

Using Super-Resolution Microscopy to Study Subcellular Structures in Cardiac Mouse Myocytes

Joseph Isaac S. Wong

Abstract

This PRIME project focused on the imaging of cardiac mouse myocytes using a super-resolution imaging technique, called dSTORM (direct stochastic optical reconstruction microscopy). Using this technique, fixed cardiac cells isolated from caveolin-3 knockout and wild type animals were immunostained, imaged, and analyzed in order to discern differences in Ryanodine Receptor between the two. These differences include characteristics such as cluster size, morphology, and distribution. Since the qualitative comparisons yielded very subtle differences, quantitative analysis efforts were postponed until after the program ended. Though the comparison of the knockout and wild-type animals was inconclusive, noticeable diameter increases in Ryanodine Receptor and t-tubule junctional areas in wild-type cells was observed and investigated. Because these were readily and qualitatively discernable, collaboration between UC San Diego and University of Auckland to quantitatively investigate these features is planned for the present.

Introduction

Microscopy has been used to study biological structures since the 17th century, and has progressed in image quality and resolution throughout history to the present day. Because structure and function share an important and inseparable relationship, possessing detailed knowledge of a cell's structure can provide a great amount of information about its various functions. By improving image quality and resolution, increasingly more detail can be obtained, leading to more information about function. Continuing to delve into subcellular features, however, there is a physical limit to what traditional light microscopy can resolve: this is called the diffraction barrier. Typically, the optical resolution of conventional light microscopy is limited to approximately half of the wavelength of light used. This means that even with an ideal confocal microscope setup, lateral resolution is limited to

~300nm and axial resolution to ~600nm (7). However, with the advent of single-molecule detection techniques, the lateral and axial resolution of light microscopy has been improved 10-fold to ~30nm and sub-100nm, respectively (2, 5, 7). While several variants of these techniques exist, the one specifically used in this project is direct stochastic optical reconstruction microscopy, or dSTORM. This technique can be explained and explored in more depth in the literature (4). However, the premise is that by reaching into the temporal dimension, resolution can be increased by recording single fluorophore molecules that are cycling between “on” and “off”. Because of the techniques’ construction, the limiting factor in resolution is no longer the wavelength of light used but instead the number of photons recorded (4, 6, 7). Other benefits of dSTORM include its relative simplicity in usage and sample preparation, inexpensiveness in setup, flexibility in using standard dyes, and sub-diffraction-limited lateral and axial resolution (1, 2, 4).

Procedure

Ventricular mouse myocytes were isolated by enzymatic perfusion and fixed in PBS containing 2% paraformaldehyde. They were then double-labeled with Mouse Ryanodine Receptor antibody and Rabbit Caveolin-3 antibody using a standard immunostaining protocol (1). Cells were subsequently labeled with anti-mouse Alexa 680 and anti-rabbit Alexa 750. Labeled cells were mounted in a “switching buffer” containing 0.5 mg/mL Glucose Oxidase, 40mg/mL Catalase, 10% weight/volume glucose, and 10mM β -mercaptoethylamine in 20% 10x PBS and 80% glycerol (1). Cells were then imaged using a custom-built modified TIRF microscope setup for dSTORM. Chromatic shift calibration was used to minimize chromic aberrations in the double-labeled recording. For 3D imaging, an astigmatic lens was used to create a PSF as well as image the samples. Image acquisition and analysis was facilitated by custom-written Python software, written by David Baddeley of the University of Auckland Physiology Department. For complete and detailed specifications, please see materials and methods in (1).

