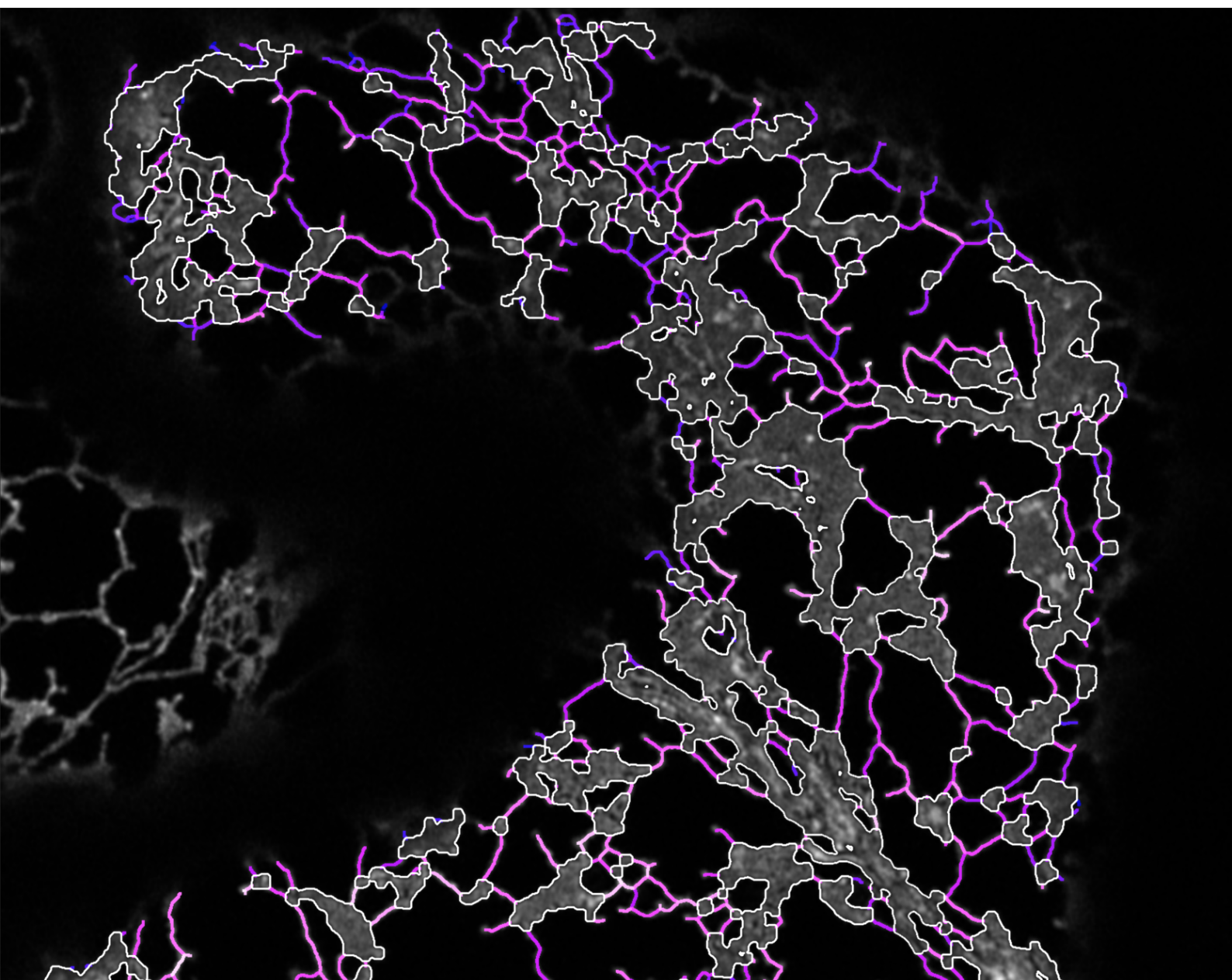


CHARLOTTE PAIN & MARK FRICKER

AnalyzER Tutorial



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Minimum system requirements

Before installing the ER network analysis programme, it is essential to ensure that you have access to a screen with a sufficient resolution to work and and download the following programmes:

- A minimum screen resolution of 1600 x 900
- **MATLAB Compiler Runtime** (www.mathworks.com) is required to install the set of shared libraries that enables execution of the compiled MATLAB application. *****Please note this can take some time**
- When running AnalyzER as a MATLAB app the following toolboxes are required:
 - Signal processing
 - Image processing
 - Statistics and machine learning
 - Curve fitting
 - Bioinformatics
 - Computer vision
- The *Bio-Formats* package has been designed to read in images from different microscope manufacturers and store them in a standardised format and should be included in the program directory during installation. Full details are available on the open microscopy website:

<http://www.openmicroscopy.org/>

If installed separately, the `bioformats_packages.jar` program needs to be available on the search path or installation directory of the matlab programs. `bioformats_package.jar` is available from:

<http://downloads.openmicroscopy.org/bio-formats>

- The latest version of Java needs to be installed, and is available from:
<https://java.com/en/download/>
- Output of images at full resolution uses `export_fig.m` originally written by Oliver Woodford (2008-2014) and now maintained by Yair Altman (2015-). When exporting to vector format (PDF or EPS) this function requires that ghostscript is installed on your system. Ghostscript can be downloaded from:

<http://www.ghostscript.com>.

- When exporting images to eps and pdf formats, `export_fig` additionally requires `pdftops`, from the Xpdf suite of functions. This is included in the xpdf tools package and can be downloaded from:

<https://www.xpdfreader.com/download.html>

Introduction

This tutorial provides three sample images, a single channel times series, and two dual channel images that can be used to analyse ER and cisternae morphology. This tutorial has the following file structure:

The three test files are stored in the 'demo images' file. Whenever an image is loaded into the software, files containing the parameters of an analysis are automatically generated. Deletion of the parameter files (in red) results in a re-setting of the parameters to default. Images are automatically output to the images folder (blue) and result spreadsheets to the results folder (green).

```
AnalyzER tutorial
├── AnalyzER tutorial.pdf
├── demo images
│   ├── 2 channel cisternae analysis
│   ├── 2 channel tubule analysis
│   ├── Single channel timeseries
│   └── processed data
│       ├── arrays
│       ├── images
│       ├── parameters
│       │   ├── 2 channel cisternae analysis_ param
│       │   ├── 2 channel tubule analysis_ param
│       │   └── Single channel timeseries_ param
│       └── results
```

This tutorial will demonstrate how to customize the default parameters to get the best results for these images and any future images. The images used in this tutorial were taken using the LSM Zeiss 880 confocal microscope and as such the tutorial is focused on analyzing an image of this type, therefore recommendations of the most appropriate analysis methods may not apply to all image types.

Image capture requirements

As with all image analysis methods, a minimum standard of image quality is required for reliable image analysis. Ideal images show a clear ER network signal with minimum background noise and high pixel density, balanced against the need for rapid image acquisition.

Sufficient ER marker expression

In order to ensure that accurate network segmentation is possible, the fluorescent construct chosen to mark the endoplasmic reticulum (typically GFP-HDEL, RFP-HDEL or GFP-CXN) must have sufficiently strong expression to enable proper network segmentation. Poor expression results in a poorly defined ER network that cannot be properly segmented by the ER analysis software due to difficulties separating

the ER network signal from the ambient background intensity of the image.

