A GREENER WORLD: THE REVOLUTION IN PLANT BIOIMAGING

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The exploitation of fluorescent proteins has heralded a new age in the *in vivo* analysis of subcellular events, and has overcome many of the limitations that are associated with the investigation of cellular and molecular processes in plant cells. Recently, there have been many exciting applications of green fluorescent protein and its spectral derivatives in the study of plant cells.

In recent years, the green fluorescent protein (GFP) has become one of the most powerful and popular tools that are used by plant biologists. The discovery, optimization and development of GFP from the jellyfish *Aequorea victoria*[1] has revolutionized studies on cellular protein location and transport pathways. Expression of the protein allows direct *in vivo* observation of the biogenesis and dynamics of organelles, plus the transport of proteins in and between living cells. Previously, only biochemical or immunolabelling studies — which by their very nature are both invasive and lethal — could provide such information.

Reporter systems, such as those that are based on the activity of β-glucuronidase (GUS) and luciferase (Luc) have been extensively used in gene-expression studies, but they have severe intrinsic limitations in cell biology. GUS expression patterns can be analysed histochemically[2], but the assay is destructive to the material, so it is inconvenient for following and timing gene activity. Likewise, luciferin–luciferase imaging has been used in plants; for example, to characterize circadian-clock phenotypes in *Arabidopsis thaliana*[3,4]. Substrates for *in vivo* labelling are available, but they suffer from a high non-specific background. Furthermore, low levels of light emission require the use of sensitive photon-counting cameras, and the overall signal strength is not sufficient to permit subcellular imaging.

GFP is an intrinsically fluorescent protein of 238 amino acids. When fused to a native or foreign protein, it allows *in vivo* monitoring of targeted cellular structures without destruction of the sample (FIG. 1; reviewed in REFS 5,6). The exploitation of GFP technology in plant biology took a year or two longer to perfect than in other biological systems. The presence of a cryptic intron in the wild-type GFP sequence limited the use of GFP to those RNA viral systems in which expression is not controlled by splicing[7–9]. Subsequently, the native gene sequence was optimized for genomic expression in plants[10–13]. Variants with codon sequences that are optimized for non-plant expression systems became available; these, by chance, lacked the cryptic intron. Commonly used techniques for transient expression of GFP in plant cells are described in BOX 1.

In this review, we consider some of the more recent applications of GFP and its spectral derivatives in the study of plant cells, and how fluorescent protein technology covers numerous applications. These range from the relatively simple and more traditional approaches, such as locating and tracking proteins within cells, to the more complex photobleaching and quantitative fluorescence technologies that are used for the analysis of physiological events *in vivo*.

Imaging over the visible spectrum

Since GFP was cloned, considerable efforts have been made to modify the sequence to enhance various properties of the protein. However, the development of spectral variants has had the most dramatic effect on the evolution of the technology. Among these, the blue

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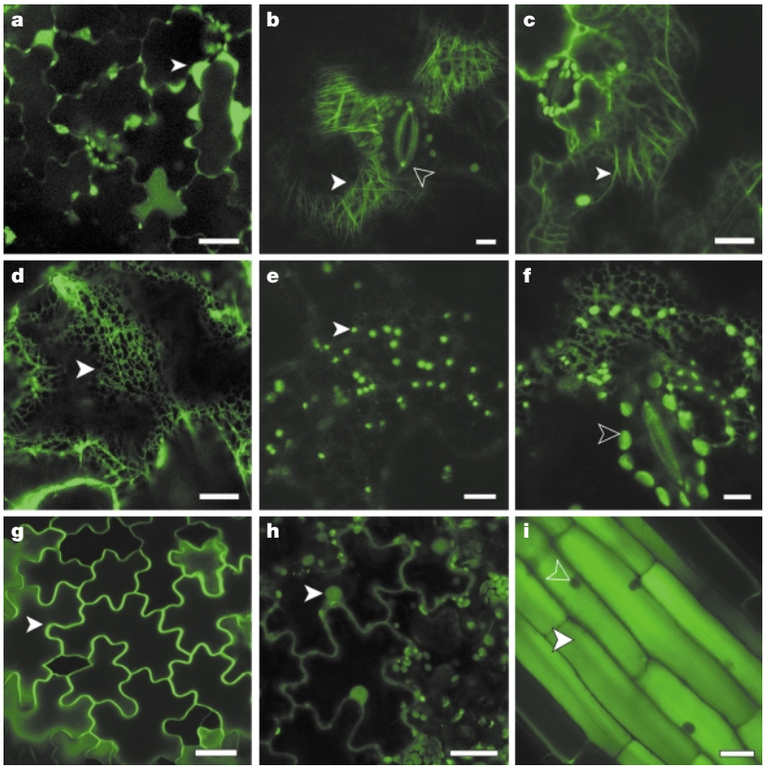


Figure 1 | **Examples of plant cellular structures that can be highlighted *in vivo* by GFP.** **a** | Green fluorescent protein (GFP) is targeted to the apoplast (arrowhead) as a secretory form (signal-peptide–wild type GFP[39]) in *Nicotiana benthamiana*. Bar, 25 µm. **b** | A GFP–tubulin construct[88] highlights microtubules (filled arrowhead) of leaf epidermal cells of *Nicotiana tabacum*. An empty arrowhead indicates a guard cell. Bar, 10 µm. **c** | GFP that is fused to an actin-binding domain of a mouse talin[42] decorates the actin network (arrowhead) of leaf epidermal cells of *N. tabacum*. Bar, 20 µm. **d** | A maize calreticulin–GFP fusion targets the endoplasmic reticulum (ER; arrowhead) of leaf epidermal cells of *N. benthamiana* (F.B. and C.H., unpublished observations). Bar, 20 µm. **e** | Golgi bodies (arrowhead) are visible in leaf epidermal cells of *N. tabacum* that were transiently transformed with a sialyltransferase signal anchor sequence fused to GFP (ST–GFP)[26]. Bar, 8 µm. **f** | An *Arabidopsis* H/KDEL-receptor[89] fusion to GFP (AtERD2–GFP) allows Golgi bodies and ER to be visualized simultaneously in transiently transformed *N. tabacum* leaf-epidermal cells. Chloroplasts inside a guard cell are indicated (empty arrowhead). Bar, 6 µm. **g** | The plasma membrane (arrowhead) is highlighted in *N. tabacum* epidermal cells that were transiently transformed with a GFP construct based on 22 transmembrane amino acids (BP22–GFP)[44]. Bar, 25 µm. **h** | Nucleoplasm (arrowhead) in *N. tabacum* epidermal cells is highlighted with cytosolic GFP. Bar, 25 µm. **i** | Tobacco-root-cell central vacuoles (filled arrowhead) are highlighted by GFP clipped from a mammalian lamin B receptor–GFP5 fusion (S. Irons, D. Evans and F.B., unpublished results). Nuclei are visible in negative contrast (empty arrowhead). Bar, 40 µm.

