

Topic

The Colocalization module provides the facility to visualize and quantify colocalization between pairs of images. The Colocalization window contains a preview of the two images overlaid, a scatter plot and a display of statistical analysis. This technical note describes the use of this dialog and the Automator tasks for colocalization.

The Colocalization module is available as an option with Openlab 3.1 and higher.

Discussion

What is colocalization?

Colocalization in the context of digital fluorescence imaging can be described as the detection of signal at the same pixel location in each of two images. The two images are two different fluorescent channels of the same sample area.

Viewing colocalization

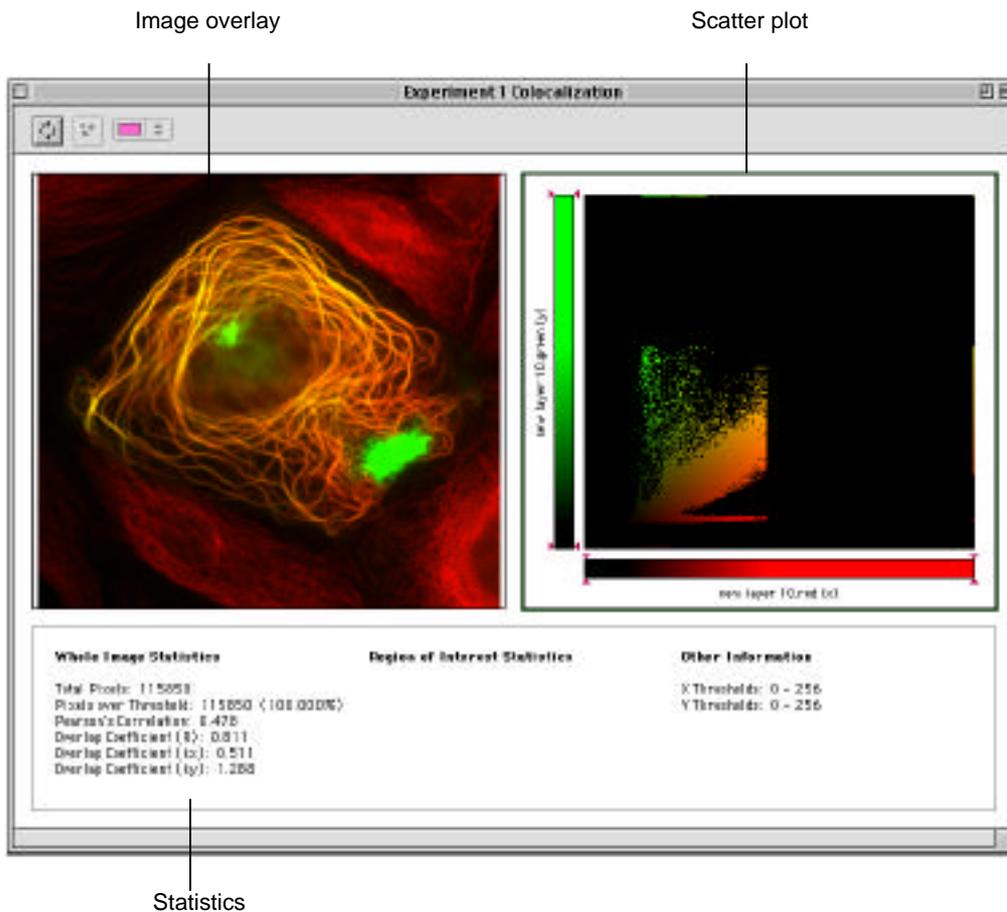
In the context of this Technical Note, a pair of images is considered to be two grayscale images of the same sample location imaged with two separate fluorescence channels. It will be much easier to visualize colocalization if each image has a different colour look up table applied.

The images that you wish to analyze must be in the same Openlab image document. Begin by selecting the pair of images in the Layers Manager.



Select **Show Colocalization** from the Image menu.

