Deconvolution of confocal images of dihydropyridine and ryanodine receptors in developing cardiomyocytes

Franklin Sedarat,1,2,* Eric Lin,1,2,* Edwin D.W. Moore,3 and Glen F. Tibbits1,2

1Cardiac Membrane Research Laboratory, Simon Fraser University, Burnaby V5A 1S6; 2Cardiovascular Sciences, British Columbia Research Institute for Children and Women’s Health, Vancouver V5Z 4H4; and 3Department of Physiology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

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Sedarat, Franklin, Eric Lin, Edwin D. W. Moore, and Glen F. Tibbits. Deconvolution of confocal images of dihydropyridine and ryanodine receptors in developing cardiomyocytes. J Appl Physiol 97: 1098–1103, 2004. First published April 2, 2004; 10.1152/japplphysiol.00089.2004.—Colocalization of dihydropyridine (DHPR) and ryanodine (RyR) receptors, a key determinant of Ca2+ release, was previously estimated in 3-, 6-, 10-, and 20-day-old rabbit ventricular myocytes by immunocytochemistry and confocal microscopy. We now report on the effects of deconvolution (using a maximum-likelihood estimation algorithm) on the calculation of colocalization indexes. Clusters of DHPR and RyR can be accurately represented as point sources of fluorescence, which enables a model of their relative distributions to be constructed using images of point spread functions to simulate their fluorescence inside a cell. This model was used to investigate the effects of deconvolution on colocalization as a function of separation distance. Deconvolution resulted in significant improvements in both axial and transverse resolutions, producing significant increases in clarity. Comparisons of intensity profiles (full-width half-maximum) pre- and postdeconvolution showed decreased dispersion of the fluorescent signal and a corresponding decrease in false colocalization as determined by fluorescence modeling. This hypothesis was extended to physiological data previously collected. The number of colocalized voxels was quantified after deconvolution, and the degree of colocalization of DHPR with RyR decreased significantly after deconvolution in all age groups: 3 days (62 ± 2% before deconvolution, 43 ± 3% after deconvolution) to 20 days old (79 ± 1% before deconvolution, 63 ± 2% after deconvolution). The data demonstrate that confocal images should be deconvolved before any quantitative analysis, such as colocalization index determination, to minimize the detrimental effects of out-of-focus light in coincident voxels.

cardiac excitation-contraction coupling; neonates; ontogeny; three-dimensional imaging

EXCITATION-CONTRACTION COUPLING in cardiac muscle requires Ca2+ influx through sarcolemmal dihydropyridine receptor (DHPR), followed by Ca2+-induced Ca2+ release through the sarcoplasmic reticulum (SR) Ca2+ release channels also known as ryanodine receptor (RyR) (4–6, 8, 15). Dyadic couplings in cardiac muscle cells are formed between RyRs and either sarcolemmal or t-tubular DHPR (1, 9, 17). The juxtaposition of DHPR and RyR in these couplings is crucial for both Ca2+-induced Ca2+ release and excitation-contraction coupling in adult myocardial cells. In newborn rabbits, myocytes have sparse SR and do not develop T tubules until 8–10 days of age; therefore the spatial relationship between DHPR and RyR is different from that of the adult heart (7, 14, 16). Using confocal microscopy and immunofluorescent labeling, we previously showed a significant increase in the degree the colocalization of DHPR-RyR as well as changes in colocalization distribution inside the cell during development. All imaging systems distort object information and/or introduce imaging artifacts that subsequently affect quantitative analysis. We expected that this distortion would lead to an overestimation of protein/fluorophore colocalization. In this study we quantified the effects of deconvolution on colocalization analysis using a fluorescence model and then applied the same deconvolution algorithms to our previously collected data.

Colocalization is a resolution-dependent descriptor, and it is often used to indicate possible protein-protein interactions. A colocalization event is defined as when one fluorophore is found in the same voxel as another fluorophore, implying that the proteins attached to the fluorophores are also within the same voxel. A colocalization event, in which two fluorophores are found together, actually refers to a range of possible separation distances. Because deconvolution increases resolution, it increases the power of colocalization analysis by limiting the range of possible separation distances. In addition to increasing the probability of predicting an actual protein-protein relationship, deconvolution also decreases the likelihood of false colocalization events. Confocal microscopy attenuates the majority of out-of-plane fluorescence emission but does not remove it completely, such that two subresolution point sources (fluorophore clusters) may generate colocalization events even if the centers of mass of the two points are well separated. These pseudocolocalization events arise because of the point spread function (PSF) of the microscope. Subresolution objects do not appear simply as blurry spheres but as blurry hourglasses, because of the numerical aperture (NA) of the objective and the pinhole radius. Because deconvolution fundamentally changes how subresolution objects appear, by transforming the hourglass shape into a bead shape, the results cannot be duplicated via other image-processing techniques such as aggressive thresholding (which affect size more so than the shape of objects).

