Fluorescent probes for living plant cells

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1 Introduction

There are several reviews that cover the biological questions that have been tackled with fluorescent techniques in plants (1-3) and there is extensive on-line documentation on the properties of the fluorescent probes themselves (e.g. Molecular Probes, http://www.probes.com). This chapter focusses on optimisation of the systems used for live cell imaging, the methods used to mount plant specimens and load them with dyes, a compendium of dyes that have proved useful in plant tissues and a series of protocols to convert qualitative observations to quantitative measurements. The hazards associated with these dyes are, in the main, not known, so all should be treated as potentially harmful.

2 Techniques for live cell fluorescence measurements

2.1 Selecting probes with high brightness

Fluorescent probes typically have de-localised electron clouds with energy levels sufficiently close that absorption of a photon of UV or visible light can excite an electron from the ground state (S_0) to the first singlet state (S_1). The excited electron usually loses a small amount of energy as heat and then decays back to the ground state within 10^{-8}-10^{-9} seconds, emitting a photon at a longer wavelength. The molar extinction coefficient (ε or EC, units: cm^{-1} M^{-1}) is defined as the absorption of a 1 M solution measured through a 1 cm path length, usually at the wavelength that shows the maximum absorption in a particular solvent (ε_{max}). In the case of more hydrophobic probes, the solvent used is often ethanol, DMSO or DMF, thus literature values of ε may not be entirely appropriate to the conditions expected in living cells. Some electrons decay by radiationless transitions that compete to depopulate the excited state. Alternatively, the singlet electron may convert to a triplet state (T_1) by intersystem crossing. Typically, electrons remain in T_1 for an extended period (>10^6 s) before decay to S_0 ground state (phosphorescence), as this transition also requires the spin on the electron to reverse. Occasionally, electrons in T_1 receive sufficient thermal energy to excite them back to S_1, giving rise to delayed fluorescence, occurring in around 10^{-6} s.

The probability that light will be emitted provides a measure of the relative extent to which fluorescence versus other competing energy dispersive processes occur and is termed the quantum efficiency (QE) or quantum yield (QY, ϕ) of the fluorochrome according to equation (1):

$$\mathrm{QE} = \frac{\text{number of photons emitted}}{\text{number of photons absorbed}} \qquad (1)$$

Some of the best current fluorophores have a QE of 0.7 or higher, however, the QE of many probes is rarely quoted. The QE is often dependent on the environment around the probe, meaning that changes in a physiological parameter can be inferred from changes in fluorescence.

The brightness of a fluorophore is the product of QE and ε_{max}. In most applications high values of both QE and ε are advantageous, however, at increasing concentrations, self-absorption (*A*) by the dye itself can significantly decrease the expected fluorescence yield. The extent of absorption for a dye in solution is given by the Beer-Lambert law (equation 2):

$$A = \log_{10}\left(\frac{I_0}{I_t}\right) = \varepsilon\, c\, l \qquad (2)$$

Where I_0 is the incident intensity, I_t is the transmitted intensity, ε is the molar extinction coefficient, *c* is the concentration and *l* is the path length in cm. The log ratio term means that the effect of self-absorbance can rapidly become problematic for dyes with high ε_{max} at concentrations above 10-100 µM, particularly if the dye is still present in the bathing medium during the experiment. This is often the case in cuvette-based measurements or when sampling deep into tissues with confocal imaging, for dyes that may only become fluorescent in a particular environment within the cell or after metabolism, but still absorb in their non-fluorescent form. For example, 18% of the incident beam is absorbed by 100 µM fluorescein (ε ~ 88,000) over 100 µm, increasing to 87% for a 1

mm path length and very close to 100% for a 1 cm path length. Thus, occasionally, it may be more appropriate to excite a dye away from its maximum wavelength to minimise self-absorption. Self-absorbance is also greatly reduced in multi-photon imaging, as the dye has essentially no absoption at the primary red or IR wavelength outside of the region where multi-photon excitation can occur.

At high dye concentrations the fluorescence emission may also be reduced by molecular collisions that dissipate energy by radiationless transitions before it can be emitted as fluorescence, although the dye concentrations required rarely occur in live cell imaging.

2.2 Spectral considerations

The energy levels in both S_0 and S_1 are spread slightly, so it is possible to excite the molecule with photons with a range of different energies (i.e. different wavelengths) represented by the excitation spectrum. This may differ from the absorption spectrum if not all wavelengths absorbed contribute to fluorescence. In some cases, the excitation spectrum shows additional peaks at shorter wavelengths that indicate transition of electrons from S_0 to higher excited states ($S_2 \Rightarrow S_n$).

Decay of the electron from the different energy levels in these excited states to S_0 gives rise to photons with different energies represented by the emission spectrum. The shift between the peak excitation wavelength and the peak emission wavelength is termed the Stokes shift.

The QE, and both the excitation and emission spectra are critical considerations when selecting the optimum fluorophore for a particular application. For example, in multiple labelling experiments, a fluorochrome with a large Stokes shift facilitates separation of the excitation and emission wavelengths and may even allow a single excitation wavelength to be used with two probes that can be separated by their emission spectra. Alternatively, a long tail in the emission spectrum gives bleed-through of fluorescence from the probe with the shorter emission, to contaminate the signal from the probe with the longer emission.

Rather than just a change in fluorescence intensity, the excitation or emission spectrum may well alter depending on the environment of the probe, for example upon binding of an ion to an ion-indicating probe. The ion concentration can be measured from the shift in the excitation spectrum after appropriate calibration. In practice, the shift in spectrum is quantified by taking the ratio of the emission signal collected at two different excitation wavelengths, often corresponding to the free and bound forms of the probe. This may be implemented for photometric systems (ratio photometry) or imaging systems (ratio imaging). In principle, ratio techniques give results that are independent of the dye concentration, dye leakage, optical path-length and, to some extent, the level of photobleaching. Results from ratio measurements are easier to interpret than single wavelength measurements if all the requisite controls have been conducted properly. Equally, calculating the ratio has the potential to introduce artifacts from image mis-registration, incorrect background subtraction and incorrect use of statistical averaging that give erroneous results.

2.3 Fluorescence lifetime imaging microscopy (FLIM)

Electrons do not decay instantaneously from the excited state but follow a first-order kinetic with a rate constant, k_F. The reciprocal of the rate constant gives the relaxation time or fluorescence lifetime, τ_F. One lifetime is the time required for the number of excited electrons to decrease to $1/e$ or 37% of the initial number, and is in the ns range for many fluorophors. (The fluorescence half-time is also often quoted as the time for the number to decay to 50% and equals $0.693 \times \tau_F$). The fluorescence lifetime is variable for different fluorophores and is also sensitive to the local environment around the probe. Thus measurement of τ_F using short, pulsed excitation and time-gated detectors can be used to separate signals from fluorophores with considerable spectral overlap, if they have different values of τ_F. Alternatively, it is possible to infer physiological information on the cellular environment if the lifetime alters in a predictable way. Thus a number of Ca^{2+} indicators do not show spectral shifts upon binding Ca^{2+} but do show changes in τ_F on ion binding, allowing ratio measurements in the time-resolved domain rather than the spectral domain. It is currently rather expensive and rare to combine fluorescence lifetime measurements with imaging microscopy (FLIM), so most time-resolved measurements in plant systems have used photometric measurements. For example, time-resolved measurements have been used to measure the viscosity of the cytoplasm (4) and plasma membrane (5).

2.4 Fluorescence polarisation anisotropy

Many fluorophors have extended ring structures that can be preferentially excited by light with a matching angle of polarisation (photoselection). If the molecule is immobilised, the fluorescence emission retains an amount of the initial polarisation. If the molecule can move during the lifetime of the excited state the emission is randomised to a greater extent with respect to the excitation light (motional depolarisation). The degree of polarisation (P) is usually measured by comparing the fluorescence intensity with polarisers oriented parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the direction of the exciting beam:

$$P = \frac{\left(I_{\parallel} - I_{\perp}\right)}{\left(I_{\parallel} + I_{\perp}\right)} \tag{3}$$

For this technique to work effectively it is important that the other optical components in the system preserve the polarisation angle of the excitation and emitted light.

Fluorescence polarisation measurements have only rarely been used in plant systems, but can provide information on microviscosity of membrane lipids (5) or the orientation of cellulose microfibrils (6).

2.5 Fluorescence resonance energy transfer (FRET)

Fluorescence emission may also alter if the energy of the excited state in one fluorophore (termed the donor) is transferred to a second (acceptor) fluorophore in a process called fluorescence resonance energy transfer or FRET. FRET is enhanced if the two fluorophores are within a few nanometers of each other, but falls off very rapidly at approximately the reciprocal of the sixth power of the separation distance (around 2-6 nm for 50% decrease). Typically, increased FRET is measured as a decrease in donor fluorescence coupled to an increase in acceptor fluorescence. FRET measurements provide an indication of the proximity or binding between two separate fluorescently-tagged molecules at physical distances far below the resolution limit of the light microscope. More recently, FRET probes have been developed where the separation of two spectral variants of green fluorescent protein (and hence the extent that FRET occurs) is dependent on Ca^{2+}-induced conformational changes in a linker polypeptide composed of calmodulin and a calmodulin-binding peptide (7, 8). Binding of Ca^{2+} to calmodulin causes the calmodulin to fold and associate with the CaM-binding peptide. This reduces the separation of the CFP and YFP fluorophores, increases FRET and gives rise to a shift in the emission spectrum towards the YFP peak. This important development heralds a new era of ratioable molecular fluorescent reporters whose ligand specificity, expression level, tissue and sub-cellular targetting are all encoded in gene constructs and can be readily manipulated using molecular biology techniques.

2.6 Photobleaching and fluorescence redistribution after photobleaching (FRAP)

In principle, increasing the excitation intensity gives rise to a stronger fluorescence signal and better signal-to-noise (S/N) ratio, however, increased excitation has major drawbacks in live cell imaging. The fluorescence process is cyclical allowing each fluorochrome to emit many photons, however, the small but finite amount of time spent in the excited state means the process can be saturated at sufficiently high illumination intensities. Further increasing the level of excitation does not provide any increase in fluorescence. A second consequence of excess excitation is the increased frequency of inter-system crossing to T_1. The excited electron tends to remain in T_1 for a much longer period of time compared to S_1, which substantially increases the likelihood that it will interact with another species, such as molecular oxygen, to form damaging free radicals capable of destroying the fluorophore (photobleaching) or damaging the tissue (phototoxicity).

Fluorophores have markedly different tendencies to photobleach and this may be another important consideration governing the choice of fluorochrome. Photobleaching can be reduced in fixed preparations by antifade reagents or by reducing oxygen levels, but these tricks are rarely possible with live cell preparations unless anoxia is acceptable.

In some situations a small region of the fluorophore within the cell may be intentionally photobleached with high light intensities and the subsequent change of fluorescence followed in a technique termed fluorescence redistribution after photobleaching (FRAP). FRAP has been used to determine diffusion coefficients of membrane lipids (9), ER-mediated intercellular communication (10), rates of phloem unloading (11) and the dynamics of cytoskeletal rearrangements following

microinjection of fluorescently-tagged tubulin or actin monomers (2, 12). In a variant of the FRAP technique, continued bleaching of a specific area can be used to probe connectivity with adjacent regions in, for example, the ER network or stromules between chloroplasts. In addition to the loss of signal from the bleached area, molecules diffusing from the connected regions are also bleached in a process termed fluorescence loss in photobleaching or FLIP (13).

3 Optimisation of fluorescent systems for live cell imaging

Live cell imaging is particularly demanding as both the amount of fluorochrome that can be used and the intensity of the excitation are heavily constrained by the need to minimise phototoxicity and keep the cells alive and functioning normally. This means that the level of fluorescence signal will be at the limits of detection by the human eye. The signal-to-noise ratio (S/N) and the signal-to-background ratio (S/B) become of increasing importance in such studies, particularly for quantitative measurements. With this in mind, it is worth optimising each component in the optical system for each application. The basic components of different fluorescent systems have been described in Chapter 1: the brief comments below focus on the additional requirements of a live-cell imaging system.

3.1 The light source

- Lasers provide well-collimated beams at defined wavelengths, but are limited in the range of the spectrum that can be conveniently covered in terms of cost or the technical difficulties in combining several different lasers on the same instrument.
- Mercury arc lamps have spectral lines at 366, 405, 436, 546 and 578 nm with substantial intervening regions of relatively lower, but even intensity. DC operation can be used for some lamps to increase the stability of the output.
- Xenon arc lamps are often preferred choices for multi-wavelength systems as they have an almost flat spectrum from UV to red allowing the user to select the most appropriate wavelength. Lamps need to be burnt in under conditions of high mechanical stability to prevent the arc wandering.
- Quartz-halogen lamps are cheap sources of visible wavelengths, but cannot be used for applications requiring UV.
- Super-bright light emitting diodes (LEDs) give out sufficient power to excite fluorescence and are now available at selected wavelengths from blue to IR. These may well provide a very low-cost and versatile illumination system.

Both Mercury and Xenon lamps have substantial emissions in the IR, and reflective IR blocking filters are essential to avoid overheating the sample (and expensive interference filters). Neutral density filters and intermittent illumination controlled by a shutter system or a blank filter position are often needed to minimise photobleaching and phototoxicity.

3.2 Selection of the excitation wavelength

Single-line lasers do not require an excitation filter, however, for broad-band light sources or multi-line lasers, the excitation wavelength(s) is usually selected by an interference band-pass filter, often mounted in a computer controlled filter-wheel. More recently, very flexible spectral excitation sources have been developed that use either monochromators with a galvonometer-driven holographic grating, or an acousto-optic tuneable filter (AOTF).

Interference filters are characterised by a center wavelength (CWL) and a bandwidth measured as the full width at half-maximum (FWHM) or half band width (HBW) of the peak. The number of cavities in the interference coatings affects the slope of the transition from attenuation (defined as 5% of peak transmission) to transmission (defined as 80% of peak transmission).

Judicious choice of the center wavelength, bandwidth and slope can significantly improve the ratio of fluorescence signal to background or specimen autofluorescence. A wide range of filters is available off-the-shelf and most manufacturers offer facilities for customer-specified filters (e.g. http://www.chroma.com or http://www.omegafilters.com).

3.3 The dichroic mirror

In epi-illumination microscope systems, the excitation beam is directed towards the sample using a dichroic mirror that reflects the excitation beam but transmits the longer wavelength emission. Typically, the dichroic mirror is specified as a long-pass filter with the cut-on wavelength at 50% of

the peak transmission when oriented at 45°, the angle used in the microscope. A sharp transition from reflection to transmission is essential for many fluorochromes that do not have large Stokes shift, to maximise the amount of the emission peak that can be collected. In multiple-labelling experiments, double- or triple-dichroics allow simultaneous excitation and collection of signals from different probes with no mis-registration.

3.4 Selection of the emission wavelength

On the emission side, long pass filters maximise the amount of the signal that can be collected, however, band-pass filters are a prerequisite for multi-colour applications or where sample autofluorescence contaminates the probe signal. A range of emission filters with different center wavelengths and bandwidths is invaluable to optimise signal collection for each probe in each application and it is now possible to 'try-out' some combinations of filters and probes on-line (http://fluorescence.bio-rad.com). The filters may be mounted in a filter wheel to allow rapid switching between different wavelengths. The performance of many of these filters falls off at wavelengths distant from the specified cut-on wavelength, particularly into the red or IR region and occasionally an additional blocking filter may be required to reduce transmission of these wavelengths.

3.5 Choice of measurement system

Several different techniques are available to visualise or quantify fluorescent signals. The main task is to match the measurement system with the biological question being addressed.

