Caught on Camera with Another Protein – Just Good Friends or Something More?

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Over the past 10 years, microscope systems have become more advanced yet are increasingly marketed as being simple to use. In the same time period, there has also been a general shift in microscopy provision in academic institutes from a "single lab, single microscope" model to a centralised approach, resulting in the onus on training and quality control often falling on facility staff. Here we attempt to provide an example of how the interaction between facility and research staff can lead to improvements in image quality and reduce the likelihood of a member of research staff "over interpreting" their data. In this case, we have described a work flow from the fairly simple job of identifying the localization of a single protein within the cell, through the practicalities of doing a colocalization experiment for 2 fluorescently tagged proteins, ending with the use of a fluorescence resonance energy transfer (FRET) experiment to determine whether these colocalized proteins are actually interacting.

In our previous articles in this publication we have discussed the basic principles of light and fluorescence microscopy and have suggested that the light microscope has a vital role in modern biological research. In recent years, academic institutes have increasingly moved towards a centralised approach to light microscopy provision and this is certainly the case in the College of Life Sciences at the University of Dundee, where the majority of high specification microscopy equipment and imaging software is now centrally managed.

There are a variety of fundamental benefits to be gained from centralising an imaging resource. These typically include:

1. The ability to provide expert knowledge that is retained beyond the lifespan of a typical studentship or postdoctoral appointment.
2. Efficient supervision and management of complex equipment, providing an area where research staff can interact with each other and operate equipment safely.
3. Equipment management, not only in terms of maintenance and time management, but also technical evaluation, purchasing and upgrades.
4. Effective staff management and the implementation of an effective long term financial strategy, either through co-ordination of grant applications or through a cost recovery mechanism.
5. Providing expert knowledge to users on what the best approach is for a given experiment and to provide a level of quality control.

This change in approach is borne out by the increase in articles that seek to address the practical requirements of setting up and/or running a central light microscopy facility (see Anderson *et al.*, 2007). Furthermore, a number of articles have been written that raise concerns about the validity of some published microscopy data, particularly that collected by inexperienced microscopists (e.g. North, 2006). There is also an extensive catalogue of techniques papers and protocols available for the research scientist to peruse with the aim of designing or improving their experimental techniques.

However, comparatively little appears to have been published that describes the kind of day to day interactions between research and microscopy staff that enables inexperienced microscopists to develop their skills and to acquire high quality data from the very first time they turn on a microscope.

Stumbling into a room and being expected to competently use a half a million pounds worth of hardware can be a particularly daunting experience for the new user who may literally have never seen, let alone used, a microscope before. With this as a starting point, it is unreasonable to expect this person to understand the differences between, say, a laser scanning microscope and a widefield fluorescence system or the potential benefits and shortcomings of each. Another common problem is that users may have come from an institute without a central facility and be familiar with only the specific piece of equipment available in their previous lab (usually a confocal microscope). Often there is some resistance to change to a different type of microscope, such as multiphoton or deconvolution, that might confer significant advantages, given that things had always worked perfectly well on a confocal microscope in the past. This is where central microscopy facility staff really earn their salaries, and can be the most exciting and challenging part of the job.

Where in the Cell is it?

Nowadays it is a relatively straightforward exercise to capture an image on a fluorescence microscope. Predefined methods are available on most systems

