

Intensity Correlation Analysis

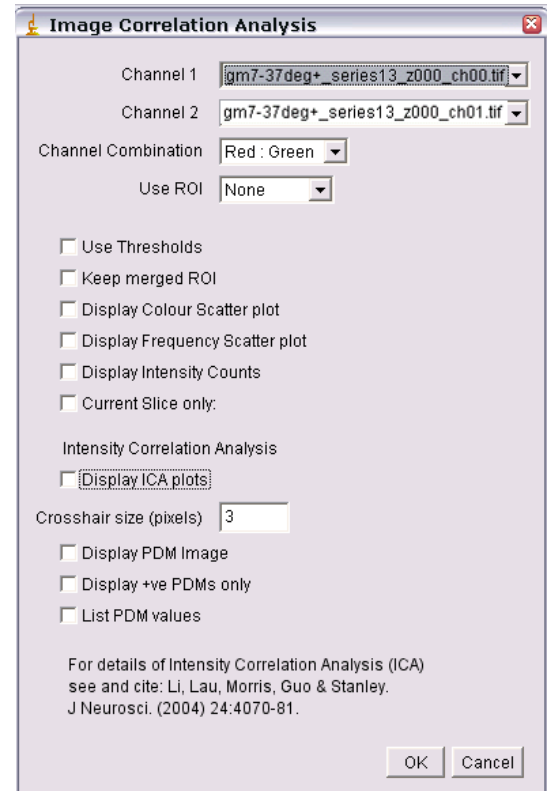
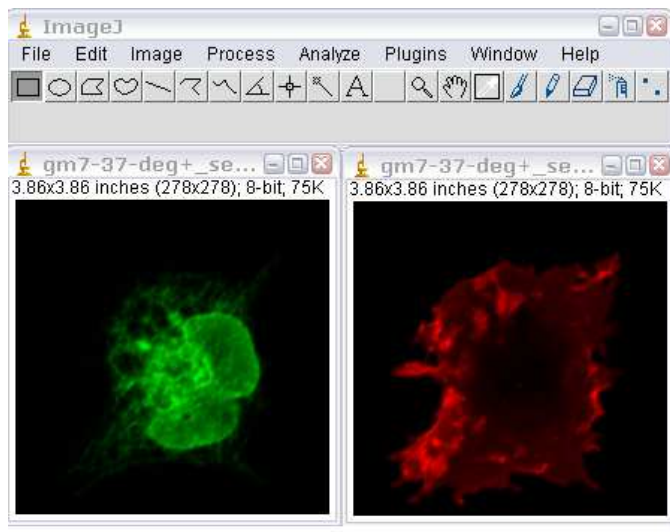
This plugin generates Mander's coefficients as well as performing *Intensity Correlation Analysis* as described by Li *et al.* To fully understand this analysis you should read:

Li, Qi, Lau, Anthony, Morris, Terence J., Guo, Lin, Fordyce, Christopher B., and Stanley, Elise F. (2004).

A Syntaxin 1, G α , and N-Type Calcium Channel Complex at a Presynaptic Nerve Terminal: Analysis by Quantitative Immunocolocalization. *Journal of Neuroscience* 24, 4070-4081.

Manders, EEM, Verbeek, FJ., and Aten, JA. (1993). Measurement of co-localisation of objects in dual-colour confocal images. *Journal of Microscopy* 169, 375-382.

1. Open your two images (File/Open).
2. You must background subtract each stack:
 - 2a. Select a BG ROI.
 - 2b. Run Plugins/ROI/BG subtract from ROI.
 - 2c. Set threshold if required.
3. Run the ICA plugin (Plugins/Colocalization Analysis/Image Correlation Analysis)



Channel Combination

Select the combination of channel colour, e.g. a TRITC vs FITC analysis would be Red:Green; a FITC vs DAPI would be Green: Blue. This determines the colouring given to the ICA plots and the merged image.

Use ROI

Choose a ROI to analyse from the drop down box. Choose either 'none' to analyse the whole image or "Channel 1:" to analyse the pixels in both channels within the ROI selected in channel 1.



Use Thresholds

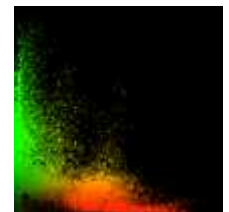
If this is selected, pixels below the image's threshold will not be included in the analysis. For instructions on how to set the threshold, see:

<http://rsb.info.nih.gov/ij/docs/menus/image.html#adjust> and

http://www.uhnresearch.ca/facilities/wcif/imagej/particle_analysis.htm#threshold

Keep merged ROI

Returns a merged image of the analysed ROI (or full image). The merged colours are determined by the "Channel combination" selected.

