REDOX IMAGING SOFTWARE V1.2

MARK FRICKER
PLANT SCIENCES, OXFORD

Contents

1
Introduction

1.1 General principles

A wealth of fluorescent reporters and imaging systems are now available to characterise dynamic physiological processes in living cells with high temporal and spatial resolution. The most reliable probes for quantitative measurements show shifts in their excitation or emission spectrum, rather than just a change in intensity, as spectral shifts are independent of optical path length, illumination intensity, probe concentration and photobleaching, and can be determined easily by ratiometric measurements at two wavelengths. A number of ratiometric fluorescent reporters, such as roGFP, have been developed that respond to the glutathione redox potential and allow redox imaging in vivo. roGFP and its derivatives can be expressed in the cytoplasm or targeted to different organelles giving fine control of measurements from sub-cellular compartments. Furthermore, roGFP can be imaged with probes for other physiological parameters, such as reactive oxygen species (ROS) or mitochondrial membrane potential, to give multi-channel, multi-dimensional 4-D (x,y,z,t) images. Live cell imaging approaches are needed to capture transient or highly-spatially localised physiological behaviour from intact, living specimens, which are often not accessible by other biochemical or genetic means. The next challenge is to be able to extract useful data rapidly from such large (GByte) images with due care given to the assumptions used during image processing.

The programs described in this manual were developed to visualise ratiometric changes in fluorescence of the redox sensitive protein, roGFP[1] and its derivatives (Fig. 1.1), expressed in transgenic cell cultures, plants and fungi. Equally, the programs can also be applied to quantify signals from any ratiometric or single-wavelength fluorescent probe in any system. The programs are written in MatLab (The Mathworks, Natick, MA) and packaged in a single compiled executable file, or available as the original matlab files, for download from www.markfricker.org.

Each program progresses through the ratio analysis step-by-step, to ensure that due consideration is explicitly given to each of the parameters used in the analysis. To aid visualization of spatial changes in roGFP, it is usual to calculate a ratio images on

[1] G. T. Hanson, R. Aggeler, D. Oglesbee, M. Cannon, R. A. Capaldi, R. Y. Tsien, and S. J. Remington. Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators. *Journal of Biological Chemistry*, 279(13):13044–53, 2004

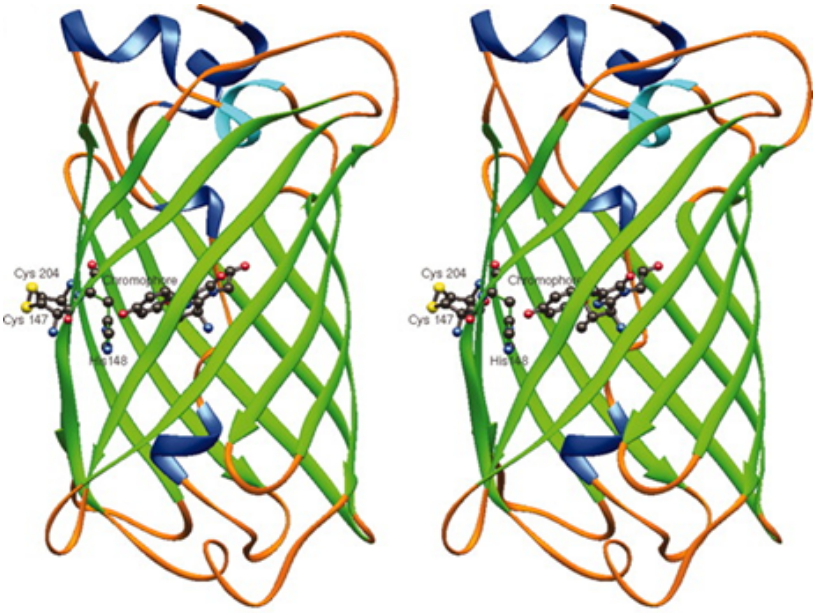


Figure 1.1: Key structural features of roGFP2, The engineered cysteines Q204C/S147C form a disulfide cross-link between adjacent β-strands near the chromophore in GFP. From Hanson et al. *2004*

a pixel-by-pixel basis from images collected at wavelengths that preferentially excite the oxidised and reduced form of the probe. For most laser-based confocal microscopes these are I_{405} and I_{488}, respectively.

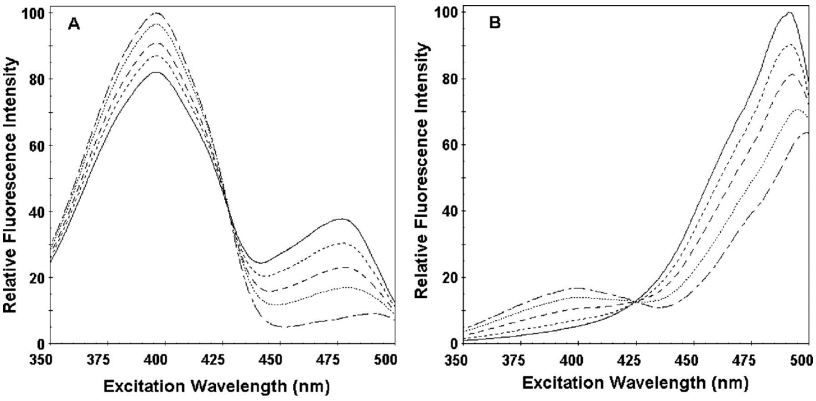


Figure 1.2: Redox equilibria of roGFPs by titration against DTT. A, fluorescence excitation spectra of roGFP1 while monitoring emission intensity at 508 nm at the following redox potential values: -0.320 (solid line), -0.294 (short dashed line), -0.286 (long dashed line), -0.275 (dotted line), and -0.249 V (dotted and dashed line). B, fluorescence excitation spectra of roGFP2 while monitoring emission intensity at 511 nm at the following redox potential values: -0.310 (solid line), -0.285 (short dashed line), -0.275 (long dashed line), -0.265 (dotted line), and -0.240 V (dotted and dashed line). Fluorescence emission intensities were normalized to the maximum intensity recorded in each series of scans. From Hanson et al. *2004*

To improve the signal-to-noise ratio (S/N), the initial images are typically smoothed in (x, y) with a spatial averaging filter, using a 3×3 or 5×5 kernel. Accurate visualisation and quantitation require measurement and subtraction of the background signal for each channel. It is also useful to mask pixels with intensity values close to background or near saturation from the ratio image, as these give a mis-leading impression of the real ratio value. These pixels are also excluded from quantitative measurements.

For pseudo-colour display, the masked ratio is coded by hue on a spectral colour scale ranging from blue (fully reduced) to red (fully oxidised), with the limits set by an *in situ* calibration or extrapolated from an *in vitro* calibration. The intensity of the ratio

image is calculated as the mean intensity for each pixel in the two channels. This effectively gives bright colours for regions with good signals that fade to background for regions of low signal.

As the signal contributing to each pixel in a ratio image is very noisy, it is preferable to make quantitative measurements from user-defined regions-of-interest (ROIs) that average the signals spatially or temporally from a larger number of pixels.

Whilst relative changes in ratio values can provide a reasonable amount of information for comparative studies, more precise calibration may be possible with experimental treatments that define the *in vivo* probe response. In addition, each experiment should ideally include an internal calibration at the end of the experiment by perfusion with a reductant, such as DTT, and an oxidant, such as H_2O_2 to drive the roGFP to a fully reduced or oxidised form, respectively. These values can be stored and applied to other data sets collected under identical conditions. Experimental details for plants can be found in Schwarzländer *et al.* (2008)[2] and Meyer and Fricker (2008)[3]. Experimental details for multi-channel measurements in fungi can be found in Samalova *et al.* (2013)[4].

The degree of oxidation (OxD) of the *roGFP* sensor is calculated from equation (1.1) after Hanson *et al.*, (2004).

$$OxD_{roGFP} = \frac{R - R_{red}}{\frac{I_{488ox}}{I_{488red}}(R_{ox} - R) + (R - R_{red})} \tag{1.1}$$

Where R is the ratio with excitation at I_{405}/I_{488} nm, after background subtraction and auto-fluorescence correction; R_{red} is the ratio of the fully reduced form following perfusion with DTT; R_{ox} is the ratio of the fully oxidised form following perfusion with H_2O_2 and I_{488ox} and I_{488red} are the emission intensities with excitation at 488nm for the fully oxidised and fully reduced forms, respectively.

The redox potential is then estimated from equation (1.2)

$$E' = E_0'^{pH} - \frac{2.303RT}{zF} \log \frac{1 - OxD_{roGFP}}{OxD_{roGFP}} \tag{1.2}$$

Where R is the gas constant ($8.315 JK^{-1}mol^{-1}$), T is the absolute temperature ($298.15K$), z is the number of transferred electrons (2), F is the Faraday constant ($9.648 \times 10^4 Cmol^{-1}$) and $E_0'^{pH}$ is the midpoint redox potential of roGFP at a given pH.

The value of $E_0'^{pH}$ is based on the standard midpoint potential E_0' at 30 °C and $pH7$, adjusted for the estimated compartment pH and experimental temperature ($20 - 25$ °C) according to Equation (1.3):

$$E_0'^{pH} = E'_{0(roGFP)} - \frac{2.303RT}{zF}(pH - 7) \tag{1.3}$$

Where the midpoint redox potential for roGFP1 (E_0') is $-288mV$ (Fig. 1.3 A)and roGFP2 (E_0') is $-272mV$ (Fig. 1.3 B).

[2] M. Schwarzlander, M.D. Fricker, C. Muller, L. Marty, T. Brach, J. Novak, L.J. Sweetlove, R. Hell, and A.J. Meyer. Confocal imaging of glutathione redox potential in living plant cells. *Journal of Microscopy*, 231:299–316, 2008

[3] A.J. Meyer and M.D. Fricker. *Imaging thiol-based redox processes in live cells*, volume 27 of *Advances in Photosynthesis and Respiration*, pages 483–501. 2008

[4] M. Samalova, A.J. Meyer, S.J. Gurr, and M.D. Fricker. Robust anti-oxidant defences in the rice blast fungus *Magnaporthe oryzae* confer tolerance to the host oxidative burst. *New Phytologist*, 201:556–573, 2013

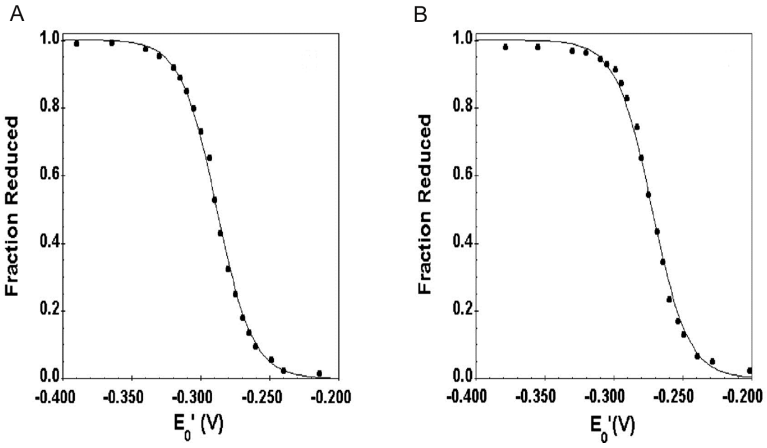


Figure 1.3: apparent redox potential values of the roGFPs were determined by plotting the fraction of reduced protein versus the ratio of DTT_{red} to DTT_{ox} or the equivalent redox potential values and fitting the data to a titration curve. From Hanson et al. *2004*

1.2 *Ratio Analysis Software Overview*

An overview of the functionality of the software suite is shown in Fig. 1.4. There are separate interfaces to import and pre-process images prior to ratio analysis (Fig. 1.4: import module); conduct a basic ratio analysis (Fig. 1.4: basic ratio analysis module), include additional fluorescence channels to allow correlation between multiple physiological parameters (Fig. 1.4: advanced ratio analysis module), which also allows with pixel-population measurements to handle complex structures that are not readily sampled by manual measurements from discrete regions-of-interest; and object-based segmentation approaches, coupled with morphological characterisation to correlate organelle physiology with spatial landmarks (Fig. 1.4: object-based ratio analysis module).

1.2.1 *Image import and pre-processing*

The programs use a common import module (Fig. 1.4A, B) to load images from a variety of formats. Each program progresses through the ratio analysis step-by-step, to ensure that due consideration is explicitly given to each of the parameters used in the analysis. Once set, the processing and calibration parameters can be stored in a database, and re-applied to subsequent analyses or datasets.

The starting point for analysis is a multi-channel, multi-dimensional fluorescence image (Fig. 1.4A), often with a corresponding (non-confocal) bright-field image (Fig. 1.4B). Visualisation of the probe response involves calculation of pseudo-colour coded ratio images that help the user to understand the spatio-temporal dynamics in different cells or sub-cellular compartments. Ratio images are calculated on a pixel-by-pixel basis from images collected at wavelengths that preferentially excite the oxidised and reduced form of the probe. For roGFP on current laser-based confocal microscopes these are typically 405 nm and 488 nm, respectively.

Pixel-by-pixel ratioing is critically dependent on correct image registration, as even very slight misalignment of the ratio images

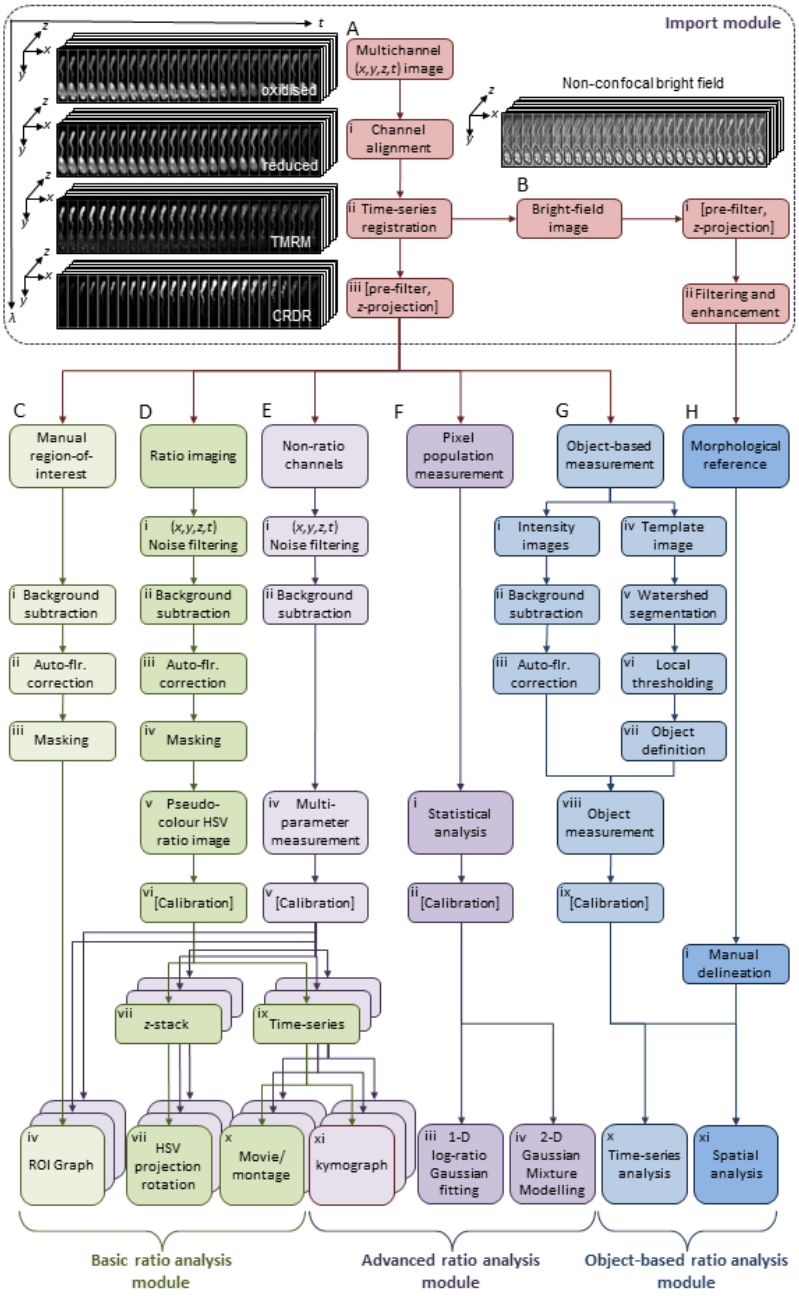


Figure 1.4: Schematic diagram showing the main elements of the redox ratio analysis software. Multi-channel, multi-dimensional images are loaded using the import module (red boxes), aligned, filtered and, if appropriate, the dimensionality reduced using projection algorithms. The Basic ratio analysis module (green boxes) is used to construct pseudo-colour coded ratio images, following background subtraction and autofluorescence correction. Measurements are made using manually defined regions-of-interest (ROIs). The Advanced ratio analysis module (purple boxes) extends the analysis to include multiple channels, and provides additional visualisation approaches, such as kymograph plots. There are also options to analyse the whole population of pixels using 1-D fits to the log-ratio data or 2-D Gaussian Mixture Models fit to the corrected intensity data. The Object-based ratio analysis module (blue boxes) uses a watershed segmentation, followed by a local intensity threshold to identify individual objects. The ratio values are then calculated from the average intensity for each object. The object ratios can be analysed as a population response, or combined with morphological information, such as cell identity or with distance from a spatial landmark, to correlate the redox behaviour with the cell response.

yields obvious fringing with completely inappropriate ratios along the boundaries. During data collection using confocal systems, this typically means that line-switching, rather than frame switching between excitation wavelengths must be used to minimise the impact of organelle or specimen movement. During data processing, it is possible to correct for minor channel misalignment by calculating the (sub)-pixel offsets that maximise the cross-correlation between the selected image(s) and a reference image, typically the channel with the brightest and best resolved features. Images are then aligned using bi-linear interpolation with these offsets (Fig. 1.4A(i)).

Likewise, time-series measurements of intact specimens in

perfusion systems often experience some level of drift in the (x,y) plane or focus shift along the z-axis. It is helpful to align the image series to ensure measurements are made from the same region of the specimen in subsequent analysis. Specimen movement or stage drift can be partially compensated by (x,y) registration using temporal cross-correlation (Fig. 1.4A(ii)).

Whilst many animal cells conveniently grow as a monolayer on a coverslip greatly facilitating imaging with a single optical section or using Total Internal Reflection Fluorescence (TIRF) microscopy, a number of optical sections are often required to capture features of interest in plant and fungal cells. When visualising morphological markers, such as organelle markers, 3-D (x,y,z) data are often visualised as a maximum intensity projection (MIP) along the z-axis for each channel. However, this is not appropriate as a precursor to quantitative measurements, particularly when ratioing two channels, as the MIP selects the 'noisiest' pixel at the extreme of the intensity distribution along the z-axis, and will pick out pixels from different positions in z for each channel, making it impossible to calculate a reliable ratio image. An average brightness projection in z, provides better noise reduction, and may be useful for simple objects that do not overlap in the z-axis. However, a more appropriate approach is to apply local noise reduction by smoothing in (x,y,z), select the z-position of the brightest voxel in one, or a sub-set of channels, and then extract the same (x,y,z) voxel from all the other channels (Fig. 1.4A(iii)). This provides a sub-sample of the original 3-D data that faithfully preserves the main features of interest in the z-axis, and is absolutely required if different channels are going to be ratioed later on to ensure that information from the same (averaged) voxels are compared. This approach also compensates to some extent for axial movement, as the brightest object is extracted irrespective of its z-position.

1.2.2 *Basic ratio analysis*

The basic ratio analysis program (Fig. 1.4C, D) is designed to analyse single (x,y) images, 3-D (x,y,t) time series or 3-D (x,y,z) z-stacks. The first processing step usually involves improving the signal-to-noise ratio (S/N) by spatial or temporal averaging (Fig. 1.4D(i)). Whilst there are a range of noise-reduction algorithms available, simple averaging using 2-D or 3-D kernels of different sizes provides a balance between S/N and spatio-temporal resolution. Averaging can be usefully combined with sub-sampling to reduce image sizes and increase processing speed, as spatial resolution is already compromised by averaging. As averaging is likely to yield non-integer values, this step also requires conversion from integer to floating-point format.

To measure intensity values correctly it is important that the images are collected with a non-zero background, so that the statistical properties of the background can be estimated accurately

and subtracted from each channel (Fig. 1.4D(ii)). In addition, auto-fluorescence bleed-through into one of the measurement channels is a common problem, particularly with plant specimens and blue/violet excitation. As auto-fluorescence has structure in the image, it cannot simply be subtracted as a single value like the background. However, it is possible to estimate the auto-fluorescence from an emission wavelength range that does not have any signal from the probe. For example, with roGFP, this is possible with excitation at 405 nm and emission at 435-485 nm. As the auto-fluorescence spectrum tends to be quite broad, a scaled version of the auto-fluorescence image can be subtracted from the probe image to correct for the auto-fluorescence bleed-through, provided the auto-fluorescence spectrum does not alter with time or treatment (Fig. 1.4D(iii)).

It is also useful to mask pixels with intensity values close to background or near saturation from the ratio image, or pixels that are drawn from regions with high local coefficient-of-variation (CV) that might correlate with edges of structures or compartments that are not well resolved, as these give a noisy and mis-leading impression of the real ratio value (Fig. 1.4D(iv)). Without masking, edge effects from ratioing low signal intensities can be substantial and can visually dominate the ratio image in plant and fungal specimens.

For pseudo-colour display (Fig. 1.4D(v)), the masked ratio is coded by hue on a spectral colour scale ranging from blue (fully reduced) to red (fully oxidised), with the limits set by an in situ calibration or extrapolated from an in vitro calibration. The most useful initial mapping is to HSV (Hue, Saturation and Value or intensity) colour space, where the ratio is coded as Hue and the average intensity from both channels gives the Value. This is converted back to RGB colour space for display, and effectively gives bright colours for regions with good signals that fade to background for regions of low signal. If the data is a time-series (Fig. 1.4D(ix)), it can be viewed as a movie or montage. If the original data is a z-stack (Fig. 1.4D(vii)), visualisation benefits from calculating rocking or tilting projections to aid 3-D interpretation via motion parallax. Conventional rotation algorithms use maximum brightness projections of RGB images. However, this is not appropriate for colour-coded ratio images. Instead, projections in HSV colour-space are used to record the (x,y,z) position of each voxel in maximum projection of the V channel alone. The corresponding voxel from the H and S channels is then used to reconstruct the rotated pseudo-colour image (Fig. 1.4D(vii)).

Whilst ratio images are a useful tool to help visualise spatial variation in signal responses, the signal-to-noise (S/N) ratio is extremely low for single pixels, even after spatial and temporal smoothing. Quantitation is better achieved by manually selecting larger regions-of-interest (ROIs), which have some morphological or physiological significance (Fig. 1C). Each ROI encompasses

many pixels with corresponding improvement in signal-to-noise ratio (S/N) at the expense of spatial resolution, that can be easily presented and interpreted in graphical form (Fig. 1.4C(iv)).

The basic ratio program also has an option (not shown in Fig. 1.4), to batch process a complete set of single (x,y) images present in one folder which is useful to compare the steady-state redox potential in multiple samples and treatments, measured over the whole image or from defined ROIs, rather than a continuous evaluation of changes in redox potential in one specimen.

1.2.3 Advanced ratio analysis

The advanced ratio program (Fig. 1.4E, F) extends the basic ratio analysis to handle 4-D (x,y,z,t) images with up-to 5 fluorescence channels, including an auto-fluorescence channel (Fig.1.4 E), and parallel bright field images (Fig. 1.4B). This allows correlation of changes in auto-fluorescence-corrected ratio images with up to two other physiological parameters, and (non-confocal) bright-field morphology. In addition to the manual ROI measurements, movies and montages available in the basic ratio analysis module, there is also an x-t kymograph option to represent the response along a manually defined transect as a 2-D image with response on the x-axis and time on the y-axis (Fig. 1.4D(xi)).

1.2.4 Pixel-population measurements

In more complex images, such as intracellular hyphal networks during fungal infection (13), picking a few ROIs across the specimen does not provide a robust, un-biased estimate of the physiological behaviour throughout the system. However, this can be achieved in the advanced module by considering the intensity or ratio information from a statistical perspective along with estimates of the goodness-of-fit (Fig. 1.4F), and either calculating multi-component 1-D Gaussian fits to the log ratio data from all pixels (Fig. 1.4F(iii)) or fitting 2-D Gaussian Mixture Models (GMM) to the corrected intensity data prior to ratioing (Fig. 1.4F(iv)). Fitting to the original intensity data or log ratio values does not take into account the relative intensity of the different pixels contributing to the distributions. Thus ratios from very dim pixels contribute as equally as ratios from very bright pixels. A better alternative is to calculate a 1-D or 2-D weighted histogram in which each data point is replicated in the data set in proportion to the average intensity at each of the two wavelengths. This ensures that a large number of dim and noisy pixels do not skew the fits. The advanced ratio module includes options to explore these data fitting approaches.

1.2.5 Object-based ratio measurements

Transgenic reporters, such as roGFP, can be easily targeted to organelles, such as chloroplasts and mitochondria, facilitating

sophisticated dissection of sub-cellular behaviour. However, these organelles are present in considerable numbers (hundreds) in most plant and fungal cells, and are highly dynamic, making it impractical to manually select individual objects, and unhelpful to consider the total pixel-population statistics described above, as these disguise individual organelle behaviour. As an alternative, it is possible to automatically segment each organelle separately, and then calculate the average ratio values on an object-by-object basis (Fig. 1.4G). A wide variety of segmentation algorithms can be used, but we have found a generic two-step procedure is routinely applicable. Objects are initially separated from each other into non-overlapping domains using watershed segmentation of an inverted template image calculated from the original intensity channels (Fig. 1.4G(iv)-(v)). Each object definition is refined using a local intensity threshold within each watershed domain, to accommodate objects with differing overall intensity (Fig. 1.4G(vi)). Once the objects are segmented, various morphological parameters can be measured automatically, along with the average intensity at each wavelength needed to calculate the ratio values. If the objects do not move during the time-series, object responses can be visualised as an 'object'-time plot analogous to a kymograph (Fig.1.4G(x)). In parallel, the (non-confocal) bright-field image can be processed separately to highlight morphological features of interest, using a combination of noise filtering, projection, contrast enhancement, and manual delineation (Fig. 1.4B,H). Key landmarks can then be established to correlate physiological response with cell or organelle identity, morphology, or localised event (Fig. 1.4G(xi)).

1.2.6 *Software interface menu*

All the programs are accessed from a simple menu-bar (Fig. 1.5), and follow the general principles set out above, but are tailored to slightly different experimental configurations.

The **Basic Ratio Program** is designed to analyse 3-D (x,y,t) time series or (x,y,z) z-stacks images. The **Batch Processing Program** can be used to process a complete set of single (x,y) images present in one folder. Each program can also store or access the processing and calibration parameters used in a corresponding probe database which can be interrogated separately using the **Set up probe database** button, and have various output options to view animations of the data, or generate publication-ready figures. The **Advanced Ratio Program** covers 4-D (x,y,z,t) images with up-to 5 fluorescence channels, and a parallel bright field image, to allow correlation of changes in autofluorescence-corrected ratio images with upto two other parameters, and the (non-confocal) bright-field morphology. The programs use a separate interface to load images from a variety of formats and also to save the parameters in a probe database that reflects the additional processing parameters used in the advanced program. Both these latter programs can be ac-

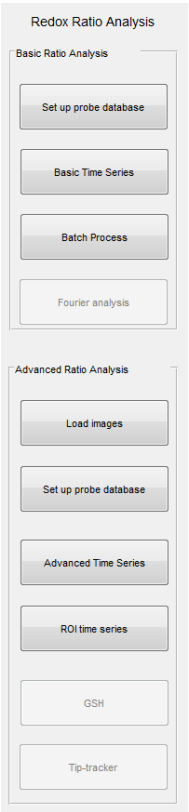


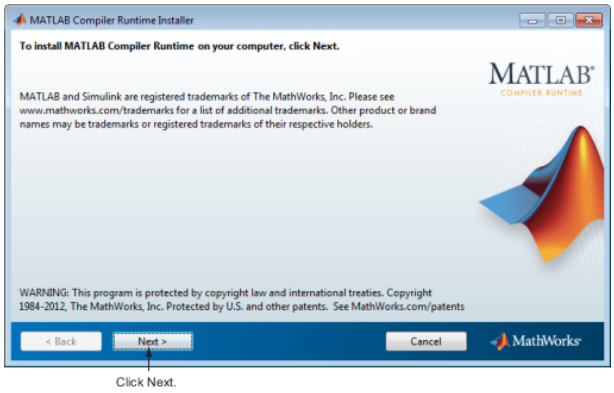
Figure 1.5: Menu bar used to run the various ratio analysis programs. Options that are not available are grayed-out, but will become available in future releases

cessed separately *via* the **Load images** and **Set up probe database** buttons on the menu bar. The **ROI Time series** is designed to allow segmentation of objects, such as mitochondria, from a time-series image and analyse fluctuations in signal or ratio for each object individually. Results can be viewed for each object or as a population response with various statistical fits to the data.

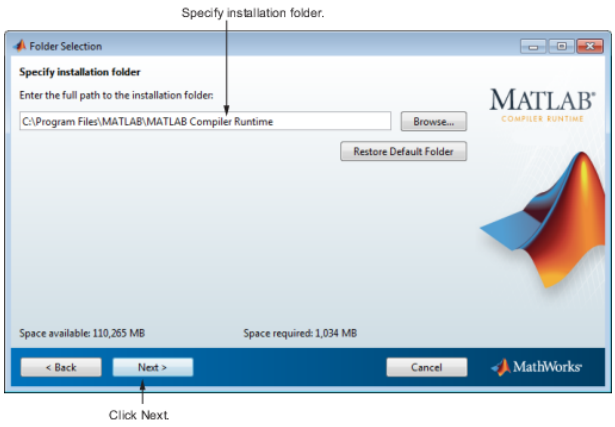
1.3 *Installation*

All the programs are packaged in a single compiled executable file for distribution. In addition, an appropriate version of the MatLab Compiler Runtime (MCR) is required to install the set of shared libraries that enables execution of the compiled MATLAB application. The MCR should automatically download from the Web when the program is installed for the first time. Alternatively MCR can be downloaded from the MathWorks Website:
`http://www.mathworks.com/products/compiler/mcr`.

To install the MCR and run the program, double-click the compiled MATLAB self-extracting archive file, named RRA_v#.exe, where # is the latest version of the program. This extracts the MATLAB Runtime Installer from the archive, along with all the files that make up the MATLAB runtime. Once all the files have been extracted, the MATLAB Runtime Installer starts automatically. When the MATLAB Runtime Installer starts, it displays the following dialog box. Read the information and then click **Next** to proceed with the installation.



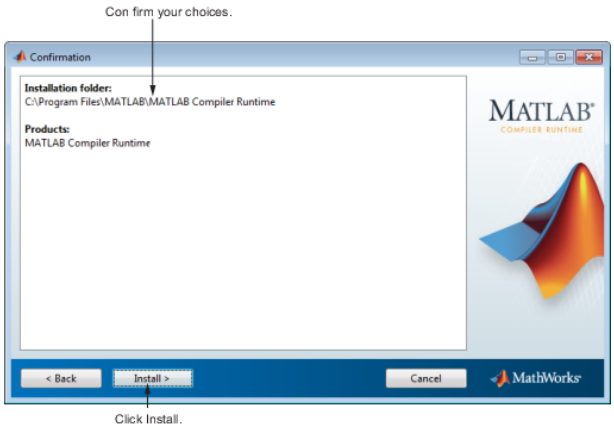
Specify the folder in which you want to install the MATLAB runtime in the Folder Selection dialog box and click **Next**.



Note: On Windows systems, you can have multiple versions of the MATLAB runtime on your computer but only one installation

for any particular version. If you already have an existing installation, the MATLAB runtime Installer does not display the Folder Selection dialog box because you can only overwrite the existing installation in the same folder.

