

TECHNIQUES FOR MOLECULAR ANALYSIS

Using intrinsically fluorescent proteins for plant cell imaging

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Received 23 August 2005; revised 15 November 2005; accepted 28 November 2005.

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Summary

The intrinsically fluorescent proteins (IFPs), such as the green, cyan and yellow fluorescent proteins, have revolutionized how we can image the dynamics of cellular events. Intrinsically fluorescent proteins have been used as reporter genes to monitor transcriptional regulation, as targeted markers for organelles and subcellular structures, in fusion proteins to directly observe protein motility and dynamics, and in sensors designed to show changes in cellular environments ranging from pH to protein kinase activity. The IFPs hold tremendous potential to reveal the dynamic processes that underlie plant cell function; however, as with all technology there are artifacts and pitfalls inherent in their use. In this review, we highlight some of the practical issues in using IFPs for live cell imaging. These include choice of the appropriate IFP, dealing with autofluorescence, photobleaching and phototoxicity, and application of approaches such as fluorescence resonance energy transfer (FRET), fluorescence lifetime imaging (FLIM) and fluorescence recovery after photobleaching (FRAP) to gain high-resolution data about protein dynamics within the cell. We also discuss some of the more common artifacts associated with these fluorescence imaging approaches and suggest controls that should help both spot these problems and suggest their solutions.

Keywords: fluorescence microscopy, FRAP, FRET, fusion protein, green fluorescent protein, IFP.

Introduction

The Holy Grail of cell biology is to learn how the myriad proteins in a cell dynamically interact to perform cellular function. In this quest, researchers have traveled various technological avenues that include biochemistry, immuno-cytochemistry and fluorescent analog cytochemistry (i.e. microinjection of fluorescently tagged proteins). While each approach continues to be used, these traditional methods require extensive training and expertise to yield interpretable information. With the discovery of green fluorescent protein (GFP; and its derivatives and orthologs, which we collectively refer to in this article as intrinsically fluorescent proteins or IFPs), a new era has been heralded. It is now possible to engineer IFP tags onto a protein of interest and to non-invasively watch its dynamics. This technology is relatively easy to use, obviating the need for the extensive training in cell biology that is associated with the more traditional methods. The potential for this approach to increase our understanding of protein dynamics is huge and already tremendous advances have been made. However, as with

any technology, the possibility for artifactual results does exist. The purpose of this article is to present an overview of the power of the IFPs in monitoring cellular dynamics in plants and to discuss some important considerations for using IFP to their full potential. It is not our intent to provide a comprehensive review on the use of IFPs and imaging approaches in plant cell biology, but rather to provide a brief guide that will help the new investigator see the potential of this technology, avoid some common problems and quickly design the best experimental approach for their particular course of study. It is our hope that this information will help others to avoid many of the time-consuming mistakes that can impede effective progress in the understanding of protein dynamics in plants.

Intrinsically fluorescent proteins and their use as reporters

Most proteins fluoresce strongly only when they bind a separately synthesized prosthetic group. However, the IFPs

are unique in that their fluorophore is composed of modified amino acid residues within the polypeptide chain (Figure 1; Tsien, 1998). Although these proteins are naturally formed in jellyfish and corals, the fluorophore is capable of forming in the plant cell. The use of IFPs has therefore become widespread as these proteins provide genetically encoded labels to non-invasively monitor the spatial and temporal dynamics of fusion proteins in plants. The native *Aequorea Victoria* GFP is a single domain protein of 238 amino acids with a molecular weight of approximately 27 kDa. The polypeptide folds such that the fluorophore is centrally located within a barrel (a so-called β -can, Figure 1) that protects it from the bulk solvent. This makes GFP relatively environmentally insensitive and remarkably resistant to denaturation, even surviving aldehyde fixation for imaging in tissue sections (Chalfie *et al.*, 1994). Deletion of >7 amino acids from the C-terminal or more than the N-terminal Met leads to loss of fluorescence (Dopf and Horiagon, 1996), likely due to failure of the protein to fold correctly. However, both the N- and C-termini of the protein are exposed on the surface of the β -can (Figure 1) and so are available to make protein fusions that do not disrupt GFP formation. Thus, most successful GFP fusions utilize the N- or C-terminal as a site of fusion to the protein of interest.

The diversity of intrinsically fluorescent proteins

The original GFP was isolated from the jellyfish *Aequorea victoria* and has major excitation peaks at 395 and 470 nm and emits green fluorescence at 520 nm (Tsien, 1998). Early attempts to express this native GFP in *Arabidopsis* suffered from a lack of expression (or low expression levels) but various genetic modifications (Chiu *et al.*, 1996; Haseloff *et al.*, 1997) increased the utility of this protein for use in plants. Although such earlier work was limited to utilizing the green fluorescent properties of GFP, a plethora of mutated versions of this protein have now been developed that alter these spectral characteristics (Table 1). This mutant screening has led to the availability of multiple blue, cyan, green, yellow and red-shifted fluorescent variants of the progenitor GFP as well as versions where the excitation wavelengths are shifted, such as a UV excited GFP. In addition, screening other fluorescent marine organisms has led to the identification of a range of other IFPs related to GFP, including the red emitting DsRed (Table 1). Thus, there are now a multitude of IFPs with varied excitation and emission characteristics (Miyawaki *et al.*, 2003; Shaner *et al.*, 2004). Therefore, before embarking on the long and often tortuous path to making, expressing and analyzing an IFP fusion protein, it is essential to carefully select the appropriate IFP for the fusion. The protein fusion to be generated, the likely subcellular locale of the GFP, the optical characteristics of the cells to be studied, the available microscope to be used for visualization and the intrinsic

optical characteristics of the IFPs themselves should all significantly impact on this decision. Each of these areas will be discussed in this article.

Chimeric gene design, promoter choice and validation of the reporter

The location of the IFP in the chimera, the use of linker sequences as spacers between the IFP and the protein of interest, promoter choice, and how to validate the fidelity of the chimera are all critical parameters to the success of an IFP imaging experiment.

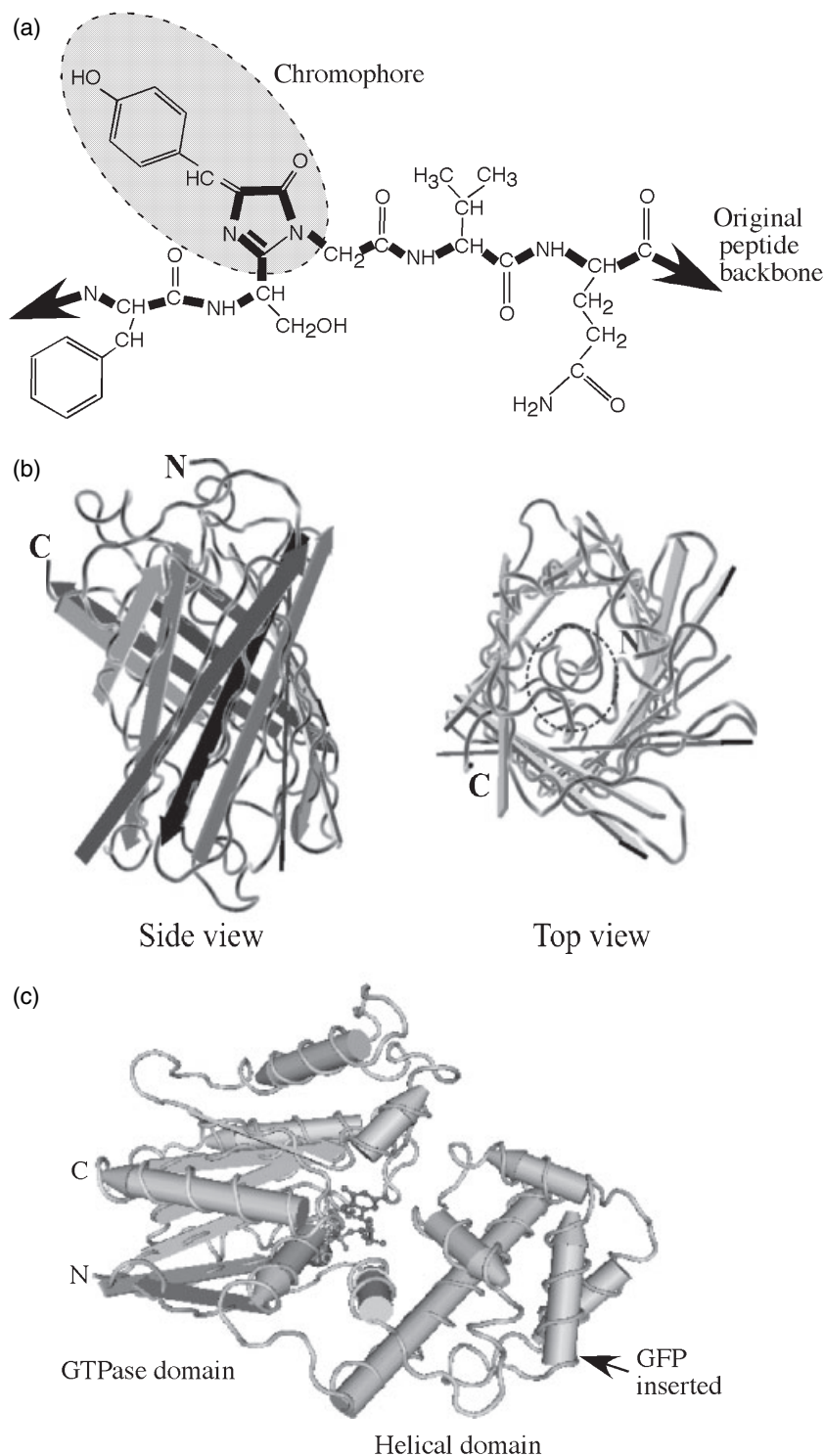
Most chimeric constructs place the IFP on either the N- or the C-termini of the protein. If structure/function data is available to guide the initial placement of the IFP, then this should be considered (e.g. if one end of the protein of interest is known to dock tightly with an interacting partner, then the other end should be chosen for initial experiments). For example, when localizing the calmodulin-like domain protein kinase CPK1 in *Arabidopsis*, Dammann *et al.* (2003) used C-terminal fusions to GFP to avoid interfering with the potential N-terminal acylation sites [Gly in position 2 (for myristoylation) and the Cys in position 5 (for palmitoylation)] that are likely involved in targeting this protein. Indeed, deletion of these predicted acylation sites shifted CPK1-GFP from a peroxisomal to a cytosolic locale, indicating how critical the free N-terminus of this protein was for correct targeting.