ANTHOZOAN
An organism that belongs to the Coelenterata, which includes the corals and sea anemones. The three principal groups or orders are *Acyonaria*, *Actinaria* and *Madreporaria*.

CHROMOPHORE
The part of a coloured molecule that is responsible for light absorption over a range of wavelengths. In the case of fluorescent proteins, the absorbed light is re-emitted at a longer wavelength, which gives rise to the fluorescent colour.

(BFP), cyan (CFP) and yellow (YFP) fluorescent proteins, red-shifted GFP and the new fluorescent proteins from different species (TABLE 1) are particularly well suited for numerous applications in plant cell biology. These include single and multiple fluorescent labelling, fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP), fluorescence correlation spectroscopy (FCS) and fluorescence resonance energy transfer (FRET), all of which are described in more detail later.

Successful imaging of multicoloured cellular structures relies on faithful spectral separation of the fluorescence emission. Commonly, the fluorescent protein couples that are used for these studies are CFP–YFP and GFP–YFP. This is because the best spectral separation from GFP is obtained with a fluorescent protein that is shifted towards the red part of the visible spectrum. Co-localization analysis with the GFP–YFP couple is better achieved with GFP5 and enhanced YFP (EYFP), rather than EGFP and EYFP, as GFP5 has a peak excitation that is further towards the blue region of the spectrum than EGFP. Attempts to generate a GFP that emits towards the red part of the visible spectrum have resulted in a red-shifted GFP, with emission at 529 nm in the green–yellow region[14].

What seemed to be a real red revolution was the identification of an ANTHOZOAN species (*Discosoma* sp.) that produces GFP-like proteins with emissions in the orange–red region at 583 nm (DsRed [drFP583])[15] and 593 nm (dis2RFP)[16], and the subsequent production of a red-shifted mutant with maximum emission at 616 nm[16]. Random and directed mutageneses have been used to generate DsRed variants with different fluorescence properties, accelerated CHROMOPHORE formation and enhanced solubility[17–20].

DsRed has spectral properties that make it ideal for dual colour labelling alongside GFP. So far, however, there are only a few reports on the use of DsRed for *in vivo* studies in plants. This lack of popularity for using the protein in such studies might be due to the interference with chlorophyll fluorescence, and to the well-documented multimerization of DsRed[21,22].

The feasibility of DsRed for expression in the plant cytosol, endoplasmic reticulum (ER) and vacuole has been assessed[23]. In transiently transformed BY-2 cells and stably transformed tobacco plants, cytosolic expression of DsRed gave rise to readily detectable levels of red fluorescence. Such fluorescence was seen in chloroplasts of stable plants after judicious use of filters to separate out chlorophyll fluorescence. DsRed was also found not to interfere with morphogenesis, plant development and fertility.

The red fluorescent protein has also been targeted to the ER by splicing the 40 amino-acid residues of the amino-terminal region and the 84 amino-acid residues of the carboxy-terminal region of BIP to the amino- and carboxy-terminal regions of DsRed, respectively[24]. However, expression of an ER-targeted DsRed-HDEL was found to precipitate in large aggregates inside the ER when expressed transiently in tobacco leaf epidermal cells (F.B. and C.H., unpublished observations). Targeting of DsRed to the Golgi apparatus has been obtained with fusions to full-length[24,25] rat SIALYLTRANSFERASE or the last 52 amino acids of this protein[26].

The potential of multifluorescent plants

Fluorescent proteins have been used as visual genetic labels at the whole-plant, tissue and cell levels.

Transgenic screening. GFP offers a fast and easy-to-use non-destructive tool with which the efficiency and timing of gene expression and organ development can be evaluated. It has been used, for example, as a visual marker for the development of wheat[27], *Brassica napus*[28]

Box 1 | Rapid transient expression of fluorescent-protein constructs

Plant tissues can be both transiently and more permanently genetically transformed. Transient transformation is easy, generates fast expression and represents a good platform for the testing of constructs before stable transformation. Stable transgenics are more laborious and take longer to achieve, but they can generate expression throughout the plant. Although expression might not be uniform, it can be achieved in tissues that would otherwise be difficult to access by transient methodology.

Expression in protoplasts. **This is a relatively easy approach to testing fluorescent-protein constructs. It allows a rapid test, in simple vectors, within a few hours and can produce material for biochemical analysis[80,81]. Limitations are due to loss of cell polarity and structure, plus the need to keep preparations sterile if long-term observations are to be made. The plasma membrane can be permeabilized to permit the internalization of DNA by treatment with polyethylene glycol or by electroporation. Transformation rates of up to 80% can be achieved.**

Agrobacterium*-mediated expression.* **An extremely effective method for monitoring expression of constructs in leaves is simply to pressure-inject a suspension of *Agrobacterium tumefaciens* into leaf cells through open stomata[6,26,80,82]. A single colony of *A. tumefaciens* (for example, strain GV3101 (pMP90RK)) that carries a binary expression vector of interest is cultured and washed in an infiltration medium that contains ACETOSYRINGONE, and diluted to an appropriate density (O.D. 0.01–0.05 at 600 nm). The bacterial suspension is inoculated using a 1-ml syringe without a needle by gentle pressure through the lower epidermal surface. Expression of GFP constructs can be monitored after 24 hours for up to 6 days.**

Virus-mediated expression. **As viruses replicate to high levels in plant cells, they can readily be exploited as expression vectors[7,43]. Various viruses have been engineered to express GFP, including potato virus X, tobacco and cowpea mosaic viruses and Gemini viruses[83]. In the virus vector, the coat-protein subgenomic promoter is duplicated, which drives the expression of any inserted foreign genes. Leaves are inoculated with infectious viral transcripts. If the virus is systemic, plants can be studied over extended periods from 3–4 days after inoculation.**

Gene guns or biolistics. **This technique is particularly suitable for transient expression in a wide range of cell types, from leaf[84] and onion epidermis[85] to pollen and suspension cultures[41]. With the biolistic method, plant material is bombarded with small (~1 nm) gold particles that are coated with DNA, and genes can be transferred into a range of species, independent of their genotype.**

Microinjection. **Although perhaps not a routine technique for testing constructs, microinjection offers the opportunity to study expression in single cells[86]. With the newly developed Galinstan expansion FEMTOSYRINGE (GEF), needle tips of a diameter as small as 0.1 μm can be used. So, it is now possible to inject directly into organelles such as chloroplasts. Low concentrations of plasmids (3.5 molecules per femtolitre) must be used, but these have been sufficient to introduce GFP constructs into tobacco-leaf chloroplasts, giving visible expression after 24 hours.**

BIP
One of the main chaperones of the endoplasmic reticulum that binds to nascent or unfolded polypeptides and ensures correct folding before the protein continues through the secretory pathway.

HDEL
Tetrapeptide composed of histidine, aspartic acid, glutamic acid and leucine that participates in the retrieval of soluble proteins from the Golgi to the endoplasmic reticulum.

