



Colocalisation Analysis Tools in ImageJ

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What are you trying to colocalise?

- Size
- Shape

Resolution

Be aware of the resolution of the system you are using. If you want to analyse submicron structures, you must use an objective lens with the appropriate magnification and numerical aperture.

Wide field fluorescence versus confocal laser scanning microscopy

If you are looking at “thin” structures, e.g. 5 microns or less, then you should be able to use wide field fluorescence microscopy. For thicker specimens, confocal microscopy is necessary because of the improved axial resolution.

Dynamic range

You should use the whole dynamic range of the detector, e.g. 0-255, 0-4095. There should not be any saturation.

Potential problems

Bleedthrough/Crosstalk

- Also known as spectral overlap.
- Red and green most commonly used combination of fluorophores and in many cases, bleedthrough is a problem. If you are using Cy3 or TRITC as your “red” fluorophore, it is likely that some “red” signal is being captured in your “green” image.
- Check using a single-labelled control, that you are detecting only one label in each channel (filterblock, PMT, etc.).

- Use filters with band pass barrier filters (for green fluorophore) or if using confocal microscopy, make sure you also use a band pass barrier filter or if using the SP2, make sure the emission range is narrow.
- For confocal microscopy, use sequential acquisition not simultaneous.
- Check with controls.

Chromatic aberration

- Some objectives are not well corrected for different wavelengths.
- Focal plane may differ.
- Check using fluorescent microspheres

Pixel shift

- Due to filter blocks (dichroic filter).
- Check using fluorescent microspheres.
- Can correct for this in software.
- Will be a constant shift.

Photon (shot) noise

- Inherent in the system
- Poisson (random) distribution
- Need to optimize signal-to-noise
- Take a background image so that you can determine the degree of noise.

Background

- From autofluorescence, non-specific staining
- Need to minimize.

Uneven illumination

- Acquire background image for correction.

FRET

- Can occur as a consequence of fluorophores being too close together.

Image Processing

- Median filter: to remove noise.
- Background subtraction: to remove background or reduce it. Various methods as detailed in last seminar. Need to remove non-zero background.
- Watershed filter to separate individual objects.
- Deconvolution: can improve signal-to-noise and also improve resolution.

Controls

- Single label positive controls
- Controls for autofluorescence.
- Negative controls
- Double positive controls if possible.

Images from colour cameras

If you are using an RGB camera, then even if you have a single-labelled “green” fluorophore (e.g. FITC), you will see that the “red” channel of the image shows some signal present. In this case, it is NOT bleedthrough because there is no second fluorophore. It is actually the fluorescence from your green label because its spectrum goes into the “yellow” range as well. This means that for colocalisation analysis, you have 2 choices:

- 1) Make the whole image grayscale.
- 2) Use RGB Split and just use the green channel.

If you are intending to do colocalisation analysis but have not yet started collecting your data, then it may be better to use a monochrome camera because it has a wider dynamic range and higher sensitivity. The alternative is to use confocal microscopy. In this situation, the selection of the z position is critical and must be done in an unbiased manner. Or a z series should be collected in a systematic manner.

Use single controls to check filter blocks are suitable.

Images from confocal microscope

- Use sequential acquisition. Use single controls to check and define settings.

Colour images

- Split into individual channels.
- *Image – Color – RGB Split.*

Confocal images

Should be indexed colour or grayscale.

Monochrome images

Use directly.

Analysis Tools

RGB overlays

- Usually show colocalised pixels either in yellow or in white.
- Show spatial distribution.
- Colour shown very dependent on relative intensities of individual channels therefore vulnerable to changes in grayscale intensity due to image processing routines.

Scatter diagrams

- Similar to flow cytometric fluorograms.
- Each axis represents one label, e.g. red along x axis and green along y axis. Intensities are plotted along the axis from 0-255 (8bit images).
- Can provide information about crosstalk, bleaching, background, etc. depending on distribution shape and spread of pixels.
- Colours can be shown in “true” colour, e.g. red for red image, green for green image and colocalised pixels as yellow or as frequency diagrams with pseudocolour LUTs.

Methods that rely on user-defined thresholds

- User decides what pixel value (grayscale) is the minimum value for positive staining.
- Values below are negative or background signal.
- You can set the thresholds by examining the histograms or by using a ROI and measuring the background intensity.
- You can also capture a background image (without cells/tissue) to use as a reference.
- Subjective approach and can be difficult to reproduce.

Spatial/distance approaches (Lachmanovich 2003)

- Identifies objects as colocalised if centre of mass of one object falls within the area of another (overlap method).
- Identifies objects as colocalised if distance between the two objects centre of mass is less than the resolution (e.g. light microscopy 0.2 microns) – nearest neighbour approach.
- Less dependent on intensity.

- Randomised images can be generated to test significance.

Methods using randomised images

- Allow you to demonstrate the degree of overlap that would be expected by random chance.
- Not dependent on intensity of individual channels.

Colocalisation Plugins

Some good documentation on colocalisation is available from Tony Collins at the McMaster Biophotonics Facility http://www.macbiophotonics.ca/PDF/MBF_colocalisation.pdf Tony was previously based at WCIF but is now employed at MBF and is continuing to update his website. He has his own compilation of ImageJ with bundled plugins.

