

## Protocol for the Isolation and Super-resolution dSTORM

### Imaging of RyR2 in Cardiac Myocytes

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**[Abstract]** Since its inception, super-resolution microscopy has played an increasingly important role in the discovery and characterization of nanoscale biological structure. dSTORM, which is one of the most commonly applied methods, relies on stochastic photoswitching of fluorophores to recreate a super-resolution image. The cardiac field has particularly benefitted from the application of this technique, as it has enabled sub-diffraction-limit visualization of calcium release units (CRUs) and the fundamental structures that trigger contraction. Acquisition of such images requires careful, reproducible sample preparation, and consistent imaging conditions maintained for the duration of the experiment. Here we present standardized methods for the production of dSTORM images of the Ca<sup>2+</sup> release channel Ryanodine Receptor type-2 (RyR2) in cardiac myocytes. The presented protocols specifically focus on steps involved in primary cardiac myocyte isolation, sample preparation, and imaging with details provided for experimental solutions and microscope settings. This discussion is followed by an overview of various analysis techniques to discern RyR2 organization within clusters and CRUs.

**Keywords:** Super-resolution, dSTORM, Langendorff, Cell Isolation, Image processing

**[Background]** In recent years, super-resolution microscopy has seen a rapid rise in popularity. A variety of super-resolution techniques have been described which enable optical resolution well below the diffraction limit of light, in some cases approaching that obtainable by electron microscopy. Together, the advent of these techniques has led to an explosion of new research into nanoscale biological structure, domains, and protein interactions. One popular super-resolution technique is direct Stochastic Optical Microscopy (dSTORM), which pairs the benefits of relatively simple sample handling with an ~10x improvement in resolution in comparison with standard confocal microscopy (van de Linde *et al.*, 2011). The trade-off, however, is an increased acquisition time as well as complexity of analysis which can seem daunting to those starting in the field. While recent commercial systems from the major imaging companies such as Zeiss, Nikon, and Olympus have made dSTORM more accessible to biologists, the technique still requires careful planning of experiments and accurate, reproducible protocols to ensure the production of high-quality images.

The Ryanodine Receptor type 2 (RyR2) protein is a homo-tetrameric Ca<sup>2+</sup> release channel localized within the sarcoplasmic reticulum of cardiac myocytes, which is an important target for super-resolution

structural studies (Jayasinghe *et al.*, 2012; Soeller and Baddeley, 2013; Asghari *et al.*, 2014; Hiess *et al.*, 2015; Hou *et al.*, 2015; Munro *et al.*, 2016). Indeed, the RyR is well-suited to such studies, owing to its large size and its tendency to agglomerate into functionally important 'clusters'. Most of these clusters have sizes that are just below the resolution of conventional microscopes. Because of these desirable features, the RyR can also serve as a useful example protein for introducing methods in sample preparation and imaging in a more general context. Here we present standardized methods to produce high-quality dSTORM images using the Carl Zeiss Elyra P1 dSTORM setup, with the RyR2 as a model target. The outlined protocols include methods for primary cardiac myocyte isolation, sample preparation, and imaging. Further discussion is provided regarding the analysis of RyR2 organization, including techniques for discernment of RyR clusters and, in turn, Ca<sup>2+</sup> Release Units (CRUs) which are functional groupings of RyR clusters thought to underlie Ca<sup>2+</sup> sparks (Inui *et al.*, 1987).

## **Materials and Reagents**

### A. Consumables

1. 18 gauge disposable needle (BD, catalog number: 301900)
2. Weigh boat
3. Falcon tubes 50 ml, 15 ml (Corning, catalog numbers: 430829, 430791)
4. Pasture pipette (VWR, catalog number: VWRI612-1684)
5. 1.5 Coverslips on dish (MATTEK, catalog number: P35G-0.170-14-C)

Coverslip preparation: Coverslips for the experiment also require prior preparation. To obtain very clean coverslips, we wash coverslips first with EtOH and allow them to dry. Apply 200 µl of prepared Poly-L-Lysine (0.01%) solution to the coverslip and incubate overnight at 4 °C or 2 h at 37 °C. The Poly-L Lysine coating allows cell adhesion when plated.