- Fluorimetry is useful for populations of (single) cells in suspension. Sampling is rapid (interval c. 0.05 s or better) and spectra are easy to measure. Autofluorescence is easy to correct in a parallel sample or prior to dye loading. There is no spatial resolution and heterogeneous responses from different cells cannot be distinguished. Signals from dead and dying cells also included.
- Flow cytometry is also appropriate for populations of single cells or (robust) protoplasts in suspension. Sampling is rapid, but of a different cell for each data point. Many systems allow measurement of multiple parameters simultaneously and there is potential for preparative sorting of cells by their response. There is no sub-cellular spatial resolution other than through targetting of the probe and heterogeneous responses in the population appear as an increase in variance (see *Chapter #*).
- Micro-photometry is typically used for microscope-based measurements on entire single cells or occasionally sub-cellular regions in large cells. An average spatial measurement is usually defined by a (variable) mechanical aperture and the specimen or aperture may be moved to sample different regions or different cells. Sampling is rapid (interval 0.05s or better minimum, typically 1s in practice). An average autofluorescence correction from comparable unloaded cells is straightforward. More sophisticated systems can be coupled to a spectral analysis system. Photometry measurements are prone to errors from heterogenous dye distribution (or redistribution).
- Camera imaging is useful to map sub-cellular spatial heterogeneity and/or variation in populations of cells and provides visual cues on the morphology and well-being of the cells. Sub-cellular regions can be measured typically down to 0.3-0.4 µm in (x,y), however (z) is poorly defined and can give rise to quantitation errors. The best systems use cooled, back-thinned CCD detectors with a fast digital (12-bit) readout. Dual-excitation or dual-emission involves sequential switching of the excitation or emission wavlength. This gives a delay between the two wavelengths and can cause artifacts in ratioing applications if the specimen grows, moves, alters shape or the cytoplasm is streaming. Simultaneous dual-emission imaging is possible with either a high-quality colour camera, split-view optics onto a single camera faceplate or two monochrome cameras with appropriate coupling optics. The sampling interval is typically every 1-2 s - more expensive systems can run at video rate, but in practice an extended integration period is often required to increase the S/N ratio. Autofluorescence subtraction is difficult as the autofluorescence may also have structure within the image and cannot, therefore, be subtracted as a single value.
- Confocal microscopy is particularly useful for large single cells, populations of cells or cells in intact tissue when the out-of-focus blur significantly reduces the image contrast and interfers with quantitative measurements. The (x,y) and (z) resolution are relatively well defined (best around 0.2 x 0.2 x 0.6 µm, typically 0.4 x 0.4 x 1.2 µm). The fastest temporal resolution is dependent on

the instrument and the volume sampled, but range from milliseconds for a line scan, seconds for 2-D section and seconds to minutes for 3-D data stack. Sophisticated sampling is also possible, for example to photobleach user-defined areas in FRAP experiments.

- Multi-photon microscopy also achieves optical sectioning, however, instead of using the absorption of a single photon at a short wavelength to excite an electron from S_0, it is possible to combine the energy of two or even three red or IR photons to achieve the same electronic transition if the excitation photons arrive within the order of 10^{-12}-10^{-15} s. Pulsed femto- or pico-second lasers give a flux density that is sufficiently high at the focal point for two-photon excitation to take place, providing optical sectioning without the use of the pinhole required for confocal imaging and reduces photobleaching. UV-fluorophors can also be excited with red or IR light, in the range 660-1047nm, that generally penetrates further into intact specimens and is likely to do less damage to the tissues, provided that the specimen shows negligible absorption at the primary wavelength. Typically the peak for the two-photon excitation spectrum is broader and blue-shifted in comparison to the equivalent single-photon excitation spectrum (14).

4 Securing the specimen for microscopy

The specimen needs to be securely fixed down to prevent movement during microscope observation, perfusion and especially if microinjection is to be attempted (see Chapter #). The procedure to immobilise roots in Phytagel is given in *Protocol 1* (15). Other techniques include:

- Immobilisation in agarose: Embed tissues in 1-2 % (w/v) low melting point agarose (gel point 26-30 °C; Sigma type VII) warmed to 40 °C; or mix equal volumes of cell/protoplast suspension with 1-2% agarose at 40 °C on a pre-warmed coverslip.
- Immobilisation in gelatin: Concentrations of gelatin up to 18% (w/v) in nutrient media are useful for embedding single cells. Samples are mixed with molten gelatin warmed to 40°C and spread thinly on a prewarmed coverslip.
- Immobilization in alginate: Alginic acid (~1.5% w/v) forms a gel at room temperature in the presence of excess (mM) $CaCl_2$ that can be used to trap protoplasts or cells. The high Ca^{2+} concentrations may perturb the cell physiology.
- Using silicone vacuum grease (M494, ICI), Vaseline (Chesebrough-Pond's Ltd, London, UK) or molten 'Valap' (1:1:1 vaseline: lanolin: paraffin) as an adhesive. Leave a clear window in the grease for observation with an inverted system.
- Contact adhesives (e.g. Dow Corning no. 355, Reading, UK; Secure B401, Factor II, Lakeside, AZ, USA).
- Adhesive tape (e.g. Cellux) can be fixed to the microscope slide with the sticky side up using double-sided tape. For microinjection, the tissue can be injected dry or covered with an inert oil such as Voltalef PCTFE Oil (Atochem, Pierre-Bénite, France). The oil prevents evapouration and improves the optics of the system for observation (16).
- Coating the coverslip with poly-L(or D)-lysine. Poly-lysine can be applied at 0.01-0.1 % (w/v) in 10 mM Tris-HCl, pH 8.0 for 5-60 min, followed by washing (see *Chapter #*). Alternatively slides pre-coated with poly-L-lysine are available (Sigma).
- Suction pipettes with 10-20 µm diameter tips can be used to hold protoplasts or single cells. These may be filled with inert solutions (e.g. silicone fluid Dow Corning 200/100 CS).
- Mechanical clips or restraints can be used to secure large cells or tissues (e.g. 17).
- Rosettes of *Arabidopsis* plants can be mounted with the stem in water-filled silicon tubing inserted into a small (15x30x15 mm) box on the microscope stage (18).
- Epidermal fragements can be trapped under folding 100/100 mesh (100 lines per inch) EM grids (Ted Pella Inc., Redding, CA) and held down by vacuum grease (19).

It is critical to ensure that the immobilization protocol does not markedly affect cell or tissue responses. For example, poly-lysine may induce K^+-channel activity (20) and hot agarose or gelatin may heat shock protoplasts before it cools enough to form a gel. All embedding and immobilization procedures are likely to reduce diffusion to and from the tissue as well as rapid exchange of media, although this is generally less of a problem with mechanical restraints.

Protocol 1. Growth of *Arabidopsis thaliana* seedlings in Phytagel for *in situ* observation of roots

Reagents

- Nutrient medium: 3 mM KNO_3, 2 mM $Ca(NO_3)_2.4H_2O$, 0.5 mM $MgSO_4.7H_2O$, 1 mM $(NH_4)_2PO_4$, 1 mg ml^{-1} thiamine, 0.5 mg ml^{-1} pyridoxine-HCl, 0.5 mg ml^{-1} nicotinic acid, 0.56 mM *myo*-inositol, 2.3 mM MES, 0.1 g l^{-1} sucrose, 25 µM KCl, 17.5 µM H_3BO_3, 1 µM $MnSO_4.H_2O$, 1 µM $ZnSO_4.7H_2O$, 0.25 µM $CuSO_4.5H_2O$, 0.25 µM $(NH)_4MoO_{24}.4H_2O$ and 25 µM Fe-Na EDTA, pH 5.7.

Method

1. Autoclave the media supplemented with 1% Phytagel (Gellan gum agar substitute, Sigma) for 25 min at 121°C and pour 2 ml to 1 mm depth onto autoclaved cover slips (48 x 65 mm) contained in sterile 90-mm Petri dishes. The gel should polymerize at room temperature within 10 min.
2. Surface sterilise seeds of *Arabidopsis* in 95% ethanol (5 min), followed by 10% sodium hypochlorite (5 min) and five washes in sterile water.
3. Plant seeds by pushing through the gel onto the surface of the cover slip and chill for 24 h at 4 °C.
4. Germinate *Arabidopsis* seeds for 4 days under continuous light (36 µmol m^{-2} s^{-1}) at 22-24 °C with the coverslip at an angle of 45° to promote growth of the root down to and then along the surface of the coverslip.
5. Prior to dye loading, ensure the gel matrix is full hydrated by adding excess nutrient media for 15 min.

5 Perfusion systems

The best clarity or brightness of image for live cell imaging is achieved with high numerical aperture (NA) water-immersion microscope objectives and immersion of the specimen in aqueous buffer. Submersion reduces light scattering from highly reflective surfaces in the sample and may assist in efficient dye loading and application of many stimuli and calibration solutions. Perfusion is required to prevent anoxia developing and allow addition of test compounds or calibration solutions. As a guide, rapidly respiring cells, such as guard cells, become anoxic within 10-30 min without perfusion (21). The composition of the bathing medium should ideally mimic the environment around the cells *in vivo*, particularly with respect to ionic composition, water potential and gaseous environment.

In the simplest case solutions can be exchanged by drawing excess solution under the coverslip or through the chamber by capillary action onto an absorbant tissue. A more typical perfusion system comprises:

- A perspex chamber with a coverslip held onto the base with glue or grease mounted on the stage of an inverted microscope. Alternatively, layers of electrical insulation tape stuck onto the coverslip provide a rapid and convenient means to cut out chambers of varying geometry.
- A gravity feed from 50 ml plastic syringes coupled together with manually operated stopcocks (Sigma). The flow rate can be adjusted by varying the height of the syringes. More reproducible control of flow rate can be achieved with a constant head apparatus or peristaltic pumps.
- For rapid switching between solutions, the manifold combining the solutions should be as close to the chamber as possible.
- The inlet and outlet tubes to the chamber can be constructed from cut-off stainless steel syringe needles positioned using coarse micromanipulators or magnetic stage clamps.
- The outlet is connected to a vacuum-assisted sipper with a water trap.
- It is important that the rate of suction is high and the diameter of the outlet syringe small to minimise oscillations in the flow. The noise of the sipper also provides an auditory cue that the perfusion is functioning even if the experiment is in darkness.
- Upright microscope systems with water-immersion objectives and focussing of the objective rather than the stage, can also be used for thick, opaque specimens such as roots or leaves.

Partially closed perfusion systems can be set up with a rectangular chamber partially covered by a small square coverslip to leave open wells at either end. This gives a faster flow across the specimen with fewer un-stirred regions and better transmission optics, however, the positioning of the inlet and outlet tubes is more critical to maintain a smooth flow through the chamber. Completely sealed chambers are more difficult to set up and are most appropriate for cells or tissues that are being cultured under sterile conditions.

6 Loading strategies for plant cells

The objective is to introduce the probe to give good signal to noise without causing toxic effects or significantly disturbing the cell physiology. In principle, membrane permeant dyes can be loaded directly in solution whilst membrane impermeant dyes require additional technique to pass this barrier. In practice, many plant tissues load poorly due to the presence of a cuticle or suberised cell walls that restrict diffusion of the dye to the cells, or through binding of the dye in the cell wall. A variety of strategies have emerged, but there are no simple rules as to which will be most effective with a particular tissue. Population loading techniques are considered here, whilst microinjection techniques are dealt with in *Chapter #*.

6.1 Extracellular and permeant intracellular dyes

It may simply be sufficient to apply such dyes to the medium around the cells of interest where the dye may remain or be taken up by the cells and partitioned within the cytoplasm and organelles. In many cases, however, the cuticle still prevents direct access to the apoplast and the dyes have to be introduced via the transpiration stream, through small wound sites, by pressure from a syringe through open stomata or following vacuum infiltration (*protocol 2*).

Protocol 2. Loading dyes by vacuum infiltration of leaf pieces.

Method

1. Cut leaf into small pieces between 4-25 mm^2, depending on the size and accessibility of the intercellular air spaces within the leaf, or punch out discs with a cork borer.
2. Place tissue pieces in the dye solution at the working concentration in a suitable container (e.g. eppendorf tube) and place in a dessicator attached to a water pump. Apply vaccum for 2-5 min. Alternatively, place tissue pieces in a syringe containing liquid, evacuate with the plunger and ensure the tissue is immersed on release of the vacuum.
3. Repeated infiltration cycles may be necessary for some tissues[a].
4. Pieces that have been infiltrated will become more transparent and sink down.
5. Successful infiltration can also be checked by co-infiltration with a cell-impermeant dye, such as propidium iodide, which stays in the apoplast and the intercellular air spaces unless cells have been damaged.

[a.] It should be noted that vaccum infiltration degasses the solutions and is likely to give rise to anoxic conditions.

6.2 Ester loading

Most of the ion selective dyes and a number of other probes are membrane impermeant due to one or more charged carboxyl groups. Esterification of the carboxyl groups in the molecule with acetate or acetoxymethyl (AM) groups masks their charge and renders the dye membrane permeant. Hydrolysis by intracellular esterases releases the free dye in an active form in the cytoplasm (*protocol 3*).

Protocol 3. Loading dyes as acetoxymethyl-ester or acetate-ester derivatives.

Method

1. Make a 1 mM stock solution of the ester form of the dye in dye DMSO. Store aliquots of dye at -20°C in darkness. Many dyes are available packaged in dry DMSO.
2. Dilute the indicator stock solution to 1-20 µM with deionized water or media immediately prior to use[a]. Keep unused dye on ice in the dark prior to use.
3. Incubate cells for 10-120 min[b,c,d] at room temperature[e].
4. Wash out excess dye prior to observation.

[a.] Addition of 0.01%-0.2% (w/v) Pluronic F-127 may maintain dye solubility and aid tissue penetration.

[b.] Varying the external pH in the range pH 5-8 may facilitate cytoplasmic loading of some probes.

[c.] Pre-incubation for 0.5-1 h in 0.1% β-escin (saponin) (22) or 0.1% digitonin (23) may improve loading.

[d.] Pre-incubation for 0.5-1 h with esterase inhibitors, such as 0.1 mM eserine (Sigma), may also help prevent external hydrolysis of the dye and improve loading (22), although care is required as esterase inhibitors can be highly toxic to humans.

[e.] Changing the temperature to either 4°C or raising it to 30°C (22) may improve loading.

Although widely used, there are numerous potential problems with the ester loading technique, including:

- Labelling of any compartment that has appropriate esterases (e.g. mitochondria and vacuoles).
- Release of acetic acid and formaldehyde within the cell following hydrolysis of AM-esters
- Incomplete hydrolysis releasing partially activated fluorescent intermediates with different spectral properties.
- Sequestration in the vacuole or other compartments after release in the cytoplasm.

6.3 Low pH loading

Reversible protonation of the carboxyl groups at low external pH can be used to mask their charge and hence allow the dye to cross the plasma membrane. The protons dissociate at the higher pH of the cytoplasm and the dye is effectively trapped in the cell in its anionic form (*protocol 4*). This approach has been particularly useful for plant cells that are tolerant of low (pH 4.5) external pH and tend to load poorly with AM esters. However, these pH values are close to when the dyes become insoluble, requiring a fine balance between protonation to allow uptake versus precipitation. Certain cells do not survive low pH treatment and the physiological consequences of pH stress on pH regulation and signalling need careful scrutiny.

Protocol 4. Low pH loading of root tissues

Method

1. Make a 1 mM stock solution of the salt form of the dye in deionized water. Free-acid forms of the dye have to be titrated to ~ pH 7.0 with KOH to make them soluble. Aliquot into small volumes and store at -20°C in darkness.
2. Dilute the stock solution prior to use to a final dye concentration of 20 - 50 µM in 25 mM dimethyglutaric acid (DMGA), pH 4.5.
3. For tissues or cells embedded in a gel, hydrate the gel matrix by adding excess nutrient media for 15 min prior to adding the dye.
4. Add the dye and incubate tissue in the dark for 1 to 2 h[a,b,c] at room temperature.
5. Wash out unloaded dye by rinsing in fresh media[d,e].