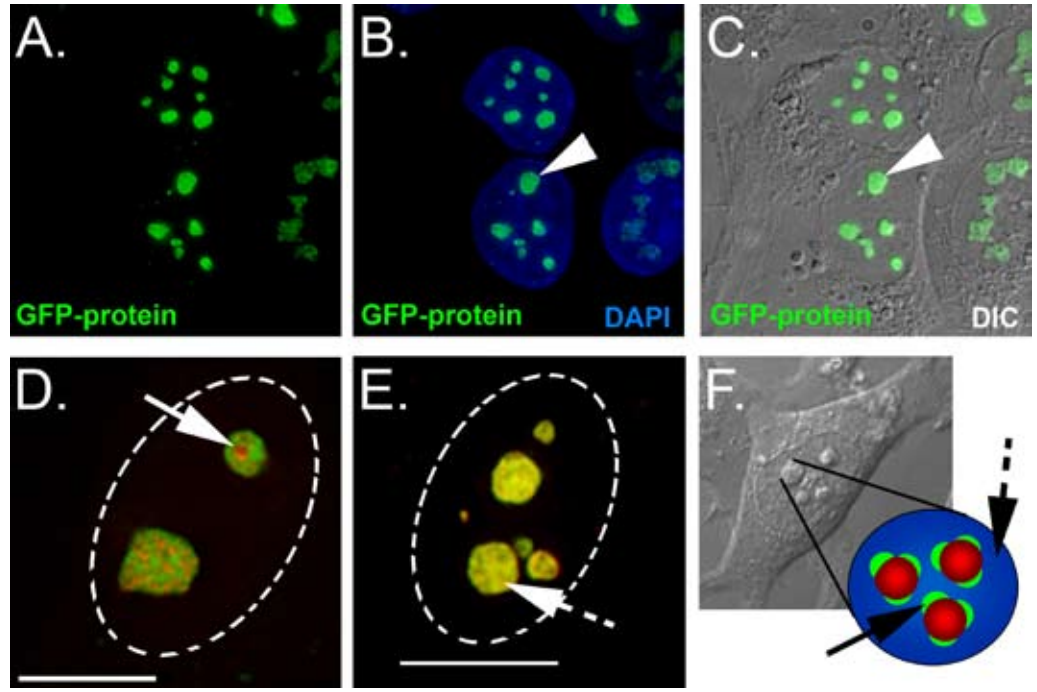


Fig. 1. Determining intracellular localization of a GFP-tagged or immunostained protein. A facility user arrived with the micrograph shown in panel A, representing the localization pattern of their GFP-tagged protein in HeLa cells. The protein was determined to be predominantly nuclear, as judged by its exclusive localization within the Hoechst 33342-stained chromatin region (B). Specifically, it accumulated in regions that are less chromatin-dense, suggesting localization to nucleoli (arrowhead). This was confirmed by a DIC image that clearly shows the protein localizing within the nucleus and limited to the phase-dense regions that are known to be nucleoli (C; arrowhead). More detailed subnucleolar localization was determined by counterstaining cells expressing the GFP-protein with antibodies to markers for specific regions within this structure. In Panels D-E the nucleus is defined by a hashed circle, and the GFP-protein (green) shown to colocalize with a TRITC-stained marker for the granular component (E; hashed arrow), but not the dense fibrillar component (D; arrow). Panel F shows the clear visualization of cytoplasm, nuclei and nucleoli in a DIC image, and a diagram of the subnucleolar structure, indicating the granular component (hashed arrow) and dense fibrillar component (arrow).

such that a new user might only need to turn on a computer, focus a slide and click the "acquire" button in order to collect an image of reasonable quality. However, acquiring meaningful data requires a good understanding of optics and fluorophores, an understanding of how artefacts might be generated, the experience to recognize and control for them, as well as an obsession for detail bordering on the anally retentive. These skills can take many years to learn, but increasingly it falls to the collective experience of central microscopy facility staff to ensure that data collected on an institute's microscopes is scientifically robust and of publishable quality. It is often the case that, due to the requirement to publish quickly, a member of research staff might arrive at a microscope without the time to fully understand the complexities of the equipment they are using to acquire data or the experience to avoid the plethora of pitfalls awaiting the unwary.

In this first "microscopy case study" we have chosen to explore one of the basic and, on the face of it, simplest of questions the light microscope is frequently used to answer. Namely, where is my protein of interest located in my cell and what is it doing there? We attempt to describe the work flow from this fairly basic single protein localization experiment, through imaging of 2 FP-tagged proteins (i.e. colocalization) and finally how to determine whether these colocalized proteins interact.