A search of the literature for orthologs that have been successfully fused to an IFP in other species can also be informative. However, in many cases it is not obvious which end of the protein should be chosen and only empirical testing of the placement will reveal which is optimal. Similarly, in some cases, neither an N- nor a C-terminal fusion is appropriate. For example, for the G-alpha G-protein subunit, both termini must be free for biological activity and, in this case, insertion of GFP into an internal cytoplasmic loop proved to be optimal, with the decision of insertion site driven by detailed knowledge of the domain structure of the protein (Figure 1c; Hughes *et al.*, 2001).

The IFP sequences are rarely fused directly to the coding sequences of the protein of interest. Instead, an intervening linker is typically added, both for cloning convenience as well as to increase the molecular mobility at the junction. Conventional wisdom dictates these linker sequences be rich in amino acids whose structure allows flexibility, e.g. glycines and alanines although serine residues are often interspersed to increase the solubility of the polyglycine regions (Nagai and Miyawaki, 2004). In practice, the use of multicloning sites to allow in-frame insertions often results in charged and hydrophobic residues being included in the final chimeric gene. Successful fusions may contain none, or upwards of 20 linker residues. It is difficult to predict if a linker will be required (for example, in our labs the majority

Figure 1. Structure of the green fluorescent protein (GFP) chromophore, GFP protein and internal GFP insertion into the G-alpha subunit of heterotrimeric G-proteins.

The chromophore (a) is derived from cyclization around amino acids 65–57 (Ser–*dehydro*Tyr–Gly) forming an imidazolidone ring that only involves the original amino acids present in the apoprotein (Cody *et al.*, 1993). The crystal structure (b) shows that the bulk of the peptide sequence forms a unique beta-barrel structure, which encloses the amino acid side chains that constitute the fluorophore (indicated by the dotted circle in the top view). The N- and C-termini of GFP are exposed on the surface of the molecule, which facilitates GFP gene fusions. (c) Structure of the G-alpha subunit of heterotrimeric G-proteins showing the internal site of GFP fusion on an exposed helix. This insertion site was chosen from knowledge of the domain structure of the protein and using comparative structural analysis of conserved domains between G-alphas from different organisms (Hughes *et al.*, 2001). Protein structures were rendered using the Molecular Modeling Database from the National Center for Biotechnology Information (Chen *et al.*, 2003).



of constructs have linkers, but often more for cloning convenience than as a flexible connector). In general, adding these extra residues between IFP and the protein of interest seems not to be harmful and at least some groups report linker design to be critical to the success of a reporter (e.g. Sullivan, 1999).

The choice of promoter can be important for several reasons. First, the chimeric gene can have detrimental effects if expressed at high levels (Hashimoto, 2002), while at low expression levels no detrimental phenotype is observed (Granger and Cyr, 2001). Secondly, high expression levels of a chimeric gene may allow the chimeric

Table 1 Excitation and emission characteristics of some of the range of available intrinsically fluorescent proteins (IFPs) and representative range of pKa values

Color of fluorescence	IFP	Excitation maximum (nm)	Emission maximum (nm)	pKa
Blue	EBFP	380	440	4.9
Cyan	ECFP	433/452	475/505	6.4
	Cerulean	433	475	4.7
	AmCyan	458	489	–
Green	EGFP	480	505	6.1
	mGFP4	480	505	–
	ZsGreen	493	505	–
Green/yellow	EYFP	514	527	6.1
	Citrine	516	529	5.7
Yellow	Venus YFP	515	528	6.0
	mHoneydew*	487/504	537/562	<4.0
	ZsYellow	529	539	–
	mBanana*	540	553	6.7
Orange	mOrange*	548	562	6.5
Red	tdTomato	554	581	4.7
	DsRed	558	583	4.7
	mTangerine*	568	585	5.7
	AsRed	576	592	–
	mStrawberry*	574	596	<4.5
	mRFP1	584	607	4.5
	mCherry*	587	610	<4.5
	HcRed	588	618	–
	mGrape1*	595	620	–
	mRaspberry*	596	625	–
	mGrape2*	605	636	–
	mPlum*	590	648	–

Blue fluorescent protein (BFP) has proven difficult to use in plant cells due to low signal strength.

*Derived from mRFP1.

Data taken from: Campbell *et al.*, 2002; Griesbeck *et al.*, 2001; Llopis *et al.*, 1998; Nagai *et al.*, 2002; Patterson *et al.*, 2001; Rizzo *et al.*, 2004; Shaner *et al.*, 2004; Tsien, 2005; Wachter *et al.*, 1997 and http://www.clontech.com/clontech/gfp/pdf/LC_NFP_Features.pdf.

protein to accumulate at the proper location, saturate and then accumulate ectopically as well. Thirdly, if multiple transformations are planned, then cosuppression of one or both of the transgenes can, in theory, occur if the same promoters are used (Baulcombe, 2005). In practice, the first and second problems are often sorted out during the production and selection of stably transformed plants or cell lines because only dimly expressing cells are recovered (which may be inconvenient for imaging, but such dim lines often report wild-type behavior with higher fidelity compared with bright lines). However, if transient expression studies are carried out, then the high levels of expression hold the potential for producing a transient dominant negative phenotype and/or ectopic localization of the gene product. Hence, it is good practice to view brightly expressed genes, whether produced by transient expression or through stable transformation, with caution. The third problem, cosuppression, can be addressed by choosing different promoters for constructs that are destined for

coexpression studies. The most commonly used constitutive promoters include those from actin, ubiquitin and the cauliflower mosaic virus 35S promoter. However, these promoters often drive expression to high levels raising the possibility of overexpression and ectopic-expression-related artifacts. Relatively low level gene expression can be achieved using inducible promoters, and a range of such systems have been successfully used in plants including those responsive to dexamethasone, ethanol, tetracycline, estradiol, copper, benzothiadiazol, methoxyfenozid, herbicide safeners and temperature (Aoyama and Chua, 1997; Deveaux *et al.*, 2003; Padidam, 2003; Yoshida *et al.*, 1995). Such an inducible promoter approach provides the added advantage of providing control over when the transgene is expressed. The alternative to using constitutive or inducible promoters is driving expression of the fusion protein with the native promoter of the protein to be localized. When compared with using constitutive promoters, the use of native promoters has a higher probability of expressing the transgene in the appropriate tissue and at endogenous levels. Therefore, the use of native promoters can reduce overexpression- and ectopic-expression-related artifacts.

Intrinsically fluorescent protein-tagged constructs are typically assembled in traditional *Agrobacterium* binary plasmids (Lorence and Verpoorte, 2004) through restriction site cloning. However, new plant transformation plasmids have been developed that considerably improve the ease of construct assembly and versatility. GATEWAY™ (Invitrogen, Carlsbad, CA, USA) technology-based *Agrobacterium* binary vectors are available that utilize site-specific recombination to generate N- or C-terminal GFP fusions without the need for unique multiple cloning sites in the transformation vector and transgene (Curtis and Grossniklaus, 2003). More recently, a modular set of vectors that support N- or C-terminal fusions to a variety of IFPs have been developed (Chung *et al.*, 2005; Tzfira *et al.*, 2005). These vectors enable the user to express multiple transgenes from a single transformation vector and provide a wider choice of promoters and terminators to reduce the risk of transgene silencing.

