CREATING NEW FLUORESCENT PROBES FOR CELL BIOLOGY

Jin Zhang, Robert E. Campbell*, Alice Y. Ting*‡ and Roger Y. Tsien*§*

Fluorescent probes are one of the cornerstones of real-time imaging of live cells and a powerful tool for cell biologists. They provide high sensitivity and great versatility while minimally perturbing the cell under investigation. Genetically-encoded reporter constructs that are derived from fluorescent proteins are leading a revolution in the real-time visualization and tracking of various cellular events. Recent advances include the continued development of 'passive' markers for the measurement of biomolecule expression and localization in live cells, and 'active' indicators for monitoring more complex cellular processes such as small-molecule-messenger dynamics, enzyme activation and protein–protein interactions.

PHOTOBLEACHING
The irreversible destruction, by any one of a number of different mechanisms, of a fluorophore that is under illumination.

**Department of Pharmacology and §Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, 18–496, Cambridge, Massachusetts 02139, USA.*
‡Department of Chemistry & Biochemistry and Howard Hughes Medical Institute, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093–0647, USA.
Correspondence to R.Y.T
e-mail: rtsien@ucsd.edu
doi:10.1038/nrm976

Our understanding of biological systems is increasingly dependent on our ability to visualize and quantify signalling molecules and events with high spatial and temporal resolution in the cellular context. Advances in fluorescence microscopy and the engineering of the green fluorescent protein (GFP) from *Aequorea victoria* into mutants with improved properties and altered colours have provided the basic tools that allow the investigation of more complex processes in live cells. The primary advantages of fluorescent protein-based indicators over simple organic dyes are that they can be designed to respond to a much greater variety of biological events and signals, targeted to subcellular compartments, introduced into a wider variety of tissues and intact organisms, and they very rarely cause photodynamic toxicity. This review highlights recent advances in the development of fluorescent probes for cellular applications, and focuses on those that can resolve spatial and temporal patterns through targeting to subcellular compartments. As the number of successful genetically-encoded reporters increases, several design trends and considerations are becoming apparent (BOX 1). We highlight the most versatile and modular of these designs as the blueprints for the construction of new and better reporters.

Recent advances in fluorescent proteins

New variants of green fluorescent protein. There is a continuing effort to develop new GFP variants with altered excitation and emission wavelengths, enhanced brightness and an improved pH resistance relative to the original enhanced green (for example, S65T and EGFP), cyan (CFP), and yellow (YFP) variants[1–5]. From now on we refer to the entire class of *Aequorea*-derived fluorescent proteins as AFPs, whereas we use 'GFP' to denote green members of that family. The newest colour in the AFPs is 'CGFP', the Thr203Tyr mutant of CFP (where Thr is threonine and Tyr is tyrosine). CGFP has an excitation and emission wavelength that is intermediate between CFP and EGFP[2]. Despite its dimness and broad excitation and emission peaks, the remarkably pH-resistant CGFP might find a use in the labelling of acidic organelles. First-generation YFPs such as GFP–Ser65Gly/Ser72Ala/Thr203Tyr[6] (where Ser is serine, Gly is glycine and Ala is alanine) were notorious for their sensitivity to pH, chloride fluctuations and PHOTOBLEACHING. A second-generation enhanced YFP (YFP–Val68Leu/Gln69Lys; where Val is valine, Leu is leucine, Gln is glutamine and Lys is lysine) slightly improved acid resistance, but only in third-generation derivatives named 'Citrine'[3] (YFP–Val68Leu/Gln69Met; where Met is methionine) and 'Venus'[4] (YFP–Phe46Leu/Phe64Leu/Met153Thr/Val163Ala/Ser175Gly; where

Box 1 | **Considerations in designing and constructing AFP-based fluorescent reporters**

Choice of *Aequorea* fluorescent protein (AFP) variant(s)
- For intermolecular fluorescence resonance energy transfer (FRET)-based reporters (FIG. 5a), non-oligomerizing cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) variants that incorporate the Ala206Lys mutation (where Ala is alanine and Lys is lysine) are strongly recommended.
- For intramolecular FRET-based reporters (FIG. 5b), CFP should be paired with one of the latest generation YFPs such as Citrine or Venus. Further improvements to monomeric red fluorescent protein (mRFP) should provide a new FRET partner for GFP.
- For single-fluorophore conformation-sensitive reporters (FIG. 4), insertion at Tyr145 (where Tyr is tyrosine), or the use of a circularly permuted AFP or YFP is recommended.

Spectral response
- The dynamic response range of the reporter must span physiologically relevant conditions.
- A ratiometric response, as obtained from a FRET reporter, is preferable to a simple increase in fluorescence intensity.
- For most reporter constructs, optimization of polypeptide linkers between components of the reporter is crucial for the success of the construct or for maximizing the fluorescent response.

Spatio-temporal resolution
- Spatio-temporal resolution will be lost with a freely diffusing cytosolic reporter that responds slowly relative to the timescale of diffusion.
- Genetic targeting to a compartment or anchoring to a subcellular structure can improve spatio-temporal resolution.

Perturbation of intracellular conditions
- Depending on the design strategy and the expression level, the introduction of the reporter might perturb the cellular component of interest or be toxic.

Specificity
- The reporter must respond to only the stimulus of interest.
- Genetic targeting can help increase biological specificity.

Versatile molecular construction
- Consideration of the available structural data can provide a rational basis for reporter construction.
- Ideally, the design strategy should be transferable to other members of the same protein family and structural homologues.

Phe is phenylalanine) has the chloride sensitivity been eliminated and the sensitivity to pH changes and photobleaching improved greatly. Specifically, Citrine remains 50% fluorescent at the lowest pH (5.7) that has been reported so far for a YFP and shows a twofold increase in photostability relative to YFP–Val68Leu/Gln69Lys, whereas Venus is the brightest and fastest maturing (with reference to the development of fluorescence) YFP so far. Unfortunately, simply transferring the crucial Gln69Met mutation of Citrine to Venus did not confer improved photostability[4] on Venus, and so there is not yet a single YFP that is superior for all applications.

Fluorescent 'highlighters'. Fluorescent proteins that can be modulated photochemically are molecular 'highlighters' that allow specific organelles or protein subpopulations to be marked by brief, localized, intense illumination and then tracked in space and time. Early versions showed only a modest contrast[7] or could only be used under anaerobic conditions[8,9]. A new YFP mutant, 'PA–GFP' (GFP–Val163Ala/Thr203His; where His is histidine) undergoes up to a 100-fold increase in fluorescence (excitation at 488 nm) when illuminated at 413 nm[10]. An even more spectacular fluorescent protein is 'Kaede' from the stony coral *Trachyphyllia geoffroyi*, which can be converted from a green to a stable red fluorescent protein by irradiation with 350–400-nm light[11]. This colour change corresponds to a 2,000-fold increase in the red to green ratio and allows both the irradiated and unirradiated species to be visualized separately with excitation wavelengths (475 and 550 nm) that do not cause any further colour change. At present, Kaede exists as a tetramer; if it can be mutated to a monomer, it should match or surpass PA–GFP in its ability to reveal the dynamic trafficking of fusion proteins.

In addition to the photoenhancement of fluorescence described above, AFP fusions find use in fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP), and fluorescence correlation spectroscopy (FCS) — other powerful techniques that can assess protein trafficking and mobility in live cells (for a review, see REF. 12).

Limiting monomer–monomer interactions. The only instance when the weak tendency of AFPs to dimerize has been documented to cause artefacts or dysfunction was in the clustering of lipid-anchored AFPs on the plasma membrane[13]. At high concentrations, AFPs might interact with each other, which results in a false-positive interaction as determined by

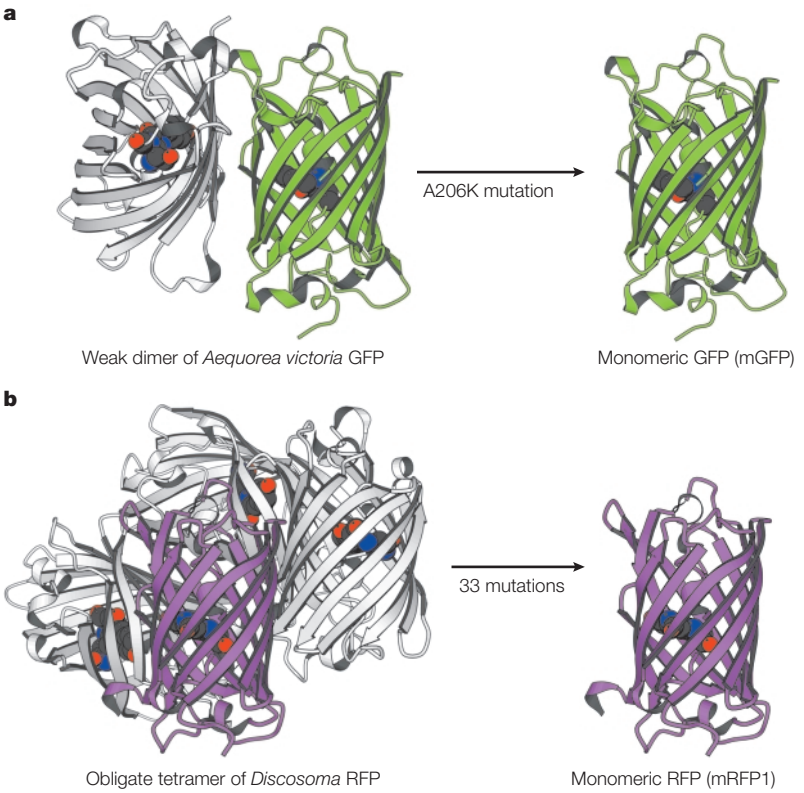

Figure 1 | **Recent advances in fluorescent proteins. a** | Introduction of the A206K mutant in *Aequorea* fluorescent proteins (AFPs) suppresses their tendency to dimerize. **b** | Several rounds of directed evolution and a total of 33 mutations were required to rescue the red fluorescence of a *Discosoma* red fluorescent protein (RFP) (DsRed or drFP583)-derived monomer following the introduction of interface-disrupting mutations.

FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET). The non-radiative transfer of energy from a donor fluorophore to an acceptor fluorophore that is typically < 80 Å away. FRET will only occur between fluorophores in which the emission spectrum of the donor has a significant overlap with the excitation of the acceptor.

QUANTUM YIELD
The probability of luminescence occurring in given conditions — expressed by the ratio of the number of photons that are emitted by the luminescing species to the number of photons that are absorbed.

FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET). This tendency to dimerize[14,15] can be reduced greatly or eliminated by mutating the hydrophobic amino acids that are in the dimerization interface to positively charged residues[13]. In AFPs, the effectiveness of these mutations increases in the order Phe223Arg < Leu221Lys < Ala206Lys (where Arg is arginine; FIG. 1a). It would now seem prudent to routinely use non-dimerizing mutants such as Ala206Lys when testing protein–protein interactions, or when AFP fusion proteins seem to be causing mis-targeting or dysfunction[13].