SIALYLTRANSFERASE
A mammalian Golgi enzyme that catalyses the transfer of *N*-acetylneuraminic (sialic) acid to an acceptor molecule, which is usually the terminal sugar residue of an oligosaccharide, glycoprotein or glycolipid.

ACETOSYRINGONE
A low-molecular-weight plant compound that stimulates the activity of *Agrobacterium vir* genes and might act as a chemotactic agent in nature.

FEMTOSYRINGE
An extremely fine microcapillary-based syringe that enables injection of attolitre to femtolitre volumes into cells and organelles with minimal structural damage.

T1 GENERATION
The first progeny that is derived from a transformed plant.

ENHANCER
DNA sequences that are present in the genomes of higher eukaryotes and of various animal viruses. They can increase the transcription of genes to messenger RNA, but alone are not sufficient to cause expression.

FLOW CYTOMETRY
An optical technique for separation, classification and quantification of fluorescent cells or particles.

and barley[29]. On the basis of differences in the intensity of GFP fluorescence, homozygous and hemizygous states of tobacco could be visualized easily in seeds and seedlings of the T1 GENERATION[30].

Motif- and enhancer-trap technology. Motif-trap technology is based on sequential screening of random DNA fragments that are fused to a fluorescent protein and expressed in cells. Those that are localized in cellular organelles are then analysed[31,32]. This tool opens up the possibility of discovering and analysing new sequences of unknown function.

Similarly, the GAL4–VP16 ENHANCER-trap technology has been applied in *Arabidopsis* (FIG. 2). The principle is based on the observation that heterologous transcription factors[33–35] can be expressed with a variable pattern in different cell lines derived from the same organism, depending on the presence of tissue-specific genomic enhancer sequences that are adjacent to the insertion site. *Arabidopsis* stable transformants that express a genetic cassette that contains a modified yeast GAL4 transcription activator, GAL4–VP16, and an ER-targeted GFP that is GAL4-responsive, were produced. A library of *Arabidopsis* lines was generated, each of which expressed GFP in a different tissue type. When plants that express this cassette are crossed with plants that express a gene that is regulated by the GAL4-responsive promoter, the gene can also be expressed in the GFP-marked cells. This approach has generated a valuable collection of marked cell lines for live-cell imaging, and is a powerful tool for studying tissue-specific expression of a range of genes[35,36] (FIG. 2; also see Jim Haseloff's web site (TABLE 2)).

Flow-cytometric analysis. FLOW CYTOMETRY is one of the best tools with which to follow gene expression rapidly, on a per-cell basis, in multidimensional cell-parameter analyses. GFP expression is particularly suited to this technique, especially for those mutants of GFP that have a unimodal absorption spectrum, such as EGFP. For example, green-fluorescent tobacco protoplasts can easily be analysed and sorted by flow cytometry[37], with spectral separation from chlorophyll autofluorescence. Moreover, GFP-based flow cytometry is a reproducible and valid method with which to characterize gene expression rapidly[38]. For example,

Table 1 | **Examples of fluorescent proteins commonly used in plants**

Fluorescent protein	Amino-acid substitutions with respect to wild-type GFP	Absorbance/ emission (nm)	References
Wild type GFP	(Chromophore: S65–Y66–G67)	396,475/508	91
mGFP5	V163A, I167T, S175G,	400,475/508	11,92
mCFP	Y66W,V163A,S175G	440/485	33
mYFP	S65G,S72A,V163A,I167T,S175G,T203Y	514/527	33
RsGFP	S65G,S72A	505/522	93
EGFP	F64L,S65T,a	488/507	94
ECFP	K26R,F64L,S65T,Y66W,N146I,M153T,V163A,N164H,N212K,a	434/474	95
EYFP	S65G,V68L,S72A,T203Y,a	514/527	95
DsRed	–	558/583	15

a denotes a variant with one valine inserted at position 2, which is not counted, and with a H231L that does not affect spectral properties. CFP, cyan fluorescent protein; GFP, green fluorescent protein; YFP, yellow fluorescent protein.

the effect of the plant hormone abscisic acid (ABA) in rice protoplasts was analysed by following the expression of GFP under the control of ABA-inducible or constitutive promoters in the presence of specific components of the ABA signalling pathway and the ABA agonist lanthanum chloride[38].

Protein and organelle fluorescence within cells

Monochromatic plants — single-protein labelling. GFP and its derivatives are being extensively used to determine the biogenesis and destination of numerous proteins in plant cells. The APOPLAST[39] (FIG. 1a), microtubules[40] (FIG. 1b), actin[41,42] (FIG. 1c) and most organelles such as the ER[39] (FIG. 1d), Golgi apparatus[43] (FIG. 1e), ER and Golgi together[43] (FIG. 1f), plasma membrane[44] (FIG. 1g), nucleus[45] (FIG. 1h), lytic and pH neutral vacuoles[46] (FIG. 1i), PEROXISOMES[47], PREVACUOLE[24], mitochondria[48,49], chloroplasts[50] and PLASTIDS[51] have been successfully tagged with GFP.

For the first time it is possible to analyse a range of organelle dynamics rapidly, and with sufficient contrast to facilitate analysis using manual or automated tracking software. By tagging organelles *in vivo* with fluorescent proteins, we can now assess their dynamics inside the whole cells. For example, GFP expression showed a dramatic tracking of leaf Golgi over the cortical ER network[43], and an example of how such movement can be analysed is provided in BOX 2.

Using fluorescent variants — multicolour plants. Although the emission spectra of GFP and its spectral variants overlap (FIG. 3), with the appropriate combinations of excitation and emission wavelengths it is possible to observe, simultaneously, two or more fluorescent proteins that are targeted to the same or to different organelles within an individual cell. Examples of multilabelling with fluorescent proteins are rare in the plant literature, but we predict that the number of reports will rapidly increase in parallel with accessibility to the new range of confocal microscopes with faithful multi-imaging capabilities. Examples of multiple labelling of organelles within cells are shown in FIG. 4, along with the corresponding filter and laser combinations.