### B. Animals

1. Mice (Breed: C57BL/6J)

### C. Reagents

1. EtOH (VWR, catalog number: 20824.296)
2. NaCl (Sigma-Aldrich, catalog number: 71376)
3. KCl (Sigma-Aldrich, catalog number: P9541)
4. HEPES (Sigma-Aldrich, catalog number: H3375)
5. MgCl<sub>2</sub>·6H<sub>2</sub>O (Sigma-Aldrich, catalog number: M2393)
6. NaH<sub>2</sub>PO<sub>4</sub> (Sigma-Aldrich, catalog number: S5011)
7. D-Glucose (Sigma-Aldrich, catalog number: 49159)
8. Isoflurane (Abbott, catalog number: B506)
9. Bovine serum albumin (BSA) (Stock dilution: 2 g/100 ml)
10. DNase, Batch Number: 57B17285 (Worthington Biochemical, catalog number: LS002006)

11. Phosphate buffered saline (PBS) (Lonza, BioWhittaker™, catalog number: BE17-512F)
12. Collagenase 2, Activity 265 units, Batch number: 45A15450 (Worthington Biochemical, catalog number: LS004176)
13. Bovine Serum Albumin (powder) (Sigma-Aldrich, catalog number: A2153)
14. CaCl<sub>2</sub> (Sigma-Aldrich, catalog number: 449709)
15. PFA (Electron Microscopy Sciences, catalog number: 19208)
16. Glycine (Sigma-Aldrich, catalog number: G7126)  
*Note: Prepare Glycine 100 mM in PBS.*
17. Triton X-100 (Sigma-Aldrich, catalog number: X100)  
*Note: Prepare 0.03% Triton X-100 (v/v) in PBS.*
18. Image iT FX signal Enhancer (Thermo Fisher Scientific, catalog number: I36933)
19. Poly-L-lysine solution 0.01% w/v (Sigma-Aldrich, catalog number: P4707)
20. Low Blocking Buffer (Thermo Fisher Scientific, catalog number: 00-4953-54)
21. NaOH (Sigma-Aldrich, catalog number: S5881)
22. Immersion oil 30 degrees (Carl Zeiss, catalog number: Immersol-518F, 30 °C)
23. Cell isolation buffer (CIV) (see Recipes for composition)
24. Collagenase solution (see Recipes for composition)
25. 4% Paraformaldehyde (PFA) solution (see Recipes for composition)

#### D. Antibodies

1. Mouse Anti-RyR1 Primary antibody (Thermo Fisher Scientific, catalog number: MA3-916)
2. Donkey Anti-mouse Alexa 647 Secondary (Abcam, catalog number: ab181292)

#### E. Mounting media

1. Vecta Shield (Vector Laboratories, catalog number: H-1000)
2. Glycerol solution 86-89% (Sigma-Aldrich, catalog number: 49781)

### **Equipment**

#### A. Mechanical apparatus

1. Peristaltic perfusion pump (Watson-Marlow Fluid Technology Group, model: 101U)
2. Standard gauge infusion tubes x3 with added three-way valve
3. Water bath (37 °C) with pump to water jacket
4. Microdissection kit
  - a. Scissors
  - b. Fine tip forceps
  - c. Curved scissors
  - d. Curved tip forceps
5. Heating plate

6. Measuring cylinder 500 ml
7. Schott Bottle 500 ml
8. Pipettes—10  $\mu$ l, 200  $\mu$ l, 1,000  $\mu$ l (Thermo Fisher Scientific, Finnpiquette)
9. Scales (Sartorius, models: BL310 and CP224S)
10. pH meter (Radiometer Copenhagen, model: pHM 92)

#### B. Imaging apparatus

1. Carl Zeiss LSM 710 inverted confocal microscope (Carl Zeiss, model: LSM 710)
2. Carl Zeiss ELYRA P1 dSTORM attachment for LSM 710 (Carl Zeiss, model: ELYRA P.1)  
Supplied with:
  - a. PLAN Apocromat 63x NA 1.41 Oil Immersion objective
  - b. PLAN Apocromat 100x NA 1.46 Oil Immersion objective
  - c. ANDOR Ixon EMCCD
  - d. Halogen lamp
  - e. TIRF filter set (SIM, TIRF, TIRF-HP, TIRF-uHP)
  - f. 200 mW 642 nm Laser and associated filters

