

Imaging of In Vitro and In Vivo Neurons in *Drosophila* Using Stochastic Optical Reconstruction Microscopy

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The *Drosophila melanogaster* brain comprises different neuronal cell types that interconnect with precise patterns of synaptic connections. These patterns are essential for the normal function of the brain. To understand the connectivity patterns requires characterizing them at single-cell resolution, for which a fluorescence microscope becomes an indispensable tool. Additionally, because the neurons connect at the nanoscale, the investigation often demands super-resolution microscopy. Here, we adopt one super-resolution microscopy technique, called stochastic optical reconstruction microscopy (STORM), improving the lateral and axial resolution to ~20 nm. This article extensively describes our methods along with considerations for sample preparation of neurons in vitro and in vivo, conjugation of dyes to antibodies, immunofluorescence labeling, and acquisition and processing of STORM data. With these tools and techniques, we open up the potential to investigate cell-cell interactions using STORM in the *Drosophila* nervous system. © 2021 Wiley Periodicals LLC.

Basic Protocol 1: Preparation of *Drosophila* primary neuronal culture and embryonic fillets

Basic Protocol 2: Immunofluorescence labeling of samples

Basic Protocol 3: Single-molecule fluorescence imaging

Basic Protocol 4: Localization and visualization of single-molecule data

Supporting Protocol: Conjugation of antibodies with STORM-compatible dyes

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INTRODUCTION

The nervous system of *Drosophila melanogaster* is an attractive model in which to study the molecular mechanisms of neuronal differentiation and proliferation, neurite outgrowth, synapse formation, and neurodegeneration (Bellen, Tong, & Tsuda, 2010; Clark, Zarin, Carreira-Rosario, & Doe, 2018; Doe, 2017). In particular, the ability to express transgenes has facilitated the use of *Drosophila* in such studies (Venken, Simpson, & Bellen, 2011). For instance, in the brain, where many neurites are entangled, it is

difficult to distinguish a single neurite from others (Scheffer et al., 2020). To resolve each neurite at high contrast, membrane markers are commonly expressed in small subsets of neurons in the brain. To this end, it is possible to take advantage of a transgenic fly in which the transcriptional activator *GAL4* is expressed under the control of a cell-type-specific enhancer (Manning et al., 2012). Complementary to this type of in vivo approach, one can also employ primary neuronal cultures from the *Drosophila* brain (Egger, van Giesen, Moraru, & Sprecher, 2013). The availability of well-defined culture media and advanced culture techniques makes it possible to dissociate individual neurons and generate primary neuronal cultures. Primary neuronal cultures have previously been used for high-resolution imaging of neurite arbors, high-throughput screening of genes for developmental brain disorders, and cell recording of neuronal activity (Campusano, Su, Jiang, Sicaeros, & O'Dowd, 2007; Del Castillo, Muller, & Gelfand, 2020; Perrimon & Mathey-Prevot, 2007). Regardless of which system—in vivo or in vitro—is chosen, analysis of complex neuron morphologies requires optical resolution below the diffraction limit of light (<250 nm), as the nanoscopic processes of neurons (e.g., dendrites <250 nm in diameter) are of high interest for imaging (Lichtman & Denk, 2011). Super-resolution microscopy techniques provide an excellent solution to this difficulty, improving the resolution beyond the diffraction limit (Huang, Bates, & Zhuang, 2009). Although many techniques have been established for super-resolution imaging of *Drosophila* samples (Gao et al., 2019; Jiang et al., 2018; McGorty, Kamiyama, & Huang, 2013; Schnorrenberg et al., 2016), stochastic optical reconstruction microscopy, also called STORM, is already widely available and can be used with an extensive array of fluorophores that we have found appropriate for our needs.

In this article, we provide detailed protocols for preparing *Drosophila* neurons and taking STORM images of various structures in the neurons. Basic Protocol 1 describes the establishment of primary neuronal cultures and the dissection of embryos for immunostaining. To label the neuronal membranes genetically, we take advantage of the *UAS-GAL4* system. We start with the appropriate genetic crosses to express membrane markers in particular neurons and train the adults to lay eggs in their new cages. Once we collect the embryos, we can either dissociate the embryos to make a primary neuronal culture or dissect them for in vivo analysis. Finally, we fix the samples before immunostaining, the approach for which can differ depending on the protein of interest (we discuss the difference in Basic Protocol 2). For immunostaining and imaging the samples, it is necessary to choose bright dyes that can overcome reduction of signal due to tissue thickness. In the Support Protocol, we describe a method of dye conjugation to antibodies for the selected far-red dye, which is optimal for STORM imaging. Basic Protocol 2 describes the immunostaining approach for these samples. Although in vivo labeling is a time-intensive process, primary neuronal cultures can be labeled and imaged within a couple of hours. Last, we describe our image acquisition approach in Basic Protocol 3 and our reconstruction approach in Basic Protocol 4. For STORM imaging, a 647-nm laser line is used to photo-switch the fluorophores. In this protocol, it is critical to optimize acquisition settings to obtain a high-resolution image. Finally, we describe our step-by-step approach to reconstructing the STORM data using the ThunderSTORM plug-in in Fiji/ImageJ.

BASIC PROTOCOL 1

PREPARATION OF *DROSOPHILA* PRIMARY NEURONAL CULTURE AND EMBRYONIC FILLETS

STORM has been previously demonstrated on primary neuronal cultures from the rat cortex (Xu, Zhong, & Zhuang, 2013), which do not require any complex sample preparation and imaging steps. In contrast, STORM imaging of the *Drosophila* nervous system is challenging. Typically, we use a whole embryo for immunofluorescence imaging due to the ease of preparation. However, the surrounding tissues and yolk outside the nervous system provide background fluorescence, which will subsequently degrade the

detection and localization of single molecules, resulting in STORM images with fewer localizations and worse resolution. To circumvent this, we dissect the embryos in fillet preparation and remove the intestines and trachea, which contribute to the background fluorescence. Here, we describe our method for culturing and plating primary cells dispersed from *Drosophila* mid to advanced gastrula embryos and dissecting late embryonic samples for STORM imaging.

Materials

UAS-myr::GFP flies (Bloomington *Drosophila* Stock Center, RRID:

BDSC_32197)

elav-GAL4 flies (Bloomington *Drosophila* Stock Center, RRID: BDSC_8765)

Yeast paste: prepare from active dry yeast (FlyStuff, cat. no. 62-103) by mixing with distilled water using 1.0 g yeast per 1.5 ml water to create a paste

Grape juice agar plates (see recipe)

50% (v/v) household bleach prepared at 1:1 ratio by mixing in distilled water (stored away from light)

70% (v/v) ethanol (for culturing neurons)

Concanavalin-A (Con-A; Sigma Aldrich, cat. no. C5275-5MG; for culturing neurons)

Supplemented SFX medium (see recipe; for culturing neurons)

Phosphate-buffered saline (PBS; for in situ preparation)

Play-Doh modeling clay (for in situ preparation)

Embryo collection cages fitting 60-mm petri dishes

Egg basket, created by cutting a conical tube horizontally and cutting out a circle from the cap, and then placing a 100- μ m nylon mesh filter between the cap and the tube.

Squirt bottle containing distilled water

Task wipers (KimTech, cat. no. 34155), sterilized

60-mm petri dishes (Corning, cat. no. 430166)

Dissection microscope (Nikon, SMZ-U)

Parafilm (Sigma-Aldrich, cat. no. P7543)

Dumont no. 5 forceps (Fine Science Tools, cat. no. 11252-20)

Coverslip, no. 1.5 thickness (Fisherbrand, cat. no. 12-541B; for culturing neurons)

Imaging spacer (Electron Microscopy Sciences, cat. no. 70327-20S; for culturing neurons)

Humid chamber or lidded Tupperware with wet tissue (for culturing neurons)

Bunsen burner (for culturing neurons)

1-ml syringe (Becton Dickinson, cat. no. 309659; for in situ preparation)

Double-sided tape (Scotch, cat. no. 665; for embryo alignment and dissection)

Pre-cleaned glass microscope slides 25 \times 75 \times 1 mm (Fisher Scientific, cat. no. S13943) with vinyl tape (Scotch, cat. no. 6143; for in situ preparation)

Micropipet puller (Narishige PC-100; for embryo alignment and dissection)

Capillary tubing with outer diameter of 1.2 mm and an inner diameter of 0.6 without filament, as dissection needle in situ preparation and suction needle

Training flies and collecting embryos

1. In an embryo collection cage, cross the *GAL4* driver flies with the *UAS* reporter flies at 25°C (Fig. 1B, left).

Having a ratio of 1:5 males to females is sufficient for all of the females to lay eggs. A typical cage containing at least 30-50 adult flies should produce a sufficient number of eggs.

