

Imaging techniques in plant transport: meeting review

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Abstract

This review covers recent advances in imaging techniques that were presented during the 11th International Workshop on Plant Membrane Biology, Cambridge, August 1998. Cell biology has been revolutionized by the arrival of green fluorescent protein (GFP) and GFP is now routinely used as a cell-lineage marker in plants, to localize proteins to subcellular compartments or as a tag to follow dynamics in endomembrane compartments. More recent developments include modification of GFP to form physiological sensors for calcium and pH. These provide a transgenic approach in parallel with conventional chemical dyes to track signalling events or follow membrane recycling pathways. Confocal microscopy has become a routine technique to visualize these fluorescent probes, particularly in intact tissues. Multi-photon microscopy may push the capability of imaging techniques further, allowing imaging at even greater depths and long-(red)-excitation of UV fluorochromes. The luminescent calcium indicator, aequorin, has been much more extensively used in plants than the transgenic fluorescent calcium indicators, and photon-counting imaging systems can now record calcium transients at video-rate in intact plants. Digital imaging, whether camera, confocal or multi-photon, provides quantitative data, but correct interpretation requires rigorous analysis of noisy and partially correlated imaging data. Statistical analysis by Bayesian inference may well become the most appropriate technique to handle such images, but has only recently been applied to ratio imaging in plants. In addition to generating images, light can also be used to manipulate intracellular events using photolysis of caged probes. The control of the location, timing and amplitude of the release allows exquisitely subtle manipulation of signalling networks. More aggressive pulses of

UV laser-light provide an equally powerful tool either to ablate whole cells completely for developmental studies or to punch-out tiny holes in the cell wall to give access to the plasma membrane for patch-clamping and electrophysiological investigation of cells *in situ*.

Key words: Green fluorescent protein, confocal microscopy, aequorin, digital imaging, light.

Introduction

Fluorescent and luminescent techniques have dramatically increased our ability to visualize, quantify and manipulate dynamic processes within living tissues and they have already provided fascinating insights into the complex spatial and temporal regulation of plant signalling systems. The types of questions that can be tackled with these approaches are increasing exponentially, most notably with the fusion of sophisticated imaging technology and molecular biology techniques. Most refreshingly, the marriage of these two disciplines may solve so many of the difficulties that previously restricted physiological imaging to a few biological systems by a few dedicated groups world-wide. The following report covers papers presented at a technical workshop on imaging techniques in plant transport organized as part of the 11th international Workshop on Plant Membrane Transport. The theme of this workshop was deliberately forward-looking to explore the potential of some of the new approaches that are rapidly gaining momentum. The areas selected for discussion reflect both recent improvements in the technology to make *in vivo* and *in planta* measurements and advances in tools to study membrane compartments and physiology. Originally the workshop was intended for about 30–50 people, but on the day the audience swelled to over 250, reflecting the growing importance and

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enormous level of interest in these topics. The numbers may have constrained the discussion to some extent but did not detract from the excellent quality of the presentations.

The GFP revolution

One of the major forces driving current progress is the availability of green fluorescent protein (GFP) from the jellyfish *Aequoria victoria*. GFP auto-catalytically forms an endogenous fluorophore by cyclization of a tripeptide sequence within the protein. No other enzymes or co-factors are required, apart from molecular oxygen. This means that expression of the GFP sequence in virtually any aerobic organism should give fluorescent protein. Unfortunately, wild-type GFP from *Aequoria* does not work in plants as there is a cryptic intron site in the sequence. Jim Haseloff (LMB, Cambridge) has engineered the wild-type sequence to remove the cryptic intron site (Haseloff *et al.*, 1997), and introduced other modifications to improve the temperature stability of the protein and alter its spectral properties (Haseloff, 1999).

Wild-type GFP emits green (509 nm) light when excited with UV (395 nm) or, to a lesser extent, blue (475 nm) light, however, the sequence has been tweaked to give different spectral variants of GFP with emission wavelengths ranging from blue to yellow. Moreover, standard molecular biology techniques can easily add targeting sequences to light up specific organelles, link GFP to other proteins to follow their dynamics *in vivo* or even generate GFP-based fluorescent indicators for other molecules. It was thought appropriate to focus a substantial proportion of the workshop on GFP-based techniques, particularly as many of the applications of conventional chemical probes have been reviewed recently (Gilroy, 1997; Hepler and Gunning, 1997; Fricker *et al.*, 1998). Despite this focus on GFP, it is worth noting that several other fluorescent protein systems have been developed recently. The phytofluors are based on reconstitution of transgenic plant phytochrome apoproteins and phycoerythrobilin (Murphy and Lagerias, 1997) and give much longer emission wavelengths than GFP. Recombinant proteins can also be made fluorescent if a short cysteine-rich alpha helix is added that can complex with a small membrane-permeant ligand (4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein) (Griffin *et al.*, 1998). This is non-fluorescent until it binds with high affinity and specificity to the tetracysteine domain to give an adduct (termed FLASH-EDT₂ for fluorescein arsenical helix binder, bis EDT adduct). This approach offers the potential advantage over GFP that the additional tag is much smaller.

Applications of GFP as a cell marker in plants

Haseloff described progress in the use of GFP as a tool to analyse cell-cell interactions in the *Arabidopsis* root

meristem. In this system, GFP is used as a live-cell lineage marker to understand how the sequence of cell divisions unfolds during development and how cell fate may be modified if local interactions between the cells are altered. To obtain cell-specific expression, Haseloff set up a promoter-trap system with a bi-functional construct containing the *mGFP5* gene, coding for ER-targeted mGFP, in front of a GAL4-responsive promoter, and a modified yeast *GAL4* sequence coding for the GAL4 transcription factor itself, with a minimal promoter. The sequence of the modified GAL4 transcription factor (*GAL4-VP16*) has altered codon usage to ensure efficient expression in *Arabidopsis*. Haseloff has generated a series of transgenic lines using *Agrobacterium*-mediated transformation with this construct and looked for localized expression of GFP in particular cell or tissue types (Fig. 1). This should only occur when the construct lands next to an enhancer capable of driving *GAL4-VP16* gene expression. The GAL4 in turn drives *mGFP5* gene expression thus marking cells where the promoter is active. Targeting to the ER appears to allow much higher expression levels than cytoplasmic GFP (Haseloff *et al.*, 1997; Haseloff, 1999). A substantial number of enhancer-trap lines are documented on the Haseloff website and many are available from the *Arabidopsis* Stock Centre. The bi-functional construct also means that any other transgene can be activated in the marked cell line if it is also equipped with a GAL4 responsive promoter. As an example, Haseloff showed results from crosses between a line where the outermost lateral root cap cells were marked with the promoterless *GAL4-VP16-mGFP5* construct and a line expressing the diphtheria toxin A chain, also on a GAL4-responsive promoter. Cells in the lateral root cap