Long-wavelength red fluorescent proteins. Long-wavelength fluorescent proteins have long been sought for multicolour protein-tracking applications and for the construction of improved FRET-based reporters. Red fluorescence should provide greater tissue penetration and better spectral separation from cellular autofluorescence than either yellow or green fluorescence. The first red fluorescent protein (RFP) to be discovered was isolated from a coral of the *Discosoma* genus (this RFP is known as DsRed or drFP583)[16] and was received with much excitement, but its use has been limited severely by a number of problems. The DsRed protein requires incubation at 37°C for more than 30 h for the red fluorescence to reach a steady-state level, and a significant fraction of the protein retains the green fluorescent 490-nm excitation peak that is associated with its 'GFP-like' intermediate. In addition, DsRed is an obligate tetramer that will almost certainly tetramerize any cellular protein to which it is fused[17,18]. If the fusion partner is a monomeric protein, tetramerization might not necessarily be detrimental, but if the partner has any tendency to oligomerize by itself, gross aggregation and precipitation of the fusion is likely. If aggregation does occur, one strategy that has proven successful for both hexameric connexin-43 (REF. 19) and dimeric thymidine kinase[20] fused to DsRed, is to co-express either the unfused or EGFP-fused target protein, but this approach requires the titration of co-expression levels and dilution of the desired red signal.

A more universal solution to the problem of oligomerization is to re-engineer the RFP using a combination of targeted and random mutagenesis to minimize the oligomerization and other limitations of wild-type DsRed[21–25]. The original commercially available form of DsRed (known as DsRed1), which incorporated mammalian codon-usage preferences, was replaced by DsRed2, which showed a 2–3-fold improvement in the speed of fluorescence maturation at 37°C and a diminished 475-nm excitation peak[22,26]. DsRed2 has, in turn, been effectively superseded by T1, which reaches its full red fluorescence within tens of minutes[23]. T1 is now commercially available as DsRed-Express (Clontech, Palo Alto, USA). The first effectively non-oligomerizing RFPs came with the development of red fluorescent tandem dimers, in which two dimer-forming subunits are concatenated with a spacer that allows them to satisfy their crucial dimer interactions through intramolecular contacts[24,27]. The most recent advance is the engineering of a completely monomeric DsRed variant (mRFP1) that matures quickly, has no residual green fluorescence and excitation and emission wavelengths that are about 25 nm longer than the previous DsRed variants[24] (FIG. 1b). At present, mRFP1 is probably the best starting point for the construction of red fluorescent fusion proteins, even though it sacrifices some QUANTUM YIELD and photostability relative to the tandem dimer that is derived from DsRed (REF. 24).

Finding more fluorescent proteins. The search for new fluorescent proteins in coelenterate marine organisms has resulted in the discovery and cloning of approximately 30 distinct fluorescent proteins, although all but a handful of these remain minimally characterized[28,29]. Several of these proteins that deserve a special mention include: the dimeric *Renilla mulleri* GFP, with its exceptionally narrow excitation (498 nm) and emission (509 nm) peaks[30]; the DsRed homologues asFP595 (REF. 31) from *Anemonia sulcata* and dsFP593 (REF. 32) from *Discosoma*, which were engineered for improved red fluorescence at 595 nm and 616 nm respectively; the dimeric but dim HcRed from *Heteractis crispa*, which was derived from a tetrameric non-fluorescent chromoprotein and emits at 618 nm[26,33]; and finally eqFP611 from *Entacmaea quadricolor*, a tetrameric red fluorescent protein that emits at 611 nm and that can be dissociated to monomers at a high dilution[34].

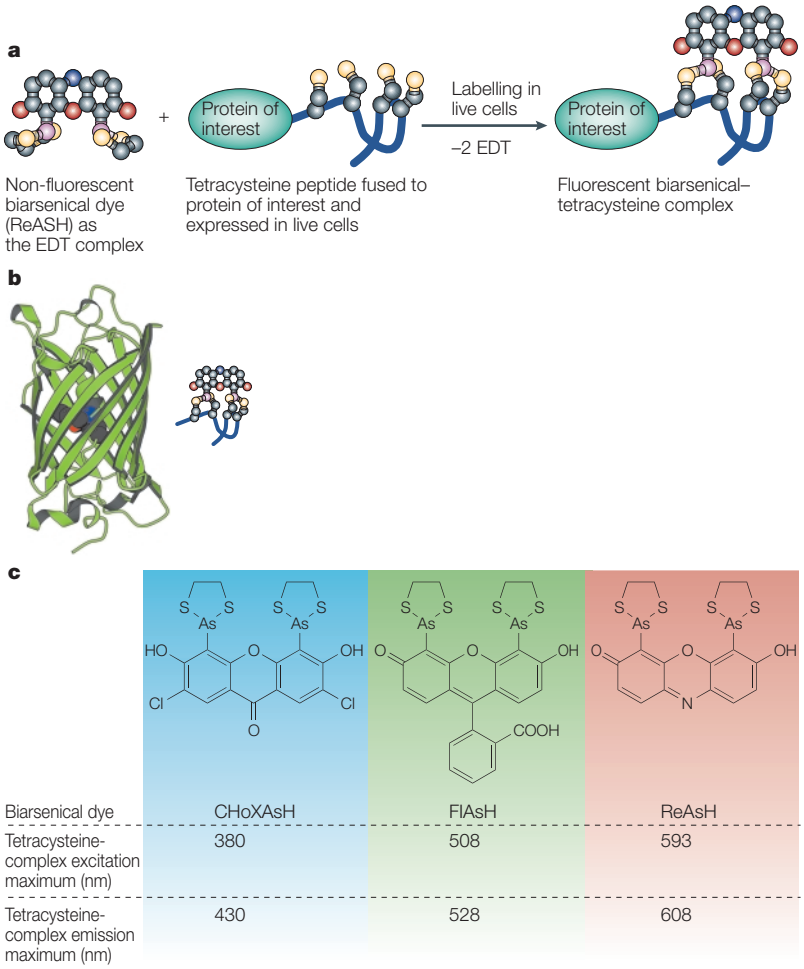

Figure 2 | **The biarsenical–tetracysteine system. a** | A non-fluorescent membrane-permeable biarsenical dye (ReAsH shown) forms specifically a fluorescent covalent complex with any intracellular protein to which a short tetracysteine-containing peptide (CCPGCC) has been genetically fused. The small size of the biarsenical dyes might be advantageous in those cases where a bulky *Aequorea* fluorescent protein (AFP) fusion disrupts normal protein function. The arsenical antidote ethanedithiol (EDT) is used to minimize the toxicity of any unbound biarsenical-dye. **b** | A comparison of the to-scale images of the green fluorescent protein (GFP) and biarsenical–tetracysteine complex. **c** | Fluorescent biarsenical labels that span the visible spectrum have been synthesized.

Recent advances in small molecule probes. Although GFP and its variants are extremely useful for tracking the expression and localization of proteins in cells, small-molecule probes with less steric bulk, faster rates of labelling and the ability to provide readouts in addition to, or other than, fluorescence are desirable.

The biarsenical–tetracysteine system. A promising alternative to GFP is the biarsenical–tetracysteine system (FIG. 2a) in which a recombinant protein that is expressed in a living cell is site-specifically labelled with a membrane-permeable biarsenical dye, which can be blue, green or red fluorescent (FIG. 2c)[35–37]. The specificity for the labelling reaction is provided by a small tetracysteine motif (at present, CCPGCC is the preferred motif) — the nucleic acid sequence of which can be fused to the gene encoding the protein of interest. The biarsenical dye and tetracysteine motif form a covalent complex in which each of the dye's arsenic atoms cooperatively binds a pair of cysteines. Toxicity is minimized by the administration of 1,2-dithiols, for example ethanedithiol (EDT) — antidotes that protect endogenous pairs of cysteines and keep the dyes largely non-fluorescent until they find their ultimate tetracysteine targets. Nevertheless, at present, background staining keeps the sensitivity and the detection limit of this method to an order of magnitude or so worse than those of GFP. The other main limitation of this method is that the target cysteines must be in their reduced form before they can bind the biarsenical dye. Although this will generally be true for cysteine residues in the reducing environment of the cytosol or nucleus, cysteines that are in the lumen of the secretory pathway or outside cells tend to oxidize spontaneously and they can only be labelled if acutely reduced.

Of the biarsenical dyes already characterized[37] (FIG. 2c), a resorufin-based red label (ReAsH) is particularly useful as it can be used for both fluorescence and electron microscopy (EM). Under intense illumination in fixed samples, ReAsH catalytically generates singlet oxygen, which oxidizes diaminobenzidine into a highly localized polymer, which is readily stained by osmium tetroxide for EM contrast. So tetracysteine tags constitute genetically targetable tags for EM that show catalytic amplification, but do not require the diffusion of large antibody molecules into fixed or frozen tissue[38].

Furthermore, sequential labelling with different biarsenical dyes can indicate the age of protein molecules. The tetracysteine motifs are labelled rapidly and saturably with one colour of a membrane-permeant biarsenical dye, such as green FlAsH (FIG. 2c), then any free dye is washed out and the live cells allowed to synthesize fresh unlabelled copies of the same tagged protein. A final exposure of the protein to a biarsenical dye of a different colour, such as red ReAsH, labels only the newly synthesized copies. This approach was used to study the life cycle of connexin-43 as it was trafficked into and out of gap junctions (FIG. 3). Newly synthesized connexins (red) enter the gap junctional plaques from their outer edges while older molecules (green) are removed by endocytosis from the plaque centres. Varying the order and timing of FlAsH versus ReAsH administration can control whether ReAsH labels exocytic vesicles that carry new connexin molecules or endocytic compartments that contain old molecules, and thereby enables each population to be visualized separately by EM[38]. The conclusion that connexins move progressively from the periphery to the centre of gap junctions agrees with the work of Lauf *et al.*[39], who photobleached gap junctions that contained GFP-tagged connexins and observed that newly synthesized connexins formed a thin fluorescent halo that thickened with time.

So, the biarsenical sequential labelling technique and photochemical marking overlap somewhat in their areas of applicability, and they can reach concordant conclusions. The biarsenical technique is unique in the small size of the tag and the ability to give EM as well as fluorescence images; the photochemically sensitive fluorescent proteins have better time-resolution, lower

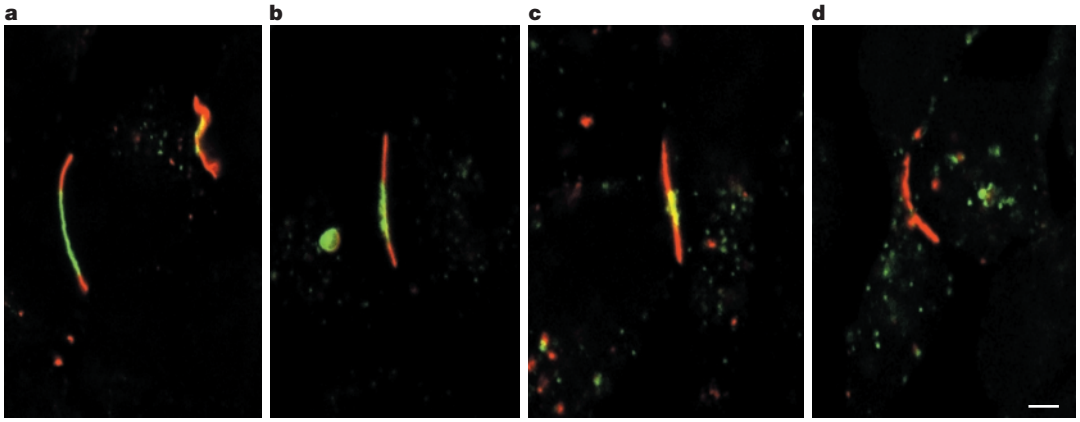

Figure 3 | **Multicolour pulse–chase biarsenical staining of gap juctions.** FlAsH and ReAsH were used to label two temporally separated pools of connexin-43 that had been fused to a tetracysteine-containing peptide (Cx43–4Cys) and then junctional plaque renewal was recorded over time[38]. HeLa cells that were expressing Cx43–4Cys were stained with FlAsH, incubated for 4 h (**a**,**b**) or 8 h (**c**,**d**), and then stained with ReAsH. Panels **a** to **d** show junctional plaques at different stages of renewal, as indicated by the different ratios of FlAsH (green) and ReAsH (red) stains. The green central zones diminish as the time interval between FlAsH and ReAsH staining increases from 4 to 8 h. Scale bar represents 1 μm. Adapted with permission from REF. 38 © 2002 American Association for the Advancement of Science.

background levels and can be marked with geometrically defined spatial patterns.