The Colocalization window, unique to the image document, appears:



The Colocalization window

The window consists of 3 parts:

Overlay image

The two channels superimposed. For images with different CLUTs applied colocalization will appear as a new color in this image, for example red and green will make yellow. It is also in this image that the binary mask, created when an ROI is drawn on the scatter plot, will be previewed.

Scatter plot

The scatter plot is a graphical representation of the intensity values found within the images.

One image of the pair is assigned the x axis, and the other image is assigned the y axis. Each axis represents the ascending gray levels of the images.

For example if a pixel in image x has an intensity value of 100 and the corresponding pixel in image y has an intensity value of 30, a point will be plotted at co-ordinates 100,30. It does not matter how many pixels in the image pair have intensity values of 100,30, only one point will be plotted on the scatter plot.

Two perfectly colocalized images, i.e. identical, will generate a scatter plot where the points fall in a line at 45 degrees to either axis.

Statistics

Pearson's Correlation and Overlap Coefficients are generated for the whole image and Colocalization Coefficients for a region of interest drawn on the scatter plot. See Calculating colocalization statistics section below for more information.

Using the Colocalization window

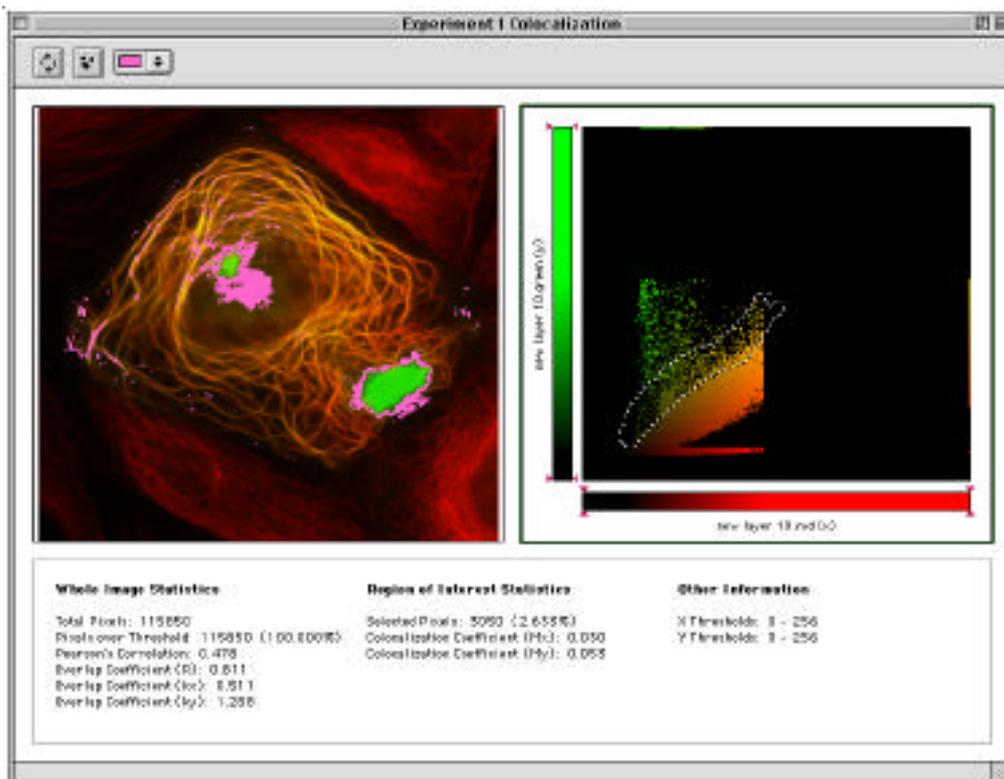
The first step in using the colocalization windows is to set thresholds for each axis so that the lower threshold is above background. It may not be necessary to change the upper threshold. If thresholds are not set pixels where both images show background intensity levels will be interpreted as colocalized.

Set thresholds by dragging the points on the color ramp for each image.

