# Basic principles of FRAP, FLIM and FRET

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## Introduction

Fluorescence microscopy offers a unique approach to the study of living and fixed cells because of its sensitivity, specificity and versatility. Fluorescence emitted from the biological sample can be simultaneously detected as both an image and as photometric data using the microscope, and has great potential for qualitative and quantitative studies on the function and structure of cells.

Stokes identified the phenomenon of fluorescence in the mid 19$^{th}$ century, and the first fluorescence microscopes were developed at the beginning of the 20th Century. These were used to study autofluorescence in organic and inorganic compounds. Imaging of secondary fluorescence (whereby specific tissues and bacteria which did not autofluoresce were labeled with a fluorescent marker and subsequently imaged) was developed in the 1930's by Haitigen, and by the 1950's, Koons and Caplan were using fluorescence microscopy to observe the location of antigens labeled with a fluorophore-tagged antibody (Wang and Lansing Taylor, 1989).

More recently, increasingly elaborate techniques including fluorescence recovery after photobleaching (FRAP), fluorescence lifetime imaging microscopy (FLIM), and fluorescence resonance energy transfer (FRET) have been developed that enable the visualization and analysis of ever more complex events in cells, organelles and sub-organelle components within the biological specimen. Our experience in this central facility, however, is that these techniques are often shrouded in jargon and can baffle the inexperienced microscopist to such an extent that they would rather try and avoid doing it altogether. This short article therefore attempts to dispel some of the mysticism surrounding FLIM, FRET and FRAP by describing the basic principles of these techniques, how they are performed on a variety of fluorescence microscopes, and what practical benefit they might be to the cell biologist.

## Back to basics – fluorescence and the light microscope

Before we consider the technicalities and practicalities of FRAP, FLIM and FRET, it is worth reminding ourselves of the principles of fluorescence and fluorescence microscopy. Each of these techniques in some way takes advantage of a particular aspect of the processes by which fluorophores are excited and damaged during excitation, or undergo non-radiative decay prior to photon emission, and a given system will necessarily have to employ different hardware to achieve this.

### *A little bit of quantum physics*

Fluorescence is a type of luminescence where light is emitted from molecules for only a short period of time following the absorption of light. When the delay between

absorption and emission is in the order of nanoseconds or less, the emitted light is called fluorescence. When this delay is in the order of microseconds, it is called delayed fluorescence, and a delay greater than this is called phosphorescence.

In fluorescence microscopy, naturally occurring autofluorescent molecules and introduced fluorophores targeted to cellular structures of interest are irradiated with high intensity light. When these molecules absorb a quantum of light, a valence electron is boosted up into a higher energy orbit, creating an excited state. When this electron returns to its original, lower energy orbit, termed the ground state, a quantum of light may be emitted (see Fig 1). Absorption occurs only at wavelengths of light whose quantum energy is equivalent to the difference in energy between the ground electronic state and the excited state. Consequently, a given fluorescent molecule will have a discrete wavelength at which it will become excited; this is know as its excitation spectrum.

Because fluorescence offers a pathway for a molecule to relax from an excited state back down to a non-excited state, it is known as a relaxation process. However, fluorescence is only one of a number of possible 'de-excitation pathways' available to molecules. One of the most fundamentally important of these is vibrational relaxation. The loss of some energy through vibrational relaxation means that less energy is available for emission as fluorescence. Because wavelength varies inversely to radiative energy, fluorescence emission is at a longer (i.e. lower energy) wavelength than the light used to excite it. This is known as Stokes Law, and the difference in emission wavelength relative to excitation wavelength is called Stokes shift (see Fig 2). Stokes shift varies among different fluorophores, so not only do fluorophores have characteristic excitation spectra, they have characteristic emission spectra as well (see Wang and Lansing Taylor, 1989 and Lakowicz et al., 1992).