#### Software

1. Zen Black software (Carl Zeiss)
2. ImageJ Software

#### Procedure

##### A. Isolation

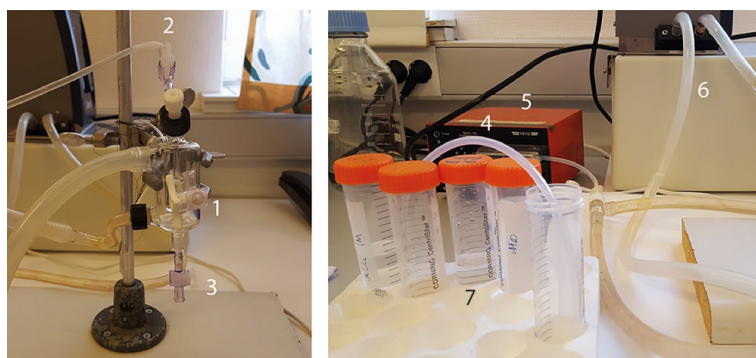
*Note: All prepared solutions should be kept on ice for the duration of the isolation protocol.*

##### 1. Setup

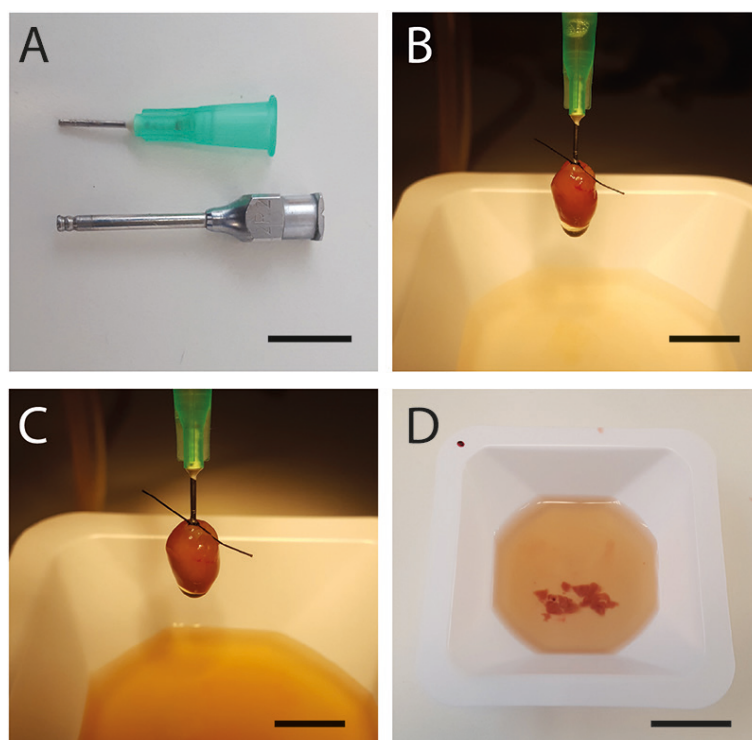
It is important that all animal usage and experimentation are approved by ethics committees with jurisdiction over the facility in which experiments will be carried out.

- a. Prior to cell isolation, the animal should be moved to the experiment room ~12 h in advance for acclimatization. If multiple animals are to be used, they should be kept separate to prevent unnecessary stress of witnessing the protocol for heart excision. Additionally, equipment should be cleaned in between each protocol.
- b. Begin by rinsing the Langendorff isolation system twice with EtOH; flushing the system at maximal pump settings.
- c. Clean the inside of the bubble trap by filling and emptying the trap.
- d. Remove excess EtOH by washing with ddH<sub>2</sub>O.
- e. Flush the system and then fill the tubing with 30 ml of CIV solution.

- f. Attach the desired canula to the bottom of the bubble trap (Figure 1). The size of the required canula is dependent on the animal to be used for isolation. For mice, we recommend using a converted 18 gauge disposable needle. It may be blunted at the tip with a small groove cut at ~1-2 mm above the bottom to enable suture placement (Figure 2A).



**Figure 1. Basic perfusion system setup.** (1) Bubble trap; (2) Perfusion fluid input; (3) Perfusion output/cannula attachment site; (4) Perfusion fluid inlet tubing; (5) Peristaltic pump; (6) Heated water jacket tubing; (7) Solutions for use in perfusion setup.