Confirm your choices and click **Install**. The MATLAB Runtime Installer starts copying files into the installation folder



Click **Finish** to exit the installer.

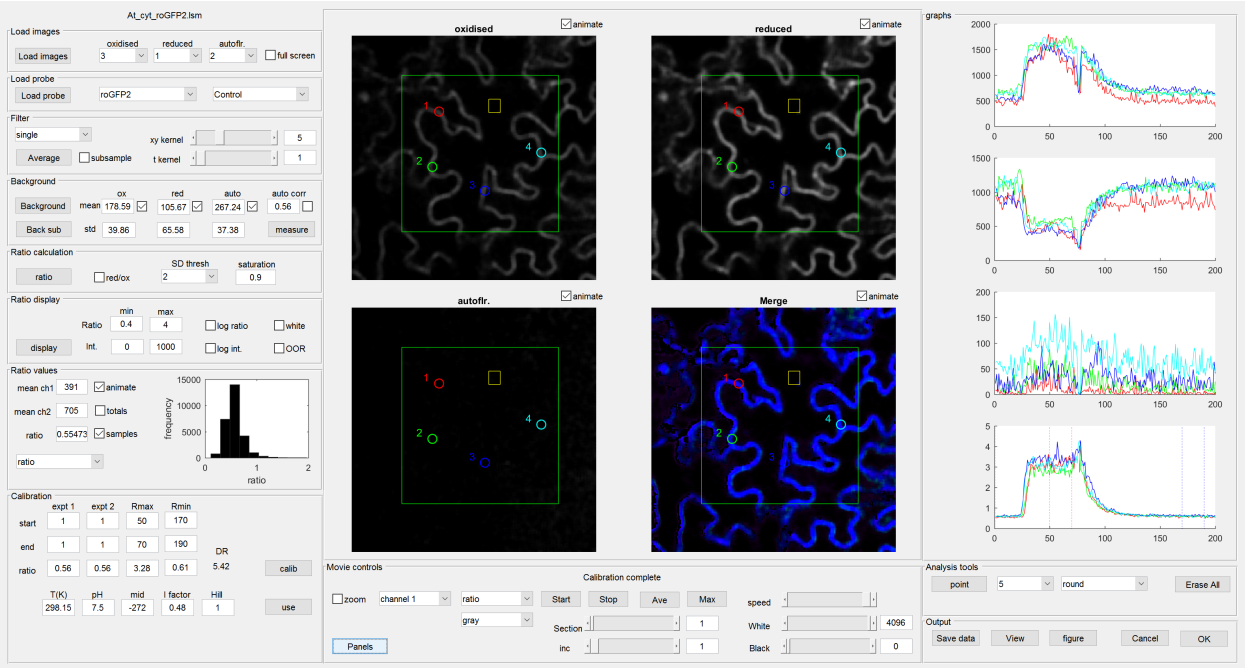
MATLAB Runtime Installer Readme File: A readme.txt file is included with the MATLAB Runtime Installer. This file, visible when the MATLAB Runtime Installer is expanded, provides more detailed information about the installer and the switches that can be used with it.

1.4 Additional files installed with the program

A number of additional files needed to run the full suite of programs are installed at the same time as the main program. These include copies of the probe databases, with a few example entries, and a number of test files.

2

Basic Time-Series Program



2.1 *Introduction*

The **Basic Ratio Program** is designed to analyse a 3-D (*x*,*y*,*t*) time-series or (*x*,*y*,*z*) *z*-stack image of roGFP fluorescence, typically with excitation at 405 nm for the oxidised form and 488 nm for the reduced form. An additional auto-fluorescence channel can be included to correct for auto-fluorescence bleed-through into the 405nm roGFP channel.

2.2 *Loading images*

The **Load images** button (Fig. 2.1) prompts the user to select a 3-D (*x*,*y*,*t*) or (*x*,*y*,*z*) image, with two or three wavelength channels to analyse. The default is the Zeiss .lsm format, but it is possible to import files in .tif, .mat or .avi format. More options are available

if the full **Load Images** interface is used in the **Advanced Ratio Analysis** (see Chapter 7). As the relationship between the order each channel appears in the image series and the physiological parameter of interest depends on the precise configuration of the microscope for each experiment, each of the three channels can be assigned to the appropriate parameter using the drop-down menus (Fig. 2.1).

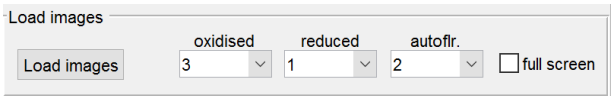


Figure 2.1: The load image control panel: Each channel in the input images is assigned to a specific physiological channel using the drop-down boxes

Thus the default order for a roGFP-based redox ratio experiment would be oxidised, reduced and auto-fluorescence. The image displays are updated to show the appropriate image. The default settings for these controls, and indeed all the parameters needed to process the images, can be set using the **Load probe** panel (Fig. 2.2, See also - Chapter 3 on setting up the probe database).

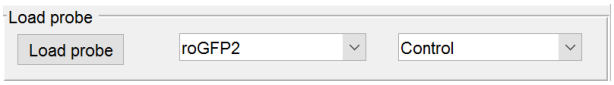


Figure 2.2: The load probe control panel: This loads the default processing parameters from a saved probe database to allow application of standard settings across all experiments for each specific probe or instrument configuration

2.3 *Image Display*

The loaded images are displayed in the four panels in the center of the interface (Fig. 2.3). Below each image are a number of drop-down menus that control which stage of processing, and which channel is being displayed. When the data are loaded initially, only the *raw* image is available for display. The **animate** checkbox controls whether the image is updated during playback.

Immediately below the display panels is the **Movie controls panel** (Fig. 2.3). The **Start** and **Stop** buttons control playback of the images selected in each of the display windows. The adjacent drop-down menu controls whether the *raw* data, *smoothed* data or background *subtracted* data are shown for the individual channels. The fourth panel shows a three channel merge of the intensity images until the pseudo-colour coded ratio image has been calculated and the *ratio* option is selected. Particular **Sections** can be selected using the slider and the increment between sections during animation controlled by the **inc** slider. The **Ave** and **Max** buttons calculate and display a z-projection if the image is a z-stack. The **White level** and **Black level** sliders and text boxes control the intensity scaling for the images displayed, whilst the **gray** drop-down menu allows the user to choose from a range of default look-up tables for the intensity images. The **zoom** checkbox toggles between the four panel display or a single full-size image of the specific channel selected in the adjacent drop-down menu.

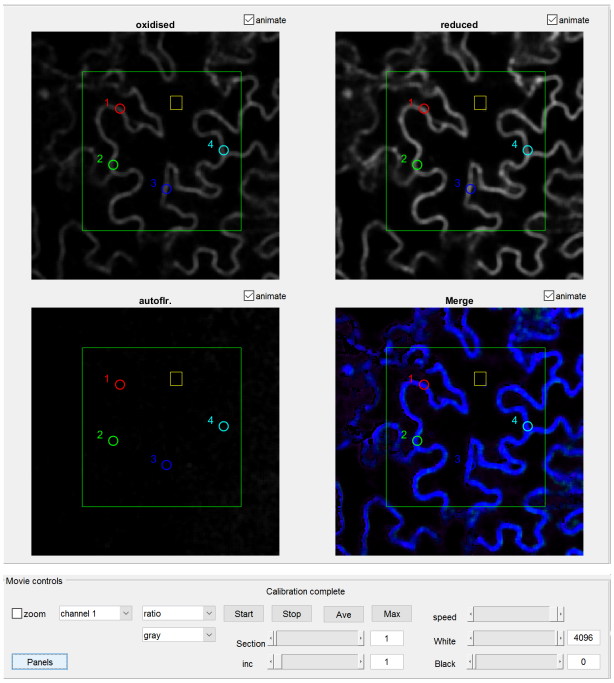


Figure 2.3: The image display and movie control panels: The three wavelength channels are displayed along with the corresponding merge or ratio image once it has been calculated. The checkboxes toggle whether the image is animated during play through of the sequence controlled by the Start/Stop buttons or the Section slider. The display controls can be used to adjust the intensity of the images

2.4 *Spatial and temporal smoothing*

Once the images have been loaded and the correct order for the channels set, the controls in the **Filter panel** are enabled (Fig. 2.4). Noise reduction is achieved at the expense of spatial and temporal resolution using the **xy kernel** and **t kernel** sliders, respectively, to set up a smoothing kernel for the appropriate dimension. The image is converted to single-precision floating point format first to prevent rounding errors.

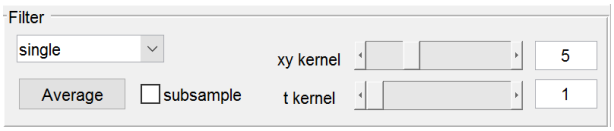


Figure 2.4: The filter panel: The controls are used to set the amount of spatial and temporal image filtering needed to increase the signal-to-noise ratio

As smoothing reduces the spatial and/or temporal resolution, there is a **subsample** checkbox, which, if active, results in a sub-sampled image at (kernel size-1)/2 in each dimension. This can significantly reduce the overall image size and increase the speed of subsequent operations. If subsampling is used, the pixel sizes are updated to reflect the pixel dimensions of the sub-sampled image.

Clicking the **Average** button applies the smoothing filter selected and the displays are updated to show the filtered image for each channel.

2.5 *Background measurement and subtraction*

The **Background** button in the **Background panel** (Fig. 2.5) prompts the user to draw a rectangular region-of-interest (ROI) in an area of background for the currently selected image. The average inten-

sity and standard deviation are returned in the text boxes for each channel. Each adjacent checkbox controls whether the background will be subtracted in the subsequent processing step. The default is active to ensure background subtraction takes place.

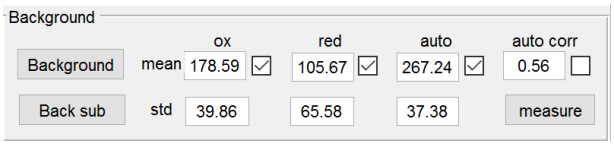


Figure 2.5: The background panel: The controls allow measurement of the background signal in each channel, and the level of bleed-through from the auto-fluorescence channel into channel 1. These values are then subtracted from the filtered images

Auto-fluorescence bleed-through into one of the measurement channels is a common problem in ratio measurements, particularly with plant specimens and blue/violet excitation. As auto-fluorescence has structure in the image, it cannot simply be subtracted as a single value like the background. However, it is sometimes possible to estimate the auto-fluorescence at an emission wavelength range that does not have any signal from the probe. For roGFP this is typically with excitation at 405 nm and emissions at 435-485 nm. As the auto-fluorescence spectrum tends to be quite broad, a scaled version of the auto-fluorescence image can be subtracted from the probe image to correct for the auto-fluorescence bleed-through.

The **measure** button allows the user to draw a rectangular ROI in a region of the image where there is auto-fluorescence, but no probe signal, to calculate the bleed-through correction factor from the auto-fluorescence channel into channel 1. The correction factor is displayed in the adjacent text box. If it is not possible to measure the bleed-through correction factor directly from the loaded image, it has to be estimated from an un-labelled control using exactly the same instrument settings and typed into the correction box. The **auto corr.** checkbox has to be active to apply the correction.

Because the background and auto-fluorescence correction subtract noisy images from each other, there is the possibility of negative values if the original intensities were close to the background. Any negative values are automatically set to zero during the calculation and excluded from further analysis.

Clicking the **Back sub** button applies the background subtraction and auto-fluorescence correction, if appropriate, and the displays are updated to show the filtered image for each channel.

2.6 *Ratio calculation*

Following background subtraction and auto-fluorescence bleed-through correction, the ratio is calculated pixel-by-pixel for the two wavelength channels as oxidised/reduced. The adjacent check box allows the user to calculate the inverse ratio in the **Ratio calculation** panel (Fig. 2.6).

A number of other options are available to mask pixels in each of the ratio channels that are too close to background or saturation

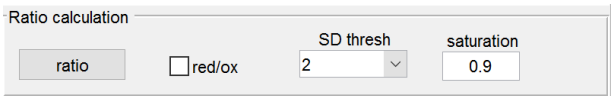


Figure 2.6: The ratio calculation panel: the controls provide various options for masking the ratio image to exclude values too close to background or saturation

to give a reliable ratio. The **SD thresh** drop-down menu allows the users to select a low intensity mask at some (integer) number of standard deviation units above background in each channel. The default is 2 SD. This will mask all areas with low pixel values, such as the vacuole or other organelles, for example.

The **saturation mask** is applied to all pixels above the proportional limit of the bit-depth of the current image in either channel and is typically set at 0.9 of the full scale.

Clicking the **ratio** button calculates the ratio, but at this stage the displays are not updated as it is customary to display the resultant ratio as a pseudo-colour coded image with a calibrated scale.

2.7 *Pseudo-colour coding of the ratio*

Once the images have been processed, the ratio data can be pseudo-colour coded to highlight the parameter of interest using the controls in the **Ratio display** panel (Fig. 2.7).

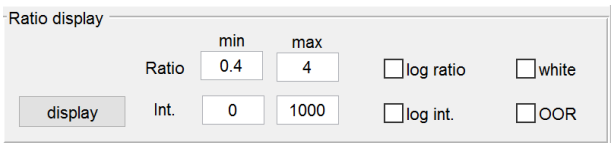


Figure 2.7: The ratio display panel: The ratio display controls are used to set the intensity scaling and colour limits for a rainbow colour scale running from the min ratio (blue) to the max ratio (red) for the pseudo-colour coded ratio image. It is also possible to set a white background for printing purposes. Pixels that fall outside the min/max limits can be displayed clamped to those limits with the out-of-range (OOR) checkbox

The most useful mapping is to HSV (**H**ue, **S**aturation and **V**alue or intensity) colour space, where the ratio is coded as Hue and the average intensity from both channels gives the value. The Hue colour scale ranges from blue (low) to red (high). The limits of the colour scale are set using the **Ratio min** and **Ratio max** textboxes, with an additional checkbox for logarithmic scaling. The **log** ratio can be useful during initial data inspection, if the appropriate scaling limits are not yet known, as the **min** and **max** can be set to cover a very broad ratio range to capture the likely response.

Likewise the intensity can be set independently from **Int. min** to **Int. max**, with an additional checkbox for logarithmic scaling.

A **white** checkbox is provided to switch the background from black (the default), which has high on-screen contrast, to white, which reduces the cost of printing figures.

The **Out-of-Range** (OOR) checkbox imposes the maximum and minimum colours on pixels that fall beyond the values set by Ratio min and max to give a more consistent display. If OOR is not checked, values that fall outside the range are displayed in greyscale.

2.8 *Displaying global results of the analysis*

The average value of all pixels included in the analysis are shown for both channels (**meanCh1** and **meanCh2**) in the appropriate text box of the **Ratio values** panel (Fig. 2.8), whilst the ratio of these means is shown in the **ratio mean** box.

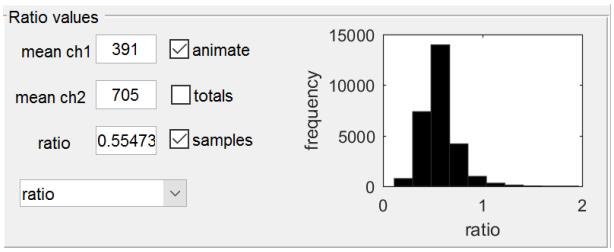


Figure 2.8: The ratio values panel: Global measurements of the average background-subtracted intensity and ratio for each channel are shown, along with a histogram of all the pixel ratio values

In addition, a histogram of the intensity or ratio distribution is shown for the current image depending on the setting of the **ratio** drop-down menu from options including *ratio*, *% oxidised* or *calibrated*. The **animate** checkbox controls whether the histogram is updated during playback of the time-serise. The **totals** checkbox controls whether the global averages are displayed on the output graphs, whilst the **samples** checkboxes controls whether the time-windows used to obtain the calibration values (See Section 2.10) are displayed on the graphs.

2.9 *Point measurements*

Whilst ratio images are a useful tool to help visualise spatial variation in signal responses, the signal-to-noise (S/N) ratio is extremely low for single pixels, even after some spatial and temporal smoothing. Quantitative measurements benefit from averaging the signal from more pixels in regions manually selected by the user that have some morphological or physiological significance. The simplest form of time-series analysis is to select a point or series of points in the image and extract the data from a user-defined local neighbourhood. The **point** button in the **Analysis tools** panel (Fig. 2.9) prompts the user to left-click to select a ROI, (or right click to exit).

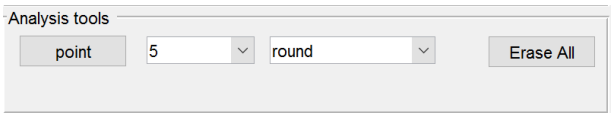


Figure 2.9: The analysis tools panel: The controls allow adjustment of the size and shape of the manually selected region-of-interest measurements

The **size** and **shape** of the ROI are controlled by the adjacent drop-down menus. The data for each ROI are displayed in the corresponding graph for each channel and the ratio in the **graphs panel** (Fig. 2.10). **The Erase All** button clears the selected points and graphs.

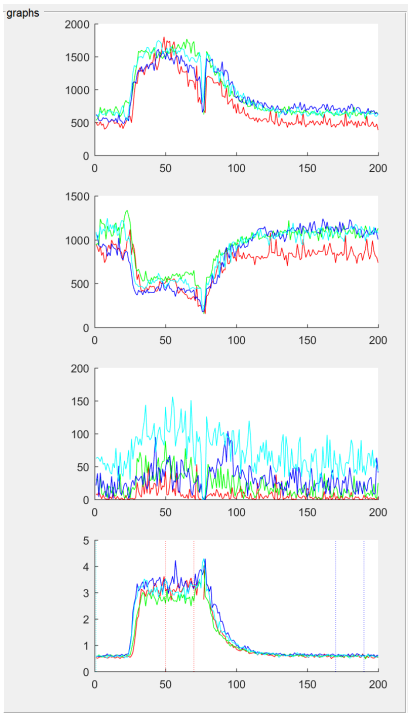


Figure 2.10: The graphs panel: Graphical output from user-defined regions-of-interest (ROIs) for the oxidised, reduced, autofluorescence and ratio channels. Note that there is considerable variation in the intensity in each ROI, but the ratio values are almost coincident, illustrating the benefit of the ratio approach. The auto-fluorescence channel is a very small fraction of the overall signal; and is compensated for by the bleed-through correction

2.10 Calibration

The **Calibration panel** (Fig. 2.11) controls the mapping between the ratio values and the physiological parameter of interest. The calibration parameters required depend on the probe used - in the case of the roGFPs these are **Rmax** when the probe is fully oxidised, **Rmin** when the roGFP is fully reduced, and the **midpoint potential**. An estimate of the **pH** in the compartment where the probe resides has to be provided in the appropriate textbox.

The **I factor** is an instrument specific value that is required to compensate for the different intensities measured from the fully reduced and fully oxidised form of the probe to allow calculation of the degree of oxidation (OxD) of the $roGFP$ sensor (equation (2.1)).

$$OxD_{roGFP} = \frac{R - R_{red}}{\frac{I_{488ox}}{I_{488red}}(R_{ox} - R) + (R - R_{red})} \tag{2.1}$$

Where R is the ratio of excitation at I_{405}/I_{488} nm, R_{red} is the ratio of fully reduced form following perfusion with DTT, R_{ox} is the ratio of the fully oxidised form following perfusion with H_2O_2 and I_{488ox} and I_{488red} are the intensities at 488 nm for the fully oxidised and fully reduced forms, respectively.

The **I factor** has to be measured from a single calibration experiment on the same sample, but can then be applied to any data collected with the same laser intensities and instrument settings.

Values for all the calibration parameters can be entered directly, or **Rmin**, **Rmax** and the **I factor** can be measured from a calibration series where the probe has been driven over the full response range using appropriate calibration solutions. Pressing the **calib** button

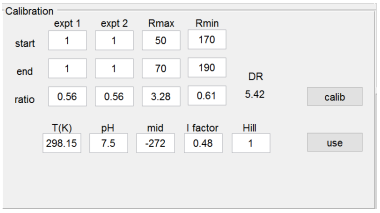


Figure 2.11: The calibration panel: The various probe and instrument calibration parameters can be entered or measured directly from a time-series experiment to allow calibration of the ratio values

prompts the user to select a ROI on the image to estimate the parameters from the data. Values are averaged for the ROI over the time period set using the **start** and **end** textboxes, for both R**min** and **Rmax**. The **use** button updates the calibration calculation if the values have been altered. The dynamic range (**DR**) of the probe is calculated from the calibration **Rmin** and **Rmax** as a check that the overall calibration is performing as expected.

The Hill coefficient (**Hill coeff.**) can be used for probes that have more complex binding kinetics.

Two additional read-outs are provided (**expt 1** and **expt 2**) to calculate a temporal average from the whole image between the **start** and **end** frames set. This is useful to provide a quick check on, for example, the resting redox potential and peak response to a treatment.

2.11 *Output*

The **Output panel** (Fig. 2.12) has buttons to save a figure of the data displayed following the point analysis. The user is prompted to select four images that will be displayed fully labelled with the ROIs overlaid, and cross-referenced to the graphs. The resulting figure (Fig. 2.13) can be saved or printed. If the image is saved as a windows metafile, it can be imported into powerpoint, un-grouped, and each element edited. The **Save data** button exports the data from the ROI point analysis to Excel. The **View** button sends the ratio image to a separate **Viewer window** (see Chapter 4)

Figure 2.12: The output panel: provides various options to save the data from different ROIs, publication quality annotated images or view movie files

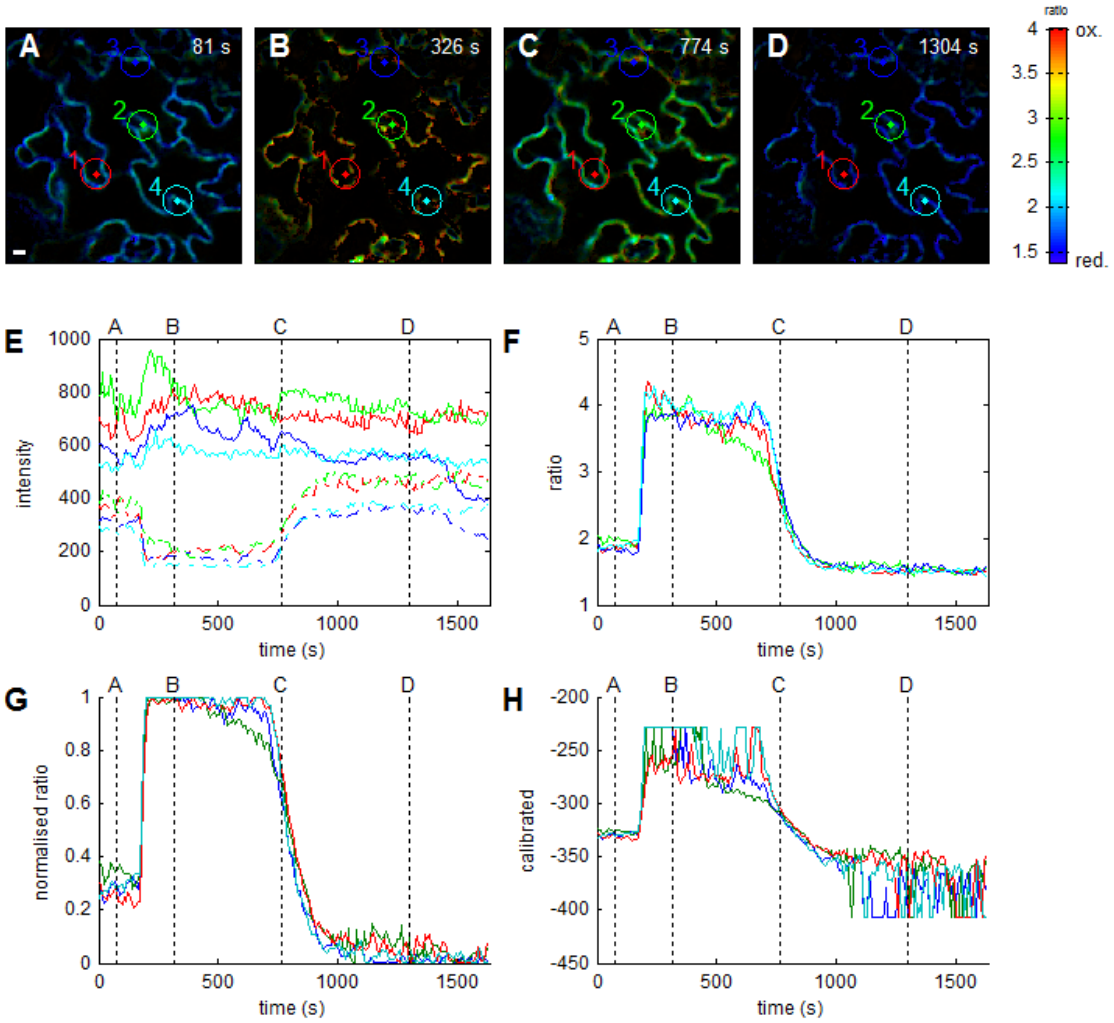
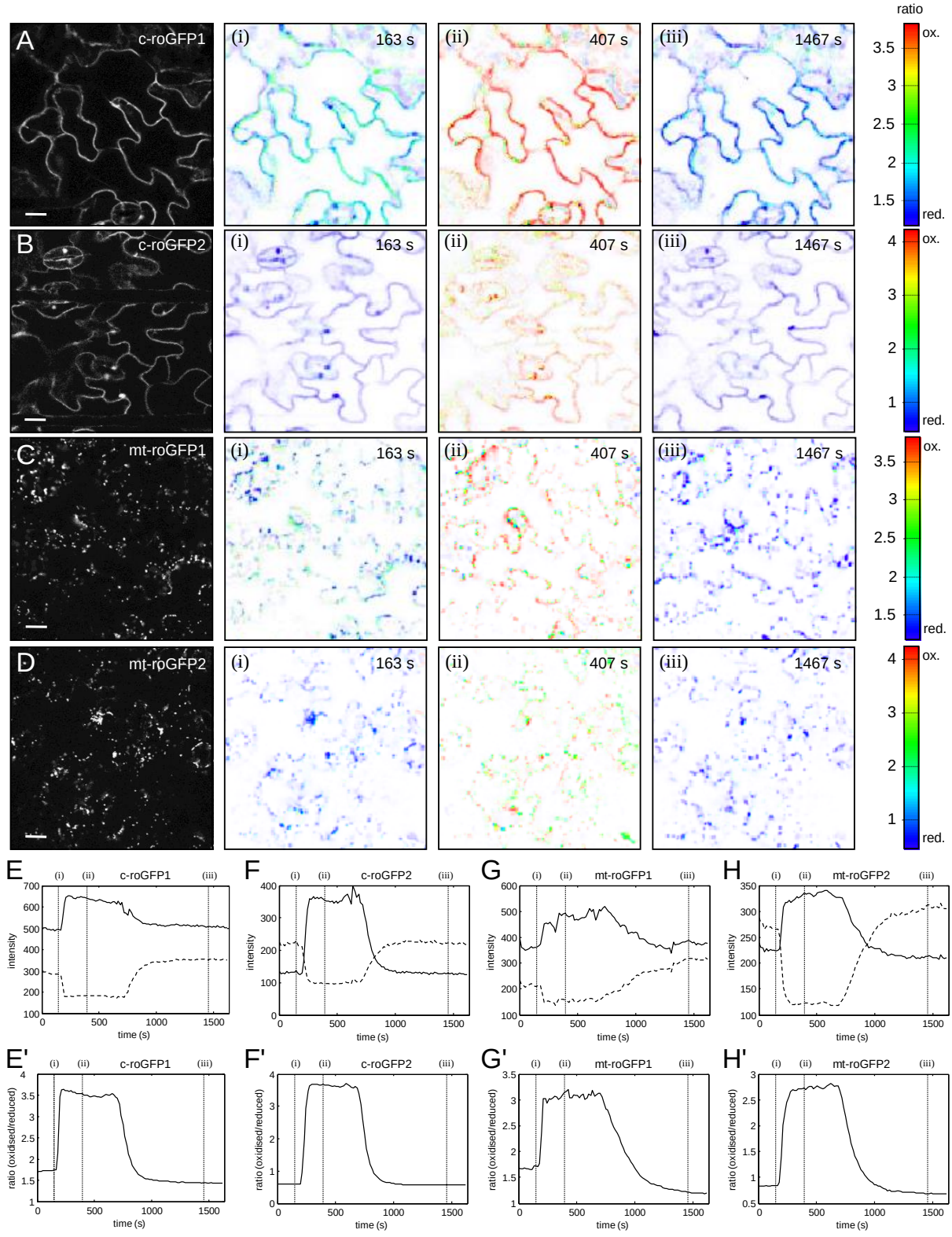


Figure 2.13: Output figure from the basic ratio analysis program showing images from four selected time-points (A-D), the intensity profiles for the ROIS selected for the oxidised channel (solid lines) and the reduced channel (dotted lines) (E), the corresponding ration (F), the % level of oxidation (G) and the conversion to the redox potential (H)



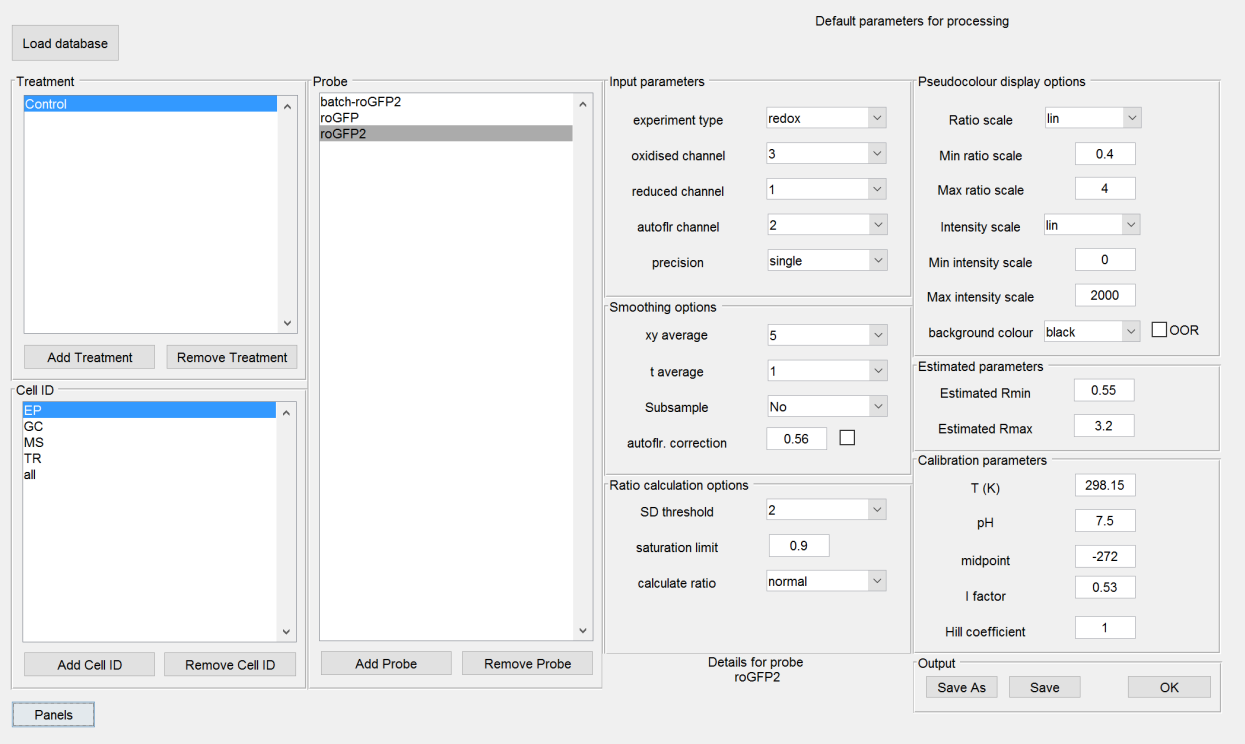
Redox potential measurements using roGFP in plants. *roGFP1 and roGFP2 expressed in different subcellular compartments of Arabidopsis leaf cells respond rapidly to induced changes in redox state. (A) cytoplasmic roGFP1, c-roGFP1; (B) cytoplasmic roGFP2, c-roGFP2; (C) mitochondrial targetted roGFP1, mt-roGFP1; (D) mitochondrial targetted roGFP2, mt-roGFP2. Ratiometric images (i-iii) show resting state (i), oxidized state (ii) and reduced*

state (iii) from typical perfusion timecourse experiments. Leaves were perfused with half-strength MS-medium (0-163 s), 10mM H2O2 (163-570 s), half-strength MS-medium (570-652 s) and then 10mM DTT (652-1630 s) at a flow rate of 4 ml min-1. Scale bars = 10 µm. The change of fluorescence intensity for both excitation wavelengths (405 nm -; 488 nm . . .) is shown in panels E-H, with the time points for the images indicated by dotted lines. (E) c-roGFP1; (F) c-roGFP2; (G) mt-roGFP1; (H) mt-roGFP2. The corresponding ratio values for the different constructs are shown in panels E′-H′. From Schwarzländer et al. (2008).