Antibody–hapten labelling. An alternative fluorescent dye-based labelling system consists of a single chain antibody which binds specifically to membrane-permeant fluorescent conjugates of its phenyloxazolinone hapten[40]. At present, because the antibody does not fold well in reducing environments, this system is best suited for use in secretory compartments and so has a complementary preference to the biarsenical–tetracysteine system. Ultimately it would be desirable to increase the affinity of the antibodies for the hapten (now an approximately 5 nM dissociation constant), and the extent to which antibody binding enhances fluorescence (~5-fold at present).

Avidin–biotin labelling. Another receptor–ligand pair that works best in the secretory compartment is the avidin–biotin pair, which is extremely popular for use *in vitro* and in histology, but has been surprisingly neglected in live cells. Chicken avidin that is expressed recombinantly in different compartments of the secretory compartment can trap biotin conjugates of fluoresceins of various pK_a values for the measurement of the pH values of those compartments, which enables the mechanism of pH regulation to be studied[41–43]. The availability of biotin conjugates and their extremely high affinity for avidin are advantages of this approach. Limitations include the tightly tetrameric nature of avidin and the essential role for biotin in the cytosol and mitochondria, such that avidin in these compartments is either toxic or biotin-saturated[44].

Phycobiliproteins. Bilin-containing proteins are important antennae for photosynthesis in cyanobacteria and photoreceptor signalling in green plants. Because they can absorb light at wavelengths that extend to the infrared, significant efforts have been made to develop biliproteins as fluorescent fusion tags — this is despite their natural oligomeric structure and their requirement for exogenous bilin cofactors or co-expression of the additional enzymes that are required for biosynthesis of the cofactor. Heterologous expression of the cyanobacterial truncated phytochrome[45], C-phycocyanin[46] and phycoerythrocyanin[47] has been achieved. The most promising class of these PHYCOBILIPROTEINS is the homodimeric phytofluors[48], which have excellent spectral properties, can be engineered as monomers and would require the co-expression of as few as three proteins[45].

Uroporphyrinogen III methylation. Another fluorescent reporter system, which seems to have been overshadowed by DsRed, is the recombinant uroporphyrinogen III methyltransferase gene (*cobA*)[49]. The cobA reporter catalyses the trimethylation of endogenous uroporphyrinogen III to generate a fluorescent small-molecule product that accumulates intracellularly. Although the cobA reporter system is not amenable to either subcellular targeting or the construction of fusion proteins, there could be certain cases in which its unique combination of a red fluorescent emission, independence from any exogenous cofactor and catalytic signal amplification will find a use.

Passive applications of fluorescent proteins

For most fluorescence imaging applications, the fluorescent label is a biologically inert participant that is used merely as a visible marker. By the very nature of their barrel-like structures (FIG. 1), which effectively shield the CHROMOPHORE from the external environment, AFPs are well suited to these more passive applications. Typical passive uses of AFPs include monitoring the appearance, degradation, location or translocation of appropriate partner proteins to which they are fused.

pK_a
The pH at which a molecule, or a particular site within a molecule, carries an ionizable H^+ 50% of the time.

PHYCOBILIPROTEINS
Proteins from blue-green algae and red algae that exhibit intense fluorescence owing to the presence of multiple bilin chromophores that are covalently attached to the protein.

CHROMOPHORE
The core portion of a molecule that is directly responsible for absorbing photons. Chromophores usually contain alternating single and double bonds.

Table 1 | **Translocating fluorescent probes**

Domains	Source proteins	Target molecules	References
PH domain	For example, Akt, ARNO, GRP1	3′ phosphoinositides including PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2	55–59
PH domain	PLC	PtdIns(4,5)P_2 and InsP_3	60,61
C1 domain	PKC	DAG	62
C2 domain	PKC	Ca^{2+}	63,64
PA domain	Raf-1	PA	65

Akt, a serine/threonine kinase, also known as protein kinase B; C1 and C2, conserved domains 1 and 2 from protein kinase C; ARNO, ADP-ribosylation factor nucleotide-binding-site opener; C1, C homology-1; C2, C homology-2; DAG, Diacylglycerol; GRP1, general receptor for 3-phosphoinositides; InsP_3, inositol 1,4,5-trisphosphate; PA, phosphatidic acid; PH, pleckstrin-homology; PKC, protein kinase C; PLC, phospholipase C; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-triphosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-biphosphate ; Raf 1, a serine/threonine kinase important in mitogen-activated signalling.

Fluorescence as a spatial marker

Perhaps the most popular application of fluorescent probes is to use fluorescence as a visible label to reveal either the static or dynamic spatial patterning of various cellular components. In 1995 it was still feasible to compile a nearly exhaustive list of AFP–fusion proteins[50], however, the number of published AFP–fusion proteins has since risen to many hundreds, if not thousands, and compilation of an updated list is far beyond the scope of this review.

Protein trap strategies. An interesting twist to the fluorescence imaging of intracellular localization is the identification of previously unknown protein targets on the basis of their pattern of localization[51]. In the so-called 'protein trap' strategies, a visual screen of cells that contain fusions of an AFP gene and a library of coding DNA sequences is used to identify cells that give rise to a pattern of interest. The DNA sequence that encodes the targeted fusion can then be cloned directly from the cell and the novel protein, which is presumably directing the localization, can be identified.

Fluorescent speckle microscopy. One particularly powerful technique for monitoring cytoskeletal dynamics is fluorescent speckle microscopy[52,53]. A fluorescently labelled protein is introduced into a cell at a very low level (~ 0.25%) relative to its endogenous counterpart, such that individual fluorescently labelled proteins can be detected with a sensitive imaging system. The dynamic assembly and disassembly of the actin cytoskeleton is shown by the 'flow' of the individual fluorescent speckles from regions of filament synthesis to regions of depolymerization. In order to get enough signal from a single protein it is necessary to either attach multiple small-molecule labels or to fuse the gene encoding the protein of interest to multiple AFP sequences[54].

Localizing the messenger. When fused to minimal protein domains that interact specifically with small-molecule messengers, AFPs can provide a straightforward readout of the cellular localization and transient production of such messengers[55–65]. For example, AFP that is fused to the pleckstrin-homology (PH) domain shows, through translocation from the general cytosol to the plasma membrane, the generation of 3′-phosphoinositides at the cell's leading edge on exposure to a chemoattractant[58,59]. Other messengers, such as phosphatidic acid and diacylglycerol (DAG), have been detected by similar methods (TABLE 1). In one elegant study, the comparison of the kinetics of translocation of full-length protein kinase C (PKC) versus the individual DAG-binding C1 domain and the Ca^{2+}-binding C2 domain led to a model for a sequential activation of PKC through a temporal coordination of the Ca^{2+} and DAG signals[64].

Localizing gene activity and transcripts. RNA localization can be visualized in live cells through fluorescence *in vivo* hybridization (FIVH)[66] or fusion of AFP to an RNA-binding protein or domain[67–70]. Using the latter approach, researchers have visualized the movement of endogenous bicoid messenger RNA during *Drosophila melanogaster* oogenesis through the use of AFP fused to Exu, a protein which accompanies bicoid mRNA during transport[69]. In a separate study, imaging of live neurons that were transfected with GFP-fused zipcode binding protein 1 (ZBP1) showed fast, bidirectional movements of granules in neurites, which can be inhibited by antisense oligonucleotides to the actin mRNA *zipcode* sequence[70]. Visualization of gene activity and changes in chromatin structure during transcription has also been achieved by creating large tandem arrays of DNA sequences (such as the *lac* operator or mouse mammary tumour virus promoter) that can be recognized with AFP-tagged DNA-binding proteins such as *lac* repressor or glucocorticoid receptor[71–74].

Fluorescence as a temporal marker

Analysing gene expression. AFPs are a favourable alternative to β-galactosidase (*lac*Z) as a marker of gene expression in tissue sections and transgenic organisms, because AFPs are self-sufficient and form their own chromophores[75]. The main disadvantage of AFP relative to enzymatic reporter systems is the absence of signal amplification. Whereas AFP is limited to a single FLUOROPHORE for each protein, a single copy of β-galactosidase, luciferase or β-lactamase[76] will catalyze the turnover of multiple substrate molecules, which allows much lower levels of gene expression to be detected. One approach to overcome this drawback is to target the AFP to a defined subcompartment of the cell and thereby use local contrast to distinguish its fluorescence from background autofluorescence.

FLUOROPHORE
A chromophore that can re-emit photons.

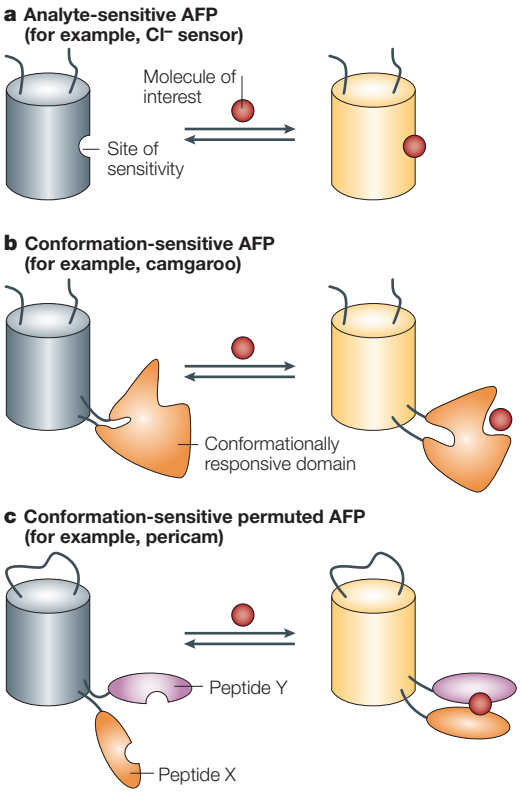

Figure 4 | **Biochemical modulation of AFP fluorescence.** **a** | *Aequorea* fluorescent protein (AFP) can be engineered to be directly sensitive to a small molecule of interest. **b** | Insertion of a conformationally responsive domain into AFP can result in a chimaera in which fluorescence is modulated by the conformational change. **c** | Alternatively, interacting proteins (or peptides) can be fused to the amino and carboxyl termini of circularly permuted AFP.

Optimizing turnover. Increasingly sophisticated reporters have been developed to monitor the temporal patterns of gene expression and protein dynamics. A limitation of AFP and similar passive markers is that the new protein cannot be distinguished from the old protein, as AFP remains fluorescent until it is degraded. Degradation can be accelerated by the fusion of AFP to degradation domains such as that of the mouse ornithine decarboxylase[77], thereby destabilizing the fluorescent protein such that it is turned over with a half-life of 2 h or less. These destabilized AFP variants minimize the accumulation of the background fluorescence that is generated by leaky, non-induced basal-level expression, which enhances their use as transcriptional reporters, albeit at the inherent cost of lower sensitivity — that is, a higher level of transcription and translation are required to produce a given level of fluorescence.