[a.] Increased temperature (30°C) and the presence of 0.1% β-escin (saponin) (22) or Pluronic F-127 (0.01-0.2% w/v) may assist dye loading in some tissues.

[b.] If dye penetration appears to be a problem, access of the dye to the tissue can be facilitated by a cutinase pretreatment (see protocol 5).

[c.] When loading some tissues, such as stomatal guard cells, incubation in low light may be required to maintain the physiological state of the cells (19).

[d.] Washing out the unloaded dye is difficult for plant roots supported in gels and may result in high background fluorescence. Carefully removing roots from the gel matrix or allowing the roots to grow into a gel-free zone prior to incubation in the dye reduces this problem.

[e.] In some intact cells the dye appears to stick in the wall, either through co-ordination with other charged groups in the apoplast or possibly through precipitation in localized regions of low pH. Charge masking with high levels of other ions might reduce this problem.

6.4 Cutinase pre-treatment and low pH loading

Many plant tissues have a relatively impermeant cuticle layer that restricts arrival of the fluorescent probes to the target cells. It is possible to partially breakdown the cuticle by treatment with cutinases and thereby enhance dye access (*protocol 5*), however, this pre-treatment is not useful for ester loading as any residual esterase activity from the cutinase is likely to hydrolyse the ester-dyes externally.

Protocol 5. Increasing the permeability of the cuticle by cutinase digestion.

Reagents

- Purified cutinase[a] (1 mg ml^{-1} equivalent to 100-300 mmol min^{-1} mg $protein^{-1}$) stored in 10 mM Tris-HCl, pH 7.6 with HCl at 4°C.

Method

1. Incubate tissues in a small drop of cutinase at cutinase activities ranging from 0.1-10 mmol min^{-1} mg^{-1} protein for 5-30 min. For larger tissues, such as an intact leaf, small drops of cutinase can be placed on the leaf surface.
2. Wash tissues in water or buffer (2x 10-30 min).
3. Load dye using the low pH method (see *protocol 4*).

[a.] Cutinase can be prepared according to Coleman *et al.* (24).

6.5 Electroporation

Pores of variable size can be selectively induced in the plasma membrane of protoplasts by short, high voltage pulses. Resealing is spontaneous, but can be slowed sufficiently at low temperature to allow diffusion of dye or other macromolecules into the cytoplasm. A cocktail of low molecular weight factors is normally included to replace cytoplasmic components diffusing out of the permeabilized protoplasts. The precise conditions for successful and reversible electropermeabilization of the plasma membrane, such as field strength (0.1-5 kV cm^{-1}, number of pulses applied (1-5), and capacitor used (1-50 µF) require careful optimization. Aim for roughly 60-80% permeabilisation efficiency and >80% viability after resealing. Many cells do not survive and the remainder are loaded with variable concentrations of dye. In addition, protoplasts should be analysed with independent cellular assays of function to ensure that protoplasting and electroporation have not altered the cellular response. Details for electroporation are given in *Chapter #.*

6.6 Loading via detergent permeabilization

Loading of dyes into large tissues, such as somatic embryos, can be facilitated by treating with a low concentration of detergents such as 0.1% (v/v) β-escin (saponin) (22) or digitonin (23) to partially permeabilize the plasma membrane. The detergent is then washed from the sample to allow the membranes to reseal.

6.7 Loading tissues with phloem-mobile probes

Oparka and colleagues have developed an ingenious technique to load a number of probes into root tissues *via* the phloem (11, 25). Probes are applied to a small wound in the leaf as acetate or AM esters at very high concentration (*protocol 6*). The dyes are loaded into the phloem and the ester groups cleaved to give free dye that is translocated to the roots. A number of dyes can be succesfully loaded including BCECF-AM, carboxy SNARF-1 DA, CFDA, calcein-AM, carboxy SNAFL-1 DA, fluorescein diacetate (FDA), HPTS-acetate and sulphofluorescein diacetate.

The pattern of symplastic dye unloading from the phloem and its subsequent distribution and sequestration can be followed using confocal optical sectioning (11, 18, 25). In the case of HPTS-acetate, the method of uptake into the phloem is unclear as the acetate group does not render the dye uncharged, however, HPTS currently provides the best symplastic tracer following unloading as it appears to be sequestered in root vacuoles far more slowly than the other probes. It should be noted that HPTS (pyranine), along with several of the other probes, has a strong pH sensitivity (pK_a ~ 7.3) and is also used as a cytoplasmic pH indicator (26,27). Dye accumulated in acidic vacuoles is less fluorescent, and, unless the isoexcitation wavelength at ~ 425 nm is used, the fluorescence intensity reflects both the dye distribution and the pH of the compartment. A variety of other dyes have also been tested that do not load via the phloem, including the Ca^{2+} and pH indicating dyes Calcium Orange AM, Calcium Green-1 AM, chloromethyl SNARF-1 acetate, Fluo-3 AM and Rhod-2 AM (25). The probability of loading dyes into the phloem can be predicted from their physicochemical properties, including molecular or ionic weight, log octanol-water partition coefficient (log *P*), conjugated bond number (CBN) and pK_a, using a structure-activity relationship (SAR) model (25).

The ability to load dyes into the phloem can also be used to observe phloem transport directly (28), although this requires removal of overlying tissues to permit access for microscopy (*protocol 7*).

Protocol 6. Loading root tissues[a] with phloem-mobile probes

Reagents

- Agarose medium: full strength Murashige and Skoog (MS) salt mixture, 3% sucrose (pH 5.8) and 1% agarose.

Method

1. Surface sterilise seeds of *Arabidopsis thaliana* (ecotype 'Columbia') in 5% sodium hypochlorite and rinse in water.
2. Plate seeds on near-vertical 1% Agarose plates.
3. Chill for 24 h at 4°C.
4. Germinate for 4-15 days under continuous light.
5. Cut-out seedlings on a block of agarose and transfer to small (32 mm) diameter plastic Petri dishes.
6. Place moist filter paper around seedling to maintain a high humidity.
7. Apply dyes at 100-500 µM from stock solutions at 10 mM in DMSO (BCECF-AM, carboxy SNARF-1 DA, CFDA, calcein-AM, carboxy SNAFL-1 DA and FDA) or 8-10 mM from stock solutions in water (HPTS-acetate and sulphofluorescein diacetate) either by:
   (i) Crimping a leaf with forceps to make a small wound and loading 0.5-1 µl probe.
   (ii) Cutting the tip from the cotyledon and continuously loading dye the cut edge via a microcapillary positioned using 'blu-tac' for support.
8. Cover Petri-dish and illuminate with white light (370 µmol m^{-2} s^{-1} for ~30 min).
9. Observe unloading of the dye from the protophloem in the tip of the primary root after 0.5-3 h using long working distance (x10 or x20) objectives.
10. Dye within selected regions of the root tip can be bleached using a 100 mW laser and 10-20 s scanning at high zoom to asses the rate of dye movement from recovery of fluorescence after photobleaching (FRAP).

[a] This technique has been used to label symplastic fields in the developing *Arabidopsis* shoot apex following loading of HPTS (2.5 mg ml^{-1}) through silicon tubing (1 mm inner diameter) attached to the cut petiole of older leaves (18).

Protocol 7. Direct observation of phloem transport

Reagents

- Bathing medium: 10 mM KCl, 10 mM $CaCl_2$ and 5 mM NaCl (unbuffered)
- Loading buffer: 10 mM KCl, 10 mM $CaCl_2$ and 5 mM NaCl, pH 6.3 with HCl

Method

1. Make two shallow, paradermal cuts in the major vein of a mature leaf from a 17-21 day old *Vicia faba* plant using a new razor blade. Cuts should be approximately 10 mm long by 2 mm wide and separated by about 3 cm.
2. Immediately add unbuffered bathing medium to the cut surfaces.
3. Mount the leaf (still attached to plant) upside down on the stage of an upright confocal microscope using double-sided tape on a convex surface.
4. Add 2 µl RH-160 (Molecular Probes) from a 25 mg ml^{-1} stock in EtOH, to the basal well to stain the plasma membrane and visualise the cell morphology.
5. Observe the intact phloem in the basal cortical window using a x63, 1.2 NA water-immersion lens with a working distance ~220 µm (ex. 567 nm, em >590 nm).
6. Blot the apical window and apply HPTS-acetate (20 mg ml^{-1}), CFDA (0.5 mg ml^{-1}) or CDCFDA 0.5 mg ml^{-1} in loading buffer[a]
7. Allow the dye to translocate for 30 min before observation (ex . 488 or 476 nm, em. >510 nm).

[a] Dyes were initially dissolved in a small volume of 500 mM KOH

7 Intracellular dye concentration, viability and toxicity

Once successfully loaded into the plant cell the fluorescent dye may interfere with the normal function of the cells as the concentration is increased. In addition, the interaction of illumination with the fluorescent dye may cause phototoxic damage, particularly if the excited dye reacts with oxygen to give highly reactive free radicals. Thus, the concentration of fluorochrome introduced should be kept low to minimise buffering effects and reduce any potential non-specific chemical or photochemical side-effects.

It is important to have accessible markers of cell function to compare in loaded and unloaded cells, such as cytoplasmic granularity, organelle morphology, cytoplasmic streaming, membrane potential, rate of cell division or elongation, response rate or level of gene expression. Alternative strategies include the monitoring of cell viability using other vital or mortal staining techniques, such as fluorescein diacetate (see section 8.1) or propidium iodide (see section 8.2), respectively, as these probe different aspects of membrane integrity and metabolic activity.

The best approach is to determine the upper limit of dye loading consistent with minimal disruption of cell physiology. Typically for ion indicators this is between 3-50 µM intracellular dye. Once the cells are loaded with the appropriate dye, the image collection protocol can be optimised. Keeping cells alive may be at odds with maximal spatial or temporal sampling and compromises have to be made to balance the spatial resolution, temporal resolution and spectral resolution. It is important to optimise the instrument to maximise the signal-to-noise ratio (S/N) and minimise phototoxicity under these conditions. It is also important to minimise the light exposure to the sample, hence even when finding the cells to study this should be done as fast as possible. The amount of excitation illumination presented at the sample is probably the most critical parameter, however, it is not easy to predict the appropriate intensities for each systems and specimen. It is useful to be able to measure the illumination intensity in the specimen plane when the imaging conditions have been optimised to act as a guide for other experiments. Typically four sets of controls should be run:

1. Sample alone with no dye or fluorescence excitation: to test the effects of the microscope perfusion regime on the physiological response of the cells studied;
2. Sample plus illumination: to test the biological effects of the excitation illumination and to measure the levels of autofluorescence;
3. Sample plus dye (but without fluorescence excitation): to test the effects of dye loading on physiological function;
4. Sample plus dye plus illumination: to test the potential phototoxic effects of illumination levels and dye concentrations.

8 Selection and use of fluorescent probes

Two main applications of live cell imaging have emerged: first to follow the morphology and dynamics of different cell compartments and organelles and second to infer physiological information from quantitative analysis of fluorescence intensity, wavelength shifts, polarisation angle or lifetime measurements. In qualitative measurements, the emphasis is on generating an image of sufficient brightness and contrast to visualise the structures of interest. Quantitative measurements are significantly more demanding if they are to be reliable. A number of criteria can act as a guide to select appropriate dyes for plant cells:

1. The excitation/emission wavelengths in relation to the spectral sensitivity of the tissue (i.e. will illumination of the dye also trigger a phytochrome or blue light response).
2. The level of autofluorescence of the tissue at the measurement wavelengths.
3. The instrument configuration for multiple-labelling or multiple-excitation or emission dyes.
4. The ease of loading the dye into a defined compartment.
5. The behaviour of the dye within the cell, including unwanted compartmentalization, metabolism and physiological perturbation.
6. Compatibility with other optical techniques, such as UV photolysis of caged compounds.

Additional criteria become important for dyes used to measure ion activities in plant cells including the k_d of the dye, i.e. how close is the dissociation constant of the dye to the ion level in the cell compartment to be monitored, and the possibility of interference by changes in other ions in the cell

of interest. Dyes with spectral shifts are preferable for ion measurements as they permit ratio measurements that distinguish fluorescence changes due to ion binding from those due to dye leakage, bleaching or uneven distribution.

In recent years, a wealth of probes based on green fluorescent protein (GFP) or its spectral variants have been developed with appropriate targetting sequences for all the major organelles in living plant cells. This technology provides an incredibly powerful, minimally invasive route to study organelle dynamics in living cells for species that can be readily transformed (29). GFP also has a high QE, excellent photostability, low phototoxicity and is available in a number of different colours. Where possible, it is recommended that a GFP-based approach is used, however, it must be bourne in mind that only a limited number of species can be readily transformed, there is a considerable overhead in time and resources required to construct and express each GFP-probe and there are currently very few GFP-probes for physiological studies. A description of the methods and full details of GFP constructs are given in *Chapter #*. The following sections provide a guide to chemical probes that have proved useful for labelling particular organelles in live cells, followed by probes that have been used to assess particular aspects of cell physiology such as Ca^{2+} signalling or pH regulation.

8.1 Vital stains

Most of the intracellular probes described in the following sections can be used as indicators that one or more aspects of cell function are still operational. In the case of the most commonly used vital dyes, such as fluorescein diacetate (FDA), fluorescence requires the action of intracellular esterases to cleave off the ester groups and an intact plasma membrane to ensure retention of the dye (30). Details of common classes of probe are given in Table 1. Esterified dyes are typically made up in dry DMSO at 1-10 mM and diluted to 1-25 µM for use (see protocol 3). Although the initial and major site of release of fluorescein from FDA may be the cytoplasm, any cell compartment that has appropriate esterases can be labelled, furthermore, fluorescein may be transported into vacuoles or leak back out across the plasma membrane in its uncharged (protonated) form. Modified forms of FDA have additional carboxyl or halogen groups to increase the retention of the probe within the cell once the acetate groups have been cleaved, although the anion forms are still effectively sequestered in the vacuole of many cell types (e,g, 31). One of the better fluorescein derivatives used in animal systems is calcein-AM, as the cleavage product is both highly charged and relatively pH-insensitive around pH 7. The Bodipy, Alexa and Oregon Green dyes are also relatively pH-insensitive and are available as ester derivatives suitable for use as vital dyes, but have not yet been widely tested in plant systems.

An alternative approach to aid cellular retention is to include a chloromethyl group that makes the released dye a target for conjugation to glutathione (GSH), if an appropriate glutathione *S*-transferase (GST) is present (see section 9.6). The glutathione-dye conjugate may remain cytoplasmic or itself be a substrate for vacuolar glutathione *S*-conjugate (GS-X) pumps.

Fluorescein derivatives have a strong pH dependent shift in their excitation spectrum. This can be exploited to load pH indicator dyes with a range of different pK_a values into particular compartments (see Section 9.3). If pH-dependent variation in fluorescence adds an unwanted variable into the analysis, probes with little or no pH-dependence, such as CellTracker Green BODIPY can be used.

Neutral Red is a weakly basic dye that has been used extensively as a vital probe in brightfield microscopy as it readily accumulates in acidic compartments, usually the large central vacuole, of living cells (e.g. 32), staining them red at acid pH (shifting to yellow at pH 6.8-8). It is also weakly fluorescent and can be imaged with excitation around 540 nm and emission at 640 nm.

8.2 Mortal stains

Mortal stains are normally membrane-impermeant and excluded from the cell unless the plasma membrane is compromised. To make identification of damaged cells more obvious, it is useful for the dye to bind strongly to intracellular components, such as DNA, to give a brightly stained feature. Propidium iodide (PI) has proved particularly useful as a mortal fluorescent probe in plants as it not only labels the nucleus in damaged cells but also stains the cell walls of healthy, intact cells and provides a good morphological marker for tissue architecture (29). In addition, PI has a broad excitation spectrum that can be excited the 488 and 514 nm Argon-ion laser lines in confocal microscopes. In addition, the UV-excitation peak around 315 nm can be excited with a UV source or

with a two-photon system. The very large Stokes shift (em_{max} 630 nm) facilitates dual labelling experiments with a single excitation wavelength and dual-emission detection. This is particularly useful in confocal and multi-photon applications where dual-emission detection can be readily implemented.

