Fluorescence lifetime imaging microscopy (FLIM) to quantify protein-protein interactions inside cells

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Abstract

Recent developments in cellular imaging spectroscopy now permit the minimally invasive study of protein dynamics inside living cells. These advances are of interest to cell biologists, as proteins rarely act in isolation, but rather in concert with others in forming cellular machinery. Until recently, all protein interactions had to be determined *in vitro* using biochemical approaches: this biochemical legacy has provided cell biologists with the basis to test defined protein-protein interactions not only inside cells, but now also with high spatial resolution. These techniques can detect and quantify protein behaviours down to the single molecule level, all inside living cells. More recent developments in time-correlated single photon counting (TCSPC) imaging are now also driving towards being able to determine protein interaction rates with similar spatial resolution, and together, these experimental advances allow investigators to perform biochemical experiments inside living cells.

Keywords

time-correlated single photon counting; TCSPC; FRET; FLIM; SNARE

Fluorescence microscopes have initiated a revolution in biomedical fluorescence imaging during recent years. In particular, laser scanning microscopy provides high image quality mainly resulting from the fact that of out-of-focus light is strongly suppressed by a pinhole, or - in case of two-photon excitation - not excited. As a result, high contrast images are obtained, permitting true 3D imaging. Moreover, the scanning technique makes detection in several wavelength channels and multi-spectral detection relatively easy. More features, such as excitation wavelength scanning, polarization imaging, and second-harmonic imaging have been added in the recent years. These multi-dimensional features make laser scanning microscopes an almost ideal choice for fluorescence imaging of biological samples [1-4]. Importantly, however, the fluorescence of organic molecules is not only characterized by the emission spectrum, but also possesses a distinctive lifetime [5]; the fluorescence lifetime is defined as the mean amount of time a fluorophore spends in the excited state after the absorption of an excitation photon. Most fluorophores commonly in use in biological imaging have fluorescence lifetimes in the order of a few nanoseconds, but the fluorescence lifetime behaves in a predictable manner if the fluorophore is involved with any interactions with the microenvironment. Including fluorescence lifetime parameters in the imaging process thus provides a direct approach to all phenomena involving energy transfer between different fluorophores and their local environment [5-9]. Typical examples are the probing of the local environment parameters of a fluorophore via lifetime changes [10, 11], sensing

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distances on the nanometer scale by FRET [7], and separation of fractions of the same fluorophore in different binding states to proteins, lipids, or DNA [12].

Fluorescence lifetime imaging is particularly attractive in combination with multi-photon excitation, as these microscopes not only provide the required pulsed excitation source for time domain fluorescence lifetime imaging microscopy (FLIM) [13]; they also avoid crosstalk of the lifetimes at different depths of thick tissue by an inherent 3-dimensional sectioning capability. Furthermore, 2-photon excitation is often suitable for multiple simultaneous fluorophore excitation, has better depth penetration through biological samples and is less phototoxic to cells than 1-photon excitation because of the use of near-infrared wavelengths as opposed to the near-UV excitation used in 1-photon excitation.

A particularly efficient energy transfer process is Förster resonance energy transfer, (FRET). This describes an interaction of two fluorophore molecules, where the emission spectrum of one dye (the so-called donor) overlaps the absorption band of the other (the acceptor). In this case the energy from the donor is transferred more-or-less immediately to the acceptor. This energy transfer in itself does not involve any light emission or absorption. FRET results in an extremely efficient quenching of the donor fluorescence and, consequently, a decrease in the donor fluorescence lifetime. The energy transfer rate from the donor to the acceptor decreases with the sixth power of the distance and thus is apparent only at distances shorter than 10 nm [14, 15]. At the critical distance where 50% of the donor energy is transferred to an acceptor - the Förster radius [16] - the donor emission and fluorescent lifetime are each reduced by 50%, and sensitized emission (*acceptor* emission specifically under *donor* excitation) is increased. Because of its utility in reporting nanometer-scale interactions, FRET has become an important tool in cell biology [2, 6, 17-21]. FRET in cell biology is used commonly to verify whether labeled proteins physically interact: by measuring the FRET 'efficiency', distances on the nm scale (a scale within the globular radii of proteins) can thus be estimated using a light microscope. Obvious difficulties in intensity-based FRET measurements in cells is that the concentrations of the donor and acceptor are variable and unknown, the emission band on the donor extends into the absorption and emission band of the acceptor, and the absorption band of the acceptor commonly extends into the absorption band of the donor. A further complication is that only a fraction of the donor molecules interact with an acceptor molecule. These effects are hard to distinguish in intensity based FRET measurements. Nevertheless, a number of FRET techniques based on intensity imaging have been developed [17, 22-24]. These techniques need several measurements, including images of cells containing only the donor and the acceptor, or are destructive and therefore not easily applicable to living cells. Intensity-based FRET approaches are thus most useful when using reporter molecules constructed from fused donor and acceptors, because the relative concentration of the donor and the acceptor is fixed and optimal. In contrast, (TCSPC)-FLIM-based FRET techniques have the benefit that the results are obtained from a single lifetime measurement of the donor. These approaches do not need calibration in different cells, and are non-destructive. Moreover, TCSPC-FLIM may able to resolve the interacting and non-interacting donor fractions.

