QUANTITATIVE CONFOCAL FLUORESCENCE MEASUREMENTS IN LIVING TISSUE

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1 Background

Fluorescent probes offer unparalleled opportunities to visualise and quantify dynamic events within single, living cells with a minimum of perturbation. Cells or monolayers maintained in culture can be readily imaged, however, measurements are often also needed from cells within intact tissues that are operating in their correct physiological context to include the effects of cell-cell interactions and the mechanical, ionic and physiological effects of the extracellular matrix (e.g. Errington et al. 1996). A range of different measurement techniques are now available to quantify fluorescence signals from reporter molecules within biological specimens, including fluorimetry, flow cytometry, microscope photometry, camera and confocal microscopy. Each system performs well for a specific range of sampling conditions and specimens, thus, several techniques in combination may be needed to provide a sufficiently flexible balance between the spatial, temporal and spectral resolution required. Several recent volumes cover many of the technical details and practical applications of these techniques (e.g. Wang and Taylor, 1989; Taylor and Wang, 1990; Mason, 1993; Matsumoto, 1993; Nuccitelli, 1994; Pawley, 1995a). Here we focus on the practical advantages and disadvantages of quantitative fluorescence measurements using confocal microscopy as a tool to study living cells in intact animal, plant and fungal tissues. (Errington et al. 1996; Fricker et al. 1994; White et al. 1996).

2 Principle and applications

2.1 Optical sectioning using confocal microscopy

In conventional camera-based fluorescence microscopy, the excitation light is needed to illuminate a large area in the *x,y* plane to allow simultaneous measurement of the fluorescence emission for all points in the focal plane. Fluorescence is also unavoidably excited from an extended volume above and below the focal plane. This fluorescence signal is not confined to the focal plane of the image but is also spread out or blurred at the face-plate of the camera. If the specimen is very thin, in the order of a few microns, this usually presents little problem for physiological measurements, however in larger cells or cells forming part of a multicellular structure, the out-of-focus blur significantly degrades the contrast and distorts any attempts at quantitative measurements. In a confocal microscope, the excitation light is focussed to one or more discrete points in the specimen. However, fluorescence is still excited in the cones of illumination above and below the focus. This out-of-focus fluorescence is prevented from contributing to the final image by a physical barrier in an imaging plane before the detector. In-focus fluorescence from the focal point passes through a small aperture in the image plane. Thus the illumination and detection points are co-aligned or ‘confocal’. A single optical section is generated by scanning the confocal point in the *x,y* plane to build up an image. A series of optical sections collected at different focus levels constitutes a three-dimensional (3-D) image. Repeated sampling in time forms a 4-D image. There are a wide range of commercially available confocal microscopes that achieve varying

degrees of optical sectioning by scanning and detecting single points, multiple points or narrow slits (Pawley, 1995a).

2.2 Quantitative imaging using confocal techniques

The main advantages of confocal microscopy in quantitative fluorescence measurements of physiological parameters arise from the well defined 3-D volume (voxel) that is sampled to form each 2-D picture element (pixel). The volume probed is always asymmetric, being at least 3-4 times longer in the axial (*z*) direction. The overall shape of the confocal probe in fluorescence is described by the point spread function (psf). Optical probe dimensions are usually given in terms of the full width at half the maximum height (FWHM) in a given direction along one of the orthogonal axes. Point-scanning confocal instruments can achieve a probe size of 0.2 µm x 0.2 µm x 0.6 µm, in *x*, *y* and *z* respectively, with a high NA (1.4) oil-immersion lens in fluorescence. Typical probe dimensions for physiological measurements are likely to be larger, in the region of 0.4 µm x 0.4 µm x 1.2 µm, as longer-working distance, lower NA lenses are often used, and the optical sectioning is relaxed by increasing the pinhole diameter.

The removal of out-of-focus blur allows discrimination of signals from sub-cellular domains, whilst contaminating signal from autofluorescence and/or stained tissues outside the focal plane is rejected. Measurements can also be made even if substantial compartmentalisation has occurred, provided the organelles can be adequately resolved. In addition, the 3-D sampling available with confocal systems allows a detailed quantitative description of any sub-cellular or cellular morphological changes associated with physiological signals.

In many instruments there is control over the degree of confocality, the area scanned, the scan speed and the number of frames averaged that provides the user with considerable flexibility in choice of sampling speed and the volume of specimen imaged. At one extreme, repeated sampling can be made of a single point in the 1 MHz range, whilst a 3-D volume of an thick tissue specimen, such as a root tip or invertebral disc for example, can be sampled at high resolution in a few minutes. Thus different facets of a given biological question can be tackled on the same instrument.

2.3 Statistics of fluorescence intensity measurements:

To make absolute photometric measurements, the relationship between the image brightness and the concentration of the fluorophore has to be calibrated. Accurate measurements of low-intensity fluorescence signals are limited by the number of photons emanating from the specimen. For a homogeneous sample, the sampled photons are drawn from a population with a mean value (S) and a distribution defined by Poisson statistics where 63% of values occur in the range $S \pm \sqrt{S}$ (Pawley, 1995b). The quantity, $\sqrt{S}$, is the intrinsic noise and provides an upper limit to the accuracy that can be achieved in any measurement. It is the task of the imaging and digitisation system to record this signal as faithfully as possible under a given set of experimental conditions. First, this involves maximising the number of photons emitted per voxel of dye that actually reach the detector. For example, increasing the NA of the lens increases the collection efficiency, whilst careful selection of wavelength filters and pinhole size may improve the contrast by optimising the optical transfer efficiency for specified wavelengths. Second, the quantum efficiency (QE) of the detector determines the number of photons that generate a measurable signal. Photomultiplier detectors have very good linearity

over a wide intensity range, but they have a low QE that is markedly wavelength sensitive. Different photocathode materials and end-window geometries can markedly increase the QE (Pawley, 1995b). Third, it is important to minimise additional noise from the electronics.