2. Before the experiment, acclimate the adult flies to the egg collection cage by allowing them to lay eggs for 1-2 days and changing the yeast-streaked agar plates

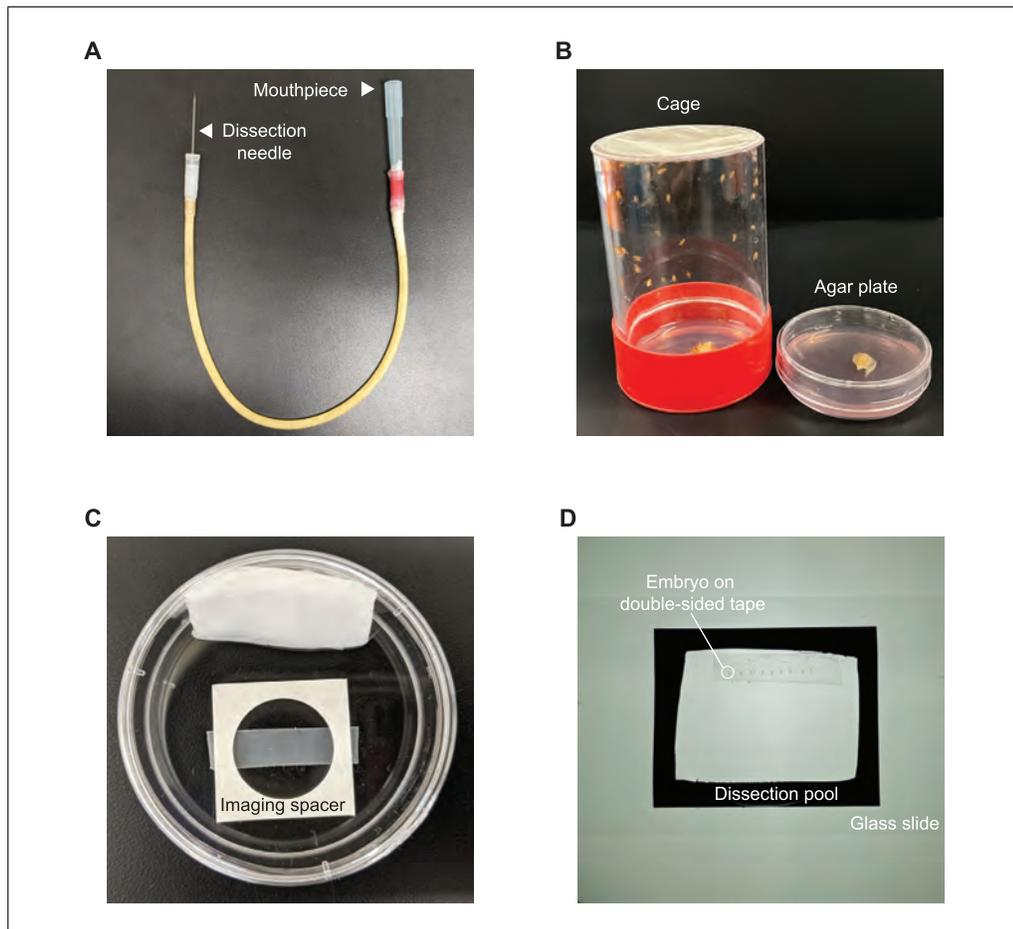


Figure 1 Equipment for sample preparation. **(A)** Tube-connected suction needle. A pipet tip (blue) attached to the tube is used as the mouthpiece (arrowhead). At the other end of the tube, a dissection needle attaches to an adaptor piece (arrowhead). **(B)** Embryo collection cage with a mesh top and yeast-streaked plate. The yeast-streaked agar plate is used by flies to lay eggs. **(C)** Coverslip preparation inside a humid chamber. The double-sided adhesive imaging spacer overlays the coverslip. Cells should be plated inside the well chamber. **(D)** Glass slide preparation with a dissection pool for dissecting the embryos. The embryos (e.g., encircled area) are aligned onto a piece of double-sided tape and submerged in saline. The dissection pool is created from vinyl tape on a clean glass slide.

at least once per day. The plates should be changed until flies can synchronously lay ~500 eggs per collection period in each plate (Fig. 1B, right).

To prepare yeast-streaked plates, spread a small amount of the yeast paste across the center of the agar plate in a thin strip ~1 cm wide; the size of the strip can be increased for larger amounts of adult flies and/or higher frequency of egg collection.

Keep the cages at 25°C with a humidity of ~65% in a 12-hr light and 12-hr dark cycle to facilitate the collection of synchronous embryos.

3. Allow the adult flies to lay eggs on a yeast-streaked agar plate for 1 hr. Collect the plate from the cage and replace with a fresh yeast-streaked plate.
4. Incubate the plates with the embryos until they reach the appropriate developmental stage.

Mid-gastrula embryos aged 5-7 hr AEL (after egg laying)—or stage 11 (Campos-Ortega, 1985)—are ideal for neuronal culture preparation. In our hands, we found that embryos aged up to 10 hr AEL (i.e., stage 12) can be plated as well.

5. Remove the eggshell from the embryos by adding 50% bleach to the embryo-containing plate for 5 min to allow access to the embryo for cell dissociation and dissection.
6. Filter the eggs through the egg basket (alternatively, a cell strainer can be used).
7. Wash the filter thoroughly with distilled water so that the embryos are cleared of chorions and yeast from the plate.

This step is especially critical for cell culture preparation to ensure that there is no contamination by yeast.

8. Disassemble the egg basket and blot the mesh filter dry with sterilized task wipers.
9. Transfer the embryos into a petri dish by flipping the mesh so that the embryos are facing the clean plate and washing them into the plate set beneath the filter.
10. Pour the water off the embryos just before collecting.

In the remainder of this protocol, we report two separate methods for in vitro and in situ sample preparation. In steps 11a-19a, we describe the preparation of primary cell cultures, and in steps 11b-20b, we describe the preparation of dissected embryos.

Collecting the embryo cell culture

- 11a. Prepare the plates by laying down a strip of Parafilm on the bottom of a 60-mm petri dish and covering a section of the plate wall with a damp task wiper. Ensure that there is enough room to later lay down the double-sided adhesive imaging spacer (of the appropriate diameter; Fig. 1C).

The strip of Parafilm underneath should roughly be the size of the coverslip plus imaging spacer. This allows easier removal of the coverslip and imaging spacer from the plate later.

- 12a. Using forceps, sterilize the coverslip by submerging it in 70% ethanol and drying it completely over the Bunsen burner. Lay the sterilized coverslip on top of the Parafilm strip inside the plate.
- 13a. Peel the sticker off one side of the spacer and press the adhesive side, facing down, onto the coverslip. Using forceps, firmly press around the circumference of the spacer. This prevents any leakage of Con-A or culture medium from the coverslip.

As an alternative, you may skip this step and instead use vacuum grease to mount the sample as described in Basic Protocol 2, steps 15 and 16.

- 14a. Spread 10 μ l of 0.5 mg/ml Con-A to produce a thin layer on the coverslip. Cover the petri dish with its lid. Place the dish in a humid chamber with humidity \sim 80%, or a lidded Tupperware container with a wet tissue inside. Incubate for 30 min at room temperature.
- 15a. Remove the Con-A from the coverslip. Add 100 μ l room-temperature supplemented SFX medium to the Con-A-coated coverslip in the plate, working near the open flame of a Bunsen burner to provide a sterile environment.
- 16a. Take a dissection needle and break off the tip until the diameter is \sim 60 μ m, or about one-third of the embryo's width, which can be checked in comparison to the embryos. Connect the needle to one end of the suction tube and add a pipet tip to the other end to act as a suction source (Fig. 1A).
- 17a. Examine the embryos, directly from step 10, under the dissection microscope. Use the pipet tip attached to the tube-connected suction needle (Fig. 1A) to suck and release the contents of the embryo to break the cells out of the vitelline membranes.

- 18a. Transfer the cells from the plate onto the center of the coverslip by gently suctioning in and out with the tube-connected suction needle; make sure any large clumps are broken up. Repeat this process until you have dissociated 20 embryos onto the center of the coverslip.

To prevent contamination, make sure to always place the lid over the plate of your coverslip.

- 19a. Place the lidded plates into the humid chamber and culture the primary cells for 1-2 days at 28°C.

Aligning and dissecting embryos for in situ preparation

- 11b. On a glass slide, create a dissection pool using vinyl tape, and place a piece of double-sided tape inside the dissection pool horizontally to align the embryos (Fig. 1D).

- 12b. Collect the embryos at 13-15 hr AEL, at which point they will have four-chambered gut morphology (i.e., be in stage 16 as described by Campos-Ortega), using forceps, and place them onto the tape with the dorsal side facing up.