I think it is fair to say that most beginner microscopists often have difficulty interpreting the localization pattern of their protein of interest,

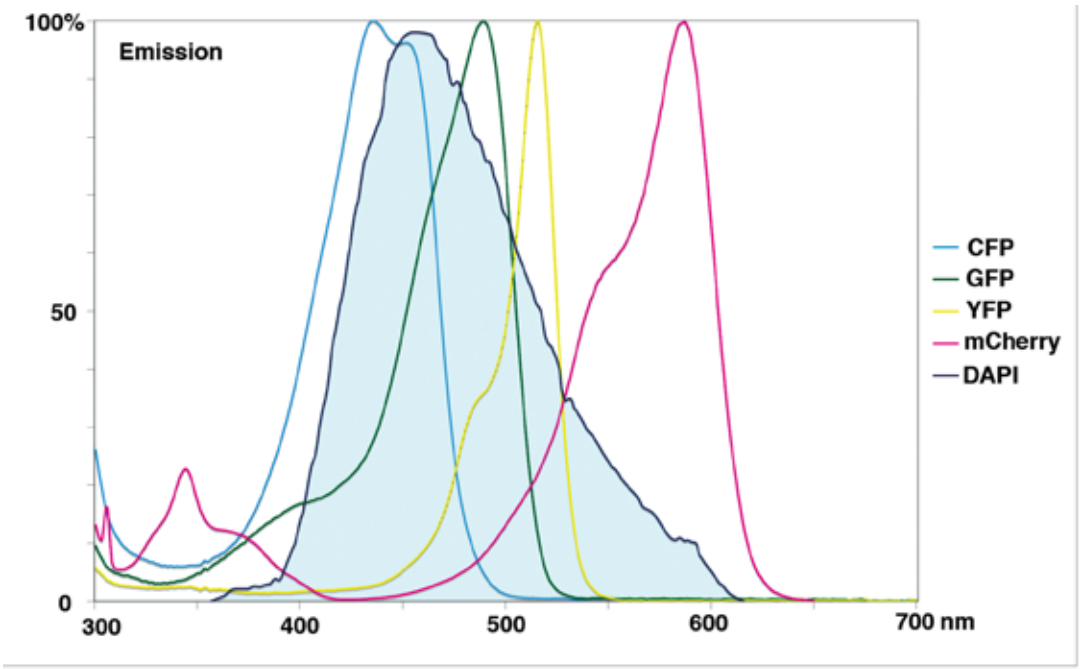


Fig. 2. Emission spectra for commonly used fluorophores. The emission spectra for DAPI is quite broad and overlaps the emission spectra for many commonly used fluorophores, as shown here by the shaded area beneath the DAPI spectra (data derived from Giepmans et al, 2006, http://www.tsienlab.ucsd.edu/Documents.htm and http://www.analytchem.tugraz.at).

often because they are unfamiliar with the structure of their cell as it appears in a micrograph or because they are not familiar with some of the techniques available to determine where their protein really is in the cell (and on the scale afforded by the light microscope).

A recent example from a user of this facility is given below. Here, a colleague arrived with a slide of cells with the localization pattern of the gfp-tagged protein shown in Figure 1A. On first impressions, this looked like an intriguing intra-nuclear localization pattern, but it might just as likely have been GFP aggregates or lysosomes in the cytoplasm. Generally, the first thing to do is determine where the protein is localizing relative to the nucleus and cytoplasm, bearing in mind that it may be found in both.

A good starting point is to stain the cells with a DNA marker such as DAPI (for fixed cells) or the cell-permeable Hoechst 33342 (for live cells) to visualize chromosomes within the nucleus. As shown in figure 1B, this particular protein is exclusively nuclear. Another, less invasive option, is to distinguish the cytoplasmic and nuclear domains structurally, by taking either a phase contrast or differential interference contrast (DIC) image (Figure 1C). "Old school" microscopy techniques such as phase contrast and DIC seem to be less popular in these days of ultrafast lasers and super-sensitive cameras, but there are 2 major benefits to using these approaches, particularly in live-cell imaging, namely; i) the wavelength of the tungsten filament lamp is far less damaging than the UV light used to excite most DNA stains, and ii) there is no danger of bleed through into your second (e.g. GFP) channel. DAPI and Hoechst 33342 have relatively long emission spectra that are likely to appear in your fluorescent protein (FP) channel. This problem will be significantly increased if the DAPI signal is very strong and the FP signal relatively weak (see Figure 2). For more information, both Nikon and Olympus have excellent microscopy primers on their websites that are well worth a look (www.olympusmicro.com/primer/index.html and www.microscopyu.com/articles/formulas/formulasindex.html).