In a system with true photon-counting, a discriminator is used to eliminate multiplicative noise in the detector: each photoelectron arising from the photocathode contributes a single pulse that is counted. This is the optimal digitisation technique for signals with a low-number of photons within the linear range of the photon-counting circuitry, as it provides a way of determining the true black-level. The number of photons that can be reliably counted in commercially available CLSMs for a 1µs pixel is around 10-20 per scan. In practice, this is often comparable to the maximum signal that is actually present under physiological imaging conditions.

In analogue mode the current from the photomultiplier can be integrated over the pixel dwell time and converted to a voltage which is usually digitised to 8 bits. The current arising from each photon depends on the statistical variation in the number of secondary electrons produced, which typically adds Gaussian noise to the signal. Thermally produced photoelectrons contribute a constant dark-current with shot noise, whilst photomultiplier and digitiser offsets will add DC-voltages, and further noise to the output. Thus, although the digitised 8-bit image has 0-255 grey-levels, there is no information about the actual number of photons contributing to the signal under these conditions, unless the system is calibrated.

To illustrate the range of signals that might be encountered a typical example under physiological imaging conditions will be considered. The maximum signal from a fluorescein molecule operating near saturation is about 10^9 photons s^{-1} and requires a laser power of ~1-2 mW at the back focal plane of the objective (Sandison et al. 1995; Tsien and Waggoner, 1995), most of which ends up passing through the sampling volume. Under physiological imaging conditions the laser power is 10-50 fold lower, to maintain cell viability, and each voxel is effectively imaged for ~1 µs in a point scanning instrument during which time each molecule would emit between 20-100 photons. A 0.4 µm x 0.4 µm x 1.2 µm 'cubic' voxel of dye at a concentration of 10 µM contains ~1200 molecules, giving 24,000-120,000 photons from the illuminated voxel. The optical transfer efficiency of a typical confocal system is 2-10 %, giving 480-12000 photons at the detector. The quantum efficiency of the photomultiplier varies with wavelength from 3-13%, giving a mean of 14-1560 photons that would actually be detected per pixel. If these were both represented within a 0-255 range, mean±√variance (noise) would be about 201±54 grey levels in the first case and about 249±6 in the second (in the absence of additive Gaussian noise). Scanning at faster rates significantly reduces the pixel dwell time and the number of photons excited per pixel resulting in significantly increased variance. Video rate imaging at 25 Hz per frame gives pixel dwell times of usually ~0.1 µs and thus a maximum number of photons per pixel of about 1.4-156 per scan, equivalent to 138±117 to 236±19 grey-levels for a point scanner.

In physiological measurements, there may be additional weak background signal from the autofluorescence in lens elements, sample chamber and specimen, so a practical definition of background (B) is a composite including non-specific fluorescence in the optics and sample (B_1), and DC-voltages from the photomultiplier dark-current (B_2); the photomultiplier offset (B_3); and the digitiser offset (B_4); etc. such that the total signal

recorded (T) is $S+B_1+B_2+B_3+B_4...B_n$. An average value of B is usually measured from a region of the specimen without fluorophore and subtracted from the total to give the mean signal (i.e. $S = T-B$). However, the overall noise (N) also contains components from all these sources as well as the shot noise associated with sampling of the signal. Noise associated with sampling photons scales as the square root of the number of photons thus the total noise $N = \Sigma(\sqrt{(S+B_1)}+N_2+N_2+N_3+N_4....N_n)$ for each sample (pixel). It is not possible to separate and remove the background noise components, thus noise from all sources contributes to an increase in the variance of the signal. To make an accurate estimate of the mean signal requires that both the background and the total intensity are recorded without clipping the intensity distributions (see section 3.1). Integration over *x,y,z* or *time* will increase the S/N ratio as the signal increases proportionally with the number of pixels averaged, whilst the noise only increases with the square root of the number.

2.4 Identifying and minimising problems associated with confocal measurements

The primary objective of quantitative physiological measurements is to maximise the signal to noise (S/N) ratio with minimal disruption to the cell physiology. The demanding optical system required to achieve optical sectioning in a confocal microscope both accentuates many of the problems associated with accurate measurements in biological tissues using conventional microscopy and poses some unique additional difficulties. In particular, problems arise when observing cells within intact or thick microscopical specimens. A digitally stored 3-D fluorescence image represents an approximation to the intensities emitted from a corresponding volume within the specimen, however, physical interactions between the specimen and both the microscope illumination and fluorescence signal significantly affect the position and volume of the confocal probe. The most important of these interactions are refractions which occur at all non-opaque boundaries through the optical path of both microscope and specimen resulting in image blurring and distortion. As refractive index varies with wavelength in most materials (dispersion), the magnitude of the spatial errors will also vary with wavelength (chromatic aberration). Blurring of the probe results in a decrease in signal accepted by a confocal detector pinhole, leading to significant attenuation of fluorescence intensity. Thus, in confocal microscopy, spatial errors also give rise to photometric errors (Hell and Stelzer, 1995; White et al, 1996). Separate calibration of such spatial and photometric errors for a given specimen is required to convert the raw digital data to a true rectilinear array of corrected intensities (Errington et al. 1996; White et al. 1996).

Given the large number of variables, it is not possible or even desirable to provide a prescriptive set of rules that can be established as generalized protocols to achieve high S/N, good specimen viability and accurate measurements for each experimental system. The points below represent guidelines that we have found useful in live cell imaging projects across a range of plant, fungal and animal specimens and are grouped under the overall scheme shown in Fig. 1. Additional tutorial material on general aspects of practical confocal microscopy can be found in Centonze and Pawley (1995). We have concentrated on the confocal instrument calibration, data collection and analysis protocols appropriate for live cell confocal imaging (see also Terasaki and Dailey, 1995). Techniques for calibration of specific dyes can be found elsewhere in this volume (see also Wang and Taylor, 1989; Mason 1993; Matsumoto, 1993; Nucitelli, 1994).