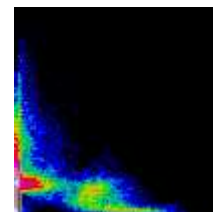


Display Colour Scatter plot

This generates a scatter-plot of red intensities vs green intensities. The colour of the scatter plot pixel represents the actual colour in the image. This does not tell you the frequency of the pixel, but is easier to relate to the original image.

Display Frequency Scatter plot

The pixels in this scatter-plot pseudocoloured so that their colour represents the frequency of the red-green pixel combination in the original image (hot colours representing high values by convention). This sort of plot contains the most information, but can be a little difficult to relate it back to the original image.



Display intensity counts

This generates a text window with the red intensities, green intensities and frequency of the intensity pairs. This can be exported to a spreadsheet program for further analysis.

Ch1	Ch2	Freq.
0	1	8509
0	2	1027
0	3	258
0	4	199
0	5	162
-	-	...

Current Slice Only

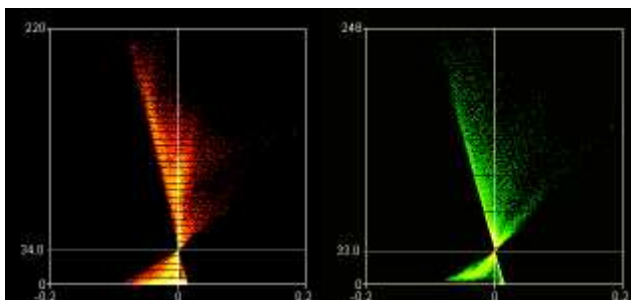
Select this if you do not wish to analyse the whole stack. Only the current slice in the Channel 1 stack (and the corresponding slice in the green channel) will be analysed.

Display ICA plots

When checked this option draws two plots, one for the red channel one for the green. The axes on the plots are the PDM values on the x-axis and the red or green intensity on the y-axis.

The PDM value is the *Product of the Differences from the Mean*, i.e. for each pixel:

$$\text{PDM} = (\text{red intensity} - \text{mean red intensity}) \times (\text{green intensity} - \text{mean green intensity})^1$$



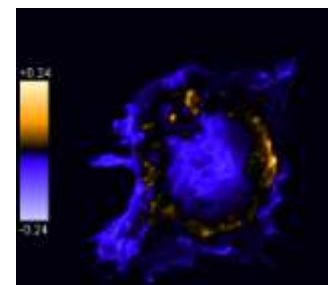
Crosshair size (pixels)

The points of the ICA plots are plotted as cross-hairs. You can select the size of the crosshair here.

Display PDM image

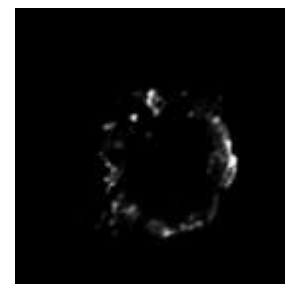
This option generates a new image where each pixel is equal to the PDM value at that location. The Image is pseudocoloured and a PDM scale bar is inserted.

For clarity, pixels that are below average in *both* channels are excluded.



Display +ves only

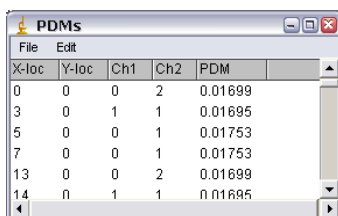
This option will generate a 2 slice stack. The first images pixels are the positive PDM values resulting from both pixels above the mean (i.e. red intensity-mean red intensity and green intensity-mean green intensity are both positive). The second slice are pixels that have pixel values in each channel which are both below the mean (i.e. red intensity-mean red intensity and green intensity-mean green intensity are both negative).



¹ PDM is equal to the value (A-a)*(B-b) as described in Li *et al.* 2004.

List PDM values

This generates a list of PDM values that can be exported to spreadsheet programs for further analysis.



