Image acquisition for colocalization using optical microscopy

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Sciven DR, Lynch RM, Moore ED. Image acquisition for colocalization using optical microscopy. Am J Physiol Cell Physiol 294: C1119–C1122, 2008. First published March 19, 2008; doi:10.1152/ajpcell.00133.2008.—Colocalization, in which images of two or more fluorescent markers are overlaid, and coincidence between the probes is measured or displayed, is a common analytical tool in cell biology. Interpreting the images and the meaning of this identified coincidence is difficult in the absence of basic information about the acquisition parameters. In this commentary, we highlight important factors in the acquisition of images used to demonstrate colocalization, and we discuss the minimum information that authors should include in a manuscript so that a reader can interpret both the fluorescent images and any observed colocalization.

The analysis and display of colocalization in optical images obtained through microscopy is now a commonly used experimental tool. In this technique, two or more molecules are labeled with fluorescent probes, and the images, acquired at the appropriate wavelengths, are overlaid. The extent of colocalization of the probes is then visualized and often quantified. This general description of a colocalization experiment covers a host of microscopic techniques, labeling methods, and image analysis algorithms that can be used in various combinations. To provide some uniformity in presentation, we are recommending several procedures for collecting colocalization data. We also present guidelines for the information that should be included in the methods section of the manuscript that will allow any reader to interpret the results. We are focusing our discussion on images acquired with wide-field and confocal microscopes since these are by far the most common approaches for image acquisition.

The Nyquist Criteria

When the term colocalization is used in optical microscopy, the objects that are being compared are usually pixels or voxels in digital images (A pixel is the smallest element in a two-dimensional digital image and a voxel is its three-dimensional counterpart). When we ask if two molecules are colocalized, we are, in effect, asking “Is the voxel illuminated by a fluorophore attached to (or part of) molecule A the same voxel as that illuminated by another fluorophore attached to molecule B?” Thus the size of the voxels is important for both planning and interpreting the experiments. When the image is digitized (voxelized), it has the same effect as placing a grid over the image and replacing the objects at each grid point (voxel) with the total intensity of all of the objects contained in it. As voxel size increases, resolution decreases so that separate structures become lumped together in the same voxel. At some point, the voxelized image will no longer accurately represent the original object and artifacts may appear. The voxel size at which an object is accurately represented by an image is given by the Nyquist criteria, which are a function of several variables: the numerical aperture of the objective, the emission wavelength of the fluorophore, the type of microscope (wide field or confocal) and, for depth, the refractive index of the medium. Further details can be found in Castleman (2) and van der Voort and Strasters (13). The formulas and a summary of appropriate values for a conventional microscope, using oil immersion lenses of various numerical apertures and illuminating wavelengths, are given in Table 1. Confocal microscopes, using laser light and a typical pinhole size of ~250 nm, have Nyquist values that are similar to those listed (13). Ideally, imaging parameters should be optimized to reach these values to obtain the best resolution for evaluating colocalization. In real systems, noise will increase the theoretical values listed in the table, and values within 10% of those tabulated will still give an acceptable result.

Voxels larger than those specified by the Nyquist criteria will under sample the image, create artifacts, and result in false colocalizations. Figure 1 shows a ventricular myocyte collected at voxel sizes satisfying the Nyquist criteria (Fig. 1, A and C) and the same image resampled with voxels four times larger in X and Y and 1.6 times in Z (Fig. 1, B and D). It is clear that not only does a large amount of colocalization appear in the under-sampled image but more importantly, structures appear that are not present in the original (compare Fig. 1, C and D). Collecting images with voxels smaller than the Nyquist values requires longer exposure times to achieve a comparable signal-to-noise ratio, increasing photobleaching and photodamage, while producing little additional information.

For proper evaluation of a manuscript, authors should therefore report the objective’s numerical aperture, the wavelengths that are being used, the voxel sizes, the refractive index of the medium and the Z spacing between image planes, and show how these values relate to the Nyquist criteria for the experiment.