Chimeric IFP genes may or may not function properly and care must be exercised when working with new genes that have not previously been fused to IFPs. In some cases, the chimeric protein may misfold and when this occurs hydrophobic domains often become exposed and protein aggregation occurs. As a consequence, punctate signals (which are characteristic of centers of protein aggregation) need to be cautiously interpreted, especially if the wild-type protein is not predicted to be compartmentalized.

If the gene of interest has a predicted cellular localization that can be easily identified (e.g. nuclear) and if the chimeric gene targets to that locale, then one has some confidence that the reporter has value for further experimentation. Even if the predicted cellular localization is seen, further

corroboration should be pursued, for example, there are instances where a GFP-chimeric gene localizes to the predicted location, but the construct can alter *in vivo* activity (Wang *et al.*, 2004). In establishing the fidelity of the IFP chimera, consideration must be given to the activity of the chimeric protein as well as to its localization. The fidelity of the observed localization pattern is important and there are several approaches that can be considered to verify if the construct is reporting a physiologically relevant cellular locale. If information is known about changes in localization of the wild-type protein that accompany physiological or developmental states, such as translocation to the nucleus, then the IFP chimera should similarly show alterations in localization patterns. Immunocytochemistry in a wild-type plant, using antibodies against the native protein, should also yield similar localization patterns as seen with the chimeric gene in transformed plants. In some cases, pharmacological approaches can also provide corroboration if it is known that treatment causes a predictable redistribution of the protein, e.g. depolymerizing actin with latrunculin B (Wang *et al.*, 2004), microtubules with an antimicrotubule herbicide (Marc *et al.*, 1998), or disrupting Golgi stacks with brefeldin A (Dixit and Cyr, 2002). However, when using such a pharmacological approach, it is essential that the treatment will lead to a known and predictable alteration in locale. In addition, relying solely on this approach to identify the structures being visualized should be avoided. For example, Brefeldin A alters Golgi structure but also affects post-Golgi compartmentation. Therefore, relying upon this treatment alone to define the Golgi is insufficient.

An alternative approach to confirming cellular location is to colocalize the fusion protein with a compatible IFP of known subcellular localization. Fortunately, there are now a host of such markers and, for Arabidopsis, stably transformed lines for many have been generated (Table 2). If such

a colocalization route is to be taken, it is critical to choose an IFP for the fusion protein that will be compatible (i.e. distinguishable from the available marker) for colocalization with these available markers. In addition, careful controls to confirm that the emission of each of the IFPs being colocalized does not bleed through into the signal from the other are critical. Should such signal overlap occur, it will give an artifactual apparent colocalization. Suggestions for the appropriate kinds of controls to detect such bleed through problems are detailed in the section on Fluorescence Resonance Energy transfer.

The gold standard for assessing if a fusion protein retains its biological function is to see if the chimeric gene can rescue the relevant null mutant *in planta*. However, this is not always practical, e.g. the null mutant is not available or has no obvious phenotype to complement, and other avenues must be explored. For example, a heterologous model organism can be considered to test function for genes that are predicted to have conserved evolutionary function. Rescue of a yeast mutant with an IFP-chimeric gene is one such alternative approach that works providing the plant protein is an authentic homolog of the yeast gene. Also, if the protein has a known and assayable biochemical activity, then the IFP construct can be expressed in bacteria and the recombinant protein isolated to see if this activity is present and similar to the wild-type version.

Other often overlooked features of IFPs that can impact on apparent localizations are the pH sensitivity of their fluorescence and possibility of degradation. Although the chromophore of these proteins is protected within the beta-barrel structure, it is accessible to protons and shows pH dependency, with lowering pH lowering fluorescence intensity. Indeed, this pH sensitivity has been used to generate IFP-based probes for intracellular pH measurements in plants. For example, both the Phlourins (Gao *et al.*, 2004) and the

Table 2 Stably expressed green fluorescent protein (GFP) markers in Arabidopsis plants

Subcellular structure	Marker used	References
Actin	fimbrin1-GFP GFP-mouse talin	Sheahan <i>et al.</i> (2004) Wang <i>et al.</i> (2004) Kost <i>et al.</i> (1998)
Centromere	Histone 3-GFP	Fang and Spector (2005)
Chloroplast/Plastid	Stroma signal peptide-GFP Rubisco-GFP	Kohler <i>et al.</i> (1997) Kwok and Hanson (2004)
Endoplasmic reticulum	ER retention signal-GFP	Haseloff <i>et al.</i> (1997) Ridge <i>et al.</i> (1999)
Golgi	<i>N</i> -acetylglucosaminyl transferase 1-GFP	Lu <i>et al.</i> (2005)
Microtubules	GFP-beta-tubulin 6 GFP-mouse MAP4	Nakamura <i>et al.</i> (2004) Granger and Cyr (2001)
Mitochondria	Mitochondrial signal peptide-GFP	Logan and Leaver (2000)
Nucleus	Histone 2B-GFP	Boisnard-Lorig <i>et al.</i> (2001)
Nuclear envelope	RanGAP-GFP	Pay <i>et al.</i> (2002)
Peroxisome	Peroxisomal targeting signal-GFP	Mathur <i>et al.</i> (2002) Mano <i>et al.</i> (2002)
Vacuole	Vacuolar syntaxin-GFP	Uemura <i>et al.</i> (2002)

H148D mutant (Fasano *et al.*, 2001) have been used in *Arabidopsis* to monitor cytosolic pH. Therefore, for IFPs predicted to be targeted to acidic compartments, judicious choice of IFP with the requisite low pKa (Table 1) is critical to ensure a bright image. This problem was exemplified in the report of Scott *et al.* (1999) who found that low enhanced green fluorescent protein (EGFP) fluorescence (pKa 6.1) when targeted to the cell wall could be alleviated simply by raising the pH of the apoplast. As further notes of caution, yellow fluorescent protein (YFP) also shows Cl⁻-dependency of fluorescence (Griesbeck *et al.*, 2001). Tamura *et al.* (2003) have also reported rapid (within tens of minutes) light- and pH-dependent degradation of GFP in the vacuole by cysteine proteases. The lack of IFP signal in the wall or vacuole could easily have been misinterpreted by either group as failure to localize to these compartments rather than pH- or protease-dependent reduction of correctly targeted IFP. It is therefore important to design your analysis of subcellular localizations with these kinds of potential challenges in mind. For example, Tamura *et al.* (2003) had to rely upon a biochemical analysis of GFP distribution to understand that their vacuolar GFP was in fact being degraded in the light.

Observational considerations

The power of IFP technology, as a non-invasive tool to observe cellular dynamics in living tissue, is contingent on judicious fluorescence microscopy to prevent unwanted physiological perturbations. Specifically, it is important to ensure that the excitation light does not result in oxidative cell damage due to reactive oxygen species generated from triplet state excited biomolecules and IFPs (Bensasson *et al.*, 1993). Both wide-field and confocal microscopy can result in significant light dosage to cells and, under harsh observation conditions, prolonged fluorescence microscopy will result in unwanted physiological perturbations and eventually cell death (Dixit and Cyr, 2003). Light-induced oxidative damage to cells occurs when free radical concentration exceeds the redox buffering capacity of the cell (Benson *et al.*, 1985; Dixit and Cyr, 2003); therefore, one of the goals of non-invasive fluorescence microscopy is to conduct observations without saturating this redox buffering capacity. In this regard, it is important to regulate the excitation light intensity, either by decreasing laser light intensity or using controlled arc lamp sources or neutral density filters, to use the lowest level possible during sample observation and image acquisition. This is particularly important because cell damage and free radical generation show a non-linear relationship to the excitation light intensity, becoming considerably pronounced as the redox buffering capacity is approached (Dixit and Cyr, 2003). Another factor to consider in conducting non-invasive fluorescence microscopy is the wavelength of the excitation light because shorter wavelength light is more energetic and so potentially more

damaging. Furthermore, cells preferentially absorb light energy in the UV and blue wavelengths, increasing the hazard of free radical generation at these wavelengths. Dixit and Cyr (2003) showed that untransformed cells are perturbed by intense illumination and this effect is increased in cells expressing an IFP, suggesting that both endogenous biomolecules of the cell and the IFPs contribute to photo-oxidative damage. Therefore, if possible, the use of IFPs such as YFP and DsRed/mRFP is preferable because they require less-damaging, longer-wavelength excitation light.

