Colocalization of Fluorescent Probes Using Image-Pro® Plus v. 5.0

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1. Introduction

Fluorescence labeling techniques are quite useful to microscopists. Not only can fluorescent probes label sub-cellular structures with a high degree of specificity, they can also do so with a very high degree of sensitivity. This combination of specificity and sensitivity permits observations of cellular events that are not always visible using traditional methodologies, such as brightfield microscopy.

Labeling specimens can occur using one or more fluorochromes. Single-wavelength fluorochromes are often used as labels to reveal structures, such as microtubules, mitochondria or nuclei. This technique works well when a researcher is interested in a single specific structure within the field of view. However, two or more labels are often used to elucidate multiple structures. Multiple probes are mainly used to view two or more different types of sub-cellular structures simultaneously, while ensuring they are visually distinct from one another. To aid with image analysis, each fluorescent wavelength is usually acquired independently and then combined with the others to create a composite image.

After image acquisition and processing, there may be some overlap when two discrete wavelengths of light occupy the same physical location within the image. This information can be of special interest to the researcher. It then becomes necessary to provide a visual representation of the location and degree of overlap between two wavelengths.

2. Acquiring Images

Fluorescence excitation occurs through either confocal scanning or wide field epifluorescence microscopy using standard illumination methods. Image capture is accomplished through standard CCD acquisition techniques. Acquisition of individual wavelengths is usually performed, followed by the application of look-up tables (color values) to the image. A composite image is then created from the colorized constituents. It is important to note that image acquisition can, and often does, occur through several optical sections.

The physical methods of excitation and acquisition are beyond the scope of this article and are not discussed in any detail. After digitization, images are imported to Image-Pro Plus v. 5.0 for colocalization analysis.

3. A Definition for Colocalization

Colocalization describes the presence of two or more types of molecules at the same physical location. Within the context of a cell or sub-cellular structure it may mean the molecules are attached to the same receptor, while in the context of digital imaging it means the colors emitted by the fluorescent molecules occupy the same pixel in the image. Measuring the degree of colocalization is performed through the use of the 'Color Composite' and 'Colocalization' functions in Image-Pro Plus.

Colocalization does not refer to the likelihood that fluorochromes with similar emission spectra will appear as overlapped in the composite image. It is important to note that cross-talk or "bleed-through" may occur if the emission spectra of the two fluorochromes are similar. Accurate colocalization determination can only occur if emission spectra are sufficiently separated between fluorochromes and the correct filter sets are used during the acquisition step. To achieve this goal, red and green wavelengths are usually selected.

Fig 1. Mouse Fibroblast Cells. Microtubules stained with FITC, mitochondria stained with MitoTracker Red, nuclei stained with DAPI. Fluorochromes were acquired separately and merged using the Process|Color Composite command in Image-Pro Plus v. 5.0.

The dyes representing these wavelengths are carefully matched to the power spectrum of the illumination source. This is done to obtain maximum excitation wavelengths while still maintaining a degree of separation between the emission wavelengths.

4. How Does All This Work?

The degree of colocalization is measured by comparing the equivalent pixel positions in each of the acquired images. To achieve this, Image-Pro Plus uses a combination of the Process|Color Composite and Measure|Colocalization commands.

4.1 Generating the Colocalization Scatterplot

The first step in the process involves displaying the image upon which the colocalization analysis will be performed. The image may be created as a composite from two single wavelength acquisitions (the preferred method), or a multi-labeled specimen acquired as a single image (Fig. 1). Then, it is determined which pair of colors will be evaluated for colocalization. Typically, red-green pairs are selected, although it is possible to select other combinations. Afterward, a scatterplot (Fig 2.) of the individual pixels from the paired images is generated. Dimmer pixels in the image are located toward the origin of the scatterplot, while brighter pixels are located farther out. Pure red and pure green pixels are colored as such, and tend to cluster more toward the axes of the plot. If colocalized pixels are present, they appear as orange to yellow, depending on the degree of colocalization, and are located toward the middle of the plot.

4.2 Analyzing the Scatterplot and Generating a Mask

Once the scatterplot has been generated, it is now possible to evaluate the presence and level of colocalization quantitatively in the paired images. In this process, the scatterplot is used in conjunction with the image to define the location of the colocalized molecules.