Diffuse Labeling

In some cases, one of the fluorophores is present in almost every voxel. This can occur when labeling a cytosolic protein or a widely distributed protein like actin. Under these conditions, colocalization by chance has almost 100% probability, making any observed or measured colocalization, not signifi-

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The under-sampled image respectively. Note the structural artifacts in $A$ of the inserts (white square) in was 6% in colocalization of caveolin-3 with vinculin, appear in the original ($A$).

$B$ demonstrates almost complete colocalization at the cell surface and 32% in $A$ and 61% in $B$ and the colocalization of caveolin-3 with vinculin was 6% in $A$ and 32% in $B$. ($C, D$) a blowup of the inserts (white square) in $A$ and $B$ respectively. Note the structural artifacts in the under-sampled image $D$ that do not appear in the original ($A, C$).

**Table 1. Lateral and axial sampling distances determined by the Nyquist criteria**

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Emission Wavelength, nm</th>
<th>NA</th>
<th>1.2</th>
<th>1.3</th>
<th>1.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI/Alexa 350</td>
<td>450</td>
<td></td>
<td>97</td>
<td>89</td>
<td>83</td>
</tr>
<tr>
<td>Cy2</td>
<td>505</td>
<td></td>
<td>105</td>
<td>97</td>
<td>90</td>
</tr>
<tr>
<td>FITC/Alexa 488</td>
<td>520</td>
<td></td>
<td>108</td>
<td>100</td>
<td>93</td>
</tr>
<tr>
<td>Cy3/Alexa 555</td>
<td>565</td>
<td></td>
<td>118</td>
<td>109</td>
<td>101</td>
</tr>
<tr>
<td>TRITC/Alexa 546</td>
<td>575</td>
<td></td>
<td>120</td>
<td>111</td>
<td>103</td>
</tr>
<tr>
<td>Texas red/Alexa 594</td>
<td>615</td>
<td></td>
<td>128</td>
<td>118</td>
<td>110</td>
</tr>
<tr>
<td>Cy5/Alexa 647</td>
<td>670</td>
<td></td>
<td>140</td>
<td>129</td>
<td>120</td>
</tr>
</tbody>
</table>

The $x$ and $y$ sampling distances are calculated (in nm) using the formula $\lambda/(4NA)$ (4) where $\lambda$ is the emission wavelength (in nm) and NA is the numerical aperture of lens. The axial ($z$) sampling distances (in italics) are for an oil ($n = 1.515$) immersion objective and are calculated with the formula, $z = \lambda/(2n (1 - \cos \alpha))$, where $\alpha = \sin^{-1} (NA/n)$ and $n$ is the refractive index of the medium (13). DAPI, 6-diamidino-2-phenylindole; TRITC, tetramethylrhodamine isothiocyanate, Cy2, Cy3, Cy5, different fluorescent cyanine dyes.

**Deconvolution**

Wide-field images contain much out-of-focus light, which will confound colocalization analysis. For this reason, it is mandatory that wide-field images be deconvolved before colocalization analysis is attempted. Authors should state which algorithm or software package was used. It should be noted that the measurement of colocalization in confocal images is also improved by deconvolution (7, 11).

**Bleed Through**

A fundamental assumption made when measuring colocalization is that the signal at each wavelength is independent of the other. Bleed through of signal from one channel to another can result in an apparent colocalization when none, in fact, exists. For example, even with modern filters, there is significant bleed through from FITC to rhodamine, or from cyan fluorescent protein to yellow fluorescent protein. Bleed through should be estimated by creating a control with labeling in one channel and none in the other and then recording the signal coming from the unlabeled wavelength(s). The resultant image should be random, having no discernable pattern, and its intensity should be less than the chosen threshold. If, however, there is significant bleed through, the control images can be used in concert with spectral linear unmixing (8, 14) to separate the two signals. Once corrected, the images can then be used for analysis. Some newer instruments have spectral unmixing built into their software and correct for bleed through at the time of acquisition, although it is important to check how well the signals are separated before accepting the results (15).

