Optical single-channel resolution imaging of the ryanodine receptor distribution in rat cardiac myocytes

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We have applied an optical super-resolution technique based on single-molecule localization to examine the peripheral distribution of a cardiac signaling protein, the ryanodine receptor (RyR), in rat ventricular myocytes. RyRs form clusters with a mean size of approximately 14 RyRs per cluster, which is almost an order of magnitude smaller than previously estimated. Clusters were typically not circular (as previously assumed) but elongated with an average aspect ratio of 1.9. Edge-to-edge distances between adjacent RyR clusters were often <50 nm, suggesting that peripheral RyR clusters may exhibit strong intercluster signaling. The wide variation of cluster size, which follows a near-exponential distribution, is compatible with a stochastic cluster assembly process. We suggest that calcium sparks may be the result of the concerted activation of several RyR clusters forming a functional "supercluster" whose gating is controlled by both cytosolic and sarcoplasmic reticulum luminal calcium levels.

Results

RyR Distribution in Peripheral Clusters. Rat ventricular myocytes, labeled with an antibody against the cardiac RyR2 and a secondary antibody carrying Alexa Fluor 647 (see Materials and Methods) were imaged using a custom-built localization microscope (13). Fig. 1A shows the typical peripheral RyR labeling pattern that was seen. Comparison between the diffraction-limited image (red) and the corresponding localization image (green) demonstrates the clear enhancement in resolution provided by localisation microscopy. At conventional, diffraction-limited resolution (~270 nm), the labeling shows only irregular double rows of puncta aligned with z-lines as described in ref. 16. From their apparent dimensions, such puncta would contain approximately 80–140 RyRs if uniformly filled by RyRs, and similar peripheral cluster sizes have been estimated from thin sectioning (17). In contrast, the high-resolution localization image shows that the red puncta are incompletely filled with RyRs, often containing several smaller clusters. Additionally, the shape and size of the RyR clusters revealed by the localization data are quite variable.

The RyR protein is a large homo-tetramer of approximately 560 kDa subunits, with a quartenfoil structure that occupies a volume of approximately 29 × 29 × 12 nm as measured in the skeletal RyR1 isoform (1). RyR2 has a similar shape and size (18), and both cardiac and skeletal isoforms are thought to form quasi-crystalline arrays in the SR membrane (1, 19). Formation of regular arrays appears to be an intrinsic property of RyRs as shown by their spontaneous formation in solution and lipid bilayers (4, 20) as well as their occurrence in preparations from skeletal muscle (21, 22). Within these arrays, the RyRs have a center-to-center spacing of approximately 30 nm. The precise two-dimensional arrangement of RyRs in mammalian cardiac muscle has not been shown to date, evidence from frog myocardium suggests a similar regular array structure (23).

With the assumption of a 30-nm unit cell, we assigned candidate RyR positions to produce a model for RyR location which matched the localization data (Fig. 1B). Note the presence of sharp cluster edges and dislocations in the data matching the model. While the achieved effective resolution of approximately...
30 nm was not quite sufficient to directly observe the regular grid structure of RyRs, the presence of sharp edges and steps in the outline of the clusters is clearly compatible with a regular array that has an approximate 30-nm unit size. This is direct evidence for a two-dimensional array-like arrangement of RyRs in cardiac muscle.

**RyR Clusters Are Small and Closely Spaced.** Because of the high contrast provided by fluorescent labeling, it was possible to automatically segment the localization data (see Materials and Methods) and obtain statistical data on cluster morphology. A total of 7,675 clusters were measured in 22 cells. Fig. 2A shows the resulting distribution of cluster diameters and nearest neighbor distances between cluster centroids, with mean values of 107 nm and 308 nm, respectively. While intercentroid distances convey the distribution pattern of clusters on the membrane, cross-signaling between RyR clusters (via calcium-induced calcium release, CICR) would depend strongly on the edge-to-edge distances between RyR arrays and about one third of clusters were within 50 nm of their nearest neighbors (see Figs. S1 and S2).