Minimizing drift in Z

Though this software is largely capable of coping with sample drift in x and y, sample drifting in z will affect image quality. During slide preparation, it is essential that the cover slide is tightly affixed to the slide to reduce sample drift. Sample drift occurs when the sample itself shifts out of the focal plane of the microscope during scanning, resulting in a loss of focus between frame collections or during single frame capturing. Sample drift results in non-uniform ER network intensity through time, perturbing ER width calculations and preventing accurate ER dynamic assessments. Any acquired time series with evidence of sample drift should not be analysed.

Saturation

To ensure accurate ER width measurements, an image must be collected without any saturation. Saturation of images, through inappropriately high gain or laser power, causes all pixels with an intensity higher than the maximum intensity value to be assigned the maximum intensity value available. As a result, the full range of pixel intensities are not captured, making accurate assessments of ER structure impossible. The measured width of tubules depends on their relationship with pixel intensity. Over-exposure corrupts these measurements by altering the distribution of pixel intensity across tubules, leading to incorrect calculations of tubule and cisternae widths during later analysis. Saturation is reduced by reducing laser power.

Pixel density

Images must be captured at a minimum pixel density of Nyquist sampling, that is the pixel size must be at least half the size of the smallest object that you wish to analyse. Practically, this requires each tubule to be at least three pixels wide, as these are the smallest structures in the ER. This will then be up-sampled within AnalyzER to prevent pixellation errors later in the analysis process.

AnalyzERs interface

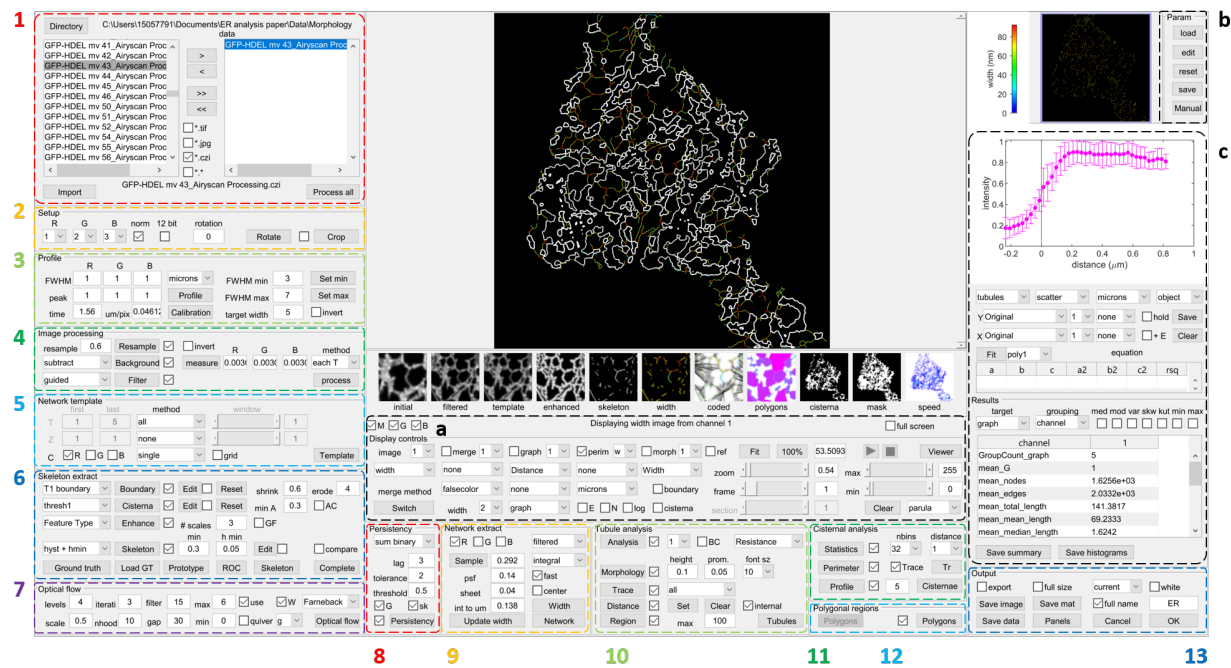


Figure 1: The Graphical User interface (GUI) of the ER analysis software

The software interface is divided into panels. In order to complete a full analysis it is essential to move through the panels in the following order (names are in the upper left corner of most panels):

Required : Panel 1: Import

Required : Panel 2: Setup

Required : Panel 3: Profile

Required : Panel 4: Image processing

Required : Panel 5: Network template

Required : Panel 6: Skeleton extract

Optional : Panel 7: Optical flow - only appropriate for time series

Optional : Panel 8: Persistence - only appropriate for time series

Required : Panel 9: Network extract

Required : Panel 10: Tubule analysis

Required : Panel 11: Cisternae analysis

Required : Panel 12: Polygonal regions

Required : Panel 13: Output

If changes are made to an earlier panel, all subsequent panels must be re-run i.e. if you have reached panel 5, but then make a change in panel 2, you must re-complete the tasks on panels 2, 3 and 4 before running panel 5.

Optional panels that can be used at any time:

- Display controls
- Data analysis
- Results

Example 1: A single channel time series

The single channel time series is a 5 frame time series of a tobacco leaf epidermal cell transiently expressing the ER lumenal marker GFP-HDEL (see fig. 2), 3 days post agrobacterium-mediated transformation ¹.

Panel 1: image input

Images are first imported into the software using the import panel in the top left (fig. 3). This panel can also be used to analyse several images once the parameters of those images have been analysed once.

1. Standard file types that can be uploaded include TIFFS, JPGs, PNGs and, with the inclusion of the bioformats package, .czi files (denoted by the *.* all files symbol)
2. First select **Directory** and navigate to the directory containing the images of interest (the 'demo images' folder)
3. Select **Select folder**
4. From the options presented, select the file type(s) of interest (for .czi files it is necessary to check the *.* box)
5. Use the single arrows to import or remove individual images of interest, and the double arrows to batch import/remove.

Process all will perform all pre-saved or default operations.

Panel 2: setup

The setup panel (fig. 4) prepared images for analysis, it controls the re-organization of the channels, the image setup within the software and controls rotation and cropping.

1. R, G and B refer to the three channel colours available within the software, while 1, 2 and 3 refer to order in which the channels are imported. To avoid confusion, it is possible to re-order the channels to assign particular channels a specific colour.
2. Norm normalises images, this must be checked as we are using airyscan images.

¹ I. A. Sparkes, J. Runions, A. Kearns, and C. Hawes. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nature Protocols*, 1(4):2019–2025, nov 2006

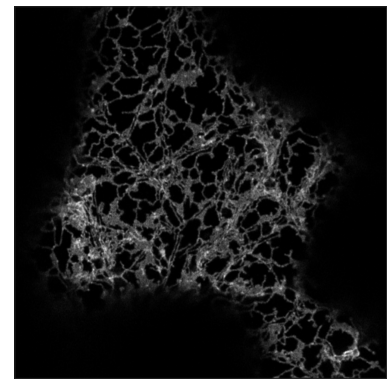


Figure 2: **Single channel example image.** The first frame of the single channel example image.

