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Multicolor Imaging: The Important Question of Co-Localization

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The use of specific fluorescent probes, combined with confocal or multiphoton microscopy and subsequent digital analysis of the images, has greatly improved the accuracy of co-localization of macromolecules in biological samples. This article addresses some of the caveats associated with the preparation and imaging of multilabeled samples and demonstrates the use of digital methods for measuring the extent of co-localization in cells.

WHAT IS CO-LOCALIZATION?

In a biological sense, co-localization describes the existence of two or more different molecules in very close spatial positions in a specimen. These are often proteins that are visualized using fluorescent antibodies or riboprobes (Figures 1 and 2). The output from most confocal microscopes is a digital image made up of a multidimensional array of many voxels (volume elements or 3-D pixels). Co-localization of two fluorescent probes, such as fluorescein (green emission) and rhodamine (red emission), is represented in the image by a voxel(s) with a green intensity value above one level and a red intensity value above another level.

The resolution of co-localization is important. Before a fluorescent staining protocol is designed to assess co-localization, it is imperative for the scientist to define the size of the cellular domain being probed. It is all about resolution. With a very low-power lens, one may be looking for the coexistence of two fluorescent probes in the same cell but be unable to determine whether the probes occupy the same or different subcellular compartments. However, with a high-power, high-numerical aperture lens, it may become quite clear that the two antibodies are bound to different subcellular structures. Typically, a green fluorescent probe for one protein and a red fluorescent probe for a second protein appear orange or yellow in regions of the merged image where they occur in the same space. However, things are not that simple.

SOME CAVEATS

Using standard epifluorescence microscopy, it is impossible to determine whether two proteins are truly co-localized or whether they simply overlap in the z dimension. The use of optical sectioning devices, such as confocal or multiphoton microscopes, has eliminated this problem.

Bleed-Through, Tissue Autofluorescence, and Nonspecific Antibody Staining

One of the biggest obstacles to the determination of co-localization is the frequent occurrence of fluorescence bleed-through between the green and red fluorescent channels. Bleed-through can be present from both the green fluorochrome into the red channel and from the red fluorochrome into the green channel. The same is true for autofluorescence or if one (or both) of the fluorescence antibodies causes a low level of nonspecific background staining.

In a dual-labeled green/red sample, these three situations will make the pixels appear yellow/orange, but this is not due to co-localization. True co-localization can only be determined when bleed-through and all nonspecific fluorescence has been removed from the images. This can be accomplished by selected sequential excitation and collection with narrow band emission filters and by the use of single-labeled controls for assessing any bleed-through and unstained controls for the degree of autofluorescence. Digital mixers available with some confocal systems allow live bleed-through correction and should be used when necessary.

The Possibility of Fluorescence Resonance Energy Transfer

Although fluorescence resonance energy transfer (FRET) can be used to monitor the very close proximity of two fluorescent molecules and, thus, potentially be a useful tool for co-localization studies, it is not desirable unless the parameters under which FRET occurs are well known. The concern about using two fluorochromes in which the emission spectrum of one overlaps with the excitation spectrum of the second is that FRET may occur without the knowledge of the investigator. This will lead to an unexpected decrease of the shorter wavelength signal and a concomitant increase in the longer wavelength signal. Fluorescence quenching of emitted signal can also occur between some pairs of fluorochromes.

SAMPLE PREPARATION

To reliably determine the degree of co-localization, very careful attention must be paid to sample preparation. Yellow/orange pixels appear if there is co-localization or any nontargeted cross-reactivity between any of the antibodies, spectral overlap of the fluorescent emissions of the fluorochromes, or autofluorescence. Therefore, knowledge of the protocol should be applied during the subsequent image analysis.

Another dilemma emerges when one tries to overcome the bleed-through issue completely; should one choose two fluorescent probes whose emission spectra are far apart (where chromatic shift can lead to false negatives) or closer together (where bleed-through can lead to false positives)? A pair of fluorochromes that have the least emission overlap possible should be selected. They should be imaged using a good-quality, color-corrected Plan Apochromatic lens to eliminate any misalignment of the images caused by chromatic shift. The best results are achieved if the green fluorochrome has a narrow emission spectrum, and the red fluorochrome has an emission spectrum in the red or far red.

