

Analysis of Colocalization using Metamorph

There are many different methods for colocalization and one approach does not necessarily fit all circumstances. Begin by consulting the literature in your field to see what others have done. Two methods of Colocalization analysis available as plugins in Metamorph, are described in this handout: Correlation Plot and Measure Colocalization. To access additional help from within Metamorph, open the dialogue box for the function and then choose Help: Help on Current Function. Note that these plugins are not included in basic versions of Metamorph. Use Metamorph on In-Neph-Image4 to access these plugins.

I. COLOCAL – Measure colocalization

The Measure Colocalization Metamorph plugin provides quantitative data about regions of overlap of two fluorescent probes in an image pair. The area, average intensity, and integrated intensity in the region of overlap can be measured and saved to a log file.

Use this command to display and save measurements of the area of overlap between two fluorescent probes, or the average or integrated intensities in the region of overlap. Typically, two source images are used. The same view of the preparation must be used for both images, but they should be acquired at different excitation or transmission wavelengths, as appropriate for the respective probes being used. If desired, you can use a Region Tool to define and select a specific region of interest for measurement. This should be defined in the Source A image. Both source images must be thresholded prior to performing the measurement.

Note: Areas of overlap will be expressed in pixels. To express the area in "real" units (square microns, millimeters, etc.), use Calibrate Distances before using this command.

How to use **Measure Colocalization**

1) *Open Images*

Open the image pair whose regions of overlap of two probes you want to compare. Image stacks or single image planes can be used. Analysis of image stacks is performed plane by plane.

Separate the channels of data if necessary using Display: Color Separate. (zeiss.lsm images)

Build stacks from individual tiffs if necessary using File: Build Stack.

2) *Subtract Background (Sometimes optional. For example, fluorescent bead images segment cleanly without this preprocessing.)*

Calculate a background for each image using a median filter of 32 x 32 using Process: Median Filter. This step is very slow (~ 15 sec/frame).

Be sure to select all planes if working with a stack. Set subsample ratio to 1.

The output will be named Median for the first image processed and Median2 for the second image processed. Keep track of which processed images go with which original images.

Subtract the background (median) images from the original images using Process: Arithmetic.

Choose operation Subtract. Choose the original image as Source 1 and the median image as Source 2. Result Depth depends on the bit depth of the input images and the operation performed. Set second source to image. Set Value to zero. The output will be named Subtract for the first image set processed and Subtract 2 for the second image set processed. The results of this step are the background subtracted images.

3) *Apply median filter (Sometimes optional. Low Pass filter may be used instead of median. This step may be done before background subtraction.)*

Some images require application of a median filter to further reduce noise before thresholding. If thresholding to highlight labeled structures is still difficult even after background subtraction, apply a median filter.

Choose Process: Median Filter and set the filter size to 3 x 3. Be sure to select all planes if working with a stack. Set subsample ratio to 1. The output will be named Median for the first image processed and Median2 for the second image processed. Keep track of which processed images go with which original images.

4) *Set threshold*

Thresholds must be set for both images for this analysis. Keep in mind that user choice of threshold level affects the results of this colocalization test. It is useful to keep a copy of the original stack open for comparison when choosing thresholds. Compare the thresholded regions to the regions of interest in the original image.

Click the bottom icon in the menu bar at the left side of the image that you wish to threshold. Choose Inclusive Threshold. The image will become red and the bottom portion of the menu bar will become red. Two sliders will become available in the left menu bar. Make sure that the top slider is set to the max position. Then, drag the bottom bar up to set the image threshold.

