

Imaging into the future: visualizing gene expression and protein interactions with fluorescent proteins

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Since its introduction into heterologous organisms as a marker of gene expression, the green fluorescent protein (GFP) has led a dramatic revolution in cell, developmental and neurobiology. By allowing breathtaking visualization of fluorescent fusion proteins as they move within and between cells, GFP has fundamentally transformed the spatial analysis of protein function. Now, new GFP technologies allow far more than simple observations of fusion protein localization. The growing family of fluorescent protein variants is enabling more sophisticated studies of protein function and illuminating wide-ranging processes from gene expression to second-messenger cascades and intercellular signalling. Together with advances in microscopy, new GFP-based experimental approaches are forging a second GFP revolution.

We begin our review at the heart of the cell, where fluorescent-protein-based methods are shedding new light on the kinetics of transcription, translation and protein metabolism. We then discuss how fluorescence resonance energy transfer (FRET) imaging is taking GFP beyond the limits of optical resolution, allowing live visualization of protein–protein interactions. We end with a glimpse of promising advances in biological imaging methods—technologies both driving and driven by new fluorescent-protein applications.

Expression profiling

Understanding the timing of transcription and protein expression is crucial when characterizing the activity of an unknown promoter sequence or studying the induction of a known reporter gene. Green fluorescent protein (GFP) has been used as a convenient transcriptional and translational reporter¹ because it is directly visible in the living cell and requires no fixation, substrates or co-enzymes. However, GFP requires many transcripts for an easily detectable signal and needs time for protein folding and fluorophore maturation. Many additions to the catalogue of GFP spectral variants^{2,3} and orthologues such as the sea coral protein DsRed^{4,5} have given added breadth to these reporter applications. Although DsRed is prone to tetramerization and has a relatively long fluorophore maturation time⁵, the introduction of DsRed2, a modified protein that is reported to have improved maturation kinetics, might begin to address these concerns.

These new variant fluorescent proteins allow simultaneous visualization of multiple fusion proteins in a cell^{6–8} or multiple cell types in a complex tissue^{9–11}. Multicolour GFP approaches can also be used to follow multiple aspects of gene expression. For example, a gene expression construct has been created¹² incorporating lac operator sites and encoding peroxisome-targeted cyan fluorescent protein (CFP). Co-expression of a yellow fluorescent protein (YFP)-fused lac repressor protein, which binds to the lac operator sites, allowed visualization of the integrated expression construct in the nucleus. Transcription was marked by visible decondensation of the YFP-associated chromatin and translation by the subsequent accumulation of CFP fluorescence in peroxisomes.

The relatively high stability of GFP in the cell, however, makes temporal analysis of protein expression difficult. In cell culture assays, 'leaky' expression from inducible systems can accumulate,

giving high background fluorescence. Similarly, *in vivo*, the dynamic expression history of a given tissue is often difficult to assess owing to the stability of residual GFP. Li *et al.*¹³ were the first to address these issues by generating destabilized variants of GFP. By fusing a PEST-containing domain of the mouse enzyme ornithine decarboxylase to the C-terminus of an enhanced GFP, they generated a GFP that was rapidly turned over by proteolysis. Cessation of translation is thus made visible as a rapid loss of fluorescence (Fig. 1a). Destabilized GFP reporters are inevitably less sensitive because higher levels of protein must be produced to achieve a detectable signal, yet, for inducible expression systems, the high rate of turnover results in reduced accumulation of leaky, uninduced GFP production. Similarly destabilized constructs have also been used to study perturbations of the ubiquitin-dependent proteolytic pathway¹⁴.

A more recent approach to the characterization of expression timing involves the use of a fluorescent protein whose spectral properties change with time. The 'fluorescent timer' protein was generated by random mutagenesis of the red fluorescent protein DsFP583 (ref. 15). This protein initially produces a green-emitting fluorophore similar to that of GFP yet, over the course of hours, undergoes an oxygen-dependent autocatalytic reaction to generate a fluorophore that emits in the red. A tissue thus indicates its fluorescent timer production history by its ratio of green to red fluorescence: tissues that have recently initiated expression appear green, those with continuous expression appear yellow to orange and those that have ceased expression appear entirely red (Fig. 1b).