3
Basic Probe Database

3.1 Accessing the processing parameters from the probe database

To standardise the way sets of experimental images are processed, it is convenient to save the parameters used for each processing step to a database. The probe database can be opened directly from the **Ratio Analysis Menu Bar** (Fig. 1.5). Alternatively, each program can call the database directly. Thus, the **Load probe** button in the **Time-Series Program** is used to open the probe database window (Fig. 3.1)



This provides a list of previously stored probe settings, and also a list of experimental treatments and cell or sub-cellular ID codes. The **Load database** button opens a standard dialog window that can be used to navigate to a particular probe database. This can be stored anywhere, so it is up to the user to decide whether to set-

Figure 3.1: Basic ratio parameter input interface: Values can be set for a particular probe and instrument combination to ensure that each set of data is processed with the same settings.

up a single database for all experiments in a specificc location, or individual databases for each experimental series that are typically stored in the same folder as the data.

Once a database is loaded, the name of the first probe in the list is highlighted and the processing parameters displayed. Selecting a different probe in the list will update the parameters displayed for that probe (Fig. 3.1).

For each experiment, it is sometimes convenient to define a treatment label that can be linked to each data point in the final spreadsheet. Additional treatments can be added using the **Add Treatment** button, which opens a dialog box for text entry, or the selected treatment can be removed using the **Remove Treatment** button. Likewise, when processing Regions-Of-Interest (ROIs), it is convenient to be able to add a specific cell identity for each ROI using the **Add Cell ID** or **Remove Cell ID** buttons as appropriate[1].

The **Add probe** button can be used to set-up a new probe, whilst the **Remove probe deletes an existing entry**.

[1] Note: the Cell ID is currently only implemented in the Batch Processing program

3.2 *Setting the default channel order*

The **experiment type** drop-down menu in the **Input parameters** panel is used to set the labels for all the controls in the ratioing program to match the type of experiment (Fig. 3.2). The order that the channels used for ratioing are collected and displayed may vary with the configuration of the instrument.

To simplify subsequent processing steps, it is convenient to specify which physiological state of the probe is linked to each channel. In addition, there is often an additional channel used to collect an auto-fluorescence image to allow correction for auto-fluorescence bleed-through into one of the fluorescence channels.

The **precision** menu defaults to single precision to reduce memory requirements, whilst still using floating point arithmetic throughout. Double precision is more accurate, but the additional precision is spurious for most biological experiments. It is not recommended that the integer options are used for ratio experiments.

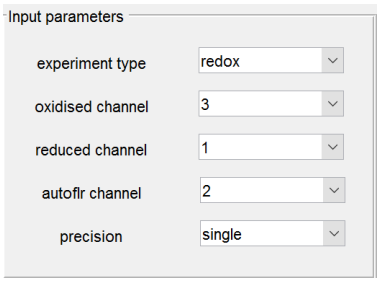


Figure 3.2: Input parameters panel: The controls can be used to set the type of experiment and associate each channel with a physiological parameter

3.3 *Setting the smoothing and sub-sampling options*

Each image can be smoothed in (x, y) using a square kernel with size set by the **xy average** drop-down menu (Fig. 3.3). Likewise a number of images can be averaged over time using the **t average** drop-down menu.

Smoothing improves the signal-to-noise (S/N) ratio, but at the expense of decreased spatial/temporal resolution. It is therefore appropriate to sub-sample the image after smoothing using the **Subsample** drop-down menu. This reduces the image size, but increases the speed of the subsequent processing steps. Visually the processed images can start to look pixelated.

The auto-fluorescence correction factor (**autoflr. correction**) is a

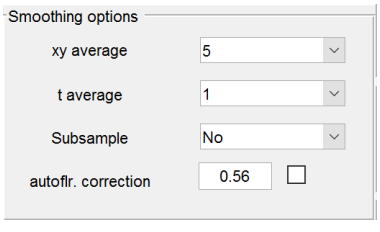


Figure 3.3: The smoothing options panel: Controls set the values for the xy *and time smoothing kernels, sub-sampling, and autofluorescence correction*

fixed value that controls the scaling of the auto-fluorescence image before subtraction from the 405nm excitation roGFP image. The value is determined from an un-labelled specimen under the same instrument settings. The adjacent checkbox controls whether or not to apply the correction.

3.4 *Masking pixels in the ratio calculation*

If the signal in either channel approaches the background intensity, the ratio will become increasingly noisy. It is useful to be able to mask out pixels close to background to exclude these noisy ratios. The masking level can be set as a number of standard deviation units above the corresponding background using the **SD threshold** drop-down menu (Fig. 3.3). The default value is 2 SD units.

Likewise, values that approach the maximum intensity may reflect pixels that are saturated and will therefore yield incorrect ratio values. The **saturation limit** excludes pixels above a fraction of the full intensity scale. The default value is 0.9. The ratio is normally calculated as ch1/ch2 (ox/red). The drop-down menu allows the *inverse* ratio to be calculated.

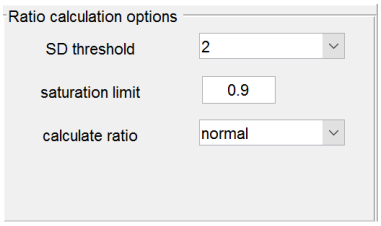


Figure 3.4: Ratio calculation options panel: The controls set the values used to mask the ratio value

3.5 *Controlling the display options*

The ratio values are mapped onto a pseudo-colour coded HSV image (Hue, Saturation, Value), where the ratio is coded by Hue and the average intensity of the two wavelengths is used to set the intensity of the ratio. The Hue images ranges from blue at the minimum value chosen for display, set by the **Min ratio scale**, through rainbow colours to red at the maximum value, set by the **Max ratio scale** (Fig. 3.5).

The mapping can be linear or logarithmic, set using the **Ratio scale** drop-down menu. The intensity (Value) of the ratio image is rescaled on a linear or logarithmic scale using the **Intensity scale** drop-down menu, with the limits set by the **Min intensity scale** and the **Max intensity scale**. If the **background colour** is set to black, colours are fully saturated giving a high-contrast image on screen. If it is set to white, the image saturation follows the intensity image so that low intensities are mapped to progressively de-saturated colours eventually giving a white background. This is useful when printing to save on black ink.

The out-of-range (**OOR**) checkbox controls whether pixels that fall outside the min and max ratio values are displayed in colour, but clamped to blue or red, respectively, or whether the pixels remain in greyscale.

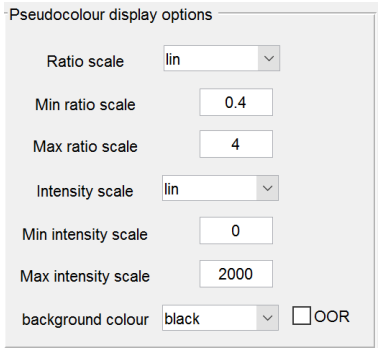


Figure 3.5: The pseudo-colour display options panel: The options used to display of the pseudo-colour coded ratio image can be set using these controls

3.6 *Calibrating the ratio values*

Whilst the ratio image colour can be scaled between arbitrary limits, calibration of the ratio requires measurement or estimation of the

actual minimum and maximum ratios for the probe. These can be determined experimentally, with an appropriate calibration protocol *in situ*, or estimated from literature values, and entered into the **Estimated Rmin** and **Estimated Rmax** boxes. (Fig. 3.6).

Additional parameters are required for a full calibration that usually have to be determined from independent experiments or are based on literature values. These include the **midpoint potential** of the probe response, the **estimated pH** of the compartment, an instrument correction factor (**I factor**) depending on the precise set-up of each channel, and, for some probes, the **Hill coefficient**, (Fig. 3.7).

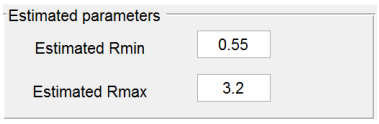


Figure 3.6: The estimated parameters panel: Sets the estimated Rmin and Rmax values for the calibrated ratio

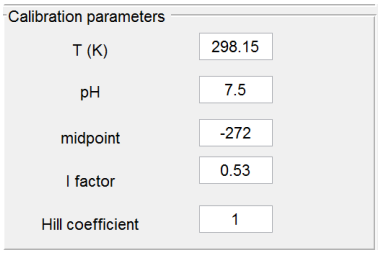
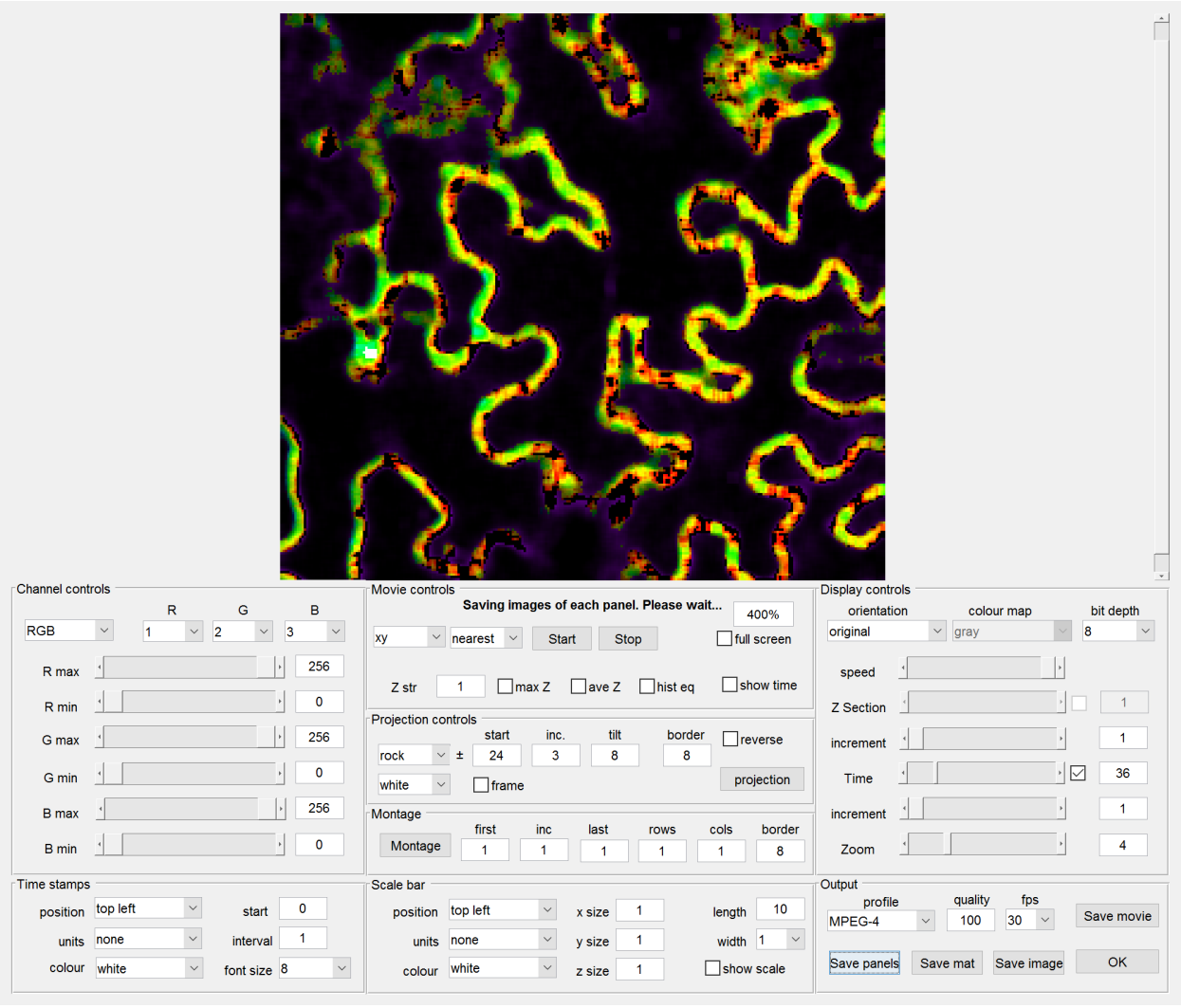


Figure 3.7: The calibration parameters panel: Sets the remaining probe and instrument calibration parameters

3.7 *Updating and saving the database*

The values chosen are returned to the probe database by clicking the **OK** button. On the probe database interface, the updated database can be saved under the existing name using the **Save** button. The **Save As** button prompts the user for a different files name and directory. The **OK** button returns the updated information (if saved) to the calling program.

4
Viewer Program



4.1 Introduction

The video **Viewer** allows the output of any processing steps to be animated on screen or saved in a variety of movie formats. Images can contain multiple channels, although a maximum of three can

be shown in RGB format. The first image in the file is displayed when the viewer opens. If the image is too large for the display window, the central portion will be displayed with scroll bars to move around the image.

4.2 *Movie playback controls*

The **Movie controls** (Fig. 4.1) can be used to **Start** or **Stop** playback. The normal image orientation is viewed in the *xy* plane. If the data has multiple *z*-sections, the orientation can be changed to view animations in *xz* or *yz* orientation using the drop-down menu. if an *xz* or *yz* view is selected, the **Z str** controls the amount of interpolation introduced to give the appropriate pixel spacing in *z*. The **show time** checkbox displays the time stamp according to the settings in the **Time stamps** panel (see section 4.6)

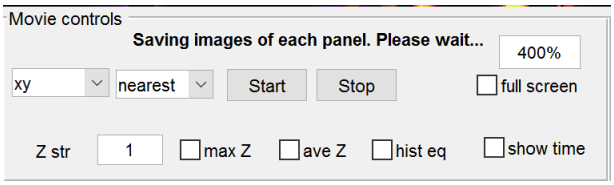


Figure 4.1: The movie controls panel: provides options to start/stop the animation or display max or average z-projections

The ave Z (**Ave**) or max Z (**Max**) checkboxes generate the corresponding projection for the current time-point if the data has multiple *z*-sections. These are applied to every image in a 4-D time series.

The **hist eq** checkbox uses contrast-limited histogram equalisation (CLAHE) to improve the overall contrast in the image. If the data has more than one *z*-section, the maximum projection is calculated first before the CLAHE enhancement.

4.3 *Display controls*

The **Display controls** panel (Fig. 4.2) can be used to display images in their **original** orientation, or as a rotated image (*90 left*, *90 right* or *180*) using the drop-down menu.

If the image is initially a single intensity channel the **colour map** drop-down menu can be used to apply a colour look-up table (LUT) to the image.

The **bit depth** controls the number of bits to scale each channel in the display, and automatically adjusts the maximum scaling of the channel intensity controls.

Note, many microscopy images contain 12 bits of information (0-4095), but are stored in 16-bit format. This means the bit-depth may be automatically set to 16 (0-65535) when the image is loaded. As a result the screen will appear black until the bit-depth is set to 12. In other situations, the software cannot pick up the correct setting for the bit-depth and defaults to a value of 1. Images may appear completely saturated until the bit-depth is set to the appropriate

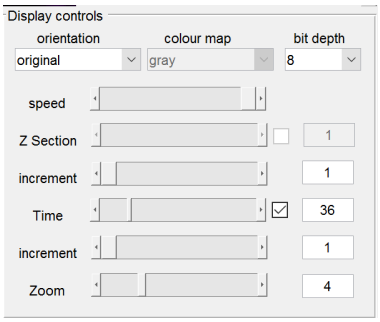


Figure 4.2: The display controls panel: set the image orientation and control the dimensions for animation

value, usually 8.

The **speed** slider adjusts the speed of playback.

The **Z Section** or **Time** sliders select a specific z-section and time point and also have **check-boxes** to control whether images are animated in this dimension during playback.

The **increment** slider controls the gap between images during playback.

The **Zoom** slider controls the scale of the image displayed. If the image is larger than the display window, horizontal and vertical scroll bars appear automatically.

4.4 *Image brightness controls*

Images are usually imported in RGB format where each channel corresponds to a different wavelength image. However, pseudo-colour coded ratio images are constructed in HSV colour space where the parameter of interest is coded as Hue, and the intensity and/or saturation are used to represent the strength of the original signals. Switching between RGB and HSV display is achieved with the **RGB** drop-down menu on the **Channel controls** panel (Fig. 4.3).

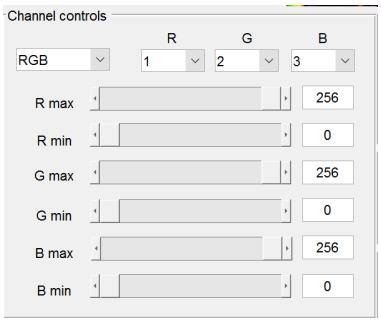


Figure 4.3: The channel controls: Select the channels to display and the minimum and maximum intensity for each channel

In RGB mode, the **R, G** and **B** drop-down menus can be used to select which channel should be displayed in which colour plane, and the sliders used to set the **minimum** and **maximum** values for each colour independently.

In **HSV** mode, the slider and drop-down labels change to represent the **Hue**, **Saturation** and intensity **Value**. This allows independent scaling of these parameters (but only yields a useful image if the data is in HSV format).

4.5 *Projection controls*

If the image contains an RGB z-stack or a series of z-stacks collected over time, the **Projection controls** panel (Fig. 4.4) can be used to construct a rocking or tilted animation of the maximum projection of each channel for each z-stack at different angles.

Figure 4.4: The projection controls: Allows calculation of tilting or rocking projections of z-stacks

The **start** text box sets the initial angle for the rotation, whilst the **inc.** text box controls the angle between projections. The **tilt** or **rock** is calculated symmetrically about zero between these limits.

The **Z str.** in the *Movie controls* panel sets a z-stretch to correct for the asymmetric sampling in x,y and z. This is calculated from the nominal z-pixel spacing divided by the x,y pixel spacing. However, additional correction may be required depending on the lens and

immersion media using to collect the original data. Ideally, values are based on a calibrated sample, such as a 15 μm fluorescent sphere, to ensure correct geometric scaling.

If the original data is an HSV ratio image, the **HSV** setting needs to be selected in the channel controls panel, and the projection will be calculated initially as a maximum projection of the intensity value (**V**). The *x*,*y*,*z*-pixel co-ordinate of this maximum brightness pixel is then used to extract the corresponding values from the Hue and Saturation channels. This approach allows projection of multi-dimensional pseudo-colour coded images.

4.6 Time stamp control

The **Time stamps** control panel (Fig. 4.5) allows the user to add a time-stamp to the display.

The **Time stamp pos.** controls the location of the time label (top left, top right, bottom left, bottom right), whilst the **start** and **interval** text boxes and **units** drop-down menu control the information to be displayed in a specific **font size** and **colour**.

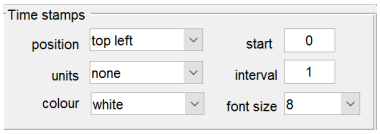


Figure 4.5: The time stamp panel: allows addition of a time-stamp to each image

4.7 Scale bar controls

The **Scale bar** control panel (Fig. 4.6) allows the user to add a scale bar to the display. The **scale bar pos.** controls the location of the scale bar, whose **length** and **width** are set by the appropriate text boxes.

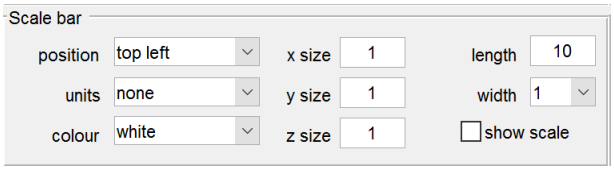


Figure 4.6: The scale bar panel: allows addition of a scale bar to each image

4.8 Montage controls

A **Montage** of selected images (Fig. 4.7), defined by the **first**, **inc.** and **last** can be constructed in a matrix format defined by the **rows** and **cols** controls, with a gap between the images defined by the **border**. The montage appears in a new window and can be saved in a variety of formats.

Figure 4.7: The montage panel: Sets up a montage of images that can be subsequently saved in a grid format

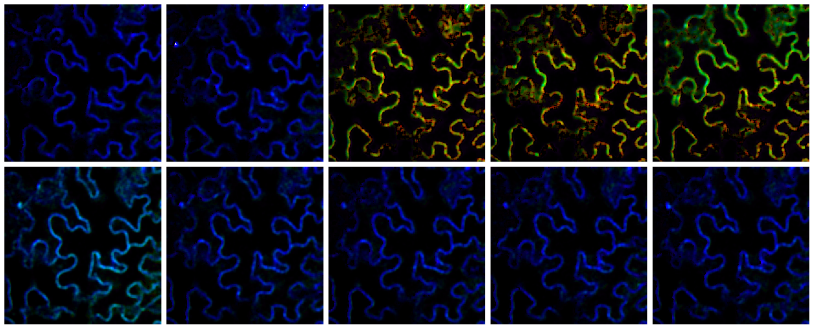


Figure 4.8: An example of a montage from a time series

4.9 Output controls

Processed images can be saved to a variety of movie formats using the **Movie** button in the **Output** panel (Fig. 4.9). This provides choice over the compression **codec**, and, if appropriate the **quality** and frames-per-second (**fps**) required. The image series can also be saved as a matlab array using the **Save mat** button, or as a single image using the **Save image** button.

The **Save panels** button saves images of all the control panels to help in construction of an application specific manual.

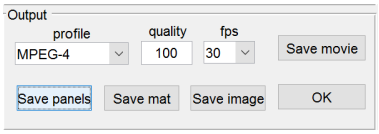
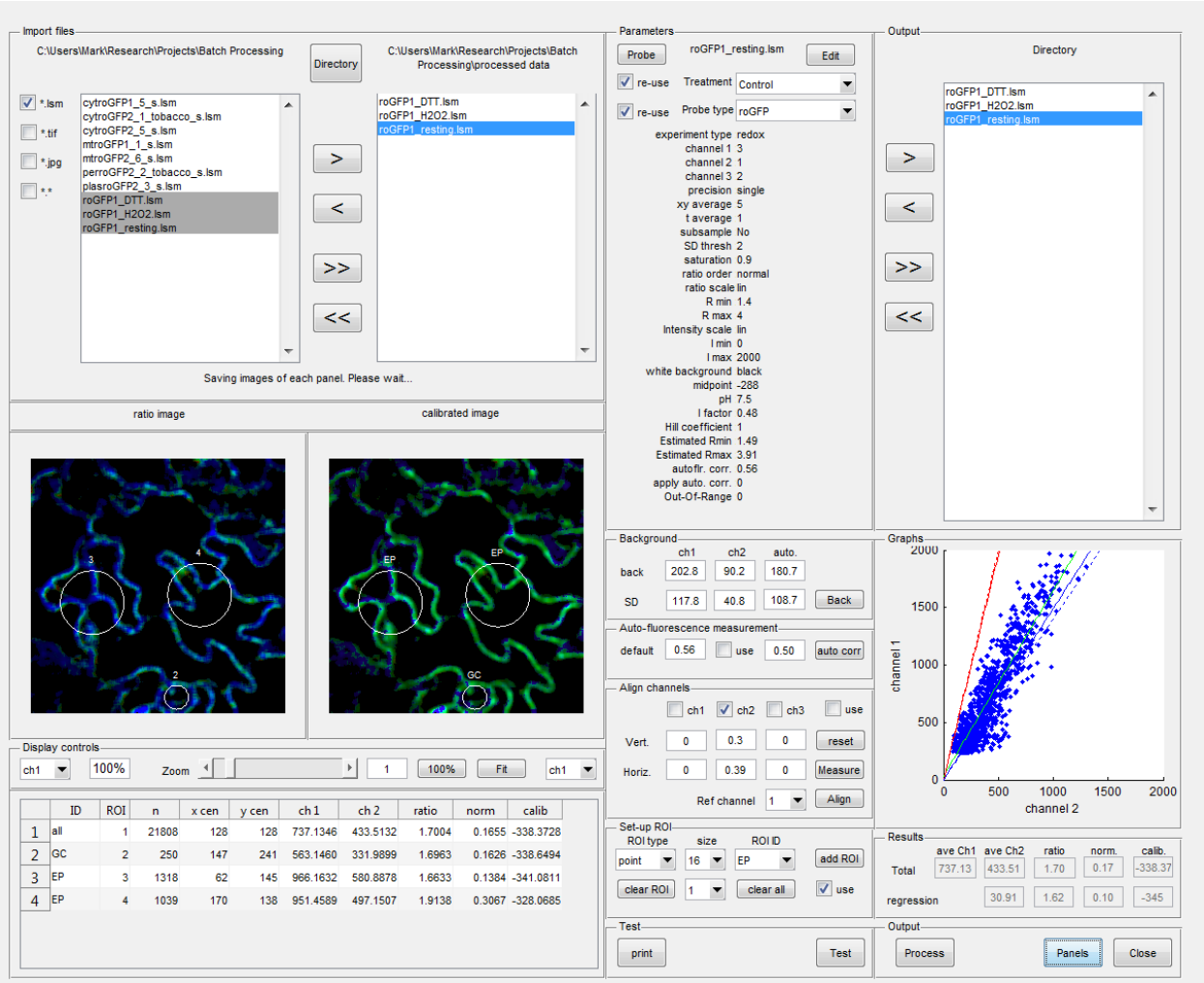


*Figure 4.9: The output panel: Saves the final movie in *.avi format*

5
Batch-Processing Program



5.1 Introduction

The batch processing program calculates the ratio image and ratio values for user-defined ROIs for a set of single time-point images in a folder using the same approach (and probe database) as the **Basic Time-Series** program (see Chapter 2). It is useful in applications

where comparison of the steady-state redox potential is required over multiple samples and treatments, measured over the whole image or from defined ROIs, rather than a continuous evaluation of changes in redox potential.

5.2 *Loading images*

The **Import files** panel (Fig. 5.1) displays a list of files in the current directory. A different directory can be selected using the **Directory** button. The user can select one or more individual files and use the **right arrow** to shift them across to the file input box. Alternatively all the files can be selected with the **double-right arrow** button. Different file extensions can be selected with the checkboxes on the left-hand side. The default is for Zeiss .lsm files. Individual files can be removed from the selected list using the **left arrow**, or all the files can be de-selected using the **double-left arrow**.

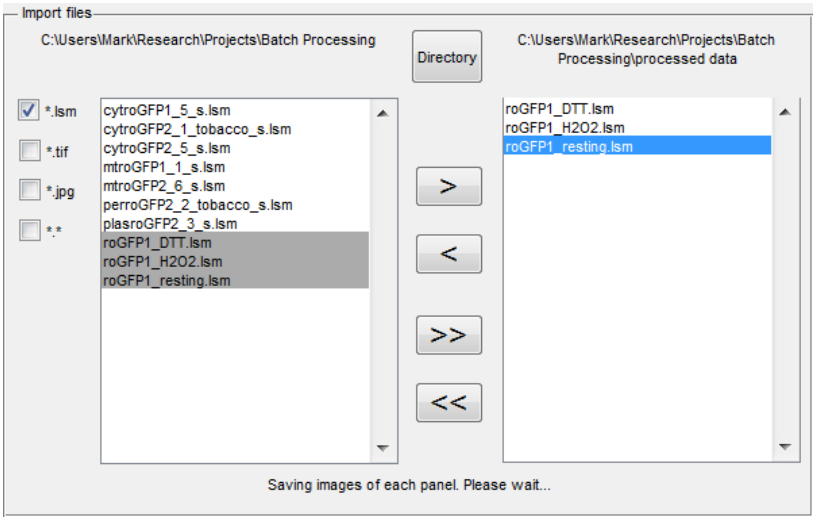


Figure 5.1: The import files panel: allows selection of a set of single (x,y) images to be imported

5.3 *Setting the processing parameters*

To process each file, the various parameters need to be set-up. The **Parameters panel** displays the values currently in use for the selected file, or is empty if no parameters have been set (Fig. 5.2). The **Probe** button prompts the user to choose a probe database with saved values. This database can be in any directory. It has the same structure as the database used for the **Basic Time Series** program (see Chapter 2), and is therefore interchangeable and can be used in either type of analysis. The **Treatment** and **Probe type** drop-down menus are then updated with the options in the probe database.

The **re-use** check boxes are ticked by default to apply the same default probe settings to subsequent images for convenience. It is possible to apply different probe settings to each image if required, and the appropriate values are stored with the processed image and

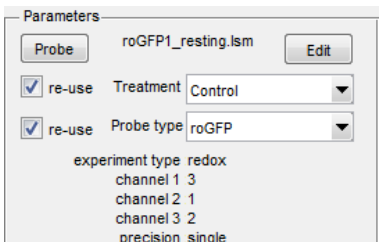


Figure 5.2: The parameters panel: Displays the parameters for processing based on the current database loaded and probe selected

saved with the results. In addition, the **ROI ID** dropdown menu in the **ROI panel** is updated with any cell types or sub-cellular compartment labels that have been entered in the probe database.

If the parameters have been previously loaded, the **Edit** button should open the appropriate database so that the values can be modified if required. A copy of these settings is stored as a matlab file in a sub-directory of the current directory when each image is processed with the same filename and a *.mat extension.

The saved file is also updated to include other parameters specific to each image analysed, such as the background values, ROIs and channel alignment that will be set later. This file is automatically re-loaded if the images are processed again, but the values can then be subject to further refinement if needed.

5.4 *Image display*

The loaded images are displayed in the two panels in the bottom left of the interface (Fig. 5.3). Initially, the two channels used for the ratio are automatically scaled and displayed. If the ratio and normalised images have been calculated, they replace the grayscale channel images during the processing steps. Alternatively, any of the channels or processed images can be selected using the dropdown menus in the **display controls** panel (Fig. 5.4).

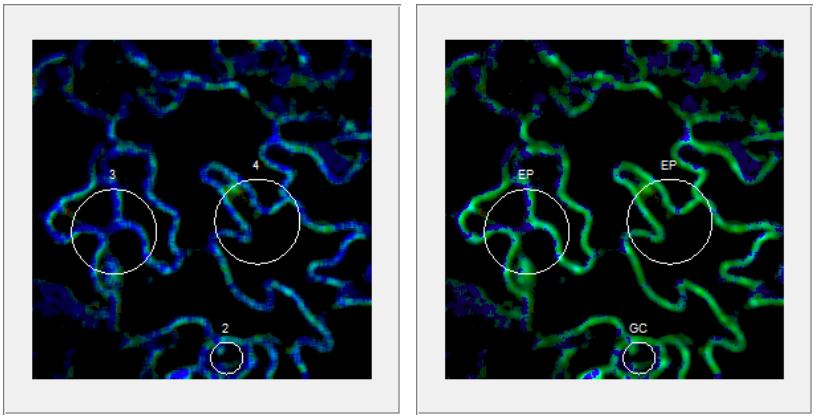


Figure 5.3: Batch-processing image display, with overlaid labelled ROIs

The images are initially displayed with a one-to-one pixel-to-screen scaling. If the image is larger than the window, horizontal and vertical scroll-bars appear automatically. The images can be zoomed using the **Zoom** slider or text box (Fig. 5.4). The **100%** button displays the images in a 1:1 pixel ratio on screen, whilst the **fit** button scales the image to fit into the display.