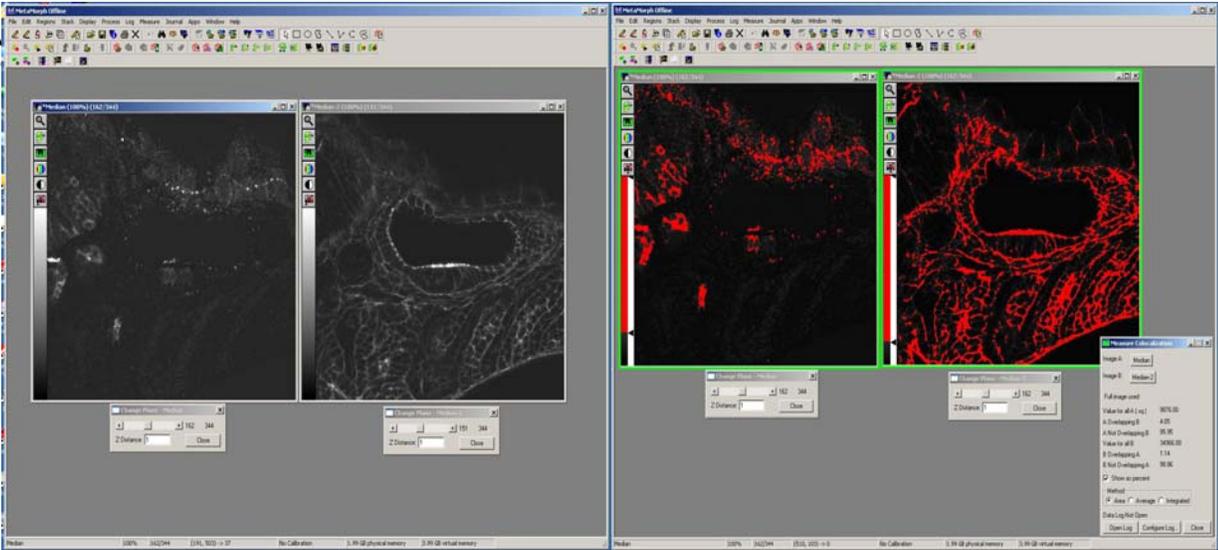
If you have skipped some of the optional steps and find that it is difficult to set the threshold level to show only the localization of your probe, then go back and include the optional steps.

5) Calculate the area of overlap – Measure Colocalization

Choose Apps: Measure Colocalization. Within the Measure Colocalization dialogue box select the images that you want to compare as Image A and Image B. If you are working with a stack, be sure that both images are set on the same plane. For this analysis, you can use the entire image plane, or you can define a region, such as a single cell or part of a cell within the image plane. The results of the test are shown in the Measure Colocalization dialogue box.

Colocalization can be analyzed either in an entire single plane, or in regions within a single plane. Regions can be created using region selection tools and must be created within the image that you have specified as Image A. When using regions, analysis is performed on the active region. A region is active if the outline is hashed and flashing.

Example 1.

Show 4.05  label B is

6) Log Data

Region of overlap colocalization data can be calculated and logged from an entire image plane or from regions within an image plane to an Excel spreadsheet.

Open Microsoft Excel. In the Measure Colocalization dialogue box, choose Open Log. In the Open Data Log dialogue, choose Dynamic Data Exchange (DDE) and click OK. In the Export Log Data dialogue, click default, then click OK. In the Measure Colocalization dialogue box, choose Configure Log. Enable desired parameters. Before logging the first dataset the Log Column Titles box should be checked to generate column titles. Choose F9:Log Data. Data will appear in the Excel spreadsheet with column titles. After logging the first dataset, choose Configure Log again. Uncheck the Log Column titles box. Subsequent data will be logged without repeating the column titles.