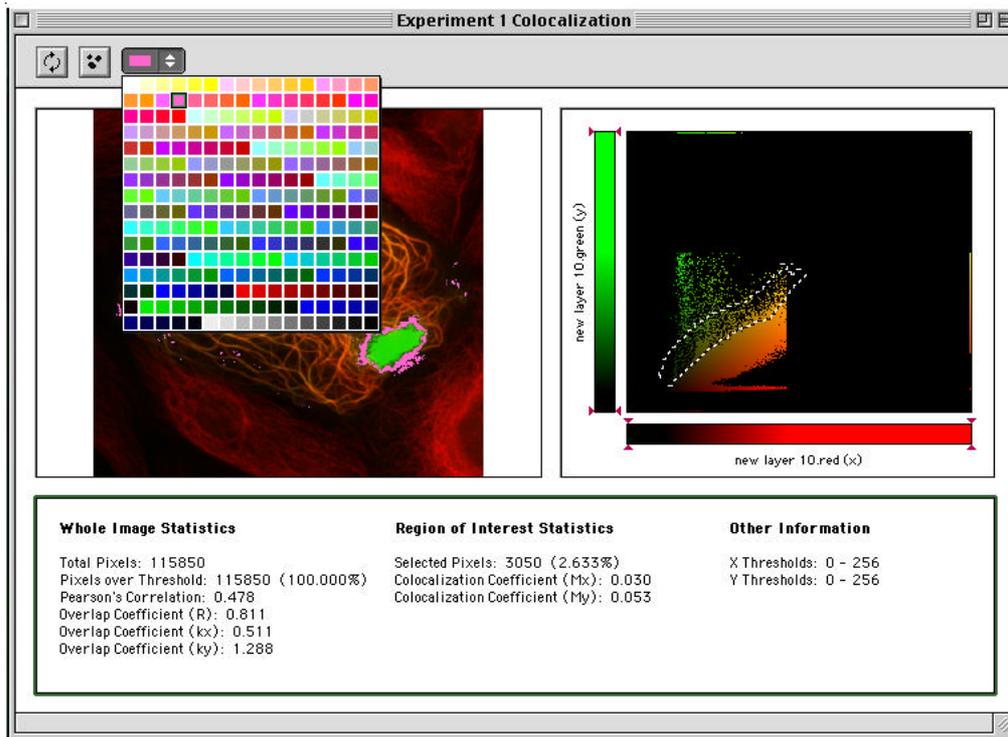
Any pixels of intensity outside threshold will be removed from the overlay image for that channel. Any point on the scatter plot that is outside threshold for either channel will be removed from the scatter plot leaving only points which are positive for both x and y, i.e.colocalized.

After setting thresholds select the whole of the scatter plot (Select all from the Edit menu or Apple A). A colored, binary mask is produced showing the areas of colocalization over the overlaid images.

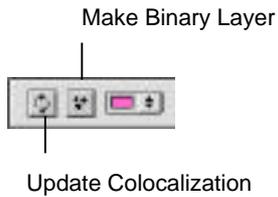
When the cursor is placed over the scatter plot it becomes a freehand ROI tool. Use this to select a portion of the colocalized pixels and view their location in the overlaid images. Click and drag the mouse to draw an ROI. Use shift-click and drag to add to the ROI, use shift+option-click to subtract from the ROI. Click outside the ROI without moving the mouse to deselect it.



Select the color of the binary mask using the color picker at the top of the window:



Once satisfied with the selection made it is possible to create a binary layer in the image document by clicking on the make Binary Layer button.



This binary layer can then be used with Openlab measurements to provide more detailed morphological and intensity information on the regions selected. The Advanced Measurements module is required for this type of analysis.