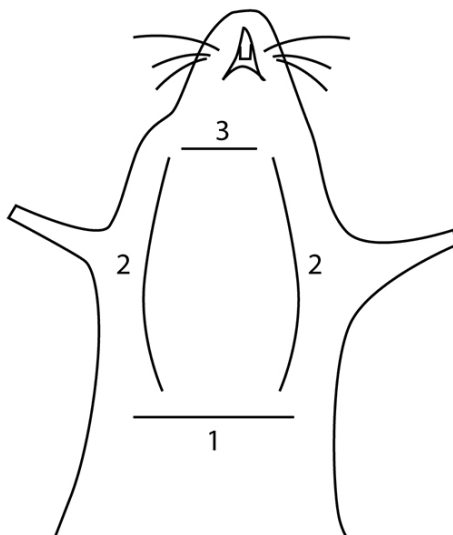


**Figure 2. Demonstration of heart cannulation and perfusion.** A. Cannula employed for aortic cannulation of the mouse and rat heart (top and bottom, respectively). B and C. A cannulated mouse heart mounted on a Langendorff setup is shown at the start (B) and end (C) of collagenase perfusion. Following successful digestion, the heart appears slightly dilated, the perfusion droplets elongate, and the perfusate becomes colored as shown. D. After successful

digestion, the heart is cut down and gently minced. The sample will now be transferred to a BSA and DNase containing solution for agitating cells from these fragments. Scale bars: A, B and C = 1 cm; D = 2 cm.

2. Heart excision and perfusion

- a. After the cannulation system is washed and readied, pour ~20 ml of isolation solution into a medium sized weigh boat and put on ice.
- b. Prepare a euthanization chamber with 2-5% Isoflurane ventilated into the chamber. Place the animal in the chamber and monitor until loss of reflex to sound and toe pinch.
- c. Remove the animal from the chamber and perform spinal dislocation to ensure euthanization. Place the animal belly up with the limbs taped onto the table (Figure 3).



**Figure 3. Illustration of basic cut positions for exposing the thoracic cavity.** The initial cut is made across the abdomen below the sternum (1), parallel cuts are made up the ribcage (2) and the chest wall is removed by cutting across at the top of the sternum (3).

- d. For removal of the heart, make cuts according to the diagram in Figure 3:
  - i. Make an initial cut across the abdomen into the cavity.
  - ii. Then make parallel cuts up the ribcage to the sides, making sure not to accidentally cut the heart.
  - iii. Remove the ribcage or alternatively lift it out of the way to expose the thoracic cavity.
  - iv. With a pair of curved forceps, lift the heart and excise behind the forceps making sure a significant portion of the aorta is retained for hanging.
  - v. Rapidly transfer the heart to the cooled CIV in the weigh boat.
- e. Rinse the heart quickly in CIV and then cannulate the aorta, making sure not to insert the cannula deeper than the coronary ostia. It is essential that the aorta is correctly identified,

as there are numerous vessels present. The aorta is usually the largest vessel seen on the ventral view of the heart.

*Note: Cannulation depth can be checked by gently tilting the heart on the cannula. The point of bending is a good indicator of the position of the cannula. To position the tip above the coronary ostia, the point of bending should be at the same level or just above the atria.*

- f. Tie the aorta onto the cannula using sutures and start perfusion at a low pump setting (Figure 1B). Slowly increase the pump speed until the drop rate is around 1 drop every 2 sec.
- g. Continue perfusion until droplets turn yellow indicating the arrival of collagenase.
- h. Switch the collection dish so that only perfused collagenase is collected. Start a timer and perfuse for a further 7 min.
- i. Following completion of digestion, excise the heart just below the atria to ensure that only ventricular cells are present for subsequent isolation steps. Place the ventricular tissue in the collecting dish together with the collagenase-containing perfusate.
- j. Dice the ventricular tissue into smaller chunks of roughly 2 x 2 mm using fine scissors. Then, with tweezers, gently agitate and pull the tissue apart to loosen cells. This should be done within roughly 2 min of excision from the base (Figure 1D).
- k. Using a Pasteur pipette with the end cut; transport the tissue chunks into a 10 ml Falcon tube with 8 ml buffer (CIV buffer + 500  $\mu$ l BSA +120  $\mu$ g DNase). The BSA will stop the digestion procedure.
- l. Pipette up and down gently with the Pasteur pipette until the solution becomes cloudy near the bottom.
- m. Using a coarse filter mesh of roughly 200-500  $\mu$ m pores, strain the contents of the tube into a 30 ml Falcon tube pipetting up and down to further dislodge cells from the main tissue mass.
- n. Once the 10 ml tube has been filtered, use a further 10 ml of CIV with BSA to dislodge more cells from the main tissue mass on the filter mesh in order to increase yield.
- o. After straining, discard the filter mesh and set the tube aside for isolated cells to settle. Typically a pellet can be seen at the end of the procedure and will grow larger over the next few minutes as more cells sediment.

