Fluorescence and luminescence techniques to probe ion activities in living plant cells

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Introduction

Fluorescent probes offer almost unparalleled opportunities to visualize and quantify dynamic events within living cells, tissues or even organs with a minimum of perturbation. Although single cells or mono-layers maintained in culture can be readily imaged, measurements are often needed from cells within intact tissues. These cells are operating in their correct physiological context of cell-wall and cell-cell interactions. Imaging technology and dye-loading approaches have now progressed to where such *in planta* experiments are a real possibility. A range of different measurement techniques is now available to quantify fluorescence signals from reporter molecules within biological specimens. These include fluorimetry, flow cytometry, microscope photometry, camera, confocal and two-photon imaging. In addition, luminescence reporters, such as aequorin, are available to measure calcium using photomultiplier-based luminometer systems or photon-counting camera systems. Each of these approaches performs well for a specific range of measurement conditions and specimens. Thus, several techniques may need to be applied to provide a sufficiently flexible balance between the spatial, temporal and spectral resolution required to understand the physiological questions in the cell(s) of interest. Although the imaging and detector technology has rapidly advanced, two real problems remain. First, the techniques to introduce ion-selective probes into plant cells are not straightforward. Second, many of the reporter dyes do not behave predictably in the plant cell cytoplasm. Transgenic approaches using aequorin measurements have circumvented many of the problems associated with loading Ca^{2+} indicators. More recently, transgenic fluorescent calcium indicators have become available that exploit fluorescence resonance energy transfer (FRET) between diferent spectral forms of 'green' fluorescent protein (Miyawaki *et al.*, 1997). These probes offer great potential to combine the advantages of protein engineering and fluorescence techniques. Currently, however, the field of fluorescence and luminescence imaging of plant cell activities remains one fraught with potential artifacts and few generalized protocols. Most studies have required an extensive period of trial and error to determine what will, and will not, work with a particular cell type. Despite these limitations, perseverance has been rewarded by fascinating glimpses into cellular regulation. Coupling these imaging approaches to techniques such as caged probe technology has begun to allow us both to observe and to manipulate these cellular processes in the ultimate test-tube setting, the cell itself. The following sections provide guidelines on how to apply these approaches to plant specimens and how to identify, and hopefully avoid, many of the unique problems associated with quantitative fluorescence imaging and manipulation of plant cells.

Tissue preparation, mounting and perfusion

The experimental systems used by plant biologists are diverse and often unique, and so the direct application of technology developed for preparing animal specimens for ion imaging has not been straightforward. Optical techniques place certain constraints on the type of tissue that can be examined. Some of the best specimens for microscopy are flat and submerged in water. Thus unicellular, filamentous or relatively flat organisms with restricted 3-D growth are readily observed, particularly if they are aquatic. Fluorescence measurements can also be made on cells one to three cell layers deep within intact tissues using confocal optical sectioning and probably twice as deep using 2-photon imaging. To expose the cells of interest even further into more bulky higher plant tissue may involve peeling, excision, dissection or the formation of protoplasts. All of these approaches will cause a certain amount of 'wound' induced artifacts that must be carefully tested for. An alternative for Ca^{2+} measurements is to monitor the luminescence from the Ca^{2+}-dependent photoprotein aequorin. Plants can be transformed with the apoaequorin gene and active aequorin reconstituted *in situ.* Thus, the Ca^{2+}-dependent aequorin signal can be imaged from entire transgenic plants (although most of the signal may well be derived from the surface layers).

The extent of the trauma induced during sample preparation is difficult to define. Plant cells normally alter their metabolic poise continuously in response to changes in their surroundings. For example, the mere act of touching the plant when mounting it in a sample chamber may elicit Ca^{2+}-signals in the stimulated cells (e.g. Knight *et al.*,1991; Legue *et al.*, 1997) and alter gene expression patterns (e.g. Braam *et al.*, 1992). Depending on the system, the extent of physiological response caused by sample preparation should be assessed from measurement of parameters such as membrane potential, cytoplasmic streaming, progression through division, growth rate or gene expression. The contribution of such perturbation to subsequent measurement may be limited by minimising the extent of the manipulation and allowing an adequate recovery time (generally 15-60 min) after mounting the sample.

Microscope-based measurements are generally preferred over cuvette-based measurements as they provide additional spatial information. This is particularly important if there are localised responses, gradients or substantial heterogeneity in the population of cells examined. In addition, continuous observation can also provide critical information on the state of the tissue and is essential in many cases to monitor responses such as stomatal closure or cytoplasmic streaming. Observation of the tissue at the cell and sub-cellular level typically requires magnification 100-600 fold. The best clarity or brightness of image is achieved with high numerical aperture (NA) microscope objectives and immersion of the specimen. To maintain viability the immersion medium is usually water. 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.

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. Continuous perfusion of this medium is preferred to simple immersion as it minimises boundary layers, reduces anoxic stress and allows rapid solution changes. For aquatic organisms, cultured cells or tissues grown in agarose, continuation of the growth conditions is usually sufficient, though the concentration of certain ions may need careful consideration. For example, Mn^{2+} can enter through Ca^{2+}-channels and quench fluorescence from intracellular Ca^{2+} sensitive dyes and may need to be omitted from the perfusion

medium. Other heavy metals may also interfere with the behaviour of both dyes and luminescent proteins.

Perfusion conditions are more difficult to define for aerial tissues as the turgor relations, apoplastic activities for ions such as H^+, Ca^{2+} and K^+, and the prevailing hormonal status are usually unknown. In addition, the waxy cuticle of aerial tissues may significantly reduce dye uptake. The consequences of immersion of aerial tissues include: (i) build up of regions depleted in O_2 and enriched in CO_2 next to non-photosynthetic cells and vice versa for actively photosynthesising tissue; (ii) dilution of apoplastic ions and equilibration of local ion gradients; and (iii) increased turgor. There are only a limited number of reports examining the significance of these changes (e.g. Mühling *et al.,* 1995).

The temperature is normally set near ambient (20-25ºC) through temperature control of the perfusion medium, without additional recourse to a temperature-regulated stage. In a non-perfused system, the sample temperature is likely to increase due to prolonged illumination and this will lead to temperature related artifacts. Good IR-blocking filters are particularly important when a Xenon light source is to be used. In addition, the composition of the air above open perfusion systems may need to be regulated and screened to prevent interference from CO_2 exhaled by the microscope operator. It can also not be stressed enough that when designing and using perfusion systems extreme care must be taken to ensure that liquid does not leak into the microscope where it may permanently damage delicate optical coatings and lens elements.

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. Various approaches may be appropriate depending on whether an upright or inverted microscope is used and the nature of the tissue, including:

1. Using silicone grease (e.g. vacuum grease, M494, ICI)as an adhesive leaving a clear window in the grease for observation and measurement.
2. Silicone contact adhesive (e.g. Corning 355 - Blatt, 1991)
3. Protoplasts, tissue culture cells and pollen tubes often adhere to clean cover-slips or more strongly after 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.
4. 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).
5. Immobilization in agarose or Phytagel™ (Gellan gum agar substitute, Sigma, Dorset): Isolated cells and protoplasts can be stabilized on cover slips with a thin film of 1-2 % (w/v) low melting point agarose (Hillmer *et al.*, 1993; Parton *et al.*, 1997). A typical protocol for *Arabidopsis* roots is as follows: roots can be grown in place by planting seeds in nutrient media (see Legue *et al*, 1997 for composition of nutrient medium) with 0.5-1% Phytagel™. The media is autoclave for 25 min and poured to 1 mm depth onto cover slips contained in sterile Petri dishes. The gel should polymerize at room temperature within 10 min. Seeds are planted by pushing through the gel onto the surface of the cover slip. This ensures that the root grows along the surface of the cover slip and therefore can be

imaged. The same planting scheme can also used for both low pH and AM-ester loading of intact *Arabidospsis* roots (Legue *et al.*, 1997; Wymer *et al.*, 1997).

6. Immobilisation in gelatin: Concentrations of gelatin up to 18% (w/v) gelatin made up in nutrient media are useful for embedding *Schizosaccharomyces pombe* or *Saccharomyces cerevisiae*. Samples are mixed with molten gelatin warmed to 40°C and spread thinly on a prewarmed coverslip.
7. Immobilization in alginate: Alginic acid at concentrations ~1.5% form a gel at room temperature in the presence of excess (mM) $CaCl_2$ that can be used to trap protoplasts or cells. However, the high Ca^{2+} concentrations may perturb the cell physiology.
8. Mechanical clips or restraints can be used to secure large cells or tissues (e.g. Plieth, 1995).

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 (Reuveni *et al.*, 1985) and hot agarose or gelatin may heat shock protoplasts before it cools enough to form a gel (Gilroy, unpublished). 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.

Micro-injection requires an open perfusion system, although cells can be loaded prior to observation and mounted in a closed perfusion system. Inverted microscopes are an ideal platform for microinjection of thin, relatively transparent tissue. Upright systems with objective rather than stage focusing, offer significant advantages for thick, opaque specimens such as roots or leaves, as observation must take place from the same side as loading. The arrival of long working distance, water immersion lenses have reduced the steric problems of microinjection on upright systems, except when a steep angle of penetration is needed (around 45° is currently possible).

Selection and use of fluorescent probes

The basic principle of intracellular ion measurement using non-protein fluorescent probes involves introduction of a chelating agent whose fluorescent properties alter with the activity of a particular ion. The change in fluorescence is measured and then converted to an estimate of the change in ion level using some form of calibration. The change may be a simple quantitative alteration in fluorescence intensity (single wavelength dye) or a shift in either the excitation or emission spectrum (ratiometric dye). A wide range of dyes are now available and their properties described in Chapter #. Applications of many of these dyes in plant cells have been reviewed recently (Gilroy, 1997). Dyes with spectral shifts are preferable as they permit ratio measurements that distinguish fluorescence changes due to ion binding from those due to dye leakage, bleaching or uneven distribution (Grynkiewicz, Poenie and Tsien, 1985).

There are several features that influence selection of a particular dye for measurements in plant cells including:

1. The ion to be measured. Dyes to monitor Ca^{2+}, H^+, K^+ and Na^+ have all been used successfully in plant cells, but many more are available.
2. The possibility of interference by changes in other ions in the cell of interest.
3. 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.

4. The dynamic range of the dye response.
5. The ease of loading the dye into a defined compartment, usually the cytosol.
6. The behaviour of the dye within the cell, including compartmentalization, metabolism and physiological perturbation.
7. 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).
8. The level of autofluorescence of the tissue at the measurement wavelengths.
9. The instrument configuration for dual-excitation or dual-emission dyes.
10. Compatibility with other optical techniques, such as UV photolysis of caged compounds.

Loading strategies for plant cells

The objective is to introduce the probe into a defined compartment, usually the cytoplasm, of as many cells as possible in sufficient concentration to give good signal to noise without causing toxic effects or significantly disturbing the cellular ion buffering. In the latter case it is important to consider not just the magnitude of the increase in buffering capacity but also whether the increase in the buffer mobility of the dye will disrupt local gradients in ion activity (Neher and Augustine, 1992). Loading the dye into the cytoplasm of plant cells has proved difficult (Cork, 1986; Bush and Jones, 1990; Callaham and Hepler, 1991; Read *et al.*, 1992; Fricker *et al.*, 1994; Gilroy, 1997). A variety of strategies have emerged, but there are no simple rules as to which will be most effective with a particular tissue. There are essentially nine approaches. The least invasive techniques that load a population of cells are preferred but unfortunately do not work with many plant cells.