Colocalization is a function of resolution and separation distance, and physiological systems, in general, cannot be used to characterize the effects of deconvolution because of their complexity and irregularity. Hence, a nonphysiological model is required to evaluate the effects of image restoration regarding colocalization.

* F. Sedarat and E. Lin contributed equally to this work.

Address for reprint requests and other correspondence: G. F. Tibbits, Cardiac Membrane Research Laboratory, Simon Fraser University, Burnaby, BC V5A 1S6, Canada (E-mail: tibbits@sfu.ca).

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To quantify the effects of deconvolution, multiple PSF images were positioned at a variety of axial and lateral separations to simulated fluorophore colocalization interactions inside a cell. Deconvolution algorithms strive to effectively reassign out-of-focus light (by conserving total actions inside a cell. Deconvolution of confocal images (see Biological samples). The beads were appropriately diluted, plated on a glass slide, and embedded in DABCO mounting medium to minimize the refractive index mismatch. Singular beads, i.e., beads with no neighbors in the field of view, were imaged on the Zeiss 410 and then aligned (centered) and averaged in Huygens Professional. Multiple (~10) bead images were acquired to provide sufficient signal-to-noise ratio for the reconstruction of the PSF. The averaged bead image was utilized as the PSF for deconvolution as well as for FWHM measurements pre- and postdeconvolution. In addition, this average bead image was also utilized as the foundational image for the fluorescence interaction’s model. Images were recorded at values greater than the Nyquist rate (100 nm lateral and 250 nm axial sampling interval) to prevent aliasing. We applied a maximum likelihood estimation (MLE) algorithm to deconvolve the confocal images. The Huygens implementation of MLE takes the following factors into consideration: NA of the microscope objective; refractive index of the medium; excitation wavelength; emission wavelength; confocal pinhole radius; pixel size; z-axis interval; microscope type (i.e., wide field, confocal, or 4π); and number of excitation photons.

Under low light-level conditions, the detector in a confocal microscope (normally a photomultiplier tube) behaves essentially as a photon counter. This conversion of fluorescence intensity to a discrete number of detected photons is described statistically as a Poisson process. The MLE algorithm computes the maximum likelihood estimate for the intensity of a Poisson process (27). This iterative process is based on the work done about three decades ago by Richardson (18) and Lucy (13) for astronomical imaging processing and is frequently referred to as R-L iteration. The relevant iterative equation for MLE is

\[ f_{new} = f_{old} \frac{\hat{h}(i,j)}{\sum \hat{h}(i,j)f_{old}(i,j)} \]

where \( f \) is the object function, \( g \) is the image, and \( \hat{h}(i,j) \) is the PSF (the fraction of light from true location \( j \) that gets scattered into observed pixel \( i \)).

The R-L iterative algorithm was shown by Shepp and Vardi (22) to converge to the maximum likelihood solution for Poisson statistics of optical data with noise from counting statistics. The number of iterations may serve as a regularization factor. In general, the remaining restoration error decreases with an increasing number of iterations. At the same time, the error due to noise amplification increases. The procedure should be stopped at an iteration number in which the sum of both errors is minimal (26).

**Methods.** Previous experiments have demonstrated that the DHPR and RyR are largely codistributed in distinct clusters throughout the membranes of T tubules and the SR, respectively. The three-dimen-

**Fig. 1.** Point spread function of a ×63/numerical aperture 1.40 oil Zeiss Plan-Apochromat objective obtained from a 200-nm fluorescent bead. A: image of fluorescent bead used to measure the full-width half-maximum (FWHM) before and after restoration. XY refers to the fact that the horizontal axis is X and the vertical axis is Y. In the lower panel, the vertical axis is the Z or axial plane. B: graph shows significant improvement in both lateral (X, Y) and axial (Z) resolutions.
sional (3D) images of these clusters were found to be indistinguishable from that of a point source object (19). Therefore, a microscope’s recorded PSF can be used to simulate a cluster of molecules. Using Optimas 5.2 image processing software, we wrote a macro in ALI (analytic language of imaging) to create multiple copies of the Alexa488 and Alexa594 fluorophores’ PSFs. These PSFs were placed at a variety of lateral and axial separation distances to analyze the effects of hourglass-shaped PSFs (raw confocal) vs. spherical-shaped PSFs (deconvolved) on colocalization. The distance between the centers of mass of the two bead images (PSF images) were varied in 100-nm increments between 0 to 1,000 nm in the z-axis and in 250-nm increments from 0 to 1,500 nm in the z-axis (see RESULTS, Fig. 2). Note that because colocalization calculations were performed on 3D stacks, colocalization events were still generated even if the spheres were well separated, especially when looking at the raw confocal beads. The colocalization index (CI) was calculated as a function of distance before and after deconvolution.