*Note: Important for observation during the perfusion period is the dilation and color change observable in the heart being perfused. Generally, there is a dilation of the overall shape as the passive forces provided by the collagen tissue are removed through its dissolution (see Figures 2B and 2C for comparison). The color of the heart also becomes paler, perfusate drips become elongated due to increase in digested collagen, and overall the tissue is softer when tested with forceps.*

## B. Fixation and plating

At this stage, it is important that the cells are not dehydrated, so a humidified chamber should be



employed during these steps.

1. Wash Poly-L-lysine pre-coated coverslips thoroughly with PBS to remove any excess Poly-L-Lysine.
2. Plate 200  $\mu$ l of cells onto each plate, with low overall density to prevent cell clumping. If a clump appears, gently agitate the plate to disperse the cells.
3. Wait for 10 to 30 min for cells to settle and adhere to the coverslip.
4. Add 200  $\mu$ l of 4% PFA solution to bring the final PFA concentration on plate to 2%; Incubate for 10 min.
5. Pipette off PFA from the coverslip and incubate for 5 min with 200  $\mu$ l PBS to wash off excess.
6. Pipette off excess PBS and apply 100 mM glycine solution for quenching of fixation. Wash again with 200  $\mu$ l PBS for 5 min. The cells at this point are ready for immunohistochemical labeling.

### C. Immunohistochemical labeling and sample mounting

There are different methods for immunohistochemical labeling that need to be adjusted according to experimental goals. Here we present the method most often used in our laboratory for immunofluorescent labeling of RyR2 for super-resolution dSTORM imaging.

1. Permeabilise cells with 200  $\mu$ l 0.03% Triton X-100 in PBS for 15 min. This treatment helps expose antigenic sites for antibody binding.
2. Wash off the permeabilizing solution by incubating the sample in 200  $\mu$ l PBS for 5 min.
3. Block the sample using 200  $\mu$ l Image iT FX signal enhancer for 1 h at room temperature.
4. Wash again with 200  $\mu$ l PBS for 5 min.
5. During the washing process, prepare 200  $\mu$ l of anti-RyR primary antibody at a 1:200 dilution in low blocking buffer.
6. Pipette off excess PBS from the sample and apply primary antibody after the wash step. Incubate primary antibodies overnight at 4 °C.
7. After incubation, remove excess antibody solution and wash three times using 15 min incubations of 200  $\mu$ l PBS.
8. Prepare 200  $\mu$ l Alexafluor 647 conjugated donkey antimouse secondary antibody at a 1:200 dilution. Apply and incubate at room temperature for 2 h.
9. After incubation, remove excess antibody and wash three times with 200  $\mu$ l PBS for 15 min. After completion of the washing steps, the cells are ready for imaging.

*Note: For dSTORM imaging, a suitable photoswitching buffer is required in the mounting media. In our experiments, commercially available Vectashield media is used diluted to 10% from stock using glycerol solution. It is noted; however, that 100% Vectashield is also usable and produces similar results as a dilution at 10% works well, it is overall much more cost efficient. Pipette off excess PBS from the wash steps and apply 200  $\mu$ l of the media solution to the sample.*



#### D. Imaging

In our laboratory, imaging is conducted using the Zeiss ELYRA P1 Super-resolution attachment on an LSM 710 confocal system. For standard dSTORM imaging, we employ either the PLAN Apocromat 63x NA 1.41 Oil Immersion objective for larger field of view (FOV) experiments or, for improved localization and smaller FOV, a PLAN Apocromat 100x NA 1.46. Oil for the immersion lens calibrated at 30 °C is used as typical sample heating from the high-intensity laser often results in sample temperature rising to approximately 30 degrees. For imaging of the 647 nm channel in the described experiment, a filter block consisting of a 670 nm dichroic long pass (LP) filter is employed, as supplied with the ELYRA imaging system. Imaging is done using an ANDOR Ixon EMCCD operating at ~-60 °C with integration time set to 50 msec. Processing of dSTORM data is achieved with the built-in processing suite in ZEN Black software.