Calculating the statistics

The statistics presented in the Colocalization window are calculated as follows:

x_i = intensity of pixel i in image x unless x_i is outside threshold in which case $x_i = 0$

y_i = intensity of pixel i in image y unless y_i is outside threshold in which case $y_i = 0$

$$\text{Pearson's Correlation} = \frac{\sum_i (x_i - x_{\text{aver}}) \cdot (y_i - y_{\text{aver}})}{\sqrt{\sum_i (x_i - x_{\text{aver}})^2 \cdot \sum_i (y_i - y_{\text{aver}})^2}}$$

Pearson's Correlation is a statistical test for a linear relationship between two variables. Pearson's Correlation can vary between -1 and 1 . A correlation of 0 means there is no linear relationship between the variables. -1 and 1 indicate perfect negative and perfect positive linear relationships respectively. Interpreting the degree of colocalization from negative values is difficult.

$$\text{Overlap Coefficient (R)} = \frac{\sum_i x_i \cdot y_i}{\sqrt{\sum_i (x_i)^2 \cdot \sum_i (y_i)^2}}$$

$$\text{Overlap Coefficient (kx)} = \frac{\sum_i x_i \cdot y_i}{(\sum_i x_i)^2}$$

$$\text{Overlap Coefficient (ky)} = \frac{\sum_i x_i \cdot y_i}{(\sum_i y_i)^2}$$

$$\text{Colocalization Coefficient (Mx)} = \frac{\sum_i x_{i,\text{coloc}}}{\sum_i x_i}$$

$$\text{Colocalization Coefficient (My)} = \frac{\sum_i y_{i,\text{coloc}}}{\sum_i y_i}$$

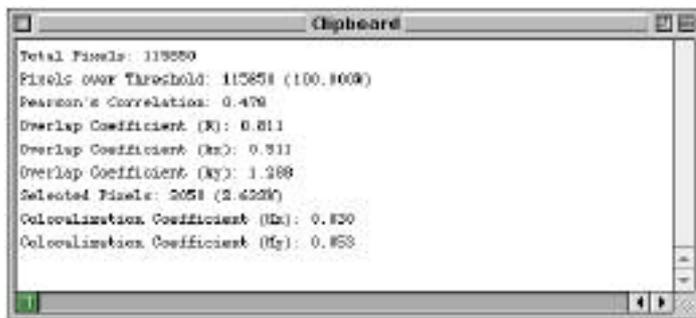
Where $x_{i,\text{coloc}} = x_i$ if y_i is within the intensity range defined by the ROI, $x_{i,\text{coloc}} = 0$ if y_i is outside the intensity range and $y_{i,\text{coloc}} = y_i$ if x_i is within the intensity range defined by the ROI, $y_{i,\text{coloc}} = 0$, if x_i is outside the intensity range.

Colocalization Coefficients may be values between 0 and 1. 0 indicates that none of the signal within thresholds in that channel exists as colocalized with the other channel. A value of 1 indicates that all of the signal within thresholds in that channel exists as colocalized with the other channel.

For further explanation of these statistics see the reference given at the end of this technical note.