Localization

Perhaps the most celebrated and widely applied function of GFP is its ability to serve as a fluorescent molecular label for proteins of interest, allowing the location and trafficking of the protein to be visualized *in vivo*. There is now an abundance of characterized GFP fusion proteins that allows live fluorescent marking of many sub-cellular structures^{6,16–27}. 'Protein trap' strategies have further used GFP to discover genes whose products have a specific intracellular location²⁸, and GFP has been used in cDNA expression screens for the same purpose²⁹. Even the intracellular transport of mRNA has been followed using GFP fusion proteins^{30–32}. Extracellularly, GFP has been shown to label viral proteins that traffic between cells³³ and has been shown to function as a label for secreted signalling proteins. Fusions of GFP to the Dpp protein can be observed to

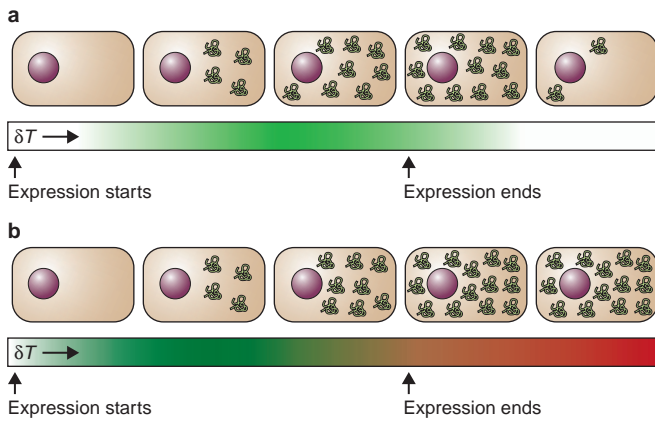


Figure 1 Two approaches for monitoring gene-expression kinetics by using fluorescent proteins: **a**, destabilized fluorescent protein¹³; **b**, fluorescent timer protein¹⁵. Destabilized fluorescent protein is degraded as expression ends, whereas the spectral properties of the fluorescent timer protein change with time.

cross many cells in *Drosophila* wing imaginal discs^{34,35} and a two-colour GFP approach has been used to demonstrate activity-dependent trafficking of brain-derived neurotrophic factor (BDNF) across synapses between cultured cortical neurons³⁶.

Seeing protein interactions using GFPs

Such studies of protein localization notwithstanding, the experimental potential of GFP is far from fully realized. The continued discovery of additional fluorescent protein variants holds terrific promise for greater understanding of protein–protein interactions. Specifically, GFPs with independent excitation spectra can be used as partners in FRET experiments. FRET occurs when the excited-state energy of a blue-shifted donor fluorophore is transferred to a red-shifted acceptor fluorophore in close physical proximity (Fig. 2a). Because FRET occurs only when GFP fluorophores are separated by physical distances on the order of 60 Å or less³⁷, compatible GFP-tagged proteins undergo FRET only when associated by direct binding or as part of a protein complex. FRET imaging thus reports spatial information about protein localization at a resolution higher than any microscope could provide optically.

For FRET experiments using two GFP molecules, the best-characterized and most commonly used proteins are CFP and YFP. Imaging protocols that detect FRET typically measure the emission of both donor CFP and acceptor YFP after the excitation of only the donor CFP. The ratio of acceptor to donor fluorescence thus reports the degree of physical association between two fusion proteins. Quantitative FRET measurement can be powerfully applied. The efficiency of FRET between two characterized GFPs can, for example, be taken as a direct measure of their separating distance using a known conversion formula—Förster's Equation³⁸, which incorporates empirically derived constants for FRET between the particular GFPs used³⁷. Such analysis has provided the basis for structural analysis of myosin bending³⁹.

In addition to ratiometric imaging, several other methods for detecting FRET have been described. These include spectral measurement approaches, in which the emission profile of an interacting FRET pair can be interpreted as an additive mixture of the emission spectra of the two component fluorophores^{40,41} (Fig. 2b). Spectral measurement approaches can also be used to investigate self-interactions of wild-type GFP protein⁴². With both ratiometric and spectral approaches to measuring FRET, however, the

experimenter must carefully assess the degree of cross-talk between fluorophores with the imaging system used. Such cross-talk might be misinterpreted as a FRET effect if the concentrations of donor to acceptor GFPs are not constant relative to one another. Once measured, however, cross-talk can be accounted for during image post-processing^{43,44}.

