Basic Ratio Imaging Tutorial

# Stage 1: Loading an image

1. Run the RRA program
2. Press the **Basic Ratio Analysis** button. Another window will open up with blank images displayed.
3. Press the **Load images** button on the top left handcorner.
4. Once the **File import** window opens, select the **At\_cyt\_roGFP2\_subsampled.lsm** file by double clicking
5. The image should load and the displays will update to show the images.

# Stage 2: Calculating the Ratio Images

1. The images are not necessarily n the correct order for processing. When the images are collected the user might collect the oxidized, reduced and auto-fluorescence channels in any sequence, so we will need to tell the software which channel is which. This is part of the information stored in the database that we will load in the next step. We also need to set all the processing parameters. These can be set manually for each step or retrieved from a database with previously stored settings. In this case we will use previously stored values.
2. Press the **Load probe** button to choose the database.
3. In the window that opens up, press the **Load database** button and navigate to the **RRA\_basic\_database** file. We do not need to make any changes at the moment so press **OK** to load the database
4. Select *roGFP2* from the drop-down menu. This will change the channel order and update the displays. All the controls setting will change to reflect the stored parameters. However, the software requires us to complete each step in sequence, so only the controls for the next step are enabled.
5. We can now apply a smoothing filter to the image that has been set as a 5x5 pixel average by pressing the **Average** button. The displays will update to show the effect of smoothing.
6. We need to measure and subtract the background signal. The **Background** button prompts you to select a region in any of the images that will be used to calculate an average background value. If you are happy with the selection, press the **Back sub** button to apply the subtraction.
7. Pressing the **ratio** button will now calculate the ratio for the background subtracted images. The status information will report that the ratios are being calculated, but at this stage the image displays will not be updated as we have not told the system how we would like the ratio to be displayed.
8. In the next step, we can set the minimum and maximum range to colour code the ratio, and also adjust the intensity of the ratio. Pressing **display** will apply these values and show the colour coded ratio.

# Step 3: Displaying the data

1. Now we have calculated a ratio, we can play through the time-series to see what has happened to our specimen using the various controls in the **Movie** panel.
2. Whilst this gives us a visual impression of the changes occurring, we also want to be able to extract some measurements from the data. The **point** button allows us to click on regions-of-interest (ROIs) in the image to extract an average profile of the intensity over time and the background subtracted ratio over time. The size and shape of the ROI can be adjusted.
3. You can now explore changing any of the settings used in the initial processing steps and the impact they have on the ratio image or ratio measurements

# Step 4: Calibration

1. In this experiment, H2O2 was added to drive the roGFP to a fully oxidized state, followed by a washout, and then addition of DTT to drive it to a fully reduced state. You can use these values to calibrate the *in vivo* probe response by estimating the ratios once the system has equilibrated.
2. In the **Calibration panel** we have to tell the software the frames to average to estimate the calibration parameters. Type ‘15’ in the **Rmax** **start** box and ‘25’ in the **Rmax end** box. Likewise type ‘55 and ‘65’ in the **Rmin start** and **Rmin end** boxes. Check the **samples** box in the panel above to overlay the sampling window on the ratio graph.
3. When you press the **calibrate** button you will be prompted to draw a ROI on the image to encompass the pixels you want to include in the calibration. The values for the other calibration parameters should update once you complete the selection.
4. These estimated ratio values for **Rmin** and **Rmax** will be used to calibrate the % oxidization of the roGFP. The other parameters in the calibration window are used to convert the % oxidation to the redox potential.
5. The calibrated values can be displayed by changing the drop-down menu in the **Ratio values** window to ‘% ratio’ or ‘calibrated’

# Step 5: Data output

1. Once you are happy with the ratioing and calibration, you can save the data and images. The **Save data** button writes out the values for each ROI to an excel spreadsheet.
2. **View** opens up another window that allows us to save the ratio as a movie. Select the **xylt** option for the type of data and press **start** to play the movie. The movie can be saved using the **Save movie** button.
3. To provide a record of the experiment, the **figure** button will generate a composite page that can be printed or saved. You will prompted for the time point of four images that will be extracted from the series, along with calibration values for the pixel size and time intervals if you wish these to be displayed correctly.
4. The **actions** tab allows you save the figure in pdf, eps and emf format. The latter can be imported into powerpoint and ungrouped to give you access to all the different elements should you wish to make a publication figure.
5. Full details of the function of all the control can be found in the manual.