**Colocalization in X-Y versus Z**

In all optical microscopes, except the 4Pi, the resolving power in $Z$ is only one-third to one-quarter of that in $X$ and $Y$ (9). The consequence is that colocalization is most powerful when performed on structures that run along the $XY$ plane, parallel to the coverslip, whereas it is least accurate for objects that are running parallel to the $Z$-axis of the microscope. The consequences of this are best seen in a structure that runs both parallel and perpendicular to the $XY$ plane. This is diagrammed in Fig. 2A, which shows a cross-section through a cell in which fluorophores of the same color are separated by 200 nm, and the two different fluorophores are interspersed at 100-nm intervals. Figure 2B shows the result of superimposing these planes—the pixels that lie in the transverse plane are not colocalized, but those at the edges, lying along the $Z$-axis appear to be. This effect can be observed in real cells. Figure 2C shows a rat ventricular myocyte labeled with antibodies specific for vinculin (green) and caveolin-3 (red). A 1-μm thick section from the cell’s center is displayed in Fig. 2C and demonstrates almost complete colocalization at the cell surface.
running parallel to the Z-axis (arrowheads). Figure 2Cii is an image of the top surface of the same cell running parallel to the XY axis, which shows <5% colocalization (boxed regions). A stereo pair of the complete data stacks (Fig. 2Ciii) shows that colocalization is confined to those regions of the sarcolemma that are oriented largely parallel to the microscope’s Z-axis, but these molecules are, in fact, not colocalized.

Sometimes the objects to be colocalized have little or no X-Y component (e.g., synapses or gap junctions), and in this case the measured colocalization has less predictive power than it would for objects oriented in the X-Y plane, since the colocalized objects could be separated by 500 nm or more (i.e., 2× the Nyquist limit in Z, see Table 1).

**Sampling the Cell**

The great advantage of using optical microscopy to assess colocalization, as opposed to other techniques including electron microscopy, is that images of entire cells or their substructures can be readily collected and analyzed, and regional variations can be visualized. When focusing an analysis on specific regions of a cell, care needs to be taken when deciding

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**Fig. 2. Edge Effects causing Colocalization.**

(A and B): A diagram showing how the effect occurs. (A) A cross-section in which fluorophores of the same color are separated by 200 nm, and the two different fluorophores are interspersed at 100 nm intervals. The grid represents 4 image planes, 250 nm apart in Z and 100 × 100 nm in X and Y. (B) Projection of the image planes onto a line of voxels; those in which colocalization occurs are colored yellow. (C) A rat ventricular myocyte labeled for vinculin (green) and caveolin-3 (red), colocalized voxels are white. 2 μm thick sections from the center of the cell (i) and the cell surface (ii). The arrowheads indicate sarcolemma that is parallel to the optical axis, and the arrows indicate sarcolemma that is perpendicular to the optical axis. The boxed regions, in which only 4% of the vinculin and 5% of the caveolin-3 were coincident, were used to numerically assess colocalization. (iii) Stereo-pair, 6° rotation, of the entire data set, 15 μm deep.
which part of the cell is sampled, because this can dramatically affect the results. Specifically, two recent papers (3, 10) have shown that regional variations in colocalization can occur in the absence of any obvious structural differences.

Guidelines for Publication

To enable readers to interpret images and to evaluate the meaning of colocalization from a given experiment, manuscripts should include the following information:

- The pixel sizes, the Z spacing between image planes, the refractive index of the medium, and how this relates to the Nyquist criteria for the experiment (Table 1).
- The objective’s magnification and numerical aperture.
- For images obtained with a confocal microscope, either the pinhole size or the axial resolution should be reported.
- The emission wavelengths of the fluorophores and the filter sets used.
- Scalebars should be included in each image group.

In addition, the following conditions should be satisfied.
- Wide-field images must be deconvolved, and the methodology reported.
- Images in which the colocalization is primarily along the Z-axis should, where possible, be examined for edge artifacts.
- Bleed through should be evaluated and corrected for, if present. If corrections are made, details of the methodology used should be provided.

Conclusion

Colocalization can be a powerful method for determining whether molecules have the potential to associate or interact, but it cannot confirm that they do. The inherent limitations of the technique mean that confirmation may require electron microscopy (1, 5) or other microscopic techniques such as fluorescence resonance energy transfer (6, 12).

GRANTS

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REFERENCES