A DsRed fluorescent timer. A 'fluorescent timer' version of DsRed has been developed as a temporal marker[78]. The variant changes from green to red fluorescent over a period of ~24 h as the 'GFP-like' intermediate that accumulates is converted to the final red species owing to a further oxidative modification of the chromophore. The temporal history of the promoter activation is therefore reflected in the ratio between green and red fluorescence. Because the timescale of the fluorescent change is fixed and the resolution is restricted to several hours, this system will probably be most useful for the analysis of developmental control genes[78] in systems where the photochemically triggered green-to-red conversion of Kaede[11] or pulse–chase labelling of tetracysteine motifs[38], which were discussed previously, cannot be applied.

Analysing protein dynamics. Through the use of innovative fusion constructs, AFPs can report the temporal dynamics of cellular processes other than promoter activation. For example, the accumulation and degradation of an AFP-based substrate has been used for the quantification of ubiquitin–proteasome-dependent proteolysis in living cells[79]. Another tactic, which has found a use in the monitoring of vesicular traffic and sorting in the secretory pathway, is based on temperature-sensitive GFP mutants that fold and mature correctly only at temperatures that are non-permissive for sorting events. By growing cells at such temperatures, a cohort of fluorescently tagged proteins can accumulate in the *trans*-Golgi network. On raising the temperature, both to allow sorting and to prevent folding of the newly synthesized GFPs, the fate of the fluorescent 'bolus' can be monitored[80]. This pulse–chase-type approach has been used recently to visualize specifically the dynamics of immature secretory granules in a much larger pool of mature secretory granules[81]. So the age and fate of protein fusions can be monitored by spontaneously slow-maturing fluorescent proteins, temperature- or illumination-sensitive fluorescent proteins or pulse–chase labelling by biarsenical ligands, all of which have their own advantages and drawbacks.

Genetically encoded biochemical sensors

In the more active applications of fluorescent proteins that are described below, biochemical parameters such as metabolite concentrations, enzyme activity, or protein–protein interactions can be detected by their effects on the fluorescence properties of the designed indicators. Such indicators can be further divided into molecules with single chromophores (FIG. 4a–c) versus composites in which the emission intensity is dependent on the energy transfer between two chromophores (FIG. 5a,b).

Modulation of fluorescent protein spectra

Exploiting pH and halide-sensitive fluorescent proteins. In general, the fluorescence of AFPs is quenched reversibly by moderate acidification. This intrinsic pH sensitivity varies between different mutants and can be exploited to measure the ambient pH[82–84]. Both intensity-modulated and ratiometric pH-sensitive variants of GFP have been engineered and fused to a vesicle membrane protein to monitor vesicle exocytosis and recycling. These 'synapto-pHluorins' report synaptic neurotransmitter secretion by detecting the abrupt pH change that occurs when the acidic interior of the

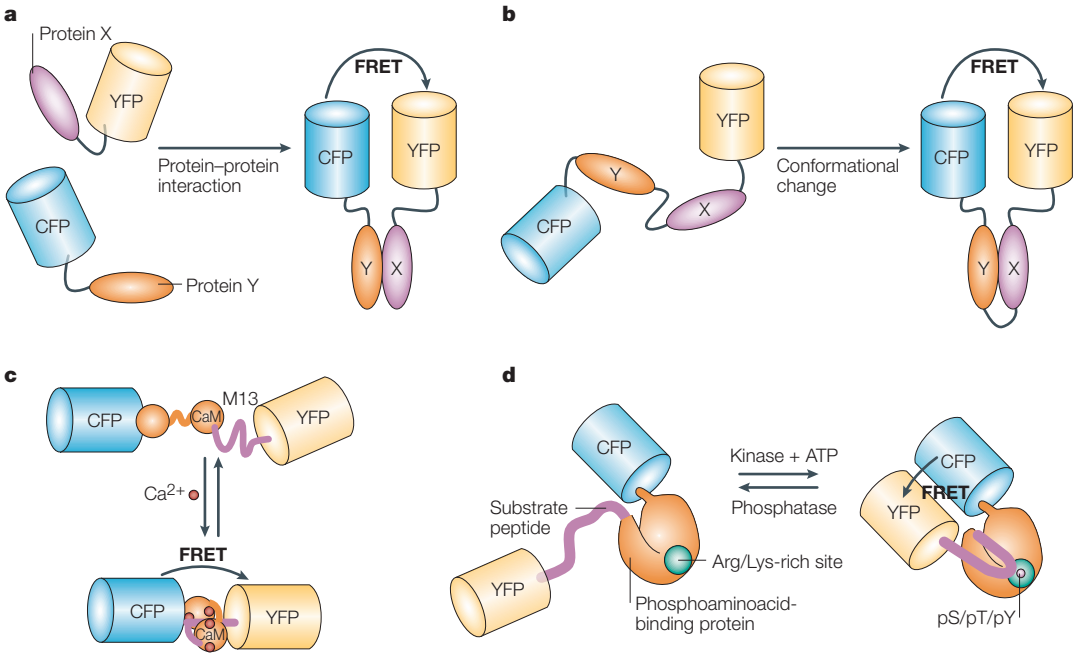

Figure 5 | **The general design of FRET-based fluorescent probes. a** | An intermolecular fluorescence resonance energy transfer (FRET)-based probe consists of two different proteins (X and Y) that are labelled with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), respectively, which interact and bring the fluorophores into close proximity, thereby increasing the FRET efficiency. **b** | An intramolecular FRET-based probe consists of either a cleavable linker or a conformationally responsive region sandwiched between a FRET pair. **c** | Cameleon is an intramolecular FRET-based probe that is used to measure intracellular Ca^{2+}. **d** | Intramolecular phosphorylation-sensitive FRET probes have been constructed with specificities for various different kinases. Arg, arginine; CaM, calmodulin; Lys, lysine; pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine. Figure 5, part **c** is reprinted with permission from REF. 105 *Nature* © (1997) Macmillan Magazines Ltd. Figure 5, part **d** is reproduced with permission from REFS 116,118 © 2002, National Academy of Sciences.

vesicle (pH ~5) is exposed to the outside of the cell (pH ~7) on fusion to the plasma membrane[84]. Some YFPs have particularly high pK_as in the range of 7 to 8, which makes them very useful for monitoring the pH of the cytosol and the mitochondrial matrix[85]. These same YFPs are quenched by halide ions, which bind selectively in the order $F^- > I^- > Cl^- > Br^-$ and raise the pK_a, which simulates acidification[86,87]. The halide-sensitive YFPs have been used in live cells to monitor Cl^- fluxes such as those mediated by the cystic fibrosis transmembrane conductance regulator (CFTR)[88]. Although the above indicators only change the intensity of fluorescence, examples of wavelength-shifting pH[89] and halide[90] indicators are also known. The responsiveness of YFPs to both protons and halides means that care has to be taken to disentangle the two perturbations. Sensitivity to pH and Cl^- can be a significant annoyance in applications where the YFP is to only be maximally fluorescent and inert (as in the passive applications discussed above). Fortunately, alternative YFPs with reduced pK_as, no halide sensitivity and increased brightness and photostability are now available[3,4].

Engineering fluorescent proteins to be sensitive to other parameters. The fluorescence of fluorescent proteins can be made directly sensitive to other signals by the introduction of specific mutations into the well-defined chromophore or barrel structure. A blue fluorescent protein (BFP) has been engineered, by the introduction of point mutations, to bind Zn^{2+} directly on the chromophore[91], but the modest fluorescence enhancement (twofold), low Zn^{2+} affinity (50 μM K_d) and slow association rate ($t_{1/2} > 4$ h) would need considerable improvement to become biologically useful. YFPs[92] and GFPs (G. T. Hanson, R. Aggeler, R. A. Capaldi, and S. J. Remington, unpublished observations) that are responsive to thiol-disulphide redox potentials have been engineered by placing two cysteine residues on adjacent β-strands so that they can form a reversible intramolecular disulphide bond. Such oxidation reduces the fluorescence of the YFP-based sensor by 2.2-fold[92], whereas it shifts the excitation maxima of the GFP-based sensors from 475–490 nm to 400 nm, which alters the excitation ratio by as much as 6–8-fold. Such indicators hold great promise, because, at present, the redox potentials of different compartments of cells are difficult to measure, but they are probably very important, heterogeneous and at least somewhat dynamic.

Most other biochemical parameters would require binding or sensing sites that are more complex than those created by point mutations in AFPs. Conformationally responsive elements, which range from short peptide motifs to full-length proteins, can be inserted into AFPs. The best-characterized example is the insertion of calmodulin (CaM) in place of Tyr145 of YFP, which

results in Ca^{2+} sensors (known as camgaroos) that increase fluorescence sevenfold on binding of Ca^{2+} (REFS 3,93). Camgaroos have proved to be useful for imaging Ca^{2+} inside mitochondria[94] and in MUSHROOM BODIES in the brain of *Drosophila*[95].

An important topological variation of this is to insert an AFP inside a conformationally responsive protein or pair of protein domains. An early example was a voltage sensor that consisted of AFP inserted into a non-conducting mutant of the Shaker K^+ channel[96]. More recently a smaller but faster response has been obtained by inserting wild-type GFP into a sodium channel[97]. Even larger responses might be obtained by inserting a circularly permuted AFP (cpAFP) rather than wild-type GFP[3,93]. In a cpAFP, the original amino and carboxyl termini are joined by a flexible linker, and new amino and carboxyl termini are introduced at one of several possible locations near the chromophore. Such permutation increases the flexibility and optical responsiveness to stresses that are applied on the new termini. Insertion of circularly permuted GFP (cpGFP) between CaM and M13 (a peptide which binds calmodulin in a Ca^{2+}-dependent fashion) yields Ca^{2+} indicators that are known as GCaMP[98] or pericams[99]. The inclusion of M13 increases the apparent Ca^{2+} affinity of the CaM by allowing the formation of ternary complexes, so that these molecules are more sensitive than camgaroos to small elevations in physiological levels of Ca^{2+}. Some pericam variants shift their excitation wavelengths on binding of Ca^{2+}, as opposed to just increasing fluorescence, which thereby enables ratiometric observation[99].

Intramolecular FRET-based indicators

FRET is a quantum-mechanical phenomenon that occurs when two fluorophores are in molecular proximity of each other (< 80 Å apart) (BOX 2). The emission spectrum of the donor fluorophore should overlap the excitation spectrum of the acceptor fluorophore, but both excitation spectra should be well enough separated to allow independent excitation. When the stoichiometry of the donor and the acceptor is fixed, as it is when they are fused in a single polypeptide chain, then the experimentally most convenient readout of FRET is the ratio of acceptor to donor fluorescence. Many reporters that are designed on the basis of intramolecular FRET changes (FIG. 5b) have been developed for measuring biochemical events in cells. Early applications used BFP as the donor and GFP as the acceptor, but the dimness and ability of BFP to be bleached soon led to its replacement by CFP, whereupon GFP had to be replaced by YFP to maintain spectral separation. When monomeric RFPs have been optimized sufficiently, GFP–RFP will probably become the next donor–acceptor pairing of choice.