Figure 5.4: Display controls

5.5 Background measurement and subtraction

The background values may vary for each set of data and therefore have to be measured from each image manually before it can be processed. The **Back** button in the **Background** panel (Fig. 5.5) prompts the user to draw a rectangular region in an area of background for the currently selected image.

The average background intensity and standard deviation are returned in the text boxes for each channel. These values are then stored, along with all the other processing parameters, in the matlab file in the processing sub-directory of the original image directory.

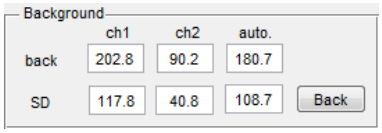


Figure 5.5: Background measurement and subtraction panel

5.6 Auto-fluorescence bleed-through correction

Auto-fluorescence bleed-through into one of the measurement channels is a common problem, particularly with plant specimens and blue/violet excitation. As auto-fluorescence has structure in the image, it cannot simply be subtracted like the background can as a single value. However, it is sometimes possible to estimate the auto-fluorescence at an emission wavelength range that does not have any signal from the measurement probe.

As the auto-fluorescence spectrum tends to be quite broad, a scaled version of this image can be subtracted from the probe image to correct for the auto-fluorescence bleed-through. The **auto corr** button (Fig. 5.6) allows the user to draw a rectangular ROI in a region of the image where there is auto-fluorescence, but no probe signal, to calculate the bleed-through correction factor from the auto-fluorescence channel into channel 1. The correction factor is displayed in the adjacent text box. If it is not possible to measure the bleed-through correction factor directly from the loaded image, it has to be estimated from an un-labelled control using exactly the same instrument settings and typed into the correction box. This is likely to be the default for batch processing, and the relevant value is stored in the probe database.

Because the background and auto-fluorescence correction subtract noisy images from each other, there is the potential to generate negative values, particularly if the original intensities were close to the background values. Any negative values are automatically set to zero during the calculation.

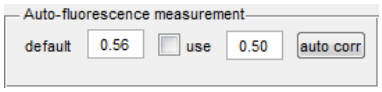


Figure 5.6: Auto-fluorescence measurement panel

5.7 Sub-pixel channel alignment

If there is any mis-alignment between the different wavelength images, substantial artefacts will be introduced in the ratio image, particularly at the edges of features. The impact of mis-alignment is less severe in the global or ROI intensity measurements, due to the spatial averaging involved.

To correct for misalignment, a reference channel (**Ref chan-**

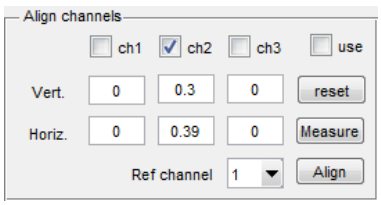


Figure 5.7: Channel alignment panel

nel) is selected, typically for the channel with the strongest signal (Fig. 5.7). The alignment for this channel is set to zero and the corresponding channel box un-checked. The other channels can be selected for alignment using the appropriate checkboxes. The **Measure** button automatically calculates the pixel offset that maximises the cross-correlation between the selected image(s) and the reference. The **reset** button restores all values and the images to their defaults. The **Align** button saves the offsets to the relevant file. These are automatically loaded up when this image is viewed again.

5.8 *Selecting Regions-of-Interest (ROIs)*

Different shaped ROIs can be selected on each image using the **ROI type** drop-down menu (Fig. 5.8). The **size** of the point and square ROIs are set using the adjacent dropdown menus. In addition, each ROI can be given a **ROI ID**, typically set to correspond to the cell type or sub-cellular compartment being analysed.

Clicking the **add ROI** button allows the user to select a set of ROIs, using a right-click to exit. The ROIs are plotted on both windows, along with their index in the left panel, and the **ROI ID** in the right panel.[1]

More complex rectangular, elliptical or polygons can be chosen from the **ROI type** dropdown menu. In this case, each additional ROI is added using the **Add ROI** button separately. Individual ROIs can be chosen deleted using the **clear ROI** button and the associated dropdown menu. The remaining ROIs are automatically re-numbered. Alternatively all the ROIs can be deleted using the **clear all** button. The **use** checkbox controls whether the values for the ROIs set will be calculated or not.

Figure 5.8: ROI panel: Setting up region-of-interest (ROIs) with ID labels

[1] The first ROI is automatically set to the whole image, so the numbering for the user-selected ROIs starts at 2

5.9 *Ratio calculations*

To check whether the processing is set-up correctly, there is a **Test** button which will process the current image using the current parameters (Fig. 5.9). If the background has not been set, the user will be prompted automatically at this point.

The results of the processing are displayed in four different forms:

- A set of values that summarise the average intensities and the ratio of the averages for all pixels that fulfil the selection criteria, along with their calibrated values (Fig. 5.10). In addition, the results of a linear regression fit to the data are presented as an offset, gradient (equivalent to the global ratio value), and the corresponding calibrated values calculated from the gradient.

Figure 5.9: Test panel: Previewing the results of the processing

Figure 5.10: Results panel: Global results averaged across the whole image

- A pseudo-colour coded ratio image (Fig. 5.11), with overlaid ROIs if these have been set.

- A 2-D scatter plot of the intensities for each pixel, with the best fit regression shown as a solid green line. The minimum and maximum calibration limits for the ratio are shown as broken blue and red lines respectively, and the minimum and maximum limits for the pseudo-colour coded ratio as solid blue and red lines, respectively (Fig. 5.12). The gradient and intercept of the regression are also displayed in the results box (Fig. 5.10). If the background subtraction and auto-fluorescence correction have been applied correctly, and all features have the same redox potential, the intercept should be zero and the slope equivalent to the average ratio.

- Results for the individual ROIs, if set, are shown in a scrolling table, which also shows the number of pixels included, the position of the centre of the ROI, and the averaged intensities from the two channels, as well as the ratio, normalised value and calibrated value (Fig. 5.9).

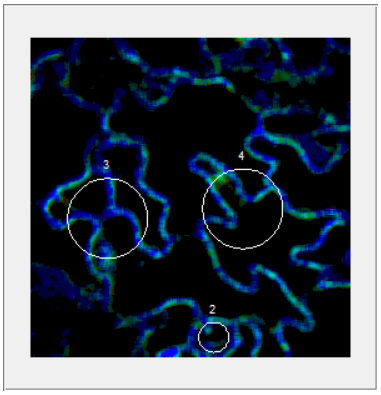


Figure 5.11: The pseudo-colour coded ratio image, overlaid with any ROIs that have been selected

	ID	ROI	n	x cen	y cen	ch 1	ch 2	ratio	norm	calib
1	all	1	21808	128	128	737.1346	433.5132	1.7004	0.1655	-338.3728
2	GC	2	250	147	241	563.1460	331.9899	1.6963	0.1626	-338.6494
3	EP	3	1318	62	145	966.1632	580.8878	1.6633	0.1384	-341.0811
4	EP	4	1039	170	138	951.4589	497.1507	1.9138	0.3067	-328.0685

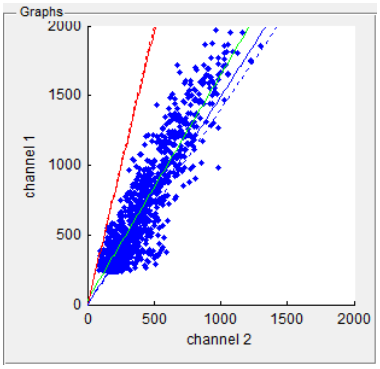


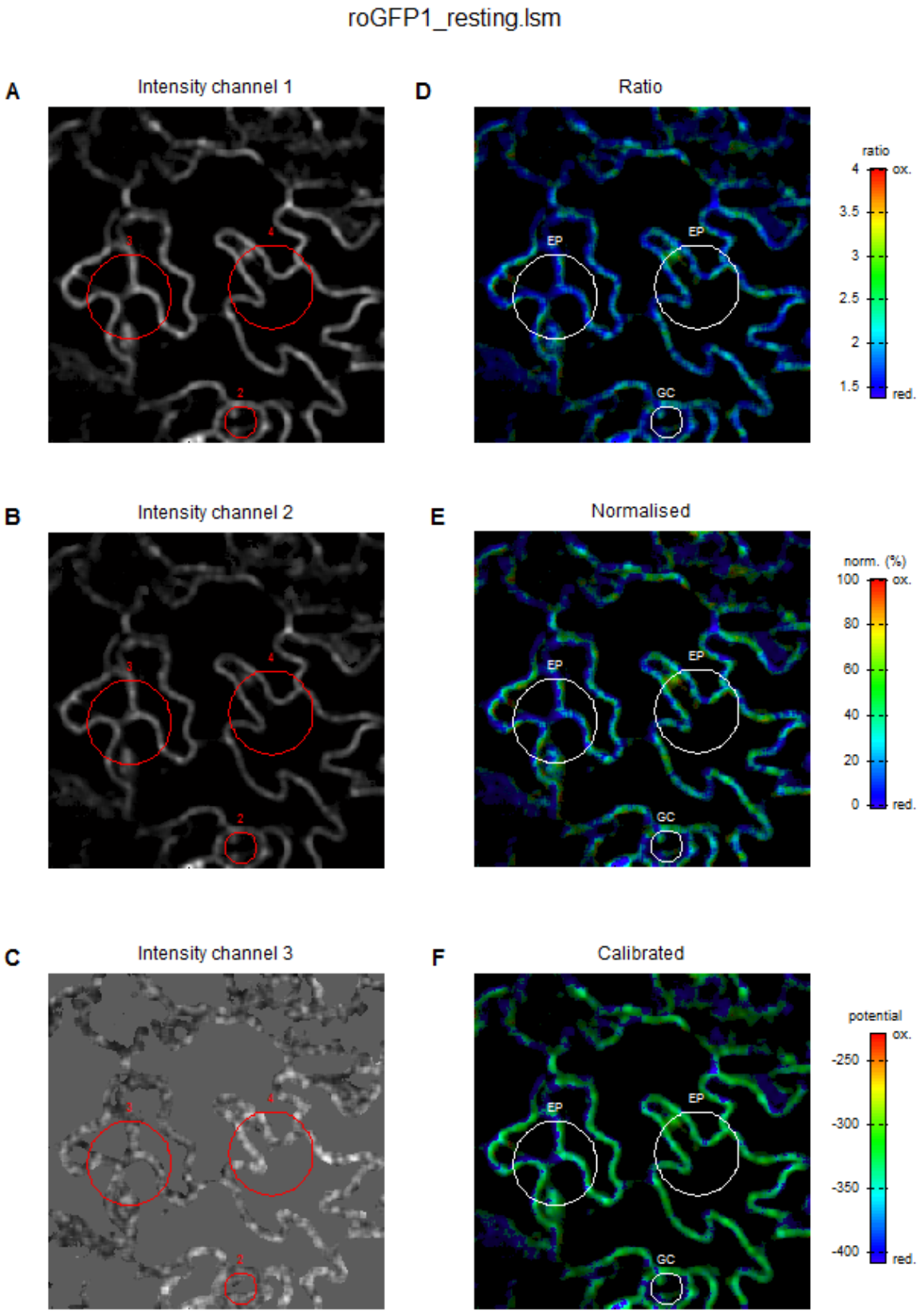
Figure 5.12: 2-D scatter-plot of the background-subtracted intensities with the ratio limits and fitted linear regression superimposed

5.10 Printing the images

The **print** button in the **test panel** automatically opens a secondary window and displays the original intensity images, with ROIs overlaid, and the ratio, normalised and calibrated images, with corresponding calibrated colour-scale (Fig. 5.10). This figure can be printed, saved or closed using the commands on the menu bar.

5.11 Processing all the files

If the processing parameters have been set, the file can be included in the output list in the **Output panel** . The single arrows move a single file at a time, whilst the double arrows include (or exclude) all of the selected files, respectively.


roGFP1_resting.lsm
A
Intensity channel 1
B
Intensity channel 2
C
Intensity channel 3
D
Ratio
ratio
4
ox.
3.5
3
2.5
2
1.5
red.
EP
EP
GC
E
Normalised
norm. (%)
100
ox.
80
60
40
20
0
red.
F
Calibrated
potential
ox.
-250
-300
-350
-400
red.
2
3
4

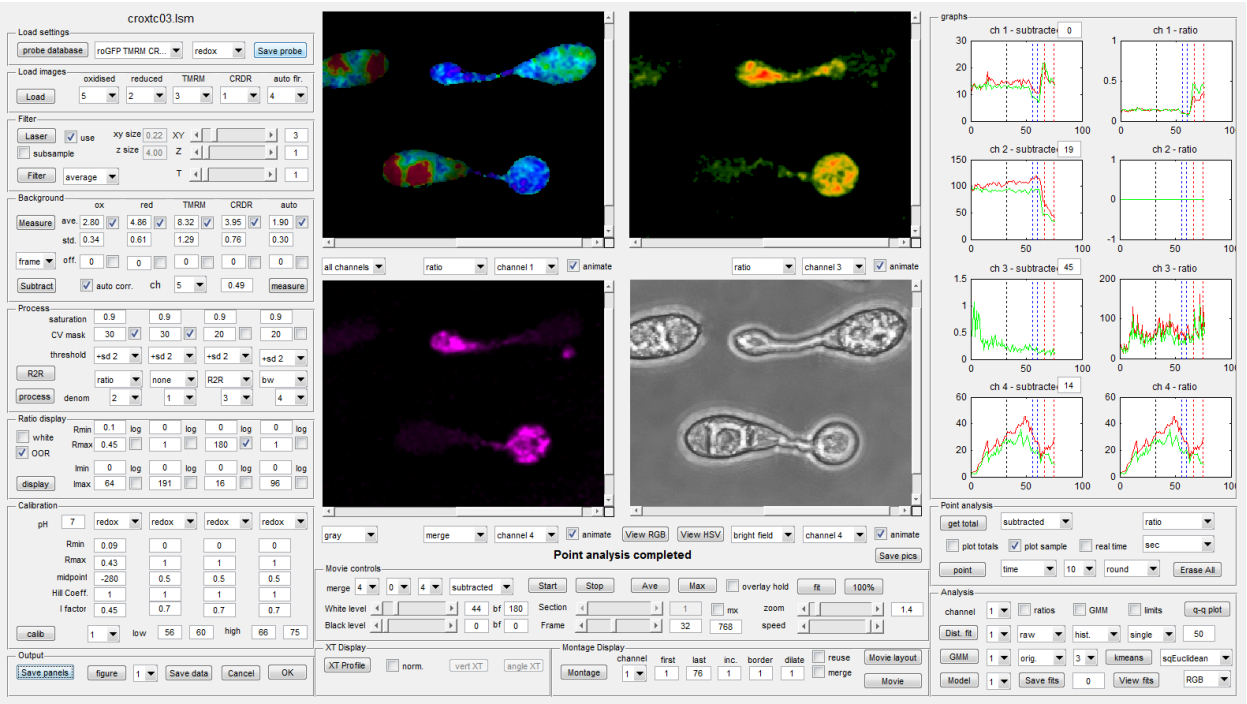
If the parameters look reasonable in the test case, the **Process** button operates on all the images in the output list (Fig. 5.13). Full-size copies of the processed ratio and calibrated images are saved, along with the calibrated figure output, and the results for the whole image or any ROIs selected in an Excel spreadsheet incrementally for each file. The format gives the name of the file, values for the ratio calculated from the average pixel intensities and from the regression line, and all of the processing parameters used (Fig. 5.14).

Figure 5.13: The output panel: processes all the files selected and saves the image and numerical data. The panels button saves an image of each panel in the interface to incorporate into a manual

file	path	probe	treatment	ID	ROI	npix	x cen	y cen	ch1 mean	ch2 mean	ratio	normalise	calibrated	intercept	fit ratio	fit norm	fit calib	ch1 back	ch2 back	ch3 back	ch1 SD	ch2 SD	ch3 SD	experimen
roGFP1_D	C:\Users\I	roGFP	Control	all	1	21977	128	128	698.3992	475.6849	1.49149	0.001282	-403.131	13.5736	1.44897	-0.03598	-360.758	172.851	96.54808	152.476	89.53024	60.75124	78.14893	redox
				GC	2	283	146	242	762.3478	529.743	1.49149	0.001282	-403.131											
				EP	3	1372	71	146	831.4719	571.0184	1.49149	0.001282	-403.131											
				EP	4	968	172	140	965.3889	657.2544	1.49149	0.001282	-403.131											
roGFP1_H	C:\Users\I	roGFP	Control	all	1	15673	128	128	982.9018	283.7135	3.464417	0.902262	-289.033	118.2533	3.0401	0.787792	-300.737	198.8235	103.0504	192.3277	109.8603	62.19947	139.0968	redox
				GC	2	190	148	242	1029.15	310.4158	3.315392	0.864785	-293.748											
				EP	3	1039	72	139	1183.423	333.8965	3.54428	0.921274	-285.986											
				EP	4	736	171	139	1303.013	352.3253	3.698323	0.956014	-278.031											
roGFP1_re	C:\Users\I	roGFP	Control	all	1	21808	128	128	737.1346	433.5132	1.700374	0.165519	-338.373	30.90903	1.616366	0.102962	-345.401	202.8352	90.16477	180.7045	117.8376	40.7727	108.6646	redox
				GC	2	250	147	241	563.146	331.9899	1.696274	0.162566	-338.649											
				EP	3	1318	62	145	966.1632	580.8878	1.663253	0.138415	-341.081											
				EP	4	1039	170	138	951.4589	497.1507	1.913824	0.306677	-328.069											

Figure 5.14: Typical Excel output

6
Advanced Ratio Program



6.1 *Introduction*

This program was developed to visualise ratiometric changes in GRX1-roGFP2 fluorescence expressed in the rice blast fungus *Magnaporthe oryzae* simultaneously with other fluorophores for reactive oxygen species or membrane potential Experimental details can be found in Samalova *et al.* (2013)[1]. It is a more sophisticated version of the original **time series program** used to quantify roGFP in plants. It can be applied equally well to any single wavelength, multi-channel or ratiometric quantitation.

6.2 *Overview of the experimental system for* Magnaporthe

GRX1-roGFP2 was developed by Gutscher *et al.* (2008)[2] to ensure

[1] M. Samalova, A.J. Meyer, S.J. Gurr, and M.D. Fricker. Robust anti-oxidant defences in the rice blast fungus *Magnaporthe oryzae* confer tolerance to the host oxidative burst. *New Phytologist*, 201:556–573, 2013

[2] M. Gutscher, A.-L. Pauleau, L. Marty, T. Brach, G.H. Wabnitz, Y. Samstag, A.J. Meyer, and T.P. Dick. Real-time imaging of the intracellular glutathione redox potential. *Nature methods*, 5: 553–559, 2008

that the kinetics of thiol interconversion between GSH and roGFP were not kinetically limited, particularly in species or cell compartments that lack a suitable glutaredoxin (GRX) (Fig. 6.1.

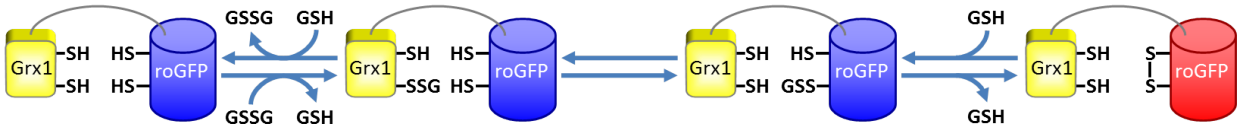


Figure 6.1: Schematic showing how tethered GRX1 assists in thiol exchange between GSH and roGFP

For GRX1-roGFP2 imaging in *Magnaporthe* spores or germlings germinated on coverslips were placed in a perfusion chamber mounted on a Zeiss LSM510META confocal microscope and Grx1-roGFP2 (Em. 505-530 nm) imaged with 405 nm and 488 nm excitation in multi-track mode with line switching. Auto-fluorescence was measured in parallel (Ex. 405 nm, Em. 435-485 nm). Reactive oxygen species were labelled with CellROX Deep Red (CRDR)[3] simultaneously with tetramethyl rhodamine methyl ester (TMRM)[4] for mitochondrial membrane potential. Cells were imaged using quadruple excitation with paired line switching at 405nm and 633nm, and 488nm and 543nm. Images were collected for wall auto-fluorescence 9Ex. 405 nm, Em. 435-485 nm), oxidised GRX1-roGFP2 (Ex. 405 nm, Em. 500-530 nm), CRDR (Ex. 633 nm, Em. 657-721 nm), reduced GRX1-roGFP2 (Ex. 488 nm, Em. 500-530 nm) and TMRM (Ex. 543 nm, Em. 561-603 nm). A non-confocal, bright field transmission image was collected in parallel (See for example Fig. 6.2)

[3] ©Molecular Probes, available from Life Technologies

[4] ®Molecular Probes, available from Life Technologies

Time series were collected at 30-120 s intervals for 15-45 min, as z-stacks of 5-10 sections taken at 0.67-3 μm intervals apart, using a Zeiss 40× 1.2 NA PlanApo water-immersion lens. Pixel sizes were 0.22-0.44 μm in x and y. Pinhole settings were adjusted individually for each channel to give an estimated optical section thickness of 2-3 μm (2-3 Airy units) for each of the wavelength combination. This provided some degree of optical sectioning, but with sufficient signal-to-noise to allow long-term physiological measurements with low laser intensities. Laser power was measured from the defocussed beam using a Newport 1815-C power meter. Values ranged from 1.3-7.3 μW. Non-confocal bright-field images were collected simultaneously with a transmission detector.

The 4-D (x,y,z,t) time-series collected in Zeiss .lsm format were imported into the software using the **Advanced Ratio File Import** program (see Chapter 7). z-stacks were averaged in x, y and z using a $3 \times 3 \times 3$ or $5 \times 5 \times 3$ kernel. The z-position of the brightest pixel in a maximum z-projection of the I_{405} channel was used to extract the corresponding z-pixel for each of the other wavelengths to give the average intensity from all channels in a volume around the same, bright pixel in x,y and z. The resultant image is termed an optimum plane projection. The average background intensity was measured adjacent to the spores on coverslips, or in the vacuole

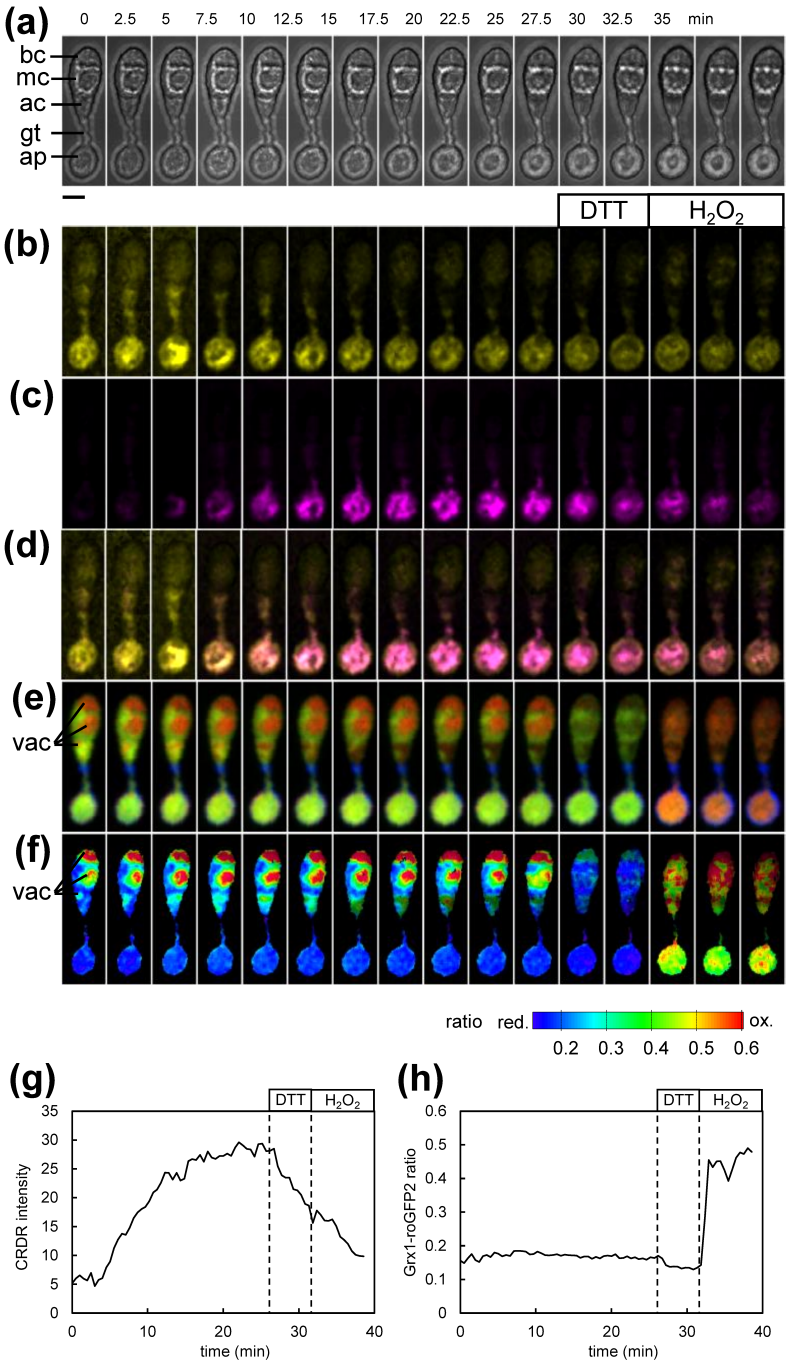


Figure 6.2: Simultaneous measurement of ROS, mitochondrial membrane potential and glutathione redox potential. Mitochondrial activity was measured using membrane-potential partitioning of TMRM during appressorium formation (a,b). ROS were visualised in parallel with CellRox Deep Red (CRDR) (c). Levels of ROS increased linearly in the cytoplasm of all three cells to a low level, but much more pronounced accumulation was observed in punctate structures in the appressorium (b,g) that overlapped with the mitochondrial TMRM signal (d). Cells were also expressing Grx1-roGFP2, colour-coded as Grx1-roGFP2$_{405}$ in red, Grx1-roGFP2$_{488}$ in green and auto-fluorescence in blue (e). The redox potential of the glutathione pool was measured simultaneously using ratio imaging following auto-fluorescence bleed-through correction (f), and showed a consistently reduced cytoplasmic redox potential during appressorial formation (f,h). During the Grx1-roGFP2 calibration, the CRDR signal was also lost (g). Fluorescent images are shown as maximum plane projections of every 5th z-stack, following smoothing with a 3x3x3 spatial average, auto-fluorescence correction and low signal masking, from a multi-channel (x,y,z,t) 4-D image series collected at 30s intervals. The bright-field image was a maximum brightness projection of the non-confocal transmission images collected in parallel. Scale bars = 5 µ m. From Samalova et al. *(2013)*

for in planta experiments, and subtracted. The auto-fluorescence bleed-through contribution into the GRX1-roGFP2$_{405}$ channel was measured from wild type spores, and the corresponding scaling factor used to subtract auto-fluorescence from the GRX1-roGFP2$_{405}$ image. Ratio images were calculated on a pixel-by-pixel basis as I_{405}/I_{488}. Pixels with intensity values less than 2 standard deviation units above background, within 10% of saturation, or where the local coefficient of variance (cv) in a 3×3 neighbourhood was above 20%, were masked.

For pseudo-colour display, the masked ratio was coded by hue on a spectral colour scale ranging from blue (most reduced) to red (most oxidised), with the limits set by the *in situ* calibration. Individual germlings were segmented using Otsu's method [5] and the extracted images rotated to give a montage of selected time-points for display.

[5] N. Otsu. A threshold selection method from gray-level histograms. *Automatica*, 11:23–27, 1975

Quantitative measurements were calculated as the ratio of the mean intensity from each channel from regions-of-interest. In situ calibration was performed using 10 mM DTT for 5 min followed by 100 mM H_2O_2 for 5 min, to drive the GRX1-roGFP2, to a highly reduced and highly oxidised form, respectively. The degree of oxidation ($OxD_{GRX1-roGFP2}$) and pH-corrected GRX1-roGFP redox potential ($E_{(Grx1-roGFP2)}$) were calculated according to (Schwarzlander et al., 2008), assuming a mid-point potential ($E_{(GRX1-roGFP2)}$) of -280 mV (Gutscher et al., 2008), and a cytoplasmic pH of pH 7.6.

For complex images with extensive networks of hyphal growth and multiple labelled compartments, a pixel-population approach was adopted. A Gaussian mixture model (GMM) with 2-4 components was fit to the pixel intensities at each wavelength, with the contribution of each pixel weighted by the average intensity. Contour maps from the GMM were overlaid on 2-D histograms generated from the weighted pixel intensities. The intensity value of the Gaussian peak at each wavelength was used to calculate the ratio, and hence degree of GRX1-roGFP2 oxidation, $E_{GRX1-roGFP2}$ and E_{GSH} for each component. (See for example Fig. 6.3)

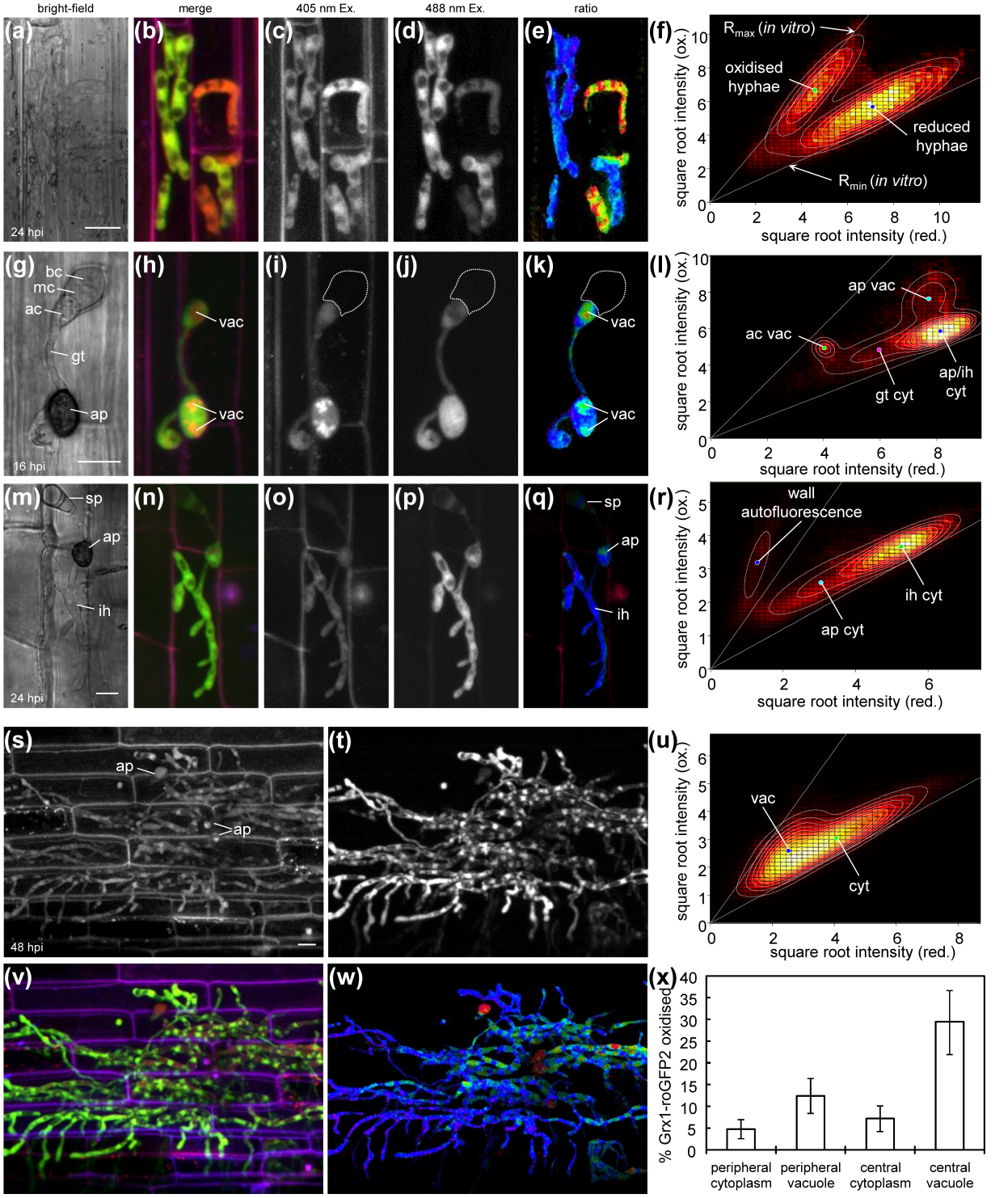


Figure 6.3: Redox relationships during infection of susceptible CO39 rice leaf sheaths with Magnaporthe oryzae *expressing GRX1-roGFP2*

Spores expressing GRX1-roGFP2 were inoculated on rice cultivar CO39 and 3-D (x,y,z) images collected with dual excitation confocal ratio imaging of GRX1-roGFP2 with excitation at 405nm (c,i,o,s) or 488 nm (d,j,p,t) and emission at 500-530nm, and additional wall auto-fluorescence (with either Ex 405nm, Em 435-485nm or Ex 543 nm,

Em 656-615nm). Bright-field images (a,g,m) were collected in parallel with a non-confocal transmission detector. Fluorescence channels were merged and colour-coded as GRX1-roGFP2$_{405}$ in red, GRX1-roGFP2$_{488}$ in green and auto-fluorescence in blue (b,h,n,v), particularly to map the level of oxidative host response, visible as a red/purple colour in the cell walls. In situ calibration was not possible, but occasionally dying hyphae with oxidised GRX1-roGFP2 were observed (a-e). The ratio image (e) and pixel population statistics, fit with a 2-component Gaussian mixture model (GMM), confirmed that the dynamic range of the probe measured during infection was comparable to that observed for spores germinated on coverslips and imaged in parallel (f). During the early stages of infection around 16 hpi (g), GRX1-roGFP2 signals from the cytoplasm in the appressorium (ap) and invasion hyphae (ih), and vacuoles (vac) in the appressorium and apical cell (ap) could be distinguished (h-j) and reported different degrees of oxidation in the ratio image (k) and population statistics, fitted with a 4-component GMM (l). At 24 h post-inoculation (hpi) (m), the majority of the signal could be attributed to cytoplasmic Grx1-roGFP2 in the invasion hyphae (n-p), although there was some host wall fluorescence arising from the host response with a very different broad spectrum (n,q). Cytoplasmic GRX1-roGFP2 was highly reduced in both the appressorium and invasion hyphae (q), and clearly separated from the auto-fluorescence in the pixel distributions (r). By 48hpi microscopic lesions were beginning to form as hyphae spread to multiple adjacent cells (s-w). Levels of vacuolation increased, particularly in the centre of the colony (v and w), but the cytoplasmic and vacuolar Grx1-roGFP2 signals were well separated using a 2-component GMM (u). 2-component GMM models were also fit to the cytoplasm and vacuoles in the centre and periphery of the colony separately, which showed a very slight increase in the EGrx1-roGFP2 but a more substantial increase in the vacuoles (x, n=17). Images are presented as maximum plane projections of 3-D (x,y,z) stacks collected at 3 µm intervals. Scale bars = 10 µm. From Samalova et al. *(2013)*

6.3 *Loading images for analysis*

The **Load** button in the **Load images** panel (Fig. 6.4) activates the **File import** window (see Chapter 7). This has a range of options to import files from different formats and with different dimensionality including multi-channel 4-D *x,y,z,t* images. Images can also be aligned, cropped and sub-sampled at this stage.

