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Review article

Super-resolution imaging of EC coupling protein distribution in the heart

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ABSTRACT

The cardiac ryanodine receptor (RyR) plays a central role in the control of contractile function of the heart. In cardiac ventricular myocytes RyRs and associated Ca^{2+} handling proteins, including membrane Ca^{2+} channels, Ca^{2+} pumps and other sarcolemmal and sarcoplasmic reticulum proteins interact to set the time course and amplitude of the electrically triggered cytosolic Ca^{2+} transient. It has become increasingly clear that protein distribution and clustering on the nanometer scale is critical in determining the interaction of these proteins and the resulting properties of cardiac Ca^{2+} handling. Such intricate near-molecular scale detail cannot be visualized with conventional fluorescence microscopy techniques (e.g. confocal microscopy) but it has recently become accessible with optical super-resolution techniques. These techniques retain the advantages of fluorescent marker technology, i.e. high specificity and excellent contrast, but have a spatial resolution approaching 10 nm, i.e. objects not much further apart than 10 nm can be distinguished, previously only attainable with electron microscopy. We review the use of these novel imaging techniques for the study of protein distribution in cardiac ventricular myocytes and discuss technical considerations as well as recent findings using super-resolution imaging. An emphasis is on single molecule localization based super-resolution approaches and their use to reveal the complexity of RyR cluster morphology, placement and relationship to other excitation–contraction coupling proteins. Super-resolution imaging approaches have already demonstrated their utility for the study of cardiac structure–function relationships and we anticipate that their use will rapidly increase and help improve our understanding of cardiac Ca^{2+} regulation. This article is part of a Special Issue entitled "Calcium Signaling in Heart".

Contents

1. Introduction

Cardiac excitation–contraction (EC) coupling [1] relies on the rapid voltage-gated opening of surface membrane L-type Ca^{2+} channels (DHPRs). The influx of Ca^{2+} via the sarcolemmal Ca^{2+} current (I_{Ca}) triggers the opening of closely apposed intracellular Ca^{2+} release channels, the ryanodine receptors (RyRs, [2]) to release further Ca^{2+} from the intracellular Ca^{2+} store, the sarcoplasmic reticulum (SR). The resulting transient increase of Ca^{2+} causes forceful contraction and is terminated by Ca^{2+} removal mechanisms that transport Ca^{2+} back into the extracellular space (mostly via the Na/Ca exchanger) and into the SR (via the SR Ca^{2+} ATPase, SERCA). While we have known the key proteins

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involved for some time their spatial distribution at the nanometer scale has increasingly become the focus of interest. Mathematical modeling has suggested that protein clustering and protein distances on the order of nanometers are critical to the tight coupling between DHPRs and RyRs (for example, [3]). This very localized aspect of protein distribution is thought to be an important determinant of the functional properties of cardiac Ca^{2+} handling, and forms the basis of the "local control" model of cardiac EC coupling (see, for example, [4]). As a result there has been strong interest to characterize the local ultrastructure and protein distribution in muscle since suitable imaging techniques became available. This includes both electron microscopy (which played an important role in discovering "dyad" junctions between sarcolemma and SR [5], and "foot structures" [6] later identified as RyRs [7]) and optical fluorescence methods, often using confocal microscopy (e.g. [8,9]) or wide-field deconvolution techniques (e.g. [10]). Recently, the motivation to characterize EC coupling structures in detail has further increased, driven by evidence that ultrastructural changes in protein distribution and membrane structures, including dyads and the fine extensions of the surface membrane, the transverse tubules (or t-tubules [11–13]) may contribute to the contractile deficit observed in heart failure (e.g. [14–17]).

Biophysically, the organization of proteins into clusters and the ultrastructural architecture of signaling compartments can profoundly affect Ca^{2+} signaling by a number of mechanisms. The tight coupling of DHPRs and RyRs as well as the local nature of activation of RyR clusters (seen functionally as Ca^{2+} sparks, [18,19]) is thought to depend upon the location of most RyR clusters within narrow dyads [5,20], junctions between SR and surface membrane (and its extension, the t-tubules [21]). The main mechanism ensuring tight coupling is the effect of spatial confinement in junctions on both the amplitude and kinetics of Ca^{2+} transients in response to channel opening as has been shown in modeling studies (e.g. [22,23]). RyR gating is Ca^{2+} sensitive and the activation of RyR clusters during EC coupling is triggered by a process termed Ca^{2+}-induced Ca^{2+}-release [24]. Cluster organization and size (quantified as the number of RyRs) are therefore biophysically expected to affect both the sensitivity to activation (more RyRs make the cluster more likely to be activated, e.g. [3]) and the termination of release (more RyRs would tend to make clusters less likely to shut down by stochastic attrition [25,26]). It is therefore clear that there is great interest in determining the size distribution and placement of RyR clusters to improve our understanding of the biophysical properties of EC coupling.