Results

Figure 1 and Figure 2 show typical wild type and knockout cells, respectively. Aside from the obvious loss of caveolin-3 in the knockout cells, visible differences in subcellular structures are very subtle or unnoticeable. Comparing wild-type and caveolin-3 cells, RyR cluster size and distribution comparisons yielded no obvious differences. Even within the same group of cells in either knockout or wild-type, there was great inherent variability in RyR cluster size and distribution. That the variability between the knockout and wild-type group was small resulted in differences being difficult to detect. Thus, qualitatively, caveolin-3 knockout cells did not exhibit an obvious difference with the wild-type cells. Because of these subtleties, a quantitative analysis would require a larger sample size to attain a reasonable power and determine statistical significance. Ultimately, quantitative analyses were postponed in favor of gathering more sample data and future collaboration between UCSD and University of Auckland.

Conclusions

As mentioned above, analysis of the data accumulated proved difficult because of the large degree of variability in image quality. Image quality—as determined by the amount of “events” or photons—varied greatly throughout all the samples in both groups. This not only includes variation in resolution but also noise, contrast, and fidelity of image. This is due to many factors and result in unequal image quality among samples. Most importantly, the variability in image quality masked the changes caused by knocking out caveolin-3, making it difficult to compare with wild-type cells.

Two main factors affect the event count and quality: original fluorescent labeling intensity and the switching buffer. If the switching buffer is not in optimal condition, the fluorophores will not cycle between their on and off states efficiently, reducing the resolving power of dSTORM. More importantly, labeling intensity determines the maximum image quality, in regards to resolution, contrast, and signal to noise ratio. Determining label intensity, however, introduces many more variables. From the cardiac

cells' natural affinity for the primary antibodies, to the specific affinities of the primary antibodies for the secondary antibodies, as well as the integrity of the immunostaining protocol, many factors determine the amount and distribution of fluorophores on the subcellular features of interest. Ultimately, the combined affinities and variations determine not only the maximum number of events but also the locations and where those events occur. Unfortunately, there is no practical way to control the issue of distribution or labeling intensity. Possible solutions to suppress these issues would be to label with a mixture of primary antibodies that target the same structure; the time period given in the PRIME program did not allow for testing this.

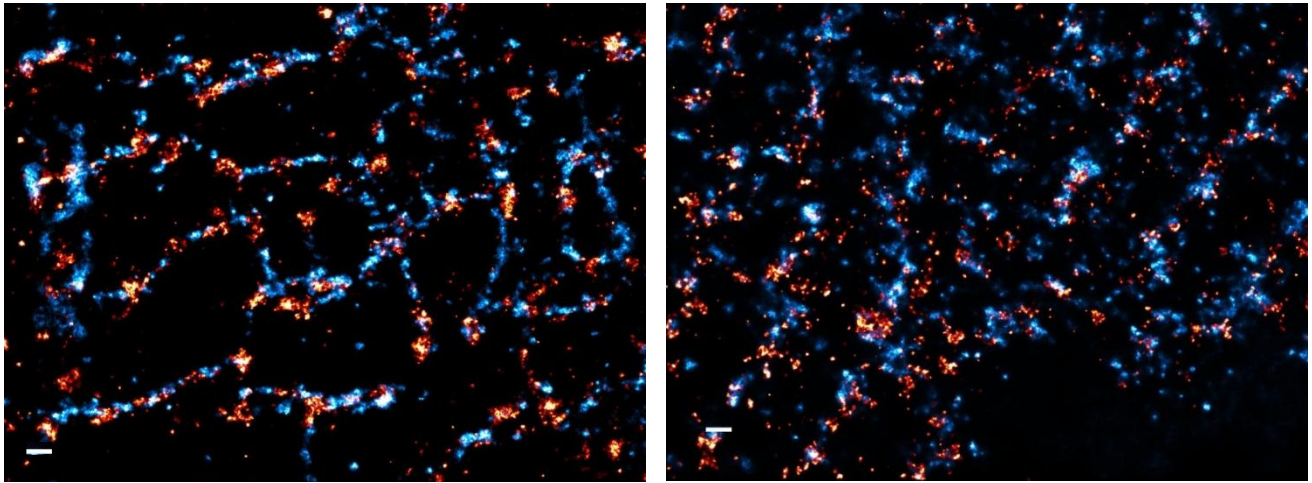
Other issues include cell drift, in which the cell or slide moves during acquisition and shifts the frame of view, resulting in shifted events. This can be partially corrected by software but the result may not correctly represent the actual cell. As a result, images corrected for drift may not be reliable in measuring cluster sizes or t-tubule diameters. Furthermore, in 3D dSTORM, rendering of the models was highly dependent on thresholding; with no unbiased way of setting threshold levels, unbiased measurements cannot be taken with the models created.

