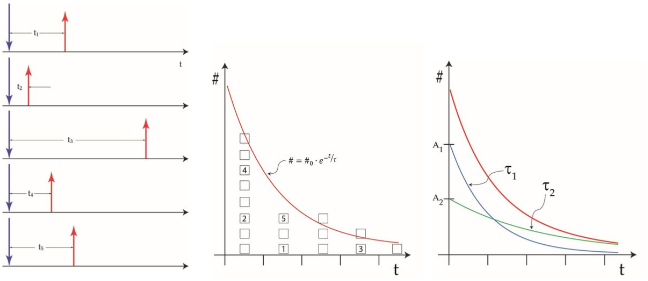

Figure 2: Measurements of arrival times, t_i, of emitted photons (red arrows) after excitation with a laser pulse (blue arrows). Here, 5 measurements are sketched. b: histogram of arrival times, including an example of the five events from above. Most counts, #, are at very short times. The histogram is fitted with an exponential decay curve (red). c: the fit reveals, for example, two single decays (blue and green curves) with decay times $τ_1$ and $τ_2$ and amplitudes A1 and A2. The sum of these would equal the red decay curve.

A fluorescence lifetime image (FLIM), therefore, does not represent an intensity in each picture element (pixel), but instead provides information about the lifetime [1]. The classical measuring method (time correlated single photon counting, TCSPC) does indeed measure single events of fluorescence. The pixel information is either an "average arrival time", i.e. the mean of all lifetime events measured in that pixel, or it is one or more characteristic times which are extracted from the arrival histogram by curve fitting (Figure 3). To reach a significant result, some 400 events per pixel should be measured.

What is FRET?

Förster Resonance Energy Transfer (FRET) is a quenching phenomenon which influences fluorescence measurements. Instead of emitting a photon, the molecule can also release the full excitation energy without emitting radiation. (quenching, Figure 1d). If the quenching molecule is again a fluorochrome, the energy, is transferred as an energy quantum by resonance without radiation from a donor (D) to an acceptor (A) fluorochrome (Figure 3). Hence, the released energy is not dissipated as heat, but stored in the excited state of the acceptor fluorochrome [2]. For FRET to occur, it is required that the excitation of the acceptor must spectrally overlap with the emission of the donor and the two molecules must also have close contact and a proper orientation. "Close" in this context means the distance should not exceed circa 10 nanometers. The closer the molecules, the higher the probability that FRET occurs.

FRET

The relation between distance and transfer-rate, k_F, is described by the equation:

$$k_F = \frac{R_0^{\,6}}{\tau_D \cdot r^6}$$

where R_0 denotes the “Förster radius” which is the distance where the efficiency of transfer is 50%, i.e. half of the excited donor molecules can transfer a quantum of energy to an acceptor. The Förster radius assumes a specific value for each donor-acceptor pair. The characteristic lifetime of the donor excitation is represented by τ_D and the actual distance between the two molecules by r. As the transfer rate is inversely proportional to the distance between the molecules raised to the power of 6, FRET occurs only if the donor and acceptor are in close contact. Still, the relationship is unique so, therefore, initially FRET was used to estimate molecular distances (a “molecular ruler”). The absolute FRET efficiency, in addition, depends on the overlap integral of donor emission and acceptor excitation and on the orientations of the transition moments of both fluorochromes.

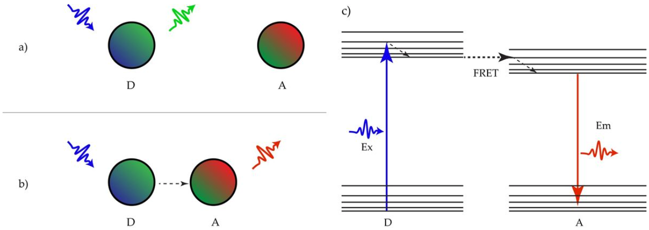

Figure 3: ordinary fluorescence and FRET: a) fluorescent donor molecule (D) can absorb a photon (blue) and emit a photon of less energy (green). b) alternatively, the energy can be transferred non-radiatively (black dotted line) to a neighboring acceptor fluorochrome (A) whose absorption band has overlapping regions with the emission band of D. The acceptor can emit a photon of lower energy (red) compared to the emission of D. The non-radiative transfer from D to A is called “Förster Resonance Energy Transfer” (FRET). c) Jablonski diagram for FRET. Donor D ist excited by a short wavelength photon Ex. The energy is transferred non-radiatively (FRET) to the acceptor A. Acceptor emits a long wavelength photon Em.