Data on the multiple labelling of cell structures with more than two fluorescent proteins have yet to be published for plants. In theory, owing to its unique excitation characteristics, DsRed could be visualized

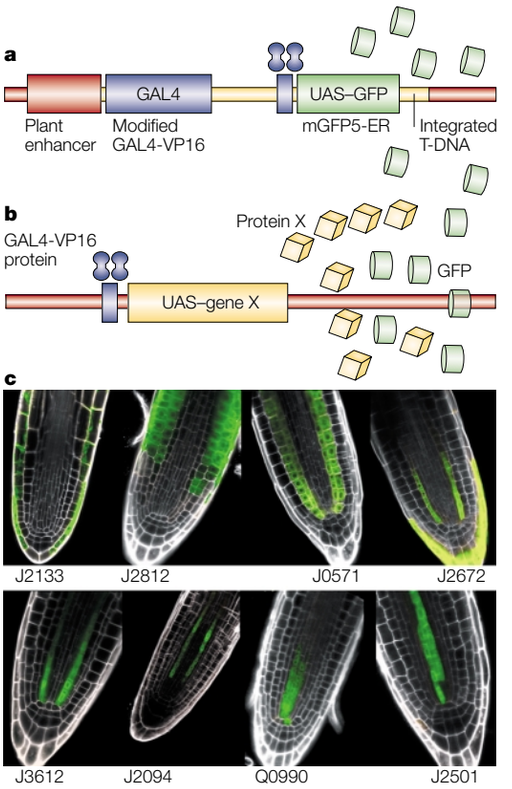


Figure 2 | **Enhancer-trap technology. a** | Diagram of a plant cassette that expresses an enhancer, which consists of the coding regions of GAL4-VP16 and green fluorescent protein (GFP), which is GAL4 responsive. When plants that express this cassette are crossed with plants that express a gene of interest under the GAL4-responsive promoter, the gene is expressed only in the GFP-marked cells (**b**). Using this system, *Arabidopsis* lines with different marked cell lines were produced (**c**). Diagrams and images courtesy of J. Haseloff (modified with permission from REF. 90 © 1999 Academic Press). UAS, upstream activation sequence.

APOPLAST
The environment that is external to the plasma membrane. This includes cell walls and intercellular spaces, through which water and solutes pass relatively freely.

PEROXISOMES
Membrane-bound organelles that contain peroxidase and catalase, sometimes as a large crystal, where oxygen is used without ATP synthesis.

PREVACUOLE
A post-Golgi organelle that is responsible for delivering cargo to the vacuole.

PLASTIDS
A family of semi-autonomous plant-cell organelles. These are surrounded by a double membrane and contain elaborate internal membrane systems, DNA, RNA and ribosomes, and reproduce by binary fission. Includes amyloplasts, chloroplasts, chromoplasts, etioplasts, leucoplasts, proteinoplasts and elaioplasts.

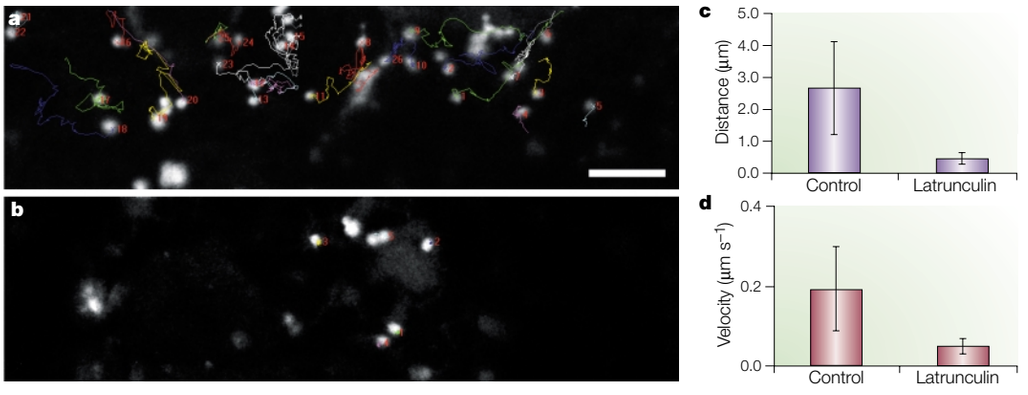
Table 2 | **Links to some plant green fluorescent protein web sites**

Organelle	Plant	Author	Web site address
Plastids in plants and suspension cultures	Tobacco	Köhler, R.	http://www.mbg.cornell.edu/kohler/kohler.html
Endoplasmic reticulum and Golgi in leaves	Tobacco, BY-2 cells *Arabidopsis*	Hawes, C.	http://www.brookes.ac.uk/schools/bms/research/molcell/hawes/gfp/gfp.html
Endoplasmic reticulum in roots	*Arabidopsis*	Haseloff, J.	http://www.plantsci.cam.ac.uk/Haseloff/IndexGFP.html
Golgi	BY-2 cells	Nebenführ, A.	http://mcdb.colorado.edu/~nebenfue/golgi/
Peroxisomes, endoplasmic reticulum, vacuoles	*Arabidopsis*	Cutler, S. & Ehrhardt, D.W.	http://deepgreen.stanford.edu

simultaneously with GFP and YFP using single excitation with a standard 488-nm laser line or using a dual-laser excitation with alternate 488 nm (for GFP and YFP) and 543 nm (for DsRed) with appropriate emission filters; or even with CFP and GFP excitation at 458 nm and using the 543-nm line for DsRed. However, such combinations would not be efficient for identifying multiple labelling within the same organelle. The development of sophisticated instrumentation for the faithful separation of fluorophores with close emission maxima has allowed the discrimination of close fluorochromes such as CFP, GFP and YFP (FIG. 5).

GFP in dead cells. Although an apparent contradiction in terms of use, GFP has been successfully exploited in immunocytochemical studies. Once fixed, GFP retains its fluorescent properties. This feature has allowed it to be used in combination with immunolabelling of organelles of interest[26,52]. This can be extremely important for confirming the identity of the target organelle[44]. Likewise, GFP itself can be immunolocalized at the ultrastructural level to determine more precisely the location of a construct within an organelle[43]. Such experiments are always recommended for the assessment of new constructs, especially where there is the possibility of mistargeting due to overexpression.

Box 2 | **Tracking organelle movement**



The rate and pattern of organelle movement can be determined from analysis of time-lapse images of labelled organelles. Manual tracking is laborious, but the high contrast and resolution, particularly of confocal images, greatly facilitates automated and semiautomated tracking procedures. With this approach, a range of parameters including instantaneous velocity, averaged velocity and distances can be determined and automatically analysed under different experimental conditions.

Examples of tracking of green fluorescent protein (GFP)-tagged Golgi bodies are given, in which Golgi move as individual stacks in a cell along an actin/endoplasmic reticulum network[43,87]. Golgi stacks have been reported to be highly mobile, alternating between random saltatory and directional movements. Data in the image were collected with a confocal microscope (Zeiss LSM510) on epidermal cells of tobacco leaves that express a sialyltransferase signal anchor sequence fused to GFP[26] (ST–GFP) using Metamorph© image software version 4.6 with motion options for semi-automated object tracking. With an intact acto-myosin cytoskeleton, Golgi bodies cover variable distances within a given time frame (figure parts a, c). Individual Golgi in the cortical region of the cell move with an average velocity of 0.19 µm s^{-1} (77 Golgi analysed within nine cells for 75 s; figure part d). The depolymerization of actin with latrunculin B drastically reduced the movement and the velocity of Golgi bodies (figure parts b–d; latrunculin treatment; 36 Golgi analysed within nine cells for 97 s).

In control cells, data are affected by a very high standard deviation. This is due to the fact that Golgi alternate randomly directional movement for short distances to higher distances and saltatory movement along the same trajectory. Bar in a, 5µm.

The cell as a laboratory

As fluorescent proteins can be used to report events in living cells, they can effectively be used to turn the cell into a miniature laboratory. So, they can be used for quantitative analysis of protein flow within or between different organelles, for the analysis of protein–protein interactions and for analysis of the ionic environment within cells.