1. Permeant dyes

The most extensively used permeant dye for Ca^{2+} measurements is chlorotetracycline (also known as aureomycin, chlortetracycline or CTC). CTC is a fluorescent, lipophilic antibiotic isolated from *Streptomyces aureofaciens* that shows enhanced fluorescence upon binding divalent and trivalent cations (Ex. 400 nm, Em 530 nm). CTC is readily membrane permeant and cells are simply loaded by incubation with 10-200 µM dye in an acidic environment (pH 4.5 - pH 6.5). CTC has provided a useful first step in identifying cells where changes in 'membrane-associated' Ca^{2+} levels occur (e.g. Caswell, 1979; Reiss and Herth, 1978; Tretyn and Kopcewicz, 1988; Timmers *et al.*, 1989). In most cases the results obtained with CTC have been reproduced with other, more sophisticated methods (see for example Reiss and Nobiling, 1986; Rathore *et al.*, 1991; Timmers *et al.*, 1991; 1996). However, CTC possesses some properties which render its use difficult and which often cause the results of Ca^{2+} measurements being questioned:

- CTC is sensitive to Mg^{2+} ions (Gupta and Berkowitz, 1989).
- CTC fluorescence increases with increasing pH: a fivefold change occurs between pH 5.5 and pH 8.5.
- CTC is a lipophilic dye which predominantly binds to membranes (Schneider *et al.*, 1983). Thus, the Ca^{2+} level monitored by the CTC fluorescence is mainly due to the Ca^{2+} ion concentration near membrane surfaces (e.g. Meindl, 1982; Polito, 1983).
- The CTC k_d for Ca^{2+} depends strongly on the polarity of the environment. For instance, it changes from 440 µM (in pure water) to 9 µM (in 70% methanol) (Caswell and Hutchison, 1971). Thus, as the polarity in living biological systems is unknown, the fluorescence of CTC cannot be calibrated in terms of absolute Ca^{2+} concentration.
- The temperature dependence of fluorescence emissions between 4°C and 35°C is linear. $(\delta F/F)/\delta T = 2.1\ \%\ ^oC^{-1}$).

- CTC is an antibiotic which exerts toxic effects especially on prokaryotes (Caswell and Hutchison, 1971). There are also some toxic effects on eukaryotes (e.g. Foissner, 1991).
- CTC stimulates an increased flux of Ca^{2+} into the cell.
- CTC increases the sensitivity of cells to damage. For example, during impalement by microelectrodes a progressive depolarization of about 1 mV min^{-1} was observed which sometimes led to death of the cell (Plieth, 1995).

2. Ester loading

Most of the ion selective dyes are impermeant due to one or more carboxyl groups in the molecule which are charged in the physiological pH range. 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. However, there are numerous problems with applying the ester loading technique to plant cells (see notes).

Protocol

1. For AM loading, prepare a 1 mM stock solution of the AM-ester indicator dye with DMSO as the solvent (AM-ester forms of indicator dyes specially packaged in dry DMSO are also available from Molecular Probes). Store aliquots of dye at -20°C and avoid exposure to light.
2. Dilute the indicator stock solution to 1-5 µM with deionized water or media prior to use and incubate cells for 10-120 min.
3. Wash out excess dye and observe cells under an epifluorescence microscope.

Notes:

- Acetoxymethyl (AM) ester loading works well with some plant cells (Gehring *et al.*, 1990a,b; Dixon *et al.*, 1989; Williams *et al.*, 1990; Fricker *et al.*, 1997b; Parton *et al.*, 1997), but many mature tissues appear to load poorly (Gilroy *et al.*, 1991; Hodick *et al.*, 1991). For example, fluorescence of BCECF is visible in intact *Arabidopsis* roots within 2-3 min of application in the AM-ester form. However, the AM-ester forms of indo-1 and calcium green-1 do not appear to work, possibly because of the larger number of ester groups (ca. 5) that are clustered together and require cleavage.
- Problems may arise if no hydrolysis or incomplete hydrolysis releases only partially activated fluorescent intermediates with different spectral properties (Scanlon *et al.*, 1987; Highsmith *et al.*, 1986; Elliot and Petkoff, 1990).
- The hydrolysis of AM-esters generates acetic acid and formaldehyde which may exert negative effects on the cells. The DMSO in the stock solution may also cause toxic effects.
- Mild detergents (e.g. 0.02%-0.2% Pluronic F-127- Gehring *et al.*, 1990b), temperature, ATP permeabilization, increased external pH (Elliot and Petkoff, 1990) and varying ionic conditions may facilitate AM-ester loading.
- The esterified dye can permeate all cell compartments and the free dye may accumulates in any organelle which has esterases (e.g. mitochondria - Almers and Neher, 1985; Cobbold and Rink, 1987; Roe *et al.*, 1990; Rathore *et al.*, 1991; Brauer *et al.*, 1996). Esterase inhibitors, such as eserine, may also help prevent external hydrolysis of the dye and improve loading (Tretyn *et al.*, 1997).
- After release in the cytoplasm dyes may be sequestered to the vacuole rapidly (sometimes within 5 to 10 min of application) and hence cannot be used for cytoplasmic

measurements (although see Kosegarten *et al.*, 1997). Instead, BCECF loaded as the AM-ester has been used successfully for ratiometric determination of vacuolar pH changes in protoplasts (Swanson and Jones, 1996) and intact roots (Brauer *et al.*, 1995). In these experiments, the dye is used well below its pK_a in a region of the ratio titration curve that is normally relatively insensitive to pH.

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 (ion sensitive) form.

Protocol

1. Make a 1 mM stock solution of the salt form of the dye using deionized water. Aliquot into small volumes. Store at -20°C and avoid exposure to light.
2. Dilute the stock solution prior to use in 25 mM dimethyglutaric acid (DMGA), pH 4.5 to a final dye concentration of 20 to 50 µM.
3. For plant 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 plants in the dark for 1 to 2 h.
5. Wash out unloaded dye by rinsing in nutrient media.

Notes:

- 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 (Gilroy *et al.*, 1991). This is not always the case (Wymer *et al.*, 1997; Legue *et al.*, 1997) but it is not clear why tissues differ. Charge masking with high levels of other ions might reduce this problem.
- Increased temperature and the prescence of saponin may assist dye loading in some tissues (Tretyn *et al.*, 1997).
- 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 (Kramer and Jones, 1996) or allowing the roots to grow into a gel-free zone prior to incubation in the dye reduces this problem.
- Low pH loading has been successful with some protoplasts types (Bush and Jones, 1987), but not others (Gilroy *et al.*, 1986).
- Certain cells do not survive low pH treatment (Elliot & Petkoff, 1990; Hodick *et al.*, 1991) and the physiological consequences of pH stress on pH regulation and signaling need careful scrutiny.
- If dye penetration appears to be a problem, access of the dye to the tissue can be facilitated by a cutinase pretreatment. Tissues are incubated with purified cutinase activities ranging from 0.1-10 µmol min^{-1} mg^{-1} protein for 5-30 min at pH 7.6 in 10 mM Tris-HCl and then washed in buffer (Fricker *et al.*, 1994). Cutinase can be prepared according to Kolattukudy *et al.* (1981) and Coleman *et al.* (1994). The cutinase gene has also been cloned and expressed (Soliday *et al.*, 1984).

4. 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 require careful optimization. Many cells do not survive and the remainder are loaded with variable concentrations of dye. A major part of any set of electroporation experiments is optimizing the electrical pulse protocol to maximize permeabilization whilst maintaining viability.

Protocol:

1. Sediment 1 ml of protoplasts at 1-5 x g for 15 min, in a 1.5 ml Eppendorf tube. Remove supernatant and resuspend in 0.8 ml of electroporation buffer (200 mM sorbitol, 300 mM mannitol, 1 mM Mg^{2+}, 100 mM KCl, 10 mM HEPES, pH 7.2). Repeat washing step and resuspend protoplasts to a final concentration of 0.5 x 10^6 ml^{-1} in buffer prechilled to 4°C. Store on ice.
2. Electroporate with systematically varied electroporation pulse characteristics: field strength (0.1- 5 kV cm^{-1}); number of pulses applied (1-5) and capacitor used (1-50 µF). Assess permeabilization efficiency as the percentage of protoplasts staining with ethidium bromide (0.1% w/v, Ex. 510 nm Em. 620 nm) within 5 min. Optimize for the lowest voltage, and briefest pulses giving 60-80% permeabilization (Gilroy *et al*., 1986).
3. Repeat steps 1-3 using the optimized pulse protocol but excluding the ethidium bromide staining. Incubate electroporated protoplasts on ice for 10 min and then add concentrated medium to restore the composition of buffer to the full strength of the normal protoplast culture medium (e.g. full strength MS medium). Cover cuvette with parafilm and mix by gently inverting the cuvette 5 times.
4. Incubate for 15 min at room temperature and transfer protoplasts to culture chamber.
5. After 1 h incubation test for viability with fluoresecein diacetate staining (FDA, Huang *et al.*, 1986). Viable protoplasts will become intensively fluorescent (Ex. 480 nm, Em. 530 nm) after 2-5 min incubation with 0.05% (w/v) FDA. Optimize the pulse protocol established in step 2 to yield >80% viability.
6. Repeat steps 1-4 until percentage permeabilization and maintained viability are optimized.
7. To load with dye repeat steps 1-4, electroporating using the optimized pulse protocol in step 2 in the presence of 20 µl of 1 mM Indo-1.

Notes

- The method is not straightforward and is likely to perturb sensitive signalling systems.
- The electroporated protoplasts need to be carefully analyzed with independent cellular assays of function to ensure the electroporation process has not altered cellular responses.
- The osmolarity of the electroporation buffer (mannitol and sorbitol concentrations) will need to be modified to be compatible with each protoplast system used.
- Ethidium bromide slowly penetrates intact cells, therefore it is important to run a non electroporated control to assess how fast this occurs with each cell type.

5. Ionophoretic Microinjection

The cell is impaled with a fine microelectrode containing charged dye and the dye is loaded into the cell by application of a small current or current pulses (see Blatt, 1991; Callaham and Hepler, 1991).

Protocol

1. Microelectrodes are pulled from filamented borosilicate glass capillaries (e.g. GC150F or GC120F, Clark Electromedical Instruments, Reading, UK) to give a tip diameter of 0.1-0.3 µm and resistances in the range of 10 to 20 MΩ.
2. Dilute the stock solution of the dye in free acid form to 0.1-1 mM with deionized water and fill the microelectrode with 2-5 µl using a 5 µl Hamilton syringe or a plastic pipette tip pulled to a long, fine taper. Backfill the rest of the pipette electrode with 1M KCl.
3. Connect the impalement electrode to a micro-iontophoresis current generator and place the reference electrode in the medium.
4. Impale the cell by carefully advancing the microelectrode. A 4-axis manipulator is very useful to help penetrate plant cell walls.
5. Loading can be achieved by diffusion from the microelectrode tip (ca. 20-30 min). Iontophoretic injection with continuous current (e.g. 2 nA for 10-20 s) or pulsed current (3-4.5 nA in 2-3 s pulses) is much more rapid.
6. Carefully withdraw the microelectrode over 3-10 min. In some cases, waiting for a few minutes before withdrawing the electrode can improve cell survival. Allow cells to recover for 30 to 60 min. Cell mortality most often occurs during pipette removal.

Notes:

- Microinjection requires a lot of patience and the number of cells that can be successfully loaded is limited. Aspects such as the angle of impalement, micropipette shape and exactly where to impale the cell all need to be optimised for each cell type. In elongated plant cells, the cytoplasm occupies a very thin region between the vacuole and the cell wall. Between 60 to 80% of injections appear to load the cytoplasm rather than the vacuole and the difference in dye distribution between the two compartments can be readily observed.
- Filtering solutions through a 0.22 µm filter before use may help to reduce tip blockage.
- Care needs to be taken to minimize the disruption caused by passing the iontophoretic current into the cell. Currents of tens of nA into a small plant cell are often highly damaging.
- Mild plasmolysis prior to injection may help reduce blockage of the electrode as less cytoplasm is forced up the electrode by the reduced turgor pressure.
- The impalement pipette can also function as a microelectrode for measurement of the plasma membrane potential to follow the extent of cell disruption and recovery during the injection procedure (van der Shoot and Lucas, 1995). Additional ions such as KCl or K-acetate may be needed to be added to the dye solution in the pipette to reduce the tip resistance and tip potentials for reliable membrane potential measurements.
- Dye can be loaded from multiple-barrelled electrodes (Blatt, 1991) with the circuit entirely between the electrodes, minimising the disturbance to the membrane potential (Grabov and Blatt, 1997). This configuration also permits simultaneous voltage-clamping and fluorescence ion measurements.