Indicators of protease activity. The earliest FRET reporters consisted of BFP and GFP fused together with a protease-sensitive linker. Proteolysis disrupts FRET by separating the donor and acceptor units[100,101]. More recent examples have used CFP and YFP to measure caspase activity during apoptosis[102,103], which includes all-or-none activation at the single-cell level[104].

Indicators for measuring changes in calcium. Genetically encoded Ca^{2+} indicators, which are known as cameleons, were constructed by sandwiching CaM, a peptide linker and M13 between CFP and YFP[3,105–107] (FIG. 5c). Increased levels of intracellular Ca^{2+} switches on the affinity of CaM for the adjacent M13 sequence, which results in a change in orientation or distance between the two fluorescent proteins and a large increase in FRET. The replacement of glutamate by glutamine residues in the Ca^{2+}-binding sites tunes the effective affinity for Ca^{2+}. The replacement of M13 by a peptide that is derived from CaM-dependent kinase kinase produces a cameleon analogue with a larger, although somewhat slower, response than the traditional cameleons[108]. Levels of free intracellular CaM–Ca^{2+} complex can be sensed by reporters in which the donor and acceptor are connected by just a CaM-binding peptide[109,110], but in this case the binding of CaM straightens the peptide linker and so decreases FRET.

Indicators for other cellular parameters. Over the past few years, this principle of indicator design has been applied to visualize the behaviour of many other signalling molecules and proteins in cells. For example, indicators for cGMP (using cGMP-dependent protein kinase)[111,112], Ras and Rap1 activity[113], and Ran activity[114] have been constructed and applied to the study of a range of cell biological phenomena.

Reporters for the activity of tyrosine kinases[115–117] and serine/threonine kinases[118] have been made by sandwiching a substrate peptide for the kinase of interest and a phosphoaminoacid-binding domain, such as Src-homology-2 (SH2) or 14-3-3, between CFP and YFP (FIG. 5d). Phosphorylation of the substrate peptide induces the formation of an intramolecular complex with the neighbouring phosphoaminoacid-binding domain, which changes the FRET. This generic concept has been adapted to create probes for Abl, Src, the epidermal growth factor (EGF) receptor, insulin receptor, protein kinase A (PKA), and PKC, (J. D. Violin, J. Z., R. Y. T., and A. C. Newton, unpublished observations) with many more underway. The FRET change is usually reversed by phosphatases, so the fluorescent substrates report continuously the balance between kinase and phosphatase activities, with time resolution in the order of a few seconds and a spatial resolution of micrometres — far better than conventional assays with radioactive phosphorus or phospho-specific antibodies. Even finer spatial discrimination is possible if the reporters are fused to specific components of scaffolding molecules. So phosphorylation kinetics can differ significantly between sites that are indistinguishable at the resolution of the light microscope[118].

A FRET-based reporter of membrane potential exemplifies a slightly different topology, in which the voltage sensor, a truncated potassium channel, is placed amino terminal to both the CFP and YFP[119]. Voltage-dependent twists of the S4 channel helix are proposed to rotate the CFP with respect to the YFP.

MUSHROOM BODIES
Two prominent bilaterally symmetrical structures in the fly brain that are crucial for olfactory learning and memory.

Intermolecular FRET-based indicators

Indicators for cyclic AMP. The first fluorescent indicator for intracellular cAMP consisted of the PKA holoenzyme, in which the catalytic and regulatory subunits were labelled with fluorescein and rhodamine, respectively, so that cAMP-induced dissociation of the holoenzyme disrupted FRET[120]. Replacement of the dyes by BFP and GFP made this system genetically encodable and eliminated the need for *in vitro* dye conjugation and microinjection[121]. Application of this probe (with CFP and YFP replacing BFP and GFP) in cardiac myocytes showed an unusual compartmentalization of cAMP[122], whereas cAMP diffused freely in other cell types such as neurons[123,124].

Detecting protein–protein interactions. Intermolecular FRET can be used in real-time to detect interactions between two protein partners (FIG. 5a). Transcription factor homo- and heterodimerization[125,126], G-protein dissociation[127] and many other interactions[128–134] have all been visualized in live cells through the attachment of donor and acceptor fluorophores to interacting protein partners.

Protein–protein interactions can also be imaged by protein complementation assays, in which the potential partner proteins are fused not to FRET donors and acceptors but to complementary fragments of GFP[135], YFP[136], or other fluorogenic reporters[137,138], the most recent of which is β-lactamase[139,140]. The interaction of the partner proteins allows the two fragments to reconstitute the fluorescence or enzymatic activity, which is analogous to yeast two-hybrid assays in which transcriptional activation is reconstituted. Protein complementation assays generally have a much lower background and a greater dynamic range than those of FRET. When the reconstituted protein is an enzyme, its ability to catalyse the turnover of several copies of a substrate provides useful amplification, but it sacrifices subcellular spatial resolution if the reaction product is diffusible. However, FRET is instantaneous, fully reversible (that is, it monitors dissociation as well as association), has a well-characterized dependence on distance (BOX 2) and orientation, does not require the partner proteins to touch each other and contributes no attraction or repulsion of its own, provided that non-dimerizing fluorescent proteins are chosen[13]. Protein complementation takes from minutes to hours for the fragments to fold, reversibility is absent or uncertain and the requirements on the conformation and affinity of the partner proteins are quantitatively ill-defined except that they must bring the two reporter fragments into the correct juxtaposition.

Notes of caution. When the FRET donor and acceptor are in two separate molecules rather than a fused chimaera, the involvement of mixed complexes between labelled and endogenous partners becomes a greater concern, and the ratio of donor to acceptor expression is no longer fixed. FRET can no longer be assessed by exciting the donor and measuring the ratio of emissions in the donor and acceptor bands. More sophisticated methods such as the mathematical processing of three images[141–144], measuring donor dequenching on photobleaching the acceptor[13,107,126,130], or FLUORESCENCE-LIFETIME IMAGING MICROSCOPY (FLIM; see below) are required to show and quantify FRET.

Intermolecular FRET can suffer from false negatives when the donor and acceptor fluorescent proteins are: perturbing the proteins to which they are fused; in close proximity but orientated unfortunately with respect to each other; too far away from each other even when their fusion partners are interacting. Concern over false negatives has probably been the main reason why intermolecular FRET has been used mainly to obtain higher spatio-temporal resolution on known interactions rather than to screen proteomes for unknown interactions. False positives could result from the weak affinity (K_d ~ 0.1 mM) of AFPs for each other, but the only known example is when CFP and YFP were anchored to the plasma membrane by fatty acyl anchors, in which case the confinement to a surface probably raised the effective concentration considerably[13]. After the dimerization was eliminated by the monomerizing mutations described above, correlation of FRET efficiency versus YFP density showed that in living cells acyl, but not prenyl, modifications promote clustering in lipid rafts on the cytosolic face of the plasma membrane[13].

Box 2 | **FRET**

Fluorescence resonance energy transfer (FRET) is a quantum mechanical phenomenon that occurs between a fluorescence donor and a fluorescence acceptor that are in molecular proximity of each other if the emission spectrum of the donor overlaps the excitation spectrum of the acceptor. Under these conditions, energy (*E*) is transferred non-radiatively from the donor to the acceptor with an efficiency defined by the equation, where *r* is the distance between the two fluorophores and R_0 (Förster distance) is the distance at which 50% energy transfer takes place (typically 20–60 Å).

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

R_0 is dependent on the extent of spectral overlap between the donor and acceptor, the quantum yield of the donor and the relative orientation of the donor and acceptor.

Excitation of a donor fluorophore in a FRET pair leads to quenching of the donor emission and to an increased, sensitized, acceptor emission. Intensity-based FRET detection methods include monitoring the donor intensity with or without acceptor photobleaching, the sensitized acceptor emission or the ratio between the donor and acceptor intensity. Methods that are based on fluorescence-decay-kinetics include determining the rate of donor photobleaching, the decrease of donor fluorescence lifetime or the appearance of new components in the acceptor decay kinetics.

FLUORESCENCE-LIFETIME IMAGING MICROSCOPY (FLIM). An imaging technique in which the lifetime, rather than the intensity, of the fluorescent signal is measured. This approach can be used to measure FRET.

FLUORESCENCE-ACTIVATED CELL SORTING
(FACS). A flow cytometry application in which live fluorescent cells are excited at a specific wavelength and then sorted into physically separated subpopulations on the basis of their fluorescence emission.

POSITRON EMISSION TOMOGRAPHY
(PET). Positron emission tomography is an imaging technique that is used to detect decaying nuclides, such as ^{15}O, ^{13}N, ^{11}C, ^{18}F, ^{124}I and ^{94m}Tc.

MAGNETIC RESONANCE IMAGING
The use of radio waves in the presence of a magnetic field to extract information from certain atomic nuclei (most commonly hydrogen, for example, in water). This technique is used to show certain types of tissue damage and the presence of tumours.

Fluorescence-lifetime imaging microscopy. Autophosphorylation of GFP-tagged PKC[145] or ErbB1 (REF. 146) has been measured by the FRET to Cy3-labelled phosphospecific antibodies, which were microinjected or applied to fixed cells and present in excess. When the acceptor is in such a large excess, FRET is best detected by the decrease in the lifetime of the donor's excited state using FLIM. FLIM and measurements of fluorescence depolarization allow the imaging of FRET between spectrally similar AFP molecules — that is, GFP–YFP or even GFP–GFP[147]. The main drawbacks of FLIM are that it sacrifices some sensitivity[134] and requires the assembly of expensive instrumentation.

Future directions

The applications of fluorescent probes will continue to expand and provide exciting new insights into the biology of living cells. Several relatively versatile reporter design strategies are now at our disposal (for example, translocation, camgaroo-, pericam- and cameleon-like strategies), but efforts to engineer new fluorophores and reporter classes must continue. For example, brighter and more red-shifted fluorescent proteins should improve the detection limits and *in vivo* applicability of AFP-based reporters. At present, all single AFP and FRET-based sensors rely on a gross structural reorganization or conformational change to produce a spectral readout. Many interesting intracellular process involve subtle conformational changes that are not amenable to the current classes of reporters, so that fluorescent protein variants with an increased sensitivity towards structural perturbations would be desirable. Another important consideration is that many of these fluorescent probes, in particular those for monitoring protein–protein interactions, function as surrogate cellular players. Visualization of their activities complement, but do not replace, measurements of endogenous components. Reporters that could assay endogenous biomolecules directly would therefore be very desirable.

Single-cell imaging with fluorescent reporters should allow the investigation of potentially interesting cell-to-cell variability that cannot be observed by the available methodologies that are based on cell population analysis[122]. Fluorescence is also an attractive readout for rapid, high-throughput approaches because of the availability of technologies such as multi-well plate readers, FLUORESCENCE-ACTIVATED CELL SORTING (FACS) and evanescent wave single-cell array technology (E-SCAT)[63].

Single-molecule spectroscopy is a young field that holds great promise. Single-molecule imaging in living cells allows the visualization of individual molecular interactions under physiological conditions and provides information that is difficult, and sometimes impossible, to obtain by conventional techniques[148]. Examples of single-molecule studies in living cells include investigations of EGF receptor dimerization[149], conformational changes in voltage-gated ion channels[150] and the mobility and aggregation of L-type Ca^{2+} channels in the plasma membrane[151].