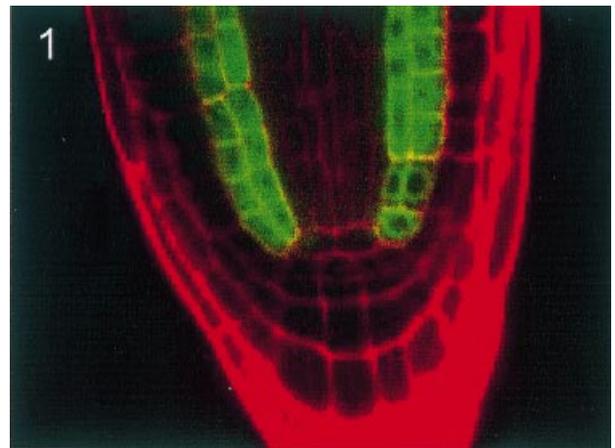


Fig. 1. GFP can be used as a live-cell lineage marker. Single confocal optical section with excitation at 488 nm of an *Arabidopsis* root tip from an enhancer-trap line (J0571) with restricted expression of mGFP5ER fluorescence (green) to the cortical and endodermal cell files. The architecture of the root is simultaneously imaged after staining with propidium iodide (red). More details of the techniques used and a catalogue of other enhancer trap lines are available from the *Arabidopsis* stock centre (from J Haseloff with permission).

expressing the diphtheria A toxin were selectively ablated in accordance with expectations. Interestingly, the underlying cells bulge outwards in the absence of the normal local interactions, somewhat akin to development of a root hair, possibly indicating that perturbation of local cell–cell signalling systems can influence the programme of cell differentiation.

The most convenient and efficient means to visualize GFP fluorescence at the moment is confocal optical sectioning and 3-D reconstruction. Confocal imaging removes the out-of-focus blur from GFP fluorescence that is not in the focal plane and provides high-contrast images even deep within growing roots. Using ER-targeted GFP, Haseloff has also been able to follow physiological processes, such as cell division and cytoplasmic streaming, in intact roots over a considerable period before photobleaching reduces the signal strength and the images become very noisy (these videos are also available on the Haseloff website (<http://brindabella.mrc-lmb.cam.ac.uk>). To add another layer of sophistication, it is possible to follow two separate processes with different spectral variants of GFP and image them simultaneously in the same cell. Haseloff demonstrated the principle using the ER-targeted GFP, as a morphological marker, and a fusion of the KNAT3 homeodomain protein (Serikawa *et al.*, 1997) to modified yellow fluorescent protein (mYFP) (also with altered codon usage and enhanced folding mutations). Transfer of the KNAT3-YFP from the cytoplasm to the nucleus could be visualized during elongation and maturation of the epidermal cells, and may reflect a developmental-stage dependent activation of the homeodomain protein.

To characterize the cellular origins of the GFP-expression patterns requires a method to map the root architecture. Conveniently, propidium iodide (PI) stains the cell walls in both live and fixed material and can be excited, although weakly, with the 488 nm line of the same argon-ion laser used for confocal imaging of GFP. PI also acts as a marker for cell viability as it stains the nucleus of any cell with a damaged membrane.

Even confocal imaging in intact tissue has problems due to the optical interactions between the specimen and the illumination which distort the focus of the imaging probe. Typically, this results in a marked attenuation of the fluorescent signal with depth into the tissues (White *et al.*, 1996). This problem is reduced in fixed and cleared material as the refractive index differences throughout the specimen are reduced, leading to less distortion of the confocal probe. Dissected embryos, seedling roots or shoot apices are all amenable to fixation, clearing and labelling with PI as a pseudo-Schiff's reagent. The architecture can be readily visualized from a complete high-resolution 3-D image of the root. Interactive visualization of this volume of data (10–100 Mbytes per root) from any angle benefits from the high powered image pro-

cessing systems that are becoming more readily available. This approach is significantly faster and simpler than conventional sectioning as the fluorescent labelling already segments the architecture of the root; images are perfectly aligned with no distortion and, as the image is already digitized, it can be readily re-sliced from any angle or viewed as a projection.

Whilst marked cell lines are a powerful tool for developmental studies, they also have other uses, most notably as an aid to identification of specific cell-types after making protoplasts in preparation for patch-clamping. Several groups reported results using this approach in posters at the meeting. Thus, Ed Kiegle *et al.* (Cambridge) used one of the Haseloff mGFP5-marked endodermal lines to compare calcium currents between endodermal and non-endodermal cells. Frans Maathuis *et al.* (York), Malcolm Bennett (Warwick) and Phil White (HRI Wellsbourne) have generated an independent series of promoter-trapped GFP lines in *Arabidopsis* and observed that inward rectifying K⁺ channels were recorded at much higher frequencies from stelar protoplasts compared to epidermal or cortical protoplasts.