PI is usually used at 5 to10 µg ml^{-1}, although higher concentrations (30 µg ml^{-1}) tend to be used when it is excited away from its excitation maximum. It is loaded into the apoplast of intact tissues along with other dyes of interest. Root tissues load readily as far as the Casparian strip, shoot and leaf tissues load more slowly and typically require vacuum infiltration (*protocol 2*). PI does penetrate intact cells slowly, so it is preferable to keep incubation times relatively short (10-20 min). Other DNA dyes, such as YO-PRO-1 (1 µM) may also prove useful as cell-impermeant DNA stain that labels nuclei in dead cells (33).

8.3 Cell permeant nuclear stains

DAPI and Hoechst dyes are excellent cell-permeable UV-excited dyes that label DNA in living cells. Confocal imaging applications usually require dyes that can be excited at longer wavelengths, such as acridine orange (AO) or the SYTO dyes (Molecular Probes). So far the green SYTO dyes have been used successfully in plants (e.g. 31, 33), however, the SYTO series encompasses a broad wavelength range, suitable for multiple-labelling applications. SYTO dyes do not exclusively label DNA and give some background cytoplasmic and mitochondrial staining. DNA stains are potential mutagens and it is recommended that double gloves be worn when handling stock solutions. Before disposal, dye solutions should be poured through activated charcoal before disposal and the charcoal incinerated.

8.4 Chloroplasts

Chlorophyll shows a strong autofluorescence *in vitro* or *in vivo*. Chlorophyll dissolved in ether can be excited at either of its major absorption peaks in the red (662 nm) or blue *Soret band* (430 nm). There is a radiationless transition from the upper (blue) excited state to the lower (red) excited state within 10^{-12} s so the fluorescence is usually >662 nm. *In vivo* there is a range of slightly different chlorophyll pigments that occur in complexes with proteins giving a red shift to the absorbance peaks and the fluorescence emission to >686 nm. In addition, the presence of accessory pigments means that chlorophyll can be excited across a much broader spectral range than its own spectrum would suggest. The amount of chlorophyll fluorescence inversely tracks the flow of electrons from the reaction centers and has been used as a powerful tool to monitor the activity of the photosystems and dark reactions *in vivo*. It is possible to monitor a number of parameters, such as the photochemical yield of PS II or the level of non-photochemical quenching, with imaging techniques at the tissue level (34), fluorimetry on individual protoplasts (21) or even imaging single chloroplasts within cells in intact leaves (35).

It is possible to use other fluorescent probes to monitor photosynthetic activity in chloroplasts. For example, fluorescein tetrazolium (Polysciences) added at 0.01-0.001% (w/v) from a 0.1% stock in methanol is reduced within the chloroplasts over a 30 min period to precipitate a fluorescein-tagged diformazan salt (36).

8.5 Mitochondria

NADH can be excited at 365 nm and fluoresces at 460 nm. In animal tissues, most of the fluorescence attributable to NADH comes from the mitochondria as cytosolic NADH fluorescence is often quenched and fluorescence from NADPH has a much lower quantum yield (37). It is possible to image NADH fluorescence using fluorimetry, camera-based, confocal or multi-photon imaging (37). As many other cellular components will also fluoresce at these wavelengths, typical treatments include changing the oxygenation state of the tissues, with anoxia leading to increased fluorescence as NADH levels increase. Although UV-imaging is not easy in plants, the development of multi-photon systems may facilitate NADH measurements in intact systems.

Several probes label mitochondria with reasonable specificity at low concentrations, including $DiOC_6$, $DiOC_7$ (38), Rhodamine 123 (39), MitoTracker and JC-1 (Table 3). The fluorescence intensity depends on the mitochondrial membrane potential that drives accumulation of the dye. In the case of JC-1, the dye molecules further stack-up at certain concentrations to form J-aggregates which shift the emission from green to red. This process appears to be promoted by specific interactions with cardiolipin in the mitochondria (J. Ermantraut, P. Spanu and M.D. Fricker, unpublished). JC-1 can, in

principle, be used as a ratiometric indicator for mitochondrial membrane potential, however, it is very difficult to calibrate the response of JC-1 in intact tissues unless the concentration of dye in the cell can be guaranteed to match that in the surrounding medium.

Inhibitors can be used to manipulate the membrane potential to give a guide to the range of response that might be expected. For example, sodium azide (1 - 10 mM, 5 min) or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 5 µM) inhibit mitochondrial electron transport and reduce fluorescence, whilst monensin (0.1 mM) stimulates fluorescence through hyperpolarisation of the membrane potential (39)

An alternative approach to label mitochondria is to use probes that require oxidation, such as 2',7'-dichlorodihydrofluorescein diacetate (2',7'-dichlorofluores*cin* diacetate, H_2DCF-DA). H_2DCF-DA is cleaved to nonfluorescent H_2DCF by esterases inside the cell. In suspension culture cells, oxidation of H_2DCF to fluorescent DCF takes place primarily in the mitochondria (40), although see Section 9.7. In contrast to dyes that respond to membrane potential, inhibition of electron transport (e.g. by a 5 min pre-incubation with 5 µM Antimycin A) gives enhanced mitochondrial fluorescence by stimulating production of reactive oxygen species (ROS).

The MitoTracker probes are cell-permeant mitochondrion-selective dyes. They also have a mildly thiol-reactive chloromethyl moiety that increases retention of the dye following fixation. MitoTrackerdyes are also available in reduced forms that are essentially non-fluorescent until they are oxidised, in a similar manner to H_2DCF. The fluorescent probe then accumulates in the mitochondria.

8.6 Vacuoles

A considerable number of vacuolar pigments, such as flavonoids and anthocyanins, show strong autofluorescence, particularly with UV-excitation. Generic classes of compound can be identified by their spectral properties and how these alter in response to alkalinisation or treatment with Naturstoffreagenz A (NRA) which induces secondary fluorescence from flavonoids (e.g. 41). Thus, the green vacuolar fluorescence of flavonoids can be enhanced by exposure of the tissue to 0.5% (w/v) NH_3 or by incubating fresh cut sections for 5 min in 0.1% w/v NRA diluted from a 2.5% (w/v) stock in ethanol (41).

Multi-photon laser scanning microscopy may be a particularly useful tool to study autofluorescence of heavily pigmented cells, as the primary infra-red excitation wavelength is affected far less by attenuation and scattering in comparison with single-photon UV or visible excitation, but can excite a wide range of anthocyanin and flavonoid components with sub-cellular resolution (A.J. Meyer and M.D. Fricker, unpublished).

Several different mechanisms can be exploited to load vacuoles with fluorescent dyes, including cation trapping, active transport of anions or glutathione *S*-conjugates. The different metabolic requirements for vacuolar accumulation of these different classes of probes can be useful to identify different types of vacuoles (42), equally, however, none of these probes can be considered as a generic marker for all types of vacuole.

Weakly basic dyes, such as acridine orange (AO), neutral red (NR) and the LysoSensor probes, are membrane permeant in their uncharged form but become trapped in acidic compartments upon protonation. AO can be excited at a range of blue/green wavelengths and accumulates in some, but not all, vacuoles depending on the pH gradient and extent of binding to intra-vacuolar components. AO also binds to other cellular components, notably DNA (see section 8.3), and can be highly phototoxic at high concentrations.

Many anionic dyes, such as Lucifer Yellow, BCECF, CDCF or carboxy-SNARF-1, are also effectively sequestered in the vacuole of some cell types when loaded as membrane permeant esters or at low pH (e.g. 30). In the case of dyes loaded as esters, localisation may reflect the distribution of the appropriate esterase activity (30). In other cases, sequestration probably involves specific transporters. These may include multi-drug-resistance pumps, glutathione (GS-X) pumps (43) or sulphonate transporters (44). At least some of these transporters can be inhibited by pre-incubation for 1 h with 1 mM probenecid (45). Probenecid has to be solubilised under alkaline conditions (pH 11.5) and then adjusted to pH 7 with HCl to give a 10 mM stock solution (45).

Probes with nucleophilic sites, such as monochlorobimane (MCB), 5-chloromethylfluorescein diacetate (CMFDA, CellTracker Green) or 7-amino-4-chloromethylcoumarin (CMAC, CellTracker Blue) are substrates for conjugation to GSH in the cytoplasm. The GS-X conjugate is then transported into the vacuole by a GS-X conjugate pump. MCB is not fluorescent until conjugated to GSH (see

section 9.6), CMFDA is not fluorescent until the acetate groups are cleaved off, whilst CMAC is fluorescent whether conjugated or not.

Proteolytic activity in vacuoles of barely aleurone protoplasts has been measured using CBZ-Phe-Arg-CMAC (7-amino-4-chloromethylcoumarin, CBZ-L-phenylalanyl-L-arginine amide, hydrochloride) (42). CBZ-Phe-Arg-CMAC is a membrane permeant, nonfluorescent protease substrate. It is initially taken up into protoplasts and conjugated to glutathione to give CBZ-Phe-Arg-CMAC-GS in the cytoplasm. CBZ-Phe-Arg-CMAC-GS is transported into both secondary and protein storage vacuoles by glutathione-S-conjugate pumps. CBZ-Phe-Arg-CMAC-GS releases CMAC when the peptide bond between the CMAC fluorophore and the adjacent arginine is cleaved. Proteolytic activity in isolated vacuoles can be inhibited by pre-incubation with the cysteine protease inhibitors E-64 or leupeptin at 100 μg ml^{-1} (42).

8.7 Endoplasmic reticulum

A variety of fluorescent probes have been used to label the endoplasmic reticulum in plants, including $DiOC_6$, Rhodamine B hexyl ester and chlortetracycline (CTC, aureomycin). Typically, cells or tissues are loaded by incubation in the dye for 5-30 min, followed by a series of washes in dye-free medium. None of these dyes are specific for the ER; typically they label mitochondria at low concentrations and can label other intracellular membrane compartments at high concentrations. In species that can be transformed, ER-targetted versions of GFP provide a more reliable ER marker (see *Chapter #*). Other probes may initially insert into the plasma membrane but become redistributed into internal membranes following flip-flop from the outer to inner leaflet of the plasma membrane. This has been suggested for NBD-phosphatidylcholine, which labels the ER after metabolism to the diacyglycerol form (NBD-DAG) by putative plasma membrane Ca^{2+}-dependent phospholipase C activity (10).

Cells labelled with the ER dyes $DiOC_6$, rhodamine B hexyl ester or AFC_{12} have been photobleached to show that ER lipids can diffuse through plasmodesmata between neighbouring cells (10). In contrast, probes that are restricted to the plasma membrane, such as NBD-SM, do not appear to move (10).

8.8 Golgi

Lipid-based fluorescent probes used to label the Golgi in animal cells, such as NBD-ceramide, do not appear to label the Golgi in plant tissues. Thus at the moment the most practical route to visualise the Golgi in living cells is through targetted GFP-derivatives (see *Chapter ##*). Fluorescent Bodipy BFA (Molecular Probes) labels the Golgi at very low concentrations (0.2 μg ml^{-1} from a 100 μg ml^{-1} stock in DMSO) and does not disrupt its morphology (C. Hawes, per comm.).

8.9 Cytoskeleton

The fungal toxins, phalloidin and phallacidin, bind to and to some extent stabilise F-actin. Several fluorescently tagged derivatives are available that can be introduced into cells by microinjection or after partial permeabilisation of the plasma membrane (46, 47, 48) (*protocol 8*). At low concentrations they do not appear to greatly perturb cell function and dynamic changes in microfilament arrays can be followed. Actin filaments may be partially stabilised using m-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS), particularly prior to fixation.

Fluorescently-tagged actin and tubulin have also been introduced into cells, usually by pressure microinjection (e.g. 12, 46, 47, 48, 49, 50, 51) (*Protocols 8, 9*) in a technique known as fluorescent analogue cytochemistry (reviewed in 2, 50).

Protocol 8. Labelling of actin filaments in living cells using fluorescent phallotoxins[a]

Reagents

- Fluorescein-phalloidin, rhodamine-phalloidin[b] or Bodipy FL phallacidin stock solutions (6.6 μM in MeOH) stored frozen in 20 μl aliquots.
- Microinjection buffer: 100 mM KCl or PBS buffer (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.0)
- Actin stabilisation buffer: 100 μM m-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS), 50 mM PIPES, pH 7.0.
- Permeabilisation buffer: 20 μM MBS, 5 mM EGTA, 5 mM $MgSO_4$, 0.01% (v/v) Nonindet P40,

1% DMSO, 50 mM PIPES, pH 6.8

Method

A - Microinjection

1. Evaporate the MeOH from the stock solutions of fluorescent phallotoxin and redissolve in buffer to 0.66 µM.
2. Sonicate and centrifuge (5 min at 10,000g).
3. Load cytoplasm to a final concentration of 0.02-0.05 µM by pressure microinjection (see Chapter #).

B - Permeabilisation

1. Lightly crosslink F-actin in actin stabilisation buffer.
2. Permeabilise and label with 0.5-20 µM labelled phallotoxin in permeabilisation buffer for 20-180 min.
3. In some tissues direct incubation in 30 nM labelled phallotoxin may give sufficient labelling.

[a.] Methods based on refs. 46, 47, and 48.

[b.] Rhodamine-phalloidin increases its fluorescence on binding F-actin, is more photostable than fluorescein-phalloidin and is better retained within cells after fixation.

Protocol 9. Labelling of microtubules epidermal cells using fluorescent analogue cytochemistry[a]

Reagents

- Sample buffer: 50 mM potassium glutamate, 0.5 mM $MgCl_2$ and 2.75 % (w/v) sucrose
- Tubulin buffer: 20 mM sodium glutamate, 0.5 mM $MgSO_4$ and 1 mM EGTA.
- Carboxyfluorescein-tubulin prepared according to Zhang *et al.* (49) or tetramethyl-rhodamine tubulin prepared according to Wymer *et al.,* (51) and frozen at -80°C at 3-10 mg ml^{-1} in tubulin buffer.

Method

1. Immobilise an epidermal peel from a pea stem on a slide using adhesive tape (Cellux) and construct a tape chamber around the specimen.
2. Wet the specimen with sample buffer and leave for 15 min to recover.
3. Select cells with cytoplasmic streaming using a x63/1.2 N.A. water immersion objective.
4. Thaw CF-tubulin stock and dilute to 5 mg ml^{-1} in tubulin buffer supplemented with 1 mM GTP on ice.
5. Load injection pipettes (0.75 µm outer diameter) by tip filling from a 2 µl drop.
6. Load cells by pressure microinjection with to a final concentration of 0.3 µM.
7. Remove injection needle within 2 min.

[a.] Modified from the methods in ref. 12 and ref. 48

8.10 The plasma membrane and endocytosis

Unlike internal membranes, the plasma membrane is accessible to the external medium either directly or after enzymatic digestion of the cell wall to form protoplasts. A number of fluorescent lipid analogues, including NBD-phosphatidylethanolamine (NBD-PE), NBD-sphingosylphosphocholine (NBD-SM) and Lucifer Yellow-cholesterol (LY-Chol), have been used to measure the both the diffusion rate of lipids in the plasma membrane and the level of plasma membrane continuity through plasmodesmata by FRAP (e.g. 10).