A more advanced technology for analysing fluorophore interactions relies on measurements of the length of time GFP remains fluorescent after excitation with a fast pulsed laser, typically <10 ns. Called fluorescent lifetime imaging microscopy (FLIM)⁴⁵, this technique can be used to monitor FRET interactions because energy transfer from donor to acceptor results in a decrease in the donor fluorescence lifetime. Importantly, this technique can be used to detect FRET between fluorophores with nearly identical emission spectra, because the change in fluorescence lifetime of the donor GFP can be analysed independently of acceptor emission. This technique is also advantageous for donor fluorophores that are prone to photobleaching.

Finally, those without access to advanced imaging systems need not be dismayed. FRET analysis can easily be used to look at spatiotemporal patterns of protein interaction using simple commercial fluorescence microscopes. A simple method for imaging FRET, for example, consists of imaging donor fluorescence from a FRET pair, specifically photobleaching the acceptor fluorophore and re-imaging donor fluorescence⁴¹. Elimination of FRET owing to destruction of the acceptor consequently results in a brighter signal from the donor. Although such an imaging method is less suitable for time-lapse or temporal analysis, it requires only simple imaging equipment, namely an emission filter that allows the imaging of primarily donor fluorescence and an excitation filter or laser line that allows selective bleaching of the acceptor. Concerns of cross-talk between fluorophores are alleviated because an increase in fluorescence following acceptor photobleaching cannot be ascribed to inadvertent excitation of the acceptor.

FRET between GFP and other fluorophores

FRET imaging can also be used to observe interactions between GFP fusion proteins and fluorescently labelled antibodies. For example, FLIM has been used to show that FRET can be detected between a protein kinase C α (PKC α)–GFP fusion protein and a Cy3.5 fluorescently labelled phosphorylation-site-specific antibody (T(P)250)⁴⁶. By microinjecting the labelled T(P)250 antibody into living cells, the phosphorylation-dependent activation of PKC could be seen as cells responded to inducing signals. Similar recent work has shown the distribution of phosphorylated epidermal growth factor (EGF) receptor in EGF-stimulated cells⁴⁷ and, recently, waves of ErbB1 receptor signal propagation following focal stimulation of cells with EGF (ref. 48).

Bioluminescence-based variations on FRET with GFPs (termed BRET), in which luciferases serve as FRET donors for GFP, have also been used to study the dynamic assembly of protein complexes in circumstances where excitation illumination of the sample is disadvantageous. For example, in the study of association between protein components of the circadian clock in cyanobacteria, *Renilla* luciferase (RLUC) has been used as an effective FRET donor in conjunction with an enhanced YFP (EYFP) acceptor to show dimerization between RLUC and EYFP-fused KaiB proteins⁴⁹. Such an approach has also been used to show modulation of β_2 -adrenergic receptor dimerization in cultured cells⁵⁰. GFP has been further used as a FRET acceptor for the chemiluminescent jellyfish luciferase aequorin⁵¹. Aequorin metabolizes coelenterazine, its luciferin, in response to binding free calcium ions. By fusing GFP directly to aequorin, a sensor was produced that reports calcium ion flux by increases in GFP fluorescence. As a resonance energy acceptor, GFP actually increases the light output of aequorin severalfold.