Once the global localization of the protein has been confirmed, it then makes sense to use markers for subnuclear or subcytoplasmic domains to narrow down the localization pattern to a more specific region. For example, the fact that the nuclear protein in Figure 1 accumulated within regions that were not stained heavily with DAPI indicated that it was most likely nucleolar, as these regions are less chromosome dense (Figure 1B). Similarly, in the DIC image (Figure 1C), the protein is shown to accumulate in the phase dense nucleolar regions of the nucleus.

Like many intracellular domains, the nucleolus itself can be divided into further subdomains. Using markers for specific subnucleolar regions, the protein was shown to be excluded from the dense fibrillar component (Figure 1D), as shown by the lack of overlap of the GFP signal (in green) with the TRITC signal of an antibody marker for this region (in red). The protein was shown to localize specifically to the granular component (Figure 1E), as shown by colocalization of the GFP signal (green) with the TRITC signal of an antibody marker for this region (red).

Although the example above is for a specific nucleolar protein, this approach to narrowing down the localization pattern of a given protein to a particular sub-cellular domain can be applied to more or less any protein located in any compartment of the cell (see Allan, 1999, for a more thorough practical overview of protein localization by fluorescence microscopy).

Determining the localization of a particular protein is, as we have seen, comparatively straightforward if potentially time-consuming. The situation can become somewhat more complicated if a microscope-user wishes to find out whether two proteins are colocalized or even interacting. Sadly, it is not simply the case that you can determine the location of Protein A and, if you see Protein B in the same place, conclude that they are colocalized, for the reasons discussed below.

Colocalization

In practical terms, colocalization can be described as the presence of the signal from 2 protein-tagged fluorophores at the same pixel location when examining multichannel fluorescence microscopy images. The channels are generated by two (or more) different fluorophores when visualizing respective antigens and within the same sample region. (Zinchuk and Zinchuk, 2006).

The complicating factor is that colocalization can only be claimed in the absence of "cross talk" or "bleed through". That is, where one is certain that the signal generated in each channel is the direct result of excitation and collection of a single fluorophore in each channel, rather than an artifact generated by light from one channel bleeding through into the second channel (North, 2006).

The first key to success is in ensuring that fluorophores are selected whose excitation and emission spectra are well separated. If their excitation spectra overlap, the wavelength of light used to excite one fluorophore will excite the second fluorophore as well (and vice versa). If the emission spectra overlap, fluorescence emitted by both fluorophores may be collected in each channel.

The second key to success is to use appropriate (and tight) excitation and emission filters in conjunction with these well separated fluorophores. A narrow, bandpass filter will undoubtedly provide superior results to a longpass filter. An example of

a poor combination of fluorophores is CFP and GFP, which are extremely difficult to separate on most fluorescence microscopes. By comparison, GFP and mCherry are a good combination and easily separated using standard FITC and TRITC filter sets. GFP and YFP can be discriminated between if narrow bandpass excitation and emission filters are used.

Finally, one must take care to discriminate between the fluorescence signal of the fluorophore and any background signal from autofluorescence.. This can be particularly problematic in plant cells containing certain pigments e.g. chlorophyll, or in animal cells rich in highly autofluorescent proteins or in cultures containing large numbers of dead cells. It is therefore important to image non-labeled samples first to establish a threshold for the autofluorescenct component, then to image single-labeled samples in BOTH channels to ensure that there is no bleed through (see Manders *et al*, 1993; Allan, 1999, and North 2006 for more information). Additional controls such as staining with preimmune sera and with fluorescent secondary antibodies alone are recommended for immunostaining experiments, to ensure that the measured signals are genuine.

Even when the fluorescent signals have been carefully measured, it is easy to overinterpret the results in a colocalization experiment. For example, users often assume that the presence of a "green" (e.g. GFP, FITC, CFP) and "red" (e.g. mCherry, TRITC, YFP) signal in the same location ("yellow" in a merged image) implies colocalization. As shown in Figure 3A, CFP and YFP alone, when co-expressed in cells, show a similar localization and the merged image appears uniformly yellow. These proteins are merely diffuse throughout the cell, however, and do not localize to specific structures or interact. Genuine colocalization is therefore difficult to demonstrate for diffuse proteins, and care must be taken that such results are not over-interpreted. It is far easier to demonstrate colocalization of proteins that accumulate at a particular location within the cell.