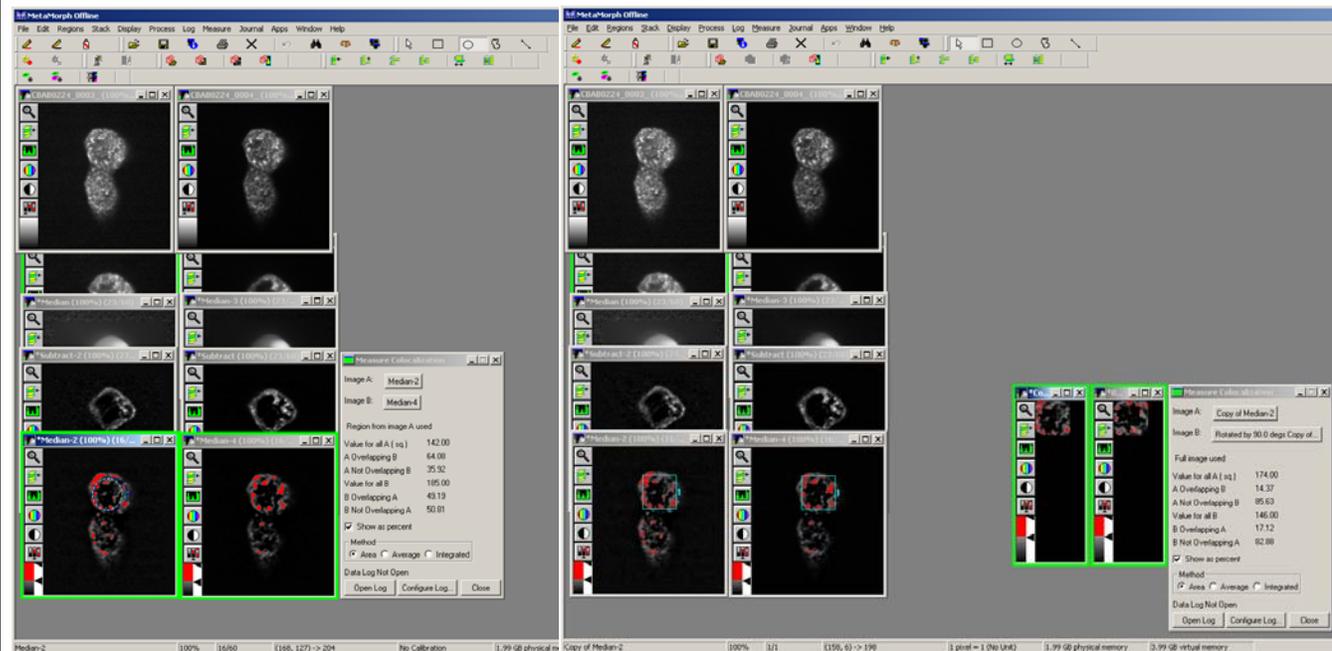
7) Are these percentages of overlap meaningful? Do I have colocalization?

A good reality check for the numbers generated by your data is to rotate one member of the pair of images by 90 degrees and calculate percent overlap again.

Using Region Tools, select a square or a round region within a cell. Choose Regions: Transfer Region to select the corresponding region in your second image. (Why square or round? Because you can rotate these shapes and still have total overlap.) Choose Display: Rotate. For one image rotate the region 0 degrees, for the other image rotate the image 90 degrees. The output will be the region of interest, rotated by the indicated amount. Measure colocalization in the output images. Compare these results to the results obtained when the images were in the correct orientation. Are the area of overlap values significantly different from the area of overlap values obtained in the original orientation? Are your area of overlap values greater than would be expected by random overlap of two unrelated probes?

Example 2

Shown below is a single slice from a stack of images stained with two labels that DO colocalize. Analysis of the images is shown in the left screenshot. The percentage of label A overlapping label B is 64.08%. The percentage of label B overlapping label A is 49.19%. In the screenshot on the right, a region within the cell has been rotated 90 degrees in one image of the image pair and the analysis is repeated. After roatation, the percentage of label A overlapping label B is 14.37% and the percentage of label B overlapping label A is 17.12%. The percentage overlap dropped when one image was rotated 90 degrees with respect to the other. If your percentage overlap is similar after rotating one image, you may not have colocalization.