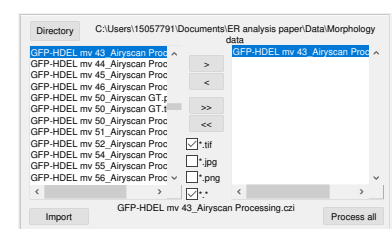


Figure 3: **Panel 1:** Import panel - the first panel used to import images into the software.



Figure 4: **Panel 2:** Image setup - modifying your image display and analysis area.

- Images are by default 8-bit however if 12-bit images are loaded, select the 12-bit option.
- Rotate - inputting a number here and selecting rotate will rotate the imported image if this is desired.
- Using the crop button allows you to draw a cropping rectangle across the image in the main panel.

TIP: cropping your image will speed up the rate of processing

Panel 3: profile

The Profile panel (fig. 7) is used to set the minimum and maximum expected tubule length. Features above the maximum tubule size will be classed as sheets, features below the minimum size will be classed as noise. It also required to input the image pixel scaling and, when using time series, the time between each frame in seconds.

To set the minimum expected tubule size:

- Select Set min
- Draw a line across a reasonable approximation of the thinnest tubule (double click to release)
- Check:** the parameter of the FWHM min (full width half maximum) box should be automatically updated.
- Check:** the graph in panel 11 a suitable tubule will produce a single peak with two dotted vertical lines (fig. 6). In the case of double peaks occurring within the dotted lines it will be necessary to repeat in a different tubule.
- Check:** also calculated and displayed will be:
 - The estimated pixel width in the FWHM min text box
 - The estimated pixel width and peak intensity (in normalised units) values in the FWHM and peak text boxes for the R,G and B channels
 - It may be appropriate to round down the automatically calculated FWHM min value
- Repeat for the maximum value using Set max (rounding up slightly to account for errors)
- Target gives a target size for the minimum tubule width that rescales the image to, for optimal results this should be an odd number, typically 3 or 5.

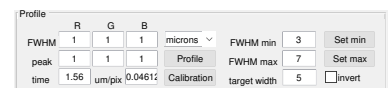


Figure 5: *Panel 4*: Profile panel

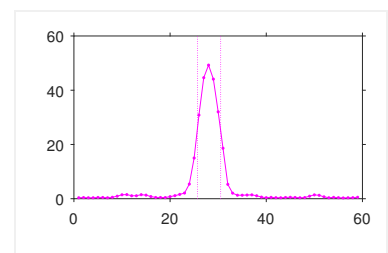


Figure 6: **FWHM max plot of an average tubule** The profile of intensity measured across an average tubule

TIP: the recommended settings for this image are min: 3, max: 7 and target: 5

To calibrate both the time between frames and the pixel spacing:

- Add the time between time frames in seconds to the box labeled time (**1.56 for the example image**)
- Add the pixel scaling, the area in microns represented by each pixel, to the box labeled um/pix (**0.046126 for the example image**).

TIP: This information can be found in the metadata of your image.

Panel 4: image processing

Image processing begins the process of noise removal and image enhancement (fig. 7). The resample factor is automatically updated by the previous panel and corresponds to the FWHM and target tubule width.

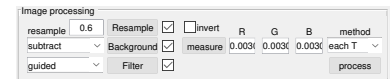


Figure 7: *Panel 4: Image processing*

1. Resample is set automatically so does not require any changes.
2. Ensure that the dropdown menu next to background displays **subtract** and then select **measure**.
 - (a) On the main figure, draw a square around a region of background.
 - (b) Double click to release.
 - (c) This will update the information in the boxes next to measure
 - (d) If using a single frame image or time series and the background region is selected outside the cell area (recommended) the dropdown box can be kept as **each T**, sampling the same region at each timepoint. If selecting within the cell this dropdown should be changed to **single** as the region may no longer have a similar intensity to the background between these frames.
3. The filter dropdown should be set to **guided**.
4. Select **Process** to run the entire panel.

Panel 5: network template

The network template is used to select the specific time points, z-stack positions and channels that will be used as a template image (fig. 8).

If a single channel, 2D single time point image is imported, the only option available will be template. If more complex images are

imported use the first/last text boxes to select relevant time points and z-slices.

Panel details:

- **T** row, select the most appropriate option to control the number of frames used in the template:
 - **Single** selects a single frame as the template, adjusting the number in the first box will chose the frame of interest, automatically set to 1
 - **Selected** selects a range of frames for analysis, adjusting the first and last values will provide the range.
 - **All** selects all frames in a timeseries
 - Projections through time are also available but not recommended.
- **Z** row, select the dropdown desired for z-stacks, similar options to above.
- **C** row, select the most appropriate option to control the channels brought forward for the template:
 - Check boxes: select the channels to be used for analysis e.g. R for a single channel image or R and G for a two channel image
 - Select the correct template method, the suggested options are:
 - * **Single** uses a single channel
 - * **Mean** generates a template with the mean intensity of two channels.
 - * **Max** generate a template using the maximum intensity from each channel.
- Select template to generate the chosen template.

The recommended settings are as follows: T - all, Z - none, C - only R selected.

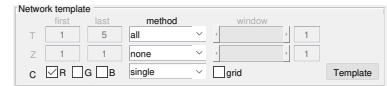


Figure 8: *Panel 5*: Network template

Panel 6: skeleton extract

The skeleton extract panel (fig. 9) is used to convert an enhanced image to a single pixel wide skeleton that runs along the center of each tubule. Cisternae are identified separately to the tubule skeleton.

Boundary

The boundary button masks the region within which the subsequent analysis steps are applied. Regions outside this mask are not included in later stages of the analysis. It is therefore essential that the mask covers the entire area of interest.

- Using the boundary dropdown menu select the most appropriate option for your image

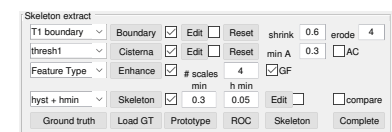


Figure 9: *Panel 6*: Skeleton extraction

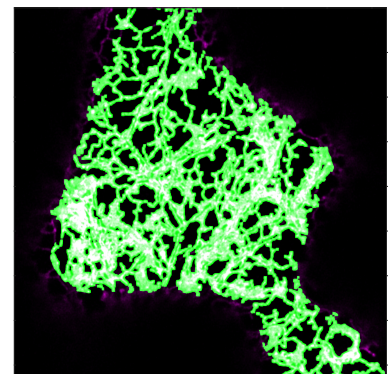


Figure 10: A T1 boundary shown in green overlaying the original image in magenta.

- The T value (1, 2 or 3) refers to the which of the image histogram partitions is used to apply the mask - higher values are more appropriate where there are very bright spots on an image, such as BFA bodies or Arabidopsis fusiform bodies
- The boundary can either have some internal regions excluded (e.g. T1 boundary) or filled (e.g. T1 fill), in this case we recommend **T1 boundary** (fig. 10).
- **Shrink**: The outermost boundary can be shrunk using the convex hull, recommended factors are between 0.5 to 0.8.
- **Erode**: The final convex hull boundary can be further eroded by a set number of pixels, recommended between 4-6 pixels.

Cisterna

The cisterna button is used to identify cisternae and remove them from the tubule image to be analysed separately. Cisternae are identified as structures that are larger than the maximum FWHM max value of ER tubules defined in the profile panel. There is an additional exclusion step where cisternae below a certain area are also excluded as they most likely represent punctae and three-way tubules as opposed to real cisternae.