Knowledge of the extinction coefficient (the amount of incident light energy absorbed by a substance), fluorescence quantum yield (the percentage of absorbed light energy emitted as fluorescence) and relative photobleaching rate of the different IFPs is also important in guiding the choice of the IFP and choosing non-invasive observation conditions (Table 3). Intrinsically fluorescent proteins with relatively higher extinction coefficients and quantum yields, and relatively lower photobleaching rates, allow observations to be conducted using lower excitation light intensity and are less likely to lead to free radical generation as compared with IFPs with relatively lower extinction coefficients and quantum yields, and relatively high photobleaching rates. For example, YFP absorbs 50% more light energy per molecule than GFP, but the two have similar fluorescence quantum yields (Table 3). Therefore, a greater proportion of the absorbed energy results in free radical formation and subsequently photobleaching, for YFP relative to GFP.

While the goal of fluorescence microscopy is to be able to observe cellular processes with the highest possible spatial and temporal resolution, the practical considerations outlined above of preventing light-induced cell damage restrict this goal in the interest of maintaining normal cellular physiology. In practice, there is an inherent trade-off between temporal (i.e. the time-lapse interval) and spatial (image quality in terms of pixel depth) resolution. One must sacrifice one or the other, according to the experimental goal, in order to maintain cell health. There are many technical factors that impact on this trade-off, such as issues of signal-to-noise and effects of specific microscopy techniques. These aspects of live cell imaging are covered in depth elsewhere in this issue by Shaw. However, in terms of

Table 3 Spectroscopic properties of some common intrinsically fluorescent proteins (IFPs)

IFP type	Extinction coefficient (M ⁻¹ cm ⁻¹)	Quantum yield (%)	Photobleaching time (% of EGFP)
EGFP	56 000	60	100
ECFP	26 000	40	85
EYFP	84 000	61	35
DsRed	75 000	79	145
mRFP1	50 000	25	19

Data taken from Patterson *et al.*, 2001 and Shaner *et al.*, 2004.

maximizing spatial resolution at a given temporal resolution, it is important to bear in mind that high excitation light intensities inflict greater damage to cells, even after brief exposure, compared with longer exposure of cells to low excitation light intensities (Benson *et al.*, 1985; Dixit and Cyr, 2003). Therefore, the use of low excitation light intensities is the most effective method to conducting non-invasive fluorescence microscopy. Similarly, reducing the frequency of imaging in a time course can dramatically reduce photo-oxidative damage.

Before embarking into the world of IFP imaging, it is also advisable to become familiar with the advantages and limitations of the available microscopy equipment. Typically, IFP imaging is conducted using wide-field (or 'regular') epifluorescence or confocal laser scanning microscopy. Wide-field microscopy is cheaper than confocal microscopy and can provide high-quality images, especially when working with cell cultures and epidermal tissue. However, out of focus fluorescence captured during wide-field microscopy can sometimes significantly reduce the signal-to-noise ratio in the image and this is particularly true when trying to visualize IFPs in intact organs such as leaves or roots. This problem can be overcome by deconvolving wide-field images, where a computer is programmed with the optical characteristics of the microscope being used and so is able to 'subtract' out-of-focus fluorescence (McNally *et al.*, 1999; Swedlow and Platani, 2002; Figure 2c). The alternatives are to use the optical sectioning ability of either the

confocal or two-photon microscopes to reduce this out-of-focus fluorescence (Feijo and Moreno, 2004; Hepler and Gunning, 1998; discussed in detail by Shaw, this issue).

As excitation light is a major source of potential perturbation to the cell and is critical to successful IFP visualization, another consideration when designing an IFP experiment that is often overlooked is the available excitation light source. The most common are mercury or xenon arc lamps (used for wide-field microscopy) and lasers (used for confocal microscopy). The output from a mercury arc lamp is not uniform for all wavelengths; but rather, it is in the form of peaks at certain wavelengths (Figure 3a). Therefore, depending on the type of IFP used, the excitation light intensity will vary (depending upon the proximity of the desired excitation wavelengths to a particular lamp emission peak). In contrast, xenon arc lamps provide a more uniform wavelength output (with a lower overall light intensity), which makes them more suitable for quantitative comparisons of fluorescence intensity across different wavelengths (Figure 3b). Lasers too display different output intensities at different wavelengths (Figure 3c) and therefore the available laser can also limit which IFP can be imaged. For example, most confocal microscopes employ a low-energy helium–neon laser that only marginally excites RFP/DsRed at 543 nm, making dim signals hard to detect. In contrast, the argon-ion laser provides a more intense 488 nm excitation peak that is much more efficient at exciting GFP and YFP. Most standard confocal microscopes do not have the 440-nm emitting laser that would be optimal for cyan fluorescent protein (CFP) imaging.

Accurate imaging of fine structures such as subcellular organelles and the cytoskeleton is critically dependent on steady imaging from a fixed focal plane. If the sample drifts up and down during acquisition of an image, or between frames of a time course, the resulting images will be compromised by this movement. Mounting the microscope on a vibration isolation table can greatly reduce focal plane drift and improve image quality. Another source of image (and biology) degradation is from desiccation of the biological sample on the microscope stage. In terms of live-cell imaging, it is therefore important to use humidity chambers (e.g. small Petri dish with a coverslip glued to the underside, containing a piece of moistened paper) to keep cultured plant cells from dehydrating over the course of the observation period. The sample can then be viewed through the coverslip. For high-resolution imaging, an additional problem is often keeping the sample close enough to the surface of the coverslip. This is because, in general, the higher the magnification of the microscope objective, the closer it must be to the object to be imaged (i.e. the shorter the working distance of the objective). Therefore, the complex shapes of organs, such as the curves and ribs of a leaf, can often hold the region of interest to be imaged too far away from the objective. Fortunately, there are many tricks to ensure you

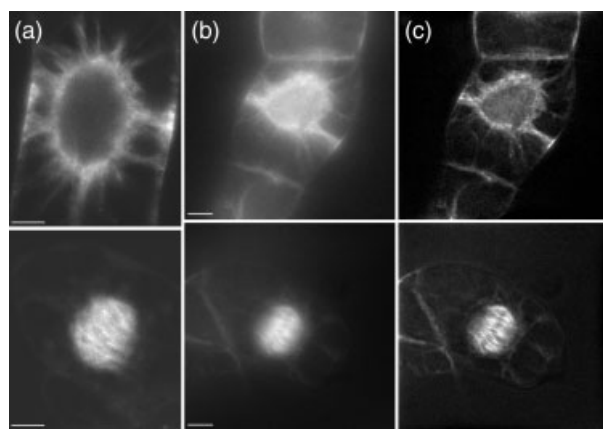


Figure 2. Image quality between wide-field and confocal fluorescence microscopy.

Images were acquired from cultured tobacco cells stably expressing green fluorescent protein (GFP)–tubulin, using either confocal laser scanning microscopy (a) or wide-field microscopy (b). Upper panels show microtubules around the premitotic nucleus and lower panels show spindle microtubules in cells at metaphase. Note there is significantly greater out-of-focus fluorescence captured during regular wide-field fluorescence microscopy than in the confocal images but this can be mathematically eliminated using deconvolution algorithms (c). Image in (a) taken using the 488-nm line of an argon laser, 488-nm dichroic mirror and 510–540 nm emission filter. Images in (b) and (c) were taken using a 100-W-Hg light source providing 460–500 nm excitation and 510–560 nm emission defined by interference filters. Scale bars = 5 μ m.

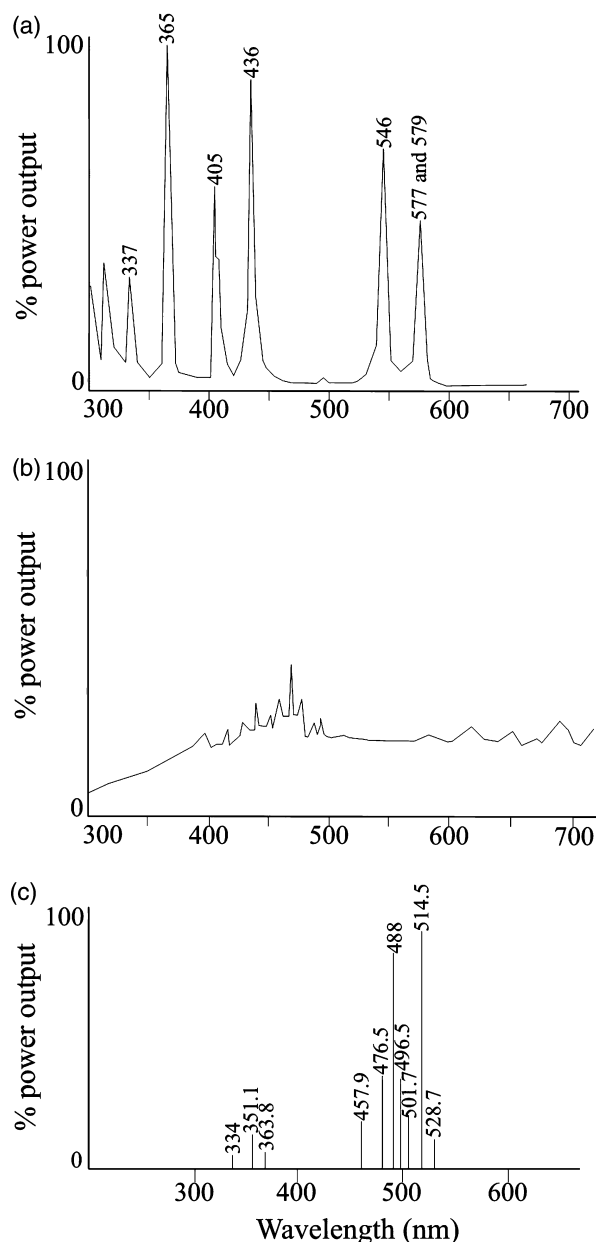


Figure 3. Emission spectra of commonly used light sources. The emission spectra of a mercury arc lamp (a), xenon arc lamp (b) and Argon ion laser (c) are shown. Note that the output power of each of the light sources is not uniform, but varies with wavelength.