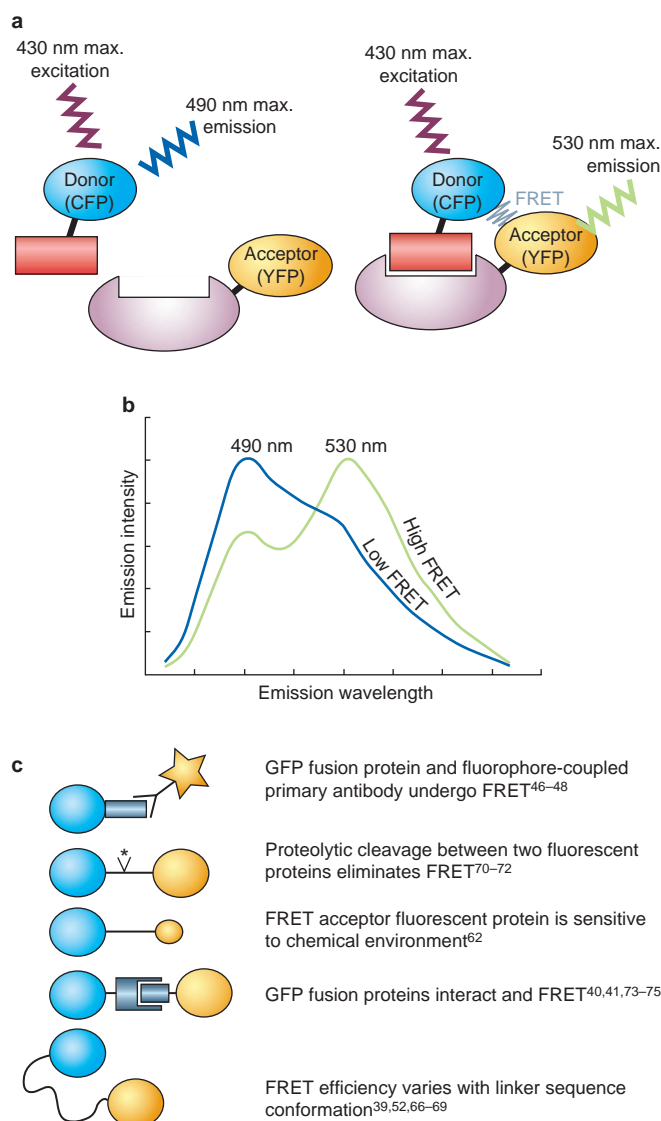


Figure 2 Principles of fluorescence resonance energy transfer with fluorescent proteins **a**, Schematic representation of FRET; **b**, schematic spectra of two fluorescent proteins undergoing FRET; **c**, architectures of some 'optical sensor' proteins and experimental approaches employing protein-based FRET.

Single gene FRET sensor constructs

The ability optically to measure changes in intracellular Ca^{2+} concentration with a genetically targetable sensor is a goal that has motivated many of the pioneering applications of FRET in biological imaging. The first published Ca^{2+} sensor proteins incorporating GFP (ref. 52) illustrates what has since become a broadly applied principle for designing FRET-based GFP reporters. Called 'CAMEleons', for their incorporation of a calmodulin domain and their ability to change colour, these calcium sensor proteins link two spectrally separable GFP variants using a domain of calmodulin and a calmodulin-binding peptide. This linker sequence changes conformation when the calmodulin domain binds Ca^{2+} . Because FRET depends on both the proximity of the donor and acceptor fluorophores, and the orientation of their relative dipoles,

the interaction of CAMEleons with Ca^{2+} leads to changes in the degree of FRET between the two constituent GFPs. Furthermore, as the two GFPs are encoded by a single gene, they are expressed at a constant level relative to one another. Thus, following calibration of the FRET response to known Ca^{2+} concentrations, the degree of FRET *in vivo* can theoretically reflect the absolute levels of Ca^{2+} present in a cell. Practical problems with the approach, however, include concerns that different sensitivities to characteristics of subcellular regions (such as pH) and different bleaching of donor and acceptor fluorophores might skew such calibrated ratiometric analysis. CAMEleons have been subject to continuing development, generating variants with decreased pH sensitivity⁵³ as well as decreased chloride sensitivity and increased photostability⁵⁴. The CAMEleons have been used to image Ca^{2+} concentration changes associated with regulated exocytosis of secretory granules⁵⁵, Ca^{2+} transients in *Arabidopsis* guard cells⁵⁶ and Ca^{2+} transients in neurons and pharyngeal muscles of intact *Caenorhabditis elegans*⁵⁷.

The design of CAMEleons has served as a general template for many other sensors of ion concentration and cell signalling events. In addition to constructs that separate GFPs with an environmentally sensitive linker protein, however, sensor constructs have also been designed using GFPs that have different sensitivities to a particular environmental characteristic. For example, some GFP variants are extremely sensitive to intracellular pH, and mutagenized GFP variants have even been used directly as non-ratiometric^{58,59} and ratiometric^{60,61} intracellular pH indicators. The chloride-ion-sensitive 'Clomeleons'⁶² are ratiometric sensor constructs in which a Cl-sensitive FRET acceptor (YFP) is fused to a non-sensitive donor (CFP). As YFP fluorescence varies greatly according to the environmental concentration of Cl^- , the ratio of donor to acceptor emission is similarly Cl^- -concentration dependent. Great potential for further design of such constructs comes from the ability to make single GFP molecules sensitive to particular aspects of the biochemical environment by 'circular permutation' and insertion of conformationally sensitive protein sequences within the β -barrel structure of GFP^{63–65}. Linking such biochemically sensitive GFPs to a stable FRET donor GFP might be an alternative method for designing FRET-based optical sensors.