The peripheral clusters were imaged in membrane areas in close contact with the coverslip and are therefore essentially flat and parallel to the image plane. Therefore, the apparent cluster area can be directly converted into the number of RyRs in the cluster (based on 1 RyR per 900 nm²). Fig. 2B shows a representative region in which segmented clusters have been color coded according to the number of RyRs they contain. The calculated cluster sizes followed an approximately exponential distribution with a mean of 13.6 RyRs (n = 22 from three animals) per cluster (see Fig. 2C). It is notable, that this result is much smaller than previous estimates of approximately 100 RyRs per cluster. There was modest cell to cell variation in cluster size (shaded region in Fig. 2C), and very little variation between animals (see Fig. S3). Although the large number of very small clusters shown in Fig. 2C is striking, Fig. 2D shows that the majority of RyRs are found in somewhat larger clusters of ≈25 RyRs. Some clusters were incompletely filled with RyRs, an observation consistent with a recent electron microscopic study which has shown interior junctions to be incompletely filled with RyRs (24).

Because some groups of clusters are in close proximity it is possible that several smaller clusters may act functionally as a calcium release unit or “supercluster” (see also Fig. S4). We therefore also calculated the total number of receptors in cluster groups that were within 100 nm of each other (edge-to-edge) and obtained a mean supercluster size of 21.6 RyRs (n = 10 cells from two animals). In agreement with a previous study (16), we find that virtually all RyR clusters occur within an approximately 700 nm wide band centered on z-lines with only few located between z-lines.

**RyR Clusters Have Complex Geometries.** A random selection of larger clusters is shown in Fig. 3A, illustrating the diversity of peripheral RyR cluster shapes. One commonly occurring feature is a crescent or doughnut shaped cluster. In some cases such shapes would be consistent with the presence of a t-tubule opening (~200 nm) and RyR clusters at the mouth of t-tubules have been seen previously in EM thin sections (25).

To quantify these cluster geometries we measured RyR cluster aspect ratios and their “circularity” (a measure of compactness, with a perfect circle having a circularity of 1) as shown in Fig. 3C and D. Most RyR clusters were moderately elongated (mean aspect ratio ~1.9, see Materials and Methods). The mean circularity of RyR clusters was approximately 0.2, implying a very complex outline (note that a rectangle with the same average aspect ratio would have a circularity of 0.7).

**Stochastic Self-Assembly Can Explain RyR Cluster Size.** Given the highly ordered double-row structure seen in skeletal muscle (26), it is possible that RyR clusters are assembled onto a structural template. The approximately exponential size distribution seen here, however, argues against the idea that RyR cluster size is very tightly controlled. To test the idea that stochastic self-assembly could explain the observed RyR cluster size distribution we used a Monte-Carlo model of cluster growth. This model incorporated a nucleation process (to seed new clusters), accretion (that adds further RyRs), and turnover. Cluster shapes resulting from one Monte-Carlo simulation are shown in Fig. 4A, which are qualitatively similar to those observed. The size distribution obtained from 1,000 simulation runs (Fig. 4B) was a close match to the measured distribution and even exhibited a similar departure from exponential behavior at low cluster sizes (compare Fig. 2C). The histograms shown here were calculated with a simple model but more complex models that explicitly consider diffusion of receptors in the SR membrane and binding between RyRs yield similar results (see SI Text and Fig. S5).
Discussion

Using localization microscopy we have produced high-resolution immunocytochemical images of RyR distribution in the periphery of cardiac myocytes. These data show that the RyR clusters have widely varying sizes and shapes, although at a lower resolution, the data are compatible with previous immunofluorescence labeling.