For excitation, highly inclined and laminated optical sheet (HiLO) illumination is implemented. With this approach, the excitation laser is projected onto a sample at an angle near the total internal reflection threshold in order to enhance contrast by reducing out-of-focus fluorescence (Tokunaga *et al.*, 2008). We employ the ELYRA built-in 200 mW 642 nm laser as the primary excitation source, with the intensity set to one of 3 available settings:

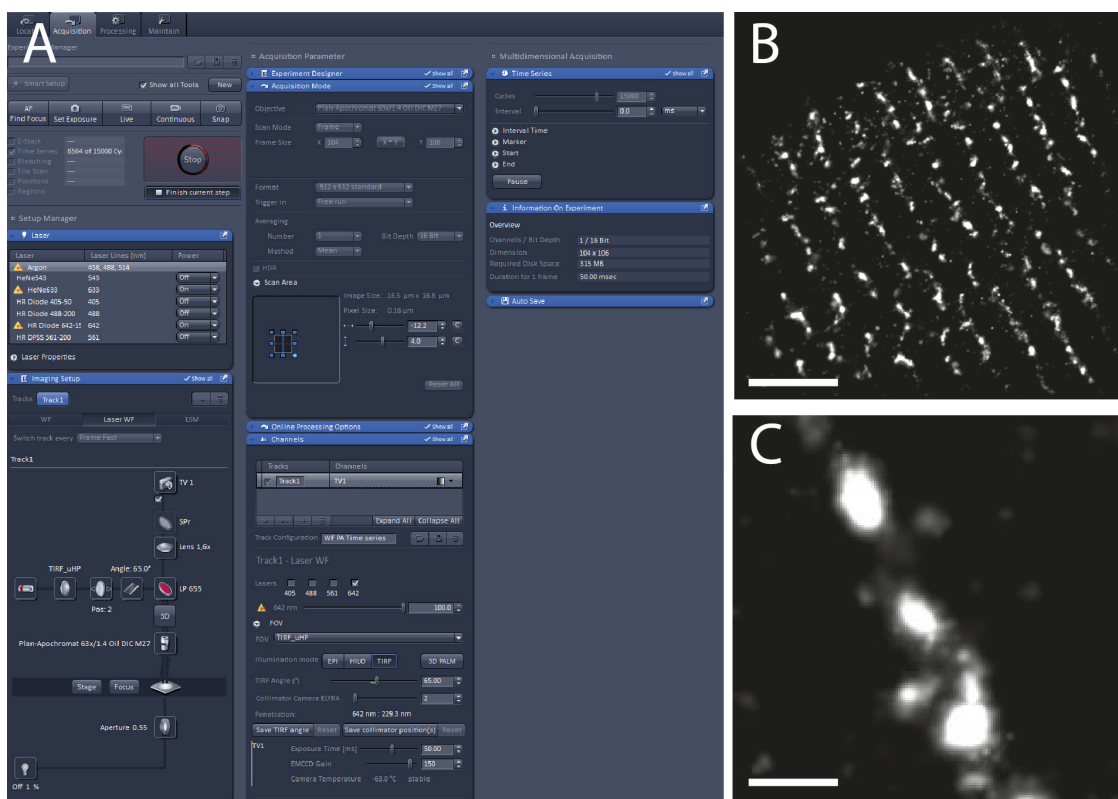
- a. Standard TIRF setting, where no additional focusing is applied.
- b. TIRF HP, a higher power setting where the intensity is increased 2x as compared to the standard TIRF setting by focusing on a smaller illuminated field.
- c. TIRF uHP, where the intensity is enhanced 8x compared with the standard TIRF setting.

The focusing of TIRF HP and TIRF uHP settings correspondingly reduce the diameter of the overall illumination field, requiring the use of a cropped section of the camera's FOV during imaging.

For a standard imaging run:

1. Sample identification and orientation are achieved with standard trans-illumination using a halogen lamp. A suitable myocyte is one with a rod-like appearance and clear sarcomere striations.
2. Once a cell has been selected, the imaging mode is switched to fluorescence with widefield laser illumination.
3. To obtain an overview and locate the ROI, laser output is reduced to 4% of maximum (as set by ZEN Black software) and the basic TIRF filter is used. The laser angle is maintained at a HiLo level of 55-65 degrees. The camera is set to 50 msec integration time with gain adjustable to produce a clear image without clipping the highlights.
4. With a region selected, the ROI is then constrained to a central 16.5 x 16.5 µm area where even illumination from the TIRF uHP focused laser spot falls.
5. The system is then placed in a dSTORM mode with TIRF uHP filter, 100% laser output, and camera integration of 50 msec. EM gain is set initially at 0 to prevent sensor damage during the bleaching period. The acquisition is set to between 15,000 and 20,000 frames (Figure 4A).