Although the wild-type and caveolin-3 knockout comparisons were not as conclusive, an offshoot of the project included investigating the local increase in diameter of t-tubules in RyR and t-tubule junctional areas (Figure 1). This hypothesis has some correlation with the work done by NCMIR in creating mesh models from electron microscopy data (3). To ensure that the diameter increase was not an artifact of 2D projection of a 3D structure, 3D dSTORM was used to improve axial resolution and produce a 3D model (Figure 3). Indeed, in the early images, these local "swellings" in the peripheral junctions appear to be present and is in agreement with 3D electron microscopy data(3). Further collaborations will investigate and characterize these features quantitatively.

Discussion

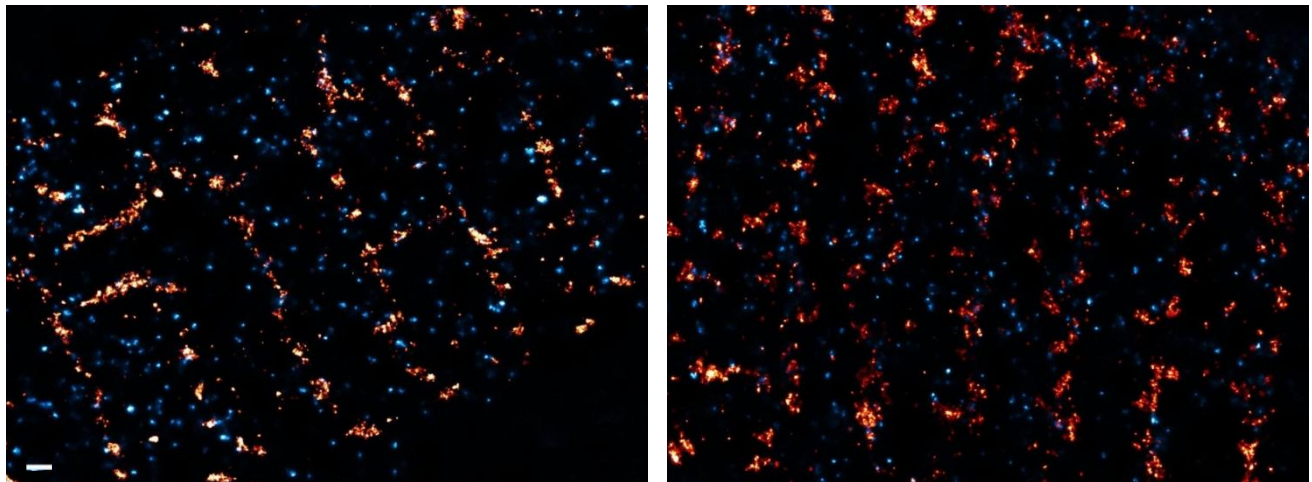
As mentioned in the introduction, structure and function share an important relationship. With increased knowledge of structure, function can be more easily understood. Likewise, from the data obtained from dSTORM, more understanding of the function of the Ryanodine Receptors can be achieved. From the images created in 2D or 3D dSTORM, one can construct computational models that represent an area or volume, respectively. Using extrapolation, that area or volume can be characterized as containing a certain number of Ryanodine Receptors. From the number of RyRs present, mathematical computations can realistically model the handling of Ca^{2+} , as was done in previous 3D electron microscopy (3). With super-resolution microscopy, however, modeling is more readily attainable with greater specificity, higher contrast, and competitive resolution. The potential of super-resolution is evident in this advantage. From its simplicity in usage, ease of sample preparation, resolving power, and in particular with dSTORM, its flexibility with traditional dyes, super-resolution microscopy provides an attractive method for breaking the diffraction barrier and, together with other imaging techniques, unlocking more knowledge about the structure and function of a cell.