For whole-body *in vivo* imaging, AFP has been used mainly as a visible localization marker[152] and a gene-expression marker[153]. Given the available wavelengths of excitation and emission, AFP imaging is still limited to surface structures (with a depth of penetration of approximately 1–2 mm) in experimental animals. A new generation of highly sensitive near infrared probes needs to be developed to complement existing non-optical probes for POSITRON EMISSION TOMOGRAPHY (PET) and MAGNETIC RESONANCE IMAGING (MRI).

1. Tsien, R. Y. The green fluorescent protein. *Annu. Rev. Biochem.* **67**, 509–544 (1998).
2. Sawano, A. & Miyawaki, A. Directed evolution of green fluorescent protein by a new versatile PCR strategy for site-directed and semi-random mutagenesis. *Nucleic Acids Res.* **28**, E78 (2000).
3. Griesbeck, O., Baird, G. S., Campbell, R. E., Zacharias, D. A. & Tsien, R. Y. Reducing the environmental sensitivity of yellow fluorescent protein. Mechanism and applications. *J. Biol. Chem.* **276**, 29188–29194 (2001).
4. Nagai, T. *et al.* A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nature Biotechnol.* **20**, 87–90 (2002).
5. Scholz, O., Thiel, A., Hillen, W. & Niederweis, M. Quantitative analysis of gene expression with an improved green fluorescent protein. *Eur. J. Biochem.* **267**, 1565–1570 (2000).
6. Ormö, M. *et al.* Crystal structure of the *Aequorea victoria* green fluorescent protein. *Science* **273**, 1392–1395 (1996).
7. Yokoe, H. & Meyer, T. Spatial dynamics of GFP-tagged proteins investigated by local fluorescence enhancement. *Nature Biotechnol.* **14**, 1252–1256 (1996).
8. Sawin, K. E. & Nurse, P. Photoactivation of green fluorescent protein. *Curr. Biol.* **7**, R606–R607 (1997).
9. Elowitz, M. B., Surette, M. G., Wolf, P. E., Stock, J. & Leibler, S. Photoactivation turns green fluorescent protein red. *Curr. Biol.* **7**, 809–812 (1997).
10. Patterson, G. H. & Lippincott-Schwartz, J. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science* **297**, 1873–1877 (2002).
11. Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H. & Miyawaki, A. An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc. Natl Acad. Sci. USA* **99**, 12651–12656 (2002).

References 10 and 11 describe a pair of photoactivatable fluorescent proteins that can be used as fluorescent 'highlighters' to mark specific organelles or protein subpopulations.

12. Lippincott-Schwartz, J., Snapp, E. & Kenworthy, A. Studying protein dynamics in living cells. *Nature Rev. Mol. Cell Biol.* **2**, 444–456 (2001).
13. Zacharias, D. A., Violin, J. D., Newton, A. C. & Tsien, R. Y. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science* **296**, 913–916 (2002).
14. Ward, W. W., Prentice, H. J., Roth, A. F., Cody, C. W. & Reeves, S. C. Spectral perturbations of the *Aequorea* green-fluorescent protein. *Photochem. Photobiol.* **35**, 803–808 (1982).
15. Yang, F., Moss, L. G. & Phillips, G. N. Jr. The molecular structure of green fluorescent protein. *Nature Biotechnol.* **14**, 1246–1251 (1996).
16. Matz, M. V. *et al.* Fluorescent proteins from nonbioluminescent *Anthozoa* species. *Nature Biotechnol.* **17**, 969–973 (1999).
17. Tsien, R. Y. Rosy dawn for fluorescent proteins. *Nature Biotechnol.* **17**, 956–957 (1999).
18. Zacharias, D. A. Sticky caveats in an otherwise glowing report: oligomerizing fluorescent proteins and their use in cell biology. *Sci. STKE.* **2002**, PE23 (2002).
19. Lauf, U., Lopez, P. & Falk, M. M. Expression of fluorescently tagged connexins: a novel approach to rescue function of oligomeric DsRed-tagged proteins. *FEBS Lett.* **498**, 11–15 (2001).
20. Soling, A., Simm, A. & Rainov, N. Intracellular localization of Herpes simplex virus type 1 thymidine kinase fused to different fluorescent proteins depends on choice of fluorescent tag. *FEBS Lett.* **527**, 153 (2002).
21. Yanushevich, Y. G. *et al.* A strategy for the generation of non-aggregating mutants of *Anthozoa* fluorescent proteins. *FEBS Lett.* **511**, 11–14 (2002).
22. Terskikh, A. V., Fradkov, A. F., Zaraisky, A. G., Kajava, A. V. & Angres, B. Analysis of DsRed mutants: space around the fluorophore accelerates fluorescence development. *J. Biol. Chem.* **277**, 7633–7636 (2002).
23. Bevis, B. J. & Glick, B. S. Rapidly maturing variants of the *Discosoma* red fluorescent protein (DsRed). *Nature Biotechnol.* **20**, 83–87 (2002).
24. Campbell, R. E. *et al.* A monomeric red fluorescent protein. *Proc. Natl Acad. Sci. USA* **99**, 7877–7882 (2002).

This paper describes the directed evolution of a monomeric RFP that overcomes the problems associated with tetrameric DsRed.

25. Knop, M., Barr, F., Riedel, C. G., Heckel, T. & Reichel, C. Improved version of the red fluorescent protein (drFP583/DsRed/RFP). *BioTechniques* **33**, 592, 594, 596–598 (2002).
26. CLONTECH Laboratories Inc. *Living Colors User Manual Vol. II: Red Fluorescent Protein* 4 (Becton, Dickinson and Company, USA, 2002).
27. Fradkov, A. F. *et al.* Far-red fluorescent tag for protein labeling. *Biochem. J.* **368**, 17–21 (2002).
28. Labas, Y. A. *et al.* Diversity and evolution of the green fluorescent protein family. *Proc. Natl Acad. Sci. USA* **99**, 4256–4261 (2002).
29. Matz, M. V., Lukyanov, K. A. & Lukyanov, S. A. Family of the green fluorescent protein: journey to the end of the rainbow. *Bioessays* **24**, 953–959 (2002).
30. Peelle, B., Gururaja, T. L., Payan, D. G. & Anderson, D. C. Characterization and use of green fluorescent proteins from *Renilla mulleri* and *Ptilosarcus guernyi* for the human cell