Here, we discuss a number of different considerations which should be taken into account when using FLIM to quantify fluorescent lifetimes in cells.

Advantages of time-correlated single photon counting (TCSPC) in imaging spectroscopy

The explosion in the use of fluorescent proteins, initially isolated from the pacific jellyfish *Aequoria victoria*, but now available from numerous sources, has made it possible for the biologist to examine the expression, distribution, colocalisation and dynamics of specific

proteins in cells [25]. All these parameters are limited by the optical resolution of the instrument; in the case of confocal laser scanning microscopes this is around 200-300 nm at best. Thus, alternative techniques have evolved to gain information about protein behaviour on the molecular scale.

From the viewpoint of detecting FRET between different fluorescent proteins, however, it is important that any overexpression of proteins is as controlled as possible. Aside from mis-targeting problems which often occur if heterologous proteins are hugely over-expressed, aggregates of (fluorescent) proteins can often result in a quenching of the fluorescent lifetime, presumably as a result of some efficient relaxational pathway. In this respect, the choice of model cell system is often also crucial. We have observed fluorescence lifetime quenching where a protein, not normally present in a particular cell-line, is over-expressed and thus cannot be correctly processed by the cell. This frequently results in large aggregates of fluorescent protein, with a reduced fluorescence lifetime compared to correctly targeted protein in a different cell type.

Finally it is obviously important to design the fluorescent fusion proteins carefully, so that the fluorophores will come within the critical distance for FRET to occur. It would be desirable to have a crystal structure available for every complex but this is simply not possible. A rational approach to fusion protein design can make experimental success more likely however.

Determination of bound *versus* unbound fractions within an interaction, inside cells

The choice of fluorophore is very important in order to resolve the maximum amount of data from a FLIM assay. For the purposes of this article, the discussion is limited to fluorescent proteins, as these have attracted a lot of attention from the FLIM field for obvious reasons.

A principal strength of TCSPC-FLIM is statistical accuracy, and this is reflected in the ability to fit two or sometimes three exponential functions to a particular fluorescence decay. The advantage of this is that it is often possible (depending on the quality of the data acquired) to determine the ratio of FRET versus non-FRET lifetimes contained within a single pixel of an image, and therefore to generate a map of interacting versus non-interacting proteins in the cell. These data may reflect the relative bound and unbound fractions within a particular protein-protein interaction reaction. It is difficult or impossible to obtain these data directly using any other FRET or FLIM approach.

Importantly for biochemists, these parameters are directly available using TCSPC-FLIM, and if the concentrations of the interacting proteins can be estimated (this is no mean feat inside cells, but it can be done), then estimates of association and dissociation constants can be made.

The resolution of a bi-exponential FRET system relies in part on the correct selection of fluorophore, particularly for the donor. Many fluorescent proteins have been shown to have complex fluorescence decays, often bi-exponential or even more complex, presumably due to protonation or some internal relaxation process, even in a non-FRET system. An example of this is enhanced cyan fluorescent protein (ECFP). This is commonly used as a FRET donor, because of its high quantum yield, long emission tail and relatively blue emission [26]. However, several fluorescence lifetime studies have revealed ECFP to have a bi-exponential lifetime [8, 27], making it difficult to resolve the interacting fraction from a non-interacting fraction in a FRET system (using FLIM); to do this, four lifetimes would need to be fit to the data (i.e. two for the non-FRET system, and two for the FRET system),

and it is beyond most FLIM measurements to acquire accurate enough data in a practical time period.