The occurrence of FRET becomes manifest by several phenomena. First, the sample (the acceptor) will emit a fluorescence color that is not expected from the applied excitation color. For the example in Figure 3, the red emission is not expected after blue excitation. This emission can be measured and compared with the original emission, a method known as “sensitized emission”. Sensitized emission indicates occurrence of FRET in a quantifiable manner. Such measurements can be performed in living material, but require complex corrections if performed in intensity mode and are, therefore, prone to all sorts of miscalibration. Here again, the lifetime measurement is the method of choice. The effect of FRET on lifetime is explained in the last section.

On the other hand, the emission of the donor will decrease, because some of the excited states transit into acceptor excitations. This phenomenon is exploited in a method called “acceptor photobleaching”, where the change of donor emission is measured upon eradication of the acceptor by photobleaching. After the acceptor is removed, the donor emission will increase. This method is only applicable for fixed samples.

FLIM FRET and biosensors

The term “biosensor” is chosen for sensors of biological origin. This could be a protein or a peptide, DNA or RNA fragments, cells, and so on. Even entire organisms can serve as biosensors, for example freshwater fish in mortality screening setups for toxic contaminations of water. In a more technical context, biosensors denote compound sensors that contain a biological part and electrical circuitry for measuring analyte concentrations. Well known are, for example, glucose biosensors, fast and simple devices used by diabetics to control blood glucose. FLIM-FRET biosensors usually indicate molecules that use fluorescence (lifetime) as a signal and FRET as a sensitive phenomenon.

By now, we have the background we need to understand how FLIM FRET biosensors work. The first part is the sensing agent: usually a protein or peptide that interacts with the analyte (the molecule of interest). Proteins and peptides can conveniently be introduced by genetic engineering into the target organism and are therefore preferred sensing molecules. A famous example is the protein calmodulin. Calmodulin can bind Ca^{2+} ions and undergoes a conformational change upon binding or unbinding.

The second part is concerned with fluorescence. We need to label the sensing molecule with a pair of fluorochromes that can do energy transfer. The fluorochromes must be connected to the sensing agent in a way that in one conformational state they are far apart and cannot perform energy transfer, but in the alternative conformational state they are close, properly oriented, and subsequently perform energy transfer. If then the sensor binds or releases the analyte, FRET will occur or disappear. We can measure binding or release by, for example, sensitized emission. If fluorescent proteins are chosen as fluorochromes, the whole biosensor can be expressed genetically and, therefore, introduced into any biological target object, even specific subcellular locations. A proper pair is for example CFP (cyan fluorescent protein) as donor and YFP (yellow fluorescent protein) as acceptor.

The last bit is the lifetime measurement. As mentioned above, sensitized emission measurements by intensity are prone to significant errors and require cumbersome calibration measurements and complex corrections. That is mainly because intensity is influenced by many other parameters than just those having an effect on the FRET process. The lifetime essentially only depends on the fluorochrome species and the molecular environment, in this case the abundancy of acceptors, which is the basis for the probing mechanism. In case of FRET, the lifetime shortens compared to pure fluorescence emission. In Figure 4, the shortening is shown by using the analogy of a water reservoir (resembling the reservoir of excited states). If the donor can only emit fluorescence (green), then there is only one drain which controls the speed of emptying the reservoir (a). If the donor can transfer the energy to an acceptor, a second drain is opened in the reservoir (red), that has the consequence, that the reservoir will empty quicker (b).