Protein movement within cells. GFP has been used to investigate protein movement between cellular compartments and between cells that are responding to an external stimulus at a specific developmental stage. One such example is analysis of the movement of PHYTOCHROME A and phytochrome B that are fused to GFP[53–55]. Differential kinetics were observed for the two proteins entering the nucleus under different conditions of light irradiation[53]. Moreover, a different requirement of quality of irradiation for protein import into the nucleus was found between *Arabidopsis* and tobacco. This was induced by continuous and pulses of far-red light in *Arabidopsis*, and only by continuous irradiation in tobacco[55]. Similarly, fluorescent proteins have been used to investigate export of proteins from the nucleus[56] and protein movement through the PLASMODESMATA[57,58].

Photobleaching shows protein movement. In most instances, the propensity of a fluorescent label to photobleach is regarded a problem, and many GFP variants have been selected because of their resistance to photobleaching. However, photobleaching can be exploited to follow and quantify cellular dynamics *in vivo*.

Fluorescence recovery after photobleaching (FRAP) is based on the principle of observing and quantifying the rate of fluorescence recovery due to the movement of fluorescent molecules into an area of the membrane or organelle that has been rendered non-fluorescent with an intense photobleaching pulse of laser light[59]. For example, this technique has been used to assess the structure and connectivity of artificial[60] and biological membranes[61]. FRAP, based on GFP as the reporter, has been used to analyse, for the first time *in vivo*, the movement of membrane proteins from the ER to the Golgi in tobacco leaves[42] (FIGS 6,7). In this system, Golgi bodies seem to be intimately associated with the cortical ER[43] (FIG. 1f), and FRAP results have shown that, despite the apparent continuity between these two organelles, protein transfer between them is reliant on BREFELDIN A- and *N*-ETHYL MALEIMIDE-sensitive factors, as well as depending on energy[42]. The technique can be used in combination with double labelling. For example, in cells that are co-transformed with CFP and YFP Golgi markers, YFP was selectively photobleached with the 514-nm laser line of the confocal microscope, while the integrity of the membranes was followed by simultaneous observation of a similar CFP construct that labelled the same stack[42] (FIG. 6g–i). This leads to the exciting possibility that FRAP experiments could be carried out on motile organelles.

Fluorescence loss in photobleaching (FLIP) is a derivative technique of FRAP that has been used to investigate the connectivity of plastids by 'STROMULES' (stroma-filled tubules[62]). FLIP involves repeated bleaching of an area of fluorescence, with the aim of depleting the whole pool of fluorescence. If two structures are interconnected, then fluorescence depletion in one can cause fluorescence loss in the interconnected one.

Stromules are particularly abundant in tissues that contain chlorophyll-free plastids, and FLIP experiments on stromules showed that these structures serve as interconnections between two or more plastids. FLIP results also showed that stromules might act as vehicles for the import and export of mobile material between plastids[62]. Similar observations were obtained with FRAP, which distinguished interconnected and independent plastids in transgenic tobacco plants that expressed GFP targeted to plastids[49].

Finally, fluorescence correlation spectroscopy (FCS) allows the quantification of particle concentration, motion and dynamics from fluctuating emission signals from a small number of molecules[63]. This technique has been used as an alternative photobleaching-based technology to measure the rate of diffusion of stroma-targeted GFP *in vivo*[64], and to investigate the dynamics of NOD FACTORS in root-hair-cell walls of *Vicia sativa* (common vetch)[65].

The two-photon excitation (2PE) FCS technique is extremely sensitive, and can detect down to the level of a single GFP molecule. It reduces the limits of detection due to photobleaching, photodamage and light

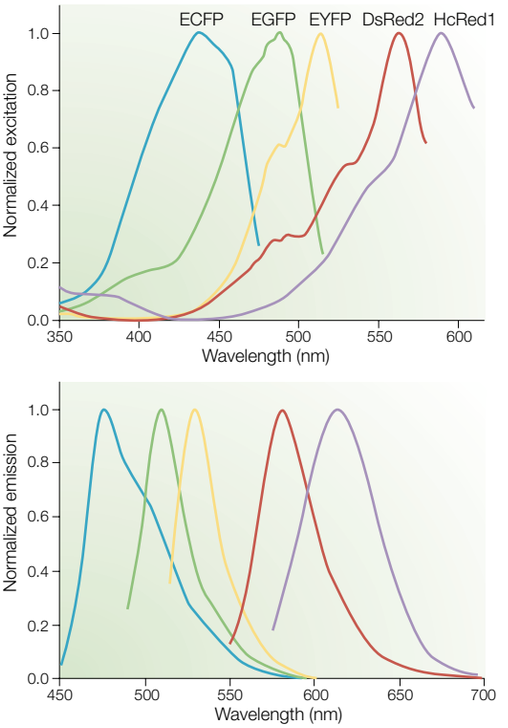


Figure 3 | **Fluorescent protein spectrum.** Absorption and emission spectra of the different fluorescent proteins. Courtesy of Clontech, Palo Alto, California.

PHYTOCHROME
A plant pigment protein that, on absorption of red light, initiates physiological responses that govern light-sensitive processes such as germination, growth and flowering.

PLASMODESMATA
Plasma-membrane-lined channels in the cell wall that interconnects adjacent plant cells. They consist of a break in the cell wall that is lined by cell membrane and contain a strand of membrane that is derived from rough ER called the desmotubule.

BREFELDIN A
A fungal metabolite that acts as a potent inhibitor of secretion. This has proved an invaluable tool in the study of membrane transport.

N-ETHYL MALEIMIDE
A sulphydryl reagent that is widely used in experimental biochemical studies to covalently modify cysteine residues in proteins.

STROMULES
Highly dynamic, chlorophyll-free, tubular-membrane interconnections between adjacent plastids, which are best observed in living tissues.

NOD FACTORS
Biologically active bacterial *Nod* gene products, such as chitin oligomers, with various modifications, including addition of *N*-linked fatty acids, which are involved in the establishment of legume-root symbioses.

DIPOLE
A molecule that has both negative and positive charges.

scattering[66]. Using this technique, it was established that the diffusion of GFP in stromules has passive-diffusion and active-transport components[64]. Moreover, GFP diffusion in the stromules is 50 times slower than diffusion of GFP in the cytosol, and 100 times slower than GFP in aqueous solutions[64]. This reflects a differential viscosity of the tubular stroma in comparison to other cellular environments, and also the interaction with freely diffusive molecules.