There are quite a lot of different plugins available Some give qualitative information only (e.g. RGB overlays), others are more quantitative using a range of algorithms.

- Colocalization
- ColocalizeRGB
- Colocalization Finder
- Colocalisation Thresholds
- JACoP

Colocalization

<http://rsb.info.nih.gov/ij/plugins/colocalization.html>

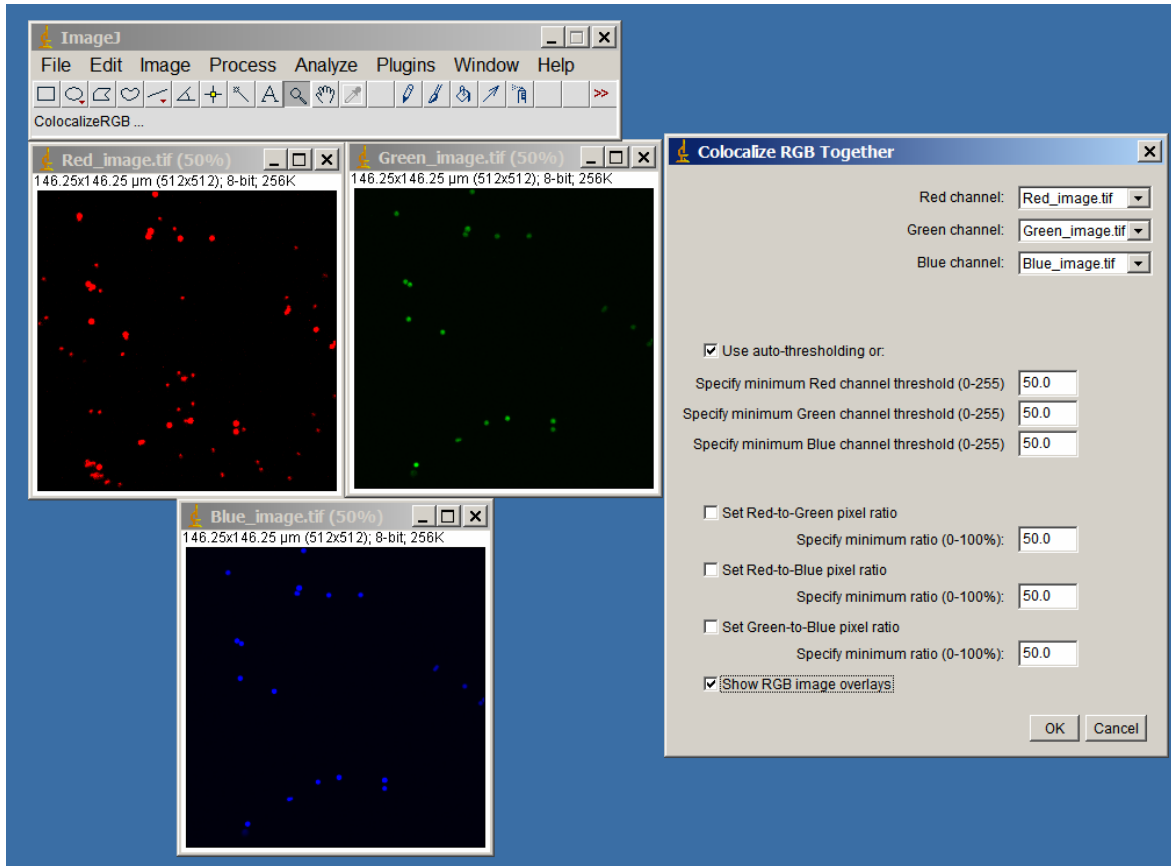
- Provides overlay images.
- Can set the thresholds.
- Works on two images.
- Also works on stacks
- Shows colocalisation if the intensity of both pixels is higher than threshold (50) and if ratio of intensity is greater than ratio setting (50%).
- Qualitative only, doesn't provide quantitative data.