***Fluorescence microscopy - what it means in practice***

The optical paths for image formation of the specimen are similar in fluorescence microscopy to those of a standard bright field microscope. The fundamental differences between bright field and fluorescence microscopy lie in the requirements of the fluorescence microscope to maximize the collection of emitted fluorescent light, while minimizing the collection of the incident excitation light. One of the key benefits of fluorescence microscopy, after all, is the increase in resolution through contrast made available by the selectivity of fluorophores to specific regions of the sample. Image contrast is critically dependent on the ability of the microscope to pass fluorescent light to the detector (typically a CCD camera or photomultiplier tube) while substantially blocking the excitation light.

Any light microscope relies on 3 components: an illumination source, a magnifying lens, and an image acquisition device. In the simplest light microscope this might consist of a candle, a convex lens, and the human eye. In a widefield microscope, the entire sample is illuminated simultaneously, and the image can be viewed directly either by eye or a camera. For a widefield fluorescence microscope, the candle is replaced by a high power lamp (typically a mercury or xenon source), which causes the fluorescently labeled

sample to emit light, and images are typically acquired using a CCD camera. As we have seen already, fluorophores have characteristic excitation spectra, so an excitation filter (usually a band pass filter) is placed between the lamp and the sample to narrow the bandwidth of light reaching the sample. We have also seen that emitted light will be at a longer wavelength than the excitation light, and therefore an emission filter (either a long pass or band pass filter) is placed between the sample and the camera to block the excitation light from the image.

Successful fluorescence excitation relies on an intense source of light. In the past 20 years or so, lasers have provided an alternative excitation source to mercury and xenon lamps, and are commonly utilised in confocal and mulitphoton laser scanning microscopy to illuminate the sample. Lasers generally produce high intensity, monochromatic light. Because the light source is monochromatic, no excitation filter is required. An emission filter is still necessary to stop the laser reaching the acquisition device. Laser scanning microscopes operate by scanning the laser over the sample and building up an image, pixel by pixel, throughout the duration of the scan by collecting emitted light with a photomultiplier tube. In the case of confocal laser scanning microscopy, the mercury lamp is replaced by the laser, the excitation filter is removed, and the camera is replaced by a photomultiplier tube. The respective merits and application requirements that have led to the development of widefield fluorescence, fluorescence deconvolution, confocal laser scanning, and multiphoton microscopes are beyond the scope of this article (see reviews in Pawley, 1995 for further information).

## FRAP – fluorescence recovery after photobleaching

Two distinct situations can occur during fluorescence imaging that lead to a decrease in fluorescence intensity, often referred to as fading. The first of these fading events is known as quenching. Fluorescence quenching is defined as a biomolecular process that reduces the quantum yield of a fluorophore without changing the fluorescence emission spectrum. It can be caused by oxidising agents, salts, heavy metals or halogen compounds as well as the transfer of energy from one fluorescent molecule to another one that is in close physical proximity. This transfer of energy is known as fluorescence resonance energy transfer, and this relationship is measured in FRET experiments, as we shall see later. The other event that causes fading is photobleaching. In this case, high light intensity in the presence of molecular oxygen causes irreversible damage to the fluorophore and inhibits it from emitting light. The deliberate bleaching of a specific region of the sample, and the rate of recovery of this photobleached region are measured in FRAP experiments. The principles, practice and merits of measuring FRAP are described below.

### *The underlying principles*

Photobleaching is defined as the permanent destruction of fluorescence by a light induced conversion of the fluorophore to a chemically non-fluorescent compound. Photobleaching requires light and molecular oxygen for most fluorophores. For live-cell

studies, the removal of oxygen is not an option, therefore the dose of light received by the sample is usually carefully controlled in fluorescence imaging to avoid bleaching.

Fluorescence recovery after photobleaching is a quantitative fluorescence technique that can be used to measure the dynamics of molecular mobility in 2D by taking advantage of the fact that most fluorophores are irreversibly bleached by incident light of very high intensity.