Care should be taken during the labeling protocol to ensure that the two fluorochromes are of similar brightness. Some factors that affect brightness include excitation efficiency, concentration, quantum yield, extinction coefficient, and efficiency of emission collection.

Traditionally, most users have tended to use a pair of fluorochromes, such as FITC and TRITC (which exhibit overlap) or FITC and Texas Red® (which overlap a great deal less). Today,

there are improved and brighter fluorochromes available, such as the Alexa™ series (Molecular Probes, Eugene, OR, USA). Many of these have very narrow emission spectra, making them more effective fluorochromes for use in co-localization experiments (e.g., Alexa 488 and Alexa 568). The combination of a green fluorochrome and a far-red fluorochrome, such as cyanine 5 or Alexa 647, works well because photomultiplier tube detectors are sensitive to far-red light (unlike the human eye). Further information on the specific excitation and emission characteristics of fluorochromes is available on an interactive fluorescence database (www.fluorescence.bio-rad.com).

Another way to overcome the issue of antibody specificity is to transfect cells with one or more vectors that express the target protein linked to blue, cyan, green, or yellow fluorescent protein or DsRed® (Clontech Laboratories, Palo Alto, CA, USA), although sequential excitation would still be required because of the large overlap in the spectra.

IMAGE ACQUISITION

One can normally make a quick assessment of co-localization in an image. If all points that are red are also green, then there is probably some bleed-through. However, if there are distinct green points, red points, and yellow/orange points, then chances are that there is some co-localization. With sequential excitation and collection, it is important to set the gain of the photomultipliers so that the red and green signals are about the same intensity but below saturation. Likewise, the black level should be adjusted to remove sub-signal noise but not background fluorescence. If bleed-through persists, it may be necessary to change the fluorochromes, perform bleed-through correction live with digital mixers, or preprocess the images before co-localization analysis. It is usually preferable to fine-tune the method of specimen preparation to the characteristics of the imaging system.

Unless one has chosen two fluorochromes with extremely well-separated emission spectra, image acquisition using a confocal microscope should be made in the sequential mode. If one is using a krypton/argon laser (or three separate lasers) with acousto-optical filter (AOTF) control, then by carefully balancing the excitation intensities, images can be produced with sufficient spectral separation, even in simultaneous mode.

Multiphoton imaging systems are particularly suited to co-localization studies because both fluorescent dyes can be ex-
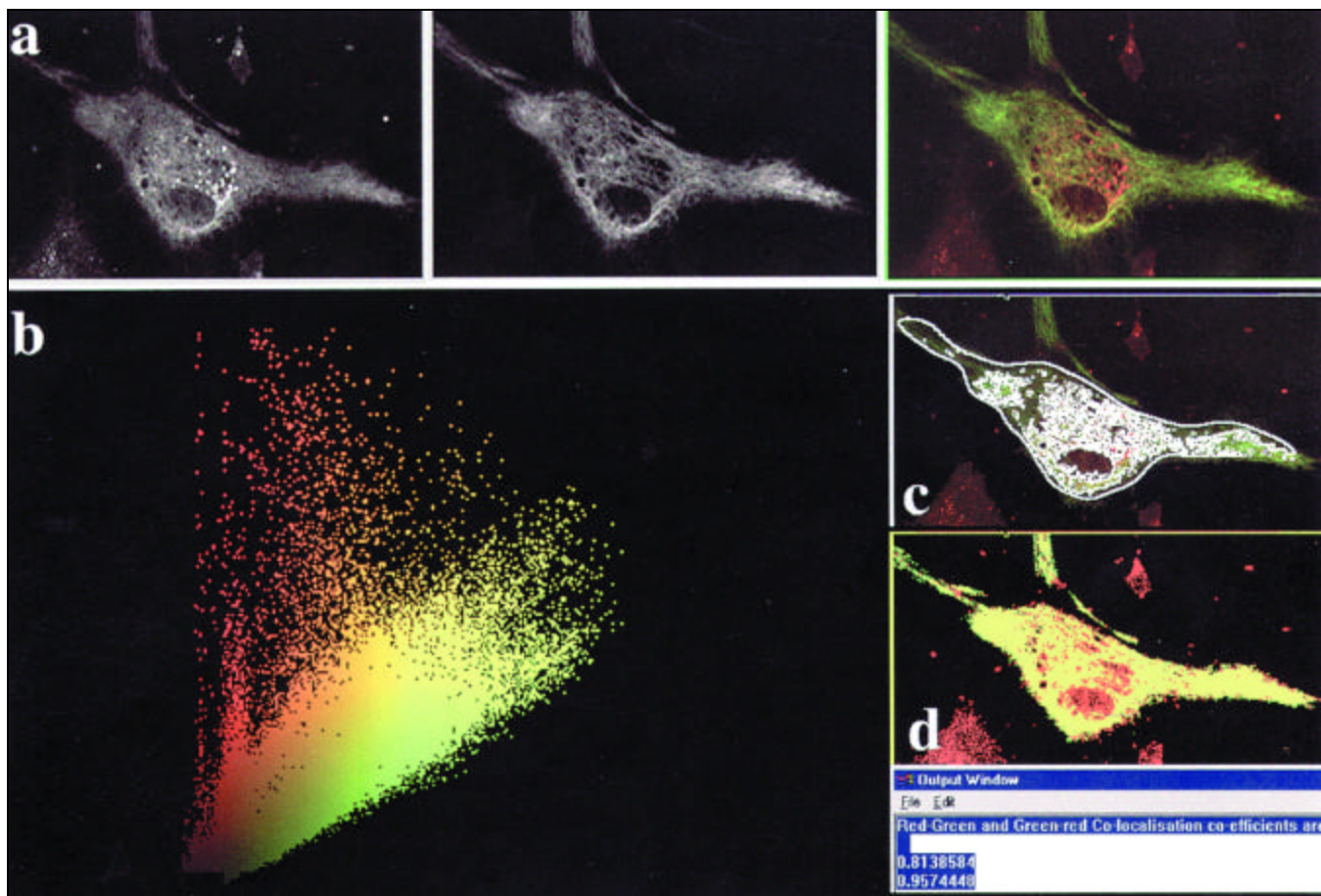