- For elongating cells, continued growth and maintenance of turgor is one way to assess viability. Unfortunately more than 50% of microinjected cells show loss of turgor, granularity of the cytoplasm and subsequent cessation of growth (Legue *et al.*, 1997).
- It may be convenient to microinject several cells and then select the best cell for measurement after the end of the recovery period. However this may not be suitable for growth related studies on whole organ since impaling several cells may cause stress to the plant and affect interpretation of results.
- Iontophoresis is not usually effective for large or uncharged molecules. Electro-osmosis of dyes may also be possible (Erhardt *et al.*, 1996) where the flow of water caused by movement of ions in the iontophoretic current carries with it large and uncharged molecules into the cell.

6. Pressure microinjection

Pressure injection can be used for large or uncharged molecules. Notably, the problem of subsequent dye compartmentation into organelles can be avoided by pressure injection of dextran-conjugated form of the dyes. The cell is impaled with a wider microelectrode and the pipette solution loaded into the cell by application of hydraulic pressure.

Protocol:

1. Pull microelectrodes from borosilicate capillaries (e.g. GC 150F) to give a tip diameter of 0.3-1 µm and back-fill with a dextran-conjugated form of the dye.
2. Insert microelectrode into a holder and connect to a continuous pressure injection system. These systems apply pressure to the pipette using either air pressure (e.g. Plieth and Hansen, 1996; Legue *et al.*, 1997) or an oil-filled displacement system (Oparka *et al.*, 1991).
3. Prior to penetration increase the holding pressure that is continuously applied to the pipette to 0.1-0.2 MPa to counteract cell turgor. The holding pressure is usually unnecessary if injecting protoplasts.
4. Impale the cell and load the dye using a series of 0.14-1.2 MPa pressure pulses or a gradual increase in pressure until the dye exits the pipette. The precise pulse protocol is highly dependent on the cell being injected.
5. Slowly withdraw the microelectrode and allow the cells to recover for 30 to 60 min.

Notes:

- Pressure injection is the easiest way to load giant cells like *Chara*, *Nitella* or *Eremosphaera* (Plieth and Hanson, 1996; Plieth *et al.*, 1997).
- Although a syringe can be used to apply the injection pressure, control over the duration and intensity of injection pressure pulses afforded by a commercial pressure injection regulator greatly improves the injection success rate.

7. Loading from patch electrodes

Fluorescent dyes, dextran-conjugated dyes and even proteins can be loaded into protoplasts in the whole cell patch-clamp configuration by diffusion of the contents of the patch pipette into the cell. The conditions for enzymatic protoplast isolation and successful gigaseal formation have to be determined for the protoplasts to be studied. The following is the protocol optimized for patch clamp loading of the dye Indo-1 into stomatal guard cell protoplasts from *Vicia faba* to simultaneously monitor Ca^{2+} levels and K^+ channel activities (Romano and

Assmann, unpublished). Precise bath and pipette solutions will need to be optimized for the cells and channel activities to be studied.

Protocol

1. The bath solution comprises 10 or 100 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ 10 mM MES, pH 5.6, osmolality adjusted to 460 mosmol Kg^{-1} with sorbitol. The patch pipette is filled with 80 mM K^+-glutamate, 20 mM KCl, 2 mM Mg-ATP, 10 mM HEPES, pH7.2 adjusted to 500 mosmol Kg^{-1} with sorbitol and 60 µM Indo-1 pentapotassium salt. Filter the pipette solution through 0.2 µm syringe filter prior to use and store on ice in the dark.
2. The resistance of the patch pipettes should be 6-10 MΩ rather than the typical 15-20 MΩ of a normal patch experiment in these protoplasts to increase the rate of dye diffusion form the pipette. Stable dye loading should occur in 5-10 min and initial seal formation is reduced by only 20% under these conditions.

Notes

- The larger pipettes required than normally used for patch clamping potentially means loss of cell constituents and channel run down may be accelerated. However, using these conditions robust Ca^{2+} signals and channel activities last for more than 1 hour in guard cell protoplasts.
- Care is needed to shield the patch clamp amplifiers from electrical noise generated by the imaging or photometry system. Also filter wheels and shutters may generate vibrations that cause seals to be lost. This problem is somewhat alleviated by mounting the microscope and patch clamp manipulators on an independent vibration isolation table and coupling the illumination system *via* fiber-optics.

8. Laser ablation and loading via patch electrodes

An alternative to enzymatic production of protoplasts for patch clamping is to reveal a small region of naked plasma membrane by laser ablation of the overlying wall. This has the advantage of maintaining the cells in its almost intact setting y*et al*lowing access to a localised region of the plasma membrane. Loading of dyes through the cell attached patch pipette is identical to that outlined above. The following is a brief outline of the protocol to laser ablate and patch a stomatal guard cell of *Vicia faba*. Details of the laser ablation equipment are reviewed in detail elsewhere (DeBoer *et al.*, 1994; Henriksen *et al.*, 1996; Henriksen and Assmann, 1997).

Protocol

1. Epidermal peels are mounted in a perfusion chamber on an inverted microscope and perfused with 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ 10 mM MES, pH 5.6
2. The guard cells are plasmolyzed in the same medium with osmolality adjusted to 460 mosmol Kg^{-1} with sorbitol.
3. The guard cell wall is ablated with 5-10 2 ns pulses of 337 nm light from the ablation laser.
4. The preparation is de-plasmolyzed using a slow decrease in osmolality provided by a linear gradient maker allowing a small 'bleb' of exposed membrane to protrude through the hole in the wall, taking care not to rupture the membrane.
5. The bleb is patch clamped as normal.

Notes

Treating the wall with the UV absorbing dye Calcofluor White may improve absorption of the laser and improve ablation efficiency (Henriksen *et al.*, 1996). Blebs of *Vicia faba* guard cells are relatively easy to patch, but this may not be true for all systems (DeBoer *et al.*, 1994).

9. Loading via detergent permeabilization

Somatic embryos have been loaded by incubation in the presence of 100 µM ion indicating dye and then treating with a low concentration of Triton X-100 (0.1% v/v) to permeabilize the plasma membrane (Timmers *et al.*, 1991). The detergent is then washed from the sample to allow the membranes to reseal. It is likely that detergent permeabilization will disrupt many cellular processes and signaling activities, at least in the short term.

It should be stressed at this point that it is important to have accessible markers of cell function to compare in loaded and unloaded cells. These may include parameters such as membrane potential, cytoplasmic streaming, growth rates, elongation rates, cell division rates, and gene expression. Alternative strategies include the monitoring of cell viability using other 'vital' staining techniques, such as fluorescein diacetate (Huang *et al.*, 1986) which probe different aspects of membrane integrity and metabolic activity.

Intracellular dye distribution and concentration

The cytoplasm in a mature plant cell typically occurs as a thin layer less than 1 µm thick sandwiched between the vacuole and the wall, with larger accumulations localised around the nucleus and chloroplasts. In young, rapidly growing cells and some specialised cells, such as tips of root hairs and rhizoids, a greater contiguous volume of cytoplasm may occur, uninterrupted by vacuoles. The distribution of dye follows the distribution of cytoplasm and often appears uneven. The nucleus appears to accumulate high concentrations of many dyes. Confocal microscopy indicates that this signal is genuinely located within the nucleus, not in the peri-nuclear cytoplasm. The majority of reliable measurements are derived from regions rich in cytoplasm where fluorescence signals are strongest.

Once successfully loaded into the plant cell the fluorescent dye may interfere with the normal function of the cells as the concentration is increased (Wagner and Keizer, 1994). In addition, the interaction of illumination with the fluorescent dye may cause photo-toxic 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 of the ion to be measured and reduce any potential non-specific chemical or photo-chemical side-effects. It is also possible to incorporate free radical scavengers such as ascorbic acid at (0.1-1 mg ml^{-1}), Trolox (vitamin E), carotenoids (see Tsien and Waggoner, 1995) or deplete oxygen levels by modification of the chamber atmosphere or adding Oxyrase (Oxyrase Inc., P.O. Box 1345, Mansfield, OH 44901). However, these treatments can dramatically affect the physiological status of the tissue, particularly the redox equilibrium of the cells and are not recommended for live preparations.

Usually the best way forward is to ascertain the upper limit of dye loading consistent with minimal disruption of cell physiology. Typically for calcium or pH dyes this is about 3-50 µM intracellular dye. Cytoplasmic dye concentrations have been estimated by comparison with the signal from known concentrations of dye confined to cell-sized volumes, either as droplets in immersion oil or enclosed in micro-cuvettes (e.g. rectangular glass capilliaries,

W5005, Vitro Dynamics Inc. Rockaway, NJ, USA). To generate droplets with a range of sizes, vigorously vortex 100 µl of immersion oil (Fisher Scientific, Type FF) with 5 µl of dye solution in a microfuge tube and transfer to a slide. In confocal measurements, the volume sampled is much better defined and measurement from calibration solutions can be directly related to dye concentrations inside cells, provided the contribution from depth- and sample-dependent attenuation is taken into account (White *et al.,* 1997; Fricker *et al.,* 1997a).

Maintaining dyes in the cytoplasm

Compartmentalisation of the dyes into organelles is a major problem with plant tissues (Callaham and Hepler, 1991; Oparka, 1991; Read *et al.,* 1992) and often limits the time window when cytosolic ion activities are faithfully reported. The tissue distribution and phloem mobility of many dyes can be partly explained using structure-activity relationships (SAR) based on parameters such as the log octan-1-ol/water partition coefficient (log*P*), pK_a, conjugated bond number (*CBN*), charge and molecular weight of the dye (Wright *et al.,* 1996). However, the models are less predictive for subcellular dye distributions, implying either specific transport steps are involved or, in the case of esterified dyes, the esterases are localised in different cells and compartments (Brauer *et al.,* 1996). Dyes are sequestered in the vacuole in many tissues with a variable time constant, from less than 10 min to longer than than 48 h. Several of the dyes are highly charged at physiological pH values, suggesting that this transport step probably involves specific transporters. These may include multi-drug-resistance pumps (e.g. Dudler and Hertig, 1992), glutathione(GS-X) pumps (Ishikawa *et al.,* 1997) or sulphonate transporters (Klein *et al.,* 1997). The signal from the vacuole is complicated by the low pH and ionic conditions, which will alter both the K_d of the dye and its fluorescent properties.

Dye may also accumulate in other organelles such as the endoplasmic reticulum, which has a high (>10 µM) lumenal Ca^{2+} activity (Bush *et al.,* 1989). However, the error introduced into cytosolic measurements is likely to be slight due to the limited amount of dye in the lumen (2% of the total in barley aleurone - Bush *et al.,* 1989; Bush and Jones, 1990), though it may present a problem in the interpretation of 'hot-spots' of Ca^{2+} seen in imaging experiments.

Dye may also be lost across the plasma membrane by an unknown mechanism, particularly during permeabilisation treatments for *in situ* calibration. Leakage rates may be 25% hr^{-1} or higher, but are highly cell type specific and must be empirically determined.

In some tissues such as pollen tubes the levels of compartmentalisation can be estimated after 'selective' permeabilisation of the plasma membrane with digitonin (100 µM) to release cytosolic dye (e.g. Fricker *et al.,* 1997b). All intracellular dye should be subsequently released by exposure to 0.1 % (v/v) Triton X-100.

Techniques to prevent or minimise compartmentalisation

1. Use of dextran linked dyes usually (but not always - see Read *et al.,* 1992) reduces compartmentalisation. The dye is usually covalently linked to a 10 kD dextran but requires pressure microinjection to load it into the cytoplasm. This approach remains the method of choice to test for compartmentalization artifacts in plant cells.
2. Changing the temperature, time and dye concentration may extend the useable window for measurements.