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 outer leaflet of the plasma membrane. The dyes do not spontaneously re-orient in the membrane so that dye appearing internally should represent internalisation of the plasma membrane and provides an estimate of the membrane flux into the cell and the compartments that trafficking is directed. Internalisation of plasma membrane has been followed after treatment with 1 µM FM1-43 for 5-60 min (52, 53). The use of protoplasts also permits parallel electrophysiological measurement of changes in membrane

capacitance. Pretreatment with Brefeldin A (10 mg ml^{-1}, 30 min) reduces the level of FM1-43 internalisation.

Other probes have been used to label surface components, for example, rhodamine-labelled fucose-specific lectin (UEA I-TRITC, Sigma) binds to densely cytoplasmic protoplasts from the hypersecretory leyer of maize root tips when used at 1 mg ml^{-1} for 4h (52). Alternatively, fluorescent markers can be covalently attached to membrane lipids or proteins (e.g. 54).

8.11 The cell wall

Many cell walls show strong autofluorescence from lignin components, particularly with excitation at UV wavelengths (e.g. 41) or using two-photon excitation from 750-800 nm. In some cases, the spectrum can be attributed to specific components, such as equisetumpyrone in *Equisetum arvense* (41).

Calcofluor White MR2 (0.1 mg ml^{-1}, 10 min) is a very bright label for cellulose, but requires UV excitation. Primulin (200 µM, 2h) is less bright, but can be excited with a 442 nm He-Cd line in confocal applications. Propidium iodide (1-10 µg ml^{-1}, 10 min) can be excited using a broad range visible wavelengths and has increasingly found applications as both a stain for cell walls and a viability indicator (see Section 8.2).

Congo Red (0.01-0.1% w/v, 10-30 min) interacts with β1-4 glucan chains and increases its fluorescence when adsorbed on polysaccharides. Dye particles are incorporated parallel to the polysaccharide surface and selectively absorb polarised excitation light. Thus, the orientation of the cellulose microfibrils can be determined from confocal fluorescence anisotropy measurements with polarised excitation at 514 nm and simultaneous dual-channel detection equipped with crossed polarisers (6).

In addition to labelling cell wall polymers, it is possible to probe the physiology of the apoplast with cell impermeant dyes, loaded directly by incubation, vacuum infiltration or through the transpiration stream. Optically, the very thin dimensions of the wall make quantitative measurements more difficult, however, the activities of a number of ions have been successfully measured and/or imaged in the apoplast (see section 9.3).

9 Physiological probes

The basics of physiological measurements are presented here, primarily for Ca^{2+} and pH. Additional information and biological applications of the techniques are covered in (1, 2, 3, 16).

9.1 Calcium

A wide range of Ca^{2+} dyes are available, but the majority of cytoplasmic measurements have used the single wavelength Ca^{2+} indicators Fluo-3 and Calcium Green-1 (notably for confocal measurements, e.g. 55), or the dual-excitation ratio dye Fura-2, notably for camera imaging experiments. CTC is the only freely membrane-permeant dye that can be used to infer changes in calcium (56) and BTC is the only probe so far used for apoplastic Ca^{2+} measurements (57). The other dyes are typically loaded as AM esters (*protocol 3*) or at low pH (*protocol 4*), however, Ca^{2+} dyes are notorious for either not loading well into plant tissues or for rapidly compartmentalising following loading. In some cell types, the level of intracellular compartmentalisation can be estimated following selective permeabilisation of the plasma membrane with 10-100 µM digitonin to release the cytosolic dye. The fluorescence decreases to a stable value over a few minutes that represents the amount of dye trapped in intracellular compartments. Subsequent addition of Triton X-100 (0.1% v/v) is sufficient to release all the intracellular dye. The fluorescence from dye accumulated in the ER can be used to estimate ER Ca^{2+} levels (58), however, the concentrations fall outside the useful reporting range of most cytosolic indicators. Attempts to measure localised plasma membrane fluxes of Ca^{2+} with the lipophilic derivative Fura-C_{18} were not successful as the dye rapidly killed the cells (17).

In cells that can be readily microinjected, problems with compartmentalisation can be avoided by pressure-injection of dextran-linked dyes (See *Chapter ##*), and this is the method of choice for chemical dyes (2, 59). The development of the transgenic cameleon reporters (7) provides an alternative approach to introduce ratioable cytoplasmic or ER Ca^{2+} reporters (e.g. 8). Currently these probes have a rather limited ratio range spanning a very large spread of Ca^{2+} concentrations, but appear to report comparable Ca^{2+} dynamics to conventional chemical dyes in stomatal guard cells (8).

9.1.1 *In vitro* calibration - calcium dyes

Calcium dyes are calibrated *in vitro* using Ca^{2+}-EGTA or Ca^{2+}-BAPTA buffers to set [Ca^{2+}], in a solution designed to mimic the plant cytosol (*protocol 10*). Calibration requires detailed knowledge of the k_d of the Ca^{2+} buffer at the pH, ionic strength and temperature of the experiment. Extensive methods to determine the K_d are given in Fabiato (60) and Bers *et al.,* (61). Once the K_d is established, *in situ* calibration is simple to perform. Calibration kits with detailed instructions are also available from companies such as Molecular Probes and World Precision Instruments (Sarasota, FL). *In vitro* calibration of single wavelength dyes is useful to define the dynamic range expected for the dye response under the collection conditions used for the experiment, but is rarely used to calibrate the Ca^{2+} response *in vivo,* as the calibration is only valid for a single dye concentration and path length. For ratio dyes, an *in vitro* calibration provides an indication of the Ca^{2+} concentration *in vivo*, if the calibration solutions matches the conditions experienced by the dye in the cytosol with respect to pH, ionic strength, viscosity, hydrophobicity, temperature and protein-binding. Unfortunately, values for K_d measured *in situ* can be markedly different from that estimated *in vitro* (62).

The spectra for dextran conjugates is often different from the free dyes and the K_d also varies from batch to batch depending on the length of the dextran molecules and the degree of substitution (62). For example, Fura-dextran has a dissociation constant around 350 nM compared to 150 nM for Fura-2 and the peak in the excitation spectrum for the Ca^{2+} free form is shifted to shorter wavelengths (from 380 nm to 364 nm) (63). In general, definition of the appropriate composition of the calibration solution can be best estimated from comparison of the dye spectra *in vivo* with that determined *in vitro* under a variety of hydrophobicity, viscosity and ionic composition regimes.

Protocol 10. *In vitro* calibration of calcium ratio dyes[a]

Equipment and reagents

- CaEGTA buffer: 100 mM KCl, 10 mM MOPS pH 7.2 and 10 mM CaEGTA made up in deionised water.
- K_2EGTA buffer: 100 mM KCl, 10 mM MOPS pH 7.2 and 10 mM K_2EGTA made up in deionised water.
- A scanning fluorimeter, such as a Perkin-Elmer LS50B with 3 ml quartz cuvettes

Method

A- Measurement of the K_d of the dye

1. Make up the indicator dye as a 1 mM stock solution in buffer without Ca^{2+} or EGTA.
2. Add 20 µl dye to 1.98 ml K_2EGTA.
3. Measure the appropriate excitation or emission spectrum of the dye in zero-Ca^{2+}.
4. Add 60 µl dye to 5.94 ml CaEGTA solution.
5. Remove 0.2 ml solution from the K_2EGTA+dye sample in the cuvette and replace with 0.2 ml of the CaEGTA+dye sample to give a total Ca^{2+} concentration of 1 mM.
6. Measure the spectrum again.
7. Continue the sequential dilution procedure according to the following table:

[CaEGTA] required (mM)	0.0	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
Volume to remove/replace (ml)	0.0	0.200	0.222	0.250	0.286	0.333	0.400	0.500	0.667	1.00	Use undiluted CaEGTA
Approximate Free Ca^{2+}(nM)	0	17	38	65	100	150	225	351	602	1,350	39,800

8. The free Ca^{2+} concentration is calculated from the K_d of EGTA for Ca^{2+} using the following equation:

$$[Ca^{2+}]_{free} = K_d^{EGTA} \times \left[\frac{CaEGTA}{K_2EGTA} \right] \quad (4)$$

9. The K_d for EGTA at 20°C, pH 7.2 and 100 mM KCl is 150.5 x 10^{-9} M however, the K_d is highly sensitive to the temperature, pH, ionic strength and presence of other ions and has to be established for other calibration solutions. Detailed protocols are given in (60, 61).

10. In addition to changing the pH and ionic strength of the calibration solution to match that expected in the cytoplasm, the viscosity may be increased by addition of 20-60 % sucrose or 500 mM mannitol and hydrophobicity altered with 25% ethanol.

11. The actual free Ca^{2+} is calculated using an iterative computer program that accounts for all the ionic interactions in the calibration buffer. A program called Maxchelator (MAXC) is freely available on the Web at http://www.stanford.edu/~cpatton/maxc.html.

B - Calibration of the response of the imaging system

12. Set up a calibration series as in part A but reserve a small volume (ca. 100 µl) at each step and adjust the volume removed and replaced accordingly. Alternatively use Ca^{2+} buffer kits with pre-diluted solutions.

13. For confocal or multiphoton measurements, the sampling volume is defined by the instrument settings and so accurate control of the pathlength in the calibration solutions is not required. For other systems, measurements should be made using volumes of calibration solution similar in size to loaded cells in the experimental apparatus by either:

(i) vortexing 100 µl of immersion oil (Fisher Scientific, Type FF) with 5 µl of dye solution in a microfuge tube to obtain cell-sized droplets;

(ii) Using flat sided rectangular capillaries (W5005, Vitro Dynamics Inc. Rockaway, NJ, USA) with a defined (50 µm) internal pathlength

14. Measure the fluorescence at both excitation or emission wavelengths for each calibration solution, subtract the value measured at each wavelength in the absence of dye, and then calculate the ratio.

15. Experimentally derived values are calibrated using the following equation, substituting in appropriate values from the *in vitro* calibration:

$$\left[Ca^{2+}\right]_{free} = K_d \cdot \left(\frac{R - R_{\min}}{R_{\max} - R}\right) \cdot \left(\frac{F_{\max\lambda 2}}{F_{\min\lambda 2}}\right) \qquad (5)$$

Where R is the measured ratio, R_{min} and R_{max} are the ratio values at zero and saturating Ca^{2+} levels, respectively, K_d is the dissociation constant and $F_{min\lambda 2}$ and $F_{max\lambda 2}$ are the fluorescent intensities at the second wavelength (the denominator of the ratio) at zero and saturated Ca^{2+}, respectively.

16. It is more convenient to re-arrange equation (5) for use with a non-linear curve fitting package as follows:

$$R = \frac{R_{\min} - R_{\max}}{(1 + 10^{-(pCa - pk_d)})} + R_{\max} \qquad (6)$$

The measured ratio at each pCa value can be fitted by this function with R_{max}, R_{min} and pK_d, as variables (63). The fitted sigmoidal response can be used to estimate the cytoplasmic Ca^{2+} concentration from the ratios measured in the living system.

[a.] Method based on Calcium Calibration Buffer Kits from Molecular Probes (62).

9.1.2 *In situ* calibration - calcium dyes

Dye loaded cells are permeabilized with a Ca^{2+}-ionophores, such as ionomycin, or 4-bromo A23187 (a non-fluorescent analogue of A23187) at the end of the experiment (*protocol 11*). Ionomycin is less effective than Br-A23187 in plant cells (59) as it requires alkaline (pH 9) rather than acidic (< pH 7) conditions normally encountered in perfusion solutions. Cytosolic Ca^{2+} is then set by extracellular Ca^{2+}-EGTA or Ca^{2+}-BAPTA buffer solutions. Ideally cytosolic conditions experienced by the dye should not have changed significantly between the calibration and *in vivo* measurements of Ca^{2+} during an experiment. In practice, it is often only practical to perform an *in situ* calibration at two points, typically at very low Ca^{2+} concentration to determine R_{min} and $F_{max\lambda 2}$, and at a saturating Ca^{2+} concentration to determine R_{max} and $F_{min\lambda 2}$.In this case the measured ratios can be converted to concentration values only if the K_d of the dye is known inside the cell. This is often not the case in plant cells.

Results from single wavelength dyes are difficult to interpret as there is no inherent correction for dye leakage, redistribution or bleaching. An alternative approach to using EGTA to deplete Ca^{2+} is to add 1 mM Mn^{2+} that permeates Ca^{2+}-channels and quenches the fluorescence. The Mn^{2+}-quench appears to give more consistent results than determining F_{min} with EGTA (64). Even so, calibration is extremely difficult to perform accurately and in general data from single wavelength dyes is more qualitatively useful than quantitatively accurate.

A second major problem in plant cells is that ionophores do not equilibrate Ca^{2+} concentrations to a sufficient or reproducible extent across the plasma membrane. Ionophore concentrations greater than 3 µM often act like detergents and damage the cell, however concentrations up to 100 µM have to be applied to penetrate into the tissues and to shift the internal ion concentration. Thus, in many cases *in situ* calibration is not a reliable method for plant cells, leaving an *in vitro* calibration as the only alternative.

Most dyes also show a pH dependence of the K_d for Ca^{2+} below about pH 7, that can lead to an error in Ca^{2+} calibrations, particularly in experiments where cytoplasmic pH is varied using for example, weak acid loading, or where steep pH gradients have been proposed e.g. in tip growing systems (65). Different calibrations may be required during the experiment or for different regions in the cell. It is possible to determine the shift in K_d of the dye in appropriate calibration solutions (once the effect of pH on the calibration buffer itself has been taken into account). The modified K_d is then used in equation 6 (63).

Protocol 11. *In situ* calibration of calcium dyes

1. Add 1-10 µM ionomycin or Br-A23187 at the end of the experiment.
2. Increase external Ca^{2+} to ~1 mM and allow the signal to stabilise at R_{max} (ratiometric dye) or F_{max} (single wavelength dye).
3. Replace the high Ca^{2+} medium and perfuse with medium containing 1 mM EGTA and allow fluorescence to stabilise at R_{min} or F_{min}.
4. An alternative to determining F_{min} for single wavelength dyes is to use Mn^{2+} to quench the fluorescence from the dye. Add 0.1-1 mM $MnCl_2$ with 10 µM ionophore for 10 min. Fluo-3 fluorescence is quenched to 0.2x F_{max}. This set point can then be used in a modified form of the equation below (64).
5. Calculate $[Ca^{2+}]_{cyt}$ using published K_d values or K_d values measured *in vitro* using equation (6) for ratio dyes or the following equation for single wavelength dyes:

$$[Ca^{2+}] = K_d \frac{(F - F_{\min})}{(F_{\max} - F)} \qquad (7)$$

9.1.3 The manganese quench technique

The fluorescence quenching observed with Mn^{2+}can also be used to discriminate between Ca^{2+} influx and Ca^{2+} mobilization from internal stores during a Ca^{2+}-response. If Mn^{2+} is present in the external medium, opening of plasma membrane Ca^{2+} channels allows Mn^{2+} influx and consequent fluorescence quenching. In the case of Fura-2, this can be readily measured at the iso-excitation wavelength around 360 nm (e.g. 66).

A variant of the Mn^{2+}-quench technique can also be used to identify release from intracellular stores. Mn^{2+} is loaded into the intracellular stores by extended incubation in Mn^{2+} followed by washing from the external medium or by microinjection into the vacuole. Under these conditions, fluorescence quenching indicates release of Mn^{2+} from the loaded stores rather than flux across the plasma membrane.

9.1.4 Dissipation of intracellular calcium gradients using buffers with varying pK_d

In root hairs, pollen tubes and rhizoids a steep tip-focussed gradient in Ca^{2+} correlates with tip growth (e.g. 55, 67). The Ca^{2+}-gradient can be dissipated by microinjection of 0.1-1 mM Ca^{2+}-BAPTA buffers with varying K_d arising from substitutions on the BAPTA moiety. These buffers are thought to

increase the mobility of Ca^{2+} in the cytoplasm, particularly if the K_d falls between the concentrations expected at the high and low points of the gradient (68, 69).