***FRAP in practice***

In practice, FRAP requires that a series of fluorescence intensity images are first collected to give a value for intensity in both the region of interest and the surrounding sample. Following this, a defined region of the sample is illuminated with high intensity light causing the fluorophore within that region to become photobleached. This creates a darker, bleached region, within the sample. Photobleached molecules are subsequently replaced by nonbleached molecules over time, and this results in an increase in fluorescence intensity in the bleach region. For a FRAP experiment to provide meaningful data, it is important that the sample is not photobleached during the pre-bleach or recovery phase of the experiment and that the camera or PMT is not saturated.

Fluorescence microscopy is often a question of compromise; an obvious example is the trade off between temporal and spatial resolution in a time course experiment (i.e. we lose spatial information if we acquire images quickly, but we lose information about the dynamic processes occurring in our biological sample if we image slowly in order to increase spatial resolution). Simply put, FRAP is a glorified time course experiment in which we must acquire data (in this case, fluorescence intensity values) quickly in order to successfully record the recovery of our bleached region. For quantitative FRAP experiments the image is often far less important and it is therefore usually the case that we trade spatial resolution for temporal resolution.

Recovery of fluorescence into the bleached area occurs as a result of the diffusional exchange between bleached and unbleached molecules. The fraction of fluorescent molecules that can participate in this exchange is referred to as the mobile fraction. The fraction of molecules that cannot exchange between bleached and nonbleached regions is called the immobile fraction. FRAP experiments have demonstrated that most fluorescently labeled cellular components do not share the continuous, unrestricted lateral diffusion associated with the diffusion characteristics of random membranes. Instead, diffusion is inhibited by a combination of constraining effects within the cell. Knowledge of the rate of molecular exchange, elucidated by FRAP, can provide important insights into the properties and interactions of molecules within the cellular environment.

FRAP experiments are commonly performed on laser scanning microscopes, in which low laser power is used to image the sample in the pre- and post-bleach phases of the experiment. In the bleach phase, a region of interest is scanned at higher laser power to cause photobleaching. Temporal resolution may be improved if a widefield system

equipped with a laser for photobleaching is used. In the example below, a widefield system equipped with a 488 nm diode laser was used to measure FRAP in a nuclear protein labeled with GFP that accumulates in the nucleolus (Fig 3).