Our data reveal that RyR cluster sizes vary widely from single RyRs to $>100$ RyRs, which seems at variance with the substantially larger estimates for the size of “couplons” within myocytes (17, 27), although the variability in junction size and feet (i.e., RyR) content has been noted in ref. 28. While this could reflect a genuine difference between peripheral and deeper RyR cluster size, it is possible that assumptions necessary for the estimation of cluster sizes from previous data led to an overestimate. For example, in both thin sectioning (17) and quantitative fluorescence imaging (27), the assumption of circularity and complete filling of the junction by close-packed RyRs has been used. Our data suggests that these key assumptions may be incorrect, and...
The RyR clusters that we observed were frequently incompletely filled with RyRs in agreement with recent studies (31). Our data (Fig. 2D) suggests that peripheral calcium sparks should most likely be triggered from clusters containing approximately 25 RyRs, a number which is in reasonable agreement with the latter estimate. This data can also be used to quantify the fraction of RyRs in the smallest clusters (32) (see Fig. 2D), but it is still unclear what the functional properties of such small RyR clusters in situ are. In connection with this point, the wide variation in cluster size (Fig. 2C) suggests that some form of autoregulation [such as local SR depletion (33) and/or luminal gating (34)] may be required to limit the site to site variability in calcium release. Were this not the case, the observed variation of mean cluster size would lead to a >5-fold variation in spark flux (8).

Our data shows that RyR clusters can be closely spaced with approximately 50% having edge-to-edge distances less than or equal to 100 nm (n = 10 cells). It is likely that clusters in such close proximity could trigger calcium release from each other. It has been estimated that a trigger level of ~10 μM is needed to explain the approximately 100-fold increase in spark rates resulting from activated RyR clusters within the timescale of EC coupling (35). Computer simulations suggest that such calcium levels may exist within 100 nm of an activated cluster (32). With these figures in mind, we can envision a calcium spark incorporating more than one of the closely spaced RyR clusters. Such an effect may reconcile the quantal nature of peripheral calcium sparks (8) with our data without requiring a subpopulation of active RyRs (≤ 6) within a cluster. This reconciliation may require that the coupling between closely spaced clusters is reduced (perhaps by membrane distortion) to prevent them from always coactivating but allow stochastic variation in the number of activated clusters. It is also possible that several RyR clusters may be connected to the same SR terminal cistern which would result in tight coupling of calcium release between clusters via the cytosolic and luminal SR calcium concentrations. Our data raises the possibility of a new level of Ca2+ signaling via a “triggered saltatory” mechanism for calcium release wherein groups of neighboring clusters form a larger supercluster (see also Fig. S4), which acts in concert to a triggering event. This idea is consistent with data from Hayashi et al. who observed that interior couplings between SR and t-tubules generally consist of several closely spaced contact zones along t-tubules containing each only relatively few RyRs (24).

We have shown that our measured cluster size distribution could be explained by a stochastic process of cluster growth. A stochastic growth process could also explain observations in skeletal muscle derived BC3H1 cells, where smaller RyR clusters of variable orientation are packed into single larger clusters in peripheral junctions (36). Although our simulations assume spontaneous nucleation, it is likely that cluster nucleation in the cell may be secondary to mechanisms mediated by additional proteins [e.g., junctophilin (37)] to ensure the correct placement of junctions. Nevertheless our models show that with the placement of a few localizing proteins appropriately sized RyR clusters may form without requiring an explicit scaffold or other process that tightly controls cluster size. We envisage that future high resolution imaging of RyR and related junctional protein distributions (and their change in response to interventions and protein modifications) will yield further insight into the process of RyR cluster formation.

Finally, we have demonstrated that high-resolution immunocytological localization microscopy can provide information on a scale that fills the gap between FRET and related techniques and conventional fluorescence microscopy. Because this method should be generally applicable to other types of proteins.
and signaling pathways, we can expect it to provide insight for a variety of cell processes.

Materials and Methods

Sample Preparation. Ventricular myocytes were enzymatically isolated from adult rats (6 weeks) as described in ref. 38 and fixed in 2% paraformaldehyde for 10 min. They were then labeled according to a standard immunofluorescence protocol (27) using a mouse-anti-RyR2 primary antibody (Ab, Affinity BioReagents, MA-3-916) and an Alexa Fluor 647 conjugated goat-anti-mouse secondary Ab (Molecular Probes/Invitrogen).

To prepare a sample slide, approximately 5 μL of the labeled cell suspension was pipetted onto a clean coverslip, approximately 15 μL “switching buffer” (15) (0.5 mg/mL glucose oxidase, 40 μg/mL catalase, 10% wt/vol glucose, and 50 mM β-mercaptoethanol in PBS, all obtained from Sigma–Aldrich) was added, a slide placed on top, and the edges sealed with nail varnish.