In the examples shown in Figures 3B-C, a facility user was able to demonstrate that proteins he had determined play a role in pre-mRNA splicing show an expected accumulation within the subnuclear domain known as interchromatin granule clusters (IGCs) or "speckles," where many splicing factors accumulate. Thus, microscopy based localization data was used to validate biochemical data. Furthermore, the proteins were shown colocalizing both in the speckles and in the nucleoplasmic pool. In order to ascertain that they were genuinely colocalized, an unlabeled sample was first imaged to ensure there was no autofluorescence, followed by imaging a CFP-only and YFP-only sample to ensure that there was minimal cross talk. This required the use of a specific CFP/YFP filter set. In a central microscopy facility the user may not always have a choice over which filter sets are installed on the microscopes, so it is important to check what is available prior to preparing samples. GFP and mCherry are a good choice for this type of colocalization experiment, as they can be imaged using the more standard FITC and TRITC filter sets.

Although the observation of colocalization of the proteins of interest gives researchers valuable clues regarding their structural and functional characteristics, it does not provide direct proof of their interaction at specific sites within the cell. For example, in the case shown in Figures 3B and C, the colocalization patterns of the three proteins fit with their expected intracellular functions and suggest that all three are part of the mammalian spliceosome machinery. However, although it is tempting to conclude that they must therefore interact directly, this cannot be proven by colocalization alone.

The spatial resolution of light microscopy is limited by the wavelength of light; in practical terms this is currently about 200 nm for most high end