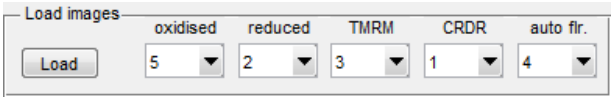


Figure 6.4: The load images panel: Provides controls to load images and assign each channel to the appropriate physiological parameter

As the relationship between the order each channel appears in the image series and the physiological parameter of interest depends on the configuration of the microscope for each experiment, up to five channels can be assigned to different parameters using the drop-down menus (Fig. 6.4). The image displays are updated to show the appropriate image.

6.4 *Image display*

The loaded images are displayed in the four panels in the centre of the interface (Fig. 6.5). Below each image are a number of drop-down menus that control which stage of processing and which **channel** is being displayed. When the data are loaded initially, only the raw image is available for display. The **animate** checkbox controls whether the image is updated during playback.

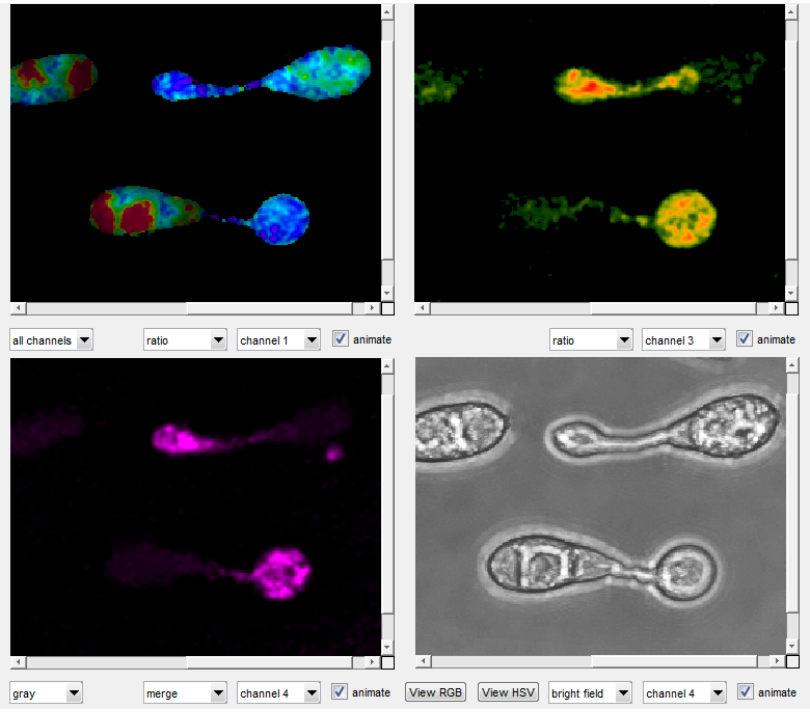


Figure 6.5: Image display panels with drop-down menus to select the channel and stage in processing. The check boxes control whether the image is animated during playback

The **View RGB** and **View HSV** buttons output the array shown

in the panel above to the **Viewer interface** (see Chapter 4. The **View RGB** format exports the underlying image without retaining any of the display contrast settings, as these can be accessed directly in the **Viewer** program. The **View HSV** is used to export the pseudo-colour coded ratio image to the **Viewer** program, and is needed if the user wishes to construct a tilt series of a pseudo-colour-coded 3-D ratio image for example. Immediately below the display panels are the movie controls (Fig. 6.6). The **Start** and **Stop** buttons control playback of the images selected in each of the display windows.

Figure 6.6: The movie controls panel: provides options to adjust the image intensity, section and/or frame number and image size, as well as start/stop animation of the image series

Alternatively, particular **z-Sections** or time **Frames** can be selected using the appropriate sliders. The **Ave** and **Max** buttons calculate and display the appropriate *z*-projection of the images for the selected section or frame. The **mx** checkbox can be ticked to calculate a maximum *z*-projection during playback of a 4-D (*x*,*y*,*z*,*t*) image. The **White level** and **Black level** sliders and text boxes control the intensity scaling for the images displayed. Adjacent to these are separate **bf** text boxes to control the scaling for the bright field image (if present) as this is likely to be very different to the fluorescence channels. The magnification of the images is controlled by the **zoom** slider, or set to fit the display windows (**fit**) or at a 1:1 pixel-to-screen scaling (**100%**). Additional controls are provided to define which channels to include in an **RGB merge** from which image in the processing sequence.

6.5 *Spatial and temporal smoothing*

Once the images have been loaded and the correct order for the channels set, the controls in the **Filter panel** are enabled (Fig. 6.7). The pixel dimensions of the original image are also displayed in the ***xy* size** and ***z* size** text boxes.

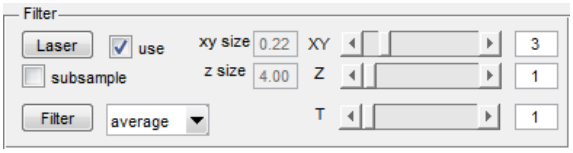


Figure 6.7: The filter panel: controls set the size of the smoothing kernels needed for noise-reduction. The Laser button also activates a new window to input values for the laser power, if these have been varied during the course of the experiment

Noise reduction is achieved at the expense of spatial and temporal resolution using the **XY**, **Z** and **T** sliders to set up a smoothing kernel for the appropriate dimension. Either an *average* or a *median* filter can be selected using the **average** drop-down menu. If the *average* filter is selected, the image is converted to single-precision floating point format first to prevent rounding errors. If *median*

filtering is selected, the median is calculated separately as a 2-D median in the *x*,*y* plane in integer format, followed by a 1-D median in the *z*-dimension and then a 1-D median over time (Note: median filtering is currently very slow). The image is then converted to single-precision floating-point format as all subsequent operations are in single precision.

As smoothing reduces the spatial and temporal resolution, there is a **subsample** checkbox, which, if checked, results in a subsample at half the kernel size-1 in each dimension. This can significantly reduce the overall image size and increase the speed of subsequent operations. If subsampling is used, the pixel sizes are updated to reflect the pixel dimensions of the sub-sampled image.

The **Laser** button opens a table of laser power values for each channel that can be edited. This allows correction for changes in laser power that might occur when using reactive dyes such as CRDR, for example, where the intensity increases as a function of time, and is likely to reach saturation if the laser power is not reduced. Once the laser power settings have been set, they can be saved and re-loaded if needed. The adjacent checkbox controls whether the laser power corrections is applied. It is not recommended that laser power setting are altered for dual-excitation ratiometric measurements as the calibrations may no longer be valid, unless care is taken to maintain exactly the same relative intensities.

Clicking the **Filter** button applies the smoothing filter selected and the displays are updated to show the filtered image for each channel.

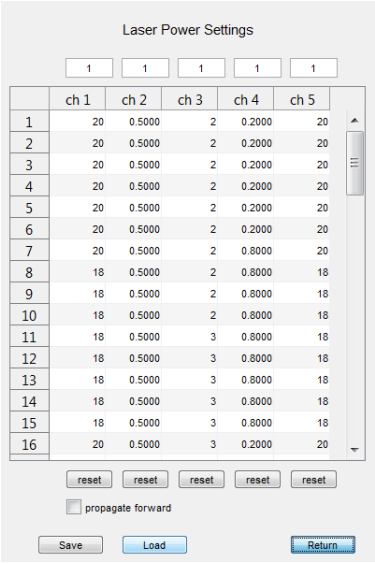


Figure 6.8: The laser power window: allows the user to input values for the laser power in each channel if these have been varied during the experiment. This is typically only needed for experiments with reactive dyes to avoid saturation as the intensity builds up over time

6.6 *Background subtraction and auto-fluorescence bleed-through correction*

The **Measure** button in the **Background panel** (Fig. 6.9) prompts the user to draw a rectangular region in an area of background for the currently selected image. The average (**ave**) intensity standard deviation (**std**) are returned in the text boxes for each channel. The adjacent **checkbox** controls whether the background will be subtracted in the subsequent processing step.

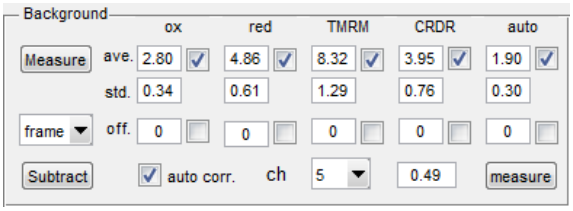


Figure 6.9: The Background panel: provides controls to measure the background fluorescence and aut-fluorescence bleed-through for each channel and subtract a single value for all time-points or on a frame-by-frame basis. For some experiments, such as imaging dyes that equilibrate between the cells and the medium, an alternative offset option is provided to subtract a pre-determined background value

In some situations there is fluorescence present throughout the specimen and medium, for example with TMRM labelling, which prevents measurement and subtraction of a background from the current image. In this case, the background has to be measured

from an un-labelled control, using the same instrument settings, and applied as an offset (**off.**) to the corresponding channel. The **checkbox** next to the **off.** textbox will turn off the measured background value and subtract the offset instead.

Auto-fluorescence bleed-through into one of the measurement channels is a common problem, particularly with plant specimens and blue/violet excitation. As auto-fluorescence has structure in the image, it cannot simply be subtracted as a single value like the background. However, it is sometimes possible to estimate the auto-fluorescence at an emission wavelength range that does not have any signal from the measurement probe. As the auto-fluorescence spectrum tends to be quite broad, a scaled version of this image can be subtracted from the probe image to correct for the auto-fluorescence bleed-through.

The **measure** button allows the user to draw a rectangular ROI in a region of the image where there is auto-fluorescence, but no probe signal, to calculate the bleed-through correction factor from the auto-fluorescence channel (**ch**), selected using the drop-down menu, into channel 1. The correction factor is displayed in the adjacent text box. If it is not possible to measure the bleed-through correction factor directly from the loaded image, it has to be estimated from an un-labelled control using exactly the same instrument settings and typed into the correction box. The **auto corr.** checkbox has to be ticked to apply the correction.

The **single** drop-down menu allows background subtraction using the values measured from a single frame, as displayed in the text boxes. If the **frame** option is selected the background for the same region-of-interest is calculated and subtracted separately for each frame in the time series.

Because the background and auto-fluorescence correction subtract noisy images from each other, there is the potential to generate negative values if the original intensities were close to the background values. Any negative values are automatically set to zero during the calculation.

Clicking the **Subtract** button applies the background subtraction and auto-fluorescence correction, if appropriate, and the displays are updated to show the filtered image for each channel.

6.7 Ratio calculation

A range of different operations can be applied to the background-subtracted images at each wavelength to extract quantitative measurements of physiological parameters using the **Process** panel (Fig. 6.10). The simplest of these is a pixel-by-pixel **ratio** of two wavelength channels, and this is the default setting in the drop-down menu for channel 1. The **denom** drop-down menu is used to select the denominator for the ratio, and the default is channel 2.

A number of options are available to mask pixels in each of the ratio channels that are too close to background or saturation to give

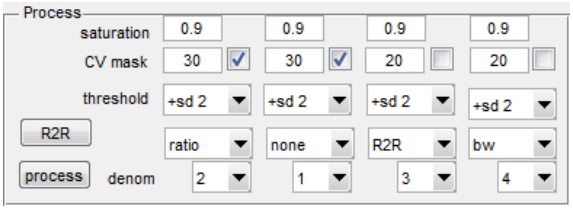


Figure 6.10: The process panel: provides a number of options to mask pixels from the processed image that are too close to background or saturation, or show too much local variance (CV). There are also a number of alternative options to process the images in addition to just ratioing against a second channel (set by the denominator drop-down menu). For example, the R2R button allows the user to define a region of the image to act as a reference to ratio against

a reliable ratio, or are drawn from regions with high local variance that might correlate with edges of structures or compartments that are not properly resolved.

The **saturation** mask is applied to all pixels above the proportional limit set, typically at 0.9 of the full scale.

The **CV** mask excludes all pixels that have a higher local coefficient-of-variation (CV) in a 3×3 neighbourhood than the threshold set as a percentage, where the CV is defined as the standard deviation divided by the mean. This is useful to exclude pixels on the boundary of objects or between cell compartments with different labelling levels.

The **threshold** drop-down menu allows the users to select a low intensity mask at some (integer) number of standard deviation units above background. The default is 2 SD.

Clicking the **process** button calculates the ratio. At this stage the displays are not updated as it is customary to display the resultant ratio as a pseudo-colour coded image with a calibrated scale (See Section 6.10).

6.8 Other processing algorithms

A number of other processing algorithms have been included in the drop-down menus. The main ones and their typical usage are described below.

- **n ratio**: The normalised ratio (n ratio) calculates the pixel-by-pixel ratio of the numerator and denominator channel selected by the drop-down menu, but then scales the resultant ratio to lie between an estimate of the minimum and maximum values for that pixel in the time series. As a single minimum or maximum value is likely to be very noise sensitive, the software takes an average value defined for **Rmin** and **Rmax** over the time intervals set using the **low** and **high** ranges in the **Calibration** panel (see Section 6.10)

- **F/Fo**: The ratio of fluorescence for each time point (F) against the initial fluorescence (Fo) provides a normalisation for different dye loadings and differences in optical path length for each part of the specimen. It can be useful to monitor the fold-change in a fluorescence signal from a single wavelength dye that only changes in intensity rather than spectrum with the physiological parameter of interest. Nevertheless, any changes in dye concentration, photo-bleaching, or optical path length will also affect

the measurement. This type of ratioing is also critically sensitive to any specimen movement.

- **F-(Fn-1)**: The difference in fluorescence between successive time points provides an estimate of the rate of change in fluorescence signal. It is typically used to measure the rate of reaction for reactive dyes. Whilst the absolute rate of reaction requires additional calibration against reference solutions, transient changes in the rate may indicate a "burst" of activity.
- **F/(Fn-1)**: Rather than calculate the absolute difference in intensity, the ratio of intensity in successive time points gives a measure of the fold change in signal and can be used to compare transients in cells with different levels of overall signal.
- **Mean**: Normalises each pixel time series to the mean (F/F_{mean})for that pixel to provide a less noise sensitive alternative to normalisation to the first time point (see **F/Fo** above).
- **Cv**: Normalisation using the difference from the mean divided by the mean provides a standardised measure of fluctuations in signal irrespective of the absolute value of the fluorescence.
- **Grey**: Simply carries the background-subtracted greyscale (**Grey**) data through to the display section without any further processing.
- **Invert**: Carries the an inverted image of the background-subtracted greyscale data through to the display section
- **norm T**: Normalises each pixel to the average of all pixels at that time point. This provides a crude way to compensate for fluctuations in illumination intensity or changes of intensity with depth.
- **R2R**: Ratio-to-a-Region (R2R) calculates the ratio of every pixel to the average of a user-defined region set using the **R2R** button. This is useful for dyes, such as TMRM, that accumulate from the medium inside a cell or sub-cellular compartment in response to a membrane potential or pH gradient. As the dye is present in the medium throughout, the normal background correction has to be replaced for this channel by an **offset** correction (see Section 6.6).

As an example, the functional connectivity between the three different cells in developing germlings of *Magnaporthe oryzae* was examined using fluorescence recovery after photobleaching (FRAP) of the GRX1-roGFP2 signal in the mid cell using the $F/F-0$ processing option to normalise the traces to the first image in the time-series, and then colour-code the ratio (Fig. 6.11). At an early developmental stage, the cells were completely isolated with no fluorescence recovery, whilst at later stages, some germlings showed rapid re-equilibration, suggesting components were able to move through the septal pores.

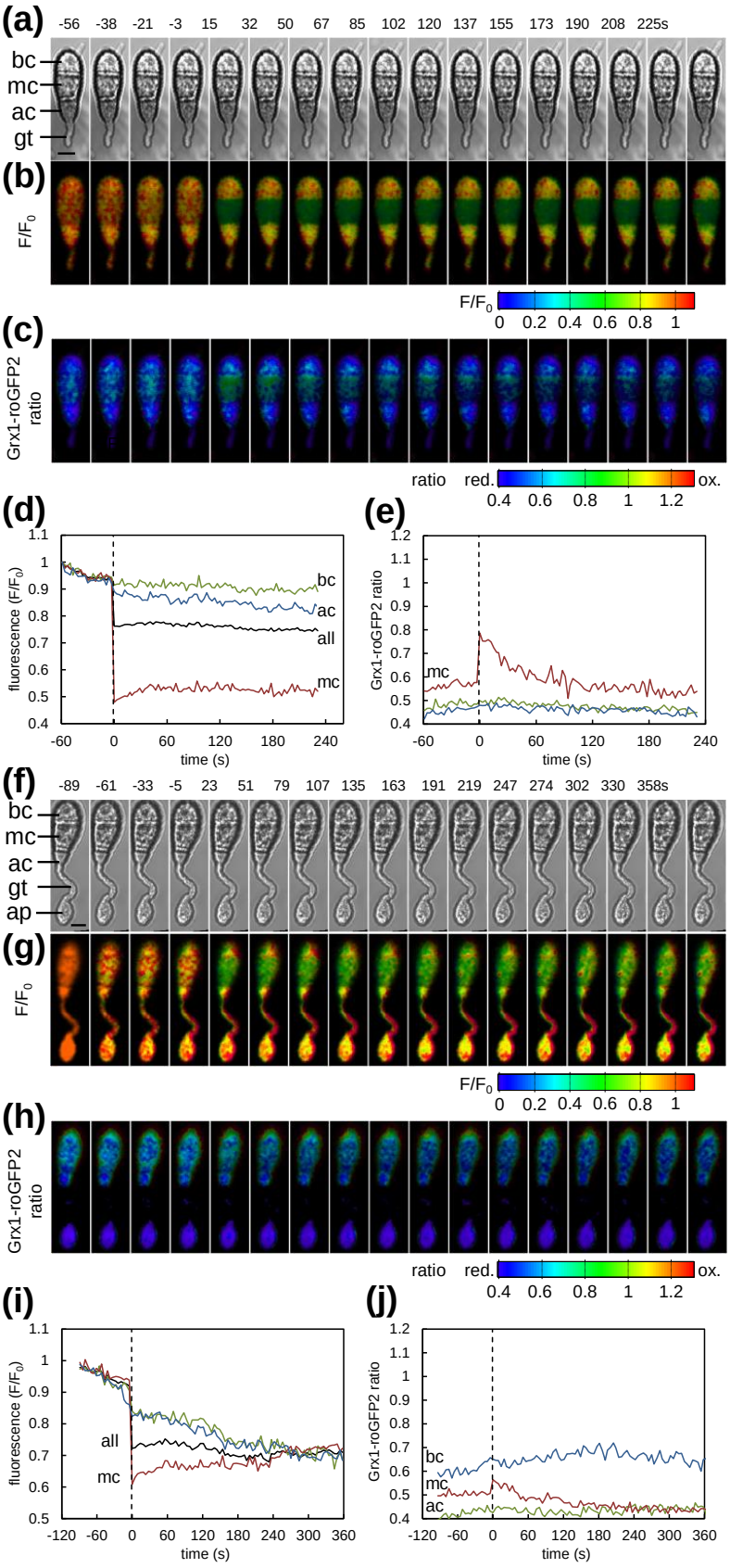


Figure 6.11: Functional connectivity between different cells in developing germlings was examined using fluorescence recovery after photobleaching (FRAP) of GRX1-roGFP2 in the mid cell. Examples are shown for growing germ tubes (a-e), and during germ tube swelling (f-j). Following a laser pulse when the 405 and 488 nm lasers were briefly increased to full power over the mid cell to bleach GRX1-roGFP2, the 488nm signal dropped to around half of the initial value (b,g), expressed as a proportion of the initial fluorescence on a pixel-by-pixel basis (F/F−0). During germination and germ tube emergence little or no recovery was observed, with only slight convergence of signals in the apical cell and middle cell over the first 30s post-bleach, whilst the total for all three cells (black line) remained constant (d). At later stages of development, GRX1-roGFP2 progressively equilibrated between two or more of the cells (g,i) and signals from all three cells converged on the average value for the germling post-bleach (i). This would be consistent with exchange through opening of the septal pores. The GRX1-roGFP2 ratio in the bleached middle cell showed transient oxidation following the bleach, that returned to resting levels over about 60s (e,j), whilst the ratio from the other cells remained constant. Fluorescent images are every 6th image from a multichannel (x,y,t) 3-D series collected at 2.3s (a-c) or 3.6s (f-h) intervals. Scale bars = 5 µm.

6.9 *Pseudo-colour coding*

Once the images have been processed, the ratio data can be pseudo-colour coded to highlight the parameter of interest in HSV (**H**ue, **S**aturation and **V**alue or intensity) colour space using the controls in the **Ratio display** panel (Fig. 6.12).

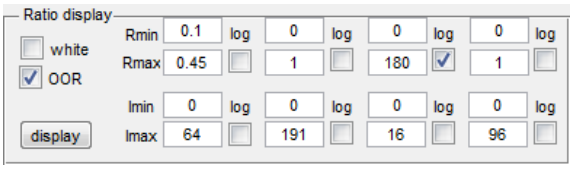


Figure 6.12: The Ratio display panel: set the scaling limits for the pseud-colour coded ratio (Rmin and Rmax) independently for each channel, and also controls the brightness of the image (Imin and Imax). In each case there is an option to use a logarithmic scaling. The white checkbox switches the background to white, whilst the OOR checkbox clamps pixels below Rmin or above Rmx at the minimum and maximum values, respectively

The Hue colour scale ranges from blue (low) to red (high). The limits of the colour scale are set independently for each channel using the **Rmin** and **Rmax** textboxes, with an additional checkbox for a logarithmic scaling. The **log** scaling can be useful during initial data inspection, if the appropriate scaling is not yet known, as **Rmin** and **Rmax** can be cover a very broad range to capture the likely response.

Likewise the Value (intensity) can be set independently from **Imin** to **Imax** for each channel, with an additional checkbox for a **log**arithmic scaling.

An checkbox is provided to switch the background from **black** (the default), which has high on-screen contrast, to white which reduces the cost of printing figures.

The Out-of-Range (**OOR**) checkbox controls impose the maximum and minimum colours on pixels that fall beyond the values set by **Rmin** and **Rmax** to give a more consistent display. If the OOR is not ticked, values that fall outside the range are visible in greyscale.

6.10 *Calibration*

The **Calibration panel** controls the mapping between the ratio values and the physiological parameter (Fig. 6.13). The type of calibration is set using the drop-down menus, with the default being **redox**. The other options include **calcium** or just a non-specific **channel**.

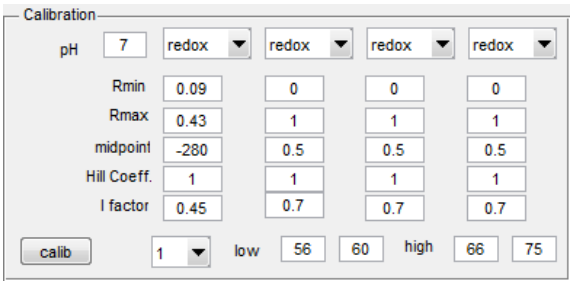


Figure 6.13: The Calibration panel: with controls to set the mapping between the ratio values (or other processing approach) and the physiological parameter for each channel

The parameters required depend on the probe used - in the case of the roGFPs these are **Rmin** when the roGFP is fully reduced,

Rmax when the probe is fully oxidised, and the **midpoint** potential. An estimate of the **pH** in the compartment where the probe resides has to be provided in the appropriate textbox.

The **Calcium** setting switches the labels from midpoint to **kd** and the binding response may also require a **Hill coefficient** different from 1.

The **I factor** is an instrument specific value that is required to compensate for the different intensities measured from the fully reduced and fully oxidised form of the probe to allow calculation of the degree of oxidation (OxD) of the *roGFP* sensor (equation (6.1)).

$$OxD_{roGFP} = \frac{R - R_{red}}{\frac{I_{488ox}}{I_{488red}}(R_{ox} - R) + (R - R_{red})} \tag{6.1}$$

Where R is the ratio of excitation at I_{405}/I_{488} nm, R_{red} is the ratio of fully reduced form following perfusion with DTT, R_{ox} is the ratio of the fully oxidised form following perfusion with H_2O_2 and I_{488ox} and I_{488red} are the intensities at 488 nm for the fully oxidised and fully reduced forms, respectively.

The **I factor** has to be measured from a single calibration experiment on the same sample, but can then be applied to any data collected with the same laser intensities and instrument settings.

Values for all the calibration parameters can be entered directly, or **Rmin**, **Rmax** and the **I factor** can be measured from a calibration series where the probe has been driven over the full response range using appropriate calibration solutions. Pressing the **calib** button prompts the user to select a **ROI** on the image to estimate the parameters from the data. Values for **Rmin** are averaged over the time period set using the **low** textboxes, while **Rmax** is averaged over the time period set by the **high** textboxes. Different time windows to estimate the calibration values can be used for each channel using the **channel** drop-down menu. Note: At present the time windows for the different channels are not saved.

6.11 Data measurement

The simplest form of time-series analysis is to select a point or series of points in the image to extract the data from a user-defined local neighbourhood. The **point** button in the **Point analysis** panel (Fig. 6.14) prompts the user to left-click to select each ROI (or right click to exit). The dimension (**time** or **z**) to extract the data from, and the **size** and **shape** of the ROI are controlled by the adjacent drop-down menus.

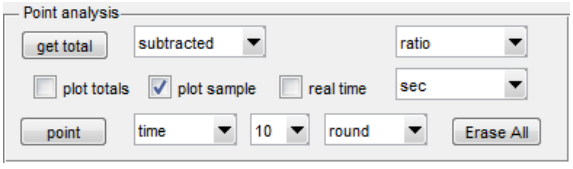


Figure 6.14: The Point analysis panel: allows the user to select different region-of-interest to display in graphical form

The **get total** button can be used to choose a ROI with a different

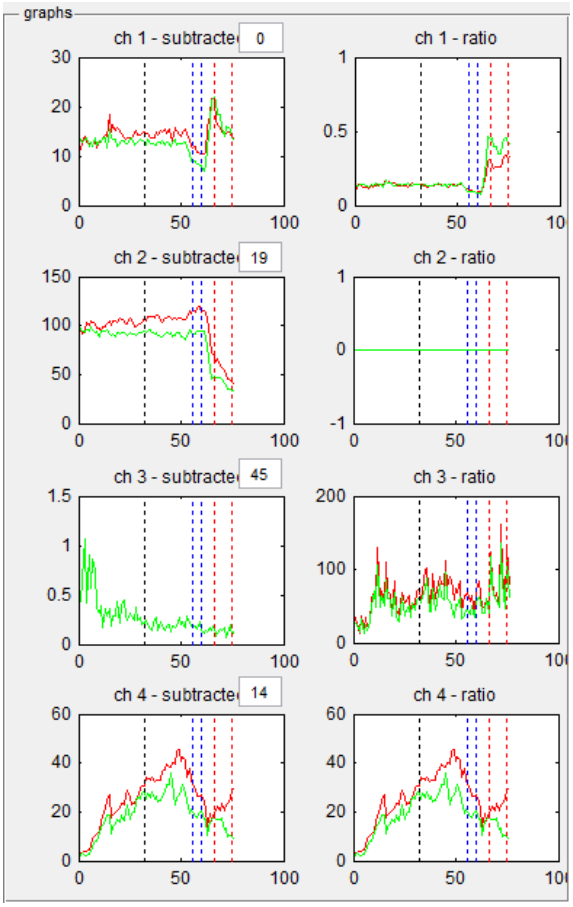


Figure 6.15: The graphs panel: Data for 2 ROIs are shown on the left column for all four channels, whilst the ratio of channels 1 and 2 for roGFP is shown in the top panel on the right (there is no ratio calculated for channel 2). Channel 3 is a TMRM signal, ratioed against the medium using the R2R function, whilst the fourth plot is the CRDR signal with no processing, but following adjustment of the laser power. The dotted blue lines indicate the time window used for measurement of Rmin for roGFP after addition of DTT to reduce the probe, whilst the dotted red lines show the time window used to estimate Rmax, following perfusion with H_2O_2

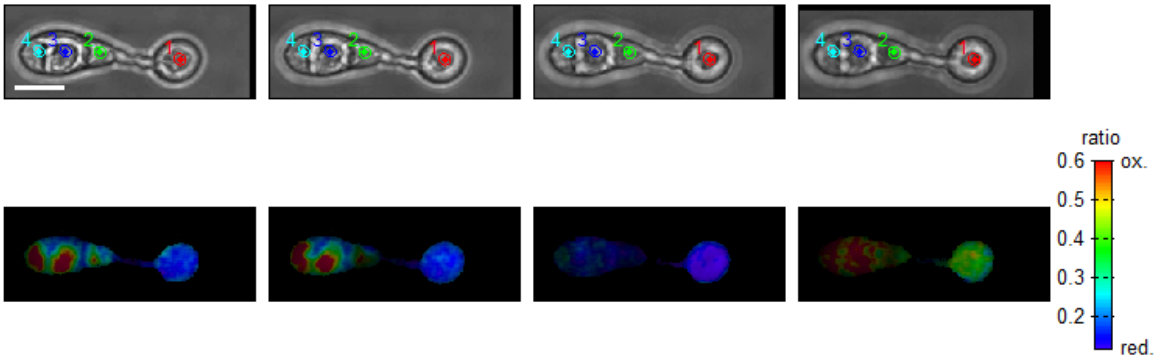
size, typically encompassing a much larger region of the image to give an estimate of the total signal. The **plot totals** checkbox toggles display of the total values in black.