6. When the imaging procedure is started, an initial bleaching period is observed where the majority of the fluorophores are pushed into the dark state. During this period, events are overlapping and not detectable.
7. As more fluorophores are bleached, EM gain for the camera should be raised to 150 (as displayed by Zen Black) typically 10-20 sec after initiation.
8. After the sequence has been acquired, the raw image stack is processed for the identification of individual fluorophores. This can be achieved within the ZEN software by supplying the signal-to-noise threshold for detection, the expected Gaussian spread, and indicating whether the multi-emitter fitting should be applied (default ratio of 6.0). For our images, the default setting is used for the detection thresholds, and the multi-emitter setting is enabled. Standard high-quality images will show > 100 k filtered events although this can depend on the target protein and the size of the area imaged. The values given are typical when staining for RyR2 using the high-quality antibodies specified above.
9. To render the image, the current method is a Gaussian overlay where detection uncertainty of the individual events is turned into a Gaussian intensity distribution, with standard pixel resolution of 10 nm (Complete rendered image seen in Figure 4B and C).
10. The intensity levels are then linearly adjusted either in ZEN or ImageJ and an appropriate look-up table is employed.



**Figure 4. Basic imaging parameters and example images.** A. Showing parameters as established in Zen Black software from Zeiss. B. Showing standard full field of view dSTORM

rendering as achieved in Zen Black. C. Zoom of a subregion of Panel B demonstrating typical resolution of RyR2 cluster ultrastructure. Scale bars: B = 4  $\mu$ m; C = 200 nm.

#### E. Image Segmentation

Segmentation is primarily carried out in ImageJ and when required for additional flexibility and efficient batch processing, by custom python scripts. Here we present the segmentation method used for measurement of cluster sizes in ImageJ.

1. Open ImageJ software and load in the image to be analyzed.
2. Under *Image > Adjust > Brightness and contrast*, adjust the observed intensities so samples are clearly visible without clipping the highlights.
3. To threshold the image for binary analysis, select *Image > Adjust > Threshold* and select the Otsu method (used by our facility) or an alternative method. Important in selection of threshold methods is to ensure the final binarised image reflects what is observed in the rendered image and that background and foreground are effectively distinguished. It is also important that the method that was used is later reported when results are used in publications. In comparative studies one method should be consistently adopted.
4. For measurement of particles, selected *Analyse > Set measure* and check the measurement parameters desired. A range of options is available; the most commonly used are the area and circularity measures.
5. Once the measurements are set, select *Analyse > Particle analysis*.
6. Because of potential nonspecific antibody binding, a minimal particle size should be set when examining structures below what is either resolvable or expected to be observed. Depending on the structures to be analyzed, a circularity limitation can also be set, however, in the context for RyR clusters, no such limit is used.
7. Once the measurements are collected, they can then be saved as tab delimited .txt or excel .xls files and further analyzed.

#### F. Considerations for imaging processing

Possibly the most difficult aspect of the acquisition procedure is deciding the best means to analyze the highly detailed data provided in dSTORM images. Indeed, these images often require additional analysis steps to discern experimentally important observations from acquisition artifacts.

Post-acquisition appraisal of image quality is highly important. Poor quality images with low event counts are often impossible to analyze or require complex processing before robust data can be extracted. Thus, it is highly recommended that poor quality images be filtered and excluded before proceeding with the analysis pipeline. Key parameters to observe are the localization precision of detected events, the event density, and sample movement during the acquisition process (drift). Each of these variables will directly affect the overall resolution of the final image, as reviewed in Deschout *et al.* (2014). In this context it is useful to point out that the field of super-resolution

imaging is developing a range of tools with the aim to robustly detect images with poor resolution and artifacts, see Banterle *et al.* (2013) and Culley *et al.* (2018).

The analysis pipeline generally begins with the separation of signal from the background by employing thresholding. This can be a contentious topic due to the often subjective nature of selecting the precise threshold point. In our experience, we have found that a modified OTSU method provides a reliable and unbiased approach which produces results in close agreement with what is visually observed. Alternatively, the threshold value can be based on simulations to identify the level necessary to reproduce the dimensions of the underlying object (Hou *et al.*, 2014).