An area of interest (AOI) is drawn on the scatterplot to indicate threshold levels of signal to be included in the analysis (Fig 3). The vertical and horizontal edges of the AOI are aligned such that they exclude background signal (clustered on the plot along the x- and y-axes). Only the signal included within the boundaries of the AOI will be included in the estimation of colocalization. By clicking within the area of interest, it is possible to see on the image the color intensities that will be included in the analysis. Three threshold masks (red, green, and yellow) appear in the image when the left mouse button is depressed. Red and green masks indicate those pixels with no color mixing, while

Fig 2. The scatterplot displays the intensity range of red and green pixels in the image, as well as the various degrees of colocalization, as shown in orange and yellow.
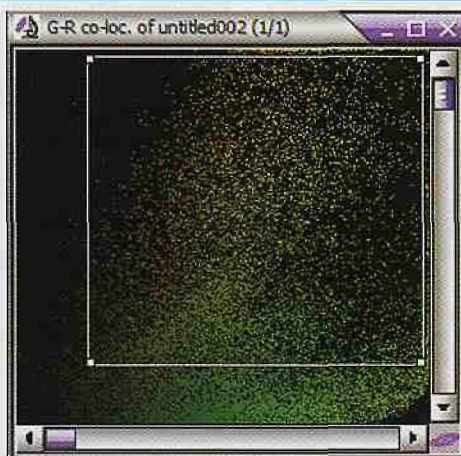

Fig 3. Area of interest (AOI) within the scatterplot. The AOI has been set to include primarily the colocalized areas, seen as orange and yellow.
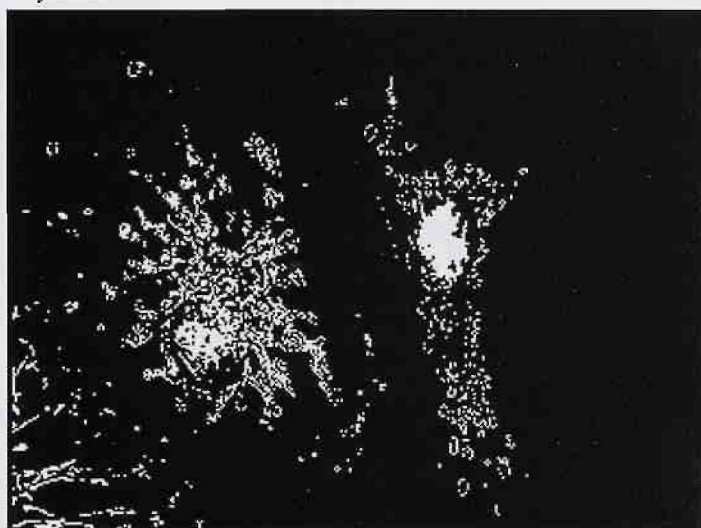

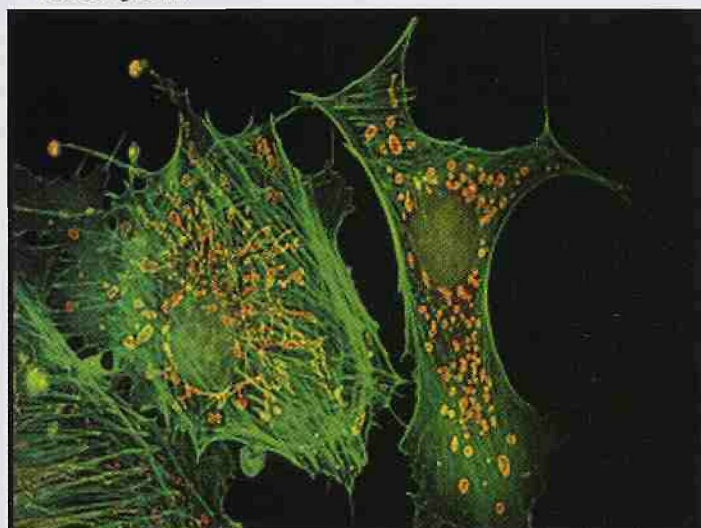Fig. 4 Colocalization mask showing overlapping regions of red and green.

Fig. 5. Composite image showing FITC-labeled fibroblasts (green), Texas Red-labeled mitochondria (red) and colocalization map (yellow)

a yellow mask indicates regions containing colocalized information. The colocalization mask (Fig. 4) may then be superimposed on the image as a colocalization map (Fig. 5).

4.3 Generating and Explaining Colocalization Coefficients

A more quantitative assessment of the colocalized areas may be performed using the information derived in the previous step. Several values are calculated, each is explained in more detail below. These values include calculations for the entire scatterplot, as well as values for pixel values contained within the AOI. Values calculated for the entire scatterplot include the following:

- Pearson's Correlation (R_r),
- Overlap Coefficient (R),
- Overlap Coefficient k_1,
- Overlap Coefficient k_2,
- Colocalization for each color channel ($m1$ and $m2$)

Values calculated for signal intensities within the AOI include:

- Colocalization of the first channel ($M1$)
- Colocalization of the second channel ($M2$)

In the following examples we call the first channel red and the second channel green.