Here we chose to observe embryos at stage 16 because synaptogenesis at the neuromuscular junction occurs during this stage; however, it is possible to dissect and image at older or younger stages as needed.

- 13b. Add PBS to prevent the embryos from desiccating.

- 14b. Using a micropipet puller at 12 V, pull the capillary tubing to create a sharp needle with a taper of ~0.4 cm in length to serve as a dissection needle. Mount the dissection needle onto a 1-ml syringe filled with some modeling clay.

- 15b. Using the newly prepared dissection needle, cut through the midline of the embryo under a dissection microscope inserting the needle from the surface of posterior end pushing to the anterior end.

- 16b. Gently lift the embryo with the needle, which should be slightly underneath the brain lobes of the embryo, and pull it up and away from the tape.

- 17b. Reposition the dissected embryo onto the glass slide. Here, the embryos should adhere to the glass slide by themselves as long as the buffer is not saturated with proteins and the embryos do not have cuticles.

Please refer to the Commentary and Troubleshooting for further details.

- 18b. Using the yolk and the intestines that come out as cushion for the dissection needle, place the epithelial tissues to either side on the glass. With this, there should be epithelial tissues on both sides of the ventral nerve cord and the brain.

- 19b. Create another tube-connected suction needle by repeating step 17a. To suction the internal organs after dissection, the diameter should be ~300 nm, which is about the size of the intestines. Using a larger or smaller tip size will change the speed of suction.

- 20b. Before moving on to the immunostaining process, clean up the sample by using a tube-connected suction needle (Fig. 1A) and blowing air or aspirating to detach and remove the dorsal tracheal trunks and any remaining intestines.

If the saline has a lot of tissues or cells floating around, you can replace it by using a micropipet to wash the sample with fresh saline to ensure the sample is clear of debris.

IMMUNOFLUORESCENCE LABELING OF SAMPLES

For immunofluorescence labeling of samples, there are a couple of considerations necessary to obtain the best quality of STORM images. One important consideration is the fixation approach applied to the samples. As a result of insufficient fixation, membrane structures and macromolecule architectures can be broken. In order to avoid this type of artifact, fixation methods for different cellular targets have been optimized through the use of certain fixatives. For instance, glutaraldehyde (GA)-based fixation for tubulin is optimal in preserving the microtubule structures, whereas fixation with paraformaldehyde (PFA) alone is sufficient for preserving membrane structures. Because of its high resolution, STORM can readily uncover disruptions resulting from insufficient fixation. Therefore, the choice of proper fixatives is crucial. Next, the density of fluorescent labels should also be considered. When immunostaining membrane structures with a membrane marker, the low density of the marker makes a STORM image discontinuous. With this inadequate labeling density, it is not easy to evaluate whether the structures are related. Here, we describe our method for fixing, immunostaining, and mounting the neuronal culture and brain tissues.

Materials

Drosophila primary culture on coverslip *or* dissected embryos on glass slide (Basic Protocol 1, step 19a or 20b)

Phosphate-buffered saline (PBS)

4% (w/v) paraformaldehyde (PFA; Electron Microscopy Sciences, cat. no. 15710) in PBS

0.1% (w/v) glutaraldehyde (GA; Sigma Aldrich, cat. no. 111-30-8) in PBS

PBS with 0.1% (v/v) Triton X-100 (TBS)

TBS + 0.06% (w/v) bovine serum albumin (BSA; TBSB)

Primary antibodies:

1:200 anti-GFP rabbit monoclonal antibody diluted in TBSB (Thermo-Fisher, cat. no. G10362, RRID: AB_2536526)

1:200 DM1A anti-tubulin antibody: (Cell Signaling Technology cat. no. 3873, RRID: AB_1904178)

1:5 anti-HRP antibody: (JacksonImmuno, cat. no. 123-005-021, RRID: AB_2338952)

Secondary antibodies: 1:200 anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 647 (AF647) dye (see Support Protocol for dye to antibody conjugation), diluted in TBSB

Imaging medium (see recipe)

Aspirator (Bio-Rad, Model cat. no. 1651754P)

Orbital shaker (CB, KJ-201BD)

Task wipers (KimTech cat. no. 34155)

Pre-cleaned glass microscope slides, 25 × 75 × 1 mm (Fisher Scientific, cat. no. S13943)

Vacuum grease or some other spacer to cushion between the coverslip and the tissues (e.g., Secure Seal Imaging Spacer from Electron Microscopy Sciences, cat. no. 70327-20S)

Clean coverslip, no. 1.5 thickness (Fisherbrand, cat. no. 12-541B)

Nail polish for sealing the sample

Permeabilization and staining

1. Using an aspirator, remove the culture medium or saline buffer and replace with 4% PFA in PBS for membrane labeling or with 3% PFA+ 0.1% GA in PBS for microtubule labeling.

2. Incubate on an orbital shaker to fix for 5 min at room temperature.
3. Wash three times with TBS for 5 min each.
4. Block the sample for 10 min for cell culture (or 1 hr for dissected embryos) at room temperature using TBSB to reduce or eliminate nonspecific binding of primary and secondary antibodies.
5. Replace the TBSB buffer with the primary antibody in TBSB.

In some cases, it may be useful to use labeled primary antibodies instead of indirect labeling with secondary antibodies; please see the Critical Parameters for a discussion of the advantages and disadvantages of each option.

For primary neuronal culture samples, incubate the primary antibody for at least 1 hr at room temperature.

For dissected embryo samples, incubate the primary antibody overnight at 4°C.

6. Before staining samples with secondary antibody, remove the primary antibody and wash three times with TBSB for 5 min each.
7. Dilute the secondary antibody IgG conjugated with AF647 dye to a working concentration in TBSB.
8. Exchange the blocking buffer with the secondary antibody using an aspirator. Ensure that the samples are not dried out during this exchange process.
9. Stain the sample by incubating at room temperature on the orbital shaker for 1-2 hr for primary neuronal culture samples or 2 hr for dissected embryo samples.
10. Wash the sample three times with TBS for 5 min each.
11. Post-fix the sample using the 4% PFA in PBS for 5 min at room temperature to crosslink the antibodies strongly and to eliminate floating dyes, which can contribute to creating background signal.
12. Finally, wash three times with TBS for 5 min each to remove the fixative from the sample.

Mounting of the immunofluorescence-labeled samples

13. Using task wipers or an aspirator, remove excess buffer from the sample, but ensure the samples do not dry out.

In case of the in vivo preparation, first remove the tapes that are on the glass slide using forceps.

14. Exchange the buffer with 100 μ l imaging medium.

Make sure to use as much imaging medium as possible to prevent any large air bubbles from forming in the next step.

15. Mount the coverslip onto the glass slide.

- a. *To mount cells:* Remove the remaining sticker from the spacer, place the glass slide on top of the spacer, and make sure that it is completely sealed.

Alternatively, apply vacuum grease to each corner of the coverslip and then place the glass slide on the coverslip.

- b. *To mount embryos:* Place the coverslip on the glass slide, and push it down to minimize the space between the coverslip and the sample until it touches the CNS.

This will allow maximum brightness of fluorophores during imaging of the tissue sample.

- Remove the excess buffer that has leaked out, seal the edges of the coverslip with nail polish, and image or incubate at 4°C until imaging.

For optimal results, it is ideal for the sample to be imaged within 3 hr after being mounted in buffer under minimal oxidation conditions.

SINGLE-MOLECULE FLUORESCENCE IMAGING

In this section, we describe the equipment and steps required for successfully acquiring raw single-molecule data. Although we use a custom-built STORM imaging system for image acquisition (Fig. 2), one can adopt any of the commercial systems currently available, e.g., N-STORM (Nikon).