3 Materials and Methods

3.1 Fluorescence intensity protocols: Fluorescence image statistics

Aim: To determine instrument settings for accurate measurements of fluorescence intensities.

1. Set up a slide-coverslip sandwich using spacers to make a chamber around 100 µm deep containing an appropriate buffer solution with and without 50 µM fluorochrome. Appropriate spacers include thin-coverslips, aluminium foil or dabs of nail varnish (Fig. 2A). All chambers should be made hours or days in advance and sealed with nail varnish to ensure maximum stability of the preparation.
2. Focus into the blank 'sea' away from the coverslip and scan a single (*x*,*y*) plane. This provides a measure of the background including autofluorescence from the medium etc.
3. Plot the frequency histogram of the pixel intensity distributions for the central ¼ of the field.
4. The black level should be adjusted so that all of the fluorescence intensity histogram is recorded above a grey-level of zero without clipping. It is now possible to determine the average background value.
5. Focus into a 'sea' containing 50 µM fluorochrome and plot the frequency histogram.
6. The histogram should have an approximately symmetrical shape and should also not be clipped. If photon-counting is used and the signal is low enough, the noise should be near to the square root of the mean number of photons. In analogue mode there is not necessarily a predicatable relationship between the mean and variance (see section 2.3).
7. The gain should be incrementally adjusted and the mean intensity plotted after inspection of the histogram distribution. If any of the histogram reaches 255, the signal will be clipped, reducing the apparent mean at higher gains. A lower dye concentration or laser setting is required and the measurement continued.
8. The linear region of the gain control can be determined as well as the effects of increasing gain on the variance of the signal.
9. A plot of the signal to noise ratio at varying pinhole diameters also provides an indication of the optimal pinhole setting to maximise the *S/N* ratio.
10. It is often useful to generate a specfic look-up table with colour coding to indicate the correct black level setting and indicate values approaching saturation - avoiding the need to keep measuring the histogram distribution.

3.2 Fluorescence intensity calibration protocols: Fluorochrome properties:

Aim: To determine the range of dye concentration that gives a linear response.

1. With the gain and black level set correctly for 50 µM fluorochrome, the effects of varying fluorophore concentration can be examined. At low fluorophore concentrations, up to *ca.* 50 µM, a linear relationship is predicted between concentration and fluorescence emission. At higher concentrations, self-absorption and self-quenching gradually decrease the relative intensity of emission towards a plateau value or even a slight decrease in emission at higher concentrations.
2. **Photobleaching:** Photobleaching also limits the total number of photons that are emitted before the fluorophore degrades to about 10^4-10^5. In principle, the rate of photobleaching can be assessed from the change in mean intensity after repeated

scanning of the fluorochrome 'sea'. However, under these conditions the dye is freely diffusible and replenishment by un-bleached molecules will reduce the apparent effects of bleaching in a fluorescent 'sea'. The rate of bleaching *in vivo* is likely to be higher as the fluorophores are constrained to a much smaller volume and will also depend markedly on the local environment, such as oxygen level etc.

3. **Saturation:** Fluorescent molecules also saturate at high laser intensities when electrons are depleted from the ground-state, particularly in fluorophores with long decay times. The maximum rate for fluorescein, for example, is about 10^9 photons s^{-1} and this typically requires laser intensities in the order of 1-2 mW at the back focal plane of the objective (Sandison et al. 1995; Tsien and Waggoner, 1995). Laser intensity settings required to keep cells viable are typically 10-50 fold lower than this so saturation is unlikely to be a problem during physiological measurements.
4. **Ratio considerations:** Excitation ratio measurements require a means to balance the intensity of the two laser lines using neutral density filters, for example, so that the fluorescence signal remains within the useable range for both wavelengths across the relevant range of dye concentration and dye responses. We have found it useful to start each set of physiological measurements with an *in vitro* calibration response for the dye across the dynamic range anticipated *in vivo*, both to adjust the relative laser intensities and define the *in vitro* calibration curve.

3.3 Confocal microscope performance protocols: calibration of image (x,y) axes

Aim: To calibrate the spatial dimensions of the image (*x*,*y*) axes.

1. Image a stage micrometer in transmission or reflection mode.
2. Measure the length of an appropriate number of graticule divisions and record the number of pixels. The pixel size (in microns) = length of the graticule units measured (in microns)/ number of pixels.
3. Repeat for each excitation wavelength available.

Lateral chromatic aberration will cause a slight change in the field size between different wavelengths which will result in mis-registration of images. In fluorescence there will also be misregistration of illumination and detection probes at each wavelength leading to signal attenuation (White et al. 1996). The degree of radial fall off across the field can be measured empirically (see section 3.5) or predicted in conjunction with measurements of the fluorescence axial-step function (see section 3.7; White et al. 1996).

3.4 Microscope performance protocols: Calibration of the image (z) axis

Aim: To determine the *z*-axis focus correction

Problem: Modern high-numerical aperture oil-immersion lenses show minimal aberration when imaging a specimen adjacent to a cover-slip. However, the changes in refractive index between the immersion medium, coverslip and aqueous perfusate introduce refraction effects that are only corrected near the coverslip. Deeper into the sample the image is degraded - marginal rays are affected to the greatest extent while axial rays are not affected at all. The result is a magnificational change in *z* and a *z*-broadening of the imaging probe. To calibrate the distortion of axial focus position due to mis-matched immersion and mountant, the distance between glass/aqueous-medium/glass boundaries in a slide-coverslip sandwich can be measured and compared with the 'correct' distance determined with a matched immersion system, usually water immersion into an aqueous medium (Errington et al. 1996; White et al. 1996). This problem is reduced if water immersion lenses can be used for the experiment and the

specimen is largely aqueous. Appropriate high NA lenses have recently become available from several manufacturers to help address this issue.