9.2 Calcium measurements using aequorin

In addition to fluorescent probes, luminescent techniques have also been extensively used to measure calcium in plants using aequorin. Aequorin is a Ca^{2+}-dependent photoprotein extensively used to measure Ca^{2+} dynamics in plants. Active aequorin is reconstitued *in vivo* from a 22 kDa apoaequorin and a low molecular weight luminophore called coelenterazine in the presence of oxygen. When calcium is bound, the coelenterazine is oxidised 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+}, for example Mg^{2+} and K^+ do not trigger luminescence, although these ions may depress the Ca^{2+}-sensitivity. Aequorin 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 proved a very versatile Ca^{2+}-reporter, particularly in intact transgenic seedlings (70, 71), although it can also be used at the single cell level with difficulty (e.g. 72). Aequorin has several potential advantages over fluorescent dyes as an indicator for Ca^{2+}. 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 targetted elsewhere. Light emission is unaffected by pH values greater than pH 7. Photo-damage associated with excitation illumination for fluorescence is also avoided. For full methodological details on the use of aequorin in plants, the reader is referred to 70 and 71.

9.3 Measurement of apoplastic, cytoplasmic and vacuolar pH

Measurement of cytoplasmic pH follows similar principles to cytosolic Ca^{2+} measurements. A range of dyes based on fluorescein has been developed with different substituent groups that alter the pK_a of the probe (Table 8). Several of these probes are available conjugated to dextrans which are useful to prevent sequestration of the dyes in cells that can tolerate pressure microinjection. These dyes increase in fluorescence around 480-500 nm and also have a pH-insensitive iso-excitation wavelength around 440 nm, facilitating dual-excitation confocal or camera ratio imaging (3, 65, 73). As dual-excitation confocal ratioing of BCECF is not straightforward to implement, an alternative solution is to co-inject BCECF-dextran 10,000 with a pH insensitive dye such as Rhodamine Dextran 10,000 and perform dual-excitation (488 and 543 nm), dual-emission (515-540 and >590) ratioing (74). The SNARF and SNAFL dyes have even more complex pH-dependent excitation and emission spectra and can be used in either dual-excitation or dual-emission mode (3, 75, 76, 77).

Apoplastic pH measurements involve loading dyes with pK_a values in the range pH 3-7, such as Cl-NERF, Cl-NERF Dextran 10,000 or FITC-Dextran 4,000, by infiltration or via the transpiration stream, at concentrations of 30-100 µM (74, 78, 79, 80). It may be necessary to keep the dye present in the medium to avoid washout from the apoplast and use confocal optical sectioning to visualise signal from the apoplast. Dual loading with a pH-insensitive dye (Rhodamine or Texas Red Dextran 3,000) has also been used to facilitate ratio pH measurements from the apoplast (74, 79). Linking the dye to dextran ensures that there is no loading into the protoplast, however, some fluorescence properties of the dextran-linked dyes differ from the free fluorophor. Thus FITC-dextran does not have a true iso-excitation wavelength, but shows strong fluorescence quenching at acidic pH values (78). Apoplastic calibration can be performed *in situ* provided the buffer strength is increased to at least 50 mM to overcome the pH buffering of the cell wall (74).

Other indicators may be useful for apoplastic pH measurements, thus Pfanz and Dietz (81) tested a range of coumarins and found that 6-glucoxy-7-hydroxycoumarin (0.1-1 mM from 1 M stock in 1 M KOH) could be loaded by vacuum infiltration into the apoplast of a range of species and tissues and remained apoplastic. Other coumarins tested had a higher membrane permeability and were judged not to be useful for apoplastic measurements (81).

Vacuolar pH measurements rely on uptake and compartmentalisation of appropriate dyes in the vacuole (e.g. esculentin, pyranine and fluorescein derivatives, CF, CDCF, BCECF). Estimates of pH have been based on the ability of intact cells to take up a variety of fluorescent pH indicators with differing K_d values. The changes in fluorescence can be attributed to pH in particular compartments on the basis of the distribution of the dye and the pH range over which the dye is responsive (26).

Care has to be taken with such measurements from intact tissues as changes in fluorescence may result from changes in other parameters, such as light scattering (27). More conventional ratioing approaches may suffer contamination of the vacuolar signal with cytoplasmic signal, and therefore represent a complex average of the pH in the two compartments (82). The calibration response of dyes in the vacuole and cytoplasm may be markedly different as the ionic strength, viscosity and protein-binding interactions will vary.

9.3.1 Calibration of ratio pH dyes

The approach for calibration of pH dyes, such as BCECF, is essentially similar to those outlined for Ca^{2+} dyes. *In vitro* calibration solutions are similar to those used for Ca^{2+}, and the ionic strength, hydrophobicity and viscosity may all be modified to better mimic the response of the dye in the cytosol. For example Feijó *et al.* (65) used 100 mM KCl, 30 mM NaCl, 500 mM mannitol, 40% (w/v) sucrose, 25 mM MES, 25 mM HEPES at varying pH values.

For *in situ* calibrations (*protocol 12*), the K^+/H^+ exchanger, nigericin, has been used as an ionophore to equilibrate internal and external pH in the presence of high K^+ (e.g. 76). Simultaneous addition of the K^+ ionophore, valinomycin, renders the clamp insensitive to the K^+-gradient and is reported to be more effective (65). However, two point *in situ* calibrations are only reliable if the K_d can be established. The overlap between *in situ* and *in vitro* calibrations can be improved by additions such as de-proteinised coconut water supplemented with 1% ovalbumin (83). pH intervals can be monitored fairly accurately even if the absolute level cannot be determined. The relatively small shift in ratio values means the limits of reliable detection lie between 0.05 and 0.15 pH units (75, 76, 83). Parallel measurements using pH sensitive microelectrodes provide a check of the fluorescence calibration.

It is also difficult to overcome the intrinsic pH buffering and cellular pH regulation using ionophores and treatments also stress cells rapidly. Alternative approaches based on equilibration of permeant weak acids and bases may be more effective and less damaging to the cells (3, 75, 76, 83, 84).

Protocol 12. *In situ* calibration of ratio pH dyes using nigericin

Method

1. Increase the external K^+ to a value close to the anticipated internal K^+, typically 100-120 mM at the end of the experiment.
2. Add nigericin to a final concentration of 10 µg ml^{-1} and adjust the pH to pH <6.0 (for BCECF)[a].
3. Allow the ratio value to stabilise (2-15 min) and measure R_{min}.
4. Shift the external pH to pH 8.5 to give R_{max} and allow the ratio to stabilise[b].
5. Estimate the calibration pH values from equation 6 substituting appropriate values for the pH dye used.
6. Determine the experimental pH values from a sigmoidal fit to the *in situ* calibration data with an assumed K_d (typically pH 7.0-7.2 for BCECF).

[a.] Addition of valinomycin (2 µM) may give a more effective clamp (65)

[b.] As it is difficult to shift pH to R_{max} and R_{min} *in situ*, an alternative calibration between 0.5-1 pH unit above and below the pK_a to cover the near-linear region of the ratio response.

9.3.2 pH-clamping with weak acids

A relatively straightforward method to acidify the cytoplasm of living cells is to apply a weak (HBA) acid to the outer medium (85). The weak acid (usually iso-butyric acid or acidic acid) has to be membrane permeable in its undissociated (lipophilic) form. Once in the cytosol the weak acid dissociates due to the higher pH in the cytoplasm (pH_c) compared with that in the outer medium (pH_o). Thus the acid anion is trapped in the cytosol after release of a proton. Assuming the plasma membrane is permeable only for the undissociated form (HBA) of the acid, an equilibrium will be established between bathing medium and cytoplasm such that the concentration of HBA will be equal in both compartments. The amount of anion that dissociates under these conditions is given by equation 8, provided no transport systems exist for the anion and that the molecule is not metabolised:

$$BA^{-}_{cyt} = \frac{10^{(pH_c - pH_o)}}{1 + 10^{(pk_d - pH_o)}} \cdot HBA_0 \qquad (8)$$

Acid anion accumulate in the cytoplasm and other compartments with an equivalent release of protons. Equation (8) gives an estimate of the amount of protons imported into the cell. Most of these protons equilibrate with cytoplasmic buffer sites. Only a few lead to the measured pH_{cyt} decrease. Normally there are other cations in the bathing medium (K^+, Na^+, Mg^{2+}, Ca^{2+}) which can also bind to BA^- forming neutral salt molecules (KBA, NaBA, $MgBA_2$, $CaBA_2$). These salt molecules are membrane permeable to an unknown extent, but will cause an overestimate in the calculated amount of protons imported into the cell.

The weak acid can be washed out of the cell and, at low concentrations, exhibits no severe side effects on the cell physiology. However, cells do respond to weak acid loading treatments and significant changes in ion transport systems have been reported (86).

9.4 Potassium

Both cytoplasmic and apoplastic potassium levels have been measured with the potassium binding fluorescent indicator (PBFI, benzofuran isophthalate). For cytoplasmic measurements. PBFI is loaded as the AM-ester from a 1 mM stock made up in DMSO:EtOH 1:7.5. The stock is first diluted 2:1 with Pluronic F-127 and then diluted in buffer to a final concentration of 8 µM. Protoplasts are loaded for 3h at 4°C (87). For apoplastic measurements, leaves are vacuum infiltrated with 50 µM PBFI buffered with 80 mM MES, 35 mM TRIS pH 6, to control for pH sensitivity of PBFI (88). Plants were grown in low Na^+ to prevent Na^+ interference in the measurements and calibration (88).

9.5 Aluminium

Morin (2,3,4,5,7-pentahydroxyflavone; ICN Biochemicals Corp., Cincinnati, OH) binds with Al^{3+} and other cations in a series of complex pH and concentration-dependent speciation reactions to form a fluorescent chelate (89). The excitation peak of Al-morin (420 nm) is pH insensitive, however, the fluorescence emission increases with increasing pH and the peak shifts from 500 nm at pH 4.5 to 515 nm at pH 6.5 (89). Morin labels the cytoplasm and nucleus in living cells to a plateau within 10-60 min (90) and the level of fluorescence is linearly related to the level of Al^{3+}, measured by graphite furnace atomic absorption spectroscopy, suggesting that morin can be used as an *in vivo* indicator for Al^{3+} uptake (90).

9.6 Measurement of cytoplasmic glutathione levels

Monobromobimane (MBB) has long been used to derivatize low molecular weight thiols to give fluorescent products that can be analysed by HPLC. The less reactive analogue monochlorobimane (MCB) can be used at 10-100 µM to fluorescently label GSH in intact cells, if the cells contain an appropriate glutathione *S*-transferase (GST) to catalyse the conjugation reaction (91). The requirement for a GST also makes MCB labelling far more specific for GSH over other thiols in comparison to MBB. Although the excitation peak of glutathione *S*-bimane (GSB) is at 395 nm, GSB can be imaged using a confocal microscope equipped with a high-powered UV (364 nm) Argon-ion laser, the 442 nm line of a HeCd laser (92) or with two-photon excitation at 770 nm (93). Labelling is followed until a plateau is reached, typically within 10-60 min. The amount of GSB is calibrated against GSB standards prepared by conjugating 1 mM MCB to excess GSH in the presence of 5 Units ml^{-1} of rabbit liver GST (Sigma). As the GSB formed in the cytoplasm is usually sequestered into the vacuole as part of the normal detoxification pathway, the volume ratio of the two compartments has to be known to calculate the initial cytoplasmic GSH concentration. This ratio can be rapidly estimated from a uniform random set of serial optical sections using the Cavalieri estimator of volume. Although this can be done manually, commercial software (Digital Sterology, Kinetic Imaging, Liverpool, UK) greatly facilitates analysis (93). An alternative approach is to prevent vacuolar sequestration of GSB by depleting cellular ATP with 1-5 mM NaN_3 in which case, the cytoplasmic fluorescence can be directly calibrated against GSB standards. Measurements of GSB fluorescence deeper into tissues also require correction for sample- and depth-dependent attenuation according to protocol 16 (92).

To demonstrate that GSH is labelled specifically the GSH level can be reduced to zero by pre-incubation with 1 mM BSO for 24 h to prevent GSH synthesis or with 10 mM CDNB (a competitive

substrate for GSH conjugation) for 10 min. Subsequent addition of MCB should not result in significant amounts of fluorescence. Labelled tissue can also be analysed by HPLC to confirm that GSH is the predominant thiol labelled and the GSH pool has been labelled to completion.

9.7 Reactive Oxygen Species

Dichlorodihydrofluorescein (H_2DCF) is non-fluorescent until oxidised to dichlorofluorescein (DCF), preferentially by H_2O_2 in the presence of peroxidases (94). H_2DCF can be loaded into cells in the diacetate form (H_2DCF-DA) and releases free dye after the ester groups are cleaved (94) (*protocol 13*). Oxidised dye accumulates in the chloroplasts, mitochondrion, cytosol and nucleus.

Protocol 13. Measurement of H_2O_2 *in situ* using H_2DCF

Method

1. Peel abaxial epidermis from the first fully expanded leaf of *Nicotiana tabacum.*
2. Load with 50 µM H_2DCF-DA (100 mM stock in DMSO) in buffer (50 mM KCl, 10 mM Tris, pH 7.2) for 10 min in darkness.
3. Wash peels by floating on fresh buffer.
4. Immobilise tissue on a microscope slide with silicon grease and immerse in 0.5 ml buffer.
5. Observe and/or measure the rate of fluorescence increase using fluorescein filter sets.
6. Reactive oxygen species can be artificially increased by:
   (i) Addition of exogenous H_2O_2, which gives a rapid and linear increase in fluorescence in the range 1 µM to 5 mM.
   (ii) Addition of Rose bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) at 50 µM. Rose bengal forms singlet oxygen ($^1O_2$) on irraditation with white light (95). In the plant, $^1O_2$ is rapidly converted to O_2^- and H_2O_2.
   (iii) Addition of xanthine (0.5 mM) and xanthine oxidase (0.2 U ml^{-1}) to form O_2^- which dismutes to give H_2O_2 spontaneously or catalysed by SOD (25 U ml^{-1}).

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10 Data analysis

Measurements from photometry systems intrinsically average the signal from a large area (volume) of the specimen. The key stages in analysing the data are to ensure that the dark-current and autofluorescence are correctly measured and subtracted before calculation of the ratio value. In photometry measurements, autofluorescence is estimated from the signal measured either prior to loading dye or after quenching the dye at the end of the experiment for the same measurement area. However, errors can be introduced into photometry measurements from uneven dye distribution within the cells. For example, changes in localised regions of the cytoplasm may be swamped by the large signal derived from the nucleus, which may comprise 30-50% of the total. A pragmatic approach to the autofluorescence problem is to calculate a mean autofluorescence value from many cells and ensure that this autofluorescence is less than 10% of the dye signal from the loaded cell.

Extracting useful data from images is somewhat more complex and there is a wide range of different analysis techniques that can be applied. Images collected at two different wavelengths can be ratioed pixel-by-pixel to generate a ratio image that compensates in principle for varying dye levels, dye leakage and bleaching, and provides a good visual indicator of the magnitude of the response and the level of spatial heterogeneity within or between cells (*protocol 14*). Graphical presentation of data derived directly from a region of the ratio image should be avoided, however. Ratio images are notoriously noisy and it is difficult to interpret the statistics from spatial averaging of the ratio values as ratioing two normally distributed populations gives a highly skewed distribution of ratio values unless expressed on a log scale. Thus it is usually more appropriate to visualise changes using ratio images, but to perform quantitative analysis on the original intensity data from the individual wavelength images directly from regions of interest (ROIs) - *protocol 15*.

Protocol 14. Processing ratio images

1. Align images taken at each wavelength in (x,y) to correct for any minor mis-registration between the two wavelength images. The extent of mis-alignment can be determined by imaging a standard fluorescent bead sample with both wavelengths.