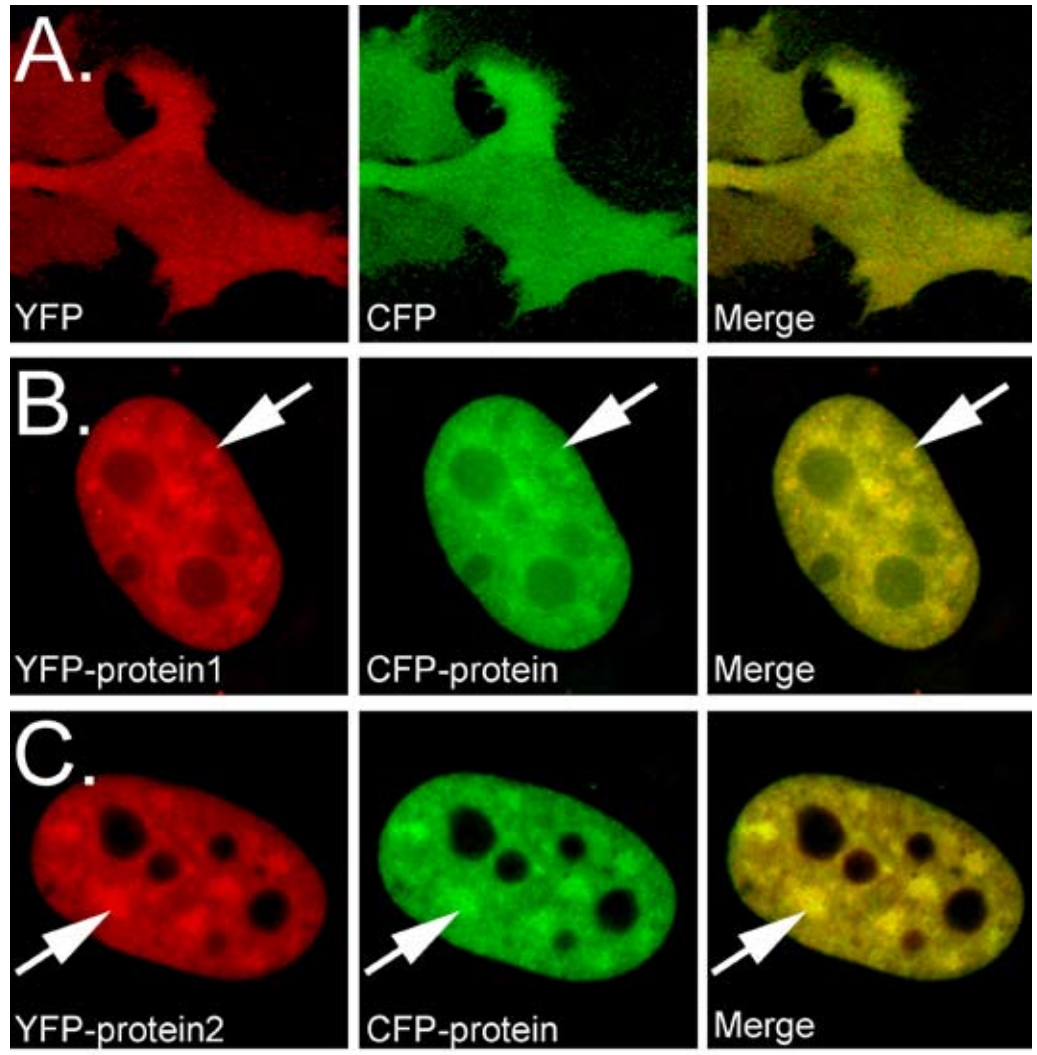
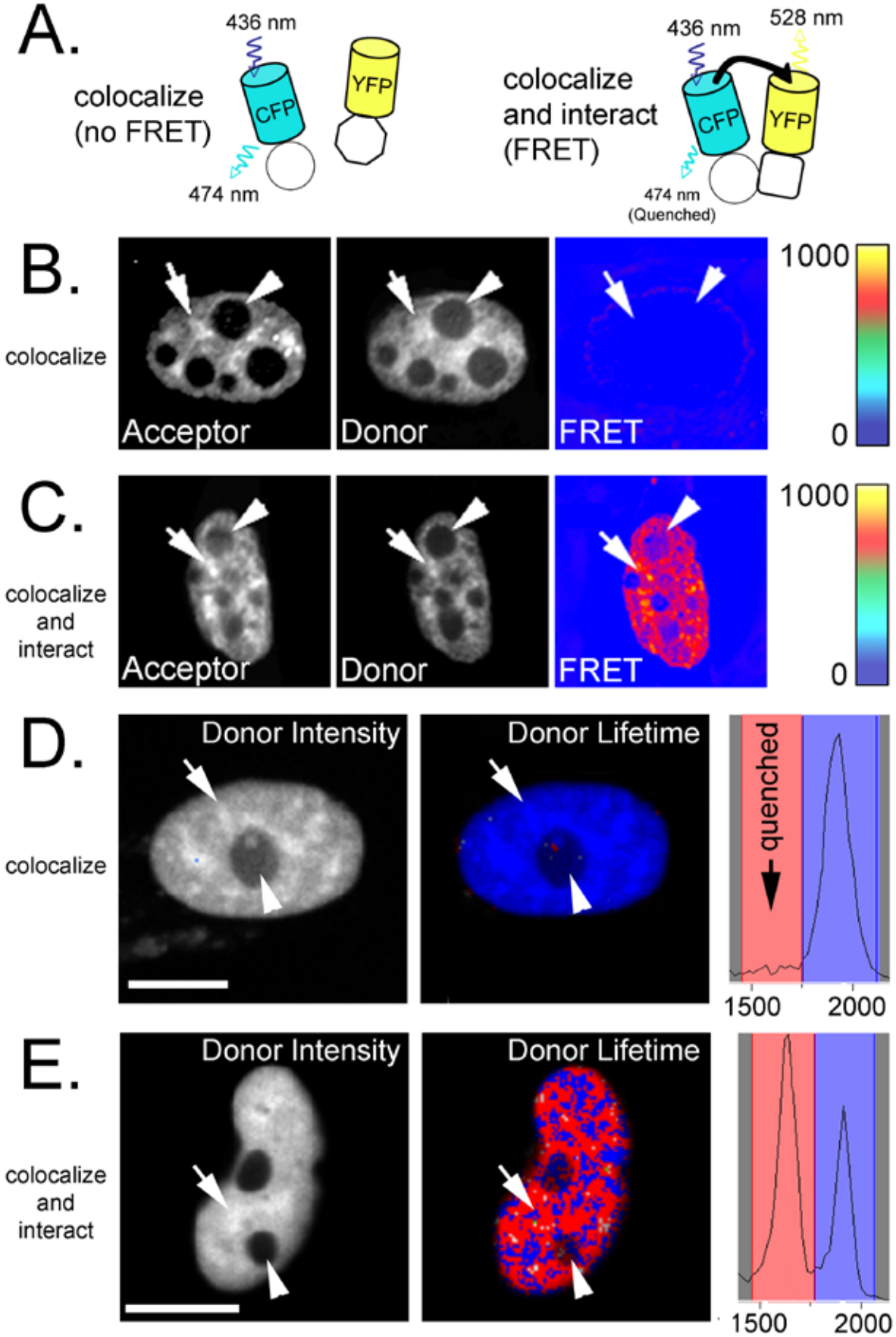