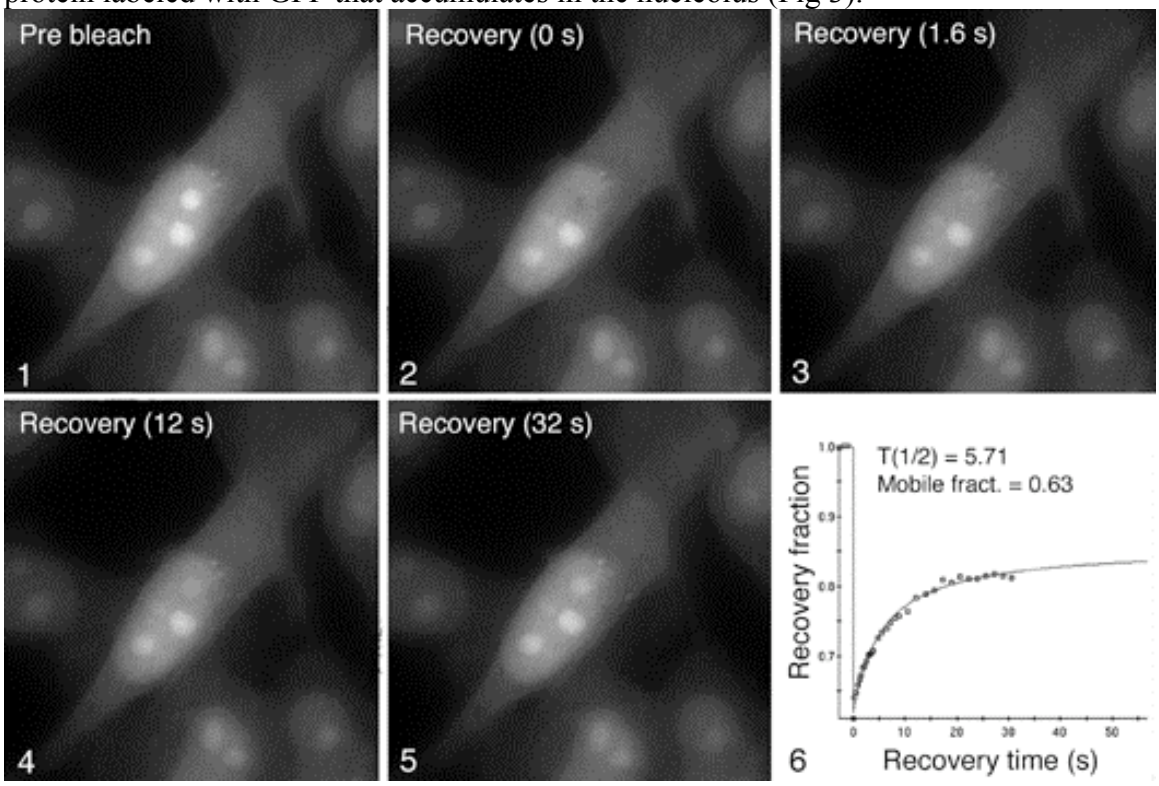


**Figure 3.** An image is taken prior to photobleaching (1), a region of interest is bleached to approximately 50% of its original intensity (2), images are acquired after the photobleach period (3-5). For qualitative FRAP, it may be enough to simply plot fluorescence intensity over time (known as the recovery curve), or just evaluate the time course images obtained. The function of true FRAP analysis, however, is to fit the recovery curve to a predefined model (6). The mobile fraction represents the fraction of recovered fluorescence. T(1/2) is the point at which the half-height recovery intersects the recovery curve. T(1/2) can then be used to calculate the diffusion coefficient (see Axelrod, 1976, for a review of FRAP analysis).

**FLIM – fluorescence lifetime imaging microscopy**

We have already seen that fluorophores have characteristic excitation and emission spectra, and that fluorophores can be distinguished by careful selection of excitation and emission filters. In the case of fluorescence lifetime imaging microscopy, these spectra are less important for resolving the fluorescent component of the sample, but instead the lifetime of the fluorescent event (i.e. the time it takes for the fluorophore to become excited and return to the ground state) is measured. The principles that make FLIM possible, and the techniques involved in measuring fluorescent lifetime are described below.

***Basic principles***

The fluorescence of organic molecules is not only characterized by their excitation and emission spectra, but also by their lifetimes. When a fluorophore absorbs a photon it goes into the excited state and returns to the ground state by emitting a fluorescence photon, converting the energy internally, or by transferring the energy to the environment, or a combination of some of these. The probability that one of these effects occurs is independent of the time after excitation. Molecular excitation is stochastic, but the lifetime of a population of molecules can be plotted. If a large number of similar molecules with similar local environments are excited by a short laser pulse, the time taken for fluorescence to decay can be plotted as a single exponential curve. Providing that no energy is transferred to the environment, the lifetime described by this curve is called 'natural' fluorescence lifetime.

If energy is transferred to the environment, the actual fluorescence lifetime is shorter than the natural fluorescence lifetime. For almost all fluorophores, the rate of energy transfer to the environment depends on the concentration of ions, oxygen, pH value or the binding of proteins in a cell. There is a direct relation between the concentrations of these ions, called fluorescence quenchers, and the fluorescence lifetime of the fluorophore. Consequently, FLIM can not only be used to discriminate between different fluorophores on the basis of their characteristic lifetimes (rather than their spectral properties) but also to distinguish among different environments within the cell based on changes in lifetime of the same fluorophore if it is present in local environments containing varying concentrations of fluorescence quenchers.