Beyond intracellular ion sensing, FRET constructs have been used to investigate the downstream events of second messenger signalling. Targeting the cAMP signalling pathway, a sensor construct has been designed based closely upon the CAMEleon model⁶⁶ in which the kinase-inducible domain (KID) of cAMP-responsive-element-binding protein (CREB) forms a linker between blue fluorescent protein (BFP) and GFP. cAMP-induced PKA-dependent phosphorylation is known to cause a conformational change in the KID, and FRET efficiency is altered in response to cAMP signalling. cGMP activity has recently been detected in a similar way. Both Sato *et al.*⁶⁷ and Dostmann and co-workers⁶⁸ have generated sensor proteins that place truncated and kinase-inactivated cGMP-dependent protein kinase between blue- and red-shifted fluorescent proteins. Unexpectedly, although both groups' constructs report a similar degree of change in FRET in response to cGMP, the orientation of this change differs between the constructs. Dostmann and co-workers report an increase in FRET in response to increasing cGMP levels, whereas Sato *et al.* report a decrease. Although any variation in FRET is useful for measuring cGMP activity, these two results reaffirm the unpredictability of outcome when designing novel FRET-based reporter proteins.

The use of FRET-based optical sensors has also recently altered our understanding of Ras family G-protein activation. To visualize Ras signalling in live cells, a single-gene construct consisting of CFP-Ras and the Raf Ras-binding domain (RBD)-YFP was fused by a protein linker domain⁶⁹. This construct was targeted to cellular membranes by the addition of a farnesyl moiety. When Ras signalling is activated by stimulating cells with EGF, RBD binds Ras, and YFP and CFP are brought into close proximity, increasing the degree of FRET interaction between the two fluorophores

and allowing spatiotemporal readout of Ras activation patterns. When visualized like this, Ras signalling was observed to occur in a wave from plasma membrane to nucleus. A similar approach has also been used to image the activation of Rap1, another Ras-family G-protein. Strikingly, Rap1 activation has been shown to radiate out from perinuclear regions. Because previous studies of Ras and Rap1 signalling were based primarily on biochemical data, these observations are a major advance in our understanding of how Ras and Rap1 pathways are activated and co-ordinated after growth factor stimulation. This work is notable for the great care taken to ensure that the FRET data reflect the real behaviour of the endogenous proteins. A strength of single-construct sensor approaches is that they limit the potentially adverse interaction of overexpressed fusion proteins with endogenous counterparts. As the RBD of Raf is physically associated with Ras-CFP in this case, it is unlikely that the Ras-GFP can interact with endogenous Raf and disrupt signalling.

Finally, protein cleavage by specific proteases underlies many signalling events and such cleavage events invite the most straightforward FRET-based sensor proteins. Several reporter constructs have been described that place FRET-interacting GFPs on either side of a protein cleavage sequence sensitive to a specific protease. Signal transduction events that activate the protease thus lead to cleavage of the reporter and loss of FRET. One protease sensor construct, for example, had a calpain-sensitive protein cleavage site between YFP and CFP (ref. 70). Calpain is activated by calcium influx and the calpain-sensitive FRET reporter has been used in cultured neurons to monitor calpain activity in dendritic spines. Also, many of these constructs have been aimed at sensing the activity of caspase endopeptidases, which are crucial components of the apoptotic cell death pathway. By placing the caspase-3-sensitive DEVD amino acid sequence between BFP and GFP, FRET could be used to monitor activity of the protease and corresponding entry into cell death⁷¹. Similar constructs have been reported using other GFP variants that have been optimized to yield a more than fivefold change of FRET in response to caspase activity⁷². The screening needs of the pharmaceutical industry have provided a major impetus for the development of apoptosis detection protocols and so many of these FRET applications have been adapted for high-throughput experimental formats.

Bimolecular FRET with GFP fusion proteins

Much initial work on the use of GFP FRET to study protein interactions was performed by Day and colleagues^{73,74}, who used the technique to characterize the heterodimerization of GFP-variant-tagged transcription factors. Such an approach requires two independently expressed GFP fusion proteins. Expressing independent concentrations of donor and acceptor GFPs means that fluorescence ratios cannot be calibrated to yield information independently of relative expression levels. Although the success rate for bimolecular FRET applications has been comparatively low, several recent reports have shown the value of this approach for following the spatial and temporal characteristics of signalling cascades and other cellular processes. For example, a bipartite protein kinase A (PKA) system for the reporting of cAMP activation has been reported⁷⁵. Following production of both a GFP-tagged catalytic α -subunit and a BFP-tagged regulatory RII β -subunit, FRET between BFP and GFP is detectable in cells, suggesting an association of both subunits into characteristic PKA holotetramers. These subunits dissociate in response to cAMP signalling and thus an increase in cAMP is measured as a decrease in FRET.