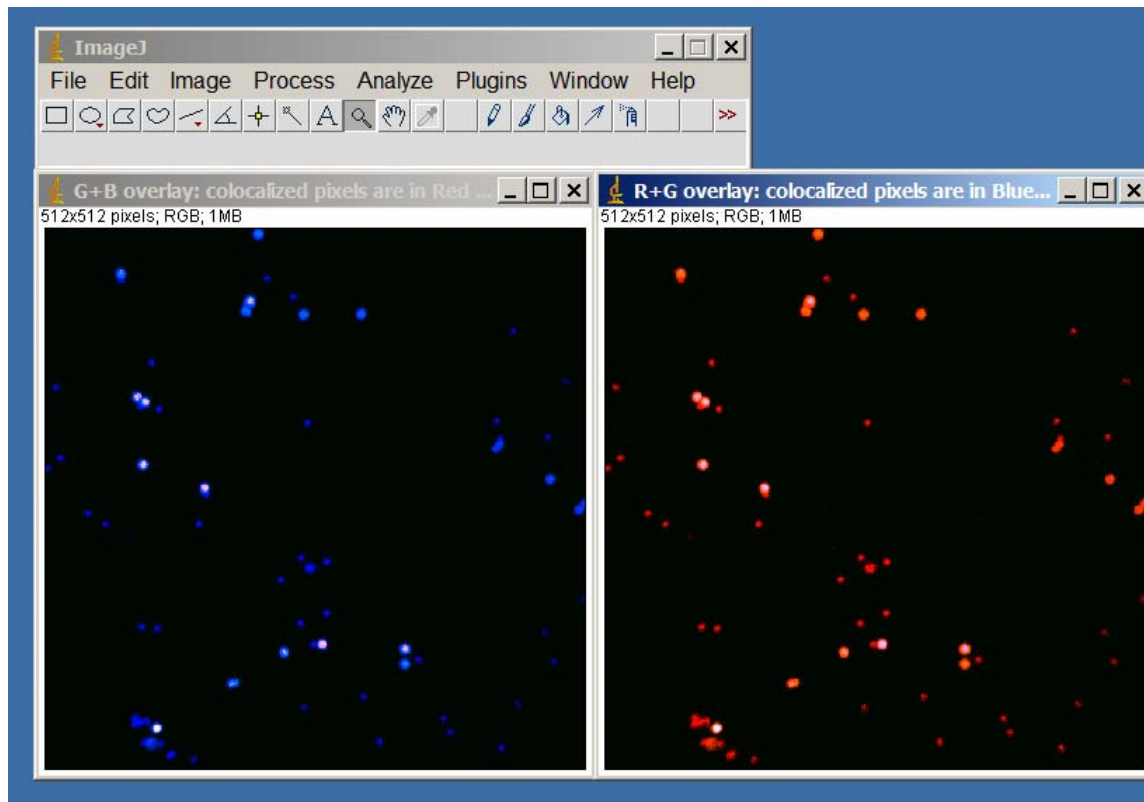
ColocalizeRGB

<http://grove.ufl.edu/~ksamn2/plugins.html#COLOC>

- Requires 3 individual images: red, green and blue (you can create a "blank" blue image if necessary).
- Works on 8bit images (colour/grayscale).

- Colocalised pixels can be displayed as white on RGB overlay images.
- Works with stacks.
- You can use automatic thresholding or select a threshold value based on your data. A minimum ratio can also be specified for each channel combination.
- Does not provide any coefficients, etc. Qualitative display only.

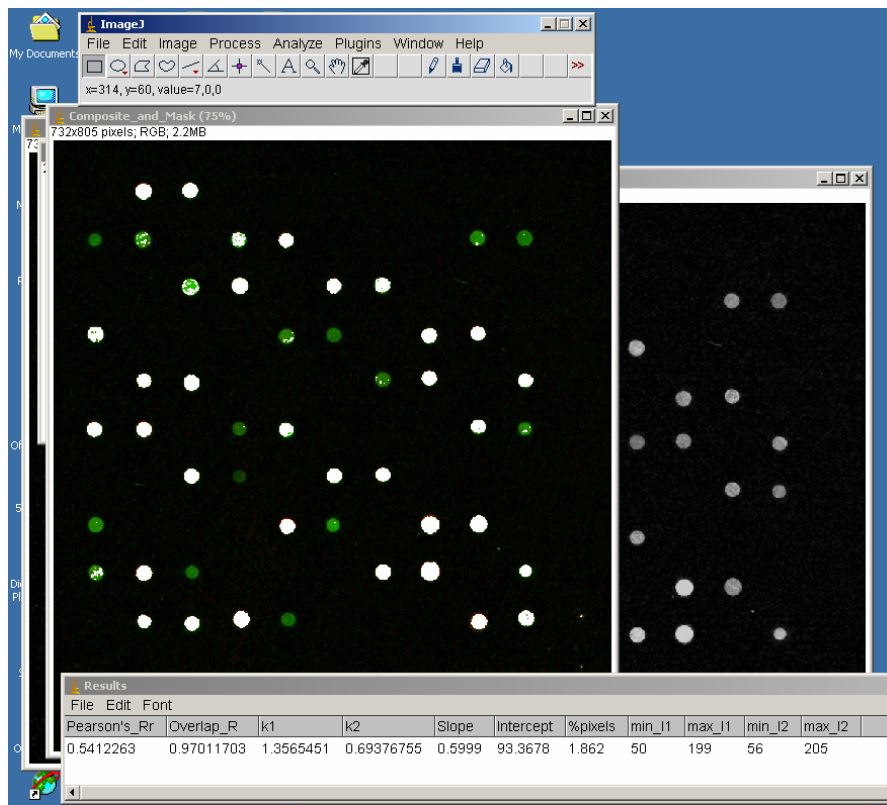




Colocalization Finder

<http://rsb.info.nih.gov/ij/plugins/colocalization-finder.html>

- Works on 2 images.
- You can make a selection using a rectangular ROI to highlight pixels on the overlay image (RGB) and if you want to, on an additional image (*Also show selected pixels on a 3rd grayscale image*). You can also limit the analysis to a minimum value (*Restrict selection to pixels of a certain ratio*).
- Provides overlay image.
- Provides Pearson's linear correlation coefficient, Overlap coefficient and Manders coefficients.