***FLIM in practice***

Several fluorescence lifetime imaging microscopes are commercially available, including systems for widefield imaging and confocal and multiphoton scanning systems. It is beyond the scope of this article to describe the fundamental differences between the approaches that have been employed by various manufacturers, however they are similar in regard to the fact that FLIM measurements in every case depend on the acquisition computer registering a 'start' signal (the point at which the sample is irradiated) and a 'stop' signal (the point at which fluorescence decay has finished).

Two photon pulsed lasers, typically employed for mulitphoton imaging, lend themselves well as light sources for FLIM because they deliver discrete and consistent bursts of light to the sample. By a happy coincidence, the lifetime of most fluorescence markers is in the order of a few nanoseconds, and most commercially available pulsed lasers deliver light every 10 nanoseconds. This provides an excellent window for lifetime imaging. It should be noted, however, that most fluorescence sources can be adapted for FLIM imaging providing the light that reaches the sample is effectively modulated (for a full account see Pawley, 1995 and Becker and Bergman, 2003).

A FLIM-enabled multiphoton microscope is shown below (Fig 4). Although this looks a little terrifying at first sight, the important parts of the system are relatively straightforward. Essentially, the pulsed laser irradiates the sample and simultaneously

sends a message to the FLIM computer telling it that everything has started. The photomultiplier tube then records fluorescence from the sample until the next laser pulse arrives, which signals that the process has stopped. In the interval between the 'start' and 'stop' signal, fluorescence from the sample will necessarily have decayed, and enables the software to calculate a lifetime value for the fluorescently-labeled components of the sample (Fig 5).

**Figure 4.** A variety of FLIM systems are commercially available for Widefield and laser scanning microscopes. In this case, a FLIM-enabled multiphoton laser scanning microscope is shown, including pulsed laser, microscope, PMTs, and computer to record and measure intensity and lifetime data.

**Figure 5.** At first site, a FLIM system can seem overly complex when in reality the basics are fairly easy to grasp. Illumination is provided by a pulsed laser (1), the pulsed laser excites the sample (2) and simultaneously sends a start signal to the computer (3). Fluorescence is emitted by the sample and recorded on the PMTs (4) while the next pulse of light leaves the laser and sends a stop signal to the computer. The process is repeated many times so that an average lifetime can be calculated for the population of excited fluorophores.

If we remember that FLIM is a measurement of the probability of fluorescence emission of a certain lifetime, then it follows that the process should be repeated may times to build up an 'average' fluorescence lifetime for every pixel that has been imaged. Consequently, as with every fluorescence imaging technique, we are faced with a tradeoff between the time it takes to acquire the image (temporal resolution) and the statistical probability that the measured lifetime is actually correct.

In the image below (Fig 6), a lifetime map has been plotted (i.e. each pixel is colour coded with a lifetime value to build up an image of differences in lifetime throughout the sample) using a multiphoton based FLIM system, and is shown next to a corresponding intensity image. Although this is a very basic example of a lifetime image, measurement of fluorescence lifetime and the construction of lifetime maps can provide the means to quantitatively measure the concentration of molecules and ions within cells, pH, and oxygen. It can also discriminate between spectrally similar fluorophores and has been used to measure fluorescence resonance energy transfer.