1. From the Cisterna row, select the chosen image histogram partition most appropriate for identifying cisternae. As with the boundary, Thresh 2 and 3 are more appropriate for images with bright regions within the ER network. We recommend thresh1 for this example image (fig. 11).
2. Min A selects the area below which cisternae will be excluded from the analysis because they are most likely extra large tubules. This is automatically set to $0.3\ \mu\text{m}$ as this is the approximate size of stable ER punctae [2].
3. AC can be selected to use the active contour to map the identified cisternae back onto the underlying image intensity, this can result in more accurate segmentation of cisternae.

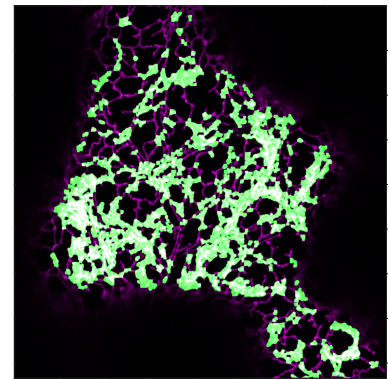


Figure 11: Cisternae identified using the Thresh1 option (green) mapped onto the original image (magenta).

Enhance

The purpose of image enhancement is to improve the contrast of the tubular elements against the image background in preparation for the next step, skeletonisation.

- Select **Feature type** from the dropdown menu, and select enhance to complete the process (fig. 12).
- Other options are available for enhancement, however feature type is usually the most appropriate.



Figure 12: An enhanced ER image

Skeleton

Skeletonisation reduces the enhanced image to a single pixel wide skeleton that runs along the center of each tubule. This skeleton is then removed from regions that have been identified as cisternae (fig. 13).

- Select 'hyst+hmin' from the drop down menu
- Recommended settings for this skeletonisation process are min = 0.3 and hmin = 0.05

Running the whole panel

Selecting skeleton will run the entire panel, re-running the boundary masking, identifying cisternae, enhancing the tubular elements and skeletonising the enhanced image and removing cisternae. This can be particularly useful if many changes have been made to the different stages in the panel.

Complete can be used to run the entire left side of the software up to this point- including processing the image, creating a template and performing all the steps required to extract the skeleton.

Panel 7: optical flow analysis on time series

Optical flow estimation of ER remodelling can only be applied to timeseries. If using a single image, this panel can be ignored entirely. 4 options for optical flow analysis (fig. 14 and 15) are available for use within the software, however only the Farneback method will be covered here. For more information visit the [MATLAB website](#). The options available for user modification change with respect to the method selected.

Setting the analysis parameters

After selecting the appropriate optical flow analysis method (in this case, Farneback) it is possible to adjust the optical flow analysis parameters. The standard parameters for Farneback analysis are:

- Levels: 4
- Iterations: 3
- Filter: 15
- Scale: 0.5
- Neighborhood: 10

These values can be modified if necessary. For further information visit the [dedicated optical flow page on the MATLAB website](#)

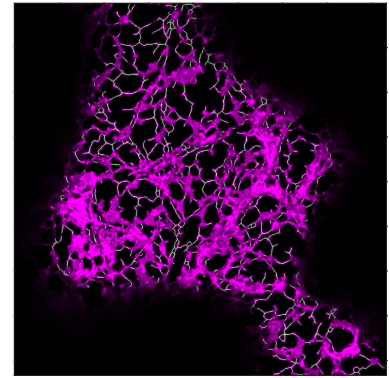


Figure 13: Skeletonised tubules (green) overlaid on the original image (magenta)

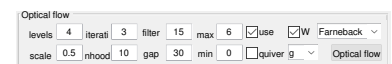


Figure 14: *Panel 7*: Optical flow analysis panel

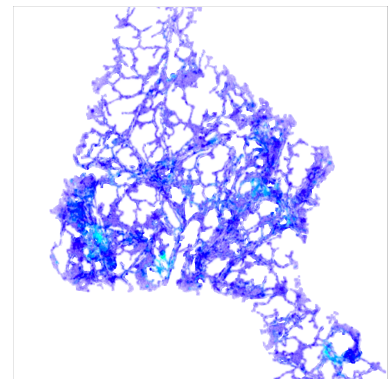


Figure 15: Optical flow mapped onto a single frame of a time series

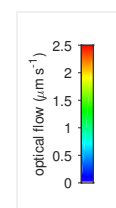


Figure 16: The colourbar of optical flow speeds associated with figure 15

Creating a normalised optical flow image

- The min and max options set the colourbar minimum and maximum values to a chosen micron/second speed. For the example timeseries we recommend a minimum value of 0 and a maximum value of 2.5.
- Use this option to standardise images for display and ensure the maximum separation within the colourbar (fig. 16).

Changing the image background

Optical flow output images are automatically displayed on a white background for ease of discrimination of the optical flow values. To display the image on a white background, simply unselect the check box denoted by **W**.

Displaying the direction of ER movement

To display the direction of ER movement simply select the **quiver** box (fig. 17). This will overlay arrows depicting the direction of movement on whatever image is currently displayed by the software, this image can be changed to one of your choice, for example the template. The value in the **gap** option controls the spacing of the arrows across the image, higher numbers result in few arrows but they will be clearer. The dropdown box gives an option on the arrow display colours available.

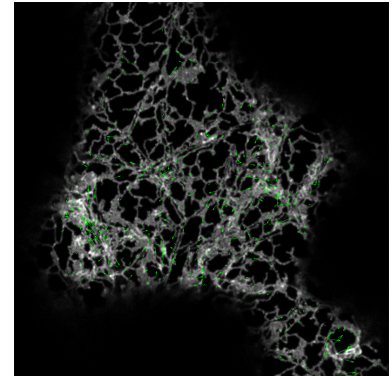


Figure 17: The direction of ER movement (green arrows) overlaid on a single image.

Panel 8: persistency analysis on time series

Persistency analysis also be applied to time series, if you are not using a time series image then this step is not required.

Recommended methods for persistency analysis (fig. 18):

1. Select your chosen display method from the dropdown list - **sum binary** is usually sufficient.
2. **Lag** is the amount of time over which persistency is assessed in terms of number of frames
3. **Tolerance** gives the number of pixels away the tubule skeleton or cisternae outline can move from the original point before it is considered to no longer be persistent e.g. a Tolerance of 2 would mean a tubule had to move more than 2 pixels from its initial position to be considered non-persistent.
4. **Threshold** is required for the identification of persistent nodes - the binary image is normalised and values below a certain fraction of the time series (recommended 0.3).
5. Select **Persistency** to run the persistency analysis (fig. 19)

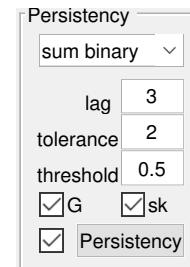


Figure 18: Panel 8: Persistency

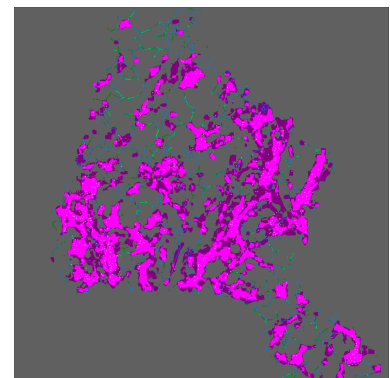


Figure 19: Sum binary persistency mapping - cisternae in magenta and tubules in green

For the example timeseries we recommend: method : **sum binary**, lag : 3, tolerance : 3, threshold : 0.3.