Figure 1. High co-localization and low bleed-through. Panel a shows cultured cells labeled with two antibodies against different cytoskeletal proteins—one has a fine, reticular distribution (green) and the other has both a fine and a punctate distribution (red). The red and green images were collected sequentially using 568- and 488-nm excitation, respectively, from a krypton/argon laser. Thus, there is very little bleed-through to consider. Panel b shows a 2-D co-localization fluorogram generated from the red and green intensities within the component images. Panel c shows an area of interest (AOI) drawn around the cell. Panel d shows a color co-localization map and the coefficients (lower).

cited by one wavelength (this is because of the extended excitation spectra associated with multiphoton absorption), and the excitation of fluorescence is limited to a tiny volume within the sample so that any fluorescence must emanate from that optical plane only (Table 1).

CO-LOCALIZATION ANALYSIS

Co-localization programs calculate two values, which represent the proportion of co-localizing objects in each component of a dual-color image (1). These values are called co-localization coefficients. The calculations are based on Pearson's correlation coefficient, which is an established means of describing the degree of overlap between patterns or images. If sufficient care is not taken during sample preparation and acquisition, then co-localization software may generate values and images that are misleading.

The coefficients generated are always between 0 and 1. A value of 0 means there is no co-localization, and a value of 1 means there is complete co-localization. A coefficient is generated for each color of a two-color merge; for example, red 0.9, green 0.45 means that the ratio of all the red intensities that show a green component, divided by the sum of all the red intensities in the selected area, is 0.9 (i.e., a very high degree of co-localization) and that the ratio of all the green intensities that

show a red component, divided by the sum of all the green intensities, is 0.45, which is half the co-localization value. Therefore, there is twice the degree of co-localization of red pixels with green than there is of green pixels with red.