Fig. 3. What is colocalization? Panel A shows individual intensity images for both CFP and YFP expressed in the same cell. When merged, as shown to the far right, these images appear to demonstrate colocalization of these two proteins (i.e. green and red channels merge into yellow), although both are simply found diffuse throughout the cell. Panels B-C show true colocalization of CFP- and YFP-tagged proteins. The CFP-tagged protein is the same in both, while a different YFP-tagged protein is co-expressed with it each time. All three of these proteins are found throughout the nucleoplasm and show accumulations in splicing speckles (arrows). The merged images show that they colocalize in both of these subnuclear regions.

fluorescence microscopy systems fitted with high numerical aperture lenses (Hell, 2007). It is worth considering that proteins are typically on the order of 3 to 10 nm in length, which equates to a substantial distance in molecular terms. So, although one might be able to say with some confidence that 2 proteins appear to be colocalized in a sub-domain of the cell, this colocalization does not prove a tight association between them.

In order to determine whether or not a closer intermolecular interaction exists (i.e. a distance of 10 nm apart or less) using the light microscope, more advanced fluorescence techniques such as fluorescence resonance energy transfer (Clegg, 1996) or fluorescence complementation (Kerppola, 2006) must be used.



Fluorescence Resonance Energy Transfer

One of the significant benefits of FRET is that the same samples used in the colocalization studies described previously can be used to probe FRET interactions. Furthermore, there are several ways of measuring FRET, some of which can be done using fairly standard microscope systems. It is therefore a more accessible technique for the typical user.

The basic principle of FRET is the transfer of energy from an excited donor fluorophore to an acceptor fluorophore in close proximity. FRET is strongly dependent on the distance between donor and acceptor, falling off with the sixth power of the distance between the two. Because of this, FRET can only occur when the proteins are within 10 nm of each other and in the proper orientation. It is also a prerequisite of energy transfer that the emission spectrum of the first fluorophore overlaps the excitation spectrum of the second fluorophore (hence common FRET pairs include CFP/YFP and GFP/mCherry). A simplified diagram is presented in Figure 4A, which demonstrates how excitation of the donor sensitizes emission from the acceptor that ordinarily would not occur. Therefore, FRET can be detected as sensitized emission of the acceptor.

In practical terms, measurement of FRET by sensitized emission requires independent control of excitation and emission filters, so that the donor (e.g. CFP) can be excited and the acceptor's (e.g. YFP) fluorescence signal collected. A laser scanning microscope fitted with an appropriate laser for CFP excitation (e.g. 405nm or 436 nm line), or one with a tunable laser, can also be used. For the examples shown in Figures 4B-C, measurements were taken using a combination of three filter sets (ECFP [excite, 436 nm; emit, 470nm], EYFP [excite, 514 nm; emit, 528 nm], and what we refer to as the FRETchannel [excite, 436 nm; emit, 528 nm]).

Although not shown here, negative controls are critical for this type of measurement, because there is significant bleedthrough of both fluorophores into the FRET channel (typically 70% of the CFP signal and 20% of the YFP signal). This crosstalk is calculated by imaging each fluorophore on its own, and then subtracted from the signal in the FRET channel when the proteins are expressed together in the same cell. As shown in Figure 4B, although these two proteins colocalize within the cell, they do not interact directly and there is no FRET signal. Note that this is the same protein pair shown in Figure 3B. The protein pair shown in Figure 3C, however, has been shown here to colocalize and interact directly (Figure 4C). Note the significant FRET signal remaining in the FRET channel when the crosstalk is subtracted. The proteins interact both in the nucleoplasmic volume and in splicing speckles (arrow), while neither is found to accumulate significantly or to interact within nucleoli (arrowhead).