1. Set-up an aqueous sea sandwiched between a slide and coverslip approximately 50-100 µm deep using spacers (see section 3.1).
2. Collect (*x*,*z*) sections at *ca.* 0.3 µm *x*,*y*-pixel spacing in reflection mode using a water immersion lens. Sampling should start *ca.* 5-10 µm outside the fluorescent medium and continue at 0.4 -0.5 µm *z*-step intervals through the medium and 5-10 µm further into the slide.
3. Repeat the sampling with the 'experimental lens' and the appropriate immersion medium.
4. Measure the intensity distribution along a *z*-transect with some averaging, e.g. 16-32 pixels wide, normal to the slide-coverslip sandwich.
5. The distance between the two major reflection peaks for the water immersion lens corresponds to the matched-refractive index situation (D_{water}).
6. The distance between the apparent reflectance peaks for the experimental lens/ immersion medium combination ($D_{expt.}$) will be greater than D_{water} for oil immersion and less for dry lenses.
7. The ratio of the distance with the water lens compared to the experimental lens ($D_{water} / D_{expt.}$) gives the *z*-correction factor that has to be applied to all measurements involving *z*. Typical correction factors range from 0.82-0.88 for oil into water (White et al. 1996) which is close to the refractive index ratio (η_{medium} / $\eta_{immersion}$).

3.5 Microscope performance protocols: useable field area

Aim: To determine the radial fall-off in intensity across the field.

Problem: The measured intensities from a homogenous fluorescent 'sea' are rarely uniform across the field. There is usually a central, circular peak and a radial fall-off in intensity towards the edge of the field. This arises from lateral spherical aberration, lateral chromatic aberration and field curvature, that combine to blur both the illumination and detection probes and shift their relative positions in *x*,*y*,*z*. The signal intensity is reduced because the blurred information is rejected at the pinhole.

1. The extent of radial fall off in signal from all sources of aberration can be measured using line transects across the (*x*,*y*) images of the fluorescent 'sea', preferably with an amount (e.g. 16-32 pixels) of averaging normal to the transect.
2. For ratio applications it is critical that this measurement is repeated for both excitation/emission combinations as the aberrations are wavelength dependent.
3. The useable confocal field of view will typically be limited to ¾ (linear dimensions) of the full field for Plan-Apo water-immersion lenses and ½ to ¼ of the field for high NA Plan-Apo oil-immersion lenses and aqueous samples. This measurement should be repeated with all lenses.

3.6 Microscope performance protocols: reflection plane-spread function

Aim: To assess the general performance of the microscope system in reflection.

The monochromatic reflection plane spread function is readily determined from reflection images of a mirrored surface. It provides an indication of optical section thickness, field curvature, wavelength-dependent shifts in axial focus position and the

effects of lens aberrations. These functions also provide a simple assessment of the microscope performance over a period of time.

1. The sample used for the empirical measurement of axial distortion can be used, although greater contrast is achieved if the glass surfaces are partially mirrored (Fig. 2B). These can easily be generated by sputter coating in an EM unit.
2. Collect axial (*x*,*z*) sections in reflection contrast over about 20 µm centered on the coverslip/medium boundary at a reasonably high resolution, e.g. 0.1 µm *z*-focus increments and an (*x*,*y*) pixel spacing of 0.03 µm for a 60x lens. Average at least 8 lines to reduce noise. Repeat for each lens and wavelength combination.
3. The spread or extent of the mean intensity averaged normal to the axis over a central portion of the field and plotted against the corrected *z* distance (see section 3.4), defines the overall sectioning characteristics (or plane *z*-response) for each monochromatic wavelength in reflection.
4. The full width at half the maximum peak height (FWHM) provides a measure of the optical section thickness. Values will be proportional to the wavelength and will also depend on pinhole size. Minimum values will be around 450 nm in the blue, increasing to around 600nm in the red.

The axial position of the maximum in the plane-spread function will also alter towards the edge of the field if there is any appreciable field curvature. In addition, the magnitude and shape of the side-lobes provides an indication of the lens aberrations. The axial displacement of the central peak between different wavelengths provides a measure of the axial chromatic aberration of the system (Fricker and White, 1992). The effect of different pinhole sizes on optical sectioning can also be readily examined to examine the *S/N* ratio versus section thickness for example.

3.7 Confocal microscope performance protocols: fluorescence axial-step function

Aim: To assess the general performance of the microscope system in fluorescence.

Fluorescence imaging involves a minimum of two wavelengths and three are used in ratio measurements. An appropriate test sample is needed to assess the performance of the microscope under these conditions. Although the fluorescence point-spread function (psf) can be derived from imaging sub-resolution fluorescence beads, good fluorescent psf measurements are difficult to achieve as they are very noisy and prone to specimen movement. An alternative strategy relies on measurement of the change in fluorescence intensity function on entering a fluorochrome ‘sea’ - the fluorescence axial-step function.

1. Set-up an aqueous fluorochrome sea sandwiched between a slide and coverslip approximately 50-100 µm deep using spacers (see section 3.1).
2. Collect axial (*x*,*z*) sections at a reasonably high resolution, e.g. 0.1 µm *z*-intervals and an (*x*,*y*) pixel spacing of 0.03 µm for a 60x lens. Average at least 8 lines to reduce noise. Sampling should start *ca.* 10 µm outside the fluorescent medium and continue 10 µm further into the medium.
3. The fluorescence step function is defined by the line transect into the fluorescent ’sea’, preferably with an amount (e.g. 16-32 pixels) of averaging normal to the transect.
4. A second measurement can also be made at the medium/slide boundary to give an indication of the fluorescence performance after traversing a reasonable depth of

aqueous medium. The overall intensity will be reduced compared to the surface profile as a result of depth-dependent signal attenuation (see section 3.8) and the boundary will be less steeply defined as the probe is blurred.