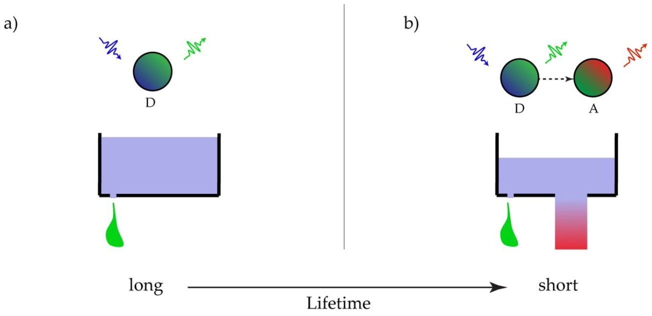

Figure 4: a): A reservoir D of donor molecules in the absence of FRET releases excitation energy only by emitting photons. Therefore, the reservoir of excited states is only depleted by one drain (green droplet) that controls the mean lifetime of the excited state. b): if acceptor molecules can absorb the excitation energy from donor molecules, then there is a second drain for emptying the reservoir of excited states. The depletion is therefore faster and, consequently, the lifetime shorter in the case where FRET occurs.

FRET kinetics

The fluorescent decay of the excited states of donor molecules, E_D, after excitation by a flash can be described by simple decay to the ground state, G_D, and emission of a fluorescence photon, p_D:

$$E_D \rightarrow G_D + p_D\,.$$

The corresponding rate equation is:

$$\frac{dE_D}{dt} = k_f \cdot E_D$$

where k_f is the reaction rate for fluorescence emission. Integration yields

$$E_D = E_D^0 \cdot e^{-k_f \cdot t} = E_D^0 \cdot e^{-t/\tau_f}$$

where E^0_D denotes the initial number of excited states after the flash and t_f the characteristic time (t_f=1/k_f). The FRET quenching process is competing with ordinary fluorescence, generating excited acceptor molecules, E_A, by a non-radiative process, hence:

$$E_D \rightarrow G_D + p_D + E_A$$

where, for this case, k_{nr} denotes the rate of non-radiative transfer:

$$\frac{dE_D}{dt} = \left(k_f + k_{nr}\right) \cdot E_D\,.$$

The apparent characteristic time, τ_{FRET}, when Förster resonant energy transfer occurs, is shorter than the characteristic time for pure fluorescence emission, t_f:

$$\tau_{FRET} = 1/\left(k_f + k_{nr}\right) < 1/k_f = \tau_f\,.$$

This difference in lifetime is employed for detecting conformational changes of the sensor upon binding of specific analytes (intramolecular FRET). Of course, any interaction between two molecules that are covered with appropriate FRET partners can be investigated in the same manner (intermolecular FRET).

Thus, if we measure the lifetime changes of the donor, we can monitor the conformational switching of the sensing agent and, with that, the change of the analyte concentration. If the bound conformation causes lower FRET, the donor lifetime will increase in relation to the analyte concentration and vice versa.

For measuring fluorescence lifetime with sufficient precision and speed for dynamical changes in vivo, the instrumentation requires both high sensitivity, allowing fast frame rates, and appropriate means for exploring the boundaries of time-correlated single-photon counting [3].

Examples for FLIM-FRET measurements

To illustrate the power of FLIM-FRET in modern biomedical science, a couple of examples will follow. FLIM FRET applications cover not only biosensors for analyte detection, but also a wide variety of interactions of proteins, peptides, nucleic acids and so forth. Very typically, the fluorescent proteins CFP and YFP or derivatives are used which have emission in cyan and yellow. For better readability, the sketches show these emissions in green and red.

Ca^{2+} ions have many important roles in cell physiology, for example, as a secondary messenger in cellular signaling. It is an obligatory constituent in muscle contraction, enables neurotransmitter release, and contributes to the membrane potential of living cells. It is also an important factor for many enzyme reactions and blood clotting.

To investigate such functions, it is necessary to have a probe that operates fast and flexible within living cells. One of the standard FLIM-FRET biosensor examples is the Ca^{2+}-indicator "cameleon", a Calcium-meter (with reference to the introductory paragraph). There are a variety of cameleons, the name indicating the Ca-sensitivity and its change in color, like a chameleon [4].