display of functional peptides. *J. Protein Chem.* **20**, 507–519 (2001).
31. Lukyanov, K. A. *et al.* Natural animal coloration can be determined by a non-fluorescent GFP homolog. *J. Biol. Chem.* **275**, 25879–25882 (2000).
32. Fradkov, A. F. *et al.* Novel fluorescent protein from *Discosoma* coral and its mutants possesses a unique far-red fluorescence. *FEBS Lett.* **479**, 127–130 (2000).
33. Gurskaya, N. G. *et al.* GFP-like chromoproteins as a source of far-red fluorescent proteins. *FEBS Lett.* **507**, 16–20 (2001).
34. Wiedenmann, J. *et al.* A far-red fluorescent protein with fast maturation and reduced oligomerization tendency from *Entacmaea quadricolor* (*Anthozoa, Actinaria*). *Proc. Natl Acad. Sci. USA* **99**, 11646–11651 (2002).
35. Griffin, B. A., Adams, S. R. & Tsien, R. Y. Specific covalent labeling of recombinant protein molecules inside live cells. *Science* **281**, 269–272 (1998).
36. Nakanishi, J. *et al.* Imaging of conformational changes of proteins with a new environment- sensitive fluorescent probe designed for site-specific labeling of recombinant proteins in live cells. *Anal. Chem.* **73**, 2920–2928 (2001).
37. Adams, S. R. *et al.* New biarsenical ligands and tetracysteine motifs for protein labeling *in vitro* and *in vivo*: synthesis and biological applications. *J. Am. Chem. Soc.* **124**, 6063–6076 (2002).
38. Gaietta, G. *et al.* Multicolor and electron microscopic imaging of connexin trafficking. *Science* **296**, 503-507 (2002).
This paper describes the application of the tetracysteine–biarsenical system in pulse–chase labelling and electron microscopy.
39. Lauf, U. *et al.* Dynamic trafficking and delivery of connexons to the plasma membrane and accretion to gap junctions in living cells. *Proc. Natl Acad. Sci. USA* **99**, 10446–10451 (2002).
40. Farinas, J. & Verkman, A. S. Receptor-mediated targeting of fluorescent probes in living cells. *J. Biol. Chem.* **274**, 7603–7606 (1999).
41. Wu, M. M. *et al.* Organelle pH studies using targeted avidin and fluorescein-biotin. *Chem. Biol.* **7**, 197–209 (2000).
42. Wu, M. M. *et al.* Studying organelle physiology using targeted avidin and fluorescein-biotin. *Methods Enzymol.* **327**, 546–564 (2000).
43. Wu, M. M. *et al.* Mechanisms of pH regulation in the regulated secretory pathway. *J. Biol. Chem.* **276**, 33027–33035 (2001).
44. Sano, T. & Cantor, C. R. Expression of a cloned *streptavidin* gene in *Escherichia coli*. *Proc. Natl Acad. Sci. USA* **87**, 142–146 (1990).
45. Gambetta, G. A. & Lagarias, J. C. Genetic engineering of phytochrome biosynthesis in bacteria. *Proc. Natl Acad. Sci. USA* **98**, 10566–10571 (2001).
46. Tooley, A. J., Cai, Y. A. & Glazer, A. N. Biosynthesis of a fluorescent cyanobacterial C-phycocyanin holo-α subunit in a heterologous host. *Proc. Natl Acad. Sci. USA* **98**, 10560–10565 (2001).
47. Tooley, A. J. & Glazer, A. N. Biosynthesis of the cyanobacterial light-harvesting polypeptide phycoerythrocyanin holo-α subunit in a heterologous host. *J. Bacteriol.* **184**, 4666–4671 (2002).
48. Murphy, J. T. & Lagarias, J. C. The phytofluors: a new class of fluorescent protein probes. *Curr. Biol.* **7**, 870–876 (1997).
49. Wildt, S. & Deuschle, U. cobA, a red fluorescent transcriptional reporter for *Escherichia coli*, yeast, and mammalian cells. *Nature Biotechnol.* **17**, 1175–1178 (1999).
50. Cubitt, A. B. *et al.* Understanding, using and improving green fluorescent protein. *Trends Biochem. Sci.* **20**, 448–455 (1995).
51. Gonzalez, C. & Bejarano, L. A. Protein traps: using intracellular localization for cloning. *Trends Cell Biol.* **10**, 162–165 (2000).
52. Waterman-Storer, C. M., Desai, A., Bulinski, J. C. & Salmon, E. D. Fluorescent speckle microscopy, a method to visualize the dynamics of protein assemblies in living cells. *Curr. Biol.* **8**, 1227–1230 (1998).
53. Watanabe, N. & Mitchison, T. J. Single-molecule speckle analysis of actin filament turnover in lamellipodia. *Science* **295**, 1083–1086 (2002).
54. Bulinski, J. C., Odde, D. J., Howell, B. J., Salmon, T. D. & Waterman-Storer, C. M. Rapid dynamics of the microtubule binding of ensconsin *in vivo*. *J. Cell Sci.* **114**, 3885–3897 (2001).
55. Watton, S. J. & Downward, J. Akt/PKB localisation and 3′ phosphoinositide generation at sites of epithelial cell–matrix and cell–cell interaction. *Curr. Biol.* **9**, 433–436 (1999).
56. Oatey, P. B. *et al.* Confocal imaging of the subcellular distribution of phosphatidylinositol 3,4,5-trisphosphate in insulin- and PDGF-stimulated 3T3-L1 adipocytes. *Biochem. J.* **344**, 511–518 (1999).
57. Gray, A., Van Der, K. J. & Downes, C. P. The pleckstrin homology domains of protein kinase B and GRP1 (general receptor for phosphoinositides-1) are sensitive and selective probes for the cellular detection of phosphatidylinositol 3,4-bisphosphate and/or phosphatidylinositol 3,4,5-trisphosphate *in vivo*. *Biochem. J.* **344**, 929–936 (1999).
58. Servant, G. *et al.* Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* **287**, 1037–1040 (2000).
59. Meili, R. *et al.* Chemoattractant-mediated transient activation and membrane localization of Akt/PKB is required for efficient chemotaxis to cAMP in *Dictyostelium*. *EMBO J.* **18**, 2092–2105 (1999).
60. Stauffer, T. P., Ahn, S. & Meyer, T. Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P_2 concentration monitored in living cells. *Curr. Biol.* **8**, 343–346 (1998).
61. Hirose, K., Kadowaki, S., Tanabe, M., Takeshima, H. & Lino, M. Spatiotemporal dynamics of inositol 1,4,5-trisphosphate that underlies complex Ca^{2+} mobilization patterns. *Science* **284**, 1527–1530 (1999).
62. Oancea, E., Teruel, M. N., Quest, A. F. G. & Meyer, T. Green fluorescent protein (GFP)-tagged cysteine-rich domains from protein kinase C as fluorescent indicators for diacylglycerol signaling in living cells. *J. Cell Biol.* **140**, 485–498 (1998).
63. Teruel, M. N. & Meyer, T. Parallel single-cell monitoring of receptor-triggered membrane translocation of a calcium-sensing protein module. *Science* **295**, 1910–1912 (2002).
64. Oancea, E. & Meyer, T. Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. *Cell* **95**, 307-318 (1998).
Reference 64 compares the kinetics of translocation of full-length PKC versus individual DAG-binding C1 domain and Ca^{2+}-binding C2 domain, which led to a model for a sequential activation of PKC through a temporal coordination between Ca^{2+} and DAG signals.
65. Rizzo, M. A., Shome, K., Watkins, S. C. & Romero, G. The recruitment of Raf-1 to membranes is mediated by direct interaction with phosphatidic acid and is independent of association with Ras. *J. Biol. Chem.* **275**, 23911–23918 (2000).
66. Dirks, R. W., Molenaar, C. & Tanke, H. J. Methods for visualizing RNA processing and transport pathways in living cells. *Histochem. Cell Biol.* **115**, 3–11 (2001).
67. Bassell, G. J., Oleynikov, Y. & Singer, R. H. The travels of mRNAs through all cells large and small. *FASEB J.* **13**, 447–454 (1999).
68. Bertrand, E. *et al.* Localization of ASH1 mRNA particles in living yeast. *Mol. Cell* **2**, 437–445 (1998).
69. Theurkauf, W. E. & Hazelrigg, T. I. *In vivo* analyses of cytoplasmic transport and cytoskeletal organization during *Drosophila* oogenesis: characterization of a multi-step anterior localization pathway. *Development* **125**, 3655–3666 (1998).
70. Zhang, H. L. *et al.* Neurotrophin-induced transport of a β-actin mRNP complex increases β-actin levels and stimulates growth cone motility. *Neuron* **31**, 261–275 (2001).
71. Muller, W. G., Walker, D., Hager, G. L. & McNally, J. G. Large-scale chromatin decondensation and recondensation regulated by transcription from a natural promoter. *J. Cell Biol.* **154**, 33–48 (2001).
72. Tsukamoto, T. *et al.* Visualization of gene activity in living cells. *Nature Cell Biol.* **2**, 871–878 (2000).
73. Straight, A. F., Marshall, W. F., Sedat, J. W. & Murray, A. W. Mitosis in living budding yeast: anaphase A but no metaphase plate. *Science* **277**, 574–578 (1997).
74. Robinett, C. C. *et al. In vivo* localization of DNA sequences and visualization of large-scale chromatin organization using lac operator/repressor recognition. *J. Cell Biol.* **135**, 1685–1700 (1996).
75. Hadjantonakis, A. K. & Nagy, A. The color of mice: in the light of GFP-variant reporters. *Histochem. Cell Biol.* **115**, 49–58 (2001).
76. Zlokarnik, G. *et al.* Quantitation of transcription and clonal selection of single living cells using β-lactamase as reporter. *Science* **279**, 84–88 (1998).
77. Li, X. *et al.* Generation of destabilized green fluorescent protein as a transcription reporter. *J. Biol. Chem.* **273**, 34970–34975 (1998).
78. Terskikh, A. *et al.* 'Fluorescent timer': protein that changes color with time. *Science* **290**, 1585-1588 (2000).
This paper describes a DsRed-derived transcriptional reporter that changes colour from green to red over a period of 24 h, thereby preserving the temporal history of promoter activation.
79. Dantuma, N. P., Lindsten, K., Glas, R., Jellne, M. & Masucci, M. G. Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. *Nature Biotechnol.* **18**, 538–543 (2000).
80. Kaether, C. & Gerdes, H. H. Visualization of protein transport along the secretory pathway using green fluorescent protein. *FEBS Lett.* **369**, 267–271 (1995).
81. Rudolf, R., Salm, T., Rustom, A. & Gerdes, H. H. Dynamics of immature secretory granules: role of cytoskeletal elements during transport, cortical restriction, and F-actin-dependent tethering. *Mol. Biol. Cell* **12**, 1353–1365 (2001).
82. Kneen, M., Farinas, J., Li, Y. & Verkman, A. S. Green fluorescent protein as a noninvasive intracellular pH indicator. *Biophys. J.* **74**, 1591–1599 (1998).
83. Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G. & Tsien, R. Y. Measurement of cytosolic, mitochondrial and Golgi pH in single living cells with green fluorescent protein. *Proc. Natl Acad. Sci. USA* **95**, 6803–6808 (1998).
84. Miesenböck, G., De Angelis, D. A. & Rothman, J. E. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* **394**, 192–195 (1998).
Reference 84 describes 'synapto-pHluorins' that report synaptic neurotransmitter secretion by detecting the abrupt pH change that occurs when the acidic interior of the vesicle (pH ~5) is exposed to the outside of the cell (pH ~7) on fusion to the plasma membrane.
85. Matsuyama, S., Llopis, J., Deveraux, Q. L., Tsien, R. Y. & Reed, J. C. Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. *Nature Cell Biol.* **2**, 318–325 (2000).
86. Wachter, R. M. & Remington, S. J. Sensitivity of the yellow variant of green fluorescent protein to halides and nitrate. *Curr. Biol.* **9**, R628–R629 (1999).
87. Wachter, R. M., Yarbrough, D., Kallio, K. & Remington, S. J. Crystallographic and energetic analysis of binding of selected anions to the yellow variants of green fluorescent protein. *J. Mol. Biol.* **301**, 157–171 (2000).
88. Jayaraman, S., Haggie, P., Wachter, R. M., Remington, S. J. & Verkman, A. S. Mechanism and cellular applications of a green fluorescent protein-based halide sensor. *J. Biol. Chem.* **275**, 6047–6050 (2000).
89. Elsliger, M.-A., Wachter, R. M., Hanson, G. T., Kallio, K. & Remington, S. J. Structural and spectral response of green fluorescent protein variants to changes in pH. *Biochemistry* **38**, 5296–5301 (1999).
90. Kuner, T. & Augustine, G. J. A genetically encoded ratiometric neurotechnique indicator for chloride: capturing chloride transients in cultured hippocampal neurons. *Neuron* **27**, 447–459 (2000).
91. Barondeau, D. P., Kassmann, C. J., Tainer, J. A. & Getzoff, E. D. Structural chemistry of a green fluorescent protein Zn biosensor. *J. Am. Chem. Soc.* **124**, 3522–3524 (2002).
92. Ostergaard, H., Henriksen, A., Hansen, F. G. & Winther, J. R. Shedding light on disulfide bond formation: engineering a redox switch in green fluorescent protein. *EMBO J.* **20**, 5853–5862 (2001).
93. Baird, G. S., Zacharias, D. A. & Tsien, R. Y. Circular permutation and receptor insertion within green fluorescent proteins. *Proc. Natl Acad. Sci. USA* **96**, 11241–11246 (1999).
94. Rapizzi, E. *et al.* Recombinant expression of the voltage-dependent anion channel enhances the transfer of Ca^{2+} microdomains to mitochondria. *J. Cell Biol.* (in the press).
95. Yu, D., Baird, G., Tsien, R. Y. & Davis, R. L. Detection of calcium transient in *Drosophila* mushroom body neurons with camgaroo. *J. Neurosci.* (in the press).
96. Siegel, M. S. & Isacoff, E. Y. A genetically encoded optical probe of membrane voltage. *Neuron* **19**, 735–741 (1997).
97. Ataka, K. & Pieribone, V. A. A genetically targetable fluorescent probe of channel gating with rapid kinetics. *Biophys. J.* **82**, 509–516 (2002).
98. Nakai, J., Ohkura, M. & Imoto, K. A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. *Nature Biotechnol.* **19**, 137–141 (2001).
99. Nagai, T., Sawano, A., Park, E. & Miyawaki, A. Circularly permuted green fluorescent proteins engineered to sense Ca^{2+}. *Proc. Natl Acad. Sci. USA* **98**, 3197–3202 (2001).
References 98 and 99 describe the insertion of circularly permuted GFP between calmodulin and M13 to yield Ca^{2+} indicators that have been dubbed 'GCaMP' and 'pericams', respectively.
100. Heim, R. & Tsien, R. Y. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence energy transfer. *Curr. Biol.* **6**, 178–182 (1996).
101. Mitra, R. D., Silva, C. M. & Youvan, D. C. Fluorescence resonance energy transfer between blue-emitting and red-shifted excitation derivatives of the green fluorescent protein. *Gene* **173**, 13–17 (1996).
102. Mahajan, N. P., Harrison-Shostak, D. C., Michaux, J. & Herman, B. Novel mutant green fluorescent protein protease substrates reveal the activation of specific caspases during apoptosis. *Chem. Biol.* **6**, 401–409 (1999).