Modifying the persistency map output

Persistency maps are automatically displayed on a grey background, for ease of discrimination of lower values of persistency. A black background can be used instead by unchecking **G**. The original skeleton (the skeleton identified in the first frame) is also displayed on persistency maps in blue. This too can be removed by unchecking the **sk** option. To apply these changes select the persistency button to re-run the entire panel.

Panel 9: network extraction

The network extraction panel (fig. 20) functions to capture the information in the underlying image intensity, such as tubule widths.

- The **RGB** tick boxes refer to which channel the the network extraction will be applied to. If you have a 2 channel image and want results from two channels you will need to select both channels.
- The adjacent drop down box is used to select the image the extraction will be applied to - leave this as filtered.
- Select sample and draw a box within a single, typical cisternae. Using this intensity we can then calculate the the calibrated tubule width
- **Fast** is used to reduce the time required to extract the network using granulometry with some loss of accuracy at low tubule widths.
- Select **width** then **network** to run the entire panel.

Panel 10: basic tubule analysis

Creates a weighted graph summarizing information of interest.

- It is sufficient to select **tubules** and run the whole panel using automatic settings (fig. 21). The additional buttons will run individual analyses:
 - **Analysis** will analyse basic tubule widths and lengths (fig. 22)
 - **Morphology** will assess the peaks and troughs of of tubule intensity and can be used to identify tubule bulges and constrictions, see chapter 2 for more details.
 - Selecting **trace** will cause a pointer to appear and will allow you to double click to select two points on the tubule graph, between which a graph of the traces will be created, see chapter 2 for more details.
 - **Distance** will allow you to set a point, outward from which you can analyse

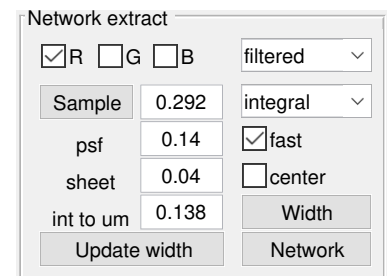


Figure 20: *Panel 9*: Network extraction

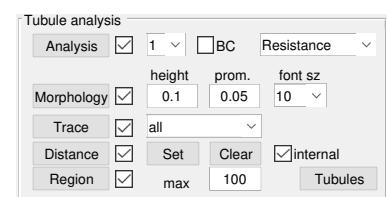


Figure 21: *Panel 10*: Tubule analysis

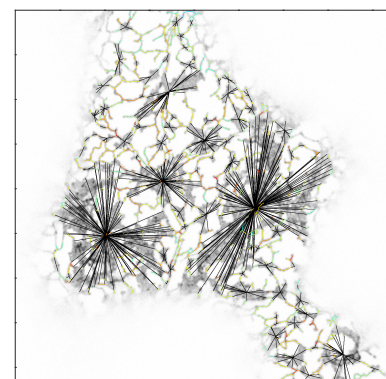


Figure 22: Coded image produced after tubule analysis.

- **Region** will allocate any signal intensity within a maximum distance of pixels (see set) from the tubules and cisternae to the nearest element.

Panel 11: basic cisternae analysis

- It is usually sufficient to select **cisternae** and run the entire panel (fig. 23).
- **Statistics** will analyse the distribution of a fluorophore across the cisternae
- **Perimeter** analyses the fluorophore distribution around the perimeter of the cisternae.
- **Profile** analyses the distribution of fluorophore perpendicular to the cisternae perimeter, see chapter 3 for more details.

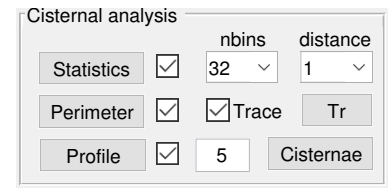
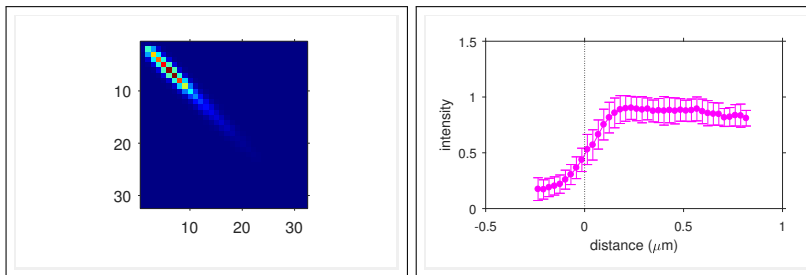


Figure 23: *Panel 11*: Cisternae analysis

Figure 24: Left: Grey-level co-occurrence matrix produced by cisternae analysis. Right: Radial fluorophore distribution normal to the cisternae perimeter.

Panel 12: polygonal regions

Polygonal regions will calculate the polygonal region statistics (fig. 25). It is sufficient to select **Polygons** on the right side of the panel.

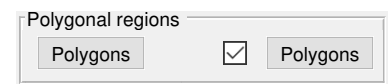


Figure 25: *Panel 12*: Polygonal region analysis

Panel 13: output

Panel 13 (fig. 26) is essential for the export of images and saving all the data produced during the analysis.

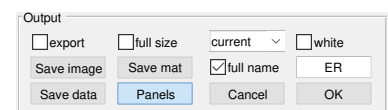


Figure 26: *Panel 13*: Output panel - for saving data and images

Exporting images

- To export the image shown on the screen, simply select **Save image**. To adjust the image being exported and how it is saved you can modify the following options:
 - **Export** is not usually recommended but it will export every image produced during the analysis
 - When ticked, **full sized** will export a full resolution image, rather than capturing the image at the resolution it is displayed.

- The **current** drop down option can be used to select specific images for export.
- Selecting **white** will put a graph on a white background
- Selecting **full name** will ensure that the name under which the image is saved includes original name of the imported name
- The box containing the word **ER** is the prefix of the name under which the image will be saved.

Saving data

Saving data can be completed by selecting save data.

TIP: Data will be overwritten unless results are moved to a new folder

Example 2: A dual channel image for tubule morphology

Over-expression of select members of the reticulon protein family can modify the structure of ER tubules, inducing membrane curvature to such an extent that luminal markers such as GFP-HDEL are forced into 'bulges' along the tubules, whilst strong expression of reticulons increases constriction^{2,3}. A sample image is therefore provided of RFP-RTN1 over-expression and GFP-HDEL, and will be used to highlight analysis of tubule traces.

Processing a multi-channel image

When processing a multi-channel image or timeseries, three panels need to be modified in order to ensure accurate segmentation and analysis. The setup panel (panel 2, fig. 27) contains an option to modify the colours in which up to three channels can be displayed. Initially the first channel captured (channel 1) will be displayed in magenta, the second channel (channel 2) will be displayed in green and the third channel (channel 3) will be displayed in blue, regardless of what colours have been assigned to the given channel in other software. Using the RGB dropdowns it is possible to re-organise the channels to assign more appropriate pseudocolouring.