can actually focus on the cells of interest. For example, cells and tissues can be immobilized onto a slide or coverslip by coating the glass surface with poly-L-lysine (MW > 300 000; Granger and Cyr, 2000, 2001). For root imaging, seeds can be germinated directly onto an agarose-coated coverslip (affixed to the bottom of a suitable chamber). The chamber can then be tilted to almost a vertical angle so that the roots grow along the agarose/glass interface (Wymer *et al.*, 1997).

In this manner, the root is stabilized and positioned directly at the surface of the coverslip. How to secure the sample, so that it is held still for imaging but yet is not being physiologically stressed, is a critical area that is often overlooked in the initial experimental design. In all cases, you want to avoid (or at least minimize) manipulation of the sample prior to imaging. For example, pulling seedlings out of agar and squashing them between a slide and coverslip is likely to induce a stress response that might confound your experiment. Similarly, prolonged incubation between a slide and a coverslip is likely to be extremely stressful for a cell due to the development of an anoxic environment.

Know your cells

Probably the single most important task before embarking on an extensive study of your chimeric IFP, is to spend some time looking at wild-type cells and tissues that express a soluble form of the IFP (e.g. GFP only). The reason for this is several fold. First, you will learn quite a bit about the cytoplasmic geometry of the plant cells that you will be studying. The cytoplasm is three-dimensionally complex and, when projected onto the two-dimensional focal plane of a microscope image, it can be a challenge to properly interpret. For example, a thin cytoplasmic strand can look like a cytoskeletal element (Figure 4a). Mitotic spindles can act as space filling scaffolds such that soluble IFPs take on the appearance of the spindle itself (Figure 4b). In addition, some compartments take up, or exclude molecules, e.g. soluble GFP can diffuse into the nucleus, but larger chimeric IFP molecules do not, based solely on size-related molecular exclusion. In all these examples, a naïve observer might conclude that a soluble IFP was accumulating in a particular location when in reality they failed to recognize cellular geometry or the idiosyncrasies of cellular compartments.

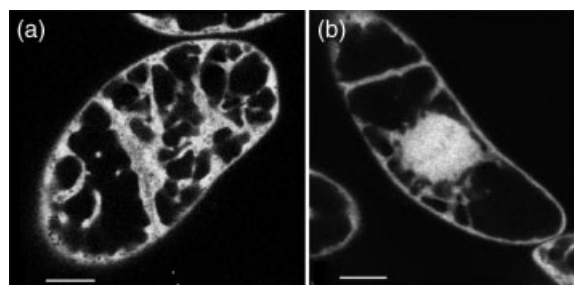


Figure 4. Soluble green fluorescent protein (GFP) reveals cytosolic compartments.

A stably transformed, 35S-promoter-driven, GFP-expressing BY-2 suspension culture cell line reveals the complexity of the cytosol of the cell (a). In a vacuolated dividing cell, the mitotic spindle occupies the phragmosome and soluble GFP intercalates into this space revealing, in negative, condensed chromosomes along the metaphase plate (b). Note in both panels how thin the cytoplasm can appear in some areas of the cell. Images were taken using a 100-W-Hg light source providing 460–500 nm excitation and 510–560 nm emission defined by interference filters. Scale bar = 10 μ m.

It is also important to spend some time looking at untransformed material because all wild-type plant material will autofluoresce to some degree (Figure 5) and the degree of autofluorescence can change with physiological state and developmental stage. Plants and cells that are grown under ideal conditions generally autofluoresce at a low level. If autofluorescence is high, this background can sometimes be filtered out with an appropriate emission filter. However, an intense autofluorescence signal will mean some IFPs are not useable with the tissue under study, e.g. the default of using GFP as the tag may not be appropriate for intensely green autofluorescent tissues and the intense red autofluorescence from chlorophyll may make many of the red-shifted GFP variants, outlined in Table 1, unusable in photosynthetically active tissues. As a further note of caution, stressed cells, in general, fluoresce brightly with a broad range of emission wavelengths (Figure 5). Indeed, this broad-wavelength character of autofluorescence can often be used as an indicator that the cells under observation may well be responding to non-optimal growth conditions. The limits placed on IFP selection imposed by autofluorescence are critical to know before spending the months needed to make the stably transformed IFP expressing lines.

When interpreting cellular structures revealed by the IFPs, the observer must always be aware of the resolution limits of

the light microscope. Point-to-point resolution in the *X,Y*-plane is limited to about 250 nm, while the *Z*-plane resolution is typically 2–3 times this for a high-quality wide-field epifluorescence microscope equipped with high numerical aperture objective lenses (for a confocal laser scanning microscope the *Z*-plane resolution can be reduced in half; Ruzin, 1999). It is important to remember that an IFP emits light from a point, and with the high quantum efficiencies of many IFPs, together with the high sensitivity of modern detection systems, many wide-field epifluorescent microscopes come close to detecting single molecules (Pierce and Vale, 1999). This means that a researcher can detect photons being emitted by 2 or 3 IFP molecules, but the volume of uncertainty as to their actual location is over 5 orders of magnitude relative to the volume occupied by a single IFP molecule itself (this is analogous to having a single fluorescing grain in a 3-kg bag of sand, but the entire bag appears to fluoresce and you cannot distinguish which grain is fluorescing). Because of this uncertainty, a thin patch of fluorescent cytoplasm, in a highly vacuolated cell, can appear similar to the plasma membrane and multiple subresolution cellular objects can appear as one. Researchers must know and understand the limitations imposed by these diffraction-limited events.

Living cells are dynamic and it is important to be mindful that movements can influence the appearance of captured images. All cellular compartments are moving, to varying degrees, and if the object under observation is changing position during image capture, then its image can be distorted. Hence, if comet streaks are observed, it is best to decrease the image capture time to see if the shape changes; if it does, then you know that the object is moving during the image acquisition. The length of the comet divided by the image acquisition time will provide you with a velocity measurement but the comet shape will not be a true reflection of the structure of the object.

The above example of image distortion due to movement highlights that IFPs are well suited to monitor the motility associated with cell function. Indeed, the unprecedented ability to study the dynamics of cellular events using IFP-based approaches means that time series studies are the rule rather than the exception. In planning a time course experiment it is important to appreciate that observational light will damage cells (for reasons discussed in Observational considerations). Avoiding this problem is simple. Do not photobleach your cells, as bleaching is indicative of the presence of unbuffered oxidative radicals that are inactivating the IFP and likely altering cell function. In practice, dropping the illumination intensity below some threshold (sometimes to 2–5% of the maximum), increasing the image acquisition time and/or adopting an appropriate sampling rate can be employed to successfully capture a time series without damaging the cell. It is also worthwhile spending time with your material so you can recognize the signs of cell

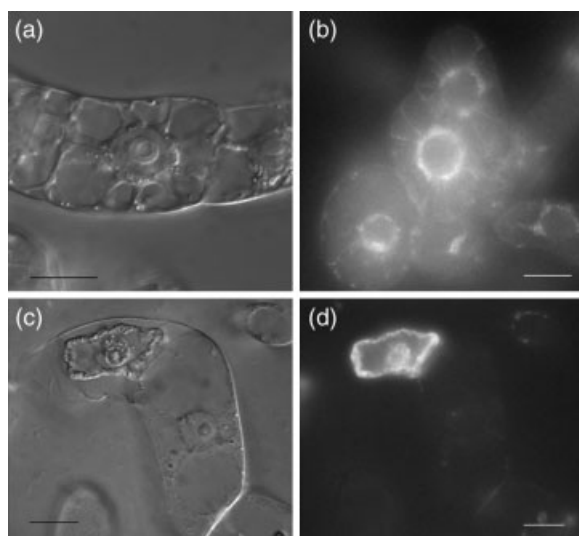


Figure 5. Untransformed, wild-type, cells autofluoresce to varying degrees. Untransformed BY-2 suspension culture cells that are healthy have cytoplasm that appears smooth by differential interference contrast (DIC) imaging, with clearly distinct cytoplasmic strands (a). However, even these healthy cells autofluoresce to some degree and extended exposure can record this (b). Dead and dying cells show a granular cytoplasm that appears distorted by DIC imaging (c) and such cells are strongly fluorescent (d). By eye, the fluorescence of the cell shown in (b) was barely discernable when looking at these cells down the microscope in a darkened room, while the cell shown in (d) was clearly seen even with the room lights on. Both (b) and (d) were taken using wide-field epifluorescence technique using 460–500 nm excitation and 510–560 nm emission filters. Scale bars = 10 μ m.

damage and experimenting with sampling rates and excitation light intensities to define what is acceptable. For example, cell division is especially sensitive to photodamage and so a reduction in the percentage of dividing cells or the presence of arrested cells (usually at prophase/prometaphase/metaphase) should be cause for alarm (Dixit and Cyr, 2003). Photodamaged cells often have cytoplasm that appears more granular compared with normal cells and often cytoplasmic strands break down, which results in highly vacuolated cells with the nucleus being tightly pressed to the side of the cell. Be sure to work with brightfield microscopy so you can familiarize yourself with how sick, dying and dead cells appear and take the necessary steps to maintain cell viability.