3.8 Microscope performance protocols: Rapid performance assessment

Aim: To assess the general performance of the microscope system rapidly in both reflection and fluorescence.

A rapid assessment of both the reflection plane-spread function and the fluorescence axial-step function can be made from single (*x*,*y*) images of a tilted specimen. This allows direct visualisation of both functions and the effects of interactive changes in imaging parameters, such as pinhole size, alignment etc. are immediately apparent.

1. Construct a wedge shaped chamber with an inclined coverslip at an angle of *ca.* 1 in 50 (Fig. 2C) containing *ca.* 50 µM fluorochrome.
2. Align the slope of the coverslip parallel with the *x*-axis of the microscope.
3. Collect single (*x*,*y*) scans in fluorescence and reflection mode and adjust the focus until the peak intensity is roughly centered in the field of view.
4. The intensity profiles along the *x*-axis represent the overall shape of the two functions. To calibrate the functions, the lateral displacement of the reflection peak is measured with changing focus by collecting an (*xz*) series through the chamber (see Section 3.4). The calibration factor = change in focus/change in *x*-position of the reflection peak

3.9 *In situ* specimen protocols: depth-dependent signal attenuation

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

Problem: The intensity response in the axial (*z*) plane is affected by the axial geometric and chromatic aberrations present along the entire optical path including the specimen. Tissues contain many additional refractive index boundaries which will all contribute to further chromatic and spherical abberation of the confocal probe geometry. The consequences of these effects will be increasing signal attenuation with increasing depth through the specimen. The complex spatial distribution of the refractive material and the overall geometry of the tissue currently prevent development of universal models for tissue-dependent attenuation. However, partial correction can be achieved by a more pragmatic approach based on determination of the axial intensity profile of a permeabilised specimen filled with a fluorescent ‘sea’ (Errington et al. 1996; White et al. 1996). The resultant response combines the effects of depth-dependent ‘sea’ response and the additional contribution of the permeabilised tissue. *In vivo*, the effects are likely to be marginally worse even than this case as a number of refractive boundries, particularly membranes, will be distorted or extracted during the fixation/permeabilisation procedure. The overall protocol is summarised in Fig. 3.

1. Place the specimen in an appropriate fixative (e.g. 2% paraformaldehyde in PBS; fresh ethanol: acetic acid (3:1); acetone or methanol at -20°C) for 30 min.
2. Specimens fixed with non-aqueous media may need to be rehydrated (e.g. through an ethanol series (70/50/30/10/PBS) for 30 min each at room temperature).

3. Incubate in PBS containing *ca*. 50 µM of the appropriate fluorochrome for 24-48 h with gentle agitation to ensure good tissue penetration.
4. Collect axial (*x,z*) sections simultaneously in fluorescence and reflection mode into two channels through the permeabilised, infused specimen. Sampling should start *ca.* 10 µm outside the fluorescent medium with a 0.3 µm *x,y*-pixel spacing, and continue at 0.4 -0.5 µm *z*-step intervals through the medium plus specimen and *ca.* 10 µm further into the slide.
5. Measure average fluorescent intensities in regions of the specimen at varying depth to determine the *in situ* 'sea' response. For plant material the most useful region to measure is in vacuoles where there is a large volume of homogeneous dye concentration.
6. The attenuation profiles are normalised to the start of the tissue, defined from the reflection images, and can be fitted with a variety of functions. Typically a quadratic function provides a reasonable agreement for simple flat tissues.
7. The inverse of the parametised equation is used to generate a correction factor to apply to succesive *z*-planes in the experimental data.

The correction for *z*-axis focus error can be applied directly to the images to view correctly scaled (*x,z*) sections, but for measurements it is more efficient to apply the correction to the numerical values extracted during the analysis (White, 1995; White et al. 1996).

In structures that have a relatively constant organisation in the *x,y*-plane a single axial correction equation may be sufficient. For tissues that show a more complex and variable organisation, a series of correction equations may be required related to each zone of the tissue. The magnitude of the attenuation will depend on the depth, lens (particularly NA), immersion medium, bathing medium and wavelength (see Errington et al. 1996; White et al. 1996). Certain lens/immersion combinations may actually give an increase in intensity initially, depending on the precise conditions that the lens has been corrected for.

3.10*In situ* specimen protocols: normalisation of fluorescence intensity against protein or DNA content.

It is often useful to be able to normalise the fluorescence intensity in a range of different units other than on a volume basis, such as µmole (mg protein)$^{-1}$. Conversion of fluorescence intensity to concentration can be readily achieved from *in vitro* calibration curves measured using identical microscope settings. If particular tissue zones, cells or compartments are segmented from the image, results can be expressed on an appropriate database, such as pmole cell^{-1}. At the moment it is rather more complex to relate fluorescence levels to other parameters such as protein levels or DNA content. The basic approach involves collection of 3-D images of protein or DNA distribution from corresponding regions of the same or an equivalent specimen and measurement of the total amount of protein/DNA. The following two protocols are given as basic approaches to labelling protein and DNA. The steps required to analyse such data are described in Section 3.17.

Aim: To label and image protein distribution to normalise quantitative fluorescence measurements for protein content.

At the end of the live experiment, material can be fixed, stained for protein and re-imaged to normalise fluorescence intensity measurements for protein content. We have used FITC labelling of amine residues as a general protein labelling reagent. A number of other

reactive groups with different amino acid specificity are available, linked to a wide range of different fluorochromes.