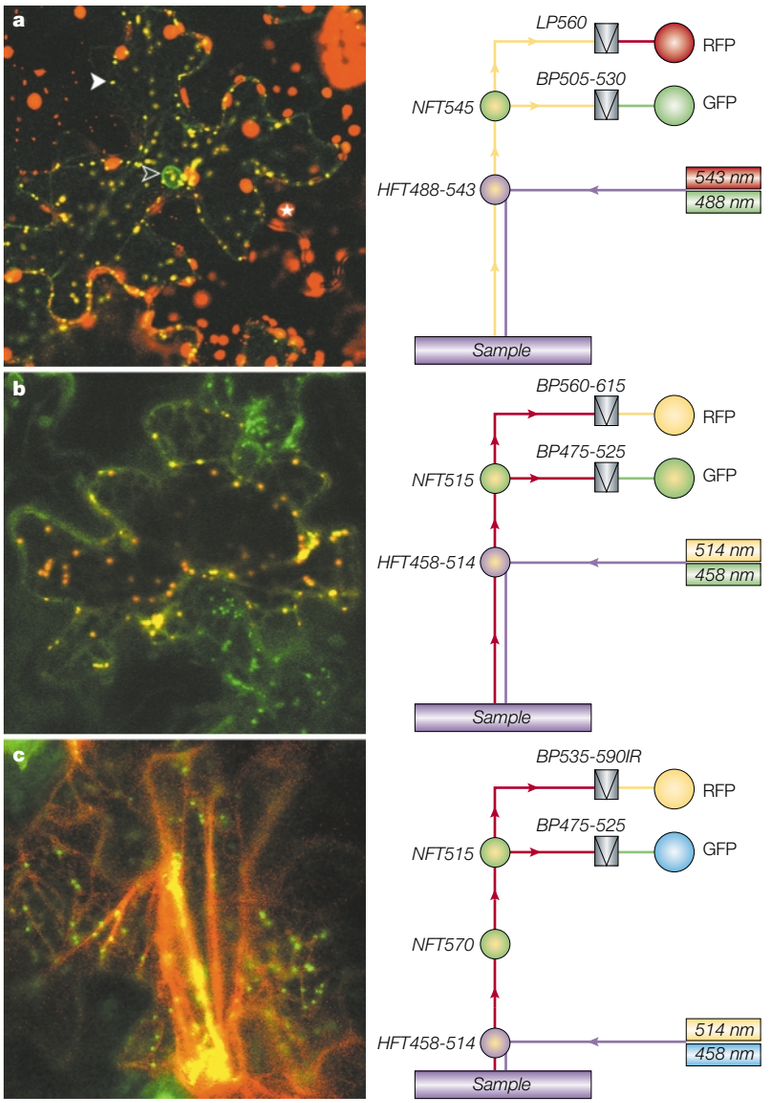


Figure 4 | **Visualization of different combinations of fluorescent proteins.** Examples of dual-colour imaging in tobacco-leaf epidermal cells. The micrographs were obtained with line switching using the multitrack facility of a Zeiss LSM510 confocal microscope with specific filter combinations to minimize crosstalk and bleed-through of fluorescence between channels (right-hand panels). **a** | GFP5 and DsRed constructs. The H/KDEL receptor homologue from *Arabidopsis thaliana* (AtERD2, in green) fused to GFP5 highlights endoplasmic reticulum (ER), Golgi (filled arrowhead, see also FIG. 1f) and nuclear envelope (empty arrowhead)[43]. DsRed fused to the last 52 amino acids of a rat sialyltransferase (ST–RFP, in red) locates to the Golgi apparatus (filled arrowhead)[26]. Chloroplasts are also visible due to their autofluorescence (star). **b** | GFP5 (ST–GFP5; in green) and EYFP (AtERD2–EYFP; in red). **c** | ECFP and EYFP. AtERD2–ECFP (in green) was co-expressed with an actin-binding domain of a mouse talin fused to EYFP[42] (in red), which decorates actin. BP, bandpass filter; GFP, green fluorescent protein; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; HFT, main dichroic beam splitter; LP, longpass; NFT, secondary dichroic beam splitter; RFP, red fluorescent protein. Excitation lines, argon ion laser.

Protein–protein interactions. One method for detecting molecular interactions *in vivo* involves fluorescence resonance energy transfer (FRET). With this technique, it is possible to use fluorescence as a spectroscopic ruler to study and quantify the interactions between cellular components. FRET is based on the transfer of the energy from an excited donor fluorophore to a neighbouring acceptor by DIPOLE–dipole interaction.

The usefulness of this technique is due to the fact that the efficiency of the energy transfer decays with the sixth power of the distance that separates the donor and acceptor fluorophores. This results in the ability to measure interactions between cellular components on a scale of 10–100 Å. Useful FRET fluorescent-protein pairs are CFP–YFP and BFP–GFP. The use of CFP as donor and YFP as acceptor is best suited for FRET experiments in living cells, as the emission spectrum of CFP overlaps the excitation spectrum of YFP, and the CFP–YFP couple shows a larger Föster distance than the BFP–GFP couple. (The Föster distance is the distance between the donor and acceptor molecules at which the average efficiency of energy transfer is 50%.) The BFP–GFP couple suffers due to the relatively poor fluorescence of BFP. However, the FRET pair of CFP–YFP is problematic because it is difficult to avoid excitation of YFP with the CFP-exciting laser, and it is hard to detect YFP emission without detecting some CFP signal. Once DsRed or substitutes are optimized for oligomerization and expression in heterologous systems, they will be good fluorescent partners to be used in combination with GFP.

Using FRET, the potential for dimerization on the plasma membrane of the *Arabidopsis* AtSERK1 protein (*Arabidopsis* somatic embryogenesis receptor kinase 1) was investigated by measuring the emission ratio in the AtSERK1–CFP and AtSERK1–YFP couple[67]. The role of the extracellular LEUCINE-ZIPPER DOMAIN of AtSERK1 in oligomerization of the receptor was tested. On elimination of this domain, the YFP–CFP emission ratio was reduced to control levels, which indicates that the domain is essential for oligomerization of the protein in the plasma membrane.

Genetically encoded fluorescent indicators that are based on GFP variants and FRET, such as the calmodulin-based 'CAMELEONS', have been developed for monitoring Ca^{2+} in whole organisms and tissues. In *Arabidopsis* guard cells, the CFP–calmodulin–YFP-based calcium indicator, yellow cameleon 2.1 (YC2.1)[68], has been expressed to measure, in a time-dependent fashion, the concentration of cytoplasmic Ca^{2+} in response to external stimuli[69]. The EYFP that was used in the original cameleons shows pH-sensitivity with a pK_a of 6.9. However, the introduction of two further mutations (V68L and Q69K) created the YC2.1 variant, which is less sensitive to pH in the physiological range[68].