Materials

Lens paper (Thorlabs, MC-5)

Immersion oil ($n = 1.52$; Cargille, cat. no. 16242)

Custom-built inverted microscope (see also Fig. 2B)

Light source:

405-nm excitation laser (Coherent, OBIS 405 nm LX 100 mW)

561-nm excitation laser (Coherent, OBIS 561 nm LS 50 mW)

647-nm excitation laser (Coherent, OBIS 647 nm LX 120 mW)

Laser control unit:

Arduino Uno

Micro-manager Arduino software (<https://micro-manager.org/wiki/Arduino>)

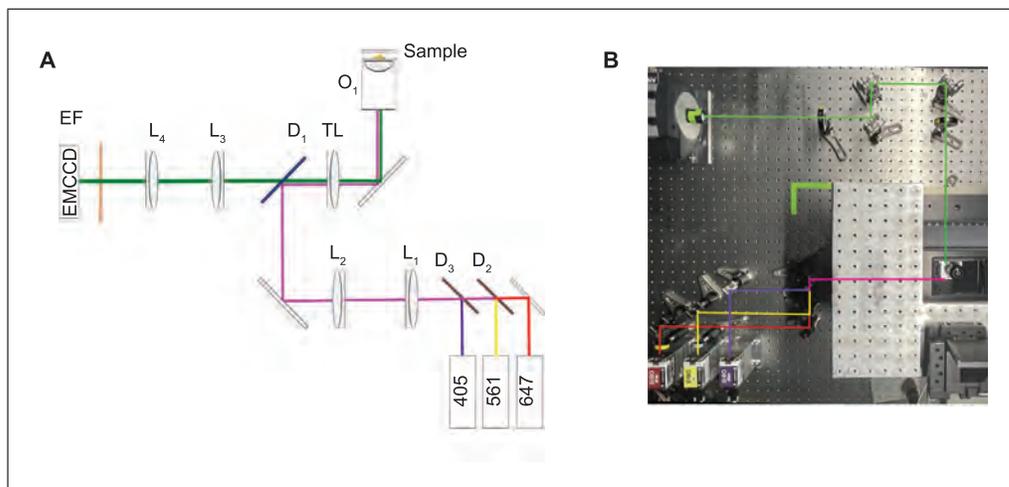


Figure 2 Microscopy setup. **(A)** Schematic of the single-molecule localization microscope. Three lasers at Wavelengths 647 nm (Coherent, OBIS LX 647-120mW), 561 nm (Coherent, OBIS LS 561-50mW), and 405 nm (Coherent, OBIS LX 405-100mW) are combined with dichroics D_2 (Thorlabs DMLP605T) and D_3 (Thorlabs, DMLP425T). Laser line filters are used to filter out spontaneous emission for the 647 laser (Semrock, LL02-657-12.5) and the 561 laser (Semrock, LL02-561-12.5). Lenses L_2 (Newport, PAC052; $\text{efl} = 100$ mm) and L_3 (Opto-Sigma, 026-1132; $\text{efl} = 120.1$ mm) expand the laser beams to a diameter ($1/e^2$) of 0.96 mm. The excitation beams are then demagnified by 60 \times by the tube lens, TL (Olympus, 180 mm), and the objective lens, O_1 (Olympus 60 \times), to a beam diameter of 16 μm . The emitted light is separated from the excitation light by dichroic D_1 (Omega Optical, XF2054, 485-555-650 TBDR). The emission from the sample is imaged on to the EMCCD Camera (Andor, Ixon-897 Life) first to 60 \times magnification by the objective lens O_1 and the tube lens TL and then by an additional 2.5 \times by lenses L_3 (120 mm) and L_4 (300 mm), for a total magnification of 150. The camera pixel size is 16 μm , so the effective pixel size at the sample is 107 nm. Two emission filters (Semrock, FF01-446/523/600/677-25 and FF01-680/42) and a notch filter (Semrock, NF01-488/647) are placed before the camera to block excitation and stray light. **(B)** Photograph of the system. The excitation paths and emission path are traced in red, yellow, purple, and green, respectively.

Spectral filters:

Excitation laser clean-up filters: 516 nm (Semrock, LL02-561-12.5)

647 nm (Semrock, LL02-647-12.5)

Dichroic mirror (Omega, XF2054,485-555-650TBDR)

Emission filters: Multi-bandpass (Semrock, FF01-446/523/600/677-25)

Red detection (Semrock, FF01-605/15)

Far-red detection (Semrock, FF01-680/42)

Notch Filters: Red detection (Chroma, ZET561NF)

Far-red detection (Semrock, NF01-488/64)

60× oil-objective lens (Plan Apo N, Olympus, N.A. 1.42)

EMCCD camera (Andor, iXon-897 Life)

Image-acquisition software (Manager 2.0, RRID:SCR_016865)

Computer workstation (Intel Xeon CPU ES-1603 v4, 2.80 GHz, 16 GB RAM) with a 500-GB solid-state hard drive

Optimizing image acquisition for cell culture and whole-mount immunofluorescence

1. At least 30 min before imaging, turn on the lasers and camera to allow components to reach a stable operating state.
2. Clean the objective with lens paper.
3. Launch image acquisition software (Manager 2.0) and let the camera temperature stabilize (to -70°C for the Andor iXon897).
4. Place a drop of immersion oil on the objective lens, and place the glass slide containing the sample in the sample holder with the coverslip facing downwards.
5. Set camera exposure, and use a white light source to bring the sample into focus.

The typical setting for the exposure of an EMCCD camera is 10 ms.

6. Record a single image using the 647-nm laser at an area on the sample where there are no cells. These images can be used later to subtract the background from the raw single-molecule data of the respective channels.
7. Using a 647-nm laser, record a wide-field image of the cell with a low laser power of 1 mW and a short exposure time of 10-20 ms.
8. Using the 647-nm laser, start the STORM imaging acquisition using empirically derived camera and laser settings. We derive these settings by inversely adjusting the exposure time and the laser power until the fluorophore emission events were far enough from each other (sparsely distributed) to be individually identified (Fig. 3A, top panel). Our typical settings for laser power and exposure time at the beginning of acquisition were an exposure time of 15 ms and laser power of 30 mW. The frame transfer mode is set to off in order to reduce noise, and the EM gain is set to 500. These settings can be found in Micromanager under Devices → Device Property Browser corresponding to each connected device (i.e., camera and lasers).

We can typically collect at least 40,000 images using the AF647 dye with these settings. The number of images to collect (i.e., count under “Time Points”) is set in the Micromanager acquisition menu with an interval of 0 s.

9. Turn on the 405-nm laser and let it illuminate the sample in continuous-wave (CW) mode to facilitate regeneration of the fluorescent dye molecules.

At the beginning of data acquisition, set the 405-nm laser power to the lowest possible setting (1 mW). Increase the laser power of the 405-nm laser by about 5 mW every 5000 frames as the acquisition time increases to record the maximum number of images until all dyes cease to photo-switch. The laser power might increase to 20 mW or more. The laser power is set in the Micromanager Device Property Browser and can be ad-

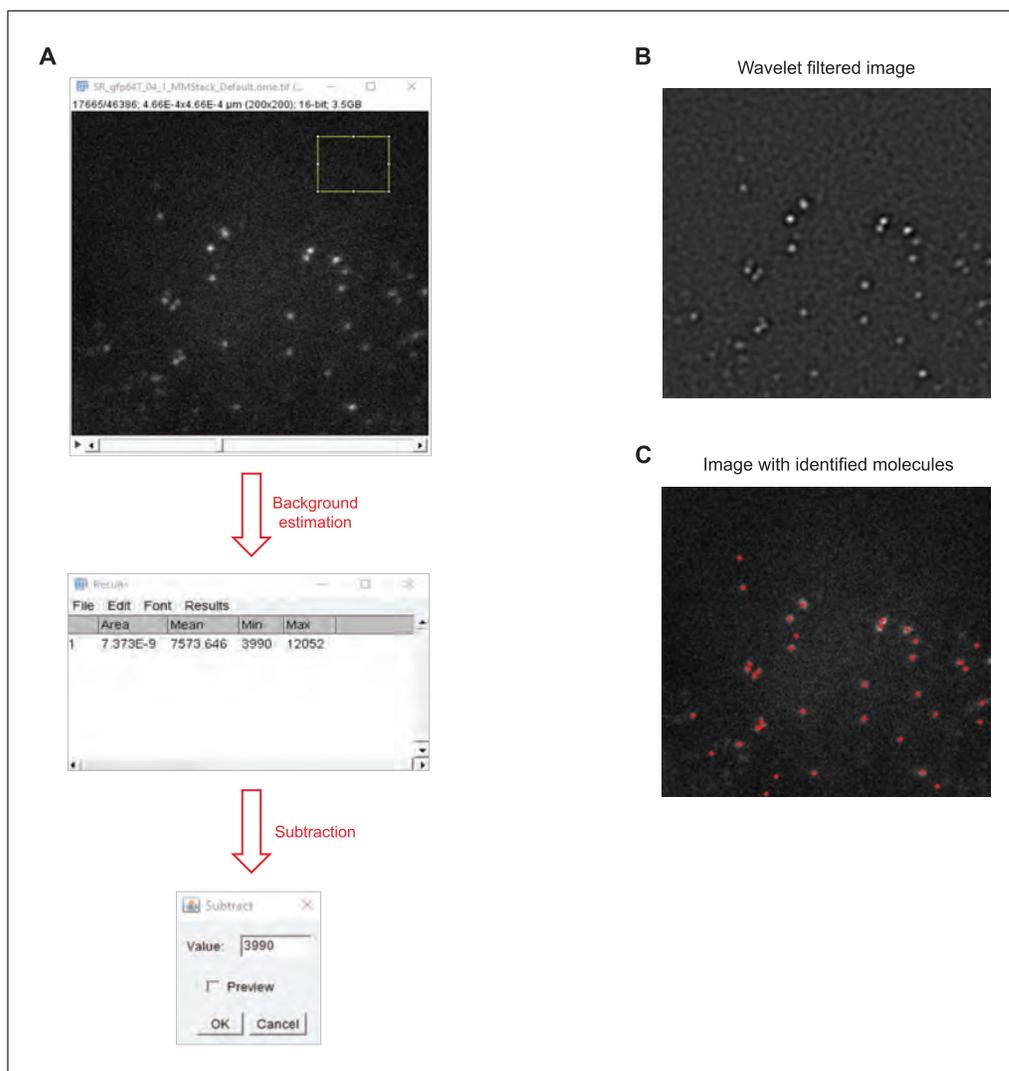


Figure 3 Workflow to detect single molecules and localize the detected molecules. **(A)** Illustration of background subtraction (Min) and peak detection (Max). The background intensity was estimated from an area with no emitting fluorophores (yellow box) using the Measure function in ImageJ, and the minimum background value was then subtracted from all frames. **(B)** Demonstration of fluorophore localization. The image shows the wavelet-filtered image that is used to find the fluorophores. **(C)** The image identifies the emitting fluorophores. The local maximum approach with “8-connected neighborhoods” is used to set parameters.

justed manually during acquisition. The two lasers are controlled by the laser control unit.