1. Fix specimen and rehydrate if necessary (see section 3.9)
2. Stain protein with 50 µM FITC in PBS for 30 min.
3. Wash in PBS x3.
4. Images are collected under the same conditions as the experimental imaging.

Aim: To label and image DNA content to normalise quantitative fluorescence measurements for DNA content.

A wide variety of DNA fluorochromes are now available with varying spectra, sensitivity, discrimination between DNA and RNA and specificity for GC versus AT base pairs. We have used Chromomycin A_3 staining to label nuclei in *Arabidopsis* roots as this method gives very little autofluorescence when excited with a 442 nm He-Cd laser. Other DNA probes should be selected and optimised for the tissue autofluorescence and excitation wavelengths available.

1. . Fix specimen and rehydrate if necessary (see section 3.9)
2. Stain in Chromomycin A_3 for 1 h in the dark:(100 µg ml^{-1} CA_3 (Sigma) in 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 140 mM NaCl, 70 mM $MgCl_2$)
3. Mount in fresh antifade: (0.1% (w/v) phenylenediamine in 90% (v/v) glycerol, 10% (v/v) 1x PBS).
4. Store slides in dark at 4°C for up to 1 week.

Note: Separate *z*-distortion and *z*-attenuation correction factors have to be determined for material mounted in glycerol-based media as these have a different refractive index to aqueous media alone.

3.11Live specimen protocols: Chamber design

Working with living cells can be difficult, however, there are specific (and even objective) decisions that can help establish the protocols needed to carry out successful experiments. Keeping cells alive, healthy and fully functional on the microscope is really the crux of live cell imaging (see Errington et al. 1996; Matsumoto, 1993; Terasaki and Dailey, 1995). Choice or design of a chamber necessitates a compromise depending on the length of observation, the nature of the experiment and the properties of the cell.

- A robust and reliable chamber is required to give reproducible imaging conditions and withstand continual z-focussing without leaking and moving. Dimensions are critical, a large viewing area enables accessibility for wide, high. NA, oil immersion lenses. However, large coverslips are more prone to warping resulting in drifts in focus.
- Temperature control is most easily achieved in a thermostatically controlled lab. with air conditioning when the chamber, media and microscope are all at equilibrium. If additional temperature regulation is required, the controller must be a proportional type as this will maintain the heater at a steady state. A simple on / off type, alternates between fully on or fully off and fluctuations in temperature may be large. Temperature regulation is most precise if the sensor sits in the medium. Chambers with large viewing areas tend to give more problems with uneven heat dispersion and increased rate of heat loss. Objectives can also be heated with coils etc. to eliminate this component as a heat dump. Apparently, the optical properties

of some lenses are not compromised up to 50°C provided the temperature is not cycled repeatedly.

- Cells may require perfusion in order to maintain appropriate levels of dissolved gases, such as O_2 and CO_2, nutrients and pH. It is important to make sure that the mechanics of slurping liquid in and out do not cause too much disturbance. It is also crucial to ensure the liquid is pre-heated before it enters the bath.
- The chamber may also have to incorporate features which enable the manipulation of the cells or tissue on the microscope such as microelectrodes or a rapid or pulsed delivery system for pharmacological agents.
- Vibration isolation tables are often essential components of the imaging system to prevent vibrations reaching the microscope optics and scan system. Such effects are often apparent as minor periodic or aperiodic signal intensity fluctuations or, in severe cases, zig-zag distortion of structures. In physiological imaging with large, sometimes open, perfusion chambers, air currents also provide a major source of specimen movement, particularly in rooms with circulating air conditioning. Robust chamber design and shielding the stage area help to minimise this effect.
- Auto-focus routines may also be used to overcome some problems with specimen movement.

3.12Live specimen protocols: Optimising the labelling environment:

The fluorescent dye may interfere with the normal function of the cells as the concentration is increased. In addition, the interaction of illumination with the fluorescent dye may cause photo-toxic damage, particularly if the excited dye reacts with oxygen to give free radicals. Thus, the concentration of fluorochrome introduced should be kept low to minimise buffering of the ion involved and 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, particulary the redox equilibrium of the cells.

Usually the best way forward is to ascertain the upper limit of dye loading consistent with *minimal* disruption of cell physiology and then manipulate the image collection conditions to maximise cell viability. Typically for calcium or pH dyes this is about 50 µM. It is important to have good 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 etc. Alternative strategies involve other ‘vital’ or ‘mortal’ staining techniques which probe different aspects of membrane integrity and metabolic activity.

3.13Fluorescence time course protocols: Optimising settings

Once the cells are loaded with the appropriate dye, the collection protocol can be optimised. In most point-scanning instruments several variables contribute to the sampling achieved within the specimen. The volume sampled depends on the lens and level of optical sectioning. The period each pixel is illuminated depends on the scan speed, however, signal will only be usefully collected for fraction of the dwell time depending on the speed of the detection electronics. The pixel spacing depends on the

lens magnification and zoom. There is usually control over the degree of confocality, the area scanned, the scan speed and the number of frames averaged. However, it is also important to minimise the light exposure to the sample, hence even when hunting around for the cells this should be done as fast as possible.