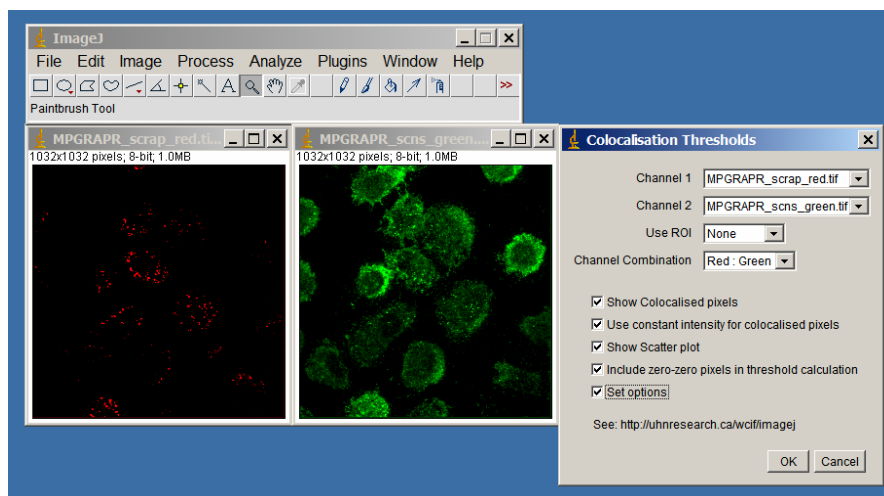


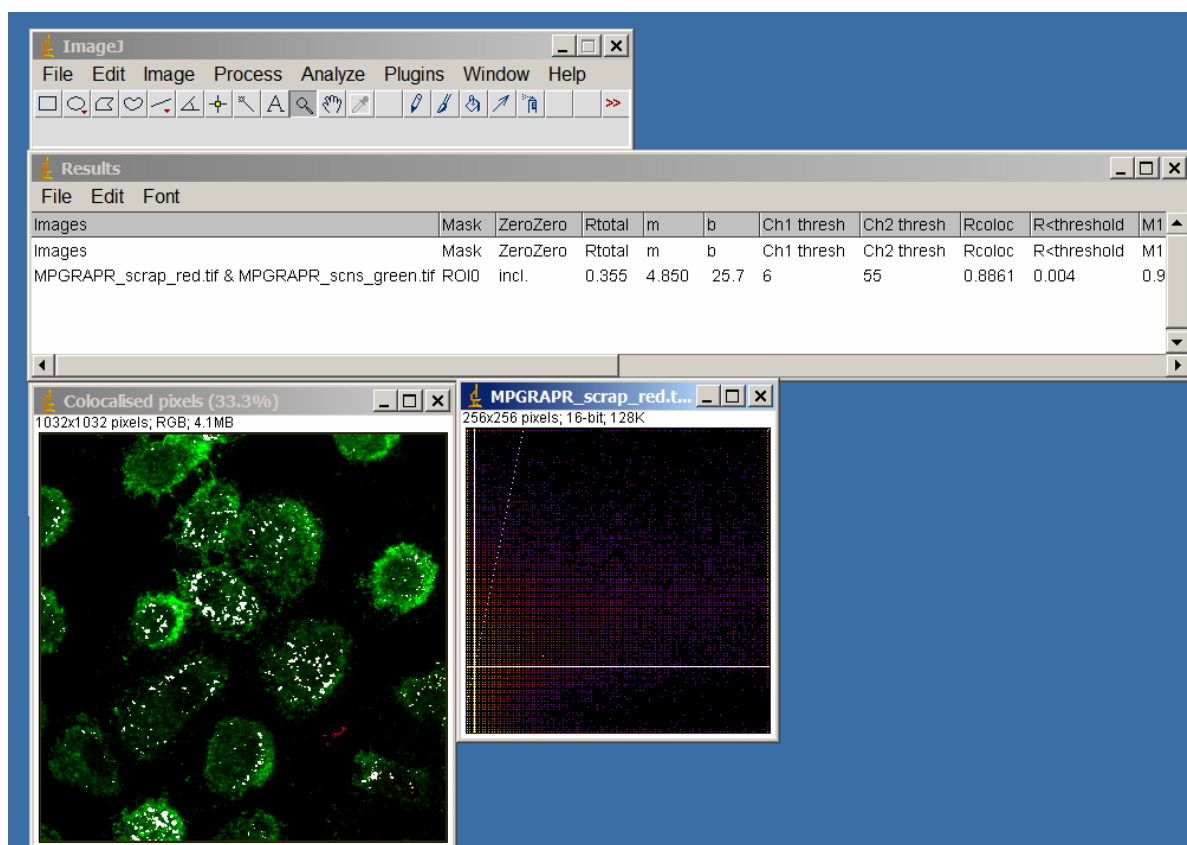
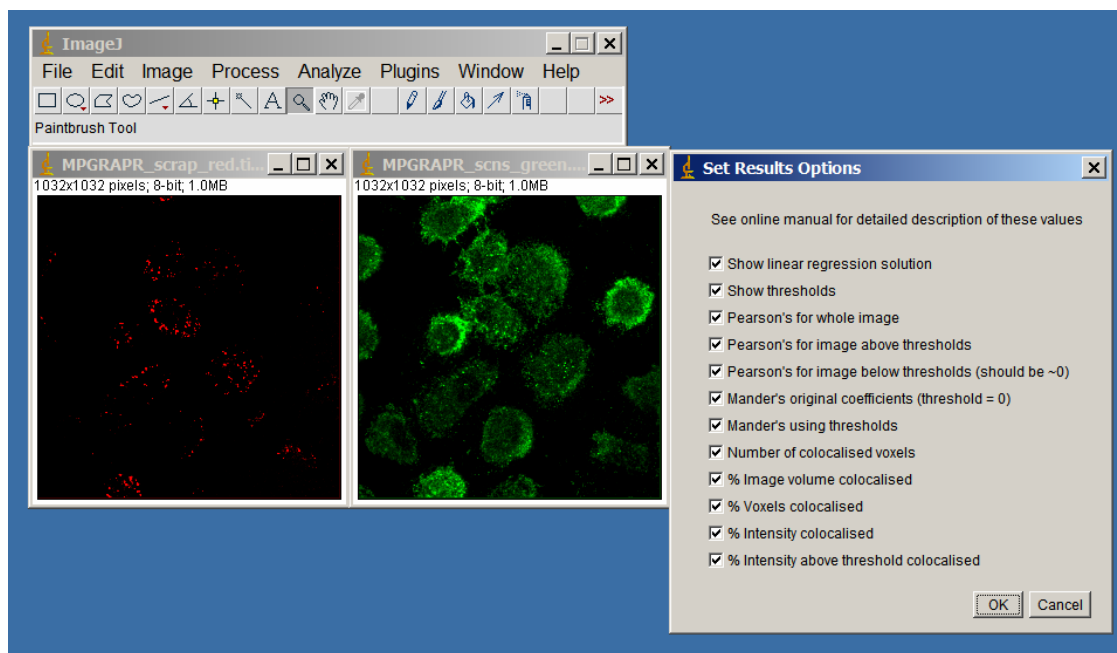
Colocalisation Threshold