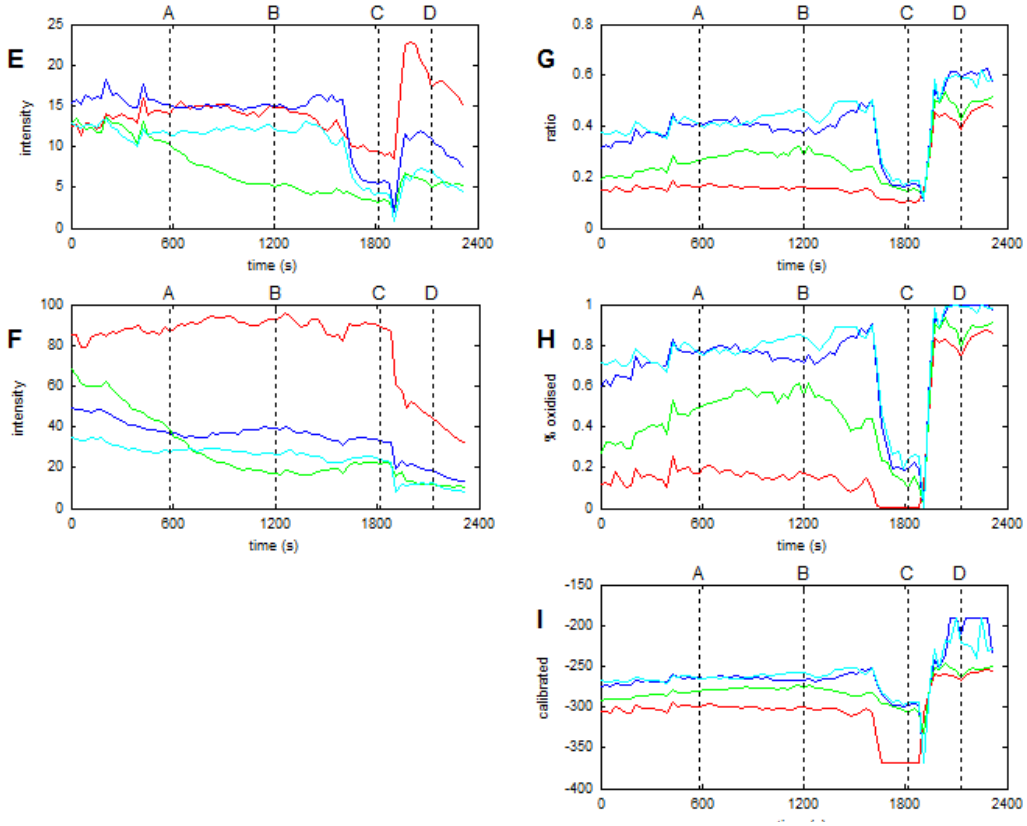
Data from each ROI over time is plotted in the two sets of graphs for each channel for the array selected using the drop-down menus below the **graph panel** (Fig. 6.15), and the corresponding point colour-coded on the image. The default for both is the *raw* data, but it is often convenient to display the *subtracted* data on the left and the *ratio* or *normalised* data on the right, once the processing is complete. .

The **x-axis** default is in frame (or section) number, but can be displayed for the **real time** using the checkbox, in **units** selected using the drop-down menu. An additional **Erase All** button clears the ROIs and graph plots. The **plot sample** checkbox is used to display the time-window limits for the internal calibration set by the current low (dotted blue) and high (dotted red) limits in the **calibration panel** (Fig. 6.15).

During animation, the current frame is shown as a dotted black line on the graphs.

The **Figure** button in the **Output panel** will generate an output version of the ROI graphs (Fig. 6.11). This can be saved in a variety of formats using the options on the menu bar. Windows extended metafile (*.emf) is useful to import into powerpoint. The figure can then be ungrouped (multiple times) to give access to the individual elements if needed.





6.12 *Kymograph analysis*

Rather than display data from a series of points, the data along a user-selected profile can be chosen using the **XT Profile** button in the **XT Display** panel (Fig. 6.16). The profile has to be drawn on the image in lower right panel and results in plots of the intensity/ratio along the profile for each timepoint on the y-axis. The **normalise** checkbox divides each channel by the maximum to make the display more visible. If the XT-images are displayed, the **Figure button** in the **Output panel** will generate an output version of the XT graphs with suitably calibrated axes (Fig. 6.12).

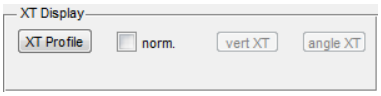
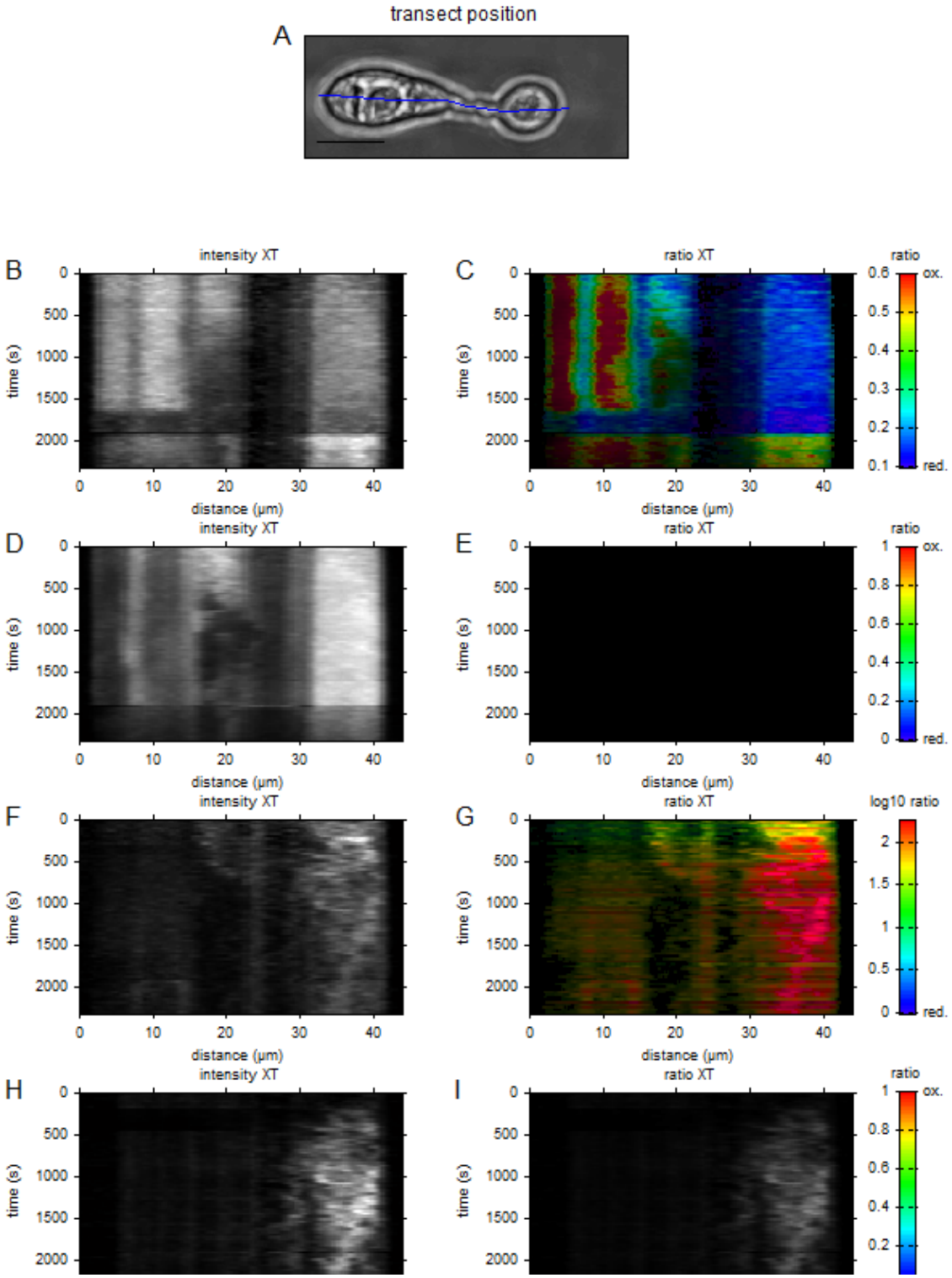


Figure 6.16: controls to extract a kymograph (XT) image



6.13 *Population statistics*

Instead of selecting particular ROIs to analyse, it is possible to examine the population statistics for all the pixels in the image using the controls in the **Analysis panel** (Fig. 6.17).

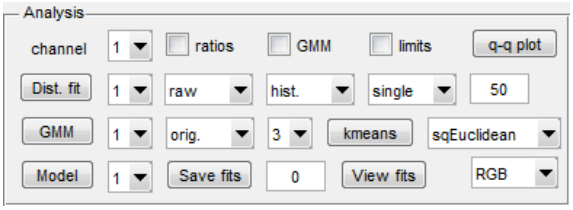


Figure 6.17: The analysis panel: Once the channel for analysis is selected, the user has a series of options on which array to visualise, whether to display a histogram, weighted histogram or 2-D scatter plot, for a single section or the entire data set, and the number of Gaussians to fit to the log ratio data. The GMM button fits a set number of 2D Gaussian Mixture Models to the original data, or after transformation on a square-root or log basis. There are additional options to view or save the fits at higher resolution

The **channel** for analysis and the stage of processing are selected from the drop-down menus, typically the **subtracted** array. The **Dist. Fit** button, displays a 2-D histogram for the two channels used to calculate the ratio, and a 1-D histogram of the log ratio values for the current section and frame (Fig. 6.18).

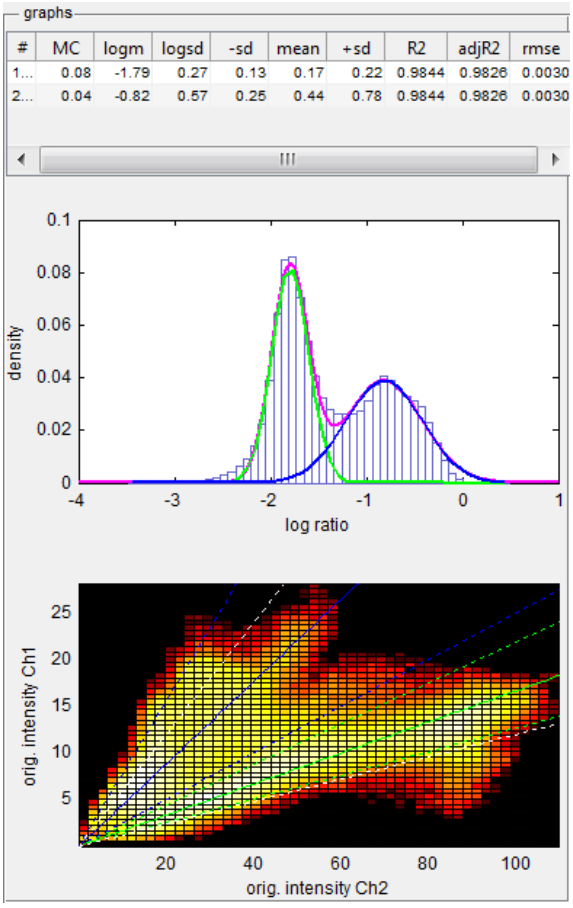


Figure 6.18: The graphs panel: The 2-D histogram is shown in the lower graph using a heat colormap, with the fits to the log ratio data for the specified number of components in the upper graph. The mean and standard deviation for the fits are presented in the results table and also plotted on the 2-D histogram as solid and dotted lines, respectively. Additional goodness-of-fit measures are also presented in the results table.

The number of **bins** in the 2-D histogram can be changed from the default of 50, using the corresponding textbox. In addition, the **log ratio data** is fit with a one or more Gaussian curves, with the **number** selected from the adjacent drop-down menu. The mean (*mCh1* and *mCh2*) for the Gaussian distribution and the standard deviation (*sd1* and *sd2*) are shown in the table above for the two channels, along with estimates of the goodness-of-fit. The standard deviation values are asymmetric as they are calculated from the log ratio data. The equivalent gradient for the ratio of mCh1/mCh2 is plotted on the 2-D histogram image if the **ratios** checkbox is ticked. The limits set using the **ratio display** for **Rmin** and **Rmax** are also

shown on the 2-D histogram if the **limits** checkbox is ticked.

Fitting to the original ratio data does not take into account the relative intensity of the different pixels contributing to the distribution. Thus ratios from very dim pixels contribute as equally as ratios from very bright pixels. The alternative is to calculate a weighted histogram (**wt hist**) in which each data point is replicated in the data set in proportion to the average intensity at each of the two wavelengths. This ensures that a large number of dim and noisy pixels do not skew the fit.

6.14 *Gaussian mixture modelling*

The **GMM** button attempts to fit a Gaussian mixture model to the 2-D histogram, which is then displayed as a contour map overlaid on the 2-D histogram .

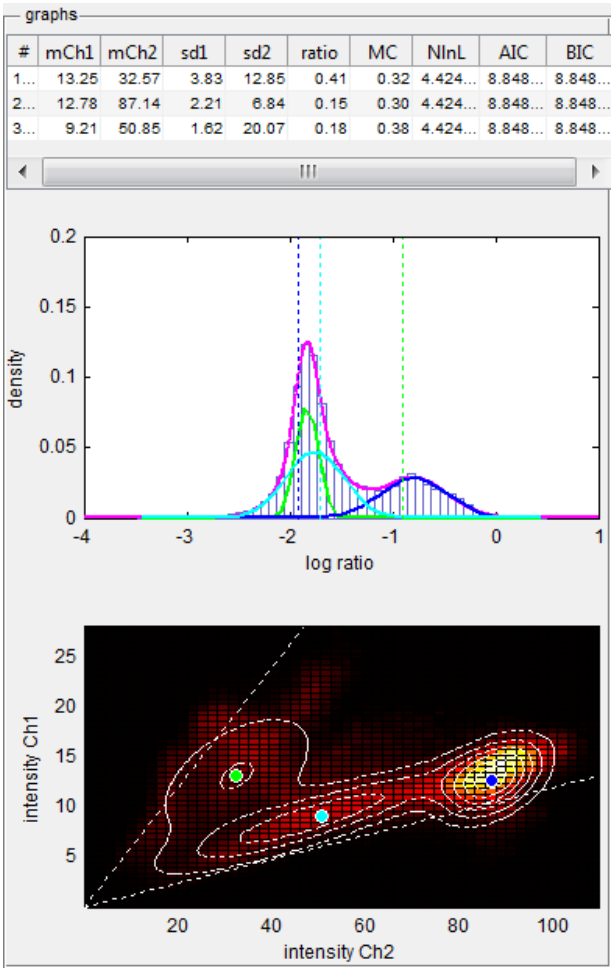


Figure 6.19: The graphs panel: The weighted 2-D histogram is shown in the lower graph with the result of a 3-component Gaussian Mixture Model (GMM) superimposed. The upper graph shows fits for a 3-component Gaussian model to the low ratio data, with the dotted lines representing the centres of the GMM components superimposed. The results table gives the values for each GMM component and the overall Goodness-of-Fit statistics

The *mean* of the GMM for each of the channels is displayed in colour on the plot, and the actual value displayed in the table, along with estimates of the goodness-of-fit, including the *log-liklihood*, Akaike Information Criterion (*AIC*) and Baysian Information Criterion (*BIC*). The number of Gaussian curves in the model is controlled by the first adjacent drop-down menu. In many cases,

the original (**orig.**) data distribution does not conform well to a 2-D Gaussian without prior transformation. It is possible to re-plot the data using a *square-root*, *log* or *inverse* transformation using the drop-down menu, and then repeat the fit. As the fitting process can get trapped in local minima, there is the option to repeat the fit multiple times with different random starting points, using the third drop-down menu and select the best fit.

The **Model** button will automatically calculate the GMM for an increasing number of Gaussian curves up to the limit set by the adjacent drop-down menu, to allow comparison between the goodness-of-fit with increasing parameters in the model. Buttons are provided to save the fitting data (**Save fit**) and view and save the contour plots (**View fit**) at higher resolution.

There is an additional button to calculate **kmeans** clustering using a variety of options set by the adjacent drop-down menu. So far this has not proved useful for ratio data.

6.15 *Data output*

The **Output panel** has buttons to save a figure of the data analysis displayed following the point analysis or kymograph analysis for the **channel** selected by the adjacent drop-down menu. The **Save data** button exports the data from the ROI point analysis to Excel.

6.16 *Image montage options*

A montage of a sub-set of the images can be output using the controls in the **Montage Display** panel for the selected channel (Fig. 6.20).

Figure 6.20: The montage display panel: a set of images at regular increments can be chosen for a given channel and displayed as a single row. The montage button prompts the user to select a subset of the image, using a simple segmented version as a guide. The movie layout provides options to assemble more complex grids of any image combination to output as a movie

The textboxes allow the user to select the **first**, **last** and **increment** between images and the **width** of the border between them (in pixels). When the **Montage button** is first pressed, a thresholded binary image of the data is displayed (Fig. 6.21) to allow the user to select which portion of the data to display. It is possible to include a larger region by drawing a line around any features required, or isolate a subset by erasing any connections to neighbouring features. These options are chosen from a dialog box that automatically appears when the segmented image appears. The remaining object(s) is then selected with a single mouse click, followed by a double click to finish.

The selected object is automatically rotated to align the long axis vertically, and then assembled and displayed in a separate window (Fig. 6.16). If the number of images in the montage needs adjustment, it is possible to **re-use** the segmented region by ticking

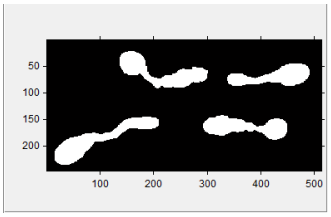
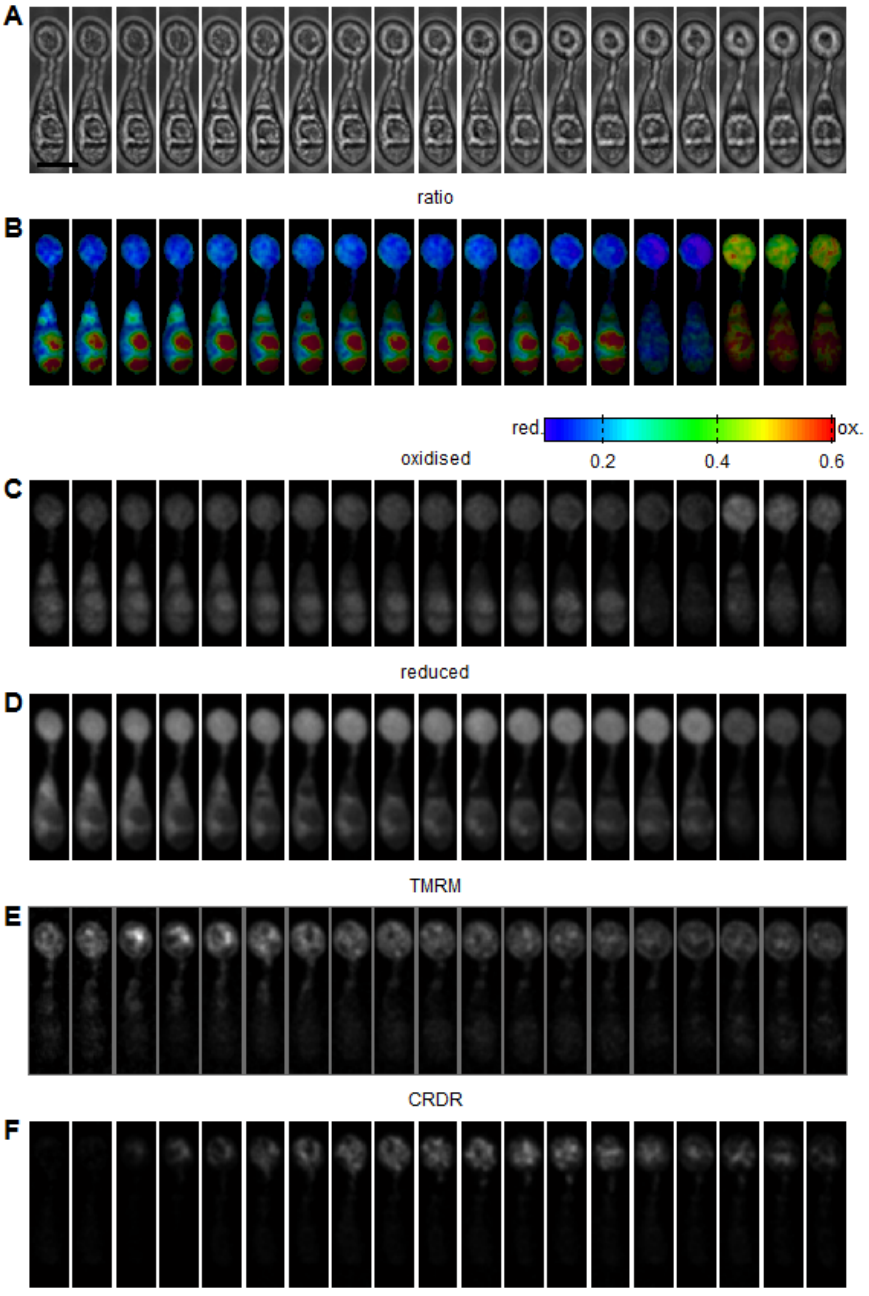
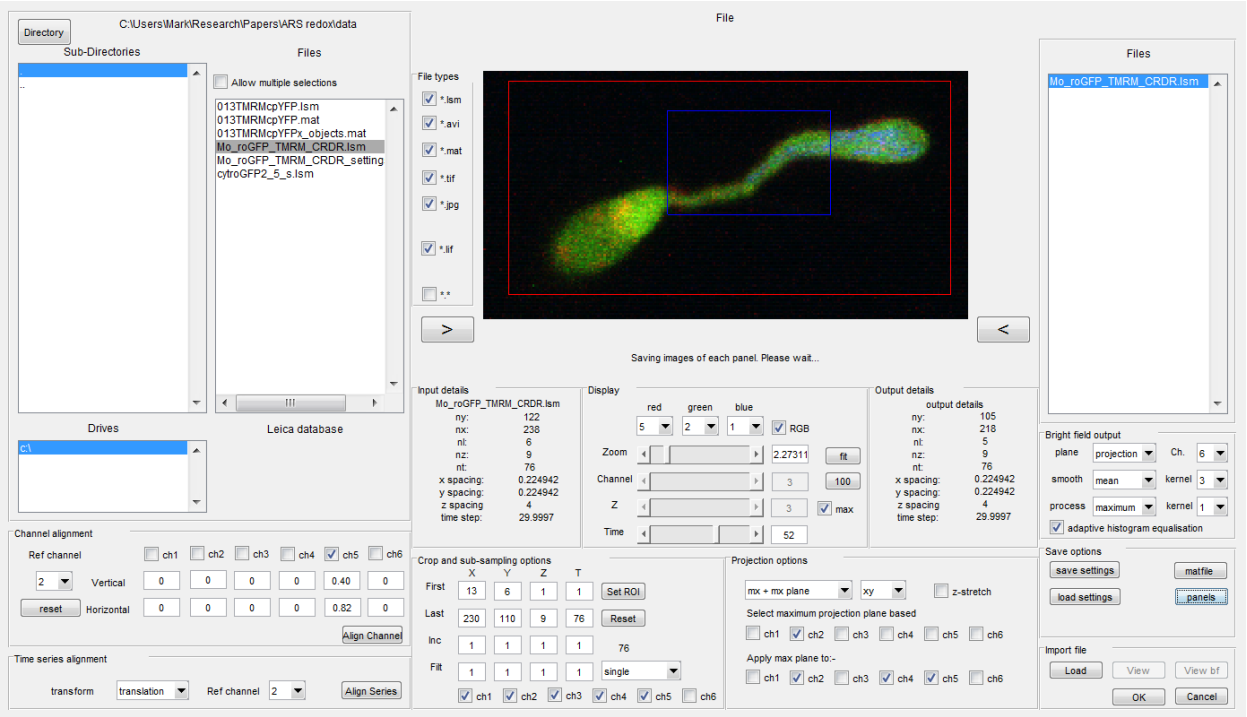


Figure 6.21: The montage button in the Montage Display panel presents a segmented version of the current image and allows the user to draw or select objects to make a composite montage image of a selected subset of data

the appropriate check box, altering the image range or increment and then pressing the **Montage** button again. The **merge** checkbox runs the montage output for the merged image rather than the ratio image for the selected channel.



7
Advanced Ratio File Import



7.1 Introduction

This software was designed to import Zeiss confocal image stacks into Matlab, particularly for ratio imaging applications. The import options allow some channel registration, time-series alignment, sub-sampling, smoothing and projection.

The import routines use the LSM file toolbox written by Peter Li and available on the Mathworks Website.

`http://www.mathworks.co.uk/matlabcentral/fileexchange/8412-lsm-file-toolbox`

An additional import filter for Leica lif databases has been included, that uses the Bio-formats program (Linkert et al. 2010[1]):

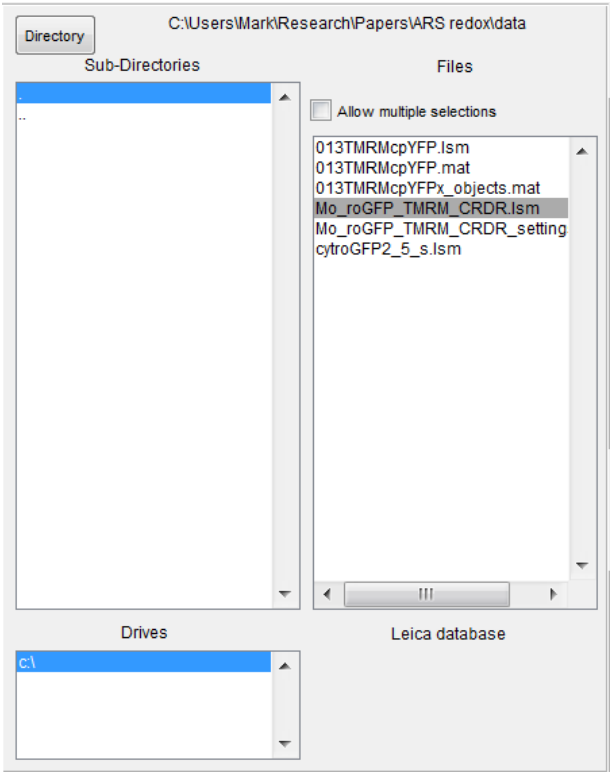
`http://www.openmicroscopy.org/site/support/bio-formats4/`
`http://loci.wisc.edu/software/bio-formats`

[1] M. Linkert, C.T. Rueden, C. Allan, J.-M. Burel, A. Moore, W.and Patterson, B. Loranger, J. Moore, C. Neves, D. MacDonald, A. Tarkowska, C. Sticco, E. Hill, M. Rossner, K. W. Eliceiri, and J. R. Swedlow. Metadata matters: access to image data in the real world. *The Journal of Cell Biology*, 189:777–782, 2010

7.2 *File Selection*

The file selection panel shows:

- a **Directory** button that opens a standard dialog box to select a different directory
- sub-directories of the current folder
- individual files in the current directory
- the currently selected file
- the available drives
- image files within a Leica "lif" database (if appropriate)



If the **Allow multiple selections** checkbox is ticked, a range of files can be loaded in one operation. This is useful to join a set of consecutive sequences from the same time-series experiment. The images must have the same dimensions in x and y, and have the same number of channels. Images are sorted in alphabetical order in the listbox and will be imported in this order. If a different order is required, each file has to be added in sequence manually.

A set of checkboxes (Fig. 7.1) are available to display a restricted set of file types or all files in the directory (*.*)

Once the required file(s) have been selected, the information on each file can be loaded by double clicking or using the **right-arrow**.

If the system can read the file format information, The file name(s) will then appear in the **output list** box, and the image

Figure 7.1: Setting the file extensions

details shown in both the **Input details** and **Output details** panels (see Fig. 7.2). If multiple files have been selected, only the details of the last file will be shown. Input and output details will be the same at this stage as no additional processing steps have happened.

If the system can not pick the pixel size information or time interval, a dialog box will appear with prompts for the user to enter the required information (see Fig. 7.3).

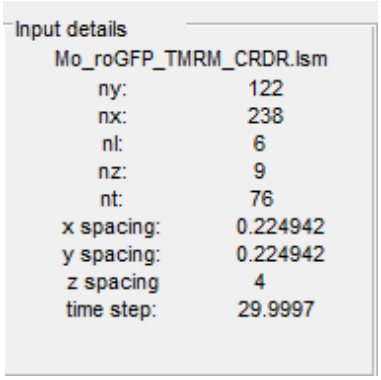


Figure 7.2: The input details panel: displays of the size and calibration values for the current image

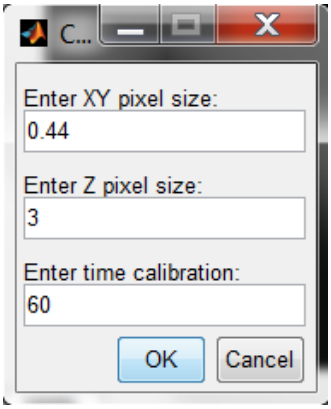


Figure 7.3: Dialog box prompting for the x,y,z spacing and the time-interval

7.3 *Importing images from a Leica database*

Leica images are stored in a single *.lif database and cannot be accessed directly. Fortunately the bioformats package has been designed to read in images from different microscope manufacturers and store them in a standardised format - See:

`http://www.openmicroscopy.org/site/support/bio-formats4/`

The latest versions of Java needs to be installed, and is available from:

`http://www.java.com/en/`

The loci_tools.jar program also needs to be available on the search path or installation directory of the matlab programs. loci_tools.jar is available from:

`http://downloads.openmicroscopy.org/bio-formats/4.4.9/`

If a "lif" database is selected, the bioformats program reads in the list of image stacks or time-series and displays these in a separate pop-up window (see Fig. 7.4).

The file is selected by double clicking in the list box and the name appears in the box underneath. Clicking the **Load file** button will import the image and display the first three channels of the first time/z-plane in the window. The **x**, **y** and **z** pixel sizes have to be entered manually into the appropriate boxes, along with the **Time** interval and **bit depth**, as these are not yet read in by the BioFormats program.

Clicking **OK**, will import the selected file into the main interface. It is also possible to view the full files at this stage using the **View file** button, or save the file as a matlab array using the **Save file** button.