10. After the acquisition ends, the images are available as an image stack in a file in `.tiff` format. This file can then be opened in ImageJ or other image-processing software for analysis.

LOCALIZATION AND VISUALIZATION OF SINGLE-MOLECULE DATA

In STORM, an algorithm is used to precisely measure the positions of single molecules from raw camera frames. The algorithm has a fundamental effect on the resolution and fidelity of the final rendered STORM image. The entire process involved in the rendering of a STORM image can be divided into five steps: detecting single molecules from raw camera frames, fitting and localizing the detected molecules, processing the positions of these molecules (e.g., drift correction, density filtering, and intensity thresholding), rendering

the final image of the molecular localizations, and finally statistically analyzing these localizations. Many software packages have been developed and published to help non-expert users perform these steps efficiently. Our protocol below gives a brief description of how to choose the critical parameters for image reconstruction using ThunderSTORM (Ovesny, Krizek, Borkovec, Svindrych, & Hagen, 2014), an open-source plug-in for ImageJ. We note that the selected parameters and options described in this protocol are not the only choices possible. Users should refer to the manual for a detailed guide to select the proper parameters based on their demands.

Materials

Fiji/ImageJ software (RRID:SCR_002285)

Software for localizing and visualizing single molecules data: e.g.,

ThunderSTORM, RRID:SCR_016897 (use version phasor-intensity-1; Martens, Bader, Baas, Rieger, & Hohlbein, 2018)

1. Drag and drop the data file collected using 647-nm laser from the file explorer to ImageJ, or select File → Open in ImageJ, browse to the data file, and press Open. This will load the entire image stack into memory.
2. Open the dark frame recorded using the 647-nm laser as mentioned in step 1 of Basic Protocol 3. Using Process → Image Calculator, subtract the dark frame from the dataset to remove the background (Fig. 3A).
3. Set the camera pixel size in the sample plane, conversion factor between photons and digital units, base level offset of the camera digitizer, and EM gain of the camera using Plugins → ThunderSTORM → Camera setup. For our setup, the pixel size is 107 nm, the gain is 500, and the conversion factor is 4.83 photons/ADU. The conversion factor and offset are specific to the camera and camera settings and can be found in the documentation that came with your camera.

Analog-to-digital units (ADU) are the integer values the camera uses to record intensity. The analog-to-digital converter (ADC) converts the electrons recorded in each pixel into a digital signal measured in ADUs, which is used to quantify the incident number of photons per pixel.

4. After opening the dataset, select Plugins → ThunderSTORM → Run analysis. Select the Wavelet filter (order 3, scale 2) to perform band-pass filtering on the dataset (Fig. 3B).
5. Under the Approximate localization of molecules tab, select Local Maximum for Method and choose a peak intensity threshold between 0.5 and 2 times the standard deviation of the first wavelet level, e.g., 1*std (Wave.F1). If the threshold is set too low, some additional peaks will be identified due to noise. If the threshold is set too high, some of the true fluorophores will be missed.
6. Select Phasor-based localization 2D as the method for sub-pixel localization of the single molecules, with the fit radius being an integer number close to $3 \times \sigma$ (Fig. 3C).

The initial size of sigma can be found by running ThunderSTORM on few images of the data sequence. A histogram of the fitted sizes of sigma (in pixels) can help to find the initial value.

7. The localized data can be visualized using averaged shifted histograms, with a magnification of 20 and 4 lateral shifts. Once the parameters for localization and visualization have been set, press OK (Fig. 4A).
8. The order of post-processing steps is specified by the user. However, we recommend the following order: filtering, density filtering (to remove outliers), and finally drift

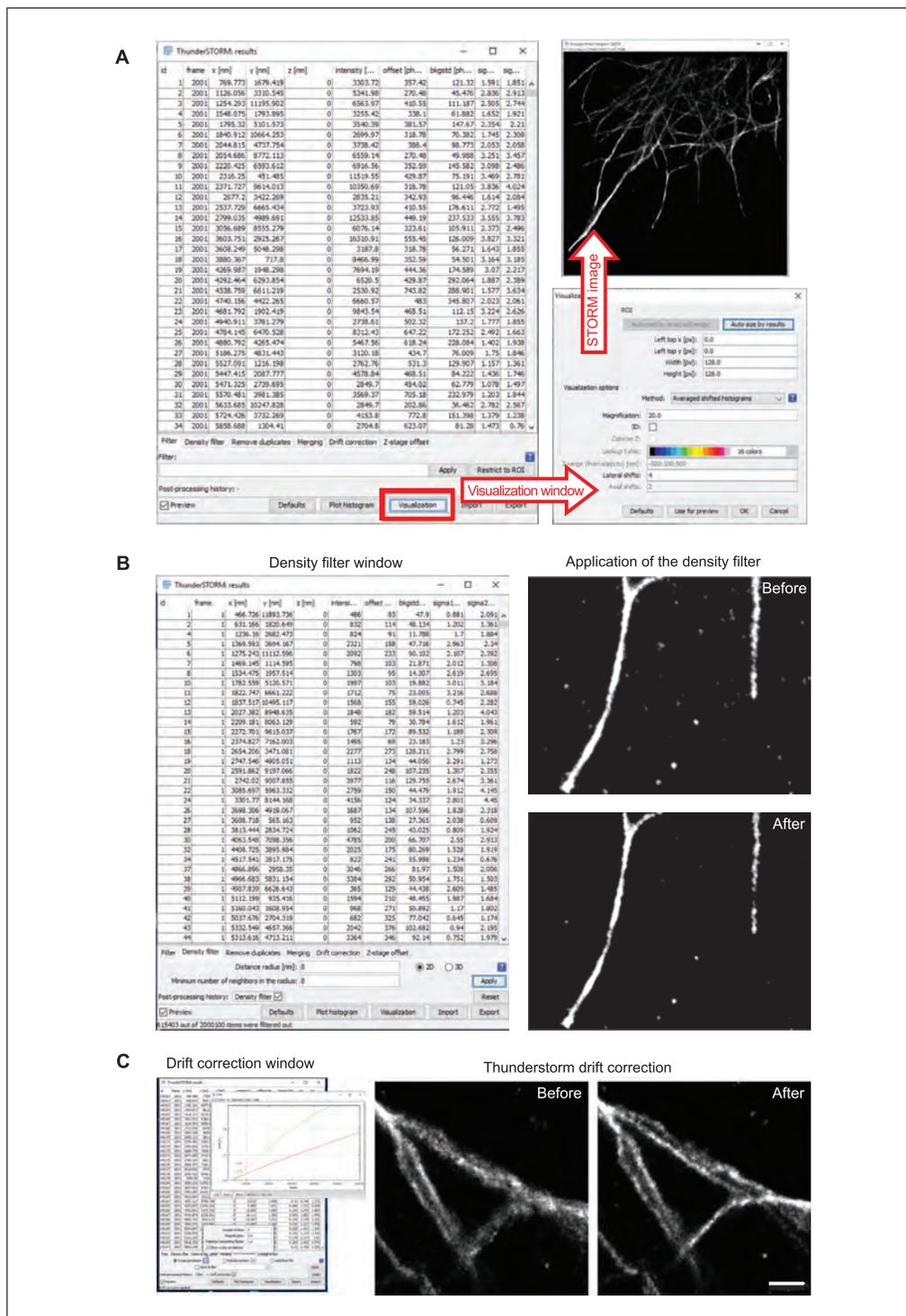


Figure 4 Visualization of single-molecule data. **(A)** Clicking on the visualization button on the ThunderSTORM results window opens the Visualization window. Set the parameters and click OK to create the image. **(B)** The density filter removes unattached fluorophores by removing localizations that are not close to other localizations and are therefore not part of a structure. Here the cutoff is 8 localizations within an 8-nm radius. As these numbers increase, more localizations are removed, which can start to affect continuous structures as well as unattached fluorophores. Left, ThunderSTORM Density filter window and settings. Right top, image of microtubules before application of the density filter. Right bottom, image after application of the density filter. Notice that there are fewer isolated spots between the microtubules, and the microtubule on the right is less

(legend continues on next page)

continuous as well. **(C)** Drift correction corrects for movement of the sample during data acquisition, which leads to a blurring of the image. We select the cross-correlation method with the following settings: number of bins, 5; magnification, 5; trajectory smoothing factor, 1.0. Left, ThunderSTORM drift correction window and plot of sample trajectory versus frame. Middle, image of microtubules before drift correction. Right, image of microtubules after drift correction. Scale bar: 0.5 μm .

correction. Typically, we will use the filtering step to remove localizations from the first frames of the acquisition when too many fluorophores are emitting. Density filtering is used to remove localizations from unattached, isolated fluorophores that do not label the structure of interest (Fig. 4B). Drift correction accounts for movement of the sample during the acquisition process.