http://www.macbiophotonics.ca/downloads/Colocalisation_Threshold.java

Also available within the MBF bundle (<http://www.macbiophotonics.ca/downloads.htm>)

- Works with 8bit images.
- Uses automatic thresholding.
- Provides a range of correlation coefficients & a scattergram.





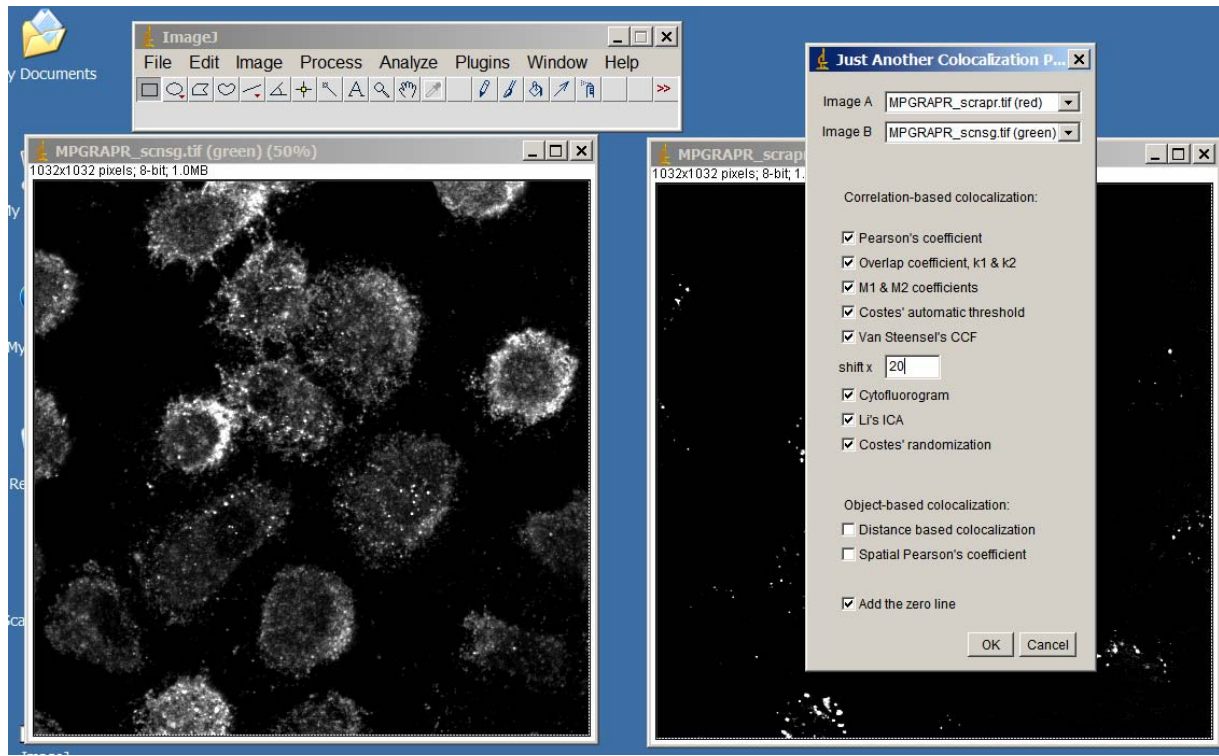
JACoP: Just Another Co-localization Plugin