A good example of the application of cameleon technology has been in the measurement of free Ca^{2+} in guard cells. Typically, measurements have previously

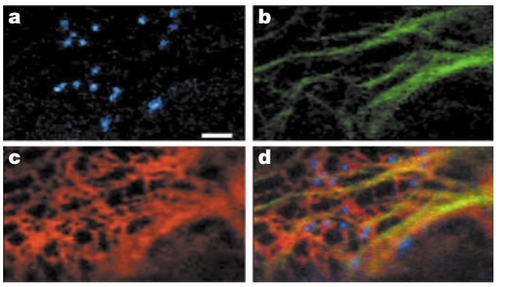


Figure 5 | **Triple colour imaging in plants.** Images were collected using the Zeiss LSM510 META detector system with deconvolution software from epidermal cells of the lower epidermis of *Nicotiana tabacum* four days after infiltration with three different *Agrobacterium* strains containing plasmids that express ST–ECFP, to label Golgi (**a**), talin–GFP5 to label the actin cytoskeleton (**b**) and EYFP–HDEL to label the endoplasmic reticulum (**c**). Spectral deconvolution then followed using reference spectra for individual constructs imaged under identical conditions. Organelles that are labelled with different fluorescent proteins are clearly resolved from each other (**d**). Images courtesy of I. Moore, University of Oxford. Scale bar, 5 μm. EYFP, enhanced yellow fluorescent protein; GFP, green fluorescent protein; ST–ECFP, sialyltransferase signal anchor sequence fused enhanced cyan fluorescent protein.

required the laborious and invasive microinjection that is needed for RATIOMETRICAL chemical dyes[70]. This has hampered progress, and the total number of Ca^{2+} measurements using fluorescent dyes over the past decade is still extremely low compared with animal systems. However, the absence of microinjection allows measurements to be made on intact tissues rather than isolated epidermis. These Ca^{2+} transients in the guard cells of transgenic *Arabidopsis* that constitutively express YC2.1 have been observed in response to both elevated external Ca^{2+} and ABA[69].

LEUCINE-ZIPPER DOMAIN
Certain DNA-binding proteins contain a motif of approximately 35 amino acids, with every seventh residue being a leucine. This facilitates dimerization of two such proteins to form a functional transcription factor.

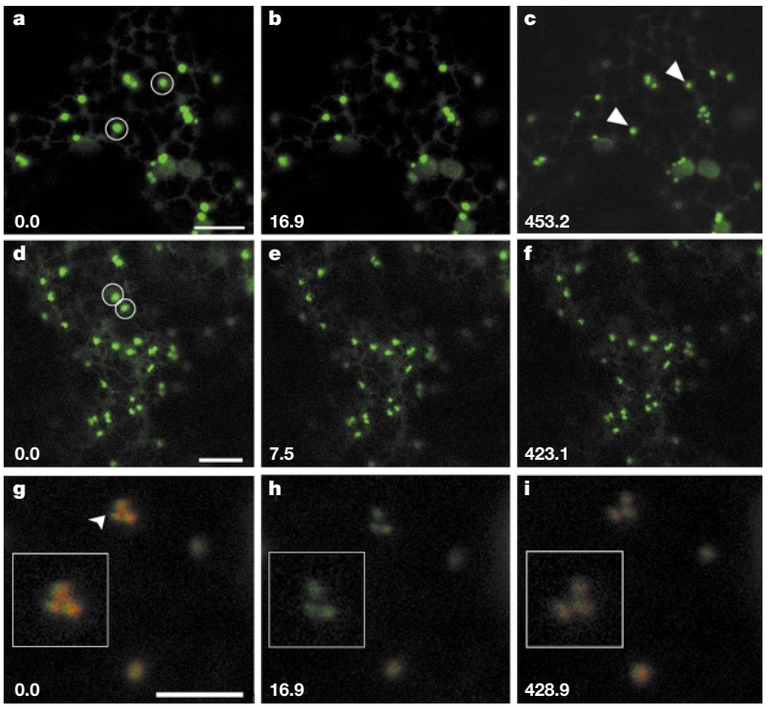


Figure 6 | **Selective photobleaching in tobacco epidermal cells. a–c** | Photobleaching of AtERD2–GFP5 that localizes in the Golgi and endoplasmic reticulum (ER). To stop Golgi movement, AtERD2–GFP5-expressing tobacco epidermal cells were treated with cytochalasin D (20 μg ml^{-1} for 30 min). Under these conditions, when Golgi bodies (**a**; circles) were bleached (bar, 4 μm) (**b**), fluorescence recovery was observed (**c**; arrowheads). **d–f** | When Golgi bodies (**d**; circles) are photobleached (**e**) in cells that were treated with the cysteine crosslinker *N*-ethyl maleimide (NEM, 20 mM for 10 mins), no recovery of fluorescence was observed (**f**). Bar, 4 μm. **g–i** | Golgi bodies (arrowhead in **g**) labelled in epidermal cells that were treated with the actin-depolymerizing agent latrunculin B (25 μm for 1 h) and co-expressing ST–ECFP (green) and ST–EYFP (red) were photobleached for ST–EYFP only (**h**). Recovery of the EYFP fluorescence was recorded against an unbleached ECFP background (**i**). A magnified portion of the whole image is shown in inset. Bar, 5 μm. Time is expressed in seconds at the bottom left of each panel. AtERD2, H/KDEL receptor homologue from *Arabadopsis thaliana*; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; ST–ECFP a sialyltransferase signal anchor sequence fused to enhanced cyan fluorescent protein.

Another example of the use of cameleons as Ca^{2+} indicators is to follow the response of *Arabidopsis* cotyledon tissue to cold shock. FIG. 8 shows that epidermal cells show a rapid but transient increase in cytosolic Ca^{2+}, whereas guard cells have a more variable response (A. Parson, N. S. White and M.F., unpublished observations).

Although the dynamic range of cameleons of <1.5-fold for a full response is lower than the >fivefold changes for common ratiometrical chemical Ca^{2+} dyes, the stability of the fluorescence and expression levels still allow measurements with a reasonable signal-to-noise ratio. The discrimination of spectral changes can also be enhanced by the measurement of fluorescence emissions across several wavelengths, rather than the two that are typically used for ratio measurements in a technique that has been called fluorescence spectral imaging[71] (SPIM).

Transgenic pH-indicators based on GFP. Fluorescence from wild-type GFP is essentially insensitive to pH in the physiological range[72], provided that this is above pH 5 (REF. 73). However, several of the spectral variants of GFP have much higher pK_a values, and show reversible pH-sensitive fluorescence at physiological pH values that are unaffected by fusion with other proteins[74]. So, it is possible to use them as *in vivo* indicators of cytoplasmic pH. Although it has not yet been applied in plant cell biology, the sensitivity of these fluorescent proteins to pH could be a useful feature to be exploited for intracellular and intercellular pH measurements. For example, vacuolar and apoplastic pH might be determined with accuracy in different tissues and developmental stages of transgenic plants.

All may not be as green as it seems...