Microscope Setup. Images were acquired on a commercial Nikon TE2000 inverted microscope with a Nikon 60×, 1.49NA oil-immersion TIRF objective (Nikon), and an Andor iXon DVB87DCS-BV electron multiplying CCD camera (Andor Technology). A bandpass interference filter (HQ710/50m, Chroma) was used to select the emitted fluorescence. To the stock microscope we have added custom illumination optics allowing high intensity laser illumination at an adjustable angle, and a custom objective holder coupled directly to the microscope stage and designed to minimize thermal and mechanical drift.

Focusing was performed using a piezo focusing unit (Physik Intrumente P-725). The laser was provided by two solid state lasers (K-Laser, VA-1-LNS-532 and VA-1-N-671) providing focal plane intensities of approximately 10¹⁶ W/m² at 543 nm (green) and 10⁹ W/m² at 671 nm (red), respectively, over a 10-μm diameter field of view. The illuminator was adjusted to generate a highly inclined light sheet (39) at an angle just below the TIRF angle.

Image Acquisition. Image acquisition was performed using a combination of the techniques described in refs. 12, 13, and 15. Cells were prebleached for a highly inclined light sheet (39) at an angle just below the TIRF angle.

Image Analysis and Reconstruction. Single molecule signals were detected and fitted in individual frames as described in ref. 13. The mean estimated localization accuracy was approximately 13 nm, corresponding to an effective diameter of the fluorophore positions belonging to a cluster, discarding any triangles with edge lengths greater than our estimated resolution (to allow for concave shapes), and summing the area of the remaining triangles. A circumference was also extracted by summing the lengths of all of the exterior edges present in the triangulation, which was used to derive the circularity (4π×area/circumference) of the clusters.

Cluster Growth Simulation. Monte-Carlo simulations of a simplified cluster growth model were performed on a 50 × 50 unit rectangular grid (unit size 30 nm), where each grid cell could either contain an RyR, or be empty. Starting with an empty grid, the probability that a grid cell would contain an RyR in the next iteration was given as the sum of a small “nucleation” probability (2.5 × 10⁻⁴) which was independent of the RyR occupancy of neighboring cells, a “growth” probability (2.5 × 10⁻² × Nighbou) which was linearly dependent on the number of four-connected neighbors a newly placed RyR would have, and a “retention” probability (0.937 if there was already an RyR present) which allows for some RyR turnover (and was tuned to match the observed mean cluster size). One hundred MC steps were performed to produce each cluster realization used in the analysis. A more complex model was also implemented (see SI Text).

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Additional Stochastic Self-Assembly Models of RyR Cluster Formation.

A simple model of cluster formation was able to reproduce the observed cluster size distribution (see main text). That model required only few free parameters at the expense of not describing in detail the diffusion of mobile RyRs in the membrane. To show that a model that includes receptor diffusion produces similar results we also constructed a second, more complex model. The model included two RyR populations, a mobile fraction that freely diffuses in the SR membrane, and a fraction of fixed receptors that are bound to (unspecified) anchoring structures. A Monte Carlo simulation was implemented on a 100 × 100 grid with unit cell size equal to the RyR size (30 nm). Diffusion of mobile receptors was implemented via movement to one of its randomly chosen four neighbouring grid cells per time step, provided that cell was not occupied by another receptor. The diffusion used periodic boundary conditions. New mobile receptors were inserted into the membrane with a probability $p_{\text{seed}}$ of $1.3 \times 10^{-5}$ per time step. Mobile receptors could become fixed by binding to randomly occurring anchoring proteins (with a probability $p_{\text{fix}} = 8 \times 10^{-4}$) or by binding to fixed RyRs with which they come into direct contact. RyRs with a single fixed neighbor had a probability of binding of 0.5. Two or more neighbouring RyRs resulted in certain binding. To generate a simulated cluster configuration 200 time steps were performed.