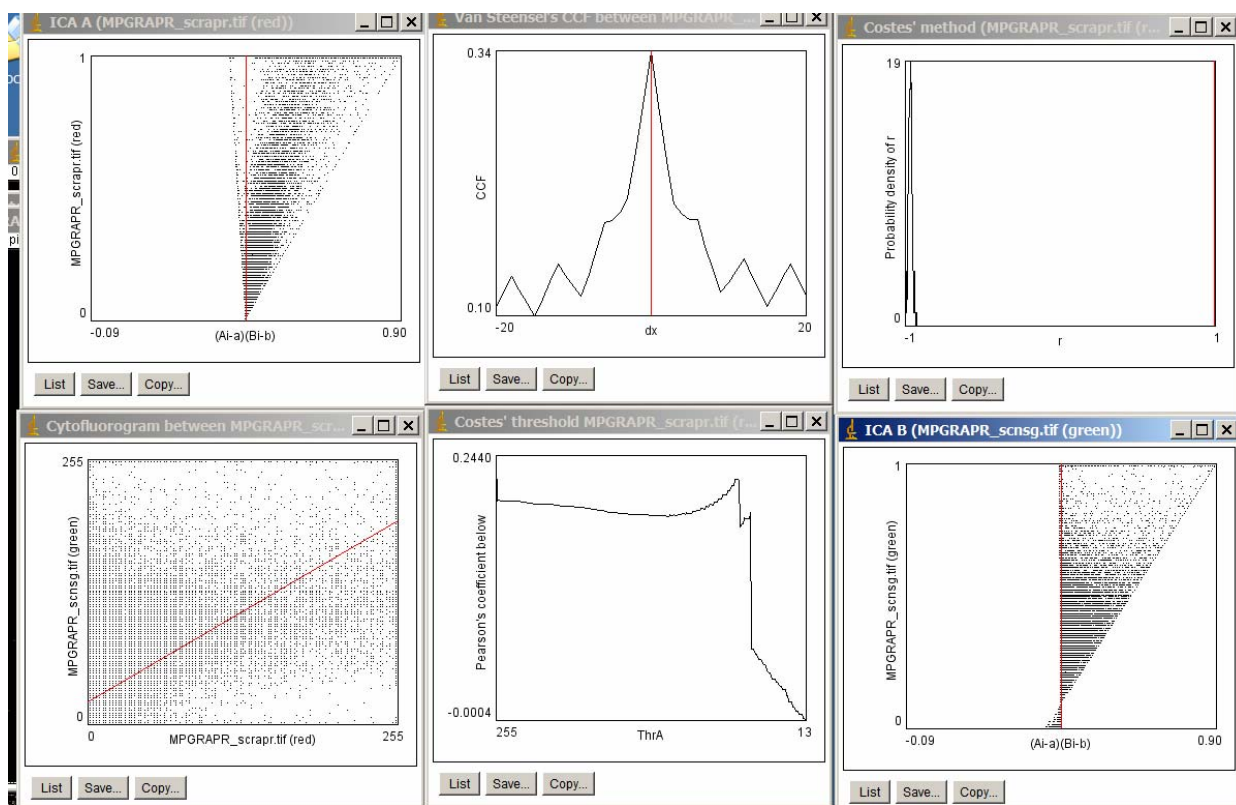
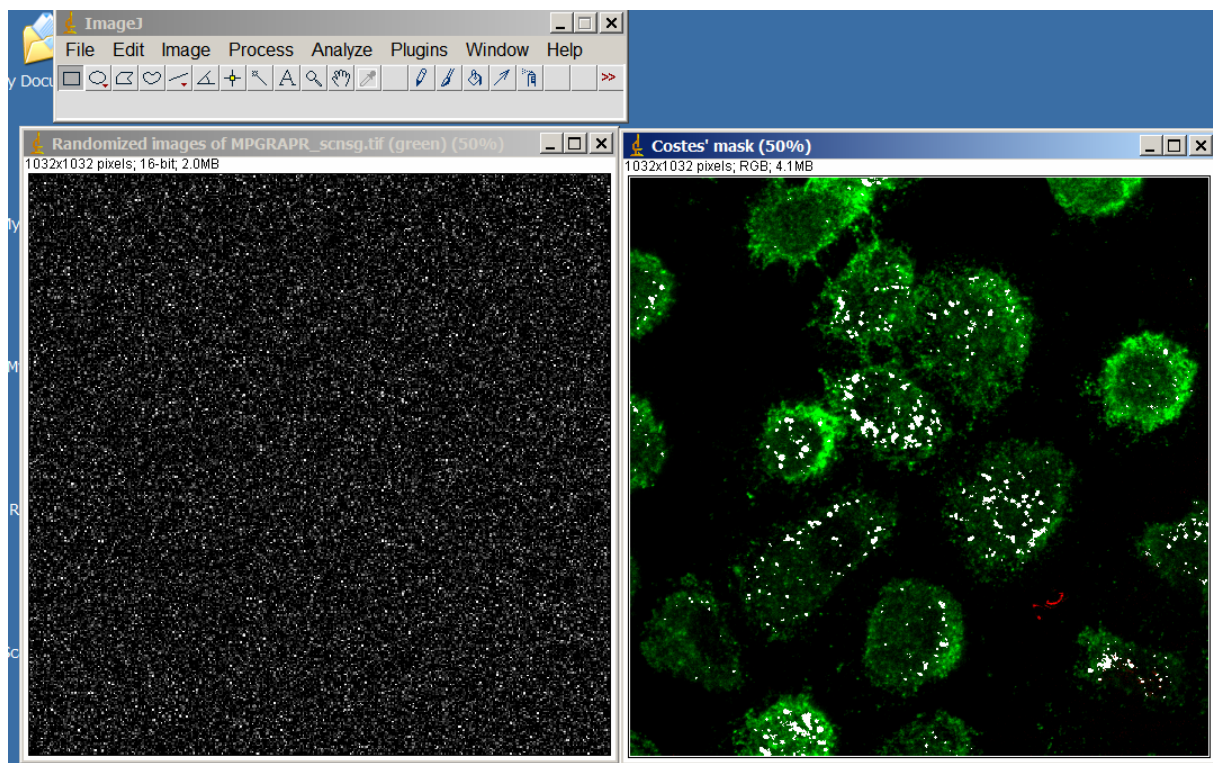
<http://rsb.info.nih.gov/ij/plugins/track/jacop.html>