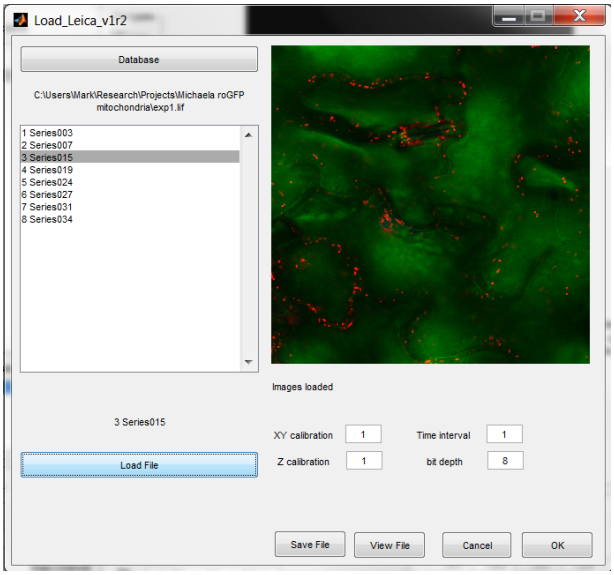


Figure 7.4: The Leica import window: Once a Leica database has been loaded, one of the image files can be selected and imported into the MatLab environment. The calibration parameters have to be set manually

7.4 *Image display*

Once the images have been loaded, the first image of a time series will be displayed in the image window (Fig. 7.5).

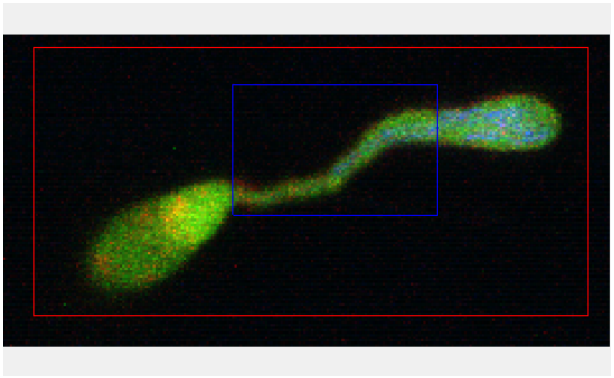


Figure 7.5: Image display panel: The image has been loaded in and an RGB version of the first three wavelength channels displayed. The red rectangle shows a user-defined region that will be cropped from the original file

If the image is a *z*-stack, the median plane will be displayed. If it is a multi-channel image, the median channel image will be displayed. If multiple separate image files have been loaded, a different file can be displayed by selecting the appropriate file from the output list box. A number of controls are available to alter how the image is displayed (Fig. 7.6).

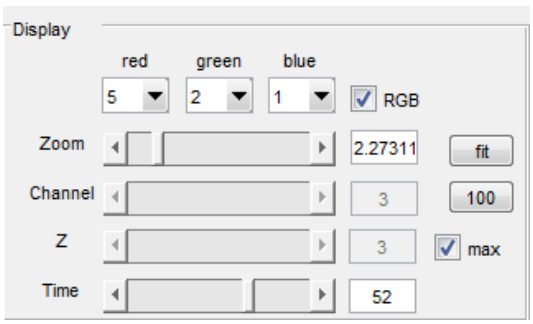


Figure 7.6: The display panel: Controls are provided to allow the user to select which channel, section or time-point to display, with further options to combine channels into an RGB image, or display a maximum projection of the z-stack (if present)

For multi-channel images, different channels can be assigned to the **R**, **G** and **B** image planes using the drop-down menus to construct a RGB image, which will be displayed if the **RGB** checkbox is active.

The image size can be increased using the **Zoom** slider. If the image is larger than the display window, horizontal and vertical scroll bars will appear. The **fit** button maximises the size of the image to fit within the display window. The **100%** button displays the image at a 1:1 image pixel to display pixel size.

The **Channel** slider displays a single channel image (if the RGB checkbox is un-ticked).

The **Z** slider scrolls through z-sections if the image is a 3-D (x,y,z) stack. The **max** checkbox displays a maximum projection of the current z-stack.

The **Time** slider scrolls through each image in the time series.

7.5 *Image crop and sub-sampling options*

The image can be cropped by entering the **First** and **Last** pixel co-ordinates independently in the **X, Y, Z** or **T** text boxes (Fig. 7.7). Alternatively, a region-of-interest (ROI) can be selected in x and y using the **Set ROI** button. This prompts the user to draw a rectangular ROI on the image, which is then displayed in red. Completion of the ROI will update the values in the text boxes. Values can be reset using the **Reset** button if required.

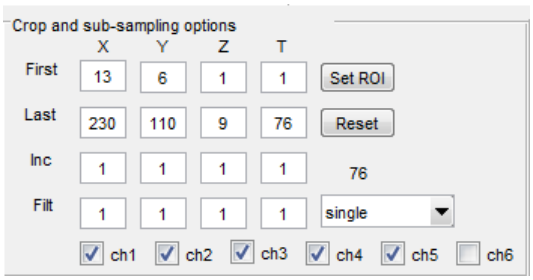


Figure 7.7: The crop panel: Provides controls to crop, sub-sample and filter images

The **Inc** text boxes allow (integer) sub-sampling independently in each image dimension.

The **Filt** text boxes allow spatial or temporal averaging over the designated number of pixels.

The image class can also be changed at this point from **integer** (the default) to single or double precision.

A series of checkboxes (**ch1**. . .**ch6**) are available to select which channels are to be included in the output image. Usually the bright-field channel is not included in this selection, as it is processed separately (see Section 7.9 - Bright-field image processing).

The file details in the **Output details** panel should update to reflect the cropping and sub-sampling chosen. If multiple images have been loaded, the crop and sub-sampling options apply to all the files.

7.6 Alignment options between wavelength images

Options are available to correct slight mis-registration in *x,y* between individual wavelength images using the **Channel alignment** controls (Fig. 7.8). A reference channel, typically containing the best image selected using the **Z** and **T** sliders, is chosen using the **Ref channel** drop-down list.

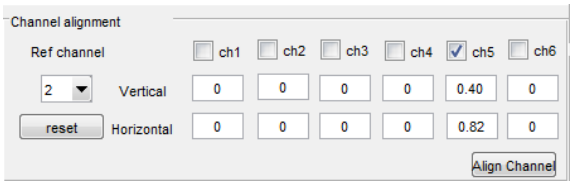


Figure 7.8: The channel alignment panel: Provides controls to allow sub-pixel registration between each channel and a selected reference channel

This acts as a template for cross correlation of any other channel, selected using the **ch1**. . .**ch6** checkboxes. The **Align Channel** button calculates the **Vertical** and **Horizontal** pixel offsets between the reference channel and the selected channels using cross-correlation across the whole image. These offsets are applied using bi-linear interpolation when the images are actually loaded.

The image display is modified during this process to display the before and after images in magenta and green.

7.7 Alignment options over time

Some level of correction for stage *x,y* drift or specimen movement can be achieved using the **Time series alignment** controls (Fig. 7.9). A reference channel is selected that has good contrast and features that are present in all images in the series, using the **Z** and **T** sliders, and, if necessary, a particular file if multiple files have been loaded simultaneously.

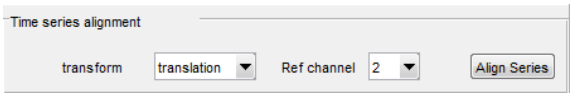


Figure 7.9: The Time series alignment panel: Provides controls to define a region for image alignment through the time-series, using a reference image

The **Align Series** button prompts the user to select a ROI on the target image that will be used as a template to calculate the *x,y* pixel offsets, rotation and scaling for the corresponding image in subsequent time-points using cross-correlation and bi-linear interpolation. The region used as a template must be within the red ROI outline, if this has been used to crop the image, and is highlighted in blue. The same offsets are applied to all channels and **z**-planes for each time point. The alignment only takes place when the images are actually loaded. A number of different transform types can be applied with increasing degrees of freedom, including *translation*, for (*x,y*) translation, *rigid*, for translation and rotation, *similarity* for translation, rotation, and scale and *affine*, for translation, rotation, scale, and shear.

7.8 Image projection options

It is possible to reduce the image dimensionality by projecting data along the z-axis using the drop-down menu in the **Projection options** panel (Fig: 7.10).

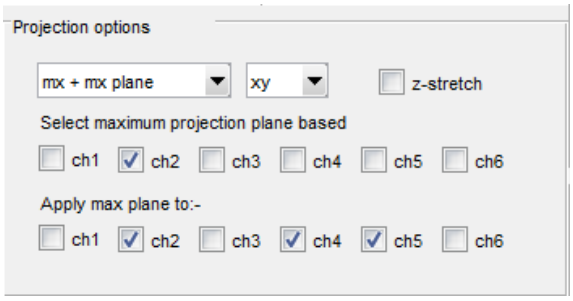


Figure 7.10: The projection panel: allows the user to reduce the image dimensionality by extracting a sub-set of the data in the z-dimension, or to re-orient the image stack to display xz or yz views. A number of different projection options can be selected and applied to different combinations of channels

- **Maximum:** displays the pixel with the maximum intensity in z for each channel (maximum intensity projection or MIP). This is a common approach to visualise data, but should not be used as a precursor to quantitative measurements, particularly when ratioing two channels, as it selects the 'noisiest' pixel at the extreme of the distribution along the z-axis, and pixels from different positions in z for different channels will appear in the projected image, making a nonsense of the ratio image.
- **Minimum:** displays the pixel with the minimum intensity in z for each channel. This is rarely useful for fluorescence images, but can be helpful for bright-field processing. Nevertheless, it is recommended that bright-field processing is handles separately (see Section 7.9 - Bright-field image processing)
- **Average:** gives an average brightness projection in z, which provides good noise reduction and may be useful for simple objects that do not overlap in the z-direction.
- **Max plane:** this gives the user the option of selecting the z-position of the brightest pixel in one-or-more channels and then extracting the same (x,y,z) pixel (voxel) from the other channels. It is recommended that the imaged is smoothed in z (as well as x and y), before this operation to ensure that the brightest pixel is more likely to correspond to the centre of the object of interest. This approach is required if different channels are going to be ratioed later on to ensure that information from the same (averaged) voxels are compared.
- **Mx + mx plane:** This provides an option to calculate the maximum plane projection, based on specific selected channel(s) that extracts the appropriate (x,y,z) voxel from a second set of channels, selected by the second set of checkboxes. The remaining channels are processed using a simple maximum. This is useful for quantitative ratioing for the max plane images, which typically involve two wavelength channels and an autofluorescence

channels for bleed through correction, and a morphological representation of the other channels from the (smoothed) maximum intensity projection.

7.9 *Bright-field image processing*

A bright-field image is often collected simultaneously with the fluorescence channels using a (non-confocal) transmission detector. Bright-field images can be processed separately to accentuate more useful information using the **Bright field output** controls (Fig: 7.11). The simplest form of processing is a **single plane**, selected by the position of the *Z*-slider from the bright field channel (**Ch.**). An amount of noise filtering can be applied using the process selected by the **smooth** drop-down list (*mean*, *median* or *Wiener*) over a square *x,y* region specified by the **kernel** drop-down list.

If a projection option is chosen, the process required can be selected from the **process** drop-down menu. The algorithms available are designed to highlight pixels that might contain the most useful information within a local neighbourhood defined by the **kernel** drop-down menu.

Whether a projection of single plane option is chosen, the contrast of the resulting image can be improved using contrast-limited adaptive histogram equalisation (CLAHE) by checking the **adaptive histogram equalisation** box.

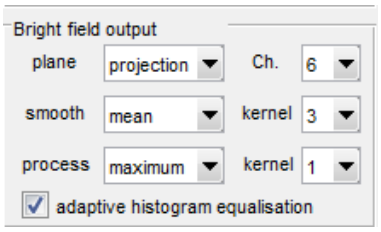


Figure 7.11: The bright field output panel: Provides controls to allow selection of single bright-field image planes or various algorithms to project the bright-field images, along with some contrast enhancement

7.10 *Saving the imported image and processing settings*

The **Save settings** button in the **Save options panel** saves the settings used for processing in a matlab file format, with the filename and a *settings* suffix (Fig: 7.12. The parameters can be re-loaded using the **Load settings** at a later stage. Note: when the settings are re-loaded, the user is prompted to re-set the alignment box for the time-series, and the cropping ROI is not available and must therefore be set-up again.

The **Save mat** button saves the processed fluorescence images and, if appropriate, bright-field images in a matlab file format. This can be re-loaded using the same interface at a later stage.

The **Save panels** button saves "png" images of each of the panels in the image and can be used to update this manual for any specific applications.

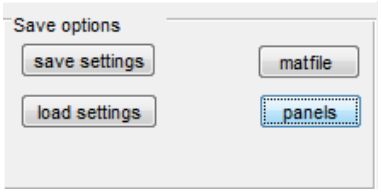


Figure 7.12: The Save options panel: allows the user to save the loaded image and the processing settings

7.11 *Importing the selected files*

Once all the processing steps have been completed, the selected files can be imported using the **Load** button (Fig: 7.13). If this is successful, the **View** button will be enabled and, if a separate bright field image has been processed, the **View bf** button. Clicking the **View** button will open a separate window with a video **Viewer** (see Chapter 4 - Viewer Program). If the image files have been

loaded satisfactorily, clicking the **OK** button will return to the main program. The **Cancel** button will return to the main program, but without exporting the processed image file.

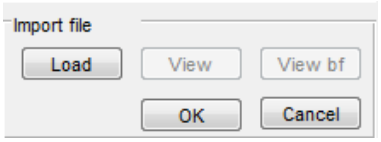
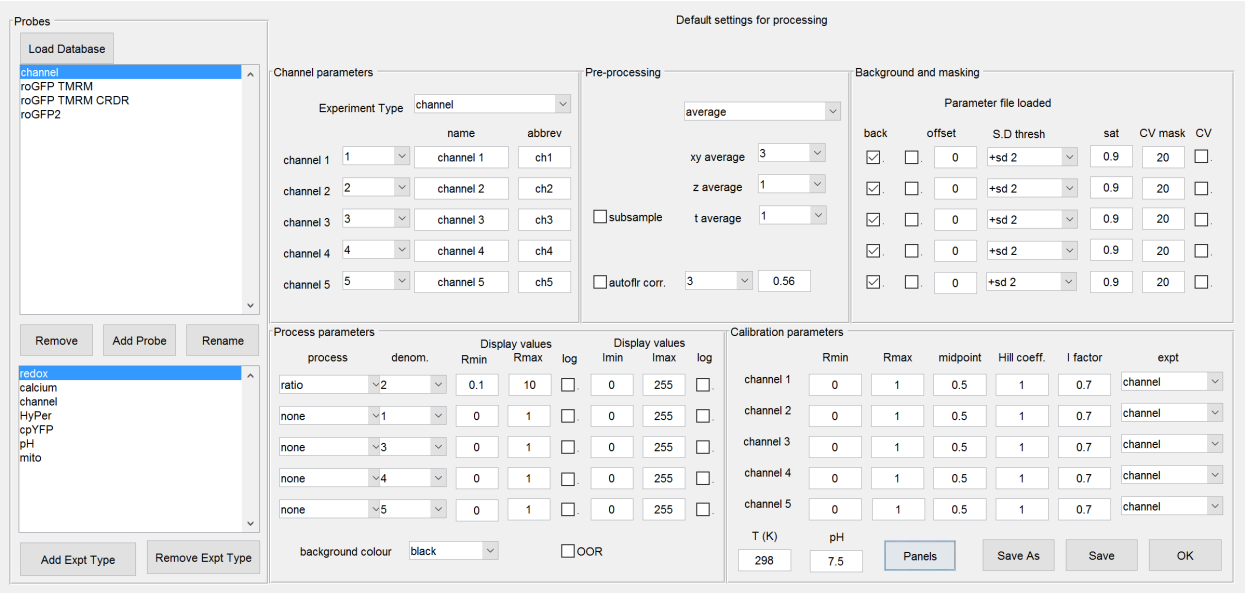


Figure 7.13: The import file panel: The Load button imports the selected image(s) and applies all the alignment, sampling, smoothing and projection parameters chosen. Once the fluorescence and bright-field images have been processed, they can be viewed with the appropriate button

8
Advanced Probe Database



8.1 Introduction

To standardise the way sets of experimental images are processed, it is convenient to save the parameters used for each processing step to a database. The advanced probe database can be opened directly from the **Ratio Analysis Menu Bar** (Fig. 1.5). Alternatively, the database can be called directly using the **probe database** button in the **Advanced Ratio Program**. Likewise, any settings that have been worked out for the current experiment can be saved to the database as another entry for re-use in the future using the **save probe** button.

The database can be stored anywhere, so it is up to the user to decide whether to set-up a single database for all experiments in a single location, or individual databases for each experimental series that are typically stored in the same folder as the data.

8.2 Channel assignment

Once the database is open, the user can step-through all the parameters needed starting with the **channels** panel (Fig: 8.1). This assigns each image channel to a physiological parameter, and also allows the user to provide both an appropriate **name** and abbreviation (**abbrev.**) for the channel that will subsequently be displayed above the relevant interface controls.

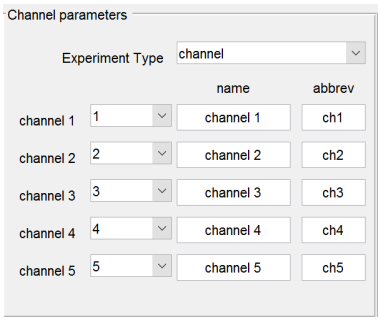


Figure 8.1: The channels panel: Provides controls to allocate each image channel to a physiological parameter and give appropriate names and abbreviations to display above the controls in the advanced ratio interface

8.3 Pre-processing controls

The **pre-processing** controls (Fig: 8.2) define the kernel size for the **xy average**, **z average** and **t average** smoothing kernels, whether to **subsample** the image after smoothing, and the auto-fluorescence bleed-through correction factor (**autoflr corr.**) and auto-fluorescence channel channel (in the adjacent drop-down menu)

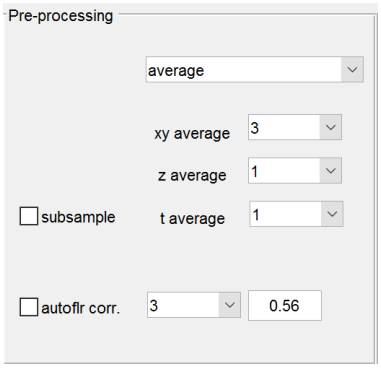


Figure 8.2: The pre-processing panel: Provides controls to filter and subsample the images

8.4 Background subtraction and masking

The **Background and masking** panel (Fig: 8.3) allows the user to select whether to apply a background correction (**back**) to each channel by checking the appropriate checkbox. During processing this will subtract the measured background for the channel. Alternatively, if it is not possible to measure the background experimentally, an **offset** can be set that will be applied if the adjacent check box is ticked. The **S.D. thresh** sets the value above the background in units of standard deviation that will be excluded from the final image and quantitative measurements. The **sat.** value, expressed as a proportion of the full intensity scale, excludes pixels that are too close to saturation, whilst the **CV mask** excludes pixels whose coefficient-of-variation (cv), defined as $SD/mean$ in a 3×3 neighbourhood, exceeds the value set.

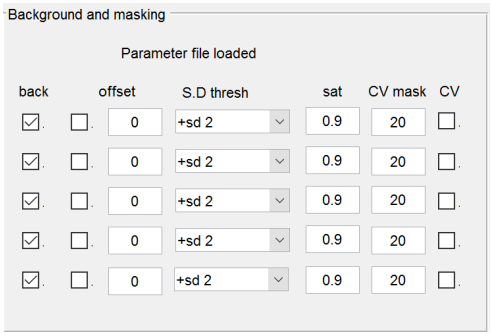


Figure 8.3: The background and masking panel: Provides a set of controls for each channel to subtract a background or offset value, and exclude pixels that are too close to background, saturation of with too high a local variance

8.5 Multi-channel processing

The **process parameters** panel provides options to **process** each channel using a range of different algorithms, from a *ratio* (the default) to a variety of approaches to normalise single channel data.

If the ratio option is chosen, the channel used as a denominator (**denom.**) has to be set.

The next set of parameters control how the pseudo-colour coded ratio is scaled between **Rmin** and **Rmax**, and **Imin** and **Imax**, with the option of a logarithmic scale in both cases. In addition, there are controls to set the **background colour** for display and printing, and whether to show pixels that fall outside the display limits, termed out-of-range (**OOR**) (Fig: 8.4)

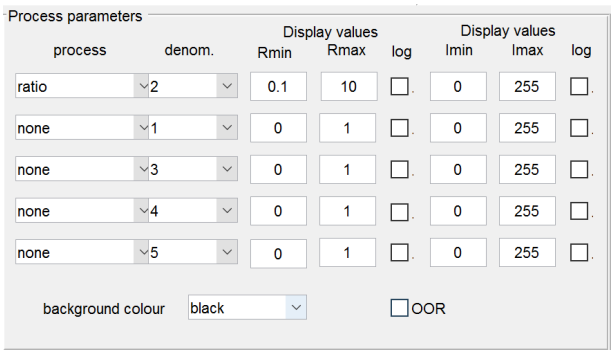


Figure 8.4: The process panel: sets out the type of processing required for each channel and also how the resultant image should be scaled in the pseudo-color ratio

8.6 Calibration

The **calibration parameters** control how the ratio or single wavelength values are mapped onto the physiological parameter of interest (Fig: 8.5). In the case of roGFP, the relevant values are set by **Rmin**, **Rmax**, the **midpoint** potential, and the instrument **I factor** (the **Hill coeff.** is not relevant and defaults to 1). The **expt** are used for the program to decide what type of calibration to apply. In addition the experimental temperature **T (k)** and **pH** for the compartment containing the reporter can be set. At the moment there are relatively few options (*redox*, *calcium* or *channel*), but this list is expected to expand to include other probes, such as cpYFP and HyPer for example.

The settings can be saved back to the current database using the **Save** button, or a new database using the **Save As** button. The **OK** button returns to the calling program. (Note: if the database has not been saved, any modified values are discarded).

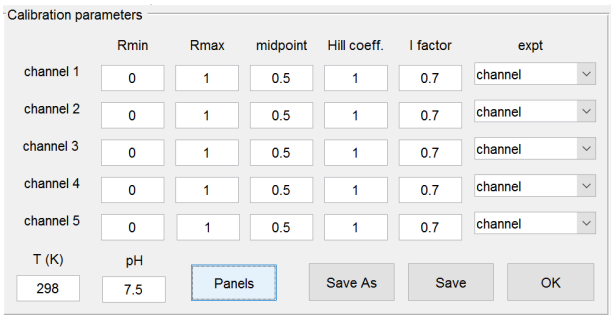
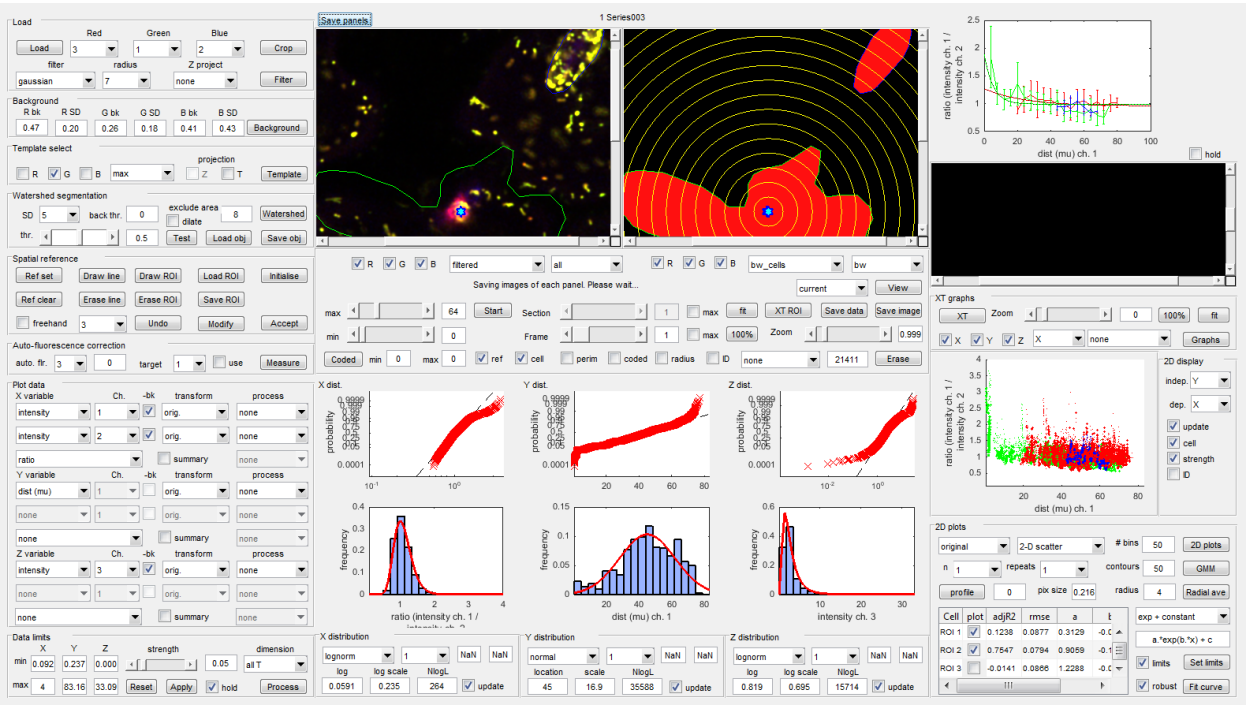


Figure 8.5: The calibration panel: provides a set of parameters for each channel that define how the ratio value relates to the physiological parameter of interest

9
Object-based segmentation and processing - population studies



9.1 Introduction

This package allows quantitative measurements from populations of discrete objects, such as mitochondria, in single images, (*x*,*y*,*z*) or (*x*,*y*,*t*) 3D images or 4D (*x*,*y*,*z*,*t*) time-series data. The objects are automatically segmented using a watershed algorithm followed by a local intensity threshold set at the mid-point between each object and the background. This ensures that objects with a range of intensity values are segmented correctly, which is not possible with a single, global intensity threshold. Once the objects are segmented, the average fluorescence intensities (and a variety of morphological parameters) are then automatically measured for each object to extract the population behaviour. The distribution of these values

can be viewed as frequency histograms, either directly or after combination through ratio or co-variance measures, and then fit with various distribution functions.

There are two major modes of operation. In the first approach, each time-point is treated as a separate sample and the objects segmented individually for that frame. This is appropriate where objects move over time, and therefore cannot be easily tracked between frames, but where their spatial position may be of importance. Thus the time-series provides a series of samples of the population with reference to their co-ordinates and other (manually-defined) reference points or features such as cell boundaries. For example, this has been used to determine the local redox potential of mitochondria adjacent to a fungal penetration site in leaf epidermis [1]

In the second approach, if the objects do not move in (*x*,*y*) over time, it is possible to extract a time-series from each object using a single ROI defined from a projection of the data over time. To aid visualisation of the data, the results can be displayed as an 'object'-time image, with results for each object as category on the x-axis, and time on the y-axis. Furthermore, to highlight any particular behaviour of interest from a population of potentially hundreds of objects, they can be sorted by a summary statistic derived from the data, such as the minimum or maximum ratio or co-variance. The object-time image can be interrogated to show graphs of the data over time, and also highlight the selected object in the image view. For example, this approach has been used to look at membrane potential pulsing in immobilised mitochondria [2,3]

[1] R. Fuchs, M. Kopischke, C. Klapprodt, G. Hause, A. Meyer, M. Schwarzländer, M. Fricker, and V. Lipka. Immobilized subpopulations of leaf epidermal mitochondria mediate pen2-dependent pathogen entry control in arabidopsis. *Plant Cell*, 28:130–145, 2016

[2] M. Schwarzlander, D.C. Logan, I.G. Johnston, N.S. Jones, A.J. Meyer, M.D. Fricker, and L.J. Sweetlove. Pulsing of membrane potential in individual mitochondria: a stress-induced mechanism to regulate respiratory bioenergetics in arabidopsis. *Plant Cell*, 24:1188–1201, 2012

[3] M. Schwarzländer, D. Logan, M.D. Fricker, and L.J. Sweetlove. The circularly permuted yellow fluorescent protein cpyfp that has been used as a superoxide probe is highly responsive to ph but not superoxide in mitochondria: implications for the existence of superoxide ŚflashesŠ. *Biochem. J.*, 437:381Ű387, 2011

9.2 *Loading and filtering images*

The **Load** button in the **Load panel** activates the **Advanced Ratio file Import** window (see Chapter 7, which allows import of images from a variety of formats. The file format for Object-based processing can be a single (*x*,*y*) image an (*x*,*y*,*t*) time-series, or an (*x*,*y*,*z*,*t*) 4-D image, with up to three channels (Fig: 9.1).

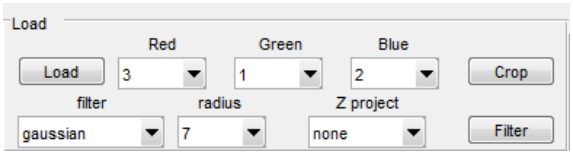


Figure 9.1: The Load panel: allows the user to load images using the Advanced Ratio File Import program, crop regions for analysis and apply various smoothing filters

For redox measurements with GRX1-roGFP2, the fluorescence channels are the oxidised and reduced channels for roGFP and a third channel for auto-fluorescence. Many other permutations are possible depending on the experiment, and we will also illustrate use of the program to analyse mitochondrial 'flashes' measured using TMRM for mitochondrial membrane potential and cpYFP for mitochondrial pH. If a bright-field image has been processed during the file-import, this is also available as a display option,

particularly to help define morphological boundaries, but is not processed further.

The image can be cropped at this point using the **Crop** button, and the order of the channels changed to match the physiological parameters of interest using the **red**, **green**, and **blue** drop-down menus. [4].

The image needs to be smoothed to facilitate object segmentation. A variety of options are available from the **filter** drop-down menu, including *average*, *gaussian*, *median*, *Wiener* or *mexican*, that are applied over a kernel with the *radius* set by the **radius** drop-down menu for circular filters (*average*, *Gaussian* and *mexican*) and ($radius.*2+1$) for square filters (*median* and *Wiener*). There is also the option to calculate an *average* or *maximum* z-projection of the data using the **Z-project** drop-down menu. The filter button applies the smoothing chosen and updates the display window (Fig. 9.2).

[4] Note: in future releases the labels and defaults for all the controls will be incorporated into a probe database in a similar manner to the basic and advanced ratio programs

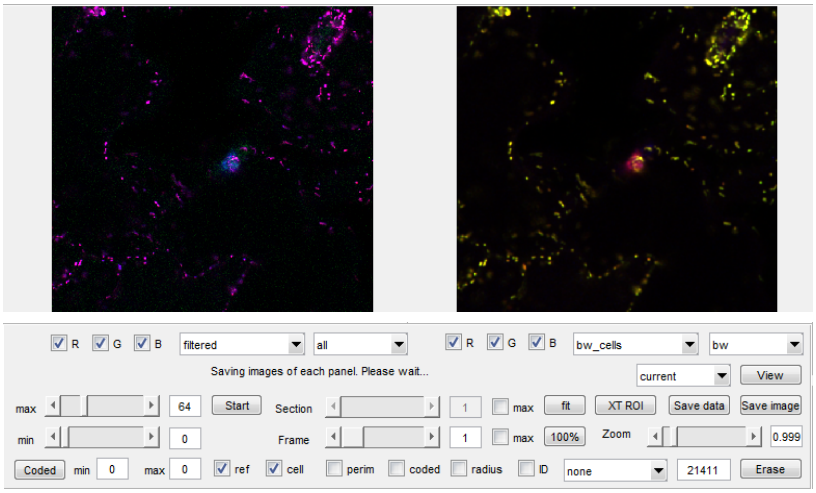


Figure 9.2: The Display controls: allow the user to select images to view, with options to change the channels displayed, the intensity and the features to overlay on the image.

The selected image is displayed in the adjacent image windows, and can be adjusted using the controls underneath (Fig. 9.2). At this stage the *initial* image is displayed in the left panel and the re-ordered, *filtered* image in the right panel.

The image to display in each panel is selected from the adjacent drop-down menu, and there are separate **R**,**G**,**B** checkboxes to display each of the colour planes. It is possible to zoom into the image using the **Zoom**, and scroll around the images in synchrony using the x and y scroll bars, or by dragging the rectangle in the overview panel. The **fit** button scales the image so that it is completely visible in the display window, whilst the **100%** button shows the image with a 1:1 pixel-to-screen mapping. The intensity of the display can be adjusted using the **max** and **min** sliders and/or adjacent textboxes. Individual **Sections** or **Frames** can be selected using the appropriate sliders and/or textboxes. Alternatively, a maximum projection along either dimension can be selected using the **max** checkbox. A number of checkbox and menu options to overlay the results of the segmentation and analysis are available and will be

referenced later.