Despite the great advantages that GFP technology offers, a word of caution is necessary. A potential risk that is encountered with any chimaeric protein, GFP-fusions included, is that it could be directed to a cellular compartment other than the one in which the target protein normally resides. This could be due to the masking of specific signals on a target protein, or misfolding of the target protein after fusion to GFP. Moreover, for the same reasons, a GFP-tagged protein might lose or alter its function. Similarly, overexpression of a functional fluorescent chimaera could have detrimental consequences on the functioning of the

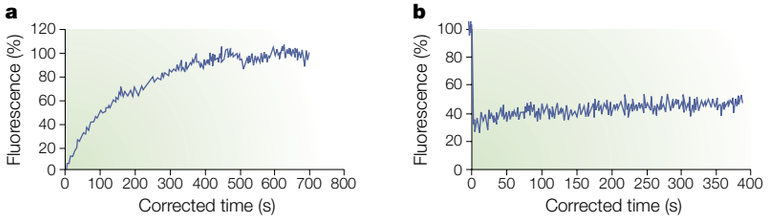


Figure 7 | **Quantification of fluorescence in FRAP experiments.** Quantification of the $t_{1/2}$ is an expression of the time that is required for fluorescence in the photobleached region to recover to 50% of the recovery asymptote (curve). The $t_{1/2}$ in FRAP experiments is carried out by scaling the post-bleach fluorescence recovery to a 0–100% scale, with the immediate post-bleach intensity being 0% and the asymptote of the recovery being 100%. Time was rescaled, with time 0 being equal to the half-time of the photobleach, as in REFS 42,85. **a** | Example of a $t_{1/2}$ recovery plot. 100% fluorescence indicates the prebleach fluorescence intensity. **b** | Representative fluorescence recovery curve of bleached Golgi in *N*-ethyl maleimide-treated cells expressed as a percentage of the initial fluorescence against time.

whole cell. Alternatively, the fusion might have a detrimental effect on the GFP fluorescence itself, or misfolding of the fluorescent protein could result in lower levels of fluorescence.

As for all light microscopy, the available resolution could limit the interpretation of image data sets. Therefore, as discussed earlier, confirmation of results of location should be obtained by co-expression with another fluorescent-protein chimaera that is known to target the organelle of interest, or by using complementary techniques such as immunofluorescence or, even better, immunogold labelling. This is particularly important when proteins are targeted to organelles such as mitochondria, plastids and Golgi, the identity of which can easily be mistaken using fluorescence microscopy if observations are made on the basis of size or morphology alone. Moreover, before attempting visualization experiments with different fluorescent proteins, we should always consider factors that might affect the performance of the proteins, such as proteolysis in certain compartments and any pH sensitivity.

Moreover, in systems in which gene knockouts can be routinely generated, such as *Schizosaccharomyces pombe* or *Saccharomyces cerevisiae*, it is straightforward to assess whether the GFP-tagged protein can functionally complement a null mutation. This is not the case in plants. Therefore, the effects of a mutant protein that is fused to GFP could still be influenced by the presence of the endogenous wild-type protein.

Finally, when working with plant material, it is necessary to consider possible interference in the detection of genuine fluorescent-protein emission due to innate chromophores such as chlorophyll and other chromophores, the spectral properties of which could overlap or share the spectrum of GFP.

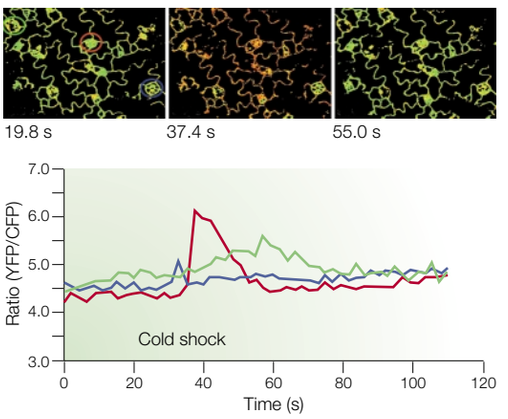


Figure 8 | **Use of cameleons in *Arabidopsis*.** Confocal ratio imaging of transgenic cameleon Ca^{2+} indicators in *Arabidopsis* cotyledons. Intact cotyledons from seedlings that express yellow cameleon 2.1 (YC2.1)[69] were imaged with excitation at 442 nm during a cold shock (upper panel). The ratio of the emission signals that correspond to the peaks for cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) provides a measure of the Ca^{2+} level throughout the tissue. There was a rapid, transient increase in cytosolic Ca^{2+}, which lasted around 10 s in all the epidermal cells (lower panel) after the application of cold water to the cotyledons. The responses from the guard cells were more variable, and ranged from a rapid response that is similar to the epidermal cells, through a longer transient of lower amplitude, to essentially no detectable change.

Towards a brighter future

There is little doubt that microscopy is now one of the most diverse and exciting of all the techniques in cell biology. The recent explosion in the use of fluorescent-protein technology has revitalized bioimaging, and allowed unprecedented discoveries from *in vivo* cellular analysis, such as communication between plastids[51] and the dynamics of the leaf Golgi apparatus[42,43]. The plant cell biology community is now using GFP as a routine investigative tool. However, multilabelling of organelles and other, more complex, applications of the fluorescent proteins are still limited to the more specialist laboratories[43]. The increasing need for *in vivo* cellular analysis will undoubtedly result in the development of more affordable technologies. For example, relatively cheap lasers are now available for the production of ABLATION MICROBEAMS and for OPTICAL-TRAP LASER TWEEZERS[75]. So, there is now the potential for microsurgery and manipulation within GFP-expressing cells.

Alongside technical developments, we can predict the development of many more fluorescent tools to probe cell physiology. For example, new GFP-like proteins[76] are now being developed. These include new far-red proteins such as HcRed from the reef coral *Heteractis crispa*[77] (FIG. 3), proteins with a decreased sensitivity to pH (for example, Venus YFP; F46L/F64L/M153T/V163A/S175G (REF. 78)), or photoactivatable GFP (G. Patterson and J. Lippincott-Schwartz, personal communication). Moreover, as the structural barrel of GFP and its fluorescent derivatives contain hydrophobic patches that are responsible for protein dimerization, experiments that aim to investigate intermolecular interactions on the basis of these fluorescent proteins will benefit from the development of the new variants, which do not dimerize[79].

CAMELEON
A fluorescent chimaera used as a Ca^{2+} indicator. Cameleons comprise BFP or CFP, calmodulin, a glycylglycine linker, the calmodulin-binding domain of myosin light chain kinase (M13) and a GFP or YFP. Binding of Ca^{2+} to the calmodulin causes intramolecular calmodulin binding to M13. This conformational change reduces the separation between the two fluorescent proteins and so increases the FRET efficiency between the shorter and the longer wavelength protein.

pK_a
The pK_a of an acid is the negative log to base 10 of its acid dissociation constant into a hydrogen ion and an anion.

RATIOMETRICAL
A property of certain fluorescent probes that improves quantitative measurements of intracellular ion levels by measuring shifts in the excitation or emission spectrum after binding to the ion as a ratio of two wavelengths. This approach compensates for changes in illumination intensity, probe concentration and optical path length that can cause errors with measurements at a single wavelength.

ABLATION MICROBEAMS
High-intensity focused laser beams that are used to selectively eliminate cellular structures.

OPTICAL-TRAP LASER TWEEZERS
Focused laser beams that are used to trap and move cellular structures.

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