Biological samples. Neonatal New Zealand White rabbits were used from four age groups: 3, 6, 10, and 20 days old. These studies conform to the guidelines of Canadian Council on Animal Care and were approved by the University Animal Care Committee at Simon Fraser University. The method of myocardial cell isolation and immunofluorescent labeling has been previously described (20). Briefly, single cardiac myocytes were isolated from neonatal rabbit hearts by enzymatic digestion. Double labeling was performed on fixed and permeabilized cardiomyocytes with anti-RyR and anti-DHPR primary antibodies. The cells were then incubated with Alexa-conjugated secondary antibodies (Alexa488 for DHPR and Alexa594 for RyR). After secondary antibodies were applied, the cells were mounted on a glass slide and embedded in DABCO mounting medium [90% glycerol, refractive index (n) = 1.47] to minimize the refractive index mismatch of the lens immersion liquid (oil, n = 1.52) to that of the specimen. The labeled cardiomyocytes were examined by use of a Zeiss LSM 410 laser scanning confocal microscope equipped with a Zeiss Plan-Apochromat ×63/NA 1.40 oil objective. An Ar-Kr 488/568 laser provided the excitation light. The confocal pinhole aperture was set to the diameter of the first Airy disk. Stacks of 50–70 focal planes were captured at 0.25-μm z-intervals through the depth of the cell. 3D images of the cells were reconstructed by using the image stacks. The Nyquist theorem, which utilizes the limitation of the microscope optics [full-width half-maximum (FWHM)] to dictate adequate sampling, was used to determined that pixel dimensions of 100 × 100 × 250 nm (x, y, z) were required to properly sample the data. Using Optima’s 5.2 image-processing software, we examined 3D images of myocardial cells to determine the degree of colocalization, or CI, of DHPR and RyR in each age group before and after image restoration. Before CI was measured, a threshold was applied to the images to exclude ~99% of the signal found in the images captured from control samples (stained with secondary antibodies without applying primary antibodies). Typically, thresholding resulted in about a 10–15% reduction in the total signal and a signal-to-noise ratio of ~15.

RESULTS

Modeling. Figure 1 shows that the measurement of the FWHM amplitude of the PSF and demonstrates significant enhancement of both lateral and axial resolution after deconvolution of confocal images. Lateral resolution improved two-fold (FWHM = 310 nm before and 150 nm after restoration).

![Fig. 2. Fluorescence interaction model. A: x,y view showing degree of colocalization pre- and postdeconvolution with increasing x-axis separation. B: x,z view showing changes in colocalization index pre- and postdeconvolution with increasing z-axis separation. Deconvolved images are shown at left and confocal images at right. Colocalization indexes are indicated below each image.](http://www.jap.org)
Axial resolution improved almost fourfold (FWHM = 850 nm before and 230 nm after restoration).

Figure 2 illustrates the arrays of fluorescent beads used for the modeling before and after restoration. By increasing the distance between the bead arrays, the CI decreased in both x- and z-axes. In raw confocal images, the separation distance along the x-axis was increased to 800–900 nm before reaching zero colocalization. Only 400–500 nm of separation were needed to reach zero colocalization in the deconvolved images. In the z-axis, a 1,500-nm separation resulted in almost zero colocalization in the raw images, as opposed to 500 nm for restored confocal images.

Figure 3A shows decreasing colocalization as a function of separation distance along the x-axis. Figure 3B shows decreasing colocalization with increasing z-distance. Both graphs show the comparison of the effects of separation distance on CI before and after restoration of confocal images. Note the significant reduction in CI in both axes after deconvolution. With the use of Origin 6.0 software, the data were fit with a first order exponential decay equation: $\text{CI} = A e^{-\lambda x}$, where CI is the colocalization index, $A$ is the maximum amplitude (with $x$ equal to 0 and maximum amplitude set to 100%), and $x$ is the separation distance (in nm). The derived correlation coefficient, $r^2$, was $>0.98$ for all curves. We used $\lambda$ as a parameter to describe CI as function of separation distance. The value of $\lambda$ significantly decreased after restoration in both axes (x-axis: $\lambda = 332 \pm 18$ nm before and $205 \pm 13$ nm after deconvolu-

Fig. 3. Graphs of colocalization index vs. distance of bead separation. A illustrates this effect in the x-axis, whereas B shows the z-axis effect.