Energy transfer from donor to acceptor depletes or "quenches" the excited state population of the donor, and FRET will, therefore, reduce the fluorescence intensity of the donor. Photobleaching the acceptor to relieve this quenching of the donor (termed "acceptor photobleaching") offers another

Fig. 4. Measuring direct protein-protein interactions by FRET. Panel A demonstrates the dependence of FRET on the close proximity of the CFP donor and YFP acceptor molecules (less than 10 nm). If the tagged proteins are close enough and in the proper orientation, excitation of CFP leads to a transfer of energy to YFP, thereby exciting it and causing it to fluoresce. This sensitized emission can be detected in the FRET channel (CFP excitation, YFP emission). The proteins shown in Panel B do not FRET and therefore it is unlikely that they interact directly, although they do colocalize. Panel C shows a pair of proteins that colocalize and also interact, as demonstrated by the strong FRET signal. A different way of measuring FRET is shown in Panels D-E. Fluorescence lifetime imaging (FLIM)/FRET takes advantage of the fact that the transfer of energy from the CFP donor can be measured as a "quenching" of its fluorescence lifetime. If a quenched pool of donor exists, the donor will therefore have two lifetimes, which in the case of CFP translates to an unquenched lifetime of approximately 1.9 ns and a quenched lifetime of approximately 1.6 ns in live mammalian cells when excited with 840 nm light. When co-expressed with two different YFP-tagged proteins, it is clear from the donor lifetime images that the CFP-tagged protein, although it colocalizes with both, interacts directly with one (E) but not the other (D). Note that both FRET techniques provide spatial information, i.e. they show an interaction profile for the proteins throughout the entire cell, on a pixel-by-pixel basis.

option for detecting FRET in vivo, although a caveat to this technique is that some photoconversion of YFP may occur; it has been shown that a signal is generated in the blue/cyan channel when YFP is bleached, even in the absence of CFP (Kirber et al, 2007). Despite this, acceptor photobleaching can be a valuable technique when there is a requirement to measure this interaction at high temporal resolution. In the present example, this was not necessary and therefore avoided. Furthermore, sensitized emission and FLIM/FRET provide superior spatial resolution; with acceptor photobleaching a single point or region of the cell is bleached which means that FRET interactions can only be measured in a selected location in the cell. By contrast, FRET measurements throughout the entire cell can be measured using sensitized emission and FLIM/FRET (leading to a "FRET map" of the cell).

FRET-induced donor quenching is also observed as a decrease in the donor's fluorescence lifetime, which is the average time that a molecule spends in the excited state before emitting a photon and returning to the ground state. Comparison of donor lifetime in the presence and absence of acceptor, termed the FLIM/FRET technique, was also used to probe potential interactions between the splicing proteins shown in Figure 3. Key advantages of this approach are the independence of measurement on probe concentration and the use of infrared excitation wavelengths, which are less damaging to cells (see Peter and Ameer-Beg, 2004, and Periasamy et al, 2002, for a full account). The results are in agreement with those found by sensitized emission in Figures 4B-C. The first pair of proteins colocalize both in the nucleoplasm and at splicing speckles (arrow), yet there is no quenching of the CFP-protein's fluorescence lifetime (Figure 4D). The second pair of proteins also colocalize, and there is significant quenching of the donor's lifetime throughout the nucleus, indicating that the two proteins are in close enough proximity for FRET to occur.

For all three of these FRET techniques, the efficiency of energy transfer can be used as a molecular ruler to determine the scale of a particular interaction (Stryer, 1978). It is important to remember, however, that an absence of FRET cannot completely rule out the possibility that both fluorophores are more than 10 nm apart, and that FRET may in fact occur where any two non-interacting proteins are highly concentrated in localized areas (see Trinkle-Mulcahy et al., 2006, for a full account). A final consideration is that the fluorescence lifetime of CFP has been demonstrated to contain a shorter component, which can complicate interpretation of the data. Using GFP and mCherry as a FRET pair for FLIM/FRET can reduce this potential for error.

Summary

In this short article we have attempted to describe one of the scenarios in which something that is, initially, fairly straightforward has developed into a relatively complicated experiment requiring a high level of knowledge and technical understanding of the microscope equipment available. Through close communication between facility staff and facility users, many of the pitfalls that face a research scientist new to these techniques can be avoided.

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