3. Choosing cells without large vacuoles or analyzing regions of large cells where the vacuole is absent minimises this difficulty, providing the experimenter is confident that small vacuoles are not being overlooked.
4. Anion channel blockers such as probenecid are known to prevent compartmentalisation of some negatively charged dyes such as lucifer yellow and FITC (e.g. Cole *et al.*, 1991), but have not yet been employed during ion measurements. The effect of these blockers on cell physiology is essentially unknown.
5. Optical-sectioning, using confocal or two-photon microscopy, can separate signals from the cytoplasm and vacuole in situations where compartmentalisation does occur and may be used to measure cytoplasmic and vacuolar ion activities simultaneously. However, the practical resolution even of these techniques means that signals from ER or small vacuoles are unlikely to be well resolved from a cytosolic background under physiological conditions.

Observation and measurement of dye fluorescence

The primary objective of quantitative physiological measurements is to maximise the signal to noise (S/N) ratio with minimal disruption to the cell physiology. A variety of measurement systems are currently available, offering a balance between cost, sensitivity, spatial resolution, temporal resolution and sampling rate. The details of these systems are described elsewhere in this book. The main principle for botanical work is to match the hardware to the experiment. Some plant processes occur over long time periods (minutes, hours and days) and involve co-ordinated interaction of many cells in the tissue, for instance the tropic responses of plant organs. Phenomena in plants that take place in the seconds to minutes or longer range do not require the sophistication of sub-second temporal resolution. Photometry systems have a significantly higher (orders of magnitude) S/N ratio that imaging systems and measurements can made at second or sub-second rates. Integration over a 1-10 s sampling interval gives respectable signal to noise for camera-based imaging applications, though intermittent sampling and shuttering of the illumination may be required to minimise photobleaching and photodamage during extended periods of data collection. The inherent S/N ratios for confocal or two-photon systems are much lower than other systems as the volume of dye sampled and the pixel dwell time are reduced. The available measurement systems are summarized in Table 1. Each has its own advantages and disadvantages related to the spatial and temporal sensitivity and cost. Use of several observation techniques provides complementary data characterising both spatial and temporal components of the processes in the cell of interest.

Table 1: Comparison of measurement techniques used to monitor ion activities in plant cells.

Measurement technique	Comments
Fluorimetry	MONITORS: Population of (single) cells in suspension. Sampling is rapid (interval c. 0.05 s or better) Spectra are easy to measure. Autofluorescence is easy to correct. DISADVANTAGES: No spatial resolution in measurement. (Although some spatial information can be collected using dyes for specific compartments or cellular domains). Heterogeneous responses cannot be distinguished. Signals from dead and dying cells also included.

Flow cytometry	MONITORS: Population of single cells in suspension. Sampling is rapid but of different cells for each data point. Potential for preparative sorting cells by response. DISADVANTAGES: No spatial resolution. Needs robust cells. Heterogeneous responses appear as an increase in variance.
Micro-photometry	MONITORS: Typically single cells. whole cells or sub-cellular regions in large cells can be measured. Average measurement defined by a (variable) mechanical aperture. The specimen or aperture may be moved to sample different regions. Sampling is rapid (interval 0.05s or better minimum, typically 1s). Requires microscope but allows simultaneous observation of cells. Autofluorescence correction straightforward. Relatively inexpensive. DISADVANTAGES: Prone to errors from heterogenous dye distribution and redistribution
Camera imaging	MONITORS: Single cells, a population of cells or cells in 'thin' tissues. Sub-cellular regions typically down to 0.3-0.4 µm in (*x*,*y*) however (*z*) is poorly defined (although it may be possible to remove out-of-focus blur by deconvolution). Dual-excitation is easy to implement with a single camera, however, simultaneous dual-emission requires split-view optics or two cameras. Allows mapping of spatial heterogeneity and transients. DISADVANTAGES: Sampling interval typically every 1-2 s. More expensive systems can run at video rate. Sampling may need to be intermittent to reduce photobleaching. May require extended integration to increase S/N. Autofluorescence subtraction is difficult. Expensive.
Confocal microscopy	MONITORS: Single cell, population of cells or cells in intact tissue. The (*x*,*y*) and (*z*) resolution are relatively well defined (maximum - 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 from milliseconds for a line scan, ms to seconds for 2-D section and seconds to minutes for 3-D data stack. Measurements are possible within intact tissue with sub-cellular resolution and reduced out-of-focus blur. Simultaneous dual-emission imaging is easy to implement. DISADVANTAGES: Excitation wavelengths are limited by available lasers. There are few ratioable visible dyes for Ca^{2+}, conversely many ratio dyes require a UV laser system. Very expensive to very, very expensive for UV systems.
2-photon imaging	MONITORS: Single cell, population of cells or cells in intact tissue. Similar spatial and temporal resolution to a confocal system with red illumination. The depth penetration into thick tissues is much better compared to a conventional confocal system. UV dyes can also be excited with long (red) wavelength illumination, minimising tissue damage. Excitation is restricted to the focal point, minimising photobleaching. DISADVANTAGES: 2-photon excitation spectra are much broader than 1-photon spectra and not yet well defined. Very, very, very expensive. May cook the specimen.

Controls for physiological measurements

Once the cells are loaded with the appropriate dye, the image collection protocol can be optimised. Keeping cells alive may be at odds with optimal sampling. Measurements of ion concentrations rarely require spatial resolution greater than ~0.5 µm in (*x,y*) although the improved (*z*) resolution in confocal and 2-photon systems is an advantage in interpreting the results. It is important to optimise the instrument to maximise 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 illumination presented at the sample is probably the most critical parameter. For example, values between 76 µW (Tsien and Waggoner, 1995) and 20 µW (Errington *et al.* 1996) are appropriate for confocal microscopy using high NA lenses to give acceptable S/N ratios and cell viability whilst maintaining adequate spatial sampling and scan speeds. It is not easy to predict the appropriate intensities for other systems and specimens, however, it is useful to be able to measure the illumination intensity when the imaging conditions have been optimised to act as a guide for other experiments. 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.

These steps require good markers of cell function and physiological response.

Data analysis

Meaurements 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. This method has found wide application in both conventional and confocal imaging (Chapters ### see also Fricker *et al.*, 1997a) and provides a good visual indicator of the magnitude of the response and the level of spatial heterogeneity within or between cells.

1. The S/N ratio in the raw images can be increased at the expense of spatial resolution by an averaging filter (e.g. averaging over a 3x3 box reduces noise by 3) or collecting the data at lower spatial resolution initially.
2. Images taken at each wavelength can be manually aligned in (*x,y*) to correct for any minor mis-registration between the two wavelength images. Objective criteria are required to perform this alignment based on, for example, imaging a standard fluorescent bead sample with both wavelengths. However, a simple image translation cannot correct for magnificational changes between two wavelengths arising from chromatic aberration.
3. The instrument background, measured in the absence of the specimen, should be subtracted from all images.
4. Correction for tissue autofluorescnce 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 different wavelengths that do not intefer with the loaded dye and subtract the appropriate 'bleed-through' component from the dye images.
5. Pixels with low values or those outside the object are normally masked and excluded from the ratio image 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 (e.g. Errington *et al.* 1996);
   (iii) A morphological boundary, such as the edge of the cell, defined from a separate image, such as a bright field view.
6. Masking is also required to exclude values in each image approaching saturation of the digitisation range. Saturation is related to an assessment of the number of photons contributing to the signal and requires knowledge of the conversion from photons to grey-levels. 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. The ratio image is calculated pixel-by-pixel and the mask applied.
8. Pseudo-colour look-up-tables are often used to enhance the viewers perception of changes, particularly in publications where grey-scale images are not reproduced well.

Notes

- Graphical presentation of data derived directly from a region of the ratio image should be avoided. Ratio images are notoriously noisy and it is difficult to interpret the statistics from spatial averaging of the ratio values. Ratioing two normally distributed populations gives a highly skewed distribution of ratio values. 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 with an average area analysis as described below.

Measurements on regions of interest

A series of regions of interest (ROI) are defined on one wavelength image and the average intensity and standard deviation measured from that region. The corresponding region is measured on the other wavelength image with appropriate image or area alignment if necessary. In some cases it is useful to segment the object using a mask prior to area measurements to facilitate measurements from regions encompassing irregular structures,

such as cytoplasmic strands or sub-cellular compartments, without recourse to detailed manual delimitation of the area. A useful objective criteria to segment the object is the 50% intensity level between the object and the background.

1. The average background values are subtracted independently for each ROI at each wavelength.
2. The average ratio is calculated and displayed graphically.
3. Error bars can be calculated from the standard deviation of the individual wavelength data from each ROI. If the 90% confidence limits of the individual wavelengths are used, the ratio values will have 81% confidence limits, assuming the variability in both populations arises only from uncorrelated noise.
4. Alternatively, an estimate of 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 (Parton *et al.,* 1997).

Notes

- Sample autofluorescence correction is almost impossible with imaging techniques as cytoplasmic streaming constantly moves organelles, potentially causing spatial re-organisation of autofluorescence. Partial correction can be made in some cases by sampling autofluorescence in corresponding regions of a similar non-loaded cell (Gilroy, *et al.*, 1991).

Calibration *in vitro* and *in situ*

The accuracy of absolute measurements depends to a large extent on calibration and becomes increasingly important when small quantitative differences rather than large qualitative changes in ion concentration between cells are thought to be significant. The best method would be to make an *in situ* calibration because all parameters in the cell which would affect the dye fluorescence and the ratio would be automatically considered. For *in situ* calibration the plasma membrane has to be made selectively permeable for the measured ion. Ionophores have been successfully used for this purpose in animal cells. These chemicals lead to an electrochemical equilibrium between bathing medium and cytoplasm (Williams and Fay 1990, Bright *et al.*, 1989). The fluorescence ratio is measured as the external concentration of the ion is varied. Often *in situ* calibration is only performed at two points, usually to determine the maximum and minimum ratio (Groden *et al.* 1991, Rathore *et al.* 1991). After application of the ionophore two different ion concentrations are established in the biological system; at a very low concentration R_{min} and F_{max2} are obtained, and at a saturating concentration R_{max} and F_{min2} are measured. However, in this case the measured ratios can be converted to concentration values only if the dissociation constant of the dye k_d is exactly known inside the cell. This is often not the case in plant cells.

A second major problem in plant cells is that ionophores do not equilibrate ion concentrations to a sufficient or reproducible extent across the plasma membrane (Bush and Jones 1987, Felle *et al.* 1992). 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. There is some confidence if both *in situ* and *in vitro* calibrations match (Brownlee *et al.* 1987; Brownlee & Pulsford 1988; Gilroy *et al.*, 1991; McAinsh *et al.* 1992; Mühling *et al.* 1995).

However, this is often not the case (Gilroy *et al.*, 1989) and it is not obvious which calibration is more appropriate. One of the most appropriate solutions to this conundrum is to independently measure the calcium level in the same system using a different technique such as a calcium-selective electrode (e.g. Felle and Hepler, 1997).

In vitro calibration - calcium dyes

Calcium dyes may be calibrated *in vitro* using Ca^{2+}-EGTA buffers to set [Ca^{2+}] in a solution designed to mimic the plant cytosol. Calibration is simple to perform and calibration kits are now available from companies such as Molecular Probes and World Precision Instruments. *In vitro* calibration of single wavelength dyes can be used to define the dynamic range expected for the dye response under the collection conditions used for the experiment. In is not possible to use an *in vitro* calibration for a single wavelength dye to calibrate the Ca^{2+} response *in vivo* as it is only valid for a single concentration and defined path length. For ratio dyes, an *in vitro* calibration provides an indication of the [Ca^{2+}] *in vivo*, but the appropriateness of the calibration depends primarily on the degree of correlation with conditions experienced by the dye in the cytosol.