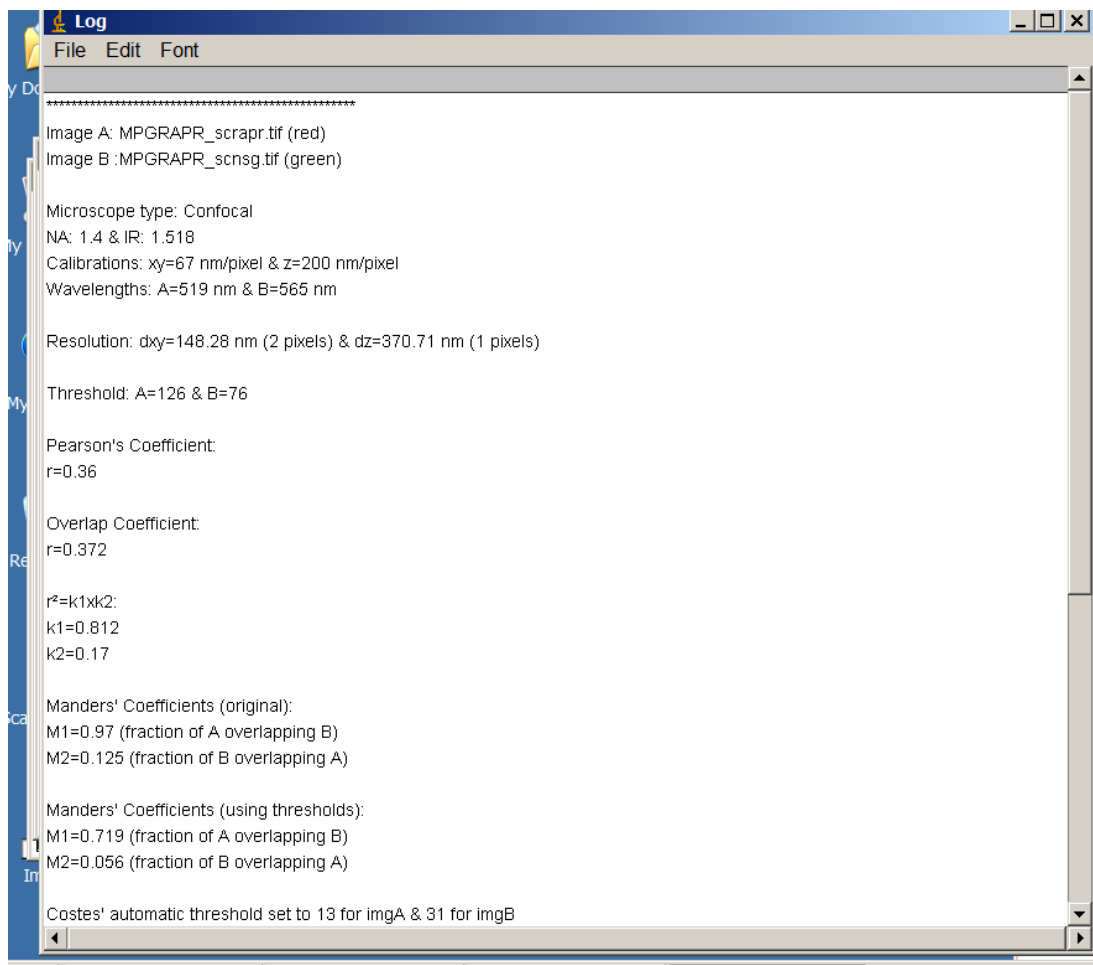
Reference: A guided tour into subcellular colocalization analysis in light microscopy, Bolte S., Cordelieres, Journal of Microscopy: 224: 213-232, 2006.