Visualization of dynamics in endomembrane morphology

Targeting GFP to the ER not only provides a convenient way to achieve high expression levels of GFP whilst minimizing cytotoxicity, but also provides an opportunity to follow the dynamics of this membrane compartment *in vivo*. Chris Hawes (Oxford Brookes) and co-workers from SCRI, Dundee have used wild-type GFP and exploited the speed of the potato virus-X transformation system to obtain high levels of expression in the endoplasmic reticulum from the viral coat promoter in tobacco leaves (Baulcombe *et al.*, 1995; Oparka *et al.*, 1995; Boevink *et al.*, 1996, 1998). By modifying the targeting and retention sequences added to the GFP, they have begun to dissect the endomembrane trafficking pathways in live cells using confocal imaging. An ER signal peptide and a KDEL sequence are sufficient to obtain ER localization of the GFP (Boevink *et al.*, 1996, 1998). In epidermal cells, the GFP-tagged ER is a motile tubular network, interspersed with lamellae of cisternal ER. Dynamic behaviour of individual tubules can be visualized as they rapidly extend, shrink or fuse with each other. Interestingly, both Haseloff and Hawes have observed GFP signals apparently from rapidly moving organelles similar in size to pro-plastids in *Arabidopsis* root tissues. It has recently been suggested that this actually represents labelling of a specialized type of dilated ER compartment in Brassicaceae rather than mis-targeting of the chimaeric proteins or ER-pro-plastid connections (Gunning, 1998).

Labelling the Golgi *in vivo* is more problematic due to the paucity of well-characterized Golgi markers in plants

that might suggest conserved targeting and retention sequences. Hawes reported on two approaches that have proved successful. Expression of GFP coupled either to 52 amino acids from the N-terminus single-pass transmembrane domain (tmd) of a rat Golgi 2,6-sialyl transferase (STtmd) or the *Arabidopsis* homologue of the yeast ERD2 HDEL receptor, leads to a predominantly punctate distribution of fluorescence superimposed onto a much weaker ER pattern (Fig. 2; Boevink *et al.*, 1998). At high zoom, the punctate structures appeared horse-shoe-shaped and around 1 μm in size. This information alone is not sufficient to identify them as Golgi stacks, however, a Golgi-localization was confirmed by cryo-EM following immuno-gold labelling of the GFP (Boevink *et al.*, 1998).

At the moment Hawes *et al.* do not have the ER and Golgi markers tagged with different spectral variants of GFP to facilitate live double-labelling studies. As an alternative approach, however, both compartments can be visualized during recovery from Brefeldin A treatment. Brefeldin A is a toxin which blocks the anterograde transport pathway to the Golgi, eventually leading to disappearance of the Golgi stack (and the punctate GFP fluorescence) as all the membrane is recycled back to the ER via the retrograde pathway. Hawes observed that during washout of the Brefeldin, the Golgi reformed, however, there was a window when the GFP was present in both the ER and Golgi allowing both compartments to be visualized simultaneously.

Given the ability to visualize both the ER and Golgi in living plant tissues for the first time, the key question is do they behave as predicted from static EM images and the models inherited from the yeast or mammalian

systems? Rather excitingly, even at this early stage, the images have led to a new model for ER to Golgi trafficking in plants. Under these, albeit slightly unusual, conditions, the Golgi units track rapidly over the ER network. Actin is critical to maintain motility of the Golgi, as movement was completely halted in the presence of cytochalasin, when the Golgi remained tethered to the cisternal ER. Interestingly, the ER network *per se* was not disrupted by cytochalasin in the same time-frame and does not appear to require an intact actin network to maintain its organization, at least in the short term. The GFP fluorescence and actin labelled with rhodamine-phalloidin were almost completely superimposed in lightly fixed material (Boevink *et al.*, 1998), suggesting that the ER sits on actin (but is stable in its absence), whilst the Golgi move along the actin, actively hunting vesicles budding from the ER network.

Imaging tools to follow the endocytic pathway

Techniques are also being developed to track dynamics of other endomembrane compartments, most notably, the various endocytic vesicle pathways from the plasma membrane. Colin Brownlee (MBA, Plymouth) provided an overview of chemical dyes, transgenic probes and electrophysiological techniques that have all been brought to bear on measurement of endocytosis in different systems (Battey *et al.*, 1996), including the recent work from the Rothman laboratory using a pH-sensitive derivative of GFP. Thus, Miesenböck *et al.* (1998) have been able to modify key amino acids adjacent to the cyclized peptide at the heart of GFP to generate two types of transgenic pH reporter, termed pHluorins. The first derivative is a dual excitation ratiometric probe with excitation peaks at 395 nm and 475 nm. The second derivative (ecliptic pHluorin) behaves essentially as a single wavelength pH probe with a quench in the 475 nm excitation peak with decreasing pH. A range of substitutions in GFP provide pH-indicators with differing pK_a values ranging from 4.8 to 7.1 (Kneen *et al.*, 1998; Llopis *et al.*, 1998).

The pHluorins and pH-sensitive GFPs can be targeted to components such as the cell surface, endosomes or the *trans* Golgi network. When linked to a vesicle membrane protein, these probes can be used to probe exocytic and endocytic events by monitoring the pH-changes in vesicles as they progress through the secretory and endosomal pathways and release or recover material from the plasma membrane. Unfortunately, the pHluorins were constructed from the wild-type GFP sequence with the cryptic intron site and so they cannot be used directly in plants. A key development would be the synthesis of plant-compatible versions of the pHluorins.

Unlike internal membranes, the plasma membrane is directly accessible to the external medium, thus it is possible to insert membrane probes directly into the

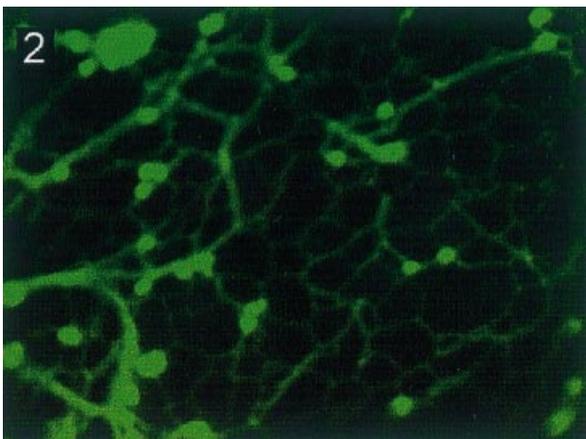


Fig. 2. Targeting GFP to organelles allows visualization of endomembrane dynamics. Viral-mediated expression of GFP tagged to the *Arabidopsis* homologue of the yeast HDEL receptor (ERD2) in leaf epidermal cells of *Nicotiana clelandii* (Boevink *et al.*, 1998). Small fluorescent Golgi stacks are visible as bright punctate structures associated with the cortical ER network. These Golgi stacks are motile and traffic on an actin network associated with the cortical ER. (CR Hawes, S Santa Cruz, K Oparka and P Boevink, unpublished data.)

plasma membrane. The cationic styryl FM1–43 and FM4–64 dyes are essentially non-fluorescent in the external medium, but become brightly fluorescent when incorporated into the external leaflet of the plasma membrane (Betz *et al.*, 1992). The dyes do not spontaneously re-orient in the membrane so that dye appearing internally should represent internalization of the plasma membrane and provides an estimate of the membrane flux into the cell and the compartments that trafficking is directed (Betz *et al.*, 1992; Murthy and Stevens, 1998).