Protocol

1. A typical buffer contains 100-120 mM KCl, 0-20 mM NaCl, 1mM $MgSO_4$, 10 mM HEPES pH 7.2, 1-10 µM dye and 10 mM EGTA plus appropriate amounts of $CaCl_2$ to give the required free [Ca^{2+}].
2. The actual free Ca^{2+} is usually calculated using an iterative computer program that accounts for all the ionic interactions in the calibration buffer (Vivaudou *et al.*, 1991; Fabiato, 1988). These authors distribute their programs free of charge.
3. Measurements can be made using volumes of calibration solution similar in size to loaded cells in the experimental apparatus.
4. Experimentally derived values are calibrated using the following equation (Grynkiewicz *et al.* 1985, Ameloot *et al.* 1993):

$$\left[Ca^{2+}\right]_{cyt} = K_x \cdot \left(\frac{R - R_{\min}}{R_{\max} - R}\right) = K_d \cdot \left(\frac{R - R_{\min}}{R_{\max} - R}\right) \cdot \left(\frac{F_{\max \lambda 2}}{F_{\min \lambda 2}}\right) \quad (1)$$

   Where R is the measured ratio, R_{min} and R_{max} are the ratio values at zero and saturating calcium levels, respectively, K_d is the (assumed) 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 calcium, respectively.
5. It is more convenient to re-arrange equation (1) for use with a non-linear curve fitting package as follows:

$$R = \frac{R_{\min} - R_{\max}}{(1 + 10^{-(pCa - pk_x)})} + R_{\max} \quad (2)$$

   The measured ratio at each pCa value can be fitted by this function with R_{max}, R_{min} and pK_x, as free running parameters (so called Boltzmann fit) (Plieth *et al.*, 1997). The fitted sigmoidal parameters can be used to estimate the cytoplasmic calcium concentration from the ratios measured in the living system:

Notes

- The *in vitro* calibration solutions should be as similar as possible to the cytoplasmic milieu. For example, when calibration curves obtained with and without Mg^{2+} were compared, the apparent dissociation constants for Fura-dextran ranged from 400-1000 nM measured with Mg^{2+} and 100-250 nM measured without Mg^{2+} (Plieth *et al.*, 1997).
- Ionic strength, viscosity and hydrophobicity of the medium have all been identified as potential factors that influence the response of these dyes (Poenie, 1990; Roe *et al.*, 1990; Uto *et al.*, 1991).
- Viscosity may be increased by addition of 20-60 % sucrose (e.g. Zhang *et al.*, 1991) or 500 mM mannitol (e.g. Dixon *et al.* 1989) and hydrophobicity altered with 25% ethanol (Russ *et al.*, 1991).
- Judicious selection of wavelengths where the dye spectrum is less susceptible to potential interference has been suggested. For example, Fura-2 can be measured using the ratio of 340 nm and 365 nm rather than the normal 340 nm and 380 nm (Roe *et al.*, 1990).
- 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 (Poenie, 1990; Owen, 1991).
- The spectra for dextran conjugates is 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 (Haugland, 1996). For example, Fura-dextran has a dissociation constant around 350 nM compared to 150 nM for Fura-K5 and the peak in the excitation spectrum for the calcium free form is shifted to shorter wavelengths (from 380 nm to 364 nm).
- Most dyes show a pH dependence of K_d (e.g. Lattanzio and Bartschat, 1991; Lattanzio, 1990; Roe *et al.*, 1990). For example, the apparent K_d of Fura becomes more and more dependent on the proton concentration below pH 7. Calcium concentrations estimated from the measured ratios have to be corrected by a pH-dependent factor X_{corr} that is detemined from an exponential fit to the K_d values determined by Lattanzio (1990). See Plieth *et al.*, (1997) for full details.

$$K_d = K_{d0} \cdot X_{corr} \tag{3}$$

$$\text{where } X_{corr} := \left(1 + 9 \cdot \exp - \left(\frac{(pH - 5{,}45)}{0{,}5}\right)\right) \tag{4}$$

The significance of the pH error will depend on the prevailing cytoplasmic pH value, which is often unknown. However, this correction becomes of increasing importance in experiments where cytoplasmic pH is varied using for example, weak acid loading, or where steep pH gradients have been proposed e.g. at the tip of algal rhizoids.

In situ calibration - calcium dyes

Dye loaded cells are permeabilized with a Ca^{2+}-ionophore or by detergent treatment at the end of the experiment. Cytosolic $[Ca^{2+}]_{cyt}$ is set by extracellular Ca^{2+}-EGTA buffer solutions. In principle, cytosolic conditions experienced by the dye should not have changed significantly between the calibration and *in vivo* measurements of $[Ca^{2+}]_{cyt}$ during an experiment. Results from single wavelength dyes are difficult to interpret as there is no inherent correction for dye leakage, redistribution or bleaching. However, dyes are available across a wide range of wavelengths and show substantial changes in fluorescence.

Protocol

1. Add 1 to 10 µM ionomycin or Br-A23187 (a non-fluorescent analogue of A23187) at the end of the experiment.
2. Increase external calcium to ~1 mM and allow the signal to stabilise at R_{max} (ratiometric dye) or F_{max} (single wavelength dye).
3. Replace the high calcium medium with medium containing 1 mM EGTA to set R_{min} or F_{min}.
4. Calculate $[Ca^{2+}]_{cyt}$ using published K_d values or K_d values measured *in vitro* using equation (2) for ratio dyes or the following equation for single wavelength dyes:

$$[Ca^{2+}] = K_d \frac{(F - F\min)}{(F\max - F)} \qquad \text{(Kao } \textit{et al.}\text{, 1989)} \qquad (5)$$

Notes

- Ionomycin has been reported to be much less effective than Br-A23187 in plant cells (Bush and Jones, 1987; Gilroy *et al.*, 1991). This may reflect its requirement for alkaline (pH 9) rather than acidic (< pH 7) conditions normally encountered in perfusion solutions (Liu and Hermann, 1978).
- An alternative to determining F_{min} for Fluo-3 using EGTA buffers is to use Mn^{2+} to quench the fluorescence from the dye. Conveniently, Mn^{2+} is efficiently transported into cells from external concentrations of 0.1-1 mM $MnCl_2$ during incubation with 10 µM ionophore for 10 min. Fluo-3 fluorescence is quenched to 8x F_{min}. This set point can then be used in a modified form of the above equation (Kao *et al.*, 1989; Minta *et al.*, 1989). Our experience in calibrating Fluo-3 in plant cells (Gilroy *et al.*, 1990) is that the Mn^{2+}-quench procedure is more consistent than determining F_{min} with EGTA. Even so, calibration is extremely difficult to perform accurately and in general data from single wavelength dyes is more qualitatively useful than quantitatively accurate.

In situ calibration - ratio pH dyes

The approach for calibration of pH dyes such as BCECF is essentially similar to those outlined for calcium dyes. 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. Dixon *et al.*, 1989; Thiel *et al.*, 1993; Fricker *et al.* 1997b; Parton *et al.*, 1997).

Protocol

1. At the end of the experiment the external $[K^+]$ is increased to a value close to the anticipated internal $[K^+]$, typically 100-120 mM.
2. Nigericin is added to a final concentration of 10 µg ml^{-1} and the pH adjusted to pH <6.0 (for BCECF).
3. The ratio values are allowed to stabilise (2-15 min) and R_{min} measured.
4. The external pH is shifted to pH 8.5 to give R_{max} and the ratio allowed to stabilise.
5. pH values are estimated from a sigmoidal fit to the *in situ* calibration data with an assumed K_d (typically pH 7.0-7.2 for BCECF) or the pH is estimated from equation 2 substituting appropriate values for the pH dye used and assuming that nigericin equilibrates pH as follows:

$$\frac{[H^+]_{in}}{[H^+]_{out}} = \frac{[K^+]_{out}}{[K^+]_{in}} \qquad (6)$$

Notes

- *In situ* calibrations give very poor agreement with *in vitro* measurements as the K_d is rather sensitive to the local environment (Dixon *et al.,* 1989). Additions such as de-proteinised coconut water supplemented with 1% ovalbumin improve the overlap between *in situ* and *in vitro* calibrations (Pheasant and Hepler, 1987).
- 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 (Pheasant and Hepler, 1987; Fricker *et al.,* 1997b; Parton *et al.,* 1997).
- As it is difficult to shift pH to R_{max} and R_{min} *in situ,* an alternative calibration between pH 6.5 and pH 7.8 can be used to cover the near-linear region of the ratio response.
- Ionophore treatments stress cells rapidly (Pheasant and Hepler, 1987). Calibration can also be based on equilibration of permeant weak acids and bases (Pheasant and Hepler, 1987; Gehring *et al.,* 1990a,b; Parton *et al.,* 1997).
- Parallel measurements using pH sensitive microelectrodes provide an independent check of the fluorescence calibration (e.g. Gibbon and Kropf, 1994)

Additional measurement techniques

The manganese quench technique

It is possible to extend the basic measurement of cytosolic calcium concentration using fluorescent dyes to identify the possible source of the Ca^{2+} leading to the increase in cytosolic Ca^{2+}. In principle there are three different sources for an increase in Ca^{2+}, namely: influx across the plasma membrane; release from internal stores, such as the vacuole, ER, mitochondria or chloroplasts, or from changes in cytoplasmic Ca^{2+} buffering capacity arising from cytosolic pH changes, for example. Manganese ions (Mn^{2+}) can permeate at least some types of Ca^{2+} channels (Striggow and Ehrlich, 1996; Fasolato *et al.,* 1993; Piñeros and Tester, 1995) and also bind Ca^{2+}-indicator dyes with a very high affinity (K_d Mn^{2+}-Fura ≈ 5 nM whilst K_d Ca^{2+}-Fura ≈ 250 nM) quenching their fluorescence (McAinsh *et al.,* 1995; Malhó *et al.,* 1995; Zottini and Zanoni, 1993; Gilroy and Jones, 1992; Thomas and Delaville, 1991). It is possible to use these properties to discriminate between Ca^{2+} influx and Ca^{2+} mobilization from internal stores by introducing Mn^{2+} into the external medium. Opening of plasma membrane Ca^{2+} allows Mn^{2+} influx and consequent fluorescence quenching. In the case of Fura, this can be readily measured at the iso-excitation wavelength around 360 nm (e.g. Plieth *et al.,* 1998).

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 (Grabov and Blatt, unpublished) or by microinjection into the vacuole (Plieth *et al.,* 1998). Under these conditions, fluorescence quenching indicates release of Mn^{2+} from the loaded stores rather than flux across the plasma membrane.

Dissapation of intracellular calcium gradients using buffers with varying pK_d

In certain cell types, most notably tip-growing cells, a steep tip-focussed gradient in calcium correlates with tip growth (e.g. Pierson *et al.,* 1996). One technique that has been used to test the importance of the Ca^{2+}-gradient is to disupt it using microinjection of calcium buffers of

varying K_d derived from substitutions on the 1,2-*bis*(o-aminophenoxy)ethane *N,N,N',N'*,-tetraacetic acid (BAPTA) moiety. These buffers are thought to increase the mobility of calcium in the cytoplasm, particularly if the K_d falls between the concentrations expected at the high and low pointsof the gradient (Speksnijder *et al.*, 1989; Pierson *et al.*, 1994).

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 (Franchisse *et al.*, 1988). The weak acid (usually 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 (assuming the anion is membrane impermeant) and a proton is released. 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:

$$BA^{-}_{cyt} = \frac{10^{(pH_c - pH_o)}}{1 + 10^{(pk_d - pH_o)}} \cdot HBA_0 \qquad (7)$$

Obviously there occurs a massive accumulation of acid anions in the cytoplasm. With the assumption that the weak acid releases in the cytoplasm as many BA^- ions as protons ($BA^-_{cyt} = \Delta H^+$), equation (9) 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.