X-loc	Y-loc	Ch1	Ch2	PDM
0	0	0	2	0.01699
3	0	1	1	0.01695
5	0	0	1	0.01753
7	0	0	1	0.01753
13	0	0	2	0.01699
14	0	1	1	0.01695

Results Window

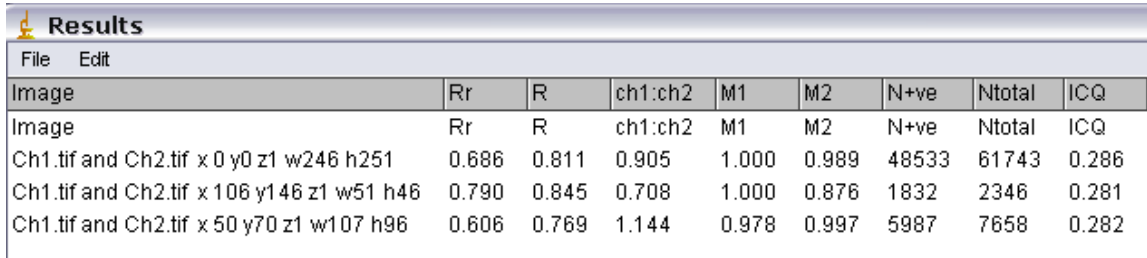


Image	Rr	R	ch1:ch2	M1	M2	N+ve	Ntotal	ICQ
Image	Rr	R	ch1:ch2	M1	M2	N+ve	Ntotal	ICQ
Ch1.tif and Ch2.tif x 0 y0 z1 w246 h251	0.686	0.811	0.905	1.000	0.989	48533	61743	0.286
Ch1.tif and Ch2.tif x 106 y146 z1 w51 h46	0.790	0.845	0.708	1.000	0.876	1832	2346	0.281
Ch1.tif and Ch2.tif x 50 y70 z1 w107 h96	0.606	0.769	1.144	0.978	0.997	5987	7658	0.282

If the Results window is closed or does not contain ICA data then the plugin will enter a “Header” row then the data row. The second time the plugin is run only the data row is entered. The column information is detailed below.

Image

The name of the images and the ROI that has been analysed is entered in the Image column. If no ROI was selected of the “Use ROI” option unchecked then the ROI describes the image dimensions.

Rr

This is the Pearson’s correlation coefficient. Zero-zero pixels are not included in this calculation.

This is a popular method of quantifying *correlation* in many fields of research from psychology to economics. In many forms of correlation analysis the values for Pearson’s will range from 1 to -1. A value of 1 represents perfect correlation; -1 represents perfect exclusion and zero represents random localisation. However, this is not the case for images. While perfect correlation gives a value of 1, perfect exclusion does not give a value of -1. Low (close to zero) and negative values for Pearson’s correlation coefficient for fluorescent images can be difficult to interpret. However, a value close to 1 does indicate reliable colocalisation.

R

This is Mander’s Overlap coefficient. This is easier than the Pearson’s coefficient to comprehend. It ranges between 1 and zero with 1 being high-colocalisation, zero being low. However, *the number of objects in both channel of the image has to be more or less equal.*

Ch1:Ch2

This value represents the red: green pixel ratio. The Overlap coefficient (**R**) is strongly influenced by the ratio of red to green pixels and should only be used if you have roughly equal numbers of red and green pixels (i.e. $N_{red} \div N_{green} \text{ pixels} \sim 1$).

M1 and M2

These split coefficients are Mander’s Colocalization coefficients for channel 1 (M1) and channel 2 (M2).

These split-coefficients avoid issues relating to absolute intensities of the signal, since they are normalised against total pixel intensity. We also get information as to how well each channel overlaps the other. There are cases where red may overlap significantly with green, but most of the may not overlap with the red.

If the assumption is made that greyscale number equates to dye molecules (*this is not necessarily correct*) then these coefficients represent the percentage of red dye molecules that share their location with a green dye molecule.

These coefficients are very sensitive to poor background correction and do not take in to account the intensity of the second channel, other than it is non-zero. For example, a bright red pixel colocalising with a faint green pixel is considered equivalent to a bright red pixel colocalising with a bright green pixel. Intuitively, a red-green pixel-pair of similar intensities should be considered “more colocalised” than a pixel pair of widely differing intensities.

N+ve

This represents the number of pixel pairs that have a positive PDM value.

Ntotal

This is the number of pixels pairs in the images that where at least one of the pixel pairs is above zero..

ICQ

This is the Intensity Correlation Quotient. If the intensities in two images vary in synchrony (i.e. they are *dependent*), they will vary around their respective mean image intensities together. So, if a pixel’s intensity is below average in the red channel (i.e. $R_i - R_{\text{mean}} < 0$); it will be below average in the green channel (i.e. $G_i - G_{\text{mean}} < 0$). Similarly, if a pixel is above average in one channel it will be above average in the other. Therefore, in an image where the intensities vary together, the *product of the differences from the mean* (PDM), will be positive. The converse is true. If the pixel intensities vary asynchronously, i.e. the channels are segregated so that when a red pixel is above average, the corresponding green pixel is below average; then most of the PDMs will be negative.