2. Imaging RyR clusters

Important insight into the distribution and size of RyR clusters has been provided by electron microscopy which has the required resolution to directly visualize RyRs which are large proteins ~30 nm in diameter [2,27,28]. RyR clusters can also be visualized using fluorescence microscopy, either using immuno-staining (e.g. [9,29]), fluorescent ligands that bind to RyRs (e.g. [30]) or as part of fusion proteins in which fluorescent proteins act as markers (e.g. [31]). An appeal of fluorescent methods is the high specificity and the contrast in fluorescent micrographs as well as the ability to investigate the relative distribution of RyRs and related EC coupling proteins using multiple, spectrally distinguishable markers. When this is combined with the optical sectioning ability of confocal microscopy (or deconvolution microscopy) very detailed volume data on the distribution of RyRs and their relative location with respect to other proteins can be obtained (e.g. [10,13,29,32]). Another advantage is the compatibility of fluorescent imaging methods with live cell imaging allowing in principle a direct comparison of the relationship of structure and function (e.g. [30]). A significant limitation of fluorescence methods has been the comparatively low spatial resolution of these optical methods. Optical imaging is subject to a fundamental limit of resolution resulting from the wave nature of light, the "diffraction-limit" [33]. Even under favorable optical conditions using high numerical aperture objectives, the smallest resolvable detail is on the order of half the wavelength of light used for imaging, typically limiting resolution to ~250 nm (for more precise formulae, see for example, [34]). This is sufficient for many purposes but cannot resolve the outline and detailed shape of RyR clusters made up of proteins ~30 nm across. Due to the inevitable optical diffraction any structures $\leq$200 nm in size appear as largely featureless spots in the fluorescence microscope and this is indeed what is seen in fluorescent micrographs of RyR labeling at high magnification (Fig. 1A).

3. Optical super-resolution imaging

For many years it had therefore been assumed that fluorescence imaging is of little utility in revealing the detailed distribution of proteins at the nanometer scale, a view that has greatly changed since the recent introduction of practical optical "super-resolution" methods. There are now a variety of fluorescence super-resolution approaches that can resolve nanoscale detail in biological structures. None of these methods invalidate the "diffraction limit" as first formulated by Abbe [33] but they provide effective workarounds to overcome it. Most importantly, these super-resolution imaging techniques are "far-field" methods (as opposed to optical "near-field" methods, such as TIRF [35]) which allow the study of samples far away from the objective and other interfaces (e.g. the coverslip) relatively deep within cells and tissues. The most widely used methods include (1) STED (stimulated emission depletion) microscopy [36], (2) single molecule imaging based approaches known under acronyms such as PALM [37,38] or STORM [39,40] and (3) structured illumination microscopy (SIM) [41,42]. Commercial implementations of all these techniques are now available and a number of recent reviews provide a good overview of the various methods and their relative strengths and weaknesses, e.g. [43–46], and we therefore discuss the various methods here only briefly.

In their commercial implementations both SIM and STED offer a somewhat lesser resolution improvement than PALM/STORM/dSTORM albeit at higher speed. SIM is typically limited to a two-fold improvement over wide-field microscopy with a final resolution of ~120 nm in the lateral direction and ~250 nm axially and can image thin (~1 μm deep) 3D stacks at ~1 Hz (see http://www.api.com/OMX-Blaze.asp). STED, like PALM/STORM/dSTORM, has theoretically unlimited resolution, and a current commercial version yields a resolution of 50–70 nm laterally with little axial resolution improvement (see http://www.leica-microsystems.com/products/confocal-microscopes/details/product/leica-tcs-sted/). Due to the degree of optical complexity involved in constructing a STED system, a homebuilt system offering significantly better resolution is a challenging undertaking. In addition, because STED is a point scanning method, its speed depends on the resolution achieved and size of the area being imaged. It can be very fast when imaging very small regions, but will be substantially slower than SIM when imaging a large field. STED speed can be best appreciated by comparison to confocal microscopy — the higher resolution of STED requires finer sampling (to fulfill the Nyquist criterion) and thus more pixels over a given area. For a resolution of $50\times50\times600$ nm (i.e. an improvement by a factor of $5\times5=25\times$ over confocal) STED will be ~25 times slower than confocal microscopy of the same region (this is potentially an under-estimate, as the improved resolution will also require more photons to be collected from the same area to achieve the same signal-to-noise ratio).

Here we will mostly focus on single molecule super-resolution microscopy based on variations of the STORM (stochastic optical reconstruction microscopy) [39] or dSTORM (direct STORM [40]) principles which we implemented for the imaging of protein distribution in cardiac myocytes. Advantages of these techniques for the imaging of cardiac myocytes, in direct comparison to SIM and STED as described above, include relatively modest hardware requirements, ease of optical alignment, the ability to use conventional fluorochromes as markers and a lateral resolution of ~20 nm in complex biological samples. We will