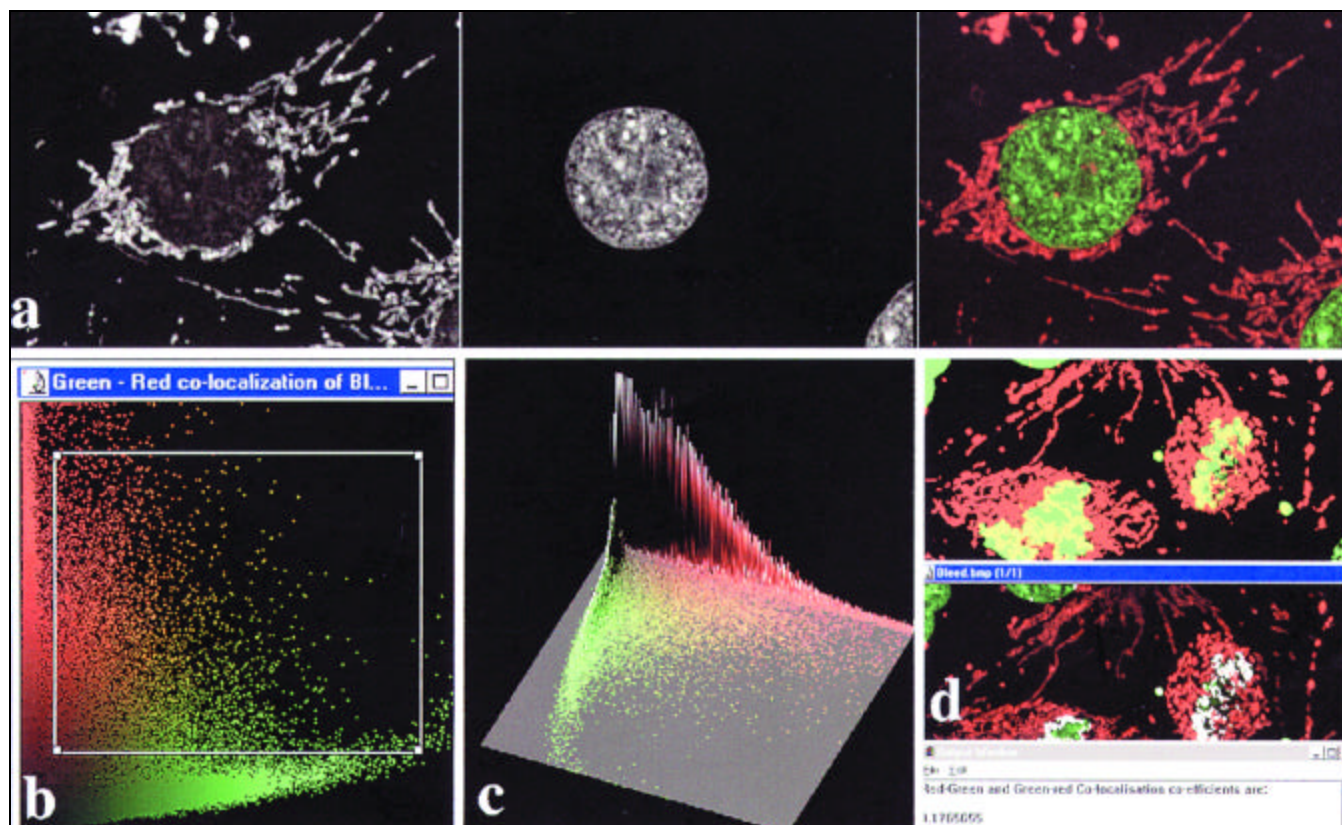For three-channel images, analysis can be done between

Table 1. Practical Tips for Co-Localization Studies

1. Ensure antibody specificity and lack of cross-reactivity.
2. Fluorochromes should have well-separated spectra to reduce the chance of bleed-through or FRET
3. Plan Apochromatic lenses should ALWAYS be used to reduce chromatic shift.
4. Emission filters should be optimized to maximize emission collection while avoiding bleed-through from other fluorochromes.
5. Use single-labeled controls to assess bleed-through.
6. Use unstained controls to check for autofluorescence.
7. Use sequential image collection where bleed-through is present.
8. Use digital mixers if available.
9. Use AOTF control (where available) of excitation intensity.
10. The multiphoton principle is better suited to co-localization than confocal studies.

Figure 2. Low co-localization and low bleed-through. Panel a shows double-labeled culture cells with a small amount of bleed-through and almost no co-localization. Panel b shows the red/green co-localization plot, using a rectangle to threshold out lower intensities in both colors. Panel c shows the same intensity plot as in panel b but with an additional third dimension that represents pixel frequency. Panel d shows a color co-localization map (upper), a mask (center), and the coefficients (lower). The sample was prepared by David Vaux and Mike Hollingshead (Oxford University, UK).

the green and red channels, the green and blue channels, and the red and blue channels to obtain coefficients for each pair of color combinations. The software should allow for independent threshold (i.e., below desired signal) selection and subtraction from each image before reading out the coefficients.