4.3.1 Pearson's Correlation

Pearson's Correlation is a well defined and commonly accepted means for describing the extent of overlap between image pairs. It is a value computed to be between -1 and 1, with -1 being no overlap whatsoever between images and 1 being perfect image registration. Pearson's Correlation takes into account only the similarity of shapes between images and does not take into account image intensity. Since a negative value can be reported using this method, it may be necessary to look at the data using other coefficients.

Pearson's Correlation is calculated according to the following formula:

$$R_r = \frac{\sum_i (S1_i - S1_{aver}) \cdot (S2_i - S2_{aver})}{\sqrt{\sum_i (S1_i - S1_{aver})^2 \cdot \sum_i (S2_i - S2_{aver})^2}}$$

where

$S1$ is signal intensity of pixels in the first channel and $S2$ is signal intensity of pixels in the second channel,

$S1_{aver}$ and $S2_{aver}$ - average intensity of first channel and second channels respectively.

4.3.2 Overlap Coefficient

As Pearson's Correlation does some averaging of pixel information and can return negative values, another method, the Overlap Coefficient, is simultaneously used to describe overlap. This method does not perform any pixel averaging functions, so correlations are returned as values between 0 and 1. This method is not sensitive to intensity variations in the image analysis. This is especially important when considering issues typical to fluorescence imaging such as camera quantum efficiency, hybridization efficiency, and sample photobleaching.

$$R = \frac{\sum_i S1_i \cdot S2_i}{\sqrt{\sum_i (S1_i)^2 \cdot \sum_i (S2_i)^2}}$$

4.3.3 Overlap Coefficients k_1 and k_2

These coefficients describe the differences in intensities of red and green. Simply put, the value k_1 is sensitive to differences in intensity for green while k_2 is sensitive to differences in intensity for red.

$$k_1 = \frac{\sum_i S1_i \cdot S2_i}{\sum_i (S1_i)^2} \quad \text{and} \quad k_2 = \frac{\sum_i S1_i \cdot S2_i}{\sum_i (S2_i)^2}$$

4.3.4 Colocalization Coefficients m_1 and m_2

Now that we have generated some information about the degree of overlap and accounted for intensity variation between the color channels, we can estimate the contribution of one color channel in the colocalized areas of the image to the overall colocalized fluorescence in the image. m_1 is used to describe the contribution of green to the colocalized area while m_2 is used to describe the contribution of red.

$$m_1 = \frac{\sum_i S1_{i,coloc}}{\sum_i S1_i} \quad \text{and} \quad m_2 = \frac{\sum_i S2_{i,coloc}}{\sum_i S2_i}$$

where

$S1_{i,coloc} = S1_i$ if $S2_i > 0$ and $S2_{i,coloc} = S2_i$ if $S1_i > 0$
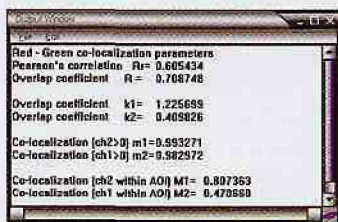
Table 1. Example of colocalization data derived from Figs. 1-5.

4.3.5 Colocalization Coefficients M_1 and M_2

Colocalization coefficients can be calculated for intensity ranges defined by AOI on the scatterplot. The coefficients for M_1 and M_2 are very similar to those generated for m_1 and m_2 but take into account only the intensities that fall within the AOI. M_1 is used to describe the contribution of green to the colocalized area, while M_2 is used to describe the contribution of red.

$$M_1 = \frac{\sum_i S1_{i,coloc}}{\sum_i S1_i} \quad \text{and} \quad M_2 = \frac{\sum_i S2_{i,coloc}}{\sum_i S2_i}, \text{ where}$$

$S1_{i,coloc} = S1$ if $S2_i$ is within thresholds defined by AOI (left and right sides of AOI in case of rectangular AOI), $S1_{i,coloc} = 0$ if $S2_i$ is outside the threshold levels.

$S2_{i,coloc} = S2_i$ if $S1_i$ is within thresholds (top and bottom margins of AOI in case of rectangular AOI), $S2_{i,coloc} = 0$ if $S1_i$ is outside the AOI. ■

These coefficients, M_1 and M_2, are proportional to the amount of fluorescence of colocalizing objects in each component of the image, relative to the total fluorescence in that component. The components are described as the red and green images, respectively.

5. References

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