Specialized Techniques for indirect assessment of dynamics

Although photobleaching must be minimized for time-course studies, the phenomenon can be of tremendous value in certain defined instances, i.e. when only small areas of the cell are subjected to controlled photobleaching. The techniques of fluorescent recovery after photobleaching (FRAP) and its related fluorescence loss in photobleaching (FLIP) both rely on the fact that photobleaching irreversibly oxidizes the IFP molecules, destroying their fluorescent character (Koster *et al.*, 2005; Lippincott-Schwartz *et al.*, 2003; Sprague and McNally, 2005; Weiss, 2004). In most instances, both techniques are most easily implemented on a confocal microscope that has suitable software, as rapid changes in laser scanning conditions are required.

In a typical FRAP experiment, the researcher defines a limited region of interest where IFP fluorescence needs to be removed, then several short, high-intensity excitation scans are applied to photo-oxidize all IFPs in the selected area. A short time series is then acquired at substantially reduced laser power (to ensure no further bleaching occurs) to follow the patterns of fluorescence recovery into the bleached region of the cell. Because photobleaching results in the permanent destruction of the fluorescence from the IFPs in the region of interest, the only way for fluorescence to reappear is by movement of unbleached fluorescent molecules back into the region (Lippincott-Schwartz *et al.*, 2003). If the IFP protein moves only by diffusion, then recovery (within the cytoplasm) will follow a function described by the inverse cube of the molecular mass of the protein (Sprague and McNally, 2005). However, if the mobility of the IFP is affected by factors other than simple diffusion (i.e. association with complexes, or partition into compartments or microdomains), then recovery will follow more complex kinetics. Thus, by monitoring the fluorescence recovery rate and pattern, inference about the dynamics and interactions of the protein within the cytoplasm can be made (Sprague and McNally, 2005).

Fluorescence-loss-in-photobleaching experiments are used to learn more about proteins that are compartmentalized and so, instead of analyzing the pattern of recovery, the researcher looks at the loss of fluorescence in one compartment relative to another (Cole *et al.*, 1996; Koster *et al.*, 2005; Phair and Misteli, 2000). For example, if a nuclear-cytoplasmic IFP protein rapidly shuttles to and from the nucleus, then bleaching of the IFP signal in a selected area of the cytoplasm will lead to decrease in the nuclear signal as the bleached molecules move into the nucleus and the unbleached molecules move out (i.e. there is a distributed loss of fluorescence between the two compartments). However, if the nuclear-cytoplasmic IFP protein has a long residence time in the nucleus (e.g. by associating tightly with chromatin) relative to the cytoplasm, then bleaching in the cytoplasm will only result in a decrease in cytoplasmic fluorescence and the nuclear fluorescence will remain relatively high (i.e. the loss of fluorescence will be unequally distributed). As with FRAP, analysis of the effects of controlled photobleaching allow deduction about protein mobility and dynamics.

The same caveat exists about interpreting both FRAP and FLIP data. The techniques inherently provide a large dose of excitation light sufficient to photobleach the IFP to be studied. From our discussion about photodamage, this should set 'alarm bells ringing' about the possibility of photo-oxidative damage and so one should photobleach the smallest area of the cell that will give interpretable results. Clearly, in these kinds of experiments extreme care has to be taken to assess whether the protein dynamics being detected are from cells damaged by the bleaching process. For example, if you photobleach a dividing cell and it arrests, then you have likely damaged the cell, but if it traverses normally through all mitotic stages then you have confidence that your bleaching light was in a sufficiently small area and did no discernable damage. When used with these kinds of appropriate controls for assessing cell viability/function, these approaches can reveal remarkable insights into how proteins move within the cell that would be difficult to monitor otherwise.

Fluorescence resonance energy transfer

Fluorescence (or Förster) resonance energy transfer (FRET) is widely used to detect protein-protein interactions *in vivo* and provides another approach to monitor subcellular protein dynamics at the 10–100 Å range: a resolution unattainable by 'standard' epifluorescence or even confocal imaging. With this approach, two interacting cell components are labeled with two different IFPs, a donor fluorophore on one protein and an acceptor on the other. When the donor is excited, some of its emitted energy is transferred to the acceptor in a non-radiative energy coupling (Jares-Erijman and Jovin, 2003; Kenworthy, 2001). If this energy

transfer from donor to acceptor is close to the excitation energy (wavelength) of the acceptor, then the acceptor itself will begin to fluoresce, i.e. excitation of the donor will lead to fluorescence emission from the acceptor (Figure 6). Fluorescence resonance energy transfer provides a measure of protein–protein interaction as the efficiency of energy transfer is highly dependent on the distance between the donor and acceptor fluorophores. Thus, the efficiency of energy transfer (E) falls as the sixth power of the intermolecular distance (R) according to $E = [1 + (R/R_0)^6]^{-1}$ (Jares-Erijman and Jovin, 2003; Kenworthy, 2001). R_0 is the Förster radius, or the distance at which FRET is 50% efficient. Typical

values of R_0 range from 20 to 100 Å, which is of the same order as typical protein dimensions; for example, GFP forms a barrel shaped protein approximately 30 Å in diameter by 40 Å in length. Thus, FRET intensity can provide a sensitive measure of the distance between two IFPs and so, by inference, the proteins to which they are fused. *In vitro*, FRET efficiency can be used to quantitatively measure the distance between molecules, leading Stryer and Haugland (1967) to dub it a 'spectroscopic ruler'. However, it is important to realize that R_0 is highly dependent on intrinsic features of the FRET partnership such as the orientation between the dipoles of the donor and acceptor molecules, the quantum