Brownlee demonstrated that, during osmotic excursions in *Fucus*, recycling of membrane can be followed from internalization of FM1–43. During normal growth little FM1–43 enters the cell, however, following osmotic perturbation to 50% normal sea water, the cell transiently expands and then exhibits volume recovery. During this excursion, the level of FM1–43 fluorescence increases as more plasma membrane is exposed and then the fluorescence becomes internalized during volume recovery.

In secretory systems such as the hypersecretory slime cells from the maize root cap, rapid vesicle cycling would be predicted to occur under normal conditions without imposed osmotic perturbation. In protoplasts from these cells, measurements of membrane capacitance and FM1–43 fluorescence internalization are possible. The internalization process is sensitive to Brefeldin A, implying that a functional Golgi is required for membrane recycling to take place.

Other approaches to measure endocytosis are based on the electrical effects of changing membrane surface area inferred from capacitance measurements and can be used to describe the underlying behaviour of the vesicle populations (Battey *et al.*, 1996). Thus, Jens-Uwe Sutter and Gerhard Thiel (Göttingen) were able to model calcium-enhanced exocytosis in protoplasts from maize coleoptiles using two pools of vesicles. One pool is adjacent to the plasma membrane and releases its contents in a Ca^{2+} -dependent step. This pool is slowly replenished from a second pool via the secretory pathway. The inserted membrane is subsequently recycled by endocytosis which shows no Ca^{2+} -dependence.

Ulrike Homann (Göttingen) used FM1–43 in conjunction with patch-clamp measurements of membrane capacitance (Smith and Betz, 1996) to follow osmotically induced changes in plasma membrane area of guard cell protoplasts. Hypo-osmotic swelling resulted in an increase in capacitance and an increase in fluorescence as more dye was incorporated into the membrane. Shrinkage reduced the membrane capacitance, but the total fluorescence did not alter in the photometry measurements which do not have the spatial resolution to separate the fluorescent signal from the internalized fluorescent membrane.

In *Neurospora*, Sabine Fischer *et al.* (Edinburgh) found that FM4–64 labels the plasma membrane immediately

and rapidly appears in the Spitzenkörper, followed by small organelles, possibly early endosomes, after a few minutes. Label did not reach the vacuole until about 20 min. The labelling kinetics would be consistent with a model where the Spitzenkörper forms part of a local cycling system distributing and recovering material from the rapidly growing tip region, rather than a massive vesicle flux throughout the entire exo- and endo-cytic pathway.

Transgenic fluorescent calcium indicators

Although it is likely that pHluorins will soon be available for expression in plants, it is not clear the extent that the basic GFP sequence can be engineered directly to produce probes for other ions or molecules of interest, such as calcium. An alternative modular strategy is to couple the GFP as a fluorescent tag to a second protein or protein domain that confers specificity. Ideally, binding of the ligand to the second domain affects the GFP fluorescence in some way to allow discrimination of free and bound forms. Roger Tsien's group has pioneered this approach using calcium-dependent changes in the level of fluorescence resonance energy transfer (FRET) between spectral derivatives of GFP (Miyawaki *et al.*, 1997). The 'cameleon' probes are chimeric proteins comprising two different colour variants of GFP, separated by calmodulin (CaM) and M13, a CaM binding domain from smooth muscle myosin light chain kinase. The distance between the donor and acceptor GFP variants is governed by the calcium-dependent conformation of the CaM/M13 linker peptide between them. At high $[\text{Ca}^{2+}]$, CaM binds Ca^{2+} and associates with the M13 peptide to bring the two GFP variants sufficiently close to promote fluorescent resonance energy transfer (FRET) between them and a shift in the emission wavelength to the characteristic longer wavelength of the acceptor fluorophore. At low calcium, the interaction between Ca^{2+} and CaM, and hence CaM and M13, is reduced. As the fluorophores are no longer sufficiently close for FRET, the emission peak shifts towards the donor emission.

The original derivative of GFP required conversion of a cryptic intron site for correct expression in plant cells. Conveniently, the codon usage of the cameleon probes has already been altered for expression in mammalian cells (Miyawaki *et al.*, 1997) and this has also removed the cryptic intron site. Andrew Parsons (Oxford) demonstrated that the mammalian constructs are correctly transcribed, translated and targeted in transient expression in *Arabidopsis* mesophyll protoplasts (Fig. 3). Fluorescence from Yellow cameleon-2 is present in the cytoplasm and has emissions characteristic of the cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) wavelengths. This cameleon can be excited using a 442 nm laser on a confocal system to permit dual-emission ratio measure-