The weak acid can be washed out of the cell and, at low concentrations, exhibits no severe side effects on the cell physiology. Thus, application of weak acids allows pH_{cyt} to be brought under experimental control and can be used to pH-clamp the cytoplasm (e.g. Thiel *et al*., 1993)

Notes:

- 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 distribution models require that no transport systems exist for the anion and that the molecule is not metabolised.
- Weak acid loading will affect the pH in all compartments of the cell and will lead to massive accumulation of anions in even more alkaline compartments, such as chloroplasts.
- It would be naïve to expect that the cell does not respond to such dramatic perturbation of pH and during extended weak acid loading treatments, significant changes in ion transport systems has been reported (Reid and Whittington, 1989).
- Iso-butyrate is considerably less smelly than butyrate.

Using recombinant aequorin for measurement of intracellular calcium in plants

Apoaequorin is a single polypeptide chain of approximately 22 kDa isolated from the coelenterate *Aequoria victoria*. Apoaequorin combines with a low molecular weight luminophore called coelenterazine to form functional aequorin in a process termed reconstitution. Molecular oxygen is also required at this stage. The aequorin molecule is similar in structure to the calcium-binding protein calmodulin and possesses 3 calcium - binding EF hand domains (analagous to the 4 EF hands of calmodulin) and a binding site for coelenterazine and 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, though these ions may depress the Ca^{2+}-sensitivity (Thomas, 1982). 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 several potential advantages over fluorescent dyes as an indicator for $[Ca^{2+}]_{cyt}$. Luminescence measurements usually have an intrinsically high signal to noise ratio as there is relatively little endogenous luminesecence under optimal conditions. However, it is important to ascertain the level of endogenous chemiluminescence as this may comprise up to 30% of the signal in plant cells (Gilroy *et al.*, 1989). 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. Supplies of the apoprotein have increased since the gene for aequorin has been cloned (Prasher *et al.*, 1985; Inouye *et al.*, 1985), although the essential co-factor coelenterazine is still expensive.

Introduction of aequorin into cells

Aequorin can be microinjected into cells, however, the method is suitable only for large cell types such as the giant green alga *Chara* (e.g. Williamson and Ashley, 1982). Pressure injection is used in preference to iontophoresis to overcome the low iontophoretic mobility of the 20kD apo-protein. Premature discharge of the aequorin in the micropipette by Ca^{2+} in the perfusion medium leads to a reduction in the amount of active probe introduced into the cell and hence the signal measured. This can be avoided by either forcing a slow constant stream of aequorin out of the pipette when approaching the cell or plugging the end of the pipette with a small amount of vegetable oil that can be expelled just prior to injection. It is also possible to inject the apoprotein only and then reconstitute *in planta*.

More recently the problems of introducing aequorin into the cytoplasm have been elegantly solved using recombinant DNA technology to transform plant cells with the cDNA for the apoprotein (Knight *et al.*, 1991; 1995). Aequorin can be reconstituted *in vivo* by adding coelenterazine to transgenic plants and it is possible to produce enough luminescence to make reliable measurements without cellular disruption. All cells produce their own Ca^{2+}-indicator and thus calcium changes can be measured in whole intact plants. *Arabidopsis* (Knight *et al*., 1996), tobacco (Knight *et al*., 1991) and the moss *Physcomitrella patens* (Russell *et al*., 1996) have all been stably transformed with an apoaequorin gene. A plasmid containing the cloned gene in a binary vector is available commercially from Molecular Probes under the name of pMAQ2. pMAQ2 encodes a constitutively expressed cytosolic form of apoaequorin, under

the control of the cauliflower mosaic virus (CaMV) promoter. This is the most commonly used form in plants. In addition, aequorin has been successfully targeted to a number of subcellular locations in addition to the cytoplasm using targeting sequences consisting of either peptide leader sequences or whole polypeptides encoding proteins which exist naturally in the chosen locale. If aequorin is to be expressed as a fusion protein it is necessary to maintain the features of the protein which are required to retain its full activity as a calcium reporter. The C-terminal proline residue of apoaequorin is essential for the long term stability of reconstituted aequorin (Watkins and Campbell, 1993). Even if the proline residue is present but linked to the N-terminal amino acid of another peptide, the resulting fusion protein is likely to be very unstable. It is therefore advisable to design aequorin fusions in which the aequorin is the C-terminal part of the protein and the other peptide the N-terminal portion. This may not always be possible, for instance in the case of endoplasmic reticulum-targeting, for which a C-terminal KDEL sequence is required. Targeted forms of apoaequorin have been engineered for use in plants, which express the protein in the chloroplast (Johnson *et al*., 1995) (pMAQ6, available from Molecular Probes), nucleus (van der Luit *et al*., submitted) and the cytosolic face of the vacuolar membrane (Knight *et al*., 1996). All of the plasmids confer kanamycin resistance and therefore the antibiotic is used in the identification of transformants (see below).

Stable transformation.

Tobacco leaf disc transformation (Draper *et al*., 1988) and *Arabidopsis* root transformation (Knight *et al*., 1997) have been successfully used to produce stable transformants. However, if mutants of *Arabidopsis* are to be transformed, it may be worth considering whether or not it would be appropriate to take the mutant through tissue culture, as some mutants are difficult to regenerate from callus. If this is a concern, the worker may choose an alternative method such as the vacuum infiltration (Bechtold *et al*., 1993) which does not include a tissue culture step.

Transient expression

Transient expression of aequorin may serve two purposes. Firstly, it may be useful to test whether or not a newly engineered aequorin construct will be transcribed and translated in a plant cell to form functional aequorin. Secondly, it is often beneficial to be able to perform calcium measurements, for instance in a mutant, as a prelude to stable transformation. For the first purpose, biolistic transformation of whole seedlings provides a very quick method of testing for luminescence from a new construct (Knight *et al.*, 1997). This could alternatively be carried out using onion epidermis (Klein *et al.*, 1987). The transiently transformed tissues can be homogenised and aequorin activity assayed *in vitro* (see later). For *in vivo* Ca^{2+} measurements in plants for which stable transformants are not yet available, PEG transformation of *Arabidopsis* protoplasts (Abel and Theologis, 1994) has proved very useful. Protoplasts from stably transformed Arabidopsis and tobacco (Haley *et al*., 1995) have been used in experiments where a uniform population of cells is required.

Measurement of luminescence

The level of light emission from recombinant aequorin is 4.30-5.16 x 10^{15} photons mg^{-1} of aequorin (Shimomura, 1991). At present the actual levels of aequorin which can be expressed in plant tissues are relatively low (a few pg protein per mg fresh weight of tissue) and therefore detection of the blue light emitted in response to calcium requires the use of very sensitive light counting equipment. A luminescence detector needs to be able to detect light

signals over a wide range varying by several orders of magnitude of intensity; from only a few photons per second to several millions. It is also important that the detector provides appropriate time resolution (Campbell, 1988).

There are two methods routinely used for measuring Ca^{2+}-transients in plants using aequorin luminescence. This first involves luminometry and provides high sensitivity and fast (sub s) time resolution. The second involves low-light-level imaging which gives additional spatial information.

Luminometry

A luminometer is the simpler and by far the cheaper of the two options. Stanley (1992) has published a survey of a great number of luminometers and imaging devices and the reader may wish to refer to this to aid in the selection of a suitable device. The luminometer can be designed to hold whole plants within a sample cuvette (Knight *et al.*, 1991). The luminometer has a light-tight sample housing containing a sample cuvette adjacent to the photomultiplier detector. A cooled photomultiplier (e.g. EMI 9235B) with a low dark current and a bi-alkali coating gives a good response in the blue with minimal background noise, especially when used with photon-counting circuitry. It is important to avoid the use of materials such as paints, silicon grease, silica gel and certain glass or plastic tubes, which luminesce themselves. Twelve mm diameter tubes (Sarstedt, Leicester, UK) are suitable for this purpose. Mirrors or silvering inside the sample housing may help reflect the light from the sample onto the detector (Campbell, 1988).

Low-level light imaging of aequorin

Low-level light imaging cameras can be purchased separately and built into an imaging system, however, there are now a growing number of 'off-the-shelf' complete imaging systems (see Stanley, 1992). The image is sent from the camera to a computer loaded with software for processing and analysis. Copies of the stored image can be printed using a colour video copy processor.

Most imaging systems are based around the charge-coupled device (CCD). CCDs are made up of an array of light sensors in which each acts as an individual light detector (Aikens, 1990). Incoming photons break bonds between the silicon atoms in the semiconductors, and generate electron hole pairs. The conversion of photon to electron is a linear process, allowing the measurement of light to be quantifiable. The electrons produced in this way are collected in a potential well; each well corresponding to a pixel on the screen of the imaging device. Exposure to light during imaging causes the CCD to acquire a pattern of electronic charge in the wells. This pattern of information is transmitted through a parallel register (of the same layout as the array) onto a serial register which transmits the information row by row to the output circuit. An image is then reconstructed by the computer. 'Blooming' may occur when there is too much charge for one well to hold (i.e. too much light has been allowed to enter the imaging device) and charge spills over to the adjacent wells.

The two types of CCD camera used for luminescence imaging can be classified by the method employed to improve the sensitivity of the camera. They are the cooled CCD and the image-intensified CCD. In the cooled CCD, sensitivity is increased by reducing the background noise by cooling the camera, allowing lower levels of light to be detected. The cooling system may be a Peltier device, circulated water, forced air or others. Improvement of the signal-to-noise ratio (S/N) is thus achieved by lowering the noise component. The disadvantage of this kind of camera is that a slow scan speed is needed and therefore rapid

changes in light emission (typical of plant calcium responses) cannot be recorded. In the image-intensified CCD, the signal from the light sensors is intensified (using microchannel plates or a phosphor-cathode sandwich) before detection and so the S/N ratio is improved by increasing the strength of the signal. This is a more sensitive device than the cooled CCD at the low levels of light expected from aequorin luminescence.

Two "off-the-shelf" intensified systems which have been used successfully to image aequorin are systems from Hamamatsu (Hamamatsu Phtonics UK Limited) e.g. the C2400-20 photon-counting camera system with an Argus-50 image processor (Knight *et al.*, 1993) or the Photek (Photek Ltd., Hastings) photon counting camera system (Campbell et al., 1996). In terms of sensitivity and ease of use, both systems are comparable. However, there is one important difference, namely that the Photek system captures luminescence image information at video rate, and this information can be accessed at video rate or above with the software supplied. This is absolutely invaluable for imaging rapid calcium-transients reported by aequorin luminescence. It also means that no *a priori* knowledge of the nature of the kinetics of luminescence is required to start an experiment. We thus favour the Photek system (see below).

in vitro reconstitution of aequorin

Not all of the kanamycin resistant plant transformants obtained will yield light, and therefore, although transformants can be screened by southern and western blotting, the most direct way of telling whether or not the plants will be of use, is to check for the ability to produce blue light after addition of coelenterazine. For this purpose, aequorin should be reconstituted in a plant extract *in vitro*.

Protocol

1. For primary transformants, select a piece of leaf tissue from individual mature plants. For T1 or T2 generations, select 3-5 green seedlings (7-8 day old tobacco or 6-7 day old *Arabidopsis*) grown on a selection plate containing nutrient media (e.g. Murashige and Skoog) supplemented with kanamycin. Place in an eppendorf tube and snap freeze in liquid nitrogen.
2. Grind tissue to a powder using a micro pestle or glass homogeniser.
3. Add 0.1 ml chilled medium (0.5 M NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.4) with 5 mM mercaptoethanol and 0.1 % (w/v) gelatin added just prior to use.
4. Homogenise further using a motorised micro pestle.
5. Add a further 0.4 ml of medium, mix and leave on ice until all samples have been processed, using a clean micro pestle for each sample.
6. Centrifuge at 13000 g for 10 minutes and retain supernatant.
7. To 100 µl of supernatant, add 1 µl of 100 µM coelenterazine and pipette up anddown to mix and to introduce air into to the reaction tube. Incubate tubes in the dark at room temperature for 3 hours.
8. Dilute 10-50 fold with 0.5 ml Tris EDTA buffer (200 mM Tris-HCL, 0.5 mM EDTA, pH 7.0) in a plastic luminometer cuvette.
9. Take up 0.5 ml 50 mM $CaCl_2$ in a 1 ml syringe covered in black electrical tape.
10. Measure the sample background then quickly inject the $CaCl_2$ and count for a further 10 seconds. Subtract background to give luminescence counts for each sample.