Exporting from the Colocalization window

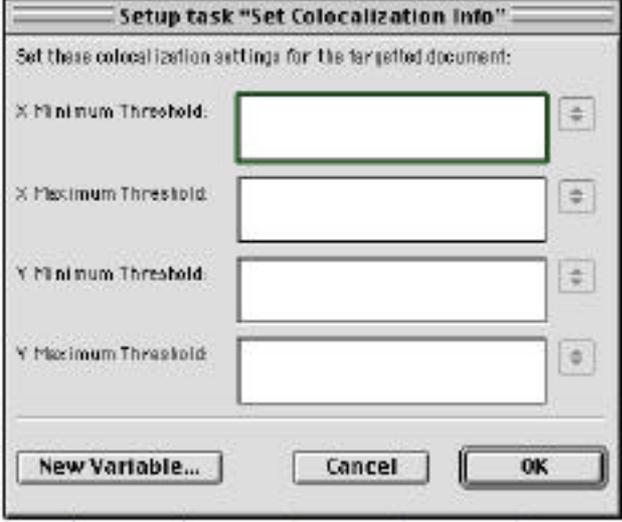
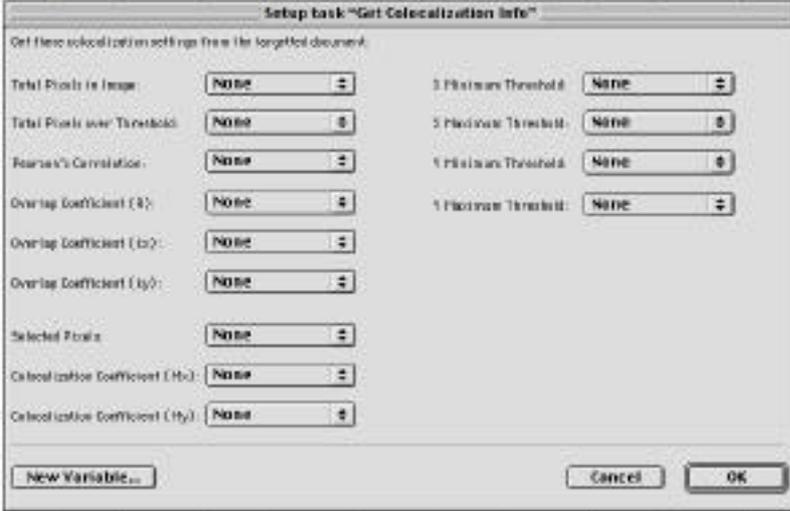
At any point, while working with the Colocalization window, the overlay image, scatter plot or statistics may be copied to the clipboard. Click on the relevant area of the window to highlight it and chose **Copy** from the Edit menu or Apple C. These can then be pasted into any application that accepts pasted text and images.



To view the colocalization of a different pair of images select the new pair of images in the Layers Manager and choose **Update colocalization** from the Image menu or click the Update colocalization button at the top of the colocalization window.

Colocalization Automator tasks

The following Automator tasks are available within the Automator under the **Colocalization** section heading.

 <p>Update Colocalization</p>	<p>Update colocalization</p> <p>This task has no set up dialog. Following image selection it will prompt the generation of overlay image, scatter plot and statistics. Update Colocalization must be performed in an automation for each pair of images before Colocalization information is extracted.</p>
 <p>Set Colocalization Info</p>	<p>Set Colocalization info</p> <p>This task sets the x and y thresholds on the scatter plot.</p> 
 <p>Get Colocalization Info</p>	<p>Get Colocalization Info</p> <p>Store any of the colocalization statistics produced as variables.</p> <p>For Selected pixels and Correlation coefficients a ROI must be drawn on the Colocalization window. This cannot be done by the Automator.</p> 

 <p>Make Colocalization Overlay</p>	<p>Make Colocalization overlay</p> <p>Makes the current colored overlay in the overlay image into a binary layer in the image document. This requires an ROI drawn on the scatter plot. This cannot be done by the automator.</p>
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Use the Select Layers tasks to choose the layers to be colocalized within the automation.

Important considerations

It is important to have adequate separation of the two fluorescence signals, with no overlap between emission of one and excitation of the other. This affects choice of filters as well as fluorochromes themselves.

The Colocalization window can only analyze the pixel values presented in the selected images. It is the users responsibility to ensure the images are valid for the study they are carrying out.

When analyzing 2D images users should be aware that fluorescence from other planes within their sample may be affecting the apparent degree of colocalization. Users may wish to deconvolve images before processing. The Openlab module 3D Restoration is appropriate for this application since it uses iterative deconvolution preserving pixel intensity values.

The module assumes that there has been no pixel shift between the capture of the two channels and that they are perfectly aligned. The Openlab module Registration allows users to correct for measured pixel shift between channels.

Adequate controls are also essential to avoid the possibility of false colocalization, for example generated by non-specific labelling.

References

M.M. Manders, P. J. Verbeek & J. A. Aten

Measurement of co-localization of objects in dual colour confocal images

Journal of Microscopy. Vol. 169, Pt 3, March 1993, pp. 375-382