After determining a suitable threshold, another important aspect to consider when proposing analysis methods is the type of structure to be investigated. For cluster-type structures such as the RyRs imaged in our experiments, standard measurements include the size of the clusters, their relative spacing, and density (Hou *et al.*, 2015). One employed approach has been to apply a 30 x 30 nm grid to the thresholded image, where a grid pixel corresponds to approximate dimensions of a single RyR. Thus, a grid position can be defined as containing an RyR if it is more than half filled with supra-threshold pixels, and RyR clusters can thereafter be defined by occupied, neighboring grid positions (Baddeley *et al.*, 2009). Of functional interest is the proposal that RyR clusters with edge-to-edge distances < 100 nm are in close enough proximity to cooperatively generate Ca<sup>2+</sup> sparks (Sobie *et al.*, 2002). Termed Ca<sup>2+</sup> release units (CRUs), these groupings can be analyzed by applying algorithms for assessing inter-cluster distances after completion of grid-based RyR localization (Jayasinghe *et al.*, 2018).

Two-colour imaging (not covered by this protocol) is a commonly used application of dSTORM which provides data on structural interfaces and interactions. Analysis of two colour images additionally requires consideration of differing photo-switching phenomena of the individual fluorophores, as this affects the event rate, density, and overall localisation precision (unless a sequential two-color imaging approach is chosen that avoids changing the dye between imaging the two targets [Werbin *et al.*, 2017; Jayasinghe *et al.*, 2018]). Optical effects of differing imaging wavelengths also typically need to be considered when carrying out distance analyses, since chromatic aberration can easily account for more than 200 nm error in alignment. For an example of this kind of analysis, please see Jayasinghe *et al.* (2015) and Munro *et al.* (2016). However, when such issues are carefully accounted for, nanoscale colocalization analyses can be performed as that has been recently done for RyR2 and its partner protein junctophilin-2 (Munro *et al.*, 2016).

## Recipes

*Note: Typically reagents are prepared in advance of the date of isolation.*

1. Cell isolation buffer (CIV)
  - a. CIV is initially made as a 10x stock relative to concentrations listed below:

Reagent	Concentration (mM)
NaCl	130.00
KCl	4.20
HEPES	25.00
MgCl <sub>2</sub> ·6H <sub>2</sub> O	0.50
NaH <sub>2</sub> PO <sub>4</sub>	0.40
D-Glucose (added later)	25.00

**Notes:**

- i. *The concentrated stock does not have glucose added and is not pH adjusted.*
  - ii. *The stock is aliquoted into 50 ml Falcon tubes and kept at -18 °C for long-term storage and later use.*
- b. Final preparation of CIV buffer is done the day prior to cell isolation, with 500 ml of standard CIV solution is made from a 50 ml 10x stock by dilution with 450 ml MilliQ H<sub>2</sub>O
  - c. Following dilution, adjust the pH to 7.4 using NaOH or HCl and then add 2.18 g D-glucose
  - d. This solution can be stored for up to 1 week at 4 °C for later use
2. Collagenase solution
- Collagenase solution is made by dissolving 2 mg powder in 2 ml of CIV used
- a. Typically, prepare 40 ml of final collagenase solution on the day of experimentation
  - b. Add 1 µl of 1 M CaCl<sub>2</sub> to 40 ml of collagenase before perfusion
3. 4% Paraformaldehyde solution (200 ml stock)
- CAUTION: Formaldehyde is highly toxic and experiments should be undertaken in a negative pressure fume hood to prevent exposure.*
- a. Add 8 g PFA powder to ~150 ml MilliQ H<sub>2</sub>O and heat on a heating plate to 60 °C, making sure the solution does not boil
  - b. To assist in depolymerization of PFA into FA, the addition of 3-4 drops of 1 M NaOH to the heated solution will allow dissolution to occur rapidly. Addition of NaOH should be done dropwise to ensure that only a minimal amount is added
  - c. Once the solution has dissolved, heat is removed and 20 ml of 10x PBS solution is added as buffer followed by pH adjustment to 7.4 through addition of HCl or NaOH
  - d. Finally, top up the solution to 200 ml using ddH<sub>2</sub>O to a final concentration of 4% (w/v)
  - e. This solution can be aliquoted and stored at -20 °C for up to 6 months

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