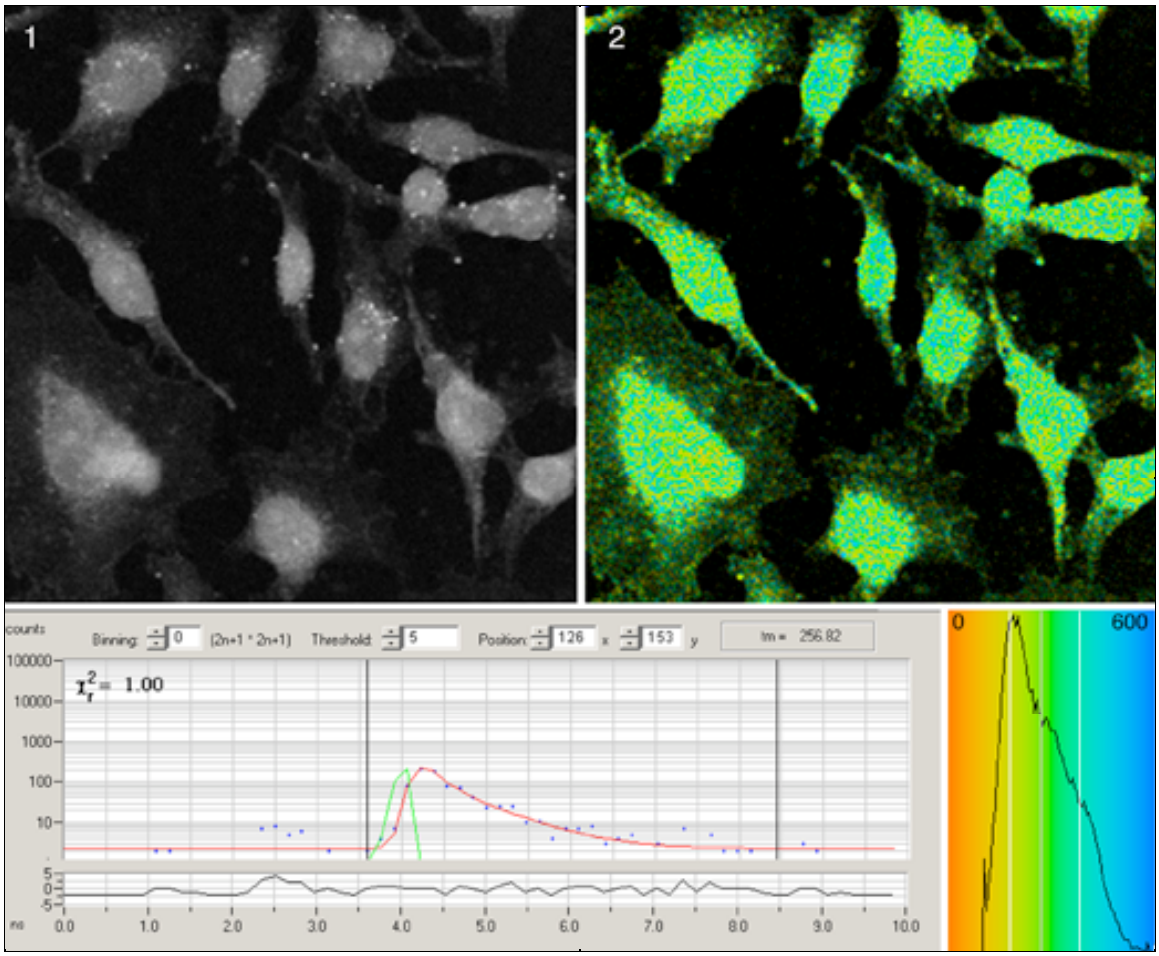


**Figure 6.** A nuclear protein has been labeled with CFP and imaged using a multiphoton FLIM system. It is helpful to compare a fluorescence intensity image (1) with the fluorescence lifetime map (2) in order to compare lifetimes among different regions of the sample. In this case, the lifetime for CFP is approximately 500 picoseconds.

**FRET – fluorescence resonance energy transfer**

It is well established that appropriate labeling of biological material and careful imaging provides a powerful tool with which to visualize the spatial distribution of cellular structures of interest. Double labeling of samples using spectrally distinct fluorophores targeted to, for example, different proteins, can also determine whether a pair of proteins are co-localized. In fact, microscope optics are so good now, that the spatial resolution of the fluorescence microscope is limited by the wavelength of light. This provides a maximum resolving power of about 5 μm.