2. If necessary, increase the S/N ratio in the raw images at the expense of spatial resolution by an averaging filter (e.g. averaging over a 3x3 box reduces noise threefold)
3. Subtract the instrument background for each wavelength, measured in the absence of the specimen, from all images.
4. Correction for tissue autofluorescence is more difficult. One approach is to measure auto-fluorescence from an adjacent region of tissue that is unloaded. An alternative is to record an autofluorescence image at a different wavelength that does not interfere with the loaded dye and subtract the appropriate 'bleed-through' component from the dye images.
5. Mask pixels with low values or those outside the object by setting the intensity to zero with a spatially defined mask. Three protocols may be used to define the mask:
   (i) An intensity value at a fixed number of standard deviation (s.d.) units above the mean background intensity, typically 2 s.d. units;
   (ii) The 50% threshold between the fluorescence intensity within the object and the background;
   (iii) A morphological boundary, such as the edge of the cell, defined from a separate image, such as a bright field view.
6. Mask regions in each image that approach saturation of the digitisation range. A pragmatic approach is to measure the distribution of intensities in a fluorescent area at about the concentration of fluorochrome encountered *in vivo* and determine the highest mean value where the distribution is not clipped.
7. Calculate the ratio image pixel-by-pixel and apply the masks.
8. Colour-code the final image using a pseudo-colour look-up-table (LUT) to enhance the viewer's perception of changes, particularly in publications where grey-scale images are not reproduced well. Display the LUT as a wedge with calibration values indicated.

Protocol 15. Quantitative analysis of ratio data from regions of interest

1. Define regions of interest on one image in the pair.
2. Measure the average pixel intensity for this region from both images.
3. Subtract the average background values independently for each ROI at each wavelength.
4. Calculate the ratio of the averaged intensities after background subtraction[a].

[a.] An alternative analysis to estimate the average ratio and the confidence limits can be made using application of Bayes theorem, where *a priori* information can be incorporated into the analysis (76).

11 Attenuation correction for optical sections deep into tissues

The intensity response in the axial (*z*) plane is affected by the axial geometric and chromatic aberrations present along the entire optical path including the specimen. Tissues contain many additional refractive index boundaries which will all contribute to further chromatic and spherical abberation of the confocal probe geometry. The consequences of these effects will be increasing signal attenuation with increasing depth through the specimen. The complex spatial distribution of the refractive material and the overall geometry of the tissue currently prevent development of universal models for tissue-dependent attenuation. However, partial correction can be achieved by a more pragmatic approach based on determination of the axial intensity profile of a permeabilised specimen filled with a fluorochrome ‘sea’ (96) - *protocol 16*. The resultant response combines the effects of depth-dependent ‘sea’ response and the additional contribution of the permeabilised tissue. *In vivo*, the effects are likely to be marginally worse even than this case as a number of refractive boundries, particularly membranes, will be distorted or extracted during the fixation/permeabilisation procedure.

The magnitude of the attenuation will depend on the depth, lens (particularly NA), immersion medium, bathing medium and wavelength (see 96). In structures that have a relatively constant organisation in the *x,y*-plane a single axial correction equation may be sufficient (96). For tissues that show a more complex and variable organisation, a series of correction equations may be required related to each zone of the tissue (92).

Protocol 16. To measure the signal attenuation with depth through a permeabilised specimen infiltrated with a fluorochrome ‘sea’.

1. Fix and permeabilise[a] the specimen in either:
   (i) 4% paraformaldehyde in PBS with 10% DMSO for 30 min
   (ii) fresh ethanol: acetic acid (3:1) for 30 min
   (iii) acetone or methanol at -20°C) for 30 min
2. Rehydrate through an ethanol series (70/50/30/10/PBS) for 30 min each at room temperature).
3. Incubate fixed and permeabilised tissue in PBS containing 10-50 µM of fluorescein or rhodamine B for 24-48 h with gentle agitation to ensure good tissue penetration.
4. Collect axial (*x*,*z*) optical sections simultaneously in fluorescence and reflection mode into two channels through the permeabilised, infused specimen, using the same imaging conditions as used for the experiment. Sampling should start *ca.* 10 µm outside the fluorescent medium and continue at 0.4 -0.5 µm *z*-step intervals through the medium plus specimen.
5. Measure the average fluorescent intensities in regions of the specimen at varying depth to determine the *in situ* 'sea' response. The most useful regions to measure are in vacuoles or spaces between cells where there is a large volume of homogeneous dye concentration.
6. Normalise the attenuation profile to the start of the tissue, defined from the reflection images.
7. Profiles can be fitted with a variety of functions: typically a quadratic or cubic function provides a reasonable description of the attenuation.
8. The inverse of the parametised attenuation profile is used to generate a correction factor to apply to succesive *z*-planes in the experimental data.

[a.] Allowing the dye to penetrate the tissues is more important than high quality tissue preservation, so crude fixation regimes are acceptable.

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Table 1. Representative classes of fluorescent[a] probes for vital staining

Dye	Ex nm	Em nm	ε 10^{-3}	Mode of action
Fluorescein diacetate (FDA)	490	514	88	Cleavage of the acetate groups by intracellular esterases releases free fluorescein that is fluorescent
5-(and-6)-carboxyfluorescein diacetate (CFDA)	492	517	75	Similar to FDA except the additional carboxy group gives better cellular retention
5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCFDA)	495	529	32	Lower pK_a (~4.8) makes carboxy-DCF is less pH sensitive than fluorescein at cytoplasmic pH values
Calcein-AM	494	517	76	Essentially pH-insensitive fluorescein derivative. Fluorescence is quenched by some heavy metals at µM concentrations.
Chloromethyl fluorescein diacetate (CMFDA, CellTracker Green™)	492	517	78	The chloromethyl group makes this a substrate for GST catalysed conjugation to GSH. The conjugate may then be a substrate for transport into the vacuole by glutathione conjugate (GS-X) pumps
CellTracker Green BODIPY (8-chloromethyl-4,4-difluoro- 1,3,5,7-tetramethyl-4-bora- 3a,4a-diaza-s-indacene)	522	529	72	Although the chloromethyl group functions in a similar manner to that in CMFDA, CellTracker Green BODIPY has no ester groups that require cleavage to give fluorescence.
Neutral Red (NR)	541	640	39	NR is a weak amine that accumulates in acidic compartments such as vacuoles. Spectra are pH dependent with a pK_a ~ 6.7.

i. The excitation and emission wavelengths, and absorption coefficient (ε) are given for the fluorescent product of each dye that is released within the cell.

Table 2. Cell permeant nuclear stains used in plant systems

Dye	Stock solution	Loading conditions	Ex nm	Em nm	ε 10^{-3}	Comments
Acridine orange	1 mg ml^{-1} in water, dark	1 µg ml^{-1} for 10-30 min	500	526	53	Labels double stranded DNA green and single stranded RNA red (Ex 460 nm, Em 640 nm). Also accumulates in acidic compartments such as the vacuole.
DAPI	0.1 mg ml^{-1}in water, 4°C, dark	0.1-1 µg ml^{-1} for 10 min	358	461	21	Preferentially binds to AT rich regions with a 20-fold increase in fluorescence
Hoechst 33342	1 mg ml^{-1} in water, 4°C, dark	0.5-5 µg ml^{-1} for 10 min	352	461	45	Preferentially binds to AT-rich regions of DNA with essentially no cytoplasmic labelling
SYTO 11	5 mM in DMSO, -20°C, dark	1-5 µM for 3 h for protoplasts	508	527	75	Similar levels of fluorescence from DNA and RNA.
SYTO 16	1 mM in DMSO, -20°C, dark		488	518	42	Twice as fluorescent bound to DNA as RNA.

Table 3. Vital probes for plant mitochondria

Dye	Stock solution	Loading conditions	Ex nm	Em nm	ε 10^{-3}	Comments
$DiOC_6$	1 mg ml^{-1} in DMSO, -20 °C, dark	0.1 µg ml^{-1}, 10-30 min	484	501	154	Labels mitochonria at low concentrations and ER at higher concentrations
H_2DCF-DA	5 mM stock in ethanol	10 µM	495	529	32	Non-fluorescent until oxidised.
JC-1	1 mg ml^{-1} in DMSO	0.1-1 µM (sparingly soluble)	514	529/ 590	195	JC-1 forms J-aggregates with Abs/Em = 585/590 nm at concentrations above 0.1 µM in aqueous solutions (pH 8.0).
MitoTracker Green FM	1 mM DMSO, -20°C, dark.	100 nM for 30-60 min in dark	490	516	119	Mitochondrial accumulation is insensitive to membrane potential, but gives weaker labelling than the other MitoTracker dyes
Mitotracker Orange CMH_2-TMRos	1 mM DMSO, -20°C, dark. Store under Ar or N_2[c]	500 nM for 15-30 min at 24 °C				Non-fluorescent until oxidised. Localises to the mitochondria and vacuole.
Mitotracker Orange CMTMRos	1 mM DMSO, -20°C, dark	100 nM for 30-60 min in dark	554	576	102	Can be fixed with 4% paraformaldehyde in PBS and retains localisation and fluorescence
Mitotracker Red CMH_2-Xros	1 mM DMSO, -20°C, dark. Store under Ar or N_2[c]	100 nM for 30-60 min in dark				Non-fluorescent until oxidised. Does not appear to label plant mitochondria well
Mitotracker Red CMXRos	1 mM DMSO, -20°C, dark	100 nM for 30-60 min in dark	579	599	116	Can be fixed with 4% paraformaldehyde in PBS and retains localisation and fluorescence
Rhodamine 123	1 mg ml^{-1} in H_2O, MeOH or 10 mg ml^{-1} in DMSO	0.1-10 µM, 10-30 min	507	529	101	More specific for mitochondria at low concentrations than $DiOC_6$

i. The related probe 3,3'-diheptyloxacarbocyanine iodide ($DiOC_7$(3)) is also reported to give good mitochondrial staining in plants (38).
ii. J. Balk, per comm.
iii. Packaged as 50 µg lyophilised solid. Store at -20°C in dark. It is best to make up the stock solution and use on the same day.

Table 4. Fluorescent probes used for vacuolar labelling

Dye	Stock solution	Loading conditions	Ex nm	Em nm	ε 10^{-3}	Comments
Acridine Orange	1 mg ml^{-1} in water	1 µg ml^{-1} for 5 min	489	520	65	Weak amine that accumulates in acidic compartments. Also used as a vital DNA stain
BCECF-AM	1 mM in DMSO	3-10 µM for 10-60 min at 18°-22°C.	503	529	90	Initially fluorescent in the cytoplasm. Can be used as a dual-excitation pH indicator
CDCF-DA	1 mM in DMSO	5 µM for 10-60 min at 18°-22°C.	504	529	90	Labels the vacuole and small vesicles in the cytoplasm. Can be used as a dual-excitation pH indicator
CMAC (CellTracker Blue)	10 mM in DMSO	100 µM 10 min	354	466	14	Conjugated to GSH and transported into the vacuole by a GS-X pump.
CMAC, CBZ-Phe-Arg	20 mM in DMSO or EtOH	20 µM, 3h	353	466	14	Cleavage of the peptide gives a substantial shift in the absorption and emission spectrum.
CMFDA (CellTracker Green)	5 mM in DMSO	50 µM 1 h	475	517	29	Conjugated to GSH and transported into the vacuole by a GS-X pump. Only fluorescent after cleavage of the acetate groups. Can be used as a dual-excitation pH indicator.
LysoSensor Yellow/Blue DND-160	1 mM in DMSO, -20°C, dark	10 µM 1h (medium pH increased to pH 8)	384	540	21	Weak amine that accumulates in acidic compartments. Ratioable pH indicator with pH-dependent shifts in both excitation and emission spectra.
Monochlorobimane (MCB)	5 mM in DMSO	50 µM 1 h	395	477	6	Only fluorescent after conjugation to glutathione. Transported into vacuole by a GS-X pump.
Neutral Red	1 mg ml^{-1} in water	70 µM 20 min	541	640	39	Spectra are pH dependent with a pK_a ~ 6.7.

Table 5. Vital probes for plant endoplasmic reticulum

Dye	Stock solution	Loading conditions	Ex nm	Em nm	ε 10^{-3}	Comments

CTC		50-200 µM for 0.5-1 h	405	530		ER, mitochondria and spherosomes. Also used as a Ca^{2+} indicator.
$DiOC_6$	1-5 mg ml^{-1} in EtOH or DMSO	1-5 µg ml^{-1} for 10-60 min	484	501	154	labels ER, mitochondria and lipid bodies
dodecanoylamino fluorescein (AFC_{12})	1-5 mg ml^{-1} in EtOH or DMSO	2 µg ml^{-1} at 4°-25° C for 10-15 min	495	518	85	labels ER, mitochondria and lipid bodies. The fluorescein moiety confers pH sensitivity to the fluorescence.
NBD-PC[a]	1 mg ml^{-1} in EtOH	40 µg ml^{-1} at 25° C for 10-15 min	465	534	22	labels intracellular membrane after conversion to NDB-DAG by a Ca^{2+}-dependent phospholipase (10)
Rhodamine B hexyl ester	1-5 mg ml^{-1} in EtOH or DMSO	1 µg ml^{-1} at 4°-25° C for 10-15 min	556	578	123	labels ER, mitochondria and lipid bodies. Shows better photostability than $DiOC_6$

iv. Available from Avanti Biochemicals, Birmingham, AL

Table 6. Vital probes for the plasma membrane

Dye	**Stock solution**	**Loading conditions**	**Ex nm**	**Em nm**	**ε 10^{-3}**	**Comments**
FM1-43	5 mg ml^{-1} in DMSO	1 µM	510	626	66	The absorption and emission shift to shorter wavelengths by 20 nm and 80 nm respectively in polar environments
NBD-PE (NBD-phosphatidyl-ethanolamine)	1 mg ml^{-1} in EtOH (may need sonication)	40 µg ml^{-1} at 4°C for 15 min.	463	536	21	Labels internal membranes with time and at higher temperatures (9)
NBD-SM (NBD-sphingosyl-phosphocholine)	1 mg ml^{-1} in EtOH (may need sonication)	2 µg ml^{-1} at 4 °C for 10-20 min	436	566	22	Does not appear to be metabolised to a DAG-derivative and remains in the plasma membrane (10)

Table 7. Fluorescent calcium dyes used in plant cells

Dye	Loading protocol	Ex (nm)		Em (nm)		K_d (μM)	Properties
		-Ca	+Ca	-Ca	+Ca		
BTC	Vacuum infiltration of apoplast	464	401	533	529	7	Dual-excitation, low affinity Ca^{2+} dye, used to measure apoplastic Ca^{2+} levels[a].
Calcium Green-1	Microinjection; low pH; AM-ester	506		531		0.19	Single wavelength dye. Brighter and more stable than Fluo-3.
Calcium Green-1Dextran 10,000	Microinjection	510		535		0.26	Membrane-impermeant version of Calcium Green well retained in the cytosol after microinjection
CTC	Direct incubation	405		530		10-440	Low affinity, permeant Ca^{2+} indicator. Also responds to hydrophobicity, pH, Mg^{2+} (56)
Fluo-3	Microinjection; low pH; AM-ester, detergent assisted	503		525		0.39	Single-wavelength dye with large (40-fold) enhancement in fluorescence on binding $Ca^{2=}$
Fura-2	Microinjection; low pH; AM-ester	363	335	512	505	0.14	Dual-excitation UV-ratioable dye
Fura-2 dextran 10,000	Pressure microinjection	364	338	500		0.24	Membrane-impermeant version of fura-2 well retained in the cytosol after microinjection
Fura-C_{18}	Pressure microinjection	364	338	500		0.15	Measures Ca^{2+} adjacent to membranes but appears to clot the cytoplasm and kill algal cells (17)
Indo-1	Microinjection; low pH; AM-ester	346	330	475	401	0.23	Dual-emission, UV-excited dye. Rather prone to photobleaching
Indo-1 dextran 3,000	Pressure microinjection	341	408	356	466	0.32	Membrane-impermeant version of Indo-1 well retained in the cytosol after microinjection
Quin-2	Microinjection, AM-ester or electroporation	353	333	495		0.06	Now superceeded by other Ca^{2+} dyes
Yellow Cameleon 2	Via transformation	433		480	535	0.07 & 11	FRET-based transgenic cytoplasmic Ca^{2+} indicator (7,8)
Yellow Cameleon 3ER	Via transformation	433		480	535	4.4	FRET-based transgenic cytoplasmic Ca^{2+} indicator targetted to the ER