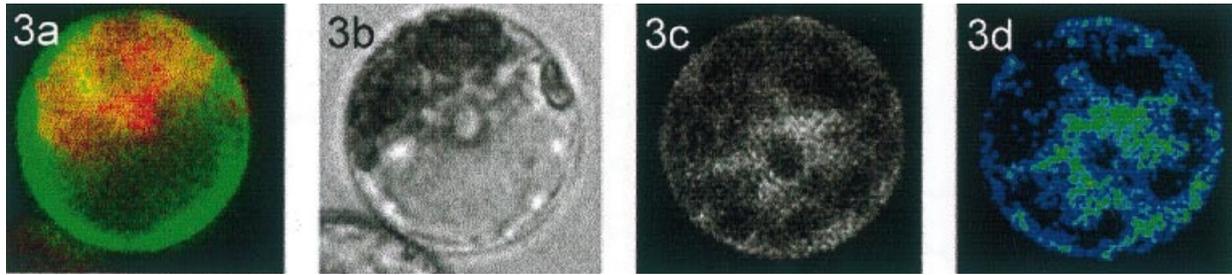


Fig. 3. Transgenic fluorescent calcium indicators (cameleons) can be expressed in plant tissues. Transient expression of yellow cameleon 2 (Miyawaki *et al.*, 1997) in the cytoplasm of an *Arabidopsis* mesophyll cell protoplast. Yellow-green cameleon fluorescence is present in the cytoplasm (a) and excluded from the chloroplasts (red fluorescence) and vacuole (b). In a single confocal optical section, fluorescence is also excluded from the nucleus (c) with excitation at 442 nm using a He-Cd laser attached to a modified BioRad MRC600 CLSM. The two emission peaks from the calcium-bound and calcium-free forms can be ratioed after background subtraction to give an estimate of cytoplasmic calcium concentration (d). (AL Parsons, MD Fricker and MR Knight, unpublished data.)

ments of cytoplasmic calcium levels. The ER-targeted Yellow cameleon-3 and Yellow cameleon-4 are correctly localized to the ER, and contain modified calmodulin sequences that lower the calcium affinity of the calmodulin. In principle, cameleon probes can be targeted to any compartment of interest in any cell type of interest using standard molecular biology techniques. This contrasts starkly with the enormous difficulties that have plagued the use of chemical calcium indicators in plant systems, where unwanted compartmentalization may completely obviate the experiment or contaminate cytoplasmic measurements. In addition, the Ca^{2+} -sensitivity of the cameleon can be altered by modifying the specificity of Ca^{2+} -binding in the EF-hands of CaM to give an appropriate K_d for the compartment under investigation. One drawback of the cameleons is the very limited ratio range that is present with the CFP/YFP couple compared to conventional ratio probes. Thus the critical next step will be to determine the overall sensitivity of calcium measurements using these probes in intact tissues.

Multi-photon microscopy—measuring fluorescence deep within living tissues

Whilst it may be possible to express fluorescent probes using transgenic techniques in any cell type, it is also necessary to excite the probe and measure the fluorescent signal under physiological conditions. Until recently, many studies on signal transduction were limited to single cells or epidermal tissues which allowed ready access both to load the dye and to image the fluorescence signals. The benefits of confocal optical sectioning in removal of out-of-focus blur have already been alluded to for visualization of GFP signals, however, even confocal imaging does not perform well more than about two cell layers into an intact living specimen.

Nick White (Oxford) introduced a new technique, termed multi-photon microscopy, which also achieves blur-removal and optical sectioning, and holds enormous promise for physiological measurements from intact tis-

ues (Xu *et al.*, 1996). In conventional fluorescence microscopy, fluorescence is unavoidably excited from an extended volume above and below the focal plane leading to out-of-focus blur. As the specimen gets thicker, the out-of-focus blur significantly degrades the contrast and distorts any attempts at quantitative measurements. In a confocal microscope, the excitation light is focused to a discrete point in the specimen. However, fluorescence is still excited in the cones of illumination above and below the focus. This out-of-focus fluorescence is prevented from contributing to the final image by a physical barrier in an imaging plane before the detector. In-focus fluorescence from the focal point passes through a small aperture (pinhole) in the image plane. Thus the illumination and detection points are co-aligned or 'confocal'. In multi-photon microscopy the illumination is approximately twice the wavelength required to excite the fluorophore with a single photon. However, the system is designed to ensure that two long wavelength photons arrive within fractions of a femto second to provide an equivalent excitation energy of a single UV or blue photon. Getting a sufficient flux of photons requires a pulsed laser system, where all the energy is squeezed into a femto second pulse. To keep the average power that the specimen receives down, the pulse is repeated at 80 MHz, i.e. with a gap about 100 times longer than the pulse. The laser power is adjusted so that the flux is only sufficient for the 2-photon excitation process at the point of focus. Thus in multi-photon microscopy no out-of-focus fluorescence is ever generated, reducing blur and photobleaching. In addition, much more emitted light from the focus point can be collected, as there is no requirement for a pinhole in the emission path, substantially increasing the detection efficiency.

The size of the volume sampled in multi-photon or confocal microscopy is around $0.3 \times 0.3 \times 1.0 \mu\text{m}$, in x , y and z , respectively, using a high NA (1.2) water-immersion lens under ideal conditions. However, in a real biological specimen, the imaging performance is degraded by all the refractive and scattering components present in

the sample. As the extent of these interactions decreases with increasing wavelength, the multi-photon system should also suffer less degradation when imaging deep into tissues, whilst being able to excite UV probes (Fig. 4).

Thus, it is anticipated that multi-photon microscopes will be able to image deeper into intact tissues, produce less specimen damage through the use of longer wavelengths and extend the range of probes that can be visualized conveniently to include UV-probes. Multi-photon imaging works well with GFP (Kohler *et al.*, 1997) and these two technologies will undoubtedly become a powerful combination.

Although it is very early days, it is also apparent that probes with excitation peaks in the far UV can be excited by infrared light in a three-photon process (Xu *et al.*, 1996; Lakowicz *et al.*, 1997). The two- or three-photon excitation spectra of common dyes do not necessarily match simple predictions from doubling or tripling of the single photon excitation spectrum. In most cases, the peaks are slightly blue shifted and may be substantially broader than the single photon spectra (Xu *et al.*, 1996). This has the advantage that several probes can be readily excited simultaneously with a two-photon system. More recently, probes specifically designed for two-photon imaging have been made (Cheng *et al.*, 1998) and highly efficient caging molecules (see below) with a large two-photon cross-section have been introduced (Adams *et al.*, 1997). Currently, the major disadvantage of the multi-photon systems is the cost of the laser at around £140 000.

Statistical analysis of fluorescence ratio images

Development of new probes and imaging technologies should increase the range of biological questions that can

be tackled using an imaging approach and most attention is directed to the hardware used to collect the images and the controls required to validate the methods. One area that is often neglected or handled inappropriately is application of rigorous analysis techniques to the data itself. As with any measurement, reliable imaging data should have estimates of the accuracy and precision for the level of spatial resolution claimed.