- The amount of laser illumination presented at the sample is probably the most critical parameter. This can be adjusted by altering the output power of the laser, inserting neutral density filters or by using an acoustic-optic modulator. If the output is measured with a power meter at the objective lens then this ensures that the optimal conditions can be achieved independent of the CLSM system. Values between 76 µW (Tsien and Waggoner, 1995) and 20 µW (Errington et al. 1996) are appropriate for high NA lenses giving acceptable S/N ratios and cell viability.
- The numerical aperture (NA) of the objective lens dictates the initial light gathering efficiency - the higher the NA the better the collecting efficiency. It should be noted that the illumination spot is smaller with higher NA lenses and this may cause increased phototoxic damage. Therefore, when changing between lenses the laser intensity may need adjustment or the integration time, scan speed etc. altered.
- Keeping cells alive may also be at odds with optimal sampling. Measurements of ion concentrations rarely require spatial resolution greater than ~0.5 µm in *x,y*, thus it is important to optimise the instrument to maximise S/N and minimise phototoxicity under these conditions. This relatively low spatial resolution may permit some flexibility in choice of objectives and pinhole settings. Pawley (1995b) also suggests using a high NA lens to maximise fluorescence collection efficiency, whilst underfilling the back focal plane to reduce the effective NA of the illumination beam giving a larger spot. The optimal pixel spacing will also be much greater than that required to give the maximum spatial resolution possible using the Nyquist criterion.
- The frame rate is determined by the frame size and scan speed, each of these parameters can be altered separately. CLSM offers many different timelapse imaging modes: *xt(time), xyt, xzt, xyzt.*, the choice of mode depends how much imaging the specimen can handle and again the temporal and spatial resolution required. In combination, the frame rate and frame size dictate the pixel-dwell time, or how long a single voxel is illuminated. In experiments where temporal resolution is critical, the area scanned can be reduced to a single line on some instruments or even a single point.
- Scanning instruments are ideally suited to simultaneous emission ratio measurements as most are equipped with two photo-multiplier detectors. Excitation ratioing requires rapid switching between different wavelengths and adds a time delay between collection of a pair of wavelength images for ratioing. With multiple lasers this requires rapid synchronised shuttering of the two beams. For multiple line lasers, wavelength selection requires filter wheels, acousto-optic wavelength selection or separation and shuttering of the individual lines.
- Integrating multiple frames or slowing the scan speed reduces noise, but in live cell imaging this increases the overall excitation exposure and may not be appropriate for rapidly changing phenomena or dynamic systems with rapid rates of streaming. At the moment it is also still not clear whether the best *S/N* regime is to have a single slow scan with no integration or several fast scans incorporating integration. The debate hinges on the recovery time of some reversibly excitated states that deplete the fluorochrome without generating fluorescence.

- Intermittent sampling with a time delay between each time point can also be used for slowly changing phenomena.
- It is very useful, if not essential, to have some form of information about the progress of the experiment on-line. Specific 'time-course' software often allows the user to specify a number of regions-of-interest (ROI) and report the average fluorescence intensities (with or without dark current and/or background subtraction) or ratios from these regions during the experiment. Graphical presentation of the intensities/ratios from these regions allow dynamic changes to be followed during the experiment and synchronised *via* on-screen annotation of the traces. Detailed analysis is better undertaken post-capture but requires additional time to save the image data.
- Some instruments have 24-bit display capacity during image collection and allow on-line 'merging' of 3 signal channels as the individual red, green and blue channels of the 24 bit display. In ratio measurements, the colour from a 2-channel 'merge' of each wavelength image can be used to provide a visual pseudo-colour indication of the instantaneous fluorescence ratio, although there is no on-screen record of the history of the experiment.

3.14Fluorescence time course protocols: Data Collection

Aim: To establish optimal collection conditions whilst maximising cell viability.

1. Find the cell(s) of interest with the pinhole aperture fully open, the laser intensity at a low setting and the scan speed to fast. (This ensures you can move the sample around and find the cells of interest as fast as possible, while exposing them to the minimal amount of laser illumination.).
2. Select the appropriate objective lens and adjust frame size and zoom to encompass the region of interest.
3. Adjust the black level settings to ensure the background is recorded
4. Close the pinhole down.
5. Select the scan speed
6. Adjust the gain to ensure the signals are not saturated.
7. Set the number of frames to be integrated.

6. Set the sampling interval between time points.

7. Four set of controls should be run: (i) sample alone with no dye or laser: to test the effects of the microscope perfusion regime in comparison with an equivalent sample under normal incubation conditions; (i) sample plus laser: to test the biological effects of intrinsic absorbance and to measure the levels of autofluorescence; (iii) sample plus dye (but without laser): to test the effects of dye loading on physiological function; (iv) sample plus dye plus laser: to test the potential phototoxic effects of illumination levels and dye concentrations. All of these steps require good markers of cell function and physiological response for comparison with unloaded cells.

3.15Data analysis protocols: Ratio image analysis

Aim: To visualise spatial and temporal fluorescence dynamics

Once the images have been collected correctly there are a wide range of different analysis techniques that can be applied to extract useful quantitative information. 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 (see Kurtz and Emmons, 1993; Mason, 1993; Diliberto et al. 1994; Girard and Clapham, 1994) and provides a good visual indicator of the magnitude of the response and the level of spatial heterogeneity within or between cells. The major steps in calculating a ratio image are given in the left column of Fig. 4.

1. The S/N ratio can be increased at the expense of spatial resolution by an averaging filter (e.g. a 3x3 box reduces noise by $\sqrt{9}$).
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. Typically the mis-alignment is less than 1-2 pixels for a dual excitation system, depending on the (*x*,*y*)-pixel spacing, and should be less than 1 pixel for a dual-emission system.
3. Measurement of the background signal is best achieved from an adjacent region of tissue that is unloaded. Background corrections should be applied for each wavelength image and time point in the series.
4. Pixels with low values or those outside the object are normally masked to exclude regions of corresponding high variance from the ratio image by setting the intensity to zero below a threshold intensity value or outside a spatially defined mask. Three protocols may be used to define the mask: (i) An intensity value at a fixed number of standard deviation units above the mean background intensity, typically 2 s.d. units; (ii) at a 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.
5. Masking is also required to exclude values approaching saturation of the 8-bit 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 (see section 2.3). A pragmatic approach is to measure the distribution of intensities in a fluorescent ‘sea’ at about the concentration of fluorochrome encountered *in vivo* and determine the highest mean value where the distribution is not clipped.
6. Background subtraction, threshold masking and saturation masking can all be accomplished in a single rapid operation by manipulation of the look-up table(LUT) (Fig. 5) and application to the image.
6. The ratio image is calculated pixel-by-pixel.
7. 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.
8. A variant on this overall scheme that avoids selection of a masking threshold is to code pixels ratios by hue and pixel intensities by brightness. In 8-bit displays this can be achieved using 6-bits for hue and 2-bits for brightness. On 24-bit displays, a wide range of hue and brightness values are available.
9. 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, as the distribution is not symmetrical. Thus it is usually more appropriate to visualise changes using ratio