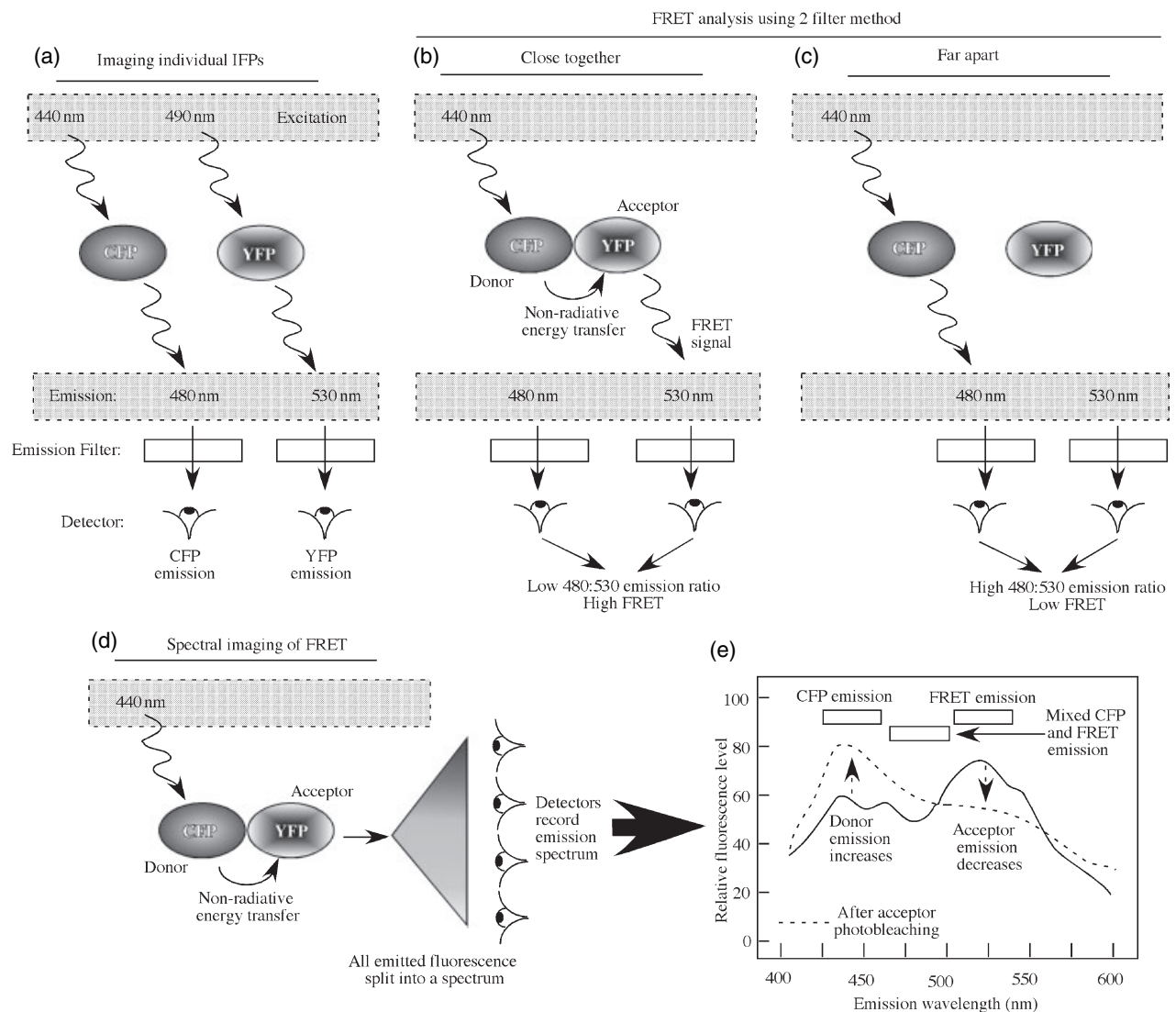


Figure 6. Fluorescence resonance energy transfer (FRET) coupling between CFP and YFP.

When the donor (CFP) and acceptor (YFP) are far apart, each fluoresces independently (a), but when brought into very close proximity, the emission energy from the donor is transferred without light emission (non-radiative or resonance energy transfer) to the acceptor, which then fluoresces (b). This means that when the CFP and YFP are close to each other, excitation of CFP will lead to YFP fluorescence or FRET. By monitoring the ratio of CFP emission to YFP emission when exciting CFP, the degree of FRET can be determined, normalized for differences in expression levels between experiments (b, c).

(d) When all emission wavelengths are recorded as a spectrum, the emission characteristics of CFP and FRET can be distinguished from areas of the spectrum where the CFP and FRET signal overlap (e). Upon acceptor photobleaching, donor emission should increase and acceptor emission (i.e. apparent FRET) should decrease.

yield and absorbancy characteristics of the donor, and the spectral overlap between acceptor and donor (Kenworthy, 2001). In addition, R_0 is sensitive to the environment in which the FRET pair is being monitored, being impacted upon by the refractive index of the medium (Suhling *et al.*, 2005). Therefore, in practice, FRET is mostly used *in vivo* as a qualitative measure that two proteins are physically interacting. This is because FRET is so highly dependent on intermolecular distance that when FRET is observed, the two labeled proteins can be inferred to be very closely associated. It is important to stress here that failure to detect FRET does not unequivocally indicate a lack of protein–protein interaction. Placement of the IFP on the fusion proteins may mean they are too far away from each other to allow FRET even if the proteins they are attached to do, in fact, interact. Hence, you need to keep in mind when designing these experiments that a positive *in vivo* FRET control may be difficult or impossible to define and, consequently, you should carefully consider how to validate your experiment. Alternative approaches to reveal protein–protein interactions such as yeast 2-hybrid (Jarillo *et al.*, 2001), split ubiquitin (Ludewig *et al.*, 2003), immunoprecipitation (Pandey and Assmann, 2004) and colocalization (Seidel *et al.*, 2004) will be required to confirm both positive and negative FRET data.

Most FRET experiments are also, to some extent, overexpression experiments in that IFP tagged versions of the proteins of interest are usually being expressed in wild-type plants. Therefore, you must pay careful attention to whether you are altering cell function through this overexpression, especially when using the 35S or a strong inducible promoter rather than using the endogenous promoters for the proteins of interest (where expression levels may more closely reflect the wild-type case). It is also critical to measure the expression levels of each partner alone in each FRET experiment to understand whether the strength of the FRET signal is accurately reflecting the degree of protein–protein interaction in the cell. For example, if the FRET donor or acceptor is expressed at low levels, the FRET signal may appear relatively weak even for strongly interacting proteins. Similarly, endogenous proteins will compete for FRET partners reducing the apparent interaction of the IFP labeled partners. In theory, if the endogenous proteins are expressed at high levels relative to the FRET constructs, FRET would not be evident precisely because the partners interact. Thus it becomes important to know the relative levels of expression of both donor and acceptor in order to place the intensity of the FRET signal in context.

Fluorescence resonance energy transfer is typically monitored by using filters that select the donor and acceptor emission wavelengths (Figure 6b,c). As FRET increases, the donor emission will reduce and the acceptor emission will increase (so-called sensitized emission). This is a very simple and cheap way to monitor FRET efficiency but there

are several potential artifacts that need to be quantified before you can confidently assign the acceptor emission to a FRET signal. Perhaps the two most common problems are (i) bleed through of the donor emission wavelengths into the FRET signal and (ii) direct excitation of the acceptor by the light used to excite the donor. These problems can be easily seen in the most common FRET partnership used at present, CFP/YFP (Figure 6). In the case of the CFP/YFP pairing, this would mean some CFP emission would contaminate the YFP FRET signal plus some of the apparent FRET signal would arise from the CFP excitation light directly exciting YFP. These artifacts are easily monitored by imaging controls that express CFP alone, YFP alone and coexpressing soluble CFP plus YFP. Quantification of the fluorescence in the resulting images will indicate how badly spectral bleed through is compromising the FRET measurement. Once identified, bleed through can be reduced by using narrow bandpass filters that precisely select a small portion of the YFP emission. However this precision will be at the expense of signal strength. Alternatively, the donor/acceptor excitations can be separated by choosing a different FRET partnership. For example, UV-GFP efficiently couples to YFP for FRET. The excitation peak of UV-GFP at 380 nm is 60 nm shorter than CFP and poorly excites YFP, reducing the possibility of direct YFP excitation. Note that, the UV-GFP emission can still appear in the FRET wavelengths highlighting that the measurements outlined above to test for donor bleed through and direct excitation of acceptor are essential to all FRET experiments.

These observations on the need to carefully choose the wavelengths of excitation and emission used to monitor FRET lead to a key question to ask about FRET signals: how much is a significant amount of FRET and what constitutes a significant change in FRET levels? This is a very hard question to answer as the degree of FRET depends on the closeness of the interaction of the two FRET partners, the orientation of the IFPs within the fusion proteins and the physical environment of the FRET partners (Kenworthy, 2001; Suhling *et al.*, 2005). Thus, there is no value that can be easily set to the maximum possible expected FRET. The absolute intensity of the FRET signal is highly dependent on the level of expression of the FRET partners, i.e. doubling the absolute expression of both partners within the cell will double the measured FRET signal. Thus, it is advantageous to normalize each FRET experiment for expression level. This is easily done by calculating the ratio of the donor emission to the FRET signal. As each signal is selected with the appropriate emission filter, this approach is often called the ‘two-filter method’. The donor signal carries information about the level of expression in the cell and so the ratio of donor signal to FRET helps to normalize the FRET signal for varied expression levels between experiments (Figure 6b,c). The next step in sophistication of analysis is the ‘three-filter method’, where not only are the donor and FRET signals

monitored but the acceptor is also directly excited and its emission recorded. A ratio of the measured FRET signal to this acceptor emission helps normalize the FRET to the level of acceptor. However, perhaps the most robust methodology to measure and normalize FRET signals is to record the emission spectrum (spectral imaging, Figure 6d,e). The spectrum can be deconstructed by software that is designed to recognize the characteristic pattern of emission wavelengths of the donor and acceptor, e.g. the CFP and YFP components of the spectrum. Thus, the spectral signatures of the donor and of the FRET signals can be robustly quantified. For confocal imaging, commercial set-ups for this include the Zeiss Meta detector (Zeiss Inc., Thornwood, NY, USA; Haraguchi *et al.*, 2002) and the Leica spectral resolving system (Leica Microsystems, Exton, PA, USA; Hiraoka *et al.*, 2002). The limitations on this technology are availability and price; the advantage is confidence in the quantitative value assigned to the FRET signal.