Notes

- The coelenterazine should be stored dry at -70°C. Aliquot 50 µl samples of 200 µM coelentrazine in MeOH into microcentrifuge tubes, vacuum dry, wrap in foil and store at -

70°C. Protect coelenterazine from light during this procedure. Remove aliquots when required and re-dissolve in methanol at the required concentration. Coelenterazine is very light-sensitive and should be protected from light at all times. When adding coelenterazine to reaction tubes, dim room lights are required.

- It is also advisable to test for activity in the debris pellet as well as the supernatant, as this will contain some of the cellular components to which the aequorin may be associated. More sophisticated subcellular fractionation may be required to localise the aequorin activity.

in vivo reconstitution of aequorin

When the plants producing the highest levels of light have been identified, an *in planta* time course of reconstitution can be carried to ascertain the optimum time to allow reconstitution to maximal levels. Subsequently, the optimum duration of reconstitution should be used for experimental plants. 6-18 hours is satisfactory for cytosolic aequorin plants and levels remain high for at least 24 hours (Knight *et al.*, 1991). Other semi-synthetic aequorins are stable for shorter periods. To be sure of achieving maximum reconstitution of aequorin in plants of other species or age, it is useful to conduct a preliminary time course study of reconstitution. The amount of aequorin reconstituted increases with the concentration of coelenterazine up to a coelenterazine concentration of at least 10 µM (Knight *et al.*, 1991).

Protocol

1. Float 7-8 day old tobacco or 6-7 day old *Arabidopsis* seedlings in as low a volume of water as is possible without causing tissue to dry out (typically 1 ml).
2. Add coelenterazine to give a final concentration of 2.5 µM, mix by gentle swirling and place in the dark at room temperature.
3. The extent of reconstitution is measured by discharging the reconstituted aequorin. Measure the luminescence counts for 10 seconds before and for 2 min after injection of 1 ml 2M $CaCl_2$ with 20% ethanol.

Notes

- Expense may prohibit the use of large amounts of tissue due to the increased amounts of coelenterazine required.

Experimental measurements

Once reconstitution of aequorin in the transformed plants has been demonstrated, and the optimum time period for reconstitution determined, intracellular calcium measurements can be made.

1. Dim room lights before switching on luminometer to reduce background counts. Take a background reading either using a wild type seedling in a cuvette or a cuvette containing 1 ml water.
2. Take a freshly reconstituted seedling (steps 1-2 above) and gently place in the bottom of a luminometer cuvette containing water, if this is to be used.
3. Allow a minute for seedling to settle. Resting level calcium will be higher just after the seedling has been moved, and luminescence counts will reflect this.
4. Fill the syringe with substance to be injected through the port.
5. Start counting luminescence counts and then inject contents of syringe.
6. If calibrating, discharge the remaining aequorin with an equal volume of 2 M $CaCl_2$, 20% ethanol at the end of the experiment.

Notes

- The syringe should be coated with black PVC tape to reduce light entry *via* the injection port.
- The time for the injected solution to mix thoroughly in the cuvette must be taken into account.
- Although magnetic stirrers speed up mixing their use should be avoided as they generate electrical noise in the photon detector. (Campbell, 1988).
- The choice of frequency at which to record luminescence counts depends on the response to be measured. Counting periods of 1 to 10 seconds may be used if low levels of luminescence are anticipated. However, for rapid responses, counting every 0.1 sec is more appropriate in order to obtain a clear picture of the dynamics of the response.
- Adequate controls must be performed as the act of injection itself may cause an elevation in cytosolic calcium. For instance, the rapid introduction of liquid into a cuvette containing a seedling will effect a touch response and consequent spike of cytosolic calcium. Similarly, introduction of cold liquids may cause a cold shock response and again an elevation in cytosolic calcium.
- The temperature at which the experiment is carried out should be monitored as any calibration formula used will only be accurate if determined at the same temperature at which measurements are made.

Imaging aequorin luminescence

Biological samples (transgenic plants) containing aequorin are imaged in a specially constructed dark box containing a three microchannel plate intensified CCD camera (Photek ICCD325-FTM800 Intensified CCD Camera, Photek Ltd, Hastings) equipped with standard Nikon photographic lenses. The normal background of this camera is approximately 10 counts s^{-1} over the whole pixel array. The camera is controlled by a HRPCS-2 camera control unit (Photek Ltd., Hastings). The camera stores the photon image, as an array of 384x288 pixels, at video rates (50Hz) or as an array of 768x576 pixels at half video rates (25Hz). The latter sampling speed has proved the most appropriate for plant measurements, as it offers the highest spatial resolution and the temporal resolution is sufficient for transients detected using aequorin. The software (IFS216 Software, Photek Ltd., Hastings) then analyses the distribution of signals in the image and extracts and stores the (x,y) co-ordinates of the individual photons at (half or full) video rates (25 or 50Hz). A user-defined number of these images are then integrated and displayed. These two features, in particular, distinguish this imaging system from those widely used for fluorescence imaging.

Regions of interest can be drawn over defined parts of the plants or seedlings and the absolute number of photons detected extracted and plotted. True photon imaging requires at least 2 microchannel plates to generate a pulse height distribution curve. The high sensitivity of the camera used here is sufficient to detect Ca^{2+} signals in seedlings and plants over integrations of greater or equal to 1 second.

Protocol

1. Reconstitute aequorin in the sample *in vivo* by treating with coelenterazine as described above.
2. Place sample inside light-tight box.
3. Set camera to 'bright-field mode'. Focus camera on sample using real-time monitor. Take an average of 4 bright-field images. Save this image.

4. Close the door of the light-tight box and ensure that the locking mechanism is closed. Set the camera to 'photon-counting mode'. Set up photon counting integration, with neutral density setting at 100%.
5. At the end of the experiment, stop the integration and save the sequential time-resolved integration (TRI) file.

Notes

- The light-tight box can be customised for particular types of experiment. A very useful feature is to integrate a Peltier element into the base to perform temperature stress experiments.
- For bright field images, the door of the light-tight box is left open and the specimen illuminated obliquely with either spectacle torches or a head-torch. The safety cut-out mechanism which will disable the camera when the door is opened needs to be overridden manually (Photek are currently developing a software 'cure' for this).
- When photon-counting, with the neutral density setting at 100%, the camera is operating at maximum gain and hence maximum sensitivity. This is fine for aequorin containing samples as this level of sensitivity is required for small responses and even the largest response will not saturate the camera. In the unlikely event that it did, the camera is equipped with an 'overbright' cut-out safety mechanism.
- The TRI sequential file contains all the imaging pixel-array information at full or half-video rate. The processing software allows this information to be recalled and displayed for a series of user-defined integrations, a significant advantage of the Photek system.

Calibration of aequorin luminescence

There are several approaches to calibrate the aequorin signal *in planta*. In the first method, cytosolic Ca^{2+} activities are calculated from aequorin luminescence by comparison of the rate of Ca^{2+}-triggered luminescence from the aequorin in the cell to the peak rate of light emission at saturating [Ca^{2+}] (Cobbold and Rink, 1987; Gilroy, *et al.*, 1989). There are significant differences, however, between *in vivo* and *in vitro* calibrations (Gilroy *et al.*, 1989). In the second method, the proportion of the total aequorin consumed at any point in time is related to the cytosolic calcium concentration by an empirically derived formula. The total amount of luminescence is measured as the integral of all luminescence during the experiment and after complete discharge of all the aequorin following permeabilisation of the tissue in the presence of high calcium.

Protocol

1. Record the total luminescence during the experiment.
2. At the end of each experiment discharge the remaining aequorin with 1 M $CaCl_2$ and 10% ethanol, and record counts for a further 1-2 minutes, or until they fall to approximately one thousandth of the maximum value recorded.
3. Measure the background luminescence from wild type seedling and subtract the average value from all the experimental data points.
4. Measure the total number of counts over the course of the whole experiment, including the discharge of aequorin at the end.
5. The rate constant, k, at each time point in the experiment is given as:

$$k = \frac{\text{luminescence counts } s^{-1}}{\text{total luminescence counts}} \qquad (8)$$

6. Conversion to calcium uses an empirically derived (Cobbold and Rink, 1987) calibration formula which is specific for the isoform of aequorin encoded by pMAQ2 (Badminton *et al.*, 1995).

$$pCa=0.332588(-logk) + 5.5593 \quad (9)$$

Notes

- These calibration coefficients were determined at 25°C. Different values are required at different temperatures. This can cause difficulties when using temperature treatments such as the cold-shock control (see below).

The cold-shock control

The cold-shock response occurs when plant tissues experience a rapid drop in temperature producing an immediate and large increase in $[Ca^{2+}]_{cyt}$. This has proven to be a very reliable response and demonstrates that functional aequorin has been reconstituted in the plant cells and that the luminometer or imaging equipment is capable of measuring Ca^{2+}-induced changes in luminescence. The drop in temperature can be achieved using a Peltier cooled stage or by placing a small piece of ice next to the seedling on the microscope stage. When the ice melts, cold water reaches and stimulates the seedling. When using the luminometer, the cold-shock response can be caused by injecting ice-cold water into the luminometer cuvette containing the plant.

Additional techniques for aequorin measurements

Semi-synthetic aequorins

Semi-synthetic aequorins are molecules made up of natural apoaequorin coupled with a chemically synthesized analogue of coelenterazine (Shimomura *et al*, 1988). Semi-synthetic aequorins have a very wide range of sensitivities to Ca^{2+} ranging from ca. 0.01 to 200 times that of natural aequorin (Shimomura, 1991). It should be noted, however, that semi-synthetic aequorins have significantly lower stability and a marked reduction in half-life compared with natural aequorins, therefore measurement of luminescence must be carried out within a few hours of reconstitution. Semi-synthetic aequorins may be reconstituted *in vitro*, for subsequent loading by traditional methods (Llinas *et al.*, 1992) but can also be reconstituted in whole plants combining chemical analogues of coelenterazine with the recombinant apoaequorin expressed in the plant tissues, (Knight *et al.*, 1993).

Semi-synthetic aequorins can be used to measure Ca^{2+} levels over a wider range than normal cytoplasmic Ca^{2+} concentrations. For instance, n-aequorin with a low sensitivity to calcium has been used in neuron cells to report very high Ca^{2+} levels (Llinas *et al.*, 1992), whereas h-aequorin which is very sensitive to calcium has been used to demonstrate very small changes in $[Ca^{2+}]_{cyt}$ which occur during the wounding response in plants (Knight *et al.*, 1993).

A variant of coelenterazine known as e-type coelenterazine confers on aequorin a bimodal emission peak. Light emission can be measured at 2 wavelengths (approx. 405nm and 465nm) and the ratio of the amounts of light is directly proportional to the calcium concentration but independent of the concentration of aequorin in the tissues, making this

method the most reliable way of quantifying calcium concentrations measured by aequorin bioluminescence (Shimomura *et al.*, 1988).

Engineered aequorins

Ten different isoforms of apoaequorin exist naturally and their sensitivities to calcium vary such that the most sensitive is about ten times more sensitive than the least (Shimomura, 1991). Other modifications in the protein structure can affect the calcium binding properties of aequorin. Aequorin has 3 cysteine residues which appear to have a role in the bioluminescence reaction either in a catalytic function or in the regeneration of active aequorin. Replacing these residues causes a reduction in the amount of luminescence produced by the protein, except when all three are replaced by serine, in which case luminescence increases (Kurose *et al.*, 1989). Single amino acid substitutions in the EF hand regions of apoaequorin produce a protein with a reduced affinity for calcium (Kendall *et al.*, 1992) . This type of aequorin can be used for making measurements in cellular locations where Ca^{2+} levels are expected to be orders of magnitude higher than in the cytosol, e.g. the mitochondria, and provides an alternative to the use of n-aequorin, with the advantage of avoiding the use of the less stable n-coelenterazine.