An exciting recent report has also showed that FRET can be used to report active signalling of G-proteins⁴⁰. G-protein signalling cascades underlie biological responses to hormones, odorants and neurotransmitters. CFP-fused $G\alpha$ and YFP-fused $G\beta$ subunits expressed in living *Dictyostelium* associate with endogenous $G\gamma$ in G-protein heterotrimers and undergo FRET. Following stimulation

with cAMP, these heterotrimers dissociate, separating fluorescent $G\alpha$ and $G\beta\gamma$ protein subunits, and eliminating FRET. Thus, FRET, detected both by ratiometric imaging and by spectrofluorimetry, allowed the first real-time measurements of G-protein dissociation and reassociation kinetics. One potential drawback of two-component FRET systems is that GFP-labelled proteins might interact with their endogenous counterparts, reducing the efficiency of FRET and potentially disrupting endogenous function. These issues are addressed by showing that GFP fusions of $G\alpha$ and $G\beta$ rescue their corresponding mutant phenotypes and by carrying out FRET analysis in a $G\alpha$ mutant background.

Yet more recently, a comprehensive analysis of protein interactions among components of the Golgi-to-ER transport pathway has been undertaken using CFP and YFP fusion protein pairs and FRET imaging⁴¹. Multiple-photon excitation and acceptor-bleaching analysis have been used to demonstrate FRET between a KDEL-motif-containing ligand (the non-toxic A subunit of a mutant cholera toxin) and the *cis*-Golgi-localized KDEL receptor ERD2. CFP- and YFP-tagged fusions of ERD2 allowed imaging of ERD2-ERD2 interactions following KDEL ligand binding. Further CFP or YFP fusion proteins of vesicle budding machinery components including ARF, ARFGAP, COPI and p24 proteins showed active rearrangement after KDEL ligand binding.

Imaging into the future

The development of new genetically encoded fluorescent labels and sensors has depended largely on the simultaneous development of new techniques for imaging cells. The advantages of live markers such as GFP have been most dramatically realized in conjunction with imaging technologies such as laser-scanning confocal microscopy, which enable researchers to work with living samples. Advances in laser-scanning microscopy, including the development of multiple-photon microscopes⁷⁶, have further pushed the boundaries of fluorescent protein imaging, allowing penetration deeper into thick tissue samples and reducing photodamage to both sample and fluorophore. The use of microscopes designed for image deconvolution⁷⁷, fast imaging⁷⁸ and imaging close to the plasma membrane⁷⁹ is further enhancing the value of GFP. In addition, these imaging technologies are now being improved to suit the needs of GFP imaging better. As a recent example, a spectral analysis technique from the aerospace industry has been adapted to image separate emission signals for multiple GFP variants⁸⁰. Several GFPs are excited simultaneously and a 32-channel spectrophotometer is then used to record an emission spectrum for each pixel in an image. By mathematical modelling of the emission spectrum of each pixel as an additive combination of several component fluorescent protein emission spectra, the contribution of each individual GFP to every pixel can be determined. With this technology, seven fluorophores can be imaged simultaneously and the nearly identical signal of FITC can be separated from that of enhanced GFP. This spectral deconvolution is of great significance for multiple-photon microscopy because variant GFPs tend to have broad multiple-photon excitation spectra, making them difficult to excite independently. This technique also holds great promise for FRET imaging following either single- or multiple-photon excitation.

New imaging technologies will also elicit new functions from fluorescent proteins. 'Second-harmonic' imaging techniques, for example, have led to a greatly sought-after technical achievement—the use of GFP to sense membrane potential with a time resolution high enough to visualize action potentials in firing neurons⁸¹. Recently such voltage measurements have been accomplished with FRET-based sensor proteins as well⁸². New imaging technologies, coupled with the increasing capability of computer software for image acquisition and analysis, will both allow and demand new advances in fluorescent-protein-based optical sensors. Image acquisition will tend towards the simultaneous acquisition of information about multiple cellular processes. What we see in our samples

will be limited only by the creativity of our optical approach and our ability to assess and understand the increasingly multilayered data we acquire. □

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