images, but to perform quantitative analysis on the original intensity data directly with an average area analysis or a transect analysis as described below.

3.16Data analysis protocols: Average area analysis

Aim: To quantify fluorescence dynamics

The basic protocol for average area measurements is shown in the middle column of Fig. 4. In some cases it is useful to segment the object using a 50% threshold mask prior to area measurements to facilitate measurements from regions encompassing irregular structures such as cytoplasmic strands, filopodia and sub-cellular compartments, without recourse to detailed manual delimitation of the area.

1. A series of 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.
2. The average background values are subtracted independently for each ROI at each wavelength.
3. The average ratio is calculated and displayed graphically.
4. Error bars can be calculated from the SD of the individual wavelength populations. If the 90% confidence limits of the individual wavelengths are used, the ratio values will have 81% confidence limits.

3.17Data analysis protocols: Transect analysis

Aim: To extract and measure changes in fluorescence along a user-defined transect.

A number of biological experiments involve systems that show well-defined growth patterns or physiological movements. We have developed a modification of the area analysis protocol that facilitates measurements of ROI that are defined with respect to a particular structure in the specimen, such as the tip of a pollen tube or leading edge of an epithelial cell (Fricker et al. 1994). It is usually appropriate to segment the object using a 50% threshold mask prior to the transect analysis. The major steps are shown in Fig. 4, right column.

1. A transect is drawn along the structure in the direction of movement and the average intensity 8-32 pixels normal to the transect measured for each wavelength image with appropriate image or transect alignment if necessary.
2. The averaged transects are used to construct successive lines in intermediate *length,time* (*l,t*) images.
3. If the region of interest has moved, the intensity profiles can be aligned vertically for the ROI and values extracted using a second transect along the vertical time axis.
4. The average background values are subtracted independently for each wavelength.
5. The average ratio for each time point is calculated and displayed graphically.

3.18Data analysis protocols: 4-D (*x,y,z,time*) single wavelength fluorescence quantitation.

Quantitative measurements from 3-D images over time requires correction of the data for axial distortion, measured using the protocol described in section 3.4, and axial-attenuation measured using the protocol described in section 3.9, to relate the measured fluorescence intensity to the ‘real’ level of fluorescent probe. A schematic representation of these stages

is shown in Fig. 6 (see Errington et al 1996). After such correction, the fluorescence is calibrated per unit volume sampled throughout the specimen. The object(s) of interest are typically segmented at the 50% intensity between an average 'internal' value and the background in each *x,y* section in turn. Alternatively, objects can be segmented at the 50% threshold from the 3-D image using a 'seed-fill' function. In many cases, additional manual or semi-automatic editing of the *x,y* sections is necessary to extract the object completely. We have not elaborated on these methods as the range of functions available and implementation depend to a large extent on the software available.

If particular zones or cells are segmented, the results can immediately be expressed on a per cell basis. It may also be appropriate to normalise the fluorescence levels to other parameters such as protein or DNA content. At the moment this is a rather complex operation and involves collection, correction and segmentation of an equivalent 3-D image of protein or DNA distribution and measurement (usually) of the total amount of protein/DNA in a corresponding region, however, the results give an incredibly detailed quantitative cellular map of fluorescence kinetics in units that can be directly compared with results from other techniques, such as flow cytometry or conventional biochemical and molecular biological analysis. This approach should have wide application in single cell biochemical measurements with the increasing range of fluorescent enzyme substrates. With the advent of GFP and its derivatives, it is also now possible to construct fluorescent chimeric fusion proteins to follow protein dynamics *in vivo*. 4-D confocal imaging allows quantitation of these events in the correct physiological and morphological context and also allows measurements on rare, highly specialised cells.

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Figure Legends

Figure 1. Schematic outline of the major stages in quantitative confocal fluorescence microscopy

Figure 2. Schematic diagrams of different slide/coverslip sandwich configurations for microscope performance measurements. (A) general fluorescence intensity measurements; (B) measurement of reflection plane spread function; (C) rapid measurement of reflection plane-spread function and fluorescence axial step function.

Figure 3. Schematic diagram of the main steps used to correct *z*-focus error and *z*-attenuation in 3-D images. The *z*-distortion factor can be applied directly to the image data to view correctly sized (*x*,*z*)-images, or used to scale the numerical data extracted from the image as part of the analysis protocol.

Figure 4. Schematic diagram of the main steps in confocal ratio analysis. Visualisation of the spatial distribution of ratio dynamics is best achieved using ratio imaging (left column). Quantitative analysis can be achieved from a region of interest (ROI) using an area average (center column) or a transect analysis (right column).

Figure 5. Manipulation of the look-up table for rapid image processing. The dotted line represents a 1:1 correspondence between input and output values. The solid line is a function that combines subtraction of a mean background value, threshold masking and saturation masking in a single operation.

Figure 6. Schematic diagram of the main steps to extract quantitative information from a four-dimensional (*x*,*y*,*z*,*time*) image and normalise the data to cell volume, protein content or DNA content.