The analysis then proceeds as normal to the network template panel (panel 5, fig. 28). Here it is necessary to select as many channels as are desired for analysis, listed by their pseudocolouring assigned in panel 2. The adjacent dropdown must then be modified to give the type of template desired - typically either an 'mean' of the two channels intensity or a 'max' of the two channels. If there is a second channel present that is not suitable for ER analysis, leaving the corresponding channel tickbox unselected will essentially remove this channel.

Finally if both channels need to be analysed in terms of tubule and cisternae statistic, all channels of interest must be selected in the network extract panel (panel 9, fig. 29). Failure to do so will result in only one channel being analysed.

² E. Breeze, N. Dzimitrowicz, V. Kriebaumer, R. Brooks, S. W. Botchway, J. P. Brady, C. Hawes, A. M. Dixon, J. R. Schnell, M. D. Fricker, and L. Frigerio. A C-terminal amphipathic helix is necessary for the in vivo tubule-shaping function of a plant reticulon. *Proceedings of the National Academy of Sciences*, 113(39):10902–10907, 2016

³ I. A. Sparkes, N. Tolley, I. Aller, J. Svozil, A. Osterrieder, S. Botchway, C. Mueller, L. Frigerio, and C. Hawes. Five Arabidopsis reticulon isoforms share endoplasmic reticulum location, topology, and membrane-shaping properties. *The Plant cell*, 22(4):1333–43, 2010



Figure 27: **Panel 2, dual channel:** Image setup - modifying your image display and analysis area for a dual channel image.

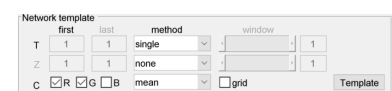


Figure 28: **Panel 5, dual channel:** example panel 5 network template to create a mean template of both channels

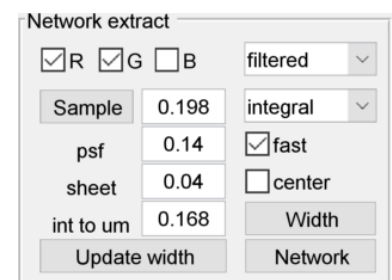


Figure 29: **Panel 9, dual channel:** Panel 9 setup for a dual channel image

Analysis of fluorophore distributions along tubules

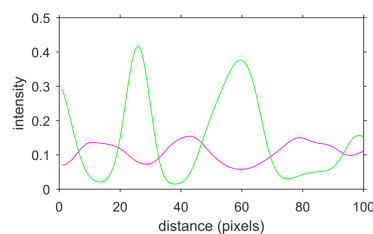
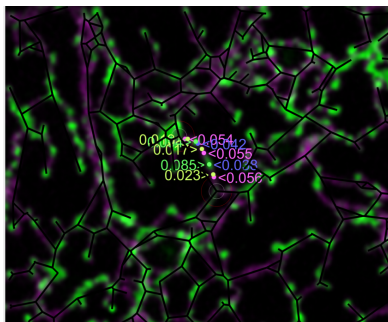
Tubule analysis is located in panel 10 ('tubule analysis') and can simply be performed by running the entire panel by selecting **Tubules** or can be broken down by using selected buttons within the panel.

Tubule morphology

Tubule morphology can be measured by using the 'morphology' button. This will produce an overlay on the displayed image identifying the peaks and bulges along tubules. Channel 1 results are right justified and coloured magenta for bulges and blue for constrictions (fig. 30). Channel 2 results are left justified and coloured green for bulges and yellow for constrictions. Which panel is displayed can be modified in panel a (central display control panel) using the morph dropdown box.

Tubule trace

Using the trace button within the panel it is possible to provide information on the distribution of fluorophores along tubules. This is achieved in two ways, either by considering the distribution along tubules across the entire cell or using selected, defined traces. Which is displayed is controlled by the dropdown menu adjacent to the Trace button.



To display all the traces (fig. 31), use the dropdown menu to select all, then use the Trace button. A graphical output will be generated in the right hand panel which displays the predominant fluorophore intensity along all tubules identified in the image, from shortest tubule to longest tubule.

Using the the dropdown menu, choose selected to measure a trace along a specific set of tubules. Then select two tubule junction and the shortest path between these two points will be traced, displaying the tubule bulges and constrictions along that pathway (fig. 32). In addition a plot will be generated on the right hand side of the software displaying the relative changes in intensity along that path, coloured by the relevant channel, with peaks in intensity identified by open circles, and troughs in intensity identified by

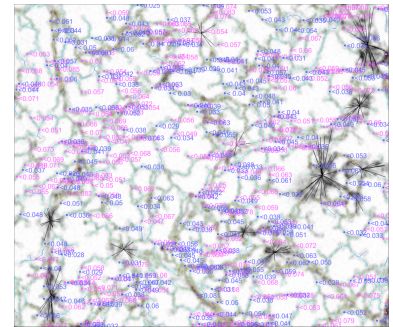


Figure 30: Tubule morphology overlay of channel 1 on an underlying image (bulges in magenta, constrictions in blue)

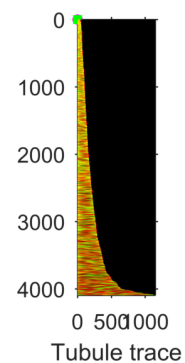


Figure 31: Sample output of the distribution of fluorophores along all tubules within the image

Figure 32: the selected trace along a single tubule. Channel 1 results are right justified and coloured magenta for bulges and blue for constrictions. Channel 2 results are left justified and coloured green for bulges and yellow for constrictions. Plotting the fluorophore intensities along a selected tubule. GFP-HDEL in green and RFP-RTN1 in magenta, with peaks identified by open circles, and troughs identified by stars.

stars (fig. ??).

Example 3: A dual channel image for cisternae analysis

The localisation of certain fluorophores across the the cisternae can also be analysed within the software. The dual channel image must be processed as above, with channels adjusted in the setup panel, and both channels selected in the network template and network extract. Upon reaching the cisternal analysis panel (panel 11), select profile. The number adjacent to the profile defines the minimum number of individual cisternae that must contribute to the measurement.

The output is a graph that plots the mean intensity moving across the border of multiple cisternae, showing peaks in intensity normal to the cisternae edges (fig. 33).

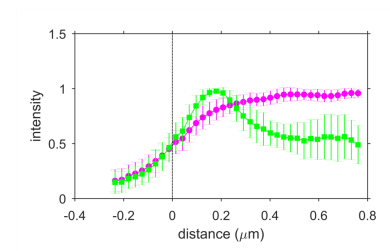


Figure 33: Example plot of both fluorophores intensity normal to the edge of a minimum of 5 cisternae.

Advanced panels

Panel a: Viewing images

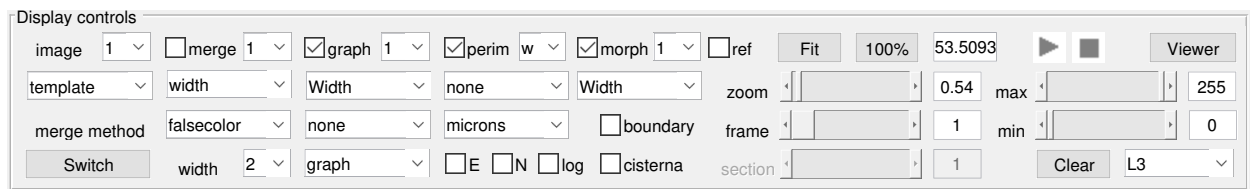


Figure 34: The full display control panel

Any combination of data, channels and timeframe can be viewed in the central panel of the software, and therefore can be exported for display. The panel is complex with multiple options and so has been split into a few panels for individual analysis.