Other approaches do exist to help determine the degree to which FRET is occurring and should be viewed as essential controls for all FRET experiments. One such common method to verify apparent FRET is acceptor photobleaching. In this approach, the acceptor IFP is bleached by directly exciting it with an intense illumination. As the acceptor is bleached it no longer acts as a FRET partner and so the fluorescence of the donor should increase (Figure 6e). This is a very important control but should be interpreted with care. As with all photobleaching experiments, by definition a large amount of energy is being directed onto the cell to effect the bleach and this may have unforeseen side effects on cell response and biology. It is also possible to photobleach both acceptor and donor with a poorly selected bleaching wavelength. A combination of spectral analysis and acceptor photobleaching provides one of the most robust ways to assign a number to the degree of FRET occurring in a sample and is the method of choice for many labs routinely conducting FRET analysis.

At present, the most widely used FRET partnership is CFP as the donor and YFP as the acceptor. This pairing has become the default due to the fact that, until recently, there were few alternatives. A quick glance at Table 1 shows this is no longer the case. Although useable for FRET, the low quantum yield of CFP and poor spectral overlap between CFP and YFP actually makes this pairing less than ideal. For example, the relatively low quantum yield of CFP relative to YFP means that a significant amount of excitation energy is required to induce FRET, with all the caveats about potential for photodamage of the cell outlined previously. YFP is also excited somewhat by CFP excitation wavelengths and so, as it has a much higher extinction coefficient than CFP, there is the possibility of considerable direct excitation of YFP, which could be interpreted as a true FRET signal if the controls for bleed through and direct acceptor excitation described above had not been carried out. Not surprisingly, there is a

large effort to either generate better acceptor/donor pairings or to develop measurement approaches that are not so sensitive to the problems of the CFP/YFP pairing. For example, brighter alternatives to CFP are available (Rizzo *et al.*, 2004), and novel YFP variants such as Venus (Nagai *et al.*, 2002) have reduced pH sensitivity and fold more efficiently than YFP. Green fluorescent protein can be used as a more effective donor than CFP in a GFP/YFP pairing. However, due to the close overlap in spectrum between these GFP and YFP, this approach is only really feasible using spectral imaging and complex spectral unmixing algorithms to define the GFP and YFP component signals (Zimmermann *et al.*, 2003). EGFP/DsRed has also been used as a superior FRET partnership with the advantage of using longer, less phototoxic wavelengths of excitation light. Unfortunately the original DsRed is slow to mature, forms a green fluorescent intermediate during maturation and exists as a tetramer, making it highly unsuited to FRET analysis. Mutagenesis has been used to generate DsRed2 and DsRed express, which mature faster. In addition, a truly monomeric DsRed, mRFP1 (Campbell *et al.*, 2002) has recently been developed that holds the promise of allowing robust GFP-DsRed FRET analysis. However, for making fusion proteins it is important to use a 'GFPized' mRFP variant. That is, the original mRFP is reported to be intolerant of N- or C- terminal fusions (Shaner *et al.*, 2004), leading to poor success in fusion proteins. When the N- and C- termini of standard GFP were added to the N- and C- termini of mRFP (producing the so called 'GFPized' versions), this problem was greatly reduced (Shaner *et al.*, 2004). mRFP1 is also the parent of a range of new IFP color variants (Table 1) that may be suited for use as acceptors and donors for a range of new FRET partnerships. Although, at present, there are no reports of testing the combinations of donor and acceptor from this new IFP palette in plants, the red-shifted form called mCherry appears to hold great promise as it shows low photobleaching, a high extinction coefficient and tolerance of N- and C-terminal fusions.

Fluorescence lifetime imaging

An alternative to monitoring fluorescence emission to quantify FRET is to capitalize on the reduction of fluorescence lifetime exhibited by a fluorescence donor undergoing FRET. This approach relies on the characteristic time that a fluorochrome exists in an excited state between absorbing a photon of excitation light but before emitting this absorbed energy as fluorescence. This excited state lasts for picoseconds to nanoseconds. However, when the fluorochrome is a donor in a FRET partnership, this excited lifetime is attenuated as the energy is rapidly transferred to the acceptor (van Munster and Gadella, 2005; Wallrabe and Periasamy, 2005). Fluorescence lifetime imaging (FLIM) equipment measures this shortening of the lifetime of the donor and so can detect

FRET. However, it does so without imaging the FRET emission of the acceptor. Therefore, unlike the standard emission measurements described above, it is independent of the concentration of the FRET pair. In addition, FLIM measurements provide a quantitative monitor of the excitation state of the donor and so a quantitative index of FRET (van Munster and Gadella, 2005; Wallrabe and Periasamy, 2005). One important consideration for FLIM is that all the information about the FRET is coming from the donor. Therefore, the levels of acceptor need to be saturating to provide an accurate measure of FRET efficiency. In addition, any cellular component that interacts with the donor and acts to accept its excitation energy (i.e. any cellular component that quenches the donor) will appear as a FRET signal. However, perhaps the major drawback of FLIM at present is accessibility to the specialized equipment and expertise needed for these measurements. Even so, FLIM does provide one of the most robust approaches to measuring FRET currently available.

Alternatives to fluorescence resonance energy transfer

Although FRET has become the fluorescence method of choice for imaging protein–protein interactions *in vivo*, alternative approaches, including bioluminescence resonance energy transfer (BRET) and the split YFP or bimolecular fluorescence complementation (BiFC) approach, also allow similar measurements to be made. In BRET, the FRET donor is replaced with a bioluminescent protein such as luciferase from *Renilla* (Subramanian *et al.*, 2004). The principle is very similar to FRET in that the emitted energy from the donor luminescent protein excites fluorescence from an extremely closely associated IFP. However, BRET is intrinsically dimmer than FRET as the donor can only emit a single photon to excite its associated acceptor, whereas in FRET the donor is a fluorochrome capable of continuously emitting light until photobleached. Thus, although BRET represents a powerful technology to monitor protein–protein interaction in a luminometer, at present it is less suited to imaging than FRET.

Another alternative to monitor protein–protein interaction is BiFC. In this approach, YFP is split into its N-terminal 155 amino acid and C-terminal 84 amino acid. If brought into close proximity, these split domains will reform an active IFP that fluoresces (Walter *et al.*, 2004). These halves can be fused to putatively interacting proteins and, should the interaction occur, YFP fluorescence will be evident. One advantage of this approach is that the two fragments of YFP are smaller than the whole IFP tags needed for FRET. Also, once the domains interact, a very stable pairing is formed and so the IFP signal is maintained, making detection easier. This is obviously a two-edged sword as this stability means transient interactions are less likely to be documented. Similarly, weak interactions may be promoted by the propensity of the two halves of the YFP to form this stable molecule leading to the possibility of generating artifactually

enhanced apparent interactions. As neither half of the interacting pair is intrinsically fluorescent, only when an interaction occurs can fluorescence be observed. Thus, unlike FRET it is impossible to visually confirm that both partners are being produced in the cell when no detectable interaction is occurring. However, BiFC does not require the precise quantification and associated equipment needed for FRET measurements and does provide a viable alternative to screen for interactions occurring *in vivo*.

Conclusions and perspectives

Since the original observation in the 1990s (Chalfie *et al.*, 1994) that GFP could be heterologously expressed and still form an IFP, live cell imaging has undergone dramatic changes. Intrinsically fluorescent proteins have truly revolutionized how we approach imaging cell activities in plants, opening the world of cell biology to a new group of researchers. We can anticipate that, as new IFPs are generated and discovered, the suite of tools available to plant cell biologists will only improve. For example, an exciting recent approach has been to 'evolve' FRET partners (Nguyen and Daugherty, 2005). CFP and YFP were reiteratively mutated and selected for improved FRET interaction to the point where the final CYpet and Ypet variants were sevenfold more efficient in FRET than their progenitors. As these IFP tools improve, the possibilities for new kinds of experiments will only grow.

However, though extremely powerful and constantly improving, this IFP-based technology will continue to have artifacts waiting for the unwary experimenter. Although some of these problems are highly specific to the IFP approach, such as the need to validate fusion protein activity or be wary of expression levels, many of the potential problems can be reduced to the theme that measurements made on stressed or perturbed cells are uninterpretable. The stress can be from the microscope illumination system, from how the plant is handled or mounted on the microscope, or from side effects of the IFP expression itself. Therefore, perhaps the most important element to any live cell imaging experiment will continue to be to get to know the cells to be imaged. Understanding where stresses originate during IFP imaging and how to spot whether your sample is becoming damaged has to be the essential first step for all IFP-based experiments. Once you satisfy yourself about the health of your cells and the fidelity of your IFP marker, you will be amazed by the world of live cell imaging and your only problem will be finding enough time to devote to microscopy.

Acknowledgements

This work was funded by grants from Department of Energy DE-FG02-91ER20050 to RC, National Science Foundation MCB-02-12099

and National Aeronautics and Space Administration NAG2-1594 to SG and a multi-user equipment grant DBI 03-01460 from NSF to RC and SG.

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