Richard Parton (York) described some of the common errors prevalent in the literature when analysing ratio images and presented an alternative approach based on the Bayesian theory of inference (Parton *et al.*, 1997). As an example, two images are normally collected for a ratio probe and the ratio image calculated pixel-by-pixel after background subtraction. The accuracy of the data depends to a large extent on the validity of the calibration curve and the estimates of error presented. Even if the sample is a uniform calibration solution, the individual pixel values still have a distribution arising from noise at both wavelengths sampled. The level of noise in most physiological images means that some form of spatial averaging is used over a number of pixels in a region of interest (ROI) to reduce the noise. This averaging determines the actual spatial resolution of the measurements, rather than the theoretical predictions of the microscope resolution.

The mean ratio value of the ROI could be calculated by either dividing the two means from each wavelength image or by averaging all the ratio values from the ratio image. The two results are not the same, however (Parton *et al.*, 1997; Fricker *et al.*, 1997, 1998). The histogram of ratio values, even from two normally distributed fluorescence images, is skewed to the right and will shift the mean derived from the ratio image accordingly. The

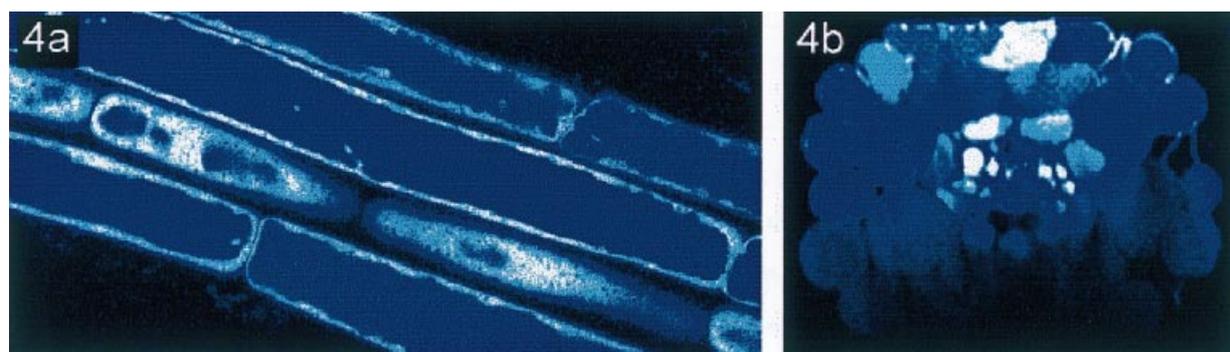


Fig. 4. Two-photon scanning optical microscopy can be used to image UV-excitable probes deep within intact tissues. Two-photon imaging of cytoplasmic glutathione (GSH) as its bimane conjugate in intact roots of *Arabidopsis thaliana*. Monochlorobimane (MCB) in its free form is non-fluorescent and cell-permeable. After reaction with glutathione it becomes cell-impermeable and fluorescent with an excitation maximum at 395 nm for single photon excitation. The fluorescent conjugate can be imaged at the cellular level deep within the intact root by two-photon microscopy with excitation light of 770 nm. Immediately after immersion of the root in 100 μ M MCB fluorescence can be observed in the cytoplasm (a) in a single (x,y) optical section. Subsequently, the conjugate gets actively transported into the vacuole (some cells in b). A vertical (x,z) optical section through the intact root provides information about cellular heterogeneity in GSH levels in all different cell-types (b). Even with two-photon imaging significant attenuation is apparent with increased depth into the tissue, however, the useful penetration depth is about 50–100% more than with single photon excitation at 442 nm. (AJ Meyer, NS White and MD Fricker, unpublished data. The multi-photon system is part of the BioRad Biological Microscopy Unit, Plant Sciences, Oxford.)

magnitude of the skew depends on the spread in the original fluorescence intensity images, including the noise and variation in dye levels. The relative spread will increase as the signal decreases, due to photobleaching, for example, or in regions of low dye loading. Thus any experiment where mean ratio values are derived from the ratio images will have systematic errors that will vary in space with dye concentration, and time if the dyes bleach or redistribute (Fricker *et al.*, 1997).

The difficulties in dealing with ratio values in conventional statistics is well known and falls under the general heading of the Fieller–Creasy problem (Kappenman *et al.*, 1970). An alternative approach to analysing ratioable probes is to use statistical analysis by Bayesian inference (Kappenman *et al.*, 1970; O'Hagan, 1994). The Bayesian approach allows probability statements to be made about unknown population parameters *a posteriori* and can accommodate both the noise distributions in the two images and a degree of correlation between pixel values. The Bayesian confidence intervals give the *a posteriori* probability that the ratio value will be within the limits given with uniform prior assumptions, and provide a measure of the precision of the measurements. The Bayesian approach is widely used elsewhere and it could be argued that it should become the *de facto* standard for live cell imaging as well. Parton and colleagues in Edinburgh have developed an appropriate application tailored to ratio imaging (Parton *et al.*, 1997) in collaboration with statisticians in the University of Edinburgh. Unfortunately, this package is not readily available as yet and interested parties should contact Richard Parton (rmp5@york.ac.uk) or Nick Read (nread@srv0.bio.ed.ac.uk) for further information.

Imaging transgenic luminescent calcium indicators

Long before chemical calcium probes or the GFP-basedameleon probes appeared, *Aequoria victoria* had generously donated its own luminescent calcium indicator to the research community. There has been a steady flow of papers using plants expressing transgenic aequorin since the first recombinant plants were made by Marc Knight and co-workers in Edinburgh (Knight *et al.*, 1991). Although Knight *et al.* demonstrated the possibility of imaging transgenic aequorin in plants in 1993, it is only recently that this has become a practical proposition with the development of appropriate camera-imaging systems.