9. Drift correction by cross-correlation can be performed by clicking the drift correction tab (Fig. 4C). Typical settings for the parameters are 5 bins, $5\times$ magnification, and 1.0 for trajectory smoothing. Cross-correlation images with detected peaks can be viewed by checking the “Show cross-correlations” checkbox to fine tune the parameters for different datasets. Successful drift correction results in an image with higher resolution and less blurring: Compare the image before drift correction (middle panel in Fig. 4C) to the image after drift correction (right panel in Fig. 4C).
10. The output of this process is the table of filtered and corrected localizations and the final super-resolution figure.

SUPPORT PROTOCOL

CONJUGATION OF ANTIBODIES WITH STORM-COMPATIBLE DYES

STORM images are constructed based on single-molecule imaging of photo-switchable fluorescent probes. Compared to labeling for epifluorescence imaging, a single fluorophore emits a weak signal, which makes its detection challenging. Additionally, tissue-induced spherical aberration and light scattering cause a further loss of fluorescence signal and thus degrade localization precision (Kamiyama & Huang, 2012). Various bright probes, including organic dyes and quantum dots, have previously been characterized to circumvent this problem. Among those probes, Alexa Fluor 647 (AF647; a far-red photo-switchable dye) is exceptionally bright, producing high-quality images of central nervous system (CNS) neurons (Dempsey, Vaughan, Chen, Bates, & Zhuang, 2011). Here, we demonstrate the conjugation of AF647 to antibodies.

Materials

- Anti-HRP (JacksonImmuno, cat. no. 123-005-021, RRID: AB_2338952)
- Phosphate-buffered saline (PBS)
- 1 M NaHCO_3 , prepared from powder (JT Baker, cat. no. 3506-01)
- Alexa FluorTM 647 NHS Ester (AF647; Thermo-Fisher, cat. no. A20006) or any other STORM-compatible dye
- Dimethyl sulfoxide (DMSO; Sigma Aldrich, cat. no. 276855)
- Secondary antibodies:
 - Anti-rabbit IgG (JacksonImmuno, cat. no. 711-001-003, RRID: AB_2340584)
 - Anti-mouse IgG (JacksonImmuno, cat. no. 115-001-003, RRID: AB_2338443)
- 0.5-ml Illustra NapTM-5 Sephadex columns (20 columns; Cytiva, cat. no. 17-0853-01)
- 1.5-ml microcentrifuge tubes (for conjugation mixture and for collecting product fractions)
- Aluminum foil
- Nutator (BD Clay AdamsTM Nutator Mixer, Becton Dickinson Diagnostics)
- Micropipets (Gilson, SKU cat. no. F167380)

1. For anti-HRP conjugation, add the following to a 1.5-ml microcentrifuge tube:
 - 60 μ l anti-HRP
 - 20 μ l PBS
 - 10 μ l of 1 M NaHCO₃
 - 1 μ l AF647 dye dissolved in 10 μ l DMSO.
2. For secondary antibody conjugation, add the following to a 1.5-ml microcentrifuge tube:
 - 20 μ l secondary antibody (anti-rabbit or anti-mouse IgG)
 - 60 μ l PBS
 - 10 μ l of 1 M NaHCO₃
 - 6 μ l (for anti-rabbit IgG) or 3 μ l (for anti-mouse IgG) of AF647 dye dissolved in 10 μ l DMSO.

The stated amount of antibody or dye may not always provide the same degree of labeling. These amounts may change depending on the conditions of the ingredients.
3. Cover the tube with aluminum foil to protect it from light.
4. Incubate 15 min at room temperature on a nutator to allow the reaction to take place.
5. During the reaction, wash the Sephadex column three times with PBS at 4°C to equilibrate the resin pores with PBS, and drain the PBS from the column.
6. After the reaction is finished, slowly add the reaction mixture to the column with a micropipet.
7. Once the mixture has set into the resin, elute with 400 μ l PBS. During AF647 conjugation, two bands can be seen forming: the lower band is the conjugated dye, and the upper band is the unconjugated dyes.
8. Collect the product in 100- μ l increments by adding PBS and eluting into different tubes.

Looking at the color makes it possible to can identify the best-conjugated fraction. Typically, these are the most dye-concentrated fractions in the middle—i.e., fractions 3 or 4.

UV/Vis absorption measurements can be used to find the degree of labeling.

Using the molar extinction coefficients and the absorptions of dyes at 280 nm, calculate the degree of labeling. The number of dye molecules per antibody (DOL) should be between 1 and 2 for STORM, and in case of quantitative studies, a DOL closer to 1 is ideal.
9. Store the antibodies up to 1 month at 4°C, or divide into aliquots in small tubes and store up to 1 year at -20°C.

REAGENTS AND SOLUTIONS

Grape juice agar plates

Prepare agar plates with Nutri-Fly Grape Agar Powder Premix Packets (FlyStuff, cat. no.47-102) following the provided instructions. Briefly, dissolve one packet of the powder mix into 500 ml water and bring to a vigorous boil using a microwave, then let sit to cool. After the temperature of the mixture has decreased to 70-75°C, pour into 60-mm Petri dishes. Once the agar has solidified, store plates are at 4°C.

Imaging medium

- 80 μ l phosphate-buffered saline (PBS)
- 20 μ l (w/v) 50% glucose
- 1 μ l 2-mercaptoethanol (Sigma Aldrich, cat. no. M3148)

1 μ l glucose oxidase with catalase, prepared by dissolving 1 mg of glucose oxidase in 10 μ l PBS (100 mg/ml, Sigma Aldrich, cat. no. G2133) and adding 2 μ l of catalase (17 mg/ml, Sigma Aldrich, cat. no. C40) solution.

2-Mercaptoethanol is toxic if inhaled; therefore, it is critical to keep it in a well-ventilated area and use a fume hood when making the imaging medium.

Typically, when preparing this buffer, we add the catalase to the glucose oxidase solution. This way, we can store the catalase aliquot of 17 mg/ml in a -20°C freezer for multiple uses in the future. This buffer should be switched out after three freeze-thaw cycles. Glucose oxidase with catalase has a much shorter lifespan in 4°C , which should be prepared freshly after 3-4 days.

Supplemented SFX medium

To 9.4 ml HyClone SFX-Insect cell culture medium (Cytiva, cat. no. SH30278.02), add:

400 μ l fetal bovine serum (R&D Systems, cat. no. S11150)

100 μ l 100 \times Penicillin-Streptomycin (R&D Systems, cat. no. B21110)

100 μ l Gibco Insulin-Transferrin-Selenium (Thermo-Fisher, cat. no. 41400045)

Divide into 200- μ l aliquots

Store up to 12 months at 4°C .

Prepare the medium in the cell culture hood. When handling the medium afterwards (such as when preparing plates), work near the Bunsen burner to avoid bacterial contamination. Use deionized, distilled water in all steps and recipes unless otherwise specified.