Section 1: Merging two images

Many images require merging two images, either from two channels or displaying two different images of interest, for example the original image and the cisternae. It is also possible to select a channel of interest for display. The merge methods of the two images are variable. Examples of different merge methods are shown below.

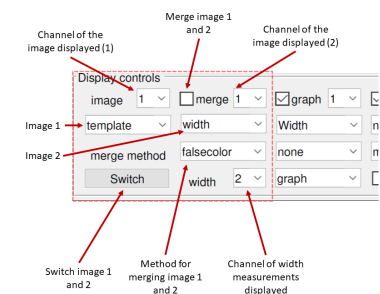


Figure 35: Focused section of display control panel 1

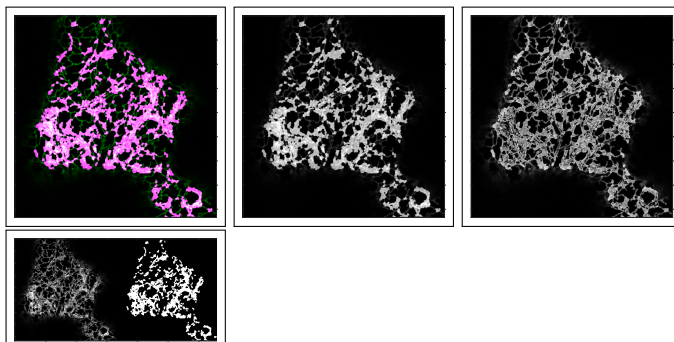


Figure 36: Top left: False colour merge, top center: Blend merge, top right: difference merge, bottom : Montage

Section 2: Displaying a graph overlay

Most data can be overlaid on a single image for demonstration purposes. This section deals with a tubular overlay, whilst the next section will deal with overlaying cisternae statistics. In order to overlay a graph it is essential to ensure that graph is selected. Then select the statistic to overlay on the image. The layout of the graph overlay can be controlled to show either a weighted, un-directed pseudocoloured graph, or the graph can follow the path of the underlying ER skeleton (see below). A colourbar will also appear in order to calibrate the results.

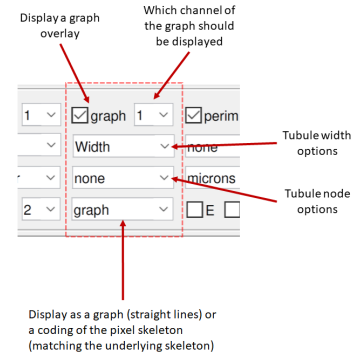


Figure 37: Focused section of display control panel 2

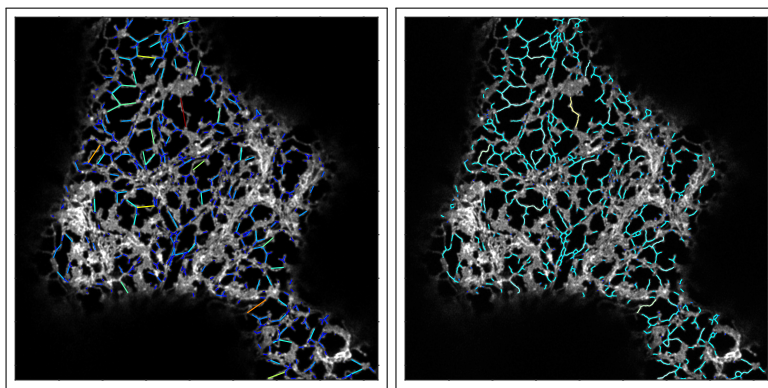


Figure 38: Left: a graphical representation of tubule lengths (set to graph), right: a graphical representation of tubule lengths (set to pixel).

Section 3: Showing cisternae and tubule morphology

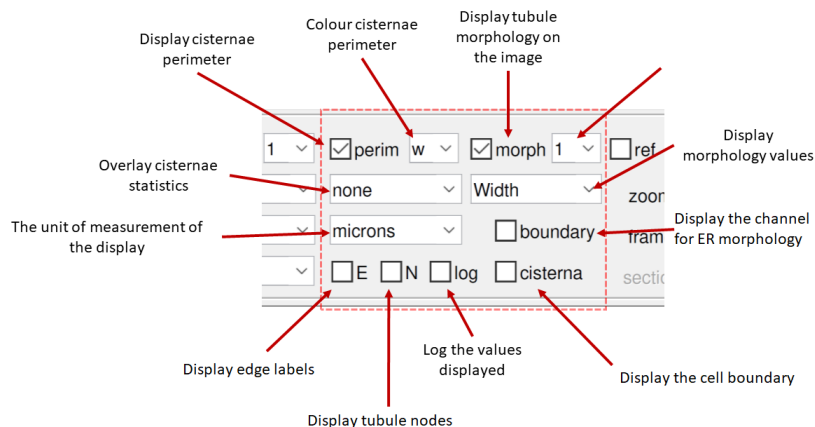


Figure 39: Focused section of display control panel 3

Cisternae statistics, such as area and geometry measurements, can also be displayed as an image overlay. In each case the values of interest are displayed in green to the right of the cisternae ID in cyan. This ID is assigned for analysis purposes and can be ignored.

The boundary of the cell defined by the convex hull and where graphs should be displayed are also controlled in this section. ER morphology peak, troughs or other values of interest can be

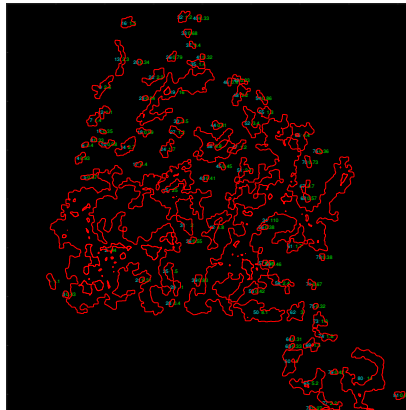


Figure 40: Outline of cisternae, ID number in cyan, cisternae area in green.

overlaid on single or two channel image.

Section 4: controlling image zoom

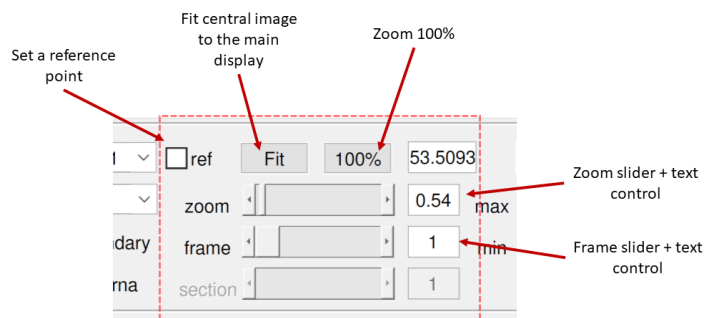


Figure 41: Focused section of display control panel 4

Image zoom as displayed in the main frame is controlled in this section. It is recommended that before exporting an image the zoom is set to 100%.