<http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2818.2006.01706.x>

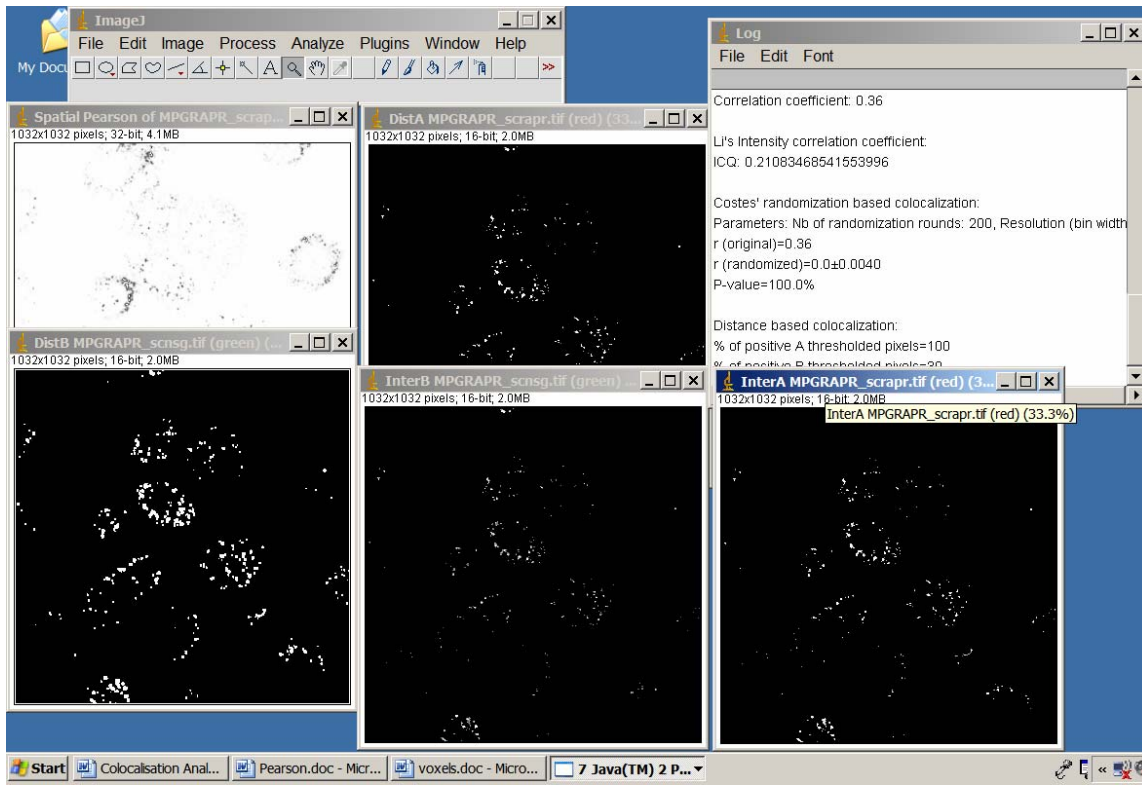
- Newly released. Comprehensive compilation of different colocalisation methodologies.
- Provides a number of different quantitative measures and correlation coefficients.







Object based analysis



Correlation coefficients

Summarised directly from: A guided tour into subcellular colocalization analysis in light microscopy, Bolte S., Cordelieres, Journal of Microscopy: 224: 213-232, 2006.

<http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2818.2006.01706.x>

Pearson's correlation coefficient

- If = 1, perfect colocalisation.
- If = 0, no colocalisation.
- If = -1 complete inverse colocalisation
- Sensitive to noise and intensity variations between fluorophores.

Manders' coefficient (overlap)

- If = 1, perfect colocalisation.
- If = 0, no colocalisation.
- Based on Pearson's coefficient.
- **M1** (proportion of green which is also red)
- **M2** (proportion of red which is also green)

Costes' approach

- Based on Pearson's coefficient.
- Estimates automatic threshold for each fluorophore and limits to this, then progressively decrements.
- Creates randomized images.
- Compares Pearson's coefficient for non-randomised with randomized and calculates significance.
- P-value (%) is inversely correlated to the probability of obtaining the specified PC by chance.
- Excludes colocalisation of pixels due to chance.
- Need to minimize noise.

Van Steensel's approach

- Based on Pearson's coefficient.

- Shifts pixels in green image in x direction relative to red image.
- Calculates Pearson's coefficient and then plots as function of pixel shift.
- Useful for small regularly shaped particles otherwise orientation may be a problem.
- Bell-shaped curve peaking at 0 = complete colocalisation.
- Partial overlap shows same shape but peak to one side.
- Complete opposite of colocalisation shows a dip at $dx = 0$.
- Intensity affects height of curve.

Li's approach

Overall difference of pixel intensities from the mean intensity of a single channel = 0.

Product should tend to zero.

Colocalising pixels, product should be positive as each difference from the mean is of the same sign.

Fits the histogram of both images to 0-1 scale (intensity).

Intensity correlation analysis = 2 graphs showing normalized intensities (0-1) as a function of the product for each channel.

X axis = covariance of current channel and y axis = intensity distribution of current channel.

If there is colocalisation, then the product is positive so dot cloud should be on the right side of the $x = 0$ line although adopting a C shape.

Spread is dependent on intensity distribution of the current channel as a function of covariance of both channels intensities.

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