COMMENTARY

Background Information

Cultured *Drosophila* neurons have been used for investigating cytoskeletal dynamics in neurons, conducting comparative analysis studies with in vivo experiments, carrying genetic RNAi screening (Mohr, Bakal, & Perrimon, 2010), and identifying cell-autonomous neuronal mechanisms such as membrane compartmentalization or presynaptic differentiation (Katsuki, Ailani, Hiramoto, & Hiromi, 2009). Although such in vitro approaches play an important role in revealing cellular phenotypes, developmental and functional findings are crucial for understanding neural circuitry development. To investigate the underlying mechanisms, we use embryonic motoneurons in vivo (Furrer, Vasenkova, Kamiyama, Rosado, & Chiba, 2007; Kamiyama & Chiba, 2009; Kamiyama et al., 2015; Sharifai et al., 2014). This system is amenable to targeted genetic manipulation in a cell-type-specific manner, allowing us to study neural specification, axon and dendritic guidance, partner selection, and synaptogenesis. In furthering our understanding of synaptogenesis, the neuromuscular junction serves as an excellent model system due to the stereotypical motoneuron targeting (Keshishian, Broadie, Chiba, & Bate, 1996). For partner recognition to result in a mature synapse, the neuronal growth cone must interact with the

muscle through actin-based hairlike structures found in both cells (Ritzenthaler, Suzuki, & Chiba, 2000). These fine structures, called filopodia, explore the local environments to match partner cells together (Fig. 5). Although some developmental time points have been identified, the nature of the filopodial interactions remains elusive because of their fine structures. However, the protocol described here can be further used for labeling the neuromuscular junction and applying dual-color STORM, so that the details of filopodial interactions can be further investigated.

Here, we demonstrate STORM, which utilizes photo-switchable organic fluorescent dyes to precisely localize individual molecules. Unlike conventional light microscopy, STORM can achieve resolutions of ~ 20 nm laterally and ~ 50 nm axially (Huang et al., 2009). There have been numerous examples of the application of STORM to biological research (Kamiyama & Huang, 2012). In particular, STORM is a valuable asset for conducting anatomical studies of filopodia. Because the filopodia are so tiny (~ 200 nm in width and ~ 5 μ m in length), their architecture can be characterized through STORM. Furthermore, we find that multi-color STORM allows us to visualize filopodia between the motoneuron and the muscle (as demonstrated by confocal microscopy;

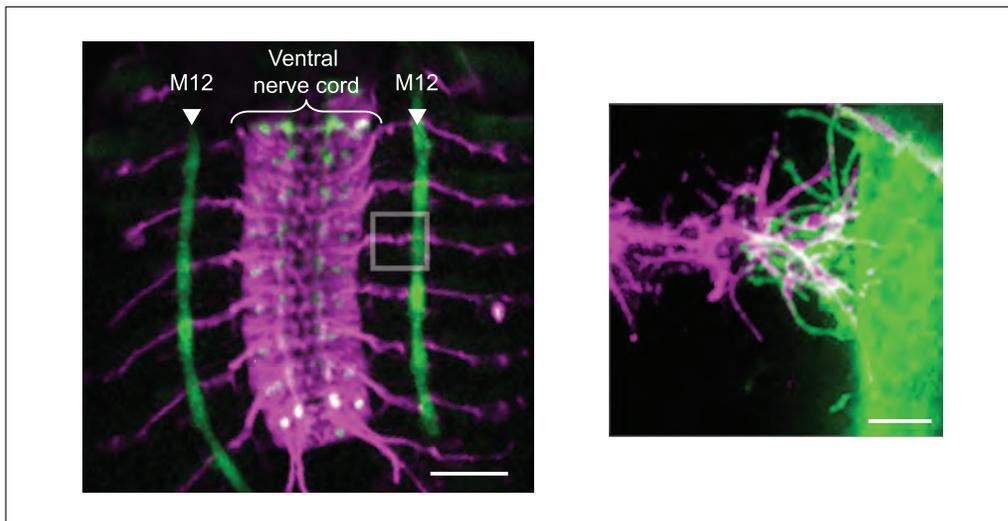


Figure 5 In vivo central nervous system. The central nervous system is labeled with anti-HRP antibody (magenta), and a single muscle (M12) is labeled using *2702-GAL4* driver with GFP (green). The boxed region highlights the neuromuscular junction of the embryo. The right panel shows a representative growth cone and muscle filopodia interaction during synaptogenesis. Once the initial contact is made, the filopodia undergo morphological changes by clustering at the synaptic site to form the synapse. Images were acquired with 10× objective (left) and 100× oil immersion objective (right). Scale bars: left, 50 μm; right, 5 μm. Genotype: *UAS-CD4-tdGFP, 2702-GAL4/TM3*.

Fig. 5). For this purpose, we have evaluated many organic dyes at different emissions (M.A.I., and D.K., unpublished data). Although the dyes are photo-switchable under specific buffer conditions, Alexa Fluor 647 exhibits the best photo-switching ability. As an alternative to the STORM technique, multiple groups have recently developed a new approach, called DNA points accumulation for imaging nanoscale topography (DNA-PAINT; Jungmann et al., 2014; Jungmann et al., 2010). DNA-PAINT is compatible with the imaging platform of standard STORM. The approach takes advantage of DNA specificity for target recognition using a docking strand of DNA conjugated to an antibody and an imager strand of DNA conjugated to a fluorescent dye. In contrast to the traditional STORM technique, the blinking effect is achieved by the transient binding of these DNA strands. We can apply different orthogonal docking sequences with unique fluorophores to the same sample, which allows the observation of multiple proteins. This approach also has the potential for multicolor imaging of *Drosophila* tissues.

Critical Parameters

Primary neuronal culture

To set up for a healthy neuronal culture, adult flies in the mating cage should be no older than 1 week. This ensures healthy embryos to begin plating neurons. Plating

an appropriate density of cells ($\sim 1.2 \times 10^5$ cells/cm²; see also Fig. 6) is key to axonal growth, secure adhesion to the coverslip, and reduced clustering and layering. However, the researcher should keep in mind that too low a cell density may risk the survival of the neurons.

Tissue preparation

For embryonic dissection, selection of the developmental age is important (Inal, Banzai, & Kamiyama, 2020). Beyond the age of ~ 15 hr after embryo laying, the embryos will start depositing cuticles, which will prevent the embryos from sticking to the glass slide. Although older embryos can also be dissected, the method described here may not be appropriate for those experiments. In addition, the number of embryos that can be dissected and placed onto the glass slide becomes limited, as the amount of protein in the buffer will be saturated and coat the glass, preventing any more embryos from sticking to the slide. When washing the embryos after dissection in the subsequent steps of the protocol, care should be taken to prevent embryos from lifting off the slide or being destroyed due to surface tension.

Fixation

We usually fix cellular proteins with 4% paraformaldehyde. In contrast, we use a mixed concentration of paraformaldehyde and glutaraldehyde to preserve microtubule structures

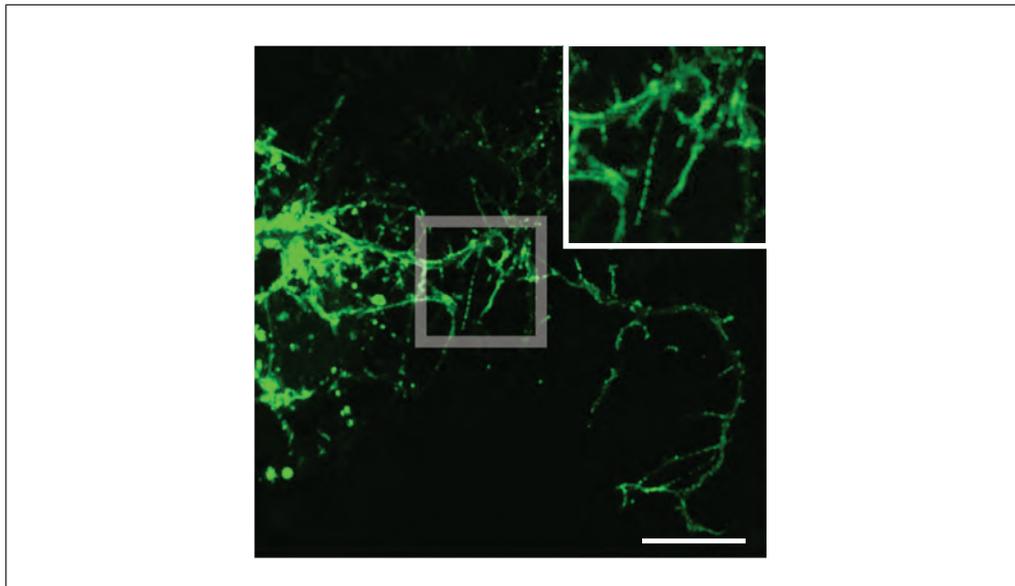


Figure 6 Neuronal cell culture expressing GFP on the plasma membrane. The boxed region details the fine structural components of the neurite. The components such as filopodia and lamellipodia are important in forming mature synapses with their partners. Scale bar: 15 μm . Genotype: *UAS-myr::GFP; elav-GAL4*.

(Kamiyama & Huang, 2012). To maintain cells in their most natural state, we optimize a fixation approach for the structure of interest. The optimal fixative for the ideal image should be whatever is best for preserving the target structure while avoiding structural artifacts.