103. Luo, K. Q., Yu, V. C., Pu, Y. & Chang, D. C. Application of the fluorescence resonance energy transfer method for studying the dynamics of caspase-3 activation during UV-induced apoptosis in living HeLa cells. *Biochem. Biophys. Res. Commun.* **283**, 1054–1060 (2001).
104. Rehm, M. *et al.* Single-cell fluorescence resonance energy transfer analysis demonstrates that caspase activation during apoptosis is a rapid process. Role of caspase-3. *J Biol. Chem.* **277**, 24506–24514 (2002).
105. Miyawaki, A. *et al.* Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* **388**, 882–887 (1997).
106. Miyawaki, A., Griesbeck, O., Heim, R. & Tsien, R. Y. Dynamic and quantitative Ca^{2+} measurements using improved cameleons. *Proc. Natl Acad. Sci. USA* **96**, 2135–2140 (1999).
107. Miyawaki, A. & Tsien, R. Y. Monitoring protein conformations and interactions by fluorescence resonance energy transfer between mutants of green fluorescent protein. *Methods Enzymol.* **327**, 472–500 (2000).
108. Truong, K. *et al.* FRET-based *in vivo* Ca^{2+} imaging by a new calmodulin–GFP fusion molecule. *Nature Struct. Biol.* **8**, 1069–1073 (2001).
109. Romoser, V. A., Hinkle, P. M. & Persechini, A. Detection in living cells of Ca^{2+}-dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence. *J. Biol. Chem.* **272**, 13270–13274 (1997).
References 105 and 109 describe the first genetically encoded indicators to be composed of conformationally responsive elements sandwiched between two fluorescent proteins that can undergo FRET — a design that has since been extended to many other systems.
110. Persechini, A. & Cronk, B. The relationship between the free concentrations of Ca^{2+} and Ca^{2+}–calmodulin in intact cells. *J. Biol. Chem.* **274**, 6827–6830 (1999).
111. Sato, M., Hida, N., Ozawa, T. & Umezawa, Y. Fluorescent indicators for cyclic GMP based on cyclic GMP-dependent protein kinase I α and green fluorescent proteins. *Anal. Chem.* **72**, 5918–5924 (2000).
112. Honda, A. *et al.* Spatiotemporal dynamics of guanosine 3′,5′-cyclic monophosphate revealed by a genetically encoded, fluorescent indicator. *Proc. Natl Acad. Sci. USA* **98**, 2437–2442 (2001).
113. Mochizuki, N. *et al.* Spatio-temporal images of growth-factor-induced activation of Ras and Rap1. *Nature* **411**, 1065–1068 (2001).
114. Kalab, P., Weis, K. & Heald, R. Visualization of a Ran-GTP gradient in interphase and mitotic *Xenopus* egg extracts. *Science* **295**, 2452–2456 (2002).
115. Kurokawa, K. *et al.* A pair of FRET–based probes for tyrosine phosphorylation of the CrkII adaptor protein *in vivo*. *J. Biol. Chem.* **276**, 31305–31310 (2001).
116. Ting, A. Y., Kain, K. H., Klemke, R. L. & Tsien, R. Y. Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells. *Proc. Natl Acad. Sci. USA* **98**, 15003–15008 (2001).
117. Sato, M., Ozawa, T., Inukai, K., Asano, T. & Umezawa, Y. Fluorescent indicators for imaging protein phosphorylation in single living cells. *Nature Biotechnol.* **20**, 287–294 (2002).
118. Zhang, J., Ma, Y., Taylor, S. S. & Tsien, R. Y. Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. *Proc. Natl Acad. Sci. USA* **98**, 14997–15002 (2001).
References 116 and 118 describe a general strategy for non-destructively imaging kinase activities in living cells using FRET-based reporters.
119. Sakai, R., Repunte-Canonigo, V., Raj, C. D. & Knopfel, T. Design and characterization of a DNA-encoded, voltage-sensitive fluorescent protein. *Eur. J. Neurosci.* **13**, 2314–2318 (2001).
120. Adams, S. R., Harootunian, A. T., Buechler, Y. J., Taylor, S. S. & Tsien, R. Y. Fluorescence ratio imaging of cyclic AMP in single cells. *Nature* **349**, 694–697 (1991).
121. Zaccolo, M. *et al.* A genetically encoded, fluorescent indicator for cyclic AMP in living cells. *Nature Cell Biol.* **2**, 25–29 (2000).
122. Zaccolo, M. & Pozzan, T. Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. *Science* **295**, 1711–1715 (2002).
The use of a genetically encodable intermolecular FRET-based cAMP indicator in cardiac myocytes showed an unusual compartmentalization of cAMP.
123. Bacskai, B. J. *et al.* Spatially resolved dynamics of cAMP and protein kinase A subunits in *Aplysia* sensory neurons. *Science* **260**, 222–226 (1993).
124. Hempel, C. M., Vincent, P., Adams, S. R., Tsien, R. Y. & Selverston, A. I. Spatio-temporal dynamics of cAMP signals in an intact neural circuit. *Nature* **384**, 166–169 (1996).
125. Periasamy, A., Kay, S. A. & Day, R. N. in *Functional Imaging and Optical Manipulation of Living Cells.* SPIE Proc. Vol. 2983 (eds Farkas, D. L. & Tromberg, B. J.) (The International Society for Optical Engineers, USA,1997).
126. Llopis, J. *et al.* Ligand-dependent interactions of coactivators SRC-1 and PBP with nuclear hormone receptors can be imaged in live cells and are required for transcription. *Proc. Natl Acad. Sci. USA* **97**, 4363–4368 (2000).
127. Janetopoulos, C., Jin, T. & Devreotes, P. Receptor-mediated activation of heterotrimeric G proteins in living cells. *Science* **291**, 2408–2411 (2001).
128. Damelin, M. & Silver, P. A. Mapping interactions between nuclear transport factors in living cells reveals pathways through the nuclear pore complex. *Mol. Cell* **5**, 133–140 (2000).
129. Ruehr, M. L., Zakhary, D. R., Damron, D. S. & Bond, M. Cyclic AMP-dependent protein kinase binding to A-kinase anchoring proteins in living cells by fluorescence resonance energy transfer of green fluorescent protein fusion proteins. *J. Biol. Chem.* **274**, 33092–33096 (1999).
130. Siegel, R. M. *et al.* Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. *Science* **288**, 2354–2357 (2000).
131. Xia, Z., Zhou, Q., Lin, J. & Liu, Y. Stable SNARE complex prior to evoked synaptic vesicle fusion revealed by fluorescence resonance energy transfer. *J. Biol. Chem.* **276**, 1766–1771 (2001).
132. Li, H. Y. *et al.* Protein–protein interaction of FHL3 with FHL2 and visualization of their interaction by green fluorescent proteins (GFP) two-fusion fluorescence resonance energy transfer (FRET). *J. Cell. Biochem.* **80**, 293–303 (2001).
133. Mas, P., Devlin, P. F., Panda, S. & Kay, S. A. Functional interaction of phytochrome B and cryptochrome 2. *Nature* **408**, 207–211 (2000).
134. Tertoolen, L. G. *et al.* Dimerization of receptor protein-tyrosine phosphatase α in living cells. *B.M.C. Cell Biol.* **2**, 8 (2001).
135. Ghosh, I., Hamilton, A. D. & Regan, L. Antiparallel leucine zipper-directed protein reassembly: Application to the green fluorescent protein. *J. Am. Chem. Soc.* **122**, 5658–5659 (2000).
136. Hu, C.-D., Chinenov, Y. & Kerppola, T. K. Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol. Cell* **9**, 789–798 (2002).
137. Rossi, F., Charlton, C. A. & Blau, H. M. Monitoring protein-protein interactions in intact eukaryotic cells by β-galactosidase complementation. *Proc. Natl Acad. Sci. USA* **94**, 8405–8410 (1997).
138. Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A. & Pelletier, J. N. Detection of protein–protein interactions by protein fragment complementation strategies. *Methods Enzymol.* **328**, 208–230 (2000).
139. Wehrman, T., Kleaveland, B., Her, J. H., Balint, R. F. & Blau, H. M. Protein–protein interactions monitored in mammalian cells via complementation of β-lactamase enzyme fragments. *Proc. Natl Acad. Sci. USA* **99**, 3469–3474 (2002).
140. Galarneau, A., Primeau, M., Trudeau, L. E. & Michnick, S. W. β-Lactamase protein fragment complementation assays as *in vivo* and *in vitro* sensors of protein–protein interactions. *Nature Biotechnol.* **20**, 619–622 (2002).
141. Gordon, G. W., Berry, G., Liang, X. H., Levine, B. & Herman, B. Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. *Biophys. J.* **74**, 2702–2713 (1998).
142. Sorkin, A., McClure, M., Huang, F. & Carter, R. Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy. *Curr. Biol.* **10**, 1395–1398 (2000).
143. Erickson, M. G., Alseikhan, B. A., Peterson, B. Z. & Yue, D. T. Preassociation of calmodulin with voltage-gated $Ca^{(2+)}$ channels revealed by FRET in single living cells. *Neuron* **31**, 973–985 (2001).
144. Chan, F. K. *et al.* Fluorescence resonance energy transfer analysis of cell surface receptor interactions and signaling using spectral variants of the green fluorescent protein. *Cytometry* **44**, 361–368 (2001).
145. Ng, T. *et al.* Imaging protein kinase Cα activation in cells. *Science* **283**, 2085–2089 (1999).
146. Verveer, P. J., Wouters, F. S., Reynolds, A. R. & Bastiaens, P. I. Quantitative imaging of lateral ErbB1 receptor signal propagation in the plasma membrane. *Science* **290**, 1567–1570 (2000).
147. Harpur, A. G., Wouters, F. S. & Bastiaens, P. I. Imaging FRET between spectrally similar GFP molecules in single cells. *Nature Biotechnol.* **19**, 167–169 (2001).
148. Weiss, S. Fluorescence spectroscopy of single biomolecules. *Science* **283**, 1676–1683 (1999).
149. Sako, Y., Minoguchi, S. & Yanagida, T. Single-molecule imaging of EGFR signalling on the surface of living cells. *Nature Cell Biol.* **2**, 168–172 (2000).
150. Sonnleitner, A., Mannuzzu, L. M., Terakawa, S. & Isacoff, E. Y. Structural rearrangements in single ion channels detected optically in living cells. *Proc. Natl Acad. Sci. USA* **99**, 12759–12764 (2002).
151. Harms, G. S. *et al.* Single-molecule imaging of l-type $Ca^{(2+)}$ channels in live cells. *Biophys. J.* **81**, 2639–2646 (2001).
152. Ito, S. *et al.* Real-time observation of micrometastasis formation in the living mouse liver using a green fluorescent protein gene-tagged rat tongue carcinoma cell line. *Int. J. Cancer* **93**, 212–217 (2001).
153. Brown, E. B. *et al. In vivo* measurement of gene expression, angiogenesis and physiological function in tumors using multiphoton laser scanning microscopy. *Nature Med.* **7**, 864–868 (2001).

Acknowledgements

We thank S. R. Adams and J. Babendure for their comments on the manuscript. This work was supported by a National Institutes of Health grant to R.Y.T. and postdoctoral fellowship to A.Y.T, a grant from the Alliance for Cellular Signaling (to J.Z. and R.Y.T.) and the Howard Hughes Medical Institute. J.Z. is supported in part by a postdoctoral fellowship from La Jolla Interfaces in Science and Burroughs Wellcome Fund, and R.E.C. is supported in part by a postdoctoral fellowship from the Canadian Institutes of Health Research.

Online links

DATABASES

The following terms in this article are linked online to:

Entrez: http://www.ncbi.nlm.nih.gov/Entrez/
Kaede
Swiss-Prot: http://www.expasy.ch/
CFTR | EGF | GFP | ZBP

FURTHER INFORMATION

Roger Y. Tsien's laboratory:
http://www.tsienlab.ucsd.edu
Alice Y. Ting's laboratory:
http://web.mit.edu/chemistry/Ting_Lab/
BD Biosciences Clontech (EGFP, YFP–S65G/V68L/S72A/T203Y, CFP, DsRed2, HcRed, Fluorescent Timer, and DsRed-Express):
http://www.clontech.com
NanoLight Technology (*Renilla mullerei* GFP):
http://www.nanolight.com
PanVera (FlAsH–EDT_2 and ReAsH–EDT_2 labelling kits):
http://www.panvera.com
Access to this interactive links box is free online.