The exploitation of fluorescence resonance energy transfer can increase the spatial resolution of the fluorescence microscope to below 10 nm. This dramatic leap in resolution is what makes FRET so appealing as a tool for studying co-localization events in biological samples, particularly when you consider that it can be performed in live cells.

***Underlying principles***

The reason FRET improves spatial resolution is because it relies on the close physical interaction of 2 fluorophores. These fluorophores are called the donor and the acceptor. FRET does not occur if the distance between these fluorophores exceeds 10 nm. Two other requirements for FRET must also be met: 1. the emission spectrum of the donor must overlap the excitation spectrum of the acceptor and 2. the donor and acceptor must be appropriately orientated to allow energy transfer (see Fig 7). Fluorescent proteins and fluorescent dyes have been successfully used as FRET pairs in the past. Commonly used FRET pairs are CFP (donor) and YFP (acceptor), and FITC (donor) and Rhodamine (acceptor). Three methods for measuring FRET are routinely used, namely; FLIM-FRET, acceptor photobleaching and sensitized emission. These techniques are discussed below.

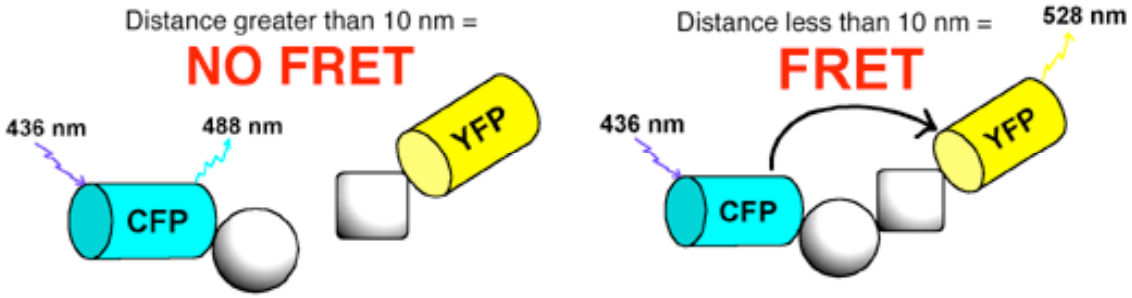


**Figure 7.** FRET depends on the close proximity of a donor and acceptor pair of fluorescent molecules in which the emission spectrum of the donor overlaps the excitation spectrum of the acceptor. Because these molecules must be within 10 nm for FRET to occur, the spatial resolution of the microscope is significantly improved.

***FLIM-FRET***

We have already seen that FLIM provides an excellent method for measuring fluorescence quenching events within cells, and that fluorescence resonance energy transfer is a very efficient fluorescence quencher. The fluorescence lifetime of a fluorophore is affected by FRET, and in the presence of a suitable acceptor, the lifetime of the donor will decrease. It is this decrease in lifetime that FLIM-FRET seeks to measure.

In such an experiment, the fluorescence decay function of a donor/acceptor population will contain the fluorescence of quenched and nonquenched molecules. Therefore the decay function is double exponential. Quantitative measurements of FRET require a value for natural lifetime of the donor, and the lifetime of the quenched donor (FRET lifetime). FRET efficiency can then be calculated based on the ratio of quenched and nonquenched fluorescence components.

Although FLIM-FRET is a robust method for calculating FRET efficiency, it can be a costly exercise both in terms of financial outlay (requiring a modulated light source and sensitive detectors) and time (to work out how all the equipment works). Fortunately, FRET can also be done using both conventional fluorescence microscopy and confocal laser scanning microscopy.

***Acceptor photobleaching***

As we have seen, when 2 appropriately labeled proteins are co-localized, fluorescence of the donor molecule is quenched because the energy that would normally be emitted as fluorescent light is transferred to the acceptor molecule. Consequently, FRET can be measured by deliberately photobleaching the acceptor molecule in order that energy from the donor can no longer be transferred to the acceptor. This leads to an increase in fluorescence of the donor molecule. All we have to do, then, is measure the fluorescence intensity of the donor molecule, bleach the acceptor, and then re-measure the intensity of the donor. The difference between these donor intensity measurements enables us to calculate FRET.