9.3 *Background measurement and subtraction*

The **Background** button in the **Background panel** prompts the user to draw a rectangle in the left display panel, which can be resized and moved by grabbing the handles on the corners and edges of the frame to encompass a suitable region of background. A double-click with the left mouse button updates the textboxes with the values for the average background (**bk**) and standard deviation (**std**) in each channel (Fig: 9.3).

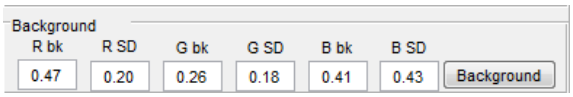


Figure 9.3: The Background panel: allows users to select a background region interactively

9.4 *Defining a template for object segmentation*

To aid a clean segmentation, it is helpful to define a template image based on the intensity information from one or more channels, set by the **R**,**G**, and **B** checkboxes in the **Template select panel**. This can be determined for each image in the time-series, or as a single template for all images based on an *average* or *maximum* projection in z (**Z proj**) or time (**T proj**) or both (Fig: 9.4).

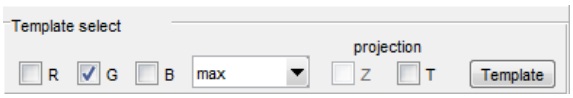


Figure 9.4: The Template select panel: is used to construct a single template image for each time-point or complete time-series as a basis for automated segmentation

Thus, for example, if the objects to be segmented are mitochondria expressing GRX1-roGFP2 in living plant cells that move between images due to cytoplasmic streaming, it would be appropriate to use both the **R**ed (oxidised) and **G**reen (reduced) channels to make sure that objects are segmented whatever their redox state, but not apply any projection, as the mitochondria move between images. Conversely, if the experimental system was isolated and immobilised mitochondria labelled with TMRM, a maximum projection over time (**T proj**), would ensure that every mitochondrion was present in the template image (see Chapter 10. Once the options have been selected, the **Template** button calculates the appropriate image and updates the right-hand display.

9.5 *Segmenting objects using a watershed function*

the watershed algorithm is used to provide an initial separation of all the objects present in the template image into non-overlapping domains. The template image is inverted, and the watershed transform finds "catchment basins" and "watershed ridge lines" in the image by treating it as a surface where light pixels are high and

dark pixels are low. The watershed ridge lines separate each object from its neighbours, but also includes any region of background in the vicinity as well. To avoid over-segmentation of noise in the background, the template image is automatically masked to only include pixels at a value set at $x\times$ SD units above background, where x is set by the **back SD** drop-down menu and the values of the background and SD are measured from the template image using the previously selected background sample rectangle (Fig: 9.5).

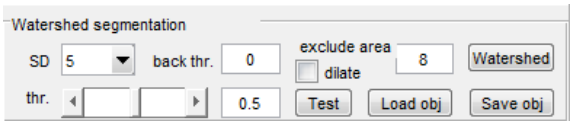


Figure 9.5: The watershed panel: segments the template image using a watershed algorithm and then applies local intensity thresholding to isolate each discrete object

A value of 5-10 SD is usually appropriate. The **Test** button calculates the watershed for the first image and displays the watershed lines superimposed in white on the segmented image.

Each object may have a different overall intensity, so individual intensity-thresholds are calculated based on the local maximum intensity of the template object and the background. Typically the threshold is set at 50% of this difference, but higher or lower values can be chosen using the **thr.** slider. The segmented object comprises all pixels in the object above the local threshold. An additional area threshold is applied using the value set in the **area exclude** box (in pixels), to remove small objects from the analysis.

The **Watershed** button applies the segmentation to all images in the series and automatically calculates a number of parameters for each segmented object, including the average intensity for each channel, the length, width, orientation, eccentricity, radius and area of the object, as well as the cell identity and distance from a reference point if defined (See Section 9.6)

This may take several minutes for complex images in a long time-series. Progress is shown in the status line and, at the end of processing, the segmented images are displayed in the right-hand panel.

The **Save obj** saves the results of the segmentation to a matlab file with a user-selected name and a *object* suffix. The **Load obj** reloads a previously saved object file. this allows the user to rapidly return to this point in the analysis in a subsequent session.

9.6 *Defining morphological reference points and features*

In some applications, one of the parameters of that may be of interest is the relative position of the segmented object(s) to a reference point or location within different cell types or tissues. The **Spatial reference panel** contains a number of controls to manually define a reference point in the image (the default for all spatial measurements is the centre), and also to outline different features, such as cell boundaries. The **initialise** button clears any existing values and sets-up the arrays needed (Fig: 9.6). The **Ref set** button

prompts the user to position the reference point using the mouse on the left-hand image panel. The point can be re-positioned if needed until double-clicked to fix it. The **Ref clear** removes the current reference point.

Figure 9.6: The reference panel: provides a series of tools to manually define a reference point on the image and various morphological

In the example shown here, the reference point is placed on the site of infection of a leaf pathogen, and is marked with a cyan star if the **ref** checkbox is active in the **display panel**. A number of mitochondria are visible nearby. (Fig: 9.7) The distance in pixels and microns is automatically re-calculated from the centroid of each object and the reference whenever it is updated.

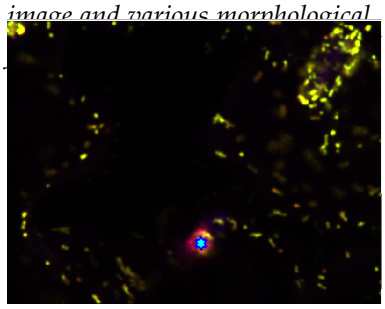


Figure 9.7: The reference point is used to mark a particular feature in the image, in this case, the site of penetration of the host cell by a fungal pathogen

Defining other features, such as cell boundaries, can be achieved using the drawing tools in the **spatial reference panel**. The **Draw ROI** button allow closed areas to be drawn on the image, which is the usual starting point for defining features of interest. Single left-button mouse clicks set each vertex, whilst a double-click closes the area. For both lines and regions, the control points at each vertex can be re-positioned if needed, by hovering over the point until a black circle appears, holding the left-button and dragging to the new location. New points can be added by hovering over the appropriate line segment and pressing **A** on the keyboard. Conversely, points can be deleted by hovering over the vertex and then clicking the right mouse button (Fig: 9.8). Once the outline is correct, a double click will update the feature and display a binary image in the right-hand panel. The **undo** button toggles between undoing or redoing the last operation. It is often helpful to toggle between different images, including a bright-field image if loaded, to decide the best position of the cell boundary.

Figure 9.8: Morphological features can be drawn using the fluorescence or bright-field images as a guide to define cell boundaries

The **erase ROI** is used to edit the feature by removing a region from the binary image that corresponds to the area defined on the left-hand panel. The **Draw line** and **Erase line**, with a drop-down menu to control the **width** of the line, can also be used to add or delete line features, respectively, to the binary image based on a user-defined feature in the left-hand panel. Normally lines are defined by a series of left-mouse clicks to set the vertex of each line segment. Alternatively, the **freehand** checkbox allows a continuous line to be drawn.

The **modify ROI** presents each currently defined feature in turn and allows the user to edit the vertex points again. A double click accepts the changes (or none) for that feature and then displays the outline of the next feature for modification.

The **Save ROI** saves the set of features defined as a matlab file, appended with a *cell* suffix. The **Load ROI** prompts the user to re-load a previously saved set of ROIs.

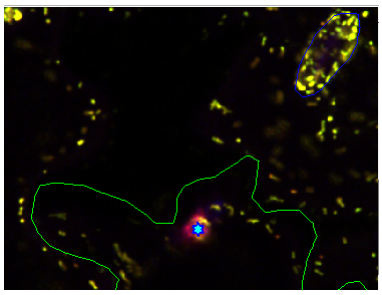


Figure 9.9: Each feature can be displayed in an overlay, with a unique colour-code

The **accept ROI** button accepts all the current ROIs and re-calculates the properties for each object based on the reference point (if set) and the cell identity (Fig: 9.9). The boundaries of each of the cell objects can be overlaid in the images if the **cell** checkbox is active in the display panel.

9.7 *Measurement and application of an auto-fluorescence correction*

As with the other ratioing programs, it is possible to apply an **auto-fluorescence correction** using the controls in the corresponding panel (Fig: 9.10).

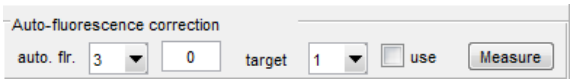


Figure 9.10: The auto-fluorescence correction panel: allows measurement of the auto-fluorescence correction factor from the image, or entry of a pre-determined value that is applied during the subsequent analysis

The **auto. flr.** drop-down menu is used to set which channel contains the auto-fluorescence image and the adjacent box contains the correction factor, whilst the **target** drop-down menu sets the channel to apply the correction to. The **Measure** button prompts the user to define a rectangular region on the left-hand image display that contains auto-fluorescence but no roGFP signal. Double-clicking calculates the bleed-through factor and updates the box. The **use corr.** checkbox must be active to apply the correction during the analysis.

9.8 *Defining data to plot*

The program allows the user to select up to three variable to explore different facets of the data in the **Plot data panel** (Fig: 9.11).

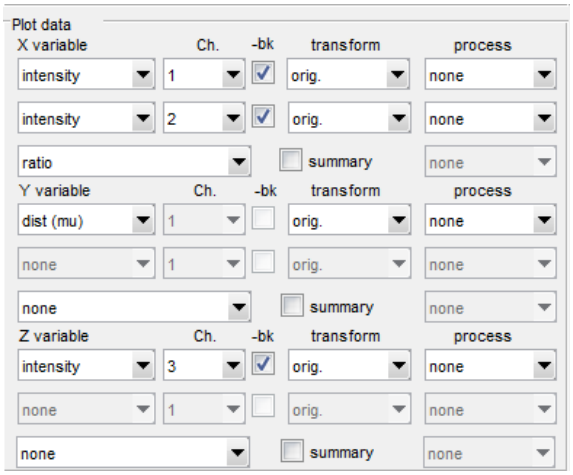


Figure 9.11: The Plot data panel: allows the selection of up to three variables for analysis, based various operations on the original data, such as transforms and/or normalisation technqiues, and combinations with other channels as ratiometric measurements or covariance estimates. The variables can also include other parameters, such as the size, shape or position of the object

For each channel, the **variable** of interest is selected from the drop-down menu for a given channel (**Ch.**), with a checkbox option to subtract the corresponding background (**-bk**), the option to apply a **transform** (*log*, *sqrt*, *inverse*) before normalising the data (if

required) using the **process** options. Normalisation can be to the mean or median (*mean*, I/I_{mean} or *med*, I/I_{med}, , respectively), the absolute deviation (*dev (mean)*, $I - I_{mean}$, or *dev (med)*, $I - I_{med}$) or the normalised deviation (*ndev (mean)*, $(I - I_{mean})/I_{mean}$ or *ndev (med)*, $(I - I_{med})/I_{med}$). The **combine** options (*none, ratio, logratio, covar*) calculate the corresponding combination with a second channel, set up with the same options. In the example shown here, the first variable (**X**) is a ratio of the intensity of channel 1 divided by channel 2, after background subtraction, the second variable (**Y**) is the distance in microns from the reference point, whilst the third variable (**Z**) is the strength (normalised intensity of the template image).

The **Process** button processes the data for each object according to the options set in the **Plot data panel** and displays a frequency histogram of the results for each variable in a separate panel (see Fig: 9.12 for the X variable for example). The controls in the **x dist panel** allow the user to fit a distribution to the histogram data, and view the results in the *probability* panel. A number of fits are possible, including *normal, lognorm, exp, weibull, extreme, rayleigh*, or multiple Gaussians (*multi*), with the number set by the adjacent drop-down menu. The parameters for the fit are displayed in the adjacent text boxes, along with an estimate of the goodness-of-fit. The labels update to reflect the type of distribution. A good fit is also discernable from the deviation between the ideal distribution (dotted line) and the data (red *) on the probability plot.

When the **Process** button in the **Data limits panel** (Fig: 9.13) is first pressed, the default is to display the intensity data for the **X** variable from a *single* time-point and z-section, set using the **dimension** drop-down menu. If the processing appears reasonable, the user can define settings for two other variables (labelled as **Y** and **Z**). If these are set, pressing **Process** again will extract the relevant data and display it in the corresponding distribution and fitting panels.

The analysis can include all the objects that have been segmented, but it is often appropriate to only consider objects that have a reasonable signal, measured as the **strength**, and expressed as a proportion of the maximum signal in the template image. The default is 0.05, which excludes objects with less than 5% of the maximum intensity in the template image. Setting a greater strength will progressively exclude dimmer objects from the analysis.

The histograms automatically scale depending on the limits calculated for the processed variable. This may occasionally yield extreme values that obscure the majority of the data, particularly with ratioing, usually reflecting noisy signals that are not particularly informative. The limits for the plots can be manually set using the **min** and **max** values in the **Data limits panel**, and applied using the **Apply limits** button, or reset to the automatically calculated values using the **Reset limits** button (Fig: 9.13). Note the **Process** button automatically resets the limits to the automatically

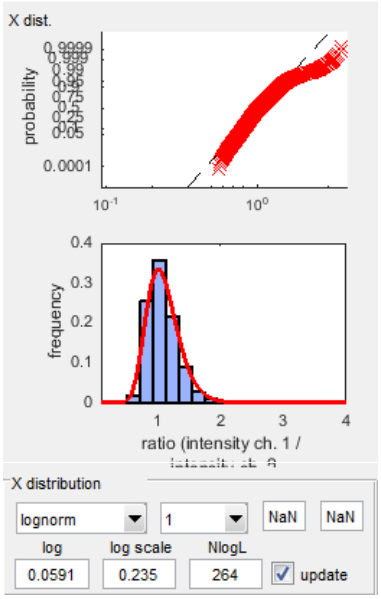


*Figure 9.12: The variable display panels: the lower panel shows a frequency histogram for the selected variable, with a superimposed curve fit in red, based on the setting in the **X distribution panel**. The fitting parameters and goodness-of-fit are shown in the corresponding textboxes, and displayed graphically as a probability plot against the selected distribution in the upper panel. The closer all points are aligned with the expected probability distribution, the better the fit*

calculated values, unless the **hold** checkbox is active.

Figure 9.13: The Data limits panel: is used to select data for plotting that is above a certain strength and within specified limits for each variable

If all the distributions and limits are reasonable, the results for all sections (*all Z*), all time points (*all T*) or both (*all ZT*) can be set using the **dimension** drop-down menu and calculated with the **Process** button. This will update all the frequency histograms, fitting parameters and probability plots, if the **update** checkboxes are active.

9.9 *Comparing different variables*

At the same time as the distributions for each calculated variable can be viewed independently, it is also possible to view the relationship between two of the variables using the **2-D plots panel**. The user can select the independent variable (**indep.**) from the drop-down menu in the **2D display panel** to plot on the *x*-axis, and the dependent variable (**dep.**) to plot on the *y*-axis (Fig: 9.14).

The type of plot is controlled by the drop-down menus in the **2D plots panel** as a *scatter*, 2D histogram (*2D hist.*), or 2D weighted histogram (*2D wt. hist.*) (Fig: 9.14).

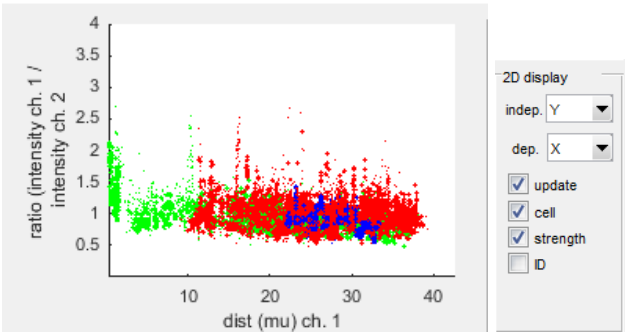


Figure 9.14: 2D plot of the X and Y variables with colour coding to reflect the cell identity and spot size to reflect the signal strength

The default is a 2D scatter plot (Fig: 9.15). If the histogram options are selected, the number of histogram bins is selected from the drop-down menu.

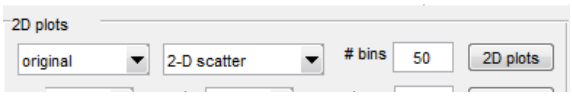


Figure 9.15: The 2D plot panel: selects the type of 2D plot, and the number of histogram bins, if the 2D histogram options are selected

If a number of cells have been defined, the **cell** checkbox colour-codes each data point by the cell ID, which also matches the colour used to display the cell outline in the image windows. The **strength** checkbox operates in conjunction with the **cell** checkbox to display data points with size scaled to the strength of the signal in the template image. The **ID** checkbox adds the ID label to each point (note plotting is extremely slow with this option active).

9.10 *Measuring the dependence on distance from the reference point*

The **radial ave.** button calculates the mean and standard deviation of the dependent variable in concentric rings moving outwards from the reference point at intervals set by the **radius** (in microns) calculated from the pixel size (**pix size**). The rings are superimposed on the left-hand display (Fig: 9.16).

The resulting data are plotted as the mean and SD error bars, with colour coding reflecting the cell identity (Fig: 9.18). The **Fit curve** button (Fig: 9.17) attempts to fit a curve using a standard set of combinations from the drop-down menu, including single or double exponentials, with constant or linear offsets, or a user-specified equation.

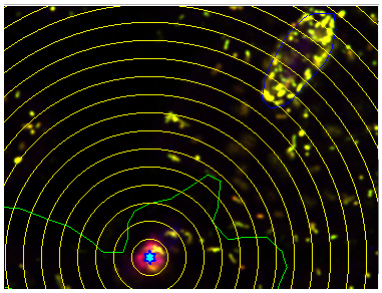


Figure 9.16: Radial average measurements are taken from concentric circles, shown in yellow) from the reference point (shown as a cyan star)

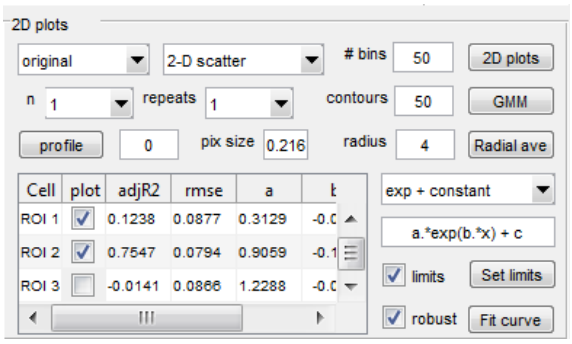


Figure 9.17: The 2D plot panel: additional controls are available to fit a pre-programmed or user-defined curve to the data, with options to constrain the values for each fitting parameter

To aid fitting, it is possible to set the limits and initial value for each of the parameters in the fit, using the **Set limits** button. this displays columns for the *initial* value and, *upper* and *lower* limits, used to constrain the fit, in the adjacent table. If the **limits** checkbox is active, the limits are applied during the fitting process. The **robust** checkbox, uses a bisquare weights method during the non-linear least-squares fitting. The results of the fit are superimposed on the graphical data, and the results for the coefficients displayed in the table with an estimate of the goodness-of-fit. The **plot** checkbox against each ROI in the table toggles the display of the fitted line on the graph.

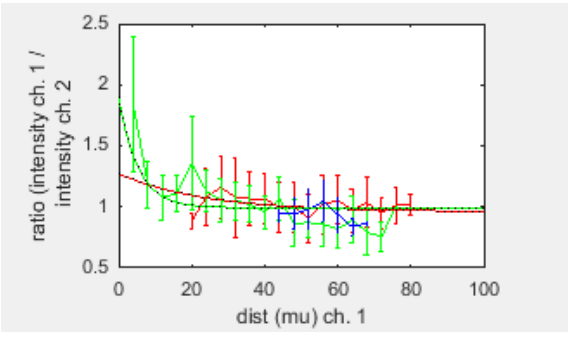


Figure 9.18: graphical presentation of the radial average results for different cells, with non-linear curve fits superimposed

9.11 *Measuring the size of objects*

To help decide the appropriate scaling for the radial average it can be useful to measure the size of particular objects in the image. The **profile** button prompts the user to draw a line over a suitable object in the left-hand panel, and automatically calculates the full-width-half-maximum (FWHM) value for each channel. These values are displayed on the image, with the corresponding number in microns (Fig: 9.19). The smallest of the values is recorded in the adjacent box .

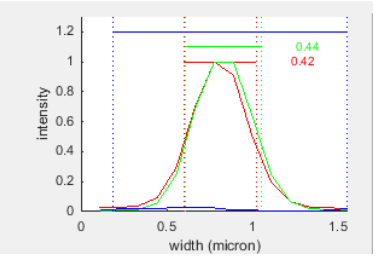
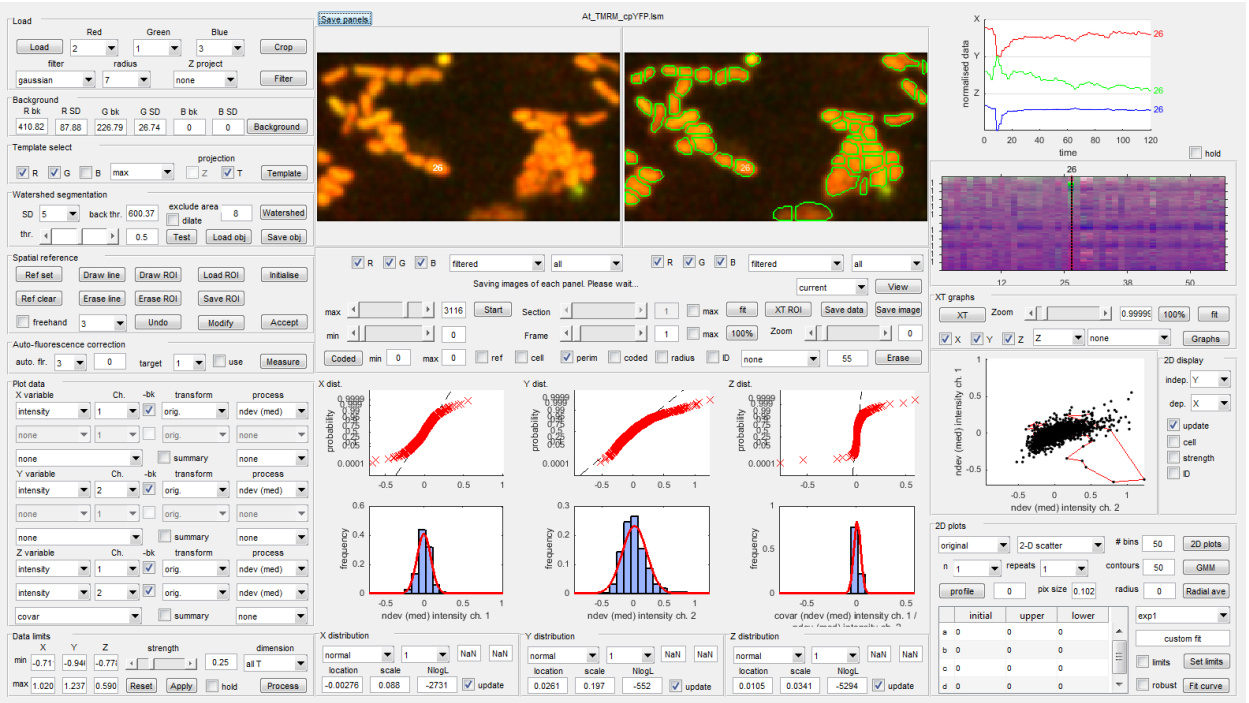


Figure 9.19: The output from the profile measurement tool, showing the intensity in each channel along the transect selected and the FWHM value

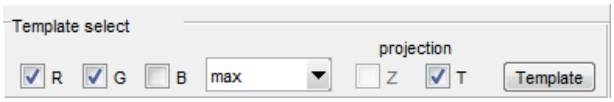
10

Object-based segmentation and processing - time-series



10.1 Introduction

In experiments where the objects do not move between frames, the behaviour of the segmented object can be easily tracked over time using a individual ROIs established from a maximum projection along the time axis using the **T proj** checkbox in the **Template select panel** (Fig: 10.1), (e.g. [1]).



The other processing steps are conducted as before, but making sure that the results for all time-points are calculated. The data for each object can then be visualised as an 'object'-time plot using

[1] M. Schwarzlander, D.C. Logan, I.G. Johnston, N.S. Jones, A.J. Meyer, M.D. Fricker, and L.J. Sweetlove. Pulsing of membrane potential in individual mitochondria: a stress-induced mechanism to regulate respiratory bioenergetics in arabidopsis. *Plant Cell*, 24:1188–1201, 2012

Figure 10.1: The template panel: using the T proj option to define a template based on the maximum projection of both the R and G channels

the **XT** button in the **XT graphs panel** (Fig: 10.2). The **X**, **Y** and **Z** checkboxes control whether to display a normalised version of the corresponding variable in the red, green and blue channels respectively of the XT image.

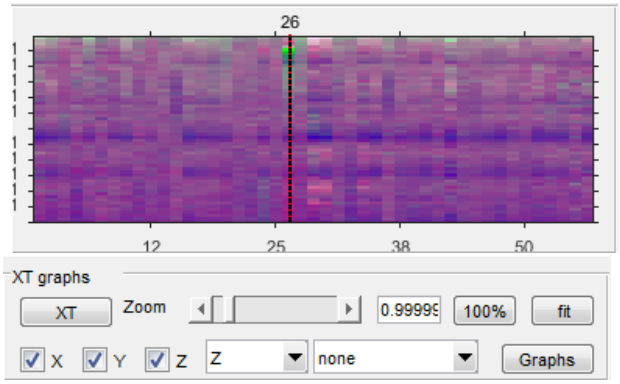


Figure 10.2: The XT graphs panel: 'Object'-time plots can be displayed with objects on x-axis and the value of each variable over time on the y-axis. More detail can be visualised by zooming or scrolling the image

Greater detail can be seen using the **Zoom** slider and scroll bars. Alternatively, the XT image can be displayed to **fit** the window or at a 1:1 pixel ratio along the time dimension using the **100%** buttons. If there is an object of interest, the **Graphs** button prompts the user to click on the object in the XT image to extract the normalised traces an display them stacked above each other for the three variables (Fig: 10.3).

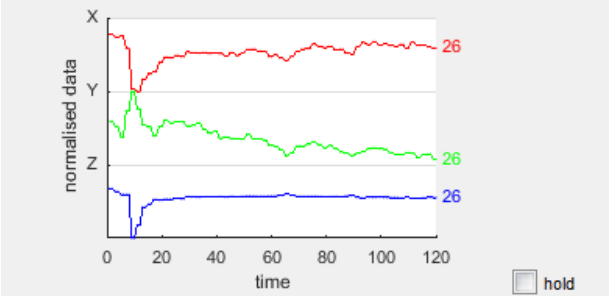


Figure 10.3: The values of the three variables can be plotted for a selected object over time. In this case, X is the normalised cpYFP signal, Y is the normalised TMRM signal and Z is the co-variance

In this example, the red line reflects the cpYFP signal, normalised to the deviation from the median (**X**), the green line represents the TMRM signal, normalised to the deviation from the median (**Y**), whilst the blue line represents the co-variance of the two signals (**Z**). During a 'mitoflash', the mitochondrial pH transiently increases (red), whilst the mitochondrial membrane potential drops (green), leading to a strong, negative co-variance (blue). during the rest of the time series, although there are fluctuations in signal, they are similar in both channels, so there is little change in the co-variance signal. If the **hold** checkbox is active, traces from successive objects are superimposed on the graph.

At the same time that the traces are displayed for the selected object, the image displays are updated to show the position of the selected object (Fig: 10.4). In this case, object 41 is labelled in white, and in the frame selected, it is clear that the green signal has dropped whilst the red is enhanced, unlike all the other mitochon-

dria. Additional information can be displayed for each object, such as the ID, if the **ID** checkbox is active, the perimeter if the **perim** checkbox is active, or a range of parameter values depending on the setting of the drop-down menu. In this case the intensity values for each object are overlaid on the right-hand panel.

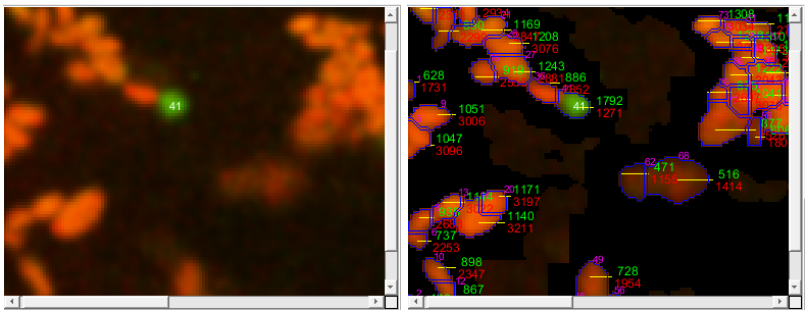


Figure 10.4: display of the ID of the object selected in the 'object'-time image in the left-panel, with additional information overlaid on the right-panel, including the perimeter of the object in blue and the intensity values for the red and green channels

When there are many segmented objects, it is often difficult to identify which objects may show interesting behaviour during the experiment. It is possible to calculate a summary statistic from the time-series for each object using the controls in the plot data panel (Fig: 10.5) that may capture the behaviour of interest.

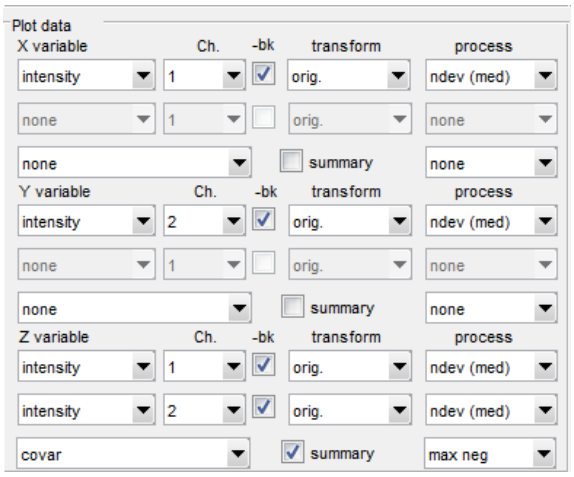


Figure 10.5: The variable display panels: If the summary box is checked, the corresponding summary statistic is calculated and displayed for as a histogram for each object. This value can also be used to rank the objects in the 'object'-time plot

A number of options are available including *mean, skewness, kurtosis, median, variance, standard deviation, coefficient-of-variation, root-mean-square, maximum negative value, maximum positive value, sum of the negative values, or sum of the positive values*. The summary variable for ranking is selected in the drop-down menu below the XT button, and the ranking order (*ascending* or *descending*) selected from the next menu. The 'object'-time image is re-ordered to show all the objects ranked according to the summary value in the 'object'-time plot (Fig: 10.6). In this case, the covariance between the normalised deviation from the median of both the TMRM and cpYFP signals has been ranked according to the maximum negative value.

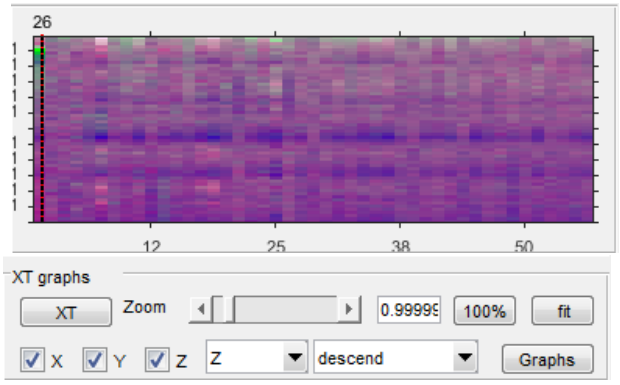


Figure 10.6: Display of the ranked objects according to the variable selected and the summary statistic calculated