II. CORR PLOT – Correlation Plot

The Correlation Plot Metamorph plugin measures and displays the correlation between the intensities of corresponding pixels in two images and provides a correlation coefficient (r) of the pixel intensity data

The intensity values of corresponding pixels in two images are used as the X and Y coordinates in the scatterplot. The Correlation Plot dialog box also displays the correlation coefficient (r) of the data. This is defined as

$$r = \frac{\sum xy}{NS_x S_y}$$

where

r = correlation coefficient,

xy = product of deviation scores,

N = sample size,

Sx = standard deviation of X (intensities in first image), and

Sy = standard deviation of Y (intensities in second image).

The range of values of the correlation coefficient is -1.0 to +1.0. A value of 1.0 shows that the data are perfectly correlated with one another. This will only happen if the two images are identical. A correlation coefficient of -1.0 is observed when there is an inverse relationship between intensities in the two images.

How to use **Correlation Plot**

1) *Open Images*

Open the image pair whose correlation coefficient you want to calculate. Image stacks or single image planes can be used.

Separate the channels of data if necessary using Display: Color Separate. (zeiss.lsm images)

Build stacks from individual tiffs if necessary using File: Build Stack.

2) *Calculate correlation coefficient.*

For this analysis, you can use the entire image plane, or you can define a region, such as a single cell or part of a cell within the image plane. Thresholding is not required, but can be used. Pixels that have intensities that are outside of the threshold range of both images will be excluded from the measurement. If thresholding is turned off in either image, all pixels in the active region will be measured. Thresholding will affect measurement of the correlation coefficient. (See details of how to process images for thresholding in the instructions for how to use the Measure Colocalization plugin.) If you have applied thresholding and want to restrict the data analysis to pixels that have not been excluded from both images by thresholding, select the Use Thresholds check box. (Pixels that are included in the threshold range in either image will be measured.) The scatterplot and displayed correlation coefficient will be updated automatically.

Choose Apps: Correlation Plot. Within the Correlation Plot dialogue box, Select the images that you wish to compare to be represented on the X and Y axis of the correlation plot. If you are working with a stack, be sure that both images are set on the same plane. A Correlation Plot is generated and the value of r, the correlation coefficient, is shown in the Correlation Plot dialogue box. To save the Correlation Plot scatterplot image, select the image and Save As a tif image.