Engineered aequorins can now be produced with different emission spectra (Ohmiya *et al.*, 1992) and these aequorins which emit different colours of light may be of use in future developments (see below).

Manipulation of intracellular events using caged probes

Caged compounds are molecules whose biological activities have been chemically masked by a photolytic 'caging' group. Illuminating the caged compound with UV light causes the cage to dissociate and the biologically active molecule to be released (Adams and Tsien 1993). Through controlling the timing, intensity, and region of illumination, the dynamics of caged probe release can be tightly regulated, allowing analysis of the spatial and temporal components of signal transduction.

The list of compounds that have been caged is extensive including: ions, ionophores, enzymes, proteins, drugs and hormones (see chapter ## for a comprehensive review of the field). Several commercial distributors provide a wide range of these compounds. However, many of the more 'exotic' caged compounds (such as caged plant hormones – see Ward and Beale, 1995) are not commercially available and must be obtained from the lab that synthesized them. The alternative is to synthesize the caged compound in house. The availability of simple nitrophenyl ethyl ester caging technology from Molecular Probes makes this a real possibility. However, as always, chemical synthesis is not for the faint of heart and a clear plan of how to separate and characterize the products of the caging reaction is essential before embarking on this kind of project. There are no good hard and fast rules for such syntheses except that access to a good synthetic chemist is almost essential.

Assuming a ready supply of your caged compound is available storage for several months is possible if the aliquoted stock is stored at -80ºC in the dark. The caged compounds are labile under UV light but working in complete darkness is usually not needed and simply handling them under subdued room lighting and storing them in the dark are adequate precautions to prevent their premature discharge.

Experimental protocols

Introduction of caged compounds into cells.

The successful approaches used to date to incorporate caged probes into plant cells have been microinjection, ester loading and electroporation. The approaches, caveats, controls, need for optimization, and difficulties with these approaches when applied to caged compounds are identical to those outlined above for fluorescent dye experiments.

Uncaging equipment

1. *The epi-illuminator of a standard fluorescence microscope*. 1-10s illumination through a narrow band UV inteference filter around 340 nm release up to 100% of the caged molecules. Localized uncaging is possible by closing the epifluorescence diaphragm to illuminate only a small spot in the center of the field of view.
2. *The UV laser of a confocal microscope*. The ability to steer and restrict the scan of such a UV laser provides a very high degree of spatial control on the uncaging process.
3. *Two(or three)-photon excitation:* Two-photon systems provide a better defined volume of released probe and most of the specimen is only illuminated with longer wavelength light.
4. *A photographic flash gun*. Remove the plastic UV absorbing lens and mount on the stage of the microscope or in place of the epi-illuminator. Care needs to be taken with this approach as the electrical surge associated with flash gun discharge can damage sensitive photodetectors. Additionally the flash gun approach is much harder to control the intensity and spatial localization of illumination.
5. *Transilluminator:* Where biochemical analysis is the goal, uncaging can be achieved by placing the loaded cells in a UV transmissive cuvette on a standard transilluminator. Uncaging efficiency can then be controlled by altering the time of exposure to UV light or attenuating the illumination with an appropriate neutral density filter.

Artifacts and controls

Being able to regulate the timing, intensity and localization of illumination become critical factors when designing the caged probe experiment. This is because only when endogenous changes are closely mimicked is true cellular regulation likely to be revealed.

This need to mimic the amplitude and spatial dynamics of endogenous changes highlights the need to monitor the site and efficiency of caged probe release *in situ*. When the caged compound is affecting a detectable cellular parameter, e.g. changes in Ca^{2+} levels caused by caged Ca^{2+}-ionophore, using fluorescent or luminescent probes to monitor this parameter can indicate how well the caged probe is working (Allan *et al.*, 1994; Gilroy, 1996; Malhó and Trewavas, 1996; Franklin-Tong *et al.*, 1996; Bibikova *et al*., 1997). When the released compound is undetectable, e.g. ABA released from caged-ABA, a useful approach to visualizing the extent of photoactivation is to co-load the cell with caged-fluorescein. Caged fluorescein is non-fluorescent until it is uncaged. Thus, the production of fluorescein in the sample is an indication of how efficiently caged compounds are being photoactivated. Although indirect, the caged fluorescein approach is a much preferable alternative to simply assuming that UV irradiation has photoactivated the caged-compound in your cell.

UV irradiation and photolysis byproducts produced during the photoactivation of the caging group can be highly damaging and may also have biological effects. Thus extensive controls for the viability of cells used in caged probe experiments are needed.

Minimal controls include repeating experiments:
(1) without the caged compound but with UV irradiation;
(2) with the caged compound but without UV irradiation;
(3) with an inactive caged analog, if available. An example of this latter control is the use of Diazo-3 as a control for Diazo-2 in experiments to manipulate cellular Ca^{2+} signaling. Both are caged Ca^{2+} chelators with almost identical chemical structures, but photoactivation of Diazo-2 produces a chelator with 5x the affinity for Ca^{2+} than Diazo-3. Similarly, a non photoactivateable analog of caged-ABA has been synthesized as a control for caged ABA experiments (Allan *et al*, 1994).

The need for a functional assay

Perhaps the most significant aspect of caged probe experiments is developing appropriate measures of cellular activities to determine the effect of the regulator released upon caged probe photoactivation. In microscope-based experiments single cell parameters such as growth (Malhó and Trewavas, 1996; Bibikova *et al.*, 1997), cell structure (Fallon *et al.*, 1993; Gilroy *et al.*, 1991; Gilroy, 1996), gene expression (Gilroy, 1996), ion channel activity (Blatt *et al.*, 1990; Romano, Gilroy and Assmann, unpublished data) and exocytosis (Gilroy, 1996) have all been used to determine whether photoactivation of a caged probe has had an effect on cell regulation.

A few plant studies have used caged probes to manipulate populations of cells where the assay of function is a biochemical change such as protein kinase activity (Fallon *et al.*, 1993). Such biochemical approaches are elegant in that they use the controllability of caged probe technology to manipulate cells but need a large number of loaded cells in order to be able to perform biochemical assays. So far electroporation and ester loading of caged probes have provided the only successful loading strategies for caged probes into large numbers of plant cells.

Probes for other compartments

A description of events in the cytoplasm gives a limited view point of dynamic metabolism in plant cells. The massive transport events occuring across the plasma membrane and tonoplast and the ionic balance in the apoplast and vacuole are all potential areas needing research. Many of the probes, particularly Ca^{2+}-dyes, have been specifically tailored to respond to cytosolic ion concentrations within a limited range of pH and ionic composition. Alternative probes or modification of the dyes (e.g. higher K_d values) are required if they are to be useful in other compartments.

Apoplast

The ionic composition of the apoplast is poorly defined, mainly as the high ion exchange capacity of the wall creates a host of polarised microenvironments for selective ion binding. Local ion activities are dependent to a large extent on the interaction between the supply of ions from the neighbouring apoplast or bathing medium, the wall polymer composition and the selective ion transport phenomena occuring in adjacent cells (Grignon and Sentenac, 1991). Membrane potentials, activity of extracellular enzymes, wall structure and binding of ligands to receptors are all likely to be affected by the apoplastic environment.

Apoplastic pH has been measured using soluble pH indicators such as umbelliferones (Pfanz and Dietz, 1987) or Cl-NERF (Taylor *et al.*, 1996). In the latter case, the signal was ratioed against the pH-insensitive signal from Texas Red dextran measured in parallel. Direct coupling of ratioable pH dyes to dextrans may also be desirable to prevent permeation or uptake by non-specific transporters in the membrane (Mühling *et al.*, 1995; Hoffmann *et al.*, 1992; 1995). Typically dyes are introduced into the apoplast *via* the transpiration stream, cut surfaces and/or vacuum infiltration.

Apoplastic [K^+] has been measured using ratio imaging of benzofuran isophthalate (PBFI) (Mühling and Sattelmacher, 1997) and gives values between 20-25 mM on the abaxial leaf surface to 5-8 mM on the adaxial surface, with higher concentrations around stomatal guard cells. These values compare with microelectrode measurements that range from 50 µM (Blatt, 1985) to 3-100 mM (Bowling, 1987) around stomatal guard cells.

The vacuole

Dynamic changes in vacuolar morphology and ion transport are of increasing interest. pH measurements rely on uptake and compartmentalisation of appropriate dyes in the vacuole (e.g. esculentin, pyranine and fluorescein derivatives, Yin *et al.*, 1990; CDCF-DA, Yoshida, 1995; BCECF, Swanson and Jones, 1996; Brauer *et al.*; 1995; CF, Davies *et al.*, 1996). 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 (Yin *et al.*, 1990). 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 (Yin *et al.*, 1996). 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 (Brauer *et al.*, 1995). The calibration response of dyes in the vacuole may also be markedly different from the response in the cytoplasm. Optical sectioning using confocal microscopy allows signals from vacuole and cytoplasm to be distinguished and may permit simultaneous measurement of ion activities in both compartments (Fricker *et al.*, 1994). Certain cells contain autofluorescent compounds in the vacuole that respond to pH, which have been imaged to follow changes in vacuolar morphology during guard cell development (Palevitz *et al.*, 1981), but not yet changes of pH in mature guard cells.

In principle, membrane permeant weak amines accumulate in acidic compartments in response to the pH gradient across the intervening membrane. Fluorescent acridine derivatives fall into this category can be readily imaged in plant vacuoles. However, many of these derivatives appear to interact with vacuolar components and their partitioning does not correctly respond to pH (Wood and Fricker, unpublished). Acridine derivatives also exhibit complex and diverse staining behaviour and can be highly photo-toxic (Gupta and De, 1988).

Future developments

The number of ions that can be imaged is increasing all the time. Dyes for potassium, sodium, magnesium, nickel, aluminium and chloride exist, but there are still only a limted number of reports on their use in plants (e.g. Lindberg, 1995; Lindberg and Strid, 1997; Mühling and Sattlemacher, 1997; Vitorello and Haug, 1997). Following the trends in recent years, we would expect more multiple parameter measurements combining calcium and/or pH measurements with electrophysiological measurements (e.g Schroeder and Hagiwara, 1990;

Grabov and Blatt, 1997; Bauer *et al.*, 1997; Felle and Hepler, 1997; Thiel *et al.*, 1997) or vibrating probe measurements (e.g. Pierson *et al.*, 1996). Interest is also developing in techniques to measure other components of the signal transduction chain, such as calmodulin distribution (Love *et al.*, 1997).

However, we feel the most significant advances are likely to arise from application of molecular genetics which offers staggering potential for precisely targetted functional analysis of key signalling and regulatory components, as well as using providing optical techniques as *in vivo* assay systems. The aequorin transformation studies have elegantly demonstrated the benefits of a molecular biological solution to calcium measurements. One recent improvement in this area is that the response can now be quantified using dual wavelength coelenterazines (Knight *et al*. 1993). The recent development (Miyawaki *et al.*, 1997) of a Ca^{2+}-sensor based on fluorescence energy transfer (FRET) between different wavelength versions of GFP is reminiscent of the introduction of the first fluorescent Ca^{2+}-indicator Quin-2 by Roger Tsien and colleagues in 1985. Quin-2 was the progenitor that heralded the explosive growth in the availability of ion-indicating fluorescent dyes. The cameleon probes can be expressed in plants and can be imaged with dual-excitation confocal systems (Fig. 1). The approach exemplified by the cameleon probes combine the advantages of fluorescent measurements with genetic engineering to target probes to different cells or sub-cellular compartments. In addition, it should be possible to engineer different linkers that modify the FRET signal in response to a wide variety of molecules other than just inorganic ions.

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