The principle benefits of this approach are that it is relatively straightforward and can be done on any fluorescence microscope with a powerful enough light source to bleach the acceptor and appropriate filter sets. Unfortunately it is not all good news. Not only can photobleaching of the acceptor cause photodamage to the sample, but in live-cell studies there is a significant threat that our FRET measurement will be invalidated by recovery of the acceptor fluorophore (just like in a FRAP experiment). In reality, this means it is probably inappropriate to use acceptor photobleaching in live-cell studies (see Bacskai et al., 2003).

***Sensitised emission***

A final possibility is to measure FRET by sensitized emission. In this case, only the donor molecule is excited and fluorescence is measured in the acceptor channel only. The theory behind this is that because the donor is excited and transfers its energy to the acceptor causing it to become excited instead, some of the signal in the acceptor channel will be the result of FRET. "Some" is the operative word, however, because in reality a fraction of this measured fluorescence will be due to direct excitation of the acceptor from the light used to excite the donor, and a fraction of measured fluorescence will be from fluorescent light coming from the donor.

The way round this is to prepare "donor-only" and "acceptor-only" samples as well as the FRET sample. First, donor excitation needs to be used to excite the acceptor-only sample and the fluorescence must be measured in the acceptor channel. Second, donor excitation needs to be used to excite the donor-only sample, and again fluorescence must be measured in the acceptor channel. This provides an essential control for our FRET calculation. Finally, the donor/acceptor FRET sample is imaged by exciting the donor and measuring fluorescence in the acceptor channel. Now we can subtract the

measurements we made for the donor-only and acceptor-only samples, and any fluorescence that is left is considered to be the result of FRET.

The low levels of light and the significant levels of crosstalk between donor and acceptor excitation and emission means that this approach is less robust than it might appear. However, if you do not have access to a FLIM microscope and you need to do FRET on live samples, this represents the only viable option. In the example below, sensitized emission has been used to measure FRET in 2 nuclear proteins (Fig 8).

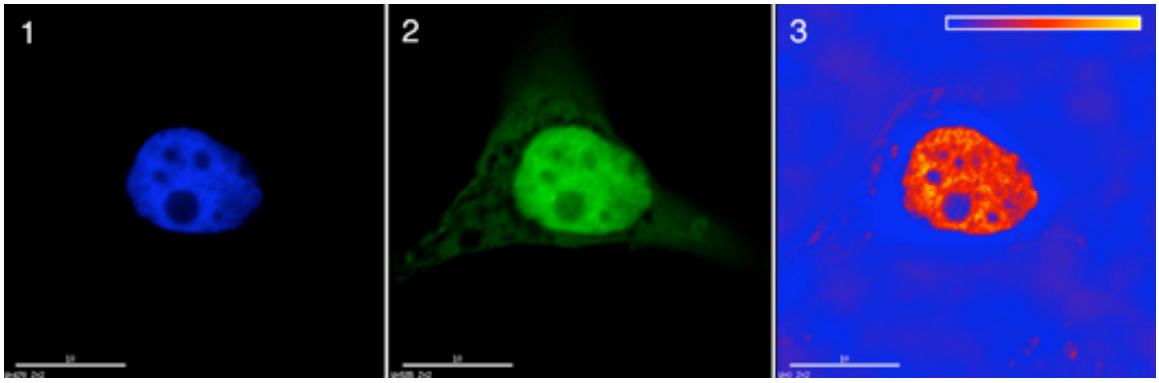


**Figure 8.** The FRET pair CFP (donor) and YFP (acceptor) were used to label 2 nuclear proteins co-localized to interchromatin granules. This was done on a widefield fluorescence microscope using standard CFP/YFP filter sets (available from Chroma). FRET efficiency varied throughout the cell, with most FRET occurring in the nucleus.

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