There are fluorescent proteins useful as FRET donors in FLIM measurements, however. Enhanced green fluorescent protein, EGFP has a high quantum yield, a mono-exponential decay in a non-FRET system and has been shown to be a good donor for red fluorescent proteins, such as dsRed and monomeric RFP [28] One disadvantage of EGFP, in our hands, is that it can be difficult to excite with a Ti;Sapphire laser, as longer NIR wavelengths are required. One advantage of EGFP for many cell biologists is that it has been in use for many years, so that cDNA constructs are often already made. More recently, the drive for a mono-exponentially decaying fluorescent protein led to the development of Cerulean, a variant of ECFP with all the advantages of a cyan donor, but a mono-exponential fluorescent decay [29]. We have used this protein extensively with some success as a FRET donor. It should be noted, however, that sometimes creating a fusion between the protein of interest and the fluorescent protein can alter the photophysical properties of the fluorophore. We have observed this when using a fusion to Cerulean. It is important therefore to check each fusion protein's lifetime properties before embarking on FRET measurements.

More recently, a 'dark' fluorescent protein was developed (REACH 1 and REACH 2; [30]). This approach has a number of advantages in FLIM experiments; the 'dark' FP can be used as an acceptor, and because it does not fluoresce, it can be used with a donor with very similar spectral properties. This is not normally feasible, because the donor and acceptor emissions need to be spectrally separated (using filtering). However, using REACH proteins with EGFP allowed the measurement of very high FRET efficiencies between a donor and an absorbing acceptor with greatly overlapping spectra. Second, the use of a 'dark' acceptor 'frees up' a band in the visible spectrum so that additional fluorophores can be used in the same experiment [30].

We have made a number of attempts to resolve bi-exponential donor fluorescence decays in a FRET system in cells. Aside from the considerations already discussed, the selection of photon counting detector is important here. Depending on the FRET system, determined by the proteins under study and thus the inter-fluorophore distance, it may not be possible to detect very short fluorescence lifetimes unless a fast detector is used. In one case, we could detect FRET between an EGFP-fused donor and a monomeric Red Fluorescent Protein (mRFP)-fused acceptor. However, using photomultiplier tube (PMT; Hamamatsu H7422) detectors, it was only possible to measure an overall quenching of the donor lifetime, not the appearance of a second, shorter FRET lifetime. These detectors have a time resolution of approximately 300 ps in our system. There are three possibilities for a mono-exponential quenching of the donor; either all the molecules are interacting in the FRET system, the interacting lifetime is too short to resolve, or the FRET versus non-FRET lifetimes are temporally similar and cannot be resolved by the fitting algorithm. We addressed this problem by using a faster multi-channel plate detector (MCP; R3809U, Hamamatsu). This detector has a much shorter transit time spread (TTS) very short lifetimes, down to approximately 25 ps. Using this detector with the same biological samples, it was possible to determine that there was indeed a very short lifetime population, with a mean lifetime of approximately 200 ps. The advantage of this is not just in the determination of the absolute lifetime, and thus potentially the inter-fluorophore distance, but in the measurement of the relative amplitudes of the interacting versus the non-interacting fraction of molecules per pixel in the image of the cell. This additional parameter, as discussed, may be of great interest to cell biologists. The disadvantage of using an MCP detector compared to a PMT is in cost, ease of use and robustness in a multi-user facility.

Acquisition speed

One accusation often leveled at TCSPC is that the data acquisition speed is too slow to be of use to cell biologists. This is a moot point; of course some biological processes are faster than others! The present state of the art is such that with fast detectors and bright samples, accurate FLIM measurement may be made in a few seconds for a 1024×1024 pixel image. Of course, the pixel resolution may be reduced, or post-acquisition data may be binned to further decrease the acquisition time at the expense of spatial resolution, and this is often satisfactory. One important consideration is that in order to fit more than one lifetime to the data (as discussed above, to resolve bound versus unbound fractions, for example), one needs more data [31]. However, for 'dynamic' measurements with spatial resolution, fitting a single exponential to a function known *a priori* to be bi-exponential can provide qualitative data over shorter time periods.

Another solution to the speed issue is as follows. A high resolution (both spatially and temporally) data set is acquired, and a region of interest (ROI) is selected. By 'parking' the laser beam on a single point, it is easily within the capabilities of a modern TCSPC system to acquire FLIM data at millisecond rates, and we are currently exploring these possibilities.

Concluding comments

FLIM is still an emerging technology, of great interest to cell biologist. One reason for lack of use of FLIM in biology laboratories is the technical difficulty associated with making reliable measurements. Although there are a number of different approaches to FLIM, some more 'turn-key' than others, TCSPC can resolve additional parameters which make the technical difficulties worthwhile. In particular, the ability to make statistically accurate estimates of the bound versus unbound fractions of fusion proteins inside living cells ought to be a very attractive prospect for cell biologists and biochemists.

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