With some programs, a fluorogram can be generated from either the entire image or a selected part of the image (Figure 1). This simply displays the intensity and distribution of different colored pixels within the merged image as a scatter plot and allows the user to select a subset of pixels that meets certain intensity criteria in both colors. These pixels may then be highlighted on the image or throughout a z series.

Example of Partial Co-Localization

The double-labeled cells shown in Figure 1 illustrate a situation in which there are some distinct red pixels but almost no distinct green pixels. All greenish pixels appear to have some red component, that is, co-localization.

Example of Extremely Low Co-Localization

Figure 2, panel a, shows an image of some double-labeled cells, which was collected simultaneously with both 488- and 568-nm excitation from a krypton/argon laser. Before thresholding, there was a tiny amount of bleed-through from the green fluorescence into the red image (i.e., nucleus). Unfortunately, there is no automatic way of deciding where the threshold intensity should be. Maximum levels of autofluorescence
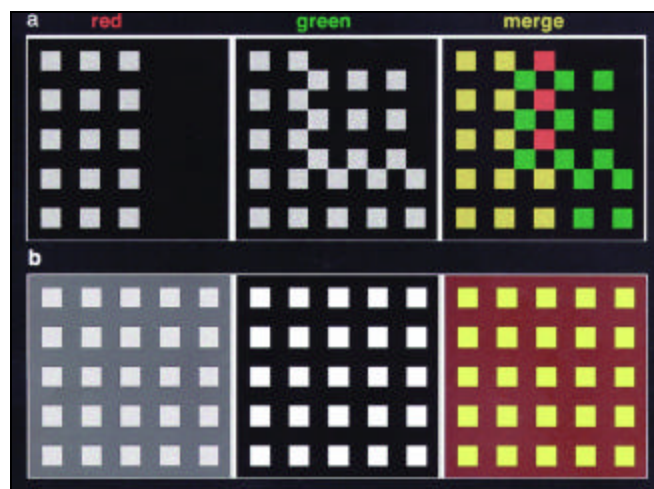

Figure 3. Co-localization simulation. Panel a shows images that have a purely red component, a purely green component, some co-localization, but no background. The red and green intensities are very similar. The co-localization analysis gives coefficients of red = 0.8 and green = 0.48. This is expected because a greater proportion of the red pixels are co-localized in comparison to the proportion of co-localized green pixels. Because the intensities are fairly uniform (i.e., there is little noise in the images), the intensity-weighted values almost exactly correspond to the overlapping areas in the image. Panel b shows a red image with high background and a green image with no background. There should be almost total co-localization when this background is removed—even though the intensity of the red and green signals is very different. The coefficients calculated without background subtraction show a falsely low red coefficient of 0.49. When the coefficients are calculated after background subtraction, both of the values are equal to 1.0, which indicates the expected value for total co-localization.

BioImaging

and nonspecific staining in both channels can be measured and used to set the thresholds, which may be different in each channel. If co-localization analysis is performed without subtracting this bleed-through, the calculation would show false co-localization. Clearer examples of partial and total co-localization are shown in Figure 3, panels a and b, respectively.

Figure 2, panel b, shows the 2-D plot of red and green intensities from the entire image and how one can use a rectangle to select out certain thresholds. Figure 2, panel c, shows the 3-D plot where the z axis displays pixel frequency. Figure 2, panel d, shows a co-localization map (upper section) where co-localized pixels are shown in yellow and non-co-localized pixels in bright red or bright green. The middle section shows how a white mask of co-localized pixels can be superimposed onto the original RGB image. The lower section shows that the co-localization coefficients are both extremely low, as they should be.

CONCLUSION

Co-localization analysis programs provide valid coefficients, provided that: *(i)* bleed-through has been avoided or subsequently removed during acquisition, *(ii)* background staining and autofluorescence have been removed from all images, and *(iii)* the analysis is done only on optical sections rather than projections. The entire process depends on optimal specimen preparation and imaging techniques.

REFERENCES

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