Apoaequorin is a single polypeptide chain of approximately 22 kDa that combines with a low molecular weight luminophore called coelenterazine to form functional aequorin in the presence of oxygen. The aequorin molecule is similar in structure to the calcium-binding protein calmodulin and possesses three calcium-binding EF hand domains (analogous to the four EF hands of calmodulin)

and a binding site for coelenterazine and oxygen. When calcium is bound, the coelenterazine is oxidized to coelenteramide and the protein undergoes a conformational change accompanied by the release of carbon dioxide and emission of blue (462 nm) light. Aequorin is highly selective for Ca^{2+} and can potentially detect free calcium levels of up to 100 μM although, in practice, most measurements are made in the range of 10 nM–10 μM .

Aequorin has several potential advantages over fluorescent dyes as an indicator for calcium. Luminescence measurements usually have an intrinsically high signal to background ratio as there is relatively little endogenous luminescence under optimal conditions. As a natural protein, aequorin is expected to be non-toxic and remain in the cytoplasm unless specifically targeted elsewhere. Light emission is unaffected by pH values greater than pH 7. Photodamage associated with excitation illumination for fluorescence is also avoided. All cells produce their own Ca^{2+} -indicator and thus calcium changes can be measured in whole intact plants. Targeted forms of apoaequorin have been engineered to express the protein in the chloroplast (Johnson *et al.*, 1995) (pMAQ6, available from Molecular Probes), nucleus, tonoplast (Knight *et al.*, 1996), mitochondria (MR Knight and C Moore, unpublished data), and the apoplast (C Plieth and MR Knight, unpublished data). Despite the success of these first generation constructs, one of the limitations in their development was a need for detailed biochemical fractionation studies to confirm their subcellular location. Knight *et al.* have exploited the benefits of both the aequorin and GFP systems in a single promoter-less fusion protein. Promoter-trapping in an analogous manner to that described for enhancer-trapping by Haseloff should generate cell-specific expression and subcellular location of aequorin that can be readily visualized using the GFP fluorescence. In addition, the GFP-aequorin construct should facilitate analysis of targeted constructs in morphological and subcellular fractionation studies.

By defining the cell-specific expression and subcellular location using molecular biology techniques, the requirement for high-resolution imaging to define the various compartments is relaxed considerably. The level of light emission from aequorin is very low compared to fluorescent signals and demands a very low-light level or photon-counting camera system to image it successfully. Knight reported results from a Photech system that can capture luminescence image information at up to video rate from intact seedlings (Fig. 5). In this system, each photon that collides with the detector face-plate has the potential to initiate a cascade of secondary electrons in the intensifier stage. The resulting cloud of electrons is spread out over a reasonable area on the detector chip, however, the associated electronics calculate the centre of the area and only record the pixel coordinates for the

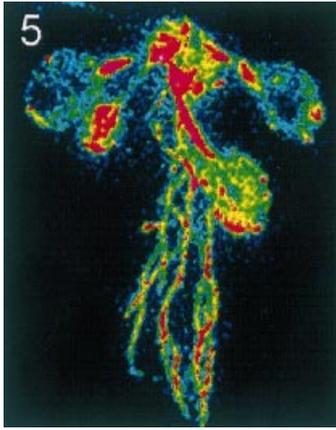


Fig. 5. Calcium dynamics in intact plants expressing aequorin can be imaged with a photon-counting camera. This figure is a pseudocolour representation of the luminescence emitted by the leaves and roots of a 3-week-old aequorin-expressing *Arabidopsis* plant in response to cooling. High levels of cytosolic Ca^{2+} occur in the areas shown as red and yellow, lower levels in those shown as blue. (H Knight and MR Knight, unpublished data.)

centre of each event detected. As only the coordinate list is retained, the massive overhead associated with saving images at video-rate is avoided. This speed and the ability to play back the sequence with varying levels of time-averaging has proved invaluable to image a range of different responses including rapid calcium-transients, differential threshold responses and root–shoot communication. It also means that no *a priori* knowledge of the nature of the kinetics of the response is required at the start of an experiment.

A highly sensitive imaging detector also facilitates screening of mutants laid out as arrays on a grid. Knight reported on the use of the camera to detect mutants with modified calcium signalling pathways. Arrays of 6×6 mutagenized seedlings were subjected to a uniform touch stimulus and those with increased or decreased luminescence selected. So far five mutants have been identified from 2000 seedlings screened, three show an increased Ca^{2+} in response to touch and two have a decreased Ca^{2+} response.

The current technical difficulties with the aequorin approach lie in the cost of the coelenterazine cofactor when planning large-scale experiments and the difficulties in calibration when imaging, particularly for targeted aequorin in different compartments. The calibration curve used has, so far, only been validated with cytosolic aequorin. With appropriately targeted aequorin, measurements from specific cell types is possible, however, it is unlikely that the level of light emission will allow detailed subcellular measurements within a realistic time-frame for most cell types. In one case, however, calcium gradients and dynamics have been visualized in single growing pollen tubes (Messerli and Robinson, 1997) and some

cytoplasmic structure has been resolved in guard cells expressing aequorin (Trewavas and Malhó, 1998).

Manipulation of intracellular events using caged probes

In parallel with the development of probes to visualize subcellular events in living tissues, optical techniques have been developed to manipulate signalling systems with the same degree of temporal and spatial resolution. Caged compounds are molecules whose biological activities have been chemically masked by a photolytic ‘caging’ group. Illuminating the caged compound with UV light or with two-photon excitation (Adams *et al.*, 1997) causes the cage to dissociate and the biologically active molecule to be released. Simon Gilroy (Penn State) described how, by controlling the timing, intensity and region of illumination, the dynamics of caged probe release can be tightly regulated and used artificially to mimic the spatial and temporal dynamics of different components in a signal transduction network (Gilroy, 1996, 1997).

The list of compounds that have been caged is extensive, including ions, ionophores, enzymes, proteins, drugs, and hormones. Several commercial distributors provide a wide range of these compounds. Many of the more ‘exotic’ caged compounds (such as caged plant hormones, see Ward and Beale, 1995; Allan *et al.*, 1994) are not commercially available, but can probably be obtained from the laboratory that synthesized them.

Loading may be difficult as many probes require microinjection, however, as the compound is not activated until the pulse of UV light, the cells can be given plenty of time to recover and the effects of the stimulus can be separated from the effects of microinjection.