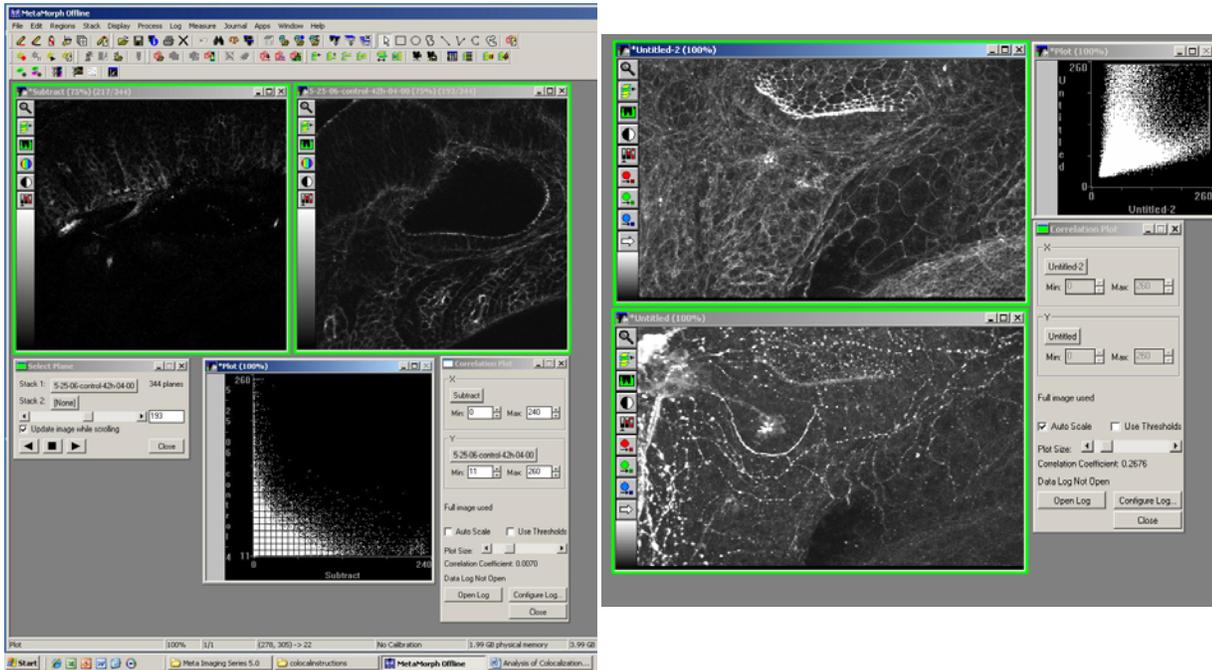
3) *Log Data*

Correlation Plot colocalization data can be calculated and logged from an entire image plane or from regions within an image plane to an Excel spreadsheet.

Open Microsoft Excel. In the Correlation Plot dialogue box, choose Open Log. In the Open Data Log dialogue, choose Dynamic Data Exchange (DDE) and click OK. In the Export Log Data dialogue, click default then click OK. In the Correlation Plot dialogue box, choose Configure Log. Enable desired parameters. Before logging the first dataset the Log Column Titles box should be checked to generate column titles. Choose F9:Log Data. Data will appear in the Excel spreadsheet with column titles. After logging the first dataset, choose Configure Log again. Uncheck the Log Column titles box. Subsequent data will be logged without repeating the column titles.

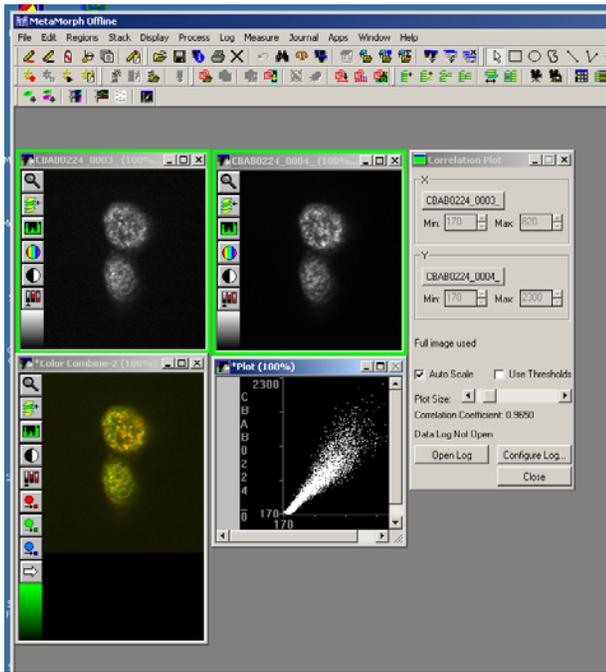
Example 3

Shown below are two examples of labels that do not colocalize. The correlation coefficient of the pair on the left is 0.007. The correlation coefficient of the pair on the right is 0.26.