The probability $p_{\text{seed}}$ of seeding new receptors was set to $1.3 \times 10^{-5}$ for the last 50 steps to simulate the cessation of the major growth phase. Cluster size distributions from 200 runs were pooled to generate cluster size histograms.

Independent support of the idea that the observed cluster size distributions reflect stochastic self-assembly of diffusible receptors was provided by comparison with an analytical model of cluster formation that was recently described in ref. 1. The model assumes that receptors are inserted into the membrane at random locations and diffuse within the membrane until captured by existing immobile clusters. While the model was originally developed for a growing population of bacterial cells the biophysical principles it is based upon are also applicable to the stochastic assembly of RyR clusters in growing heart cells. In the steady state, the probability $p(N)$ of finding a cluster containing $N$ receptors can then under fairly general conditions (which include that the cluster center-to-center distances are large compared to the cluster half-diameter) be approximated by the expression:

$$p(N) = c_1 \exp(-c_2 N + c_3 N \ln(N) - c_4 N (\ln(N))^2))$$

with unknown constants $c_1$, $c_2$, $c_3$, and $c_4$ (1). We fitted this equation with four free parameters to our observed histograms, noting that histogram frequencies are proportional to $p(N)$ in the limit of large numbers of clusters.

Fig. S1. Edge-to-edge distances between peripheral RyR clusters in a typical cell. This information is complementary to the RyR cluster center-to-center distances and important in determining the likelihood of cross-signaling between clusters during activation. Clusters are close to their nearest neighbors with approximately half of all clusters being within 100 nm of a neighboring site.
The density of neighboring clusters as a function of the distance from a cluster center. The low values at small distances can be largely attributed to size exclusion effects. For a uniform distribution of clusters, the density would then be expected to rise to a constant level and remain there. The observed distribution, however, peaks just above the cluster size, indicating a nonuniform distribution in which clusters are “bunched” together, i.e., it is more likely to find a cluster close to another one than far away from it. Looking at the small (N_{bR} < 10) and large (N_{bR} > 20) clusters separately shows that this bunching is most pronounced for smaller clusters. The right shift of the initial rise in the density of larger clusters can be attributed to the larger excluded area of these clusters.
Fig. S3. Animal to animal variability in RyR cluster size. We observed very little variability in RyR cluster size between animals as illustrated by the close agreement in the cluster size distribution observed for different animals. The mean cluster sizes in the three animals were 13.6 (n = 11 cells), 14.0 (n = 4 cells), and 13.2 (n = 7 cells) RyRs per cluster, respectively.
Fig. S4. The concept of RyR superclusters. This image shows detected clusters (white) with a 50-nm “halo” (red) indicating an “area of influence” around each cluster. This is based on the idea that within −100 nm of an active cluster $[\text{Ca}^{2+}]$ is expected to be high enough ($>10 \, \mu\text{M}$) to induce RyR activation. Contiguous regions of red thus represent groups of clusters whose edge to edge distances are $<100$ nm. These groups might be expected to act functionally as one supercluster (possibly with stochastic recruitment among members) in response to a trigger in any of the constituent clusters. (Scale bar, 1 μm.)
Fig. S5. Stochastic self-assembly of RyR clusters. (A) A Monte Carlo model that includes diffusion of single RyRs in the SR membrane yields cluster sizes and geometries, which are similar to those measured. This includes the excess over an exponential distribution at small cluster sizes. The model generated size histogram (blue line) is well fit by an analytical equation derived from a cluster growth model (red line) demonstrating the consistency of both approaches. (B) The analytical cluster model also provides a good fit (red) to the observed cluster size distribution (blue, $n = 7$ cells from one animal).
Fig. S6. Relationship between RyRs and detected event numbers. When the number of single molecule events observed within a cluster is plotted against the number of estimated RyRs a strong linear correlation is observed with a slope of approximately 7.9 events/RyR. A linear relationship between the amount of labeling and cluster size is to be expected, and supportive of our segmentation and size estimation method. The number of events per RyR (yielding a single RyR signal to noise of $\sim2.8$) is consistent with our ability to obtain reliable estimates of cluster size.