UV irradiation and photolysis by-products produced during the photoactivation of the caging group can be highly damaging to the cells and may also have biological effects. Thus extensive controls for the viability of cells used in caged probe experiments are needed to demonstrate that the UV pulse or the compound alone do not affect the response. The development of a caged probe for Ca^{2+} , azid-1, that can be efficiently photolysed using two-photon imaging (Adams *et al.*, 1997) offers great potential not only to reduce the potentially photodamaging effects of UV-irradiation, but also to confine the release to a tightly-controlled and spatially localized volume in three dimensions.

Perhaps the two most significant aspects of caged probe experiments are to develop appropriate measures of cellular activities to determine the effect of the regulator released upon caged probe photoactivation and to mimic the normal pattern of signalling dynamics. Being able to regulate the timing, intensity and localization of illumination allows control of the amplitude and spatial dynamics of the artificial signal to match the endogenous changes

and trigger true cellular regulatory events. When the caged compound is affecting a detectable cellular parameter, e.g. changes in Ca^{2+} levels caused by the caged Ca^{2+} -ionophore, the change can be visualized using an appropriate fluorescent indicator to show how well the caged probe is working (Fig. 6). When the released compound is undetectable, e.g. ABA released from caged-ABA (Allan *et al.*, 1994), a useful approach to visualizing the extent of photoactivation is to co-load the cell with caged-fluorescein. Caged fluorescein is non-fluorescent until it is uncaged. Thus, the production of fluorescein in the sample is an indication of how efficiently caged compounds are being photoactivated (Gilroy, 1996, 1997).

Laser-assisted patch-clamping

In uncaging systems, the energy of the light source is used to break relatively unstable bonds in the caging group. Many biological macromolecules absorb UV light and these bonds can also be broken with a sufficiently high laser intensity. A focused high intensity 337 nm UV laser has sufficient power to ablate portions of the cell wall (Berger *et al.*, 1994; Bouget *et al.*, 1998) or even whole cells (Van Den Berg *et al.*, 1995; Blancaflor *et al.*, 1998). This has led to the development of laser-assisted patch-clamping as an alternative to enzymatic production of protoplasts for both plants (De Boer *et al.*, 1994; Henriksen *et al.*, 1996; Henriksen and Assmann, 1997) and fungi (Roberts *et al.*, 1997). Sarah Assmann (Penn State) described the experimental system needed to ablate a small region of the wall after plasmolysis of the protoplast to avoid damage. To ablate small regions of the

wall reliably, the laser has to be parfocal with the bright-field system. Too high a power may cause excess damage outside the focused point, lower power may require multiple shots, but is less damaging overall. Few lenses are designed to be parfocal in the low-UV as well as for visible wavelengths, so the laser input optics must include prefocusing to compensate for the axial shift in the focus point. It is possible to track the position of the beam during alignment by using the laser to vaporize ink deposits on a Petri dish. As the focus becomes parfocal, the diameter of the blast area becomes smaller until a precise burn-hole is produced in the plastic.

After ablation of the cell wall, the protoplast is re-swelled inside the target cell and a small bleb of membrane protrudes through the hole. It is then possible to patch this protruding bleb. This has the advantage of maintaining the cells in an almost intact setting where intercellular signalling, cell-wall signalling and cell polarity are all maintained and can be used to explore spatial localization of different ion channels in polarized cells (Taylor *et al.*, 1996; Roberts *et al.*, 1997). Assmann reported on several new types of ion channels that have been recorded using this approach in plants (see also Roberts *et al.*, 1997, for fungi), that may have been inactivated during the harsh enzyme treatments used for protoplast preparation.

As well as allowing patching of a cell *in situ*, the attached patch pipette can be used to load in fluorescent dyes for photometry or imaging simultaneously with the electrophysiological measurements. This requires further modification of the excitation path to combine the ablation laser and the conventional fluorescence illumination system, but offers the exciting potential to combine laser-assisted patching, caging and imaging experiments.

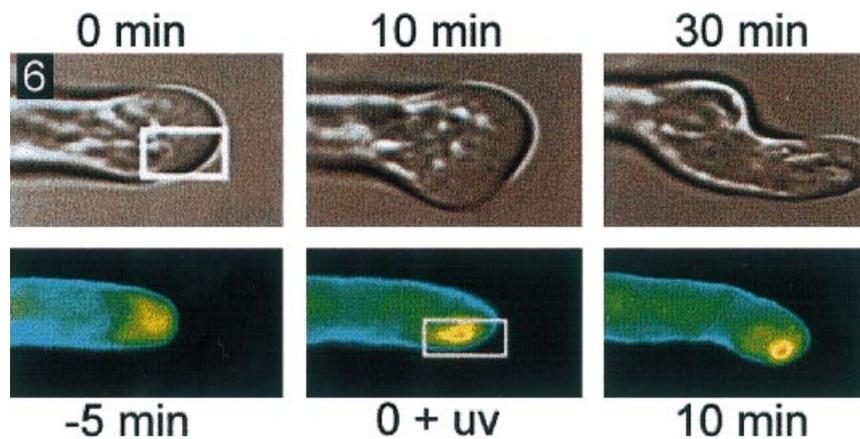


Fig. 6. Localized release of caged ionophores can be used to subtly alter calcium-regulated tip-growth in root hairs. The effect of UV activation of caged calcium ionophore on (a) growth and (b) cytoplasmic Ca^{2+} levels in root hairs of *Arabidopsis thaliana*. Note after localized UV irradiation (box) and ionophore activation, apical calcium levels in the root hair are increased laterally, and growth is transiently altered to the side. UV irradiation and calcium imaging were performed using a Zeiss LSM-410 confocal microscope. Representative view of at least nine individual root hairs (from Bibikova *et al.*, 1997, with permission).

Conclusions

There can be little doubt from the level of interest of delegates at the meeting that fluorescence and luminescence techniques have reached a watershed in plant sciences. From being something of a curiosity a decade ago they have taken centre stage in many different areas of research and are rapidly becoming indispensable tools in cell, developmental and molecular laboratories. An added bonus is that the clarity and beauty of even the raw data should also make so much more research science accessible to a wider public audience.

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