The ICQ is based on the non-parametric sign-test analysis of the PDM values and is equal to the ratio of the number of positive PDM values to the total number of pixel values. The ICQ values are distributed between -0.5 and +0.5 by subtracting 0.5 from this ratio.

Random staining: $ICQ \sim 0$; Segregated staining: $0 > ICQ \geq -0.5$; Dependent staining: $0 < ICQ \leq +0.5$

Instructions for the ImageJ-based ICA ICQ analysis module.

Installation

Download the ImageJ bundle from the TWRI Wright Cellular Imaging Facility web site.
[www.uhnresearch.ca/wcif]

Open Images

1. Review deconvolved image stack or confocal image and select 2-3 planes with clear staining.
2. For each plane save three images in TIFF format: the colour merge and single of each stain alone (16bit) for ICA analysis (the colour overlay can be created from single channel images in ImageJ after transfer: Image>Color>RGB merge)

Subtract Background (BG)

For each image:

1. Convert to 8 bit (Image>type>8bit)
2. Adjust Brightness/Contrast (Use the rectangle tool to select the region of interest. Open the tool box: 'Image>Adjust>Brightness/Contrast'. Click on 'auto'). *Note: this does not affect the values of the pixels unless you press the "Apply" button*)
3. Subtract background staining (Use the rectangle tool to select a region in a blank area of the slide. Plugins>ROI>BG subtraction from ROI, accept default '3').
4. Enlarge the area of interest (Magnifying lens button; click on the centre of the area to be enlarged. The image window size can be adjusted by selecting the rim and dragging)

Select region of interest (ROI)

If the analysis is for the whole image and not a subregion skip this step for both image pairs.

1. Use one of the shape select tools (the left hand end of buttons: rectangle, oval, polygon or freehand – I find the latter the most useful for a specific cell area) to demarcate the analysis area. The tool will automatically close off the shape when the mouse button is released.
2. Save this (File>SaveAs>selection). Freehand selections can be edited by first converting it to a poly-line with the menu command Edit>Selection>Fit spline and then by dragging the nodes.
3. For the Other image Open the area shape created for the first image (File>open>****.roi; where *** depends on the shape type (rectangle, oval etc.). The ROI can also be restored with the menu command Edit>Selection>Restore Selection (keyboard shift-E).

Set threshold

1. Select the channel 1 image window by *clicking on the title bar*.
2. Open the threshold box (Image>Adjust>Threshold). The Threshold box will depict a frequency histogram of the staining intensities.
3. Adjust the lower threshold to the onset of the histogram. This can be used to cut off background staining – but only with caution since this is a value judgment. My experience is that the threshold should be set to either the base of the histogram or the lowest value. Depending on your camera settings/image brightness, this can be displaced almost to extinction to the left. Regardless, in all cases the minimum threshold must be >0. Set the upper threshold to the maximum value (255). Click on 'Set' and accept.
4. Select the channel 2 image window by *clicking on the title bar* and set the Threshold for this ROI.

Perform ICA

1. Select Image Correlation Analysis (Plugins>Colocalisation Analysis>Image Correlation Analysis)
2. Ensure that the correct two files are listed in Channel 1 and Channel 2.

3. Select “Channel 1” from the “Use ROI” drop down box.
4. Select the colour combination of your original overlay (this is just to keep things consistent)
5. Make selections of which functions you would like to use. In any case, select “Display Frequency” “Scatter Plot” and “Display ICA plots”. The PDM image is useful to identify subregions of correlated and separated staining. I also recommend selecting the PDM values. This generates a worksheet of the pixel intensity pairs that can be used in a spreadsheet at a later date.
6. Click on OK.

A number of windows will open with graphs, images or spreadsheets (the number depends on the boxes checked in the previous screen). The Results spreadsheet window summarizes the conditions, lists the number of positive and negative PDM values and also calculates the ICQ.

Save

In order to keep the results of the analysis first save the Results and PDM values files from within and then close their windows. The remaining windows can all be saved as TIFF files in a single step as follows. SaveAs>SaveAllAsTiff which opens a selection box.

This method is recommended since attempting to save the original images one at a time will actually superimposing the modified files (e.g. 16 bit to 8 bit conversion; background subtraction) on the original raw data.

Interpretation.

Please see Li *et al* for details. However, we recommend statistical analysis of ICQ values based on multiple cells, each generating a single value. This is least likely to generate spurious analyses.