The sensing agent in the cameleon probe is the Ca-binding protein calmodulin. It undergoes a conformational change upon binding to calcium. A construct with two fluorescence proteins, e.g. CFP and YFP, constitutes the FRET-biosensor (see Figure 5).

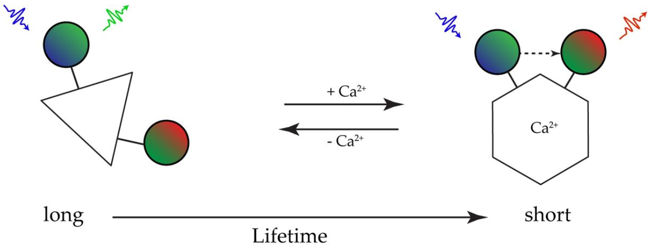

Figure 5: Left: The sensor without Calcium has the fluorescent proteins in a far distance. No FRET occurs and the lifetime is long. Right: upon binding of Ca^{2+}, the construct undergoes a conformational change that brings fluorescent proteins in close contact and allows FRET. The lifetime of the donor becomes short, which is the signal measured to monitor Ca^{2+} levels.

The cameleon Calcium sensor shows FRET upon binding of Ca2+ ions, i.e. the lifetime will shorten in the presence of calcium.

An equally famous second messenger in cellular signaling is cyclic AMP (cAMP) which was found to be the shape-coordinating agent during development of fruiting bodies of dictyostelium discoideum [5]. Cyclic AMP is the intracellular messenger of hormones that are not transferred through the cell membrane and, therefore, has a key part in carbohydrate and lipid metabolism. It is also involved in regulation of some ion channels.

Similar to cameleon, it was possible to create a biosensor by fusion of the cAMP-binding protein, Epac, with two fluorescent proteins [6][7].

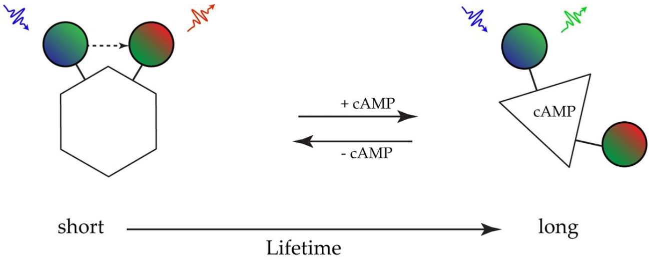

Figure 6. Left: Epac molecule in absence of cAMP. The fluorescent proteins are in close contact allowing FRET, the lifetime is short. Right: upon binding of cAMP, the construct undergoes a conformational change that brings the fluorescent proteins further away and prevents FRET. The lifetime becomes longer. That change is used as the signal for monitoring cAMP activity.

The cAMP sensor, Epac, shows FRET in the absence of cAMP, i.e. the lifetime will increase in the presence of cAMP.

One more approach is non-invasively mapping spatial and temporal changes of metabolic states in 3D cell cultures [8]. Here, a FRET-Biosensor (T2AMPKAR) is used to monitor activity of AMPK (5' adenosine monophosphate-activated protein kinase) in tumor spheroids. To sufficiently image the spheroids, excitation is performed by two-photon excitation.

These examples are just a very small fraction of what is possible with FRET measurements in biomedical research. Interaction of proteins, ligands with receptors, DNA with proteins, or segments of DNA with RNA are open to investigation with fluorescent methods. Many of them are already established for cuvette fluorescent measurement methods, but also are very good potential applications for dynamic imaging. The lifetime imaging field will, therefore, significantly increase in the near future.

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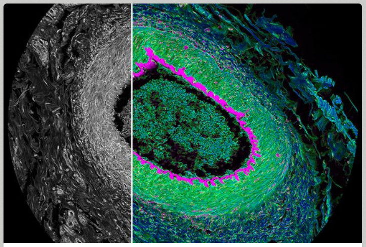

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