Figure 1



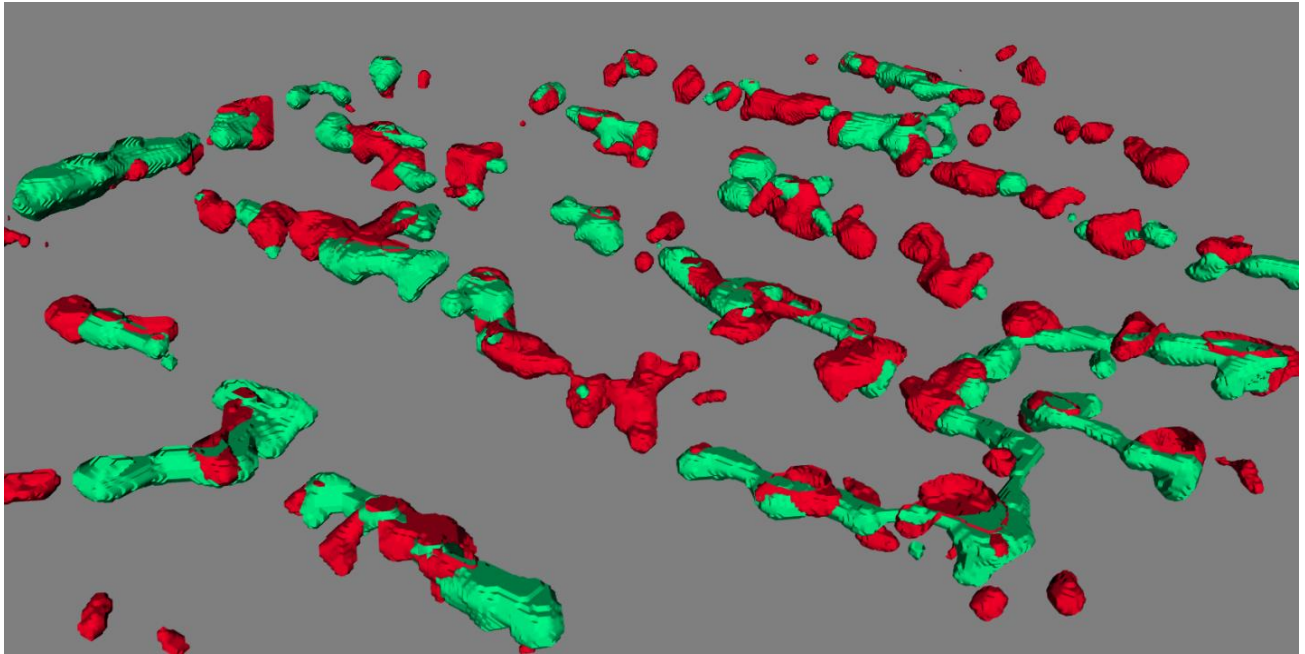
Super-resolution (dSTORM) 2D image of wild-type cardiac ventricular myocytes.
Red: Ryanodine Receptor. Cyan: Caveolin-3. Scale: 500nm.
Left: intracellular image. Right: surface image

Figure 2



Super-resolution (dSTORM) 2D image of caveolin-3 knockout cardiac ventricular myocytes.
Red: Ryanodine Receptor. Cyan: Caveolin-3. Scale: 500nm.
Left: intracellular image. Right: surface image

Figure 3



Super-resolution (dSTORM) 3D image of wild-type cardiac ventricular myocytes.
Red: Ryanodine Receptor. Green: Caveolin-3. Rendered with MC Annealing Segmentation

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