Table 8. Fluorescent pH dyes used to measure apoplastic, cytoplasmic or vacuolar pH in plant cells

Dye[a]	Ex (nm)		Em (nm)		pK_a	Comments
	Low pH	High pH	Low pH	High pH		
5-(and-6)-carboxy SNAFL-1	508	540	543	623	7.8	Dual-excitation or dual emission pH indicator
5-(and-6)-carboxy SNARF-1[a]	548	576	587	635	7.5	Dual-excitation or dual emission pH indicator
5-(and-6)-carboxy-2',7'-dichlorofluorescein	495	504	529		4.8	Dual-excitation ratio indicator using iso-excitation wavelength ~440 nm for vacuolar pH.
5-(and-6)-carboxy-4',5'-dimethylfluorescein	500	507	537		7.0	Dual-excitation ratio indicator using iso-excitation wavelength ~440 nm.
5-(and-6)-carboxyfluorescein	475	492	517		6.4	Dual-excitation ratio indicator using iso-excitation wavelength ~440 nm for slightly acidic compartments
BCECF[a]	482	503	520	528	7.0	Dual-excitation ratio indicator using iso-excitation wavelength ~440 nm. Used for cytoplasmic and vacuolar pH measurements
Cl-NERF[a]	504	514	540		3.8	Dual-excitation ratio indicator using iso-excitation wavelength ~440 nm for vacuolar pH.
DM-NERF	497	510	527	536	5.4	Dual-excitation ratio indicator using iso-excitation wavelength ~440 nm for vacuolar pH.
HPTS (pyranine)	403	454	511		7.3	Dual-excitation ratio indicator. Used for vacuolar pH measurements and as a symplastic tracer.
LysoSensor Yellow/Blue DND-160	384	329	540	440	4.2	Dual-excitation, dual-emission ratioable pH indicator used for vacuolar pH measurements.
Oregon Green 488 carboxylic acid	478	492	518		4.7	Dual-excitation ratio indicator using iso-excitation wavelength ~440 nm for vacuolar pH.

v. Most dyes can be loaded as AM- or acetate esters, as free acids at varying pH or by iontophoretic microinjection. Dextran-linked versions can be loaded by pressure microinjection and are well retained in the cytosol or can be loaded as apoplastic indicators by vacuum infiltration.

1 Gilroy, S. (1997) *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, **48**, 165.
2 Hepler, P.K. and Gunning, B.E.S. (1998) *Protoplasma*, **201**, 121.
3 Roos, W. (2000) *Planta*, **210**, 347.
4 Srivastava, A. and Krishnamoorthy, G. (1997) *Arch. Biochem. Biophys.*, **340**, 159.
5 Shripathi, V., Swamy, G.S. and Chandrasekhar, K.S. (1997) *Biochem. Biophys. Acta* **1323,** 263.
6 Verbelen, J.P. and Kerstens, S. (2000) *J. Microsc.* **198,** 101.
7 Miyawaki, A., Griesbeck, O. and Tsien, R.Y. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 2135.
8 Allen, G.J., Kwak, J.M., Chu, S.P., Llopis, J., Tsien, R.Y., Harper, J.F. and Schroeder, J.I. (1999) *Plant J.* **19,** 735.
9 Metcalf, T.N.III, Wang, J.L. and Schindler, M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 95.
10 Grabski, S., de Feijiter, A.W. and Schindler, M. (1993) *Plant Cell* **5,** 25.
11 Oparka, K.J., Duckett, C.M., Prior, D.A.M. and Fisher, D.B. (1994) Plant J. **6,** 759.
12 Hush, J.M., Wadsworth, P., Callaham, D.A. and Hepler, P.K. (1994) *J. Cell Sci.* **107,** 775.
13 Köhler, R.H., Cao, J., Zipfel, W.R., Webb, W.W. and Hanson, M.R. (1997) *Science*, **276**, 2039.
14 Xu, C., Zipfel, W., Shear, J.B., Williams, R.M. and Webb, W.W. (1996) *Proc. Natal. Acad. Sci. USA* **93,** 10763
15 Legue, V., Blancaflor, E., Wymer, C., Perbal, G., Fantin, D. and Gilroy, S. (1997) *Plant Physiol*.,. **114,** 789.
16 Wymer, C.L., Beven, A.F., Boudonck, K. and Lloyd, C.W. (1999) In: *Confocal Microscopy: Methods and Protocols.* Ed. S.W. Paddock. Humana Press. Totowa, New Jersey. Pp103.
17 Plieth, C. and Hansen, U.-P. (1996) *J. Exp. Bot.*, **47**, 1601.
18 Gisel, A., Barella, S., Hempel, F.D. and Zambryski, P.C. (1999) *Development*, **126**, 1879.
19 Allen, G.J., Kuchitsu, K., Chu, S.P., Murata, Y. and Schroeder, J.I. (1999) *Plant Cell*, **11**, 1785.
20 Reuveni, M., Lerner, H.R. and Poljakoff-Mayber, A. (1985) *Plant Physiol.*, **79**, 406.
21 Goh, C.-H., Schreiber, U. and Hedrich, R. (1999) *Plant Cell Env.*, **22**, 1057.
22 Tretyn, A., Kado, R.T. and Kendrick, R.E. (1997) *Folia Hist. Cyto.*, **35**, 41.
23 Timmers, A.C.J., Reiss, H.-D. and Schel, J.H.N. (1991) *Cell Calcium*, **12**, 515.
24 Coleman, J.O.D., Hiscock, S.J. and Dewey, F.M. (1994) *Physiol. Mol. Plant Path.*, **43**, 391.
25 Wright, K.M., Horobin, R.W. and Oparka, K.J. (1996) *J. Exp. Bot.*, **47**, 1779.
26 Yin, Z.-H., Neimanis, S., Wagner, U. and Heber, U. (1990) *Planta*, **182**, 244.
27 Yin, Z.-H., Hüve, K. and Heber, U. (1996) *Planta*, **199**, 9.
28 Knoblauch, M. and A.J.E. van Bel (1998) *Plant Cell* **10,** 35.
29 Haseloff, J. (1999) *Meth. Cell Biol.* **58**, 139
30 Huang, C.-N., Cornejo, M.J., Bush, D.S. and Jones, R.L.(1986) *Protoplasma*, **135**, 80.
31 Brauer, D., Uknalis, J., Triana, R. and Tu, S.-I. (1996) *Protoplasma*, **192**, 70
32 Timmers, A.C.J., Tirlapur, U.K. and Schel, J.H.N. (1995) *Protoplasma* **188,** 236.
33 Bethke, P.C., Lonsdale, J.E., Fath, A. and Jones, R.L. (1999) *Plant Cell*, **11**, 1033.
34 Genty, B. and Meyer, S. (1994) *Aust. J. Plant Physiol.*, **22**, 277.
35 Oxborough, K. and Baker, N.R. (1997) *Plant, Cell Env.*, **20,** 1473.
36 Robertson, D. and Earle, E.D. (1987) *Plant Cell Reports*, **6**, 70.
37 Masters, B.R. and Chance, B. (1999) In: *Fluorescent and Luminescent Probes for Biological Activity.* Ed. W.T.Mason. 2nd Ed. Academic Press, London. Pp 361-374.
38 Liu, Z., Bushnell, W.R. and Brambl, R. (1987) *Plant Physiol.* **84,** 1385.
39 Vannini, G.L., Pancaldi, S., Poli, F. and Fasulo, M.P. (1988) *Plant Cell Env*. **11,** 123.
40 Maxwell, D.P., Wang, Y. and McIntosh, L. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 8271.
41 Hutzler, P., Fischbach, R., Heller, W., Jungblut, T.P., Reuber, S., Schmitz, R., Veit, M., Weissenböck, G. and Schnitzler, J.-P. (1998) *J. Exp. Bot.*, **49**, 953.
42 Swanson, S.J., Bethke, P.C. and Jones, R.L. (1998) *Plant Cell*, **10**, 685.
43 Rea, P.A., Li, Z.-S., Lu, Y.P., Drozdowicz, Y.M. and Martinoia, E. (1998). *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 727.
44 Klein, M., Martinoia, E. and Weissenböck, G. (1997) *FEBS Lett.*, **420**, 86.
45 Cole, L., Coleman, J.O.D., Kearns, A., Morgan, G. and Hawes, C. (1991) *J. Cell Sci.*, **99**, 545.
46 Schmit, A.-C. and Lambert, A.-M. (1990) *Plant Cell*, **2**, 129.
47 Zhang, D., Wadsworth, P. and Hepler, P.K. (1993) *Cell Motil. Cytoskel.*, **24**, 151.
48 Braun, M. and Wasterneys, G.O. (1998) *Planta*, **205**, 39.
49 Zhang, D., Wadsworth, P. and Hepler, P.K. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8820.

50 Hepler, P.K., Cleary, A.L., Gunning, B.E.S., Wadsworth, P., Wasteneys, G.O. and Zhang, D.H. (1993) *Cell Biol. Int.* **17,** 127.
51 Wymer, C.L., Shaw, P.J., Warn, R.M. and Lloyd, C.W. (1997) *Plant J.*, **12**, 229.
52 Carroll, A.D., Moyen, C., Van Kesteren, P., Tooke, F., Battey, N.H. and Brownlee, C. (1998) *Plant Cell,* **10**, 1267.
53 Brownlee, C., Manison, N.F.H. and Anning, R. (1998) *Exp. Biol. Online,* **3**, 11.
54 Van Kesteren, W.J.P. and Tempelaar, M.J. (1993) *Cell Biol. Int.*, **17**, 235.
55 Camacho, L., Parton, R., Trewavas, A.J. and Malhó, R. (2000) *Protoplasma* **212**, 162.
56 Plieth, C., Sattelmacher, B., Hansen, U.-P (1998) *Planta*, **207**, 42.
57 Muhling, K.H., Wimmer, M. and Goldbach, H.E. (1998) *Physiol. Plant.*, **102**, 179.
58 Bush, D.S., Biswas, A.K. and Jones, R.L. (1989) *Planta,* **178**, 411.
59 Callaham, D.A. and Hepler, P.K. (1991) In J.G. McCormack, P.H. Cobbold, Eds., *Cellular Calcium - A Practical Approach*. Oxford University Press. Pp 383-410.
60 Fabiato, A. (1991) In J.G. McCormack, P.H. Cobbold, Eds., *Cellular Calcium - A Practical Approach*. Oxford University Press. Pp 159-176.
61 Bers, D.M., Patton, C.W. and Nuccitelli, R. (1994) *Meth. Cell Biol.*, **40**, 3.
62 Haugland, R.P. (1999) *Handbook of Fluorescent Probes and Research Chemicals.* Molecular Probes, Eugene, Oregon., 7th Ed.
63 Plieth, C., Sattelmacher, B. and Hansen, U.-P. (1997) *Protoplasma,* **198**, 107.
64 Kao, J.P.Y. (1994) *Meth. Cell Biol.*, **40**, 155.
65 Feijó, J.A., Sainhas, J., Hackett, G.R., Kunkel, J.G. and Hepler, P.K. (1999) *J. Cell Biol.*, **144**, 483.
66 Plieth, C., Sattelmacher, B., Hansen, U.-P. and Thiel, G. (1998) *Plant J.*, **13**, 167.
67 Felle, H.H. and Hepler, P.K. (1997) *Plant Physiol.*, **114**, 39.
68 Speksnijder J.E., Miller, A.L., Weisenseel, M.H., Chen, T.H. and Jaffe, L.F. (1989) *Proc. Natl. Acad. Sci. USA,* **86**, 6607.
69 Pierson, E.S., Miller, D.D., Callaham, D.A., Shipley, A.M., Rivers, B.A., Cresti, M. and Hepler, P.K. (1994) *Plant Cell,* **6**, 1815.
70 Knight, H. and Knight, M.R. (1995) *Meth. Cell Biol.*, **49**, 201.
71 Knight, H., Trewavas, A.J. and Knight, M.R. (1997) In: *Plant Molecular Biology Manual* (eds. SB Gelvin & Schilperoort) Kluwer Academic Publishers. **C4**, 1.
72 Messerli, M. and Robinson, K.R. (1997) *J. Cell Sci.*, **110**, 1269.
73 Scott, A.C. and Allen, N.S. (1999) *Plant Physiol.*, **121**, 1291.
74 Bibikova, T.N., Jacob, T., Dahse, I. and Gilroy, S. (1998) *Development,* **125**, 2925.
75 Roos, W., Evers, S., Hieke, M., Tschöpe, M. and Schumann, B. (1998) *Plant Physiol.* **118**, 349.
76 Parton, R.M., Fischer, S., Malhó, R., Papasouliotis, O., Jelitto, T.C., Leonard, T. and Read, N.D. (1997) *J. Cell Sci.*, **110**, 1187.
77 Messerli, M.A. and Robinson, K.R. (1998) *Plant J.*, **16**, 87.
78 Mühling, K.-H., Plieth, C., Hansen, U.-P. and Sattelmacher, B. (1995) *J. Exp. Bot.*, **46**, 377.
79 Taylor, D.P., Slattery, J. and Leopold, A.C. (1996) *Physiol. Plant.*, **97**, 35.
80 Hoffmann, B. and Kosegarten, H. (1995) *Physiol. Plant.*, **95**, 327.
81 Pfanz, H. and Dietz, K.-J. (1987) *J. Plant Physiol.*, **129**, 41.
82 Brauer D., Otto J. and Tu, S.-I. (1995) *J. Plant Physiol.*, **145**, 57.
83 Pheasant, D.J. and Hepler, P.K. (1987) *Eur. J. Cell Biol.*, **43**, 10.
84 Brauer, D., Uknalis, J., Triana, R. and Tu, S.-I. (1997) *Plant Physiol. Biochem.*, **35**, 31.
85 Franchisse, J.-M., Johannes, E. and Felle, H.H. (1988) *Biochem. Biophys. Acta,* **938**, 199.
86 Reid, R.J. and Whittington, J. (1989) *J. Exp. Bot.*, **40**, 883.
87 Lindberg, S. (1995) *Planta,* **195**, 525.
88 Mühling, K. and Sattelmacher, B. (1997) *J. Exp. Bot.*, **48**, 1609.
89 Browne, B.A., McColl, J.G. and Driscoll, C.T. (1990a) *J. Environ. Qual.*, **19**, 65.
90 Vitorello, V.A. and Haug, A. (1997) *Plant Science,* **122**, 35.
91 Coleman, J.O.D., Randall, R. and Blake-Kalff, M.M.A. (1997) *Plant Cell Env.*, **20**, 449.
92 Fricker, M.D., May, M., Meyer, A.J., Sheard, N. and White, N.S. (2000) *J. Microsc.* **198**, 162.
93 Meyer, A.J. and Fricker, M.D. (2000) *J. Microsc.* **198**, 174.
94 Allan, A.C. and Fluhr, R. (1997) *Plant Cell,* **9**, 1559.
95 Knox, P. and Dodge, A.D. (1984) *Plant Sci. Lett.*, **37**, 3.
96 White, N.S., Errington, R.J., Fricker, M.D. and Wood, J.L. (1996) *J. Microscopy,* **181**, 99.