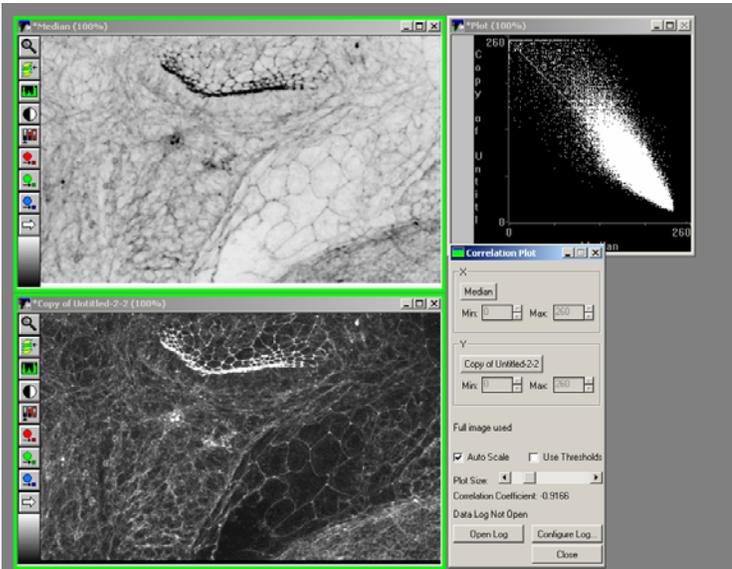
Example 4

The screenshot below shows two labels that DO colocalize. The correlation coefficient is 0.96. Also that the scatterplot is nearly linear and increasing.



Example 5

The screenshot below shows two labels that are inversely correlated. The correlation coefficient is -0.97. Note the linear decreasing shape of the correlation plot.

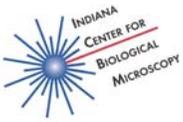


4) Are these values meaningful? Do I have colocalization?

A good reality check for the numbers generated by your data is to rotate one member of the pair of images by 90 degrees and calculate percent overlap again.

Using Region Tools, select a square or a round region within a cell. Choose Regions: Transfer Region to select the corresponding region in your second image. (Why square or round? Because you can rotate these shapes and still have total overlap.) Choose Display: Rotate. For one image rotate the region 0 degrees, for the other image rotate the image 90 degrees. The output will be the region of interest, rotated by the indicated amount. Measure Correlation Coefficient. Compare these results to the results obtained when the images were in the correct orientation.

One approach for analysis of colocalization may not fit all circumstances. Spend time critically reading the literature in your field to see how others process and interpret colocalization data. Read the literature about colocalization analysis. This is your data analysis and you are ultimately responsible for how you interpret and present your data.



Some Colocalization References that may be helpful

- Comeau, J.W., S. Costantino, and P.W. Wiseman. 2006. A guide to accurate fluorescence microscopy colocalization measurements. *Biophys J.* 91:4611-4622.
- Costantino, S., J.W. Comeau, D.L. Kolin, and P.W. Wiseman. 2005. Accuracy and dynamic range of spatial image correlation and cross-correlation spectroscopy. *Biophys J.* 89:1251-1260.
- Costes, S.V., D. Daelemans, E.H. Cho, Z. Dobbin, G. Pavlakis, and S. Lockett. 2004. Automatic and quantitative measurement of protein-protein colocalization in live cells. *Biophys J.* 86:3993-4003.
- Lachmanovich, E., D.E. Shvartsman, Y. Malka, C. Botvin, Y.I. Henis, and A.M. Weiss. 2003. Co-localization analysis of complex formation among membrane proteins by computerized fluorescence microscopy: application to immunofluorescence co-patching studies. *J Microsc.* 212:122-131.
- Landmann, L. 2002. Deconvolution improves colocalization analysis of multiple fluorochromes in 3D confocal data sets more than filtering techniques. *J Microsc.* 208:134-147.
- Manders, E.M., J. Stap, G.J. Brakenhoff, R. van Driel, and J.A. Aten. 1992a. Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy. *J Cell Sci.* 103 (Pt 3):857-862.
- Manders, E.M., F.J. Verbeek, and J.A. Aten. 1992b. Measurement of coLocalization of objects in dual-colour confocal images. *Journal of Microscopy.* 169:375-382.
- Nohe, A., and N.O. Petersen. 2004. Analyzing protein-protein interactions in cell membranes. *Bioessays.* 26:196-203.
- Smallcombe, A. 2001. Multicolor imaging: the important question of co-localization. *Biotechniques.* 30:1240-1242, 1244-1246.
- Taneja, K.L., L.M. Lifshitz, F.S. Fay, and R.H. Singer. 1992. Poly(A) RNA codistribution with microfilaments: evaluation by in situ hybridization and quantitative digital imaging microscopy. *J Cell Biol.* 119:1245-1260.