Section 5: playing movies, controlling image intensity

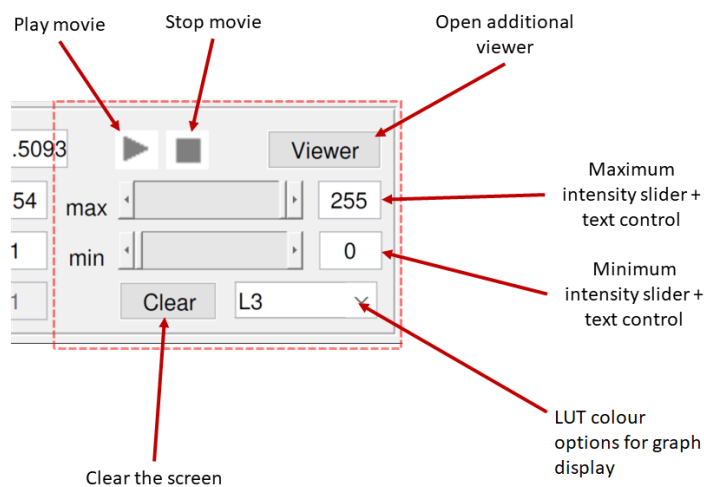


Figure 42: Focused section of display control panel 5

This final section of the panel has many functions;

- Playing and stopping loaded movies
- Transferring movies to the export viewer
- Controlling the maximum and minimum image intensity
- Clearing the image screen
- Setting the colour scheme of the image graph overlay and the colourbar.

Panel B: Parameter control

Analysis parameters are automatically loaded as either the default settings or from 'parameters' file uploading an image for analysis. using the parameter panel it is possible to modify the parameters used to analyse any particular image:

- To load a set or parameters from a different folder, select load and select the parameter file.
- Use edit to change the parameter file options
- Reset can be used to reset the parameter file associated with an image to the defaults.
- Parameters automatically save after each step in the analysis.
- The manual option allows for manual modification of the parameter file



Figure 43: Parameter control panel

Panel c: Displaying your results

Two options for within software result display and analysis are available. These analysis are applied to the image or time series once the software analysis has been completed. The first panel can be used to display a variety of graphs, whilst the second panel will provide a quick tabulated summary of the data relating to the analysed image/timeseries.

Panel 1: Displaying results as a graph

Generalized Extreme Value					
k	sigma	mu		re...	p
-0.375	0.484	-1.060	0	0	0.163

Figure 44: Graphical display results

The whole results panel for in house graph display is shown above, smaller individual sections are labeled below.

Basic selection of graph details

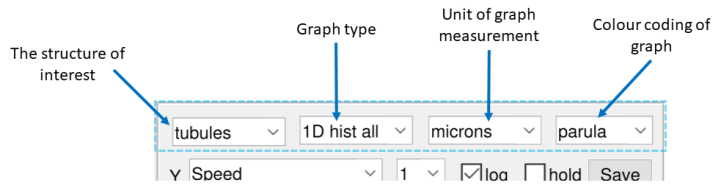


Figure 45: Graphing data panel, subsection 1

The available data display options are:

1. **Tubules:** Tubule statistics e.g. length, width, area, speed
2. **Nodes:** Tubule nodes e.g. strength, speed, persistency
3. **Polygons:** Polygonal region statistics including area and geometry measures
4. **Cisternae:** Cisternae features such as area, speed, persistency, length
5. **Morphology:** Tubule morphology results
6. **Tubule trace:** The trace of intensity along tubules
7. **Profile:** The profile of ER intensity moving across the boundary of the ER cisternae
8. **Perimeter trace:** Fluorophore distribution trace along the edge of cisternae
9. **Graph:** Network analysis of ER structure

The available graph options are:

1. **scatter:** scatter plot of the selected frame
2. **scatter all:** scatter plot of the selected results across all frames
3. **1D hist:** single statistic histogram from the selected frame
4. **1D hist all:** single statistic histogram of all frames
5. **2D hist:** a 2D histogram of two selected variables from the selected frames
6. **2D hist all:** a 2D histogram of two selected variables from all frames
7. **Time:** the mean result of a variable plotted over time

The second section of the panel can be used to select the variable of interest for each axis (Y contains the variable selected for the image display). These variables can be transformed or different channels selected depending on the desired outcome.

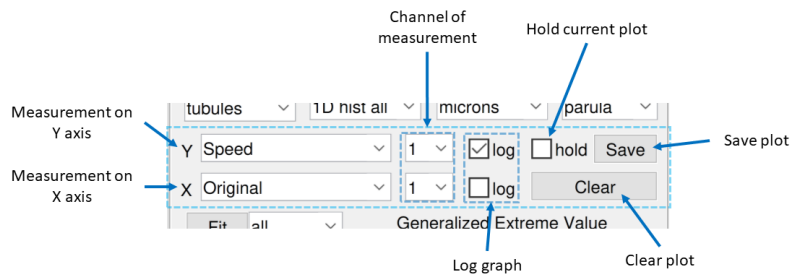


Figure 46: Graphing data panel, subsection 2

The final section of the panel are used to fit distributions to a density plot of the variable selected in the Y drop down section. Selecting all in the dropdown option will assess all available distribution fits, however a specific distribution can also be selected.

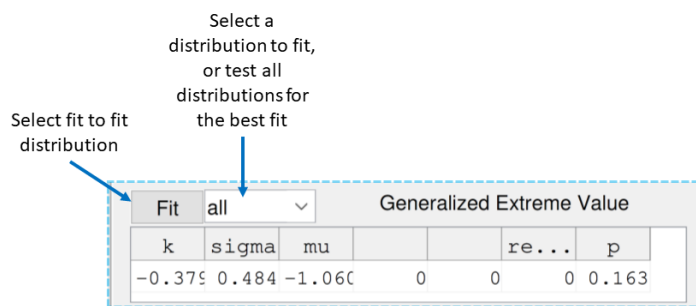


Figure 47: Graphing data panel, subsection 3

Displaying summary data

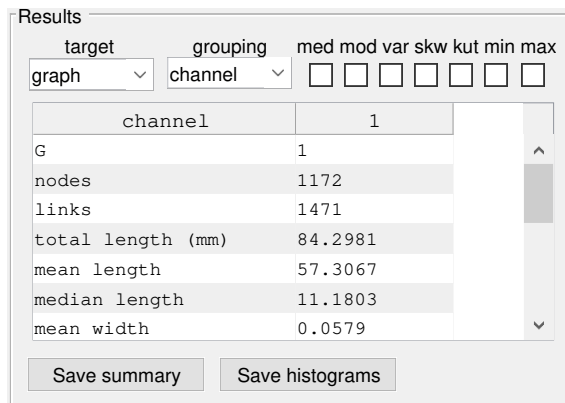


Figure 48: Summary data display panel.

Using the results panel it is possible to create a condensed summary of the data of a specific image.

- Target refers to the structure of interest
- Grouping is how the data should be grouped for example by channel or frame
- The various tick boxes select what summary statistic should be displayed, for example:
 - med - the median of the data
 - mod - the mode of the data
 - var - the variance of the data
 - skw - the skew of the data
 - kut - the kurtosis of the data
 - min - the minimum value of the data
 - max - the maximum value of the data
- Save summary can be used to save the table displayed within the software as an excel spreadsheet
- Save histograms will produce a 1D histogram of all the available statistics as a summary of the all the data

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