Fig. 4. A–C: confocal images (x-y) of the dihydropyridine receptor (DHPR) staining pattern (pseudocolored green), ryanodine receptor (RyR) staining pattern (pseudocolored red), and colocalization pattern (pseudocolored yellow) in myocardial cells isolated from a 10-day-old rabbit heart. Data are shown by superposition (summation projection) of a series of optical sections through the z-axis from the front to back surfaces of the cell. The images have been scaled by adjusting both the intensity and contrast to highlight all pixels containing signal. This adjustment makes it possible to display out-of-focus light readily, but because of the intensity scaling the background noise is also exaggerated. Note that for the purpose of colocalization analysis these images were corrected for the background and were thresholded to exclude $\sim 99\%$ of signal found in control images related to nonspecific binding of secondary antibody. A1–C1: confocal images restored by maximum likelihood estimation algorithm. Note increased clarity and significant reduction in dispersion of the signal in the deconvolved images. Scale bars indicate 5 μm.
Fig. 5. Cross-sectional (x-z) images of a rabbit myocardial cell. A: confocal image before restoration (DHPR in green, RyR in red, and colocalization in yellow). See Fig. 4 for image display information. Note the elongation of distribution patterns along the z-axis due to the lower axial (z) resolution. B: same image as in A after deconvolution. Note that there are significant improvements in z-axis elongation and noise after restoration. Scale bars for both images indicate 5 μm.

Fig. 6. Pictorial representation of two 3-dimensional bead images and the corresponding Airy disk patterns. Note the well-separated bead centers (red and green spheres) and false colocalization events (yellow) that occur axially away from the focal plane.

Table 1. Colocalization index of DHPR and RyR before and after restoration of confocal images

<table>
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<tr>
<th>Age, days</th>
<th>CI of DHPR with RyR</th>
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<tbody>
<tr>
<td></td>
<td>Confocal</td>
</tr>
<tr>
<td>3</td>
<td>62 ± 2</td>
</tr>
<tr>
<td>6</td>
<td>76 ± 1</td>
</tr>
<tr>
<td>10</td>
<td>75 ± 1</td>
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<tr>
<td>20</td>
<td>79 ± 1</td>
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Colocalization index (CI) values are means ± SE. The CI is calculated by the number of colocalized voxels divided by the total number of dihydropyridine receptor (DHPR) voxels and expressed as a percentage. RyR, ryanodine receptor. The effect of restoration on CI is the CI after deconvolution divided by the CI in the confocal image before deconvolution, expressed as a percentage.
(blurring) for a point source, the image degradation can then be reversed for a whole object or sample. The PSF was found by imaging a 200-nm fluorescent bead (subresolution bead), and the distortion was particularly obvious in the axial direction (Fig. 1).

Although the imaging properties in a confocal microscope give rise to much less blurring than a conventional microscope, the distortions will still significantly skew subsequent quantitative analyses. Colocalization without deconvolution will tend to describe a looser protein-protein relationship due to more limited resolution as well as overestimate the actual number of colocalization events due to image blurring.

Modeling. An important issue in image restoration is the quantitative assessment of the gains in resolution and the corresponding gains in the reliability of the deconvolved image compared with the original image. Physiological systems are difficult to characterize and always carry a level of uncertainty. This uncertainty prevents the accurate assessment of the effects of deconvolution on physiological systems. Because of the quantitative nature of deconvolution, the effects of deconvolution on physiological systems can be modeled computationally quantitative assessment of the gains in resolution and the quantitative nature of deconvolution, the effects of deconvolution, because of the redistribution of out-of-focus light.

Biological samples. We reexamined our confocal images of dyadic coupling formation in rabbit ventricular myocytes during the first 20 days after birth (20). Deconvolved data emphasize the importance of microstructural changes in relation to DHPR and RyR association during ontogeny. In each age group, the degree of colocalization for DHPR decreased after deconvolution, because of the redistribution of out-of-focus light as established with bead models (see Fig. 2). We have previously shown that DHPR-RyR colocalization increases with age (6–20 days) and deconvolution does not appear to change the overall distribution pattern of DHPR and RyR. This study shows that deconvolution has a positive effect on the reliability of quantitative measurements by increasing resolution and decreasing false colocalization events. We conclude that, to optimize colocalization’s predictive power of protein-protein in confocal microscopy, resolution must be maximized and false positives must be minimized by deconvolving before quantitative analysis.

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REFERENCES


6. Fabiato A. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. Am J Physiol Cell Physiol 245: C1–C14, 1983.