Immunostaining

Because the quality of a STORM image depends on labeling density, the immunostaining needs to provide the best labeling density to make it possible to take the best image. To achieve this, antibody concentrations higher than the recommended should be used. Additionally, longer incubation times allow more effective labeling. Therefore, if possible, the primary antibody should be incubated overnight at 4°C and secondary antibody for ~1-2 hr at room temperature. Selecting antibodies that are highly specific for the antigens will minimize nonspecific labeling and background signals. Furthermore, it may be helpful to use primary antibodies that are directly conjugated with the fluorophore to minimize linkage error, which results in increased apparent size of structures (Huang et al., 2009).

Antibody conjugation

The degree of antibody labeling should be between 1 and 2 for STORM imaging, and this can be measured using a spectrophotometer with the given calculation approach in the methods. The conjugation from one batch to another may not give the same DOL, and even

within the same batch, the labeling efficiency may vary. In this case, it is advisable to collect the conjugated mixture in smaller (~100 μl) fractions.

Mounting and imaging buffers

During long imaging processes, the pH value of the buffer decreases as oxygen reacts with the oxygen-scavenging component of the imaging buffer. This can hinder the photo-switching ability of the fluorophore. Therefore, the freshest buffer possible should be used and the oxygen concentration in the buffer should be minimized by sealing the sample, which can improve the lifespan of imaging buffer. Additionally, the fluorophores may lose photon counts if the sample is far away from the coverglass. Therefore, the mounting approach, especially for tissues, should minimize the distance between the sample and the coverglass (Dani, Huang, Bergan, Dulac, & Zhuang, 2010). This will evidently increase the photon counts and resolution.

Acquisition

The resolution of an image acquired using STORM is inversely proportional to the number of photons emitted by a single molecule (Huang et al., 2009). Apart from the freshness of the imaging buffer and the quality of the dye, the laser power with which the dye is excited plays a vital role in determining the number of photons emitted by the dye. Every dye has a maximum number of photons that it can

Table 1 Troubleshooting

Protocol	Problem	Possible cause	Solution
Sample preparation	Poor neuronal growth and/or differentiation	Suboptimal temperature incubation	Incubate cell culture at a higher temperature (e.g., 28°C)
	Embryo does not stick to glass slide	Unclean glass slide Embryo cuticles	Use clean glass slide Use younger embryos for dissection
	Embryo is floating or destroyed	Sample is exposed to meniscus of the buffer	When exchanging buffers, use large volumes for washes to ensure sample remains entirely covered and prevent drying
	Cells are dispersing or floating	Pressure from the objective lens forcing the cells to float if the buffer leaks out	Make sure the coverslip perimeter is well coated and dried in nail polish Adjust the objective lens to make sure it does not compress the coverslip Minimize the distance between coverslip and the slide, which will increase the working distance for the objective lens
	Filopodia are moving	Poor fixation	Increase the fixation time or the concentration of fixative
	Poor signal and/or high background	Nonspecific labeling	Use highly specific antibodies Increase antibody concentration Use blocking buffer and/or increase blocking time
Acquisition		Large distance from sample to coverslip	Minimize distance from the coverslip by pressing the coverslip down; if it is not being imaged, the ventral nerve cord can also be removed via suctioning after fixation
	Inconsistent labeling efficiency	Variable antibody degree of labeling	Collect conjugates in smaller fractions and test degree of labeling
	Low photon count from fluorophores	Excitation laser is not aligned perfectly through the objective lens.	Realign the system
		Reflectivity of mirrors in excitation path is low, causing loss of laser power before light reaches sample	Measure reflectivity of mirrors in the excitation pathway and change mirrors accordingly; we recommend using dielectric mirrors for the highest reflectivities
	Fluorophores are not turning off/blinking	Low laser power	Increase the laser power until blinking can be observed
		Buffer is not fresh	Make sure the enzymes (glucose oxidase and catalase) in the imaging buffer are freshly prepared; imaging buffer has an expiration period of 2-3 hr
	Fluorophores are not turning on	Fluorophore is photobleached Buffer is not fresh	Alternatively, use an activation laser such as UV (405 nm) and increase its laser power See above about imaging buffer

(Continued)

Table 1 Troubleshooting, *continued*

Protocol	Problem	Possible cause	Solution
Post-processing	Axons appear blurry once reconstructed	Lateral or optical drift	Ensure the stage is stabilized before acquisition Perform drift correction on reconstructed image
	Reconstructed image has too many localizations	Inaccurate intensity thresholding	Filter out unwanted molecules by changing the intensity threshold

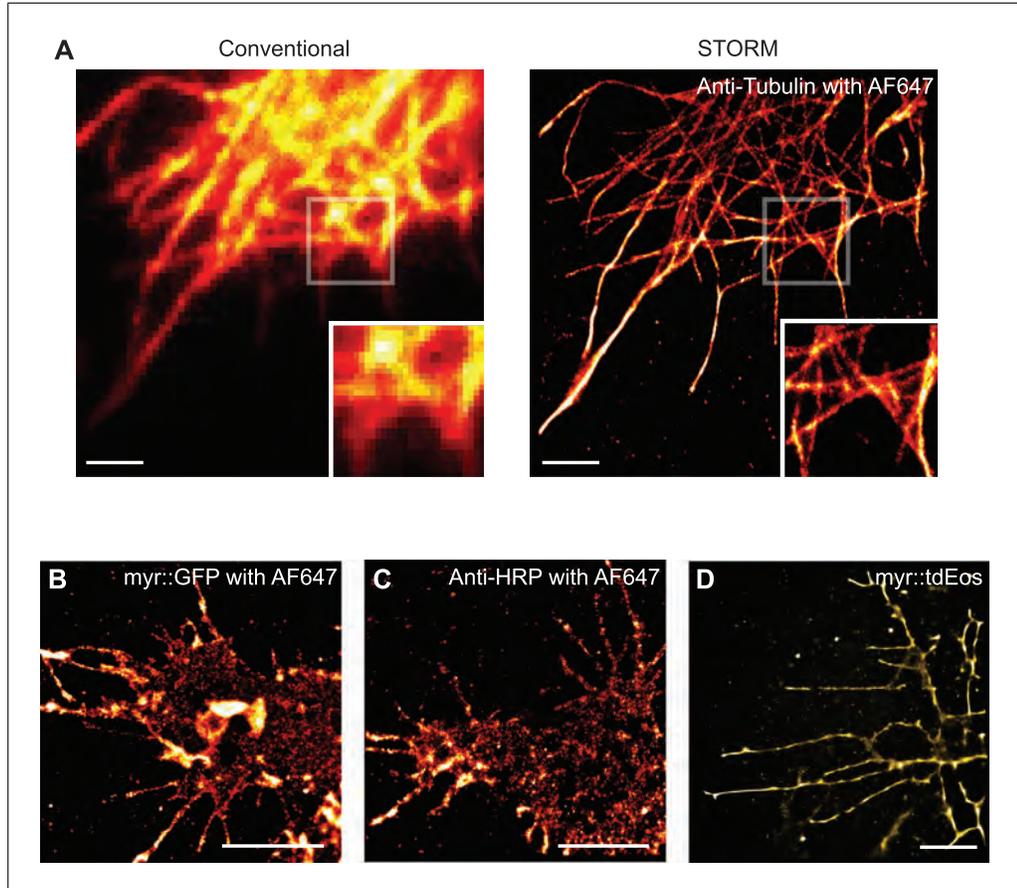


Figure 7 Reconstructed super-resolution images of various proteins labeled in primary neuron culture. **(A)** Conventional wide-field (left) and super-resolution STORM (right) images of microtubules from primary neuronal cells, immunostained with Alexa Fluor 647. The super-resolution image is reconstructed from 60,000 acquired frames. The insets show the magnified regions of the white boxes. **(B-D)** Neurons immunostained with Alexa Fluor 647 **(B and C)** or labeled with tdEos **(D)**. Scale bars: 2 μm . Genotypes: **(A)** *UAS-myr::GFP; elav-GAL4*, **(B and C)** *UAS-myr::GFP; elav-GAL4*, and **(D)** *UAS-myr::tdEos; elav-GAL4*.

emit (Dempsey et al., 2011), and the number of photons emitted by a dye is linearly related to the laser power until it reaches the maximum number of photons emitted, at which point the dye is saturated and the number of photons emitted remains constant at the maximum. Therefore, to achieve the best acquisition, it is important to excite the sample with and appropriate laser power sufficient to facilitate blinking but not too high that one bleaches the dye too fast.

Troubleshooting

Table 1 lists problems that may arise with this procedure along with their possible causes and solutions.

Understanding Results

Reconstruction of super-resolution images acquired via STORM

For reconstruction, it is necessary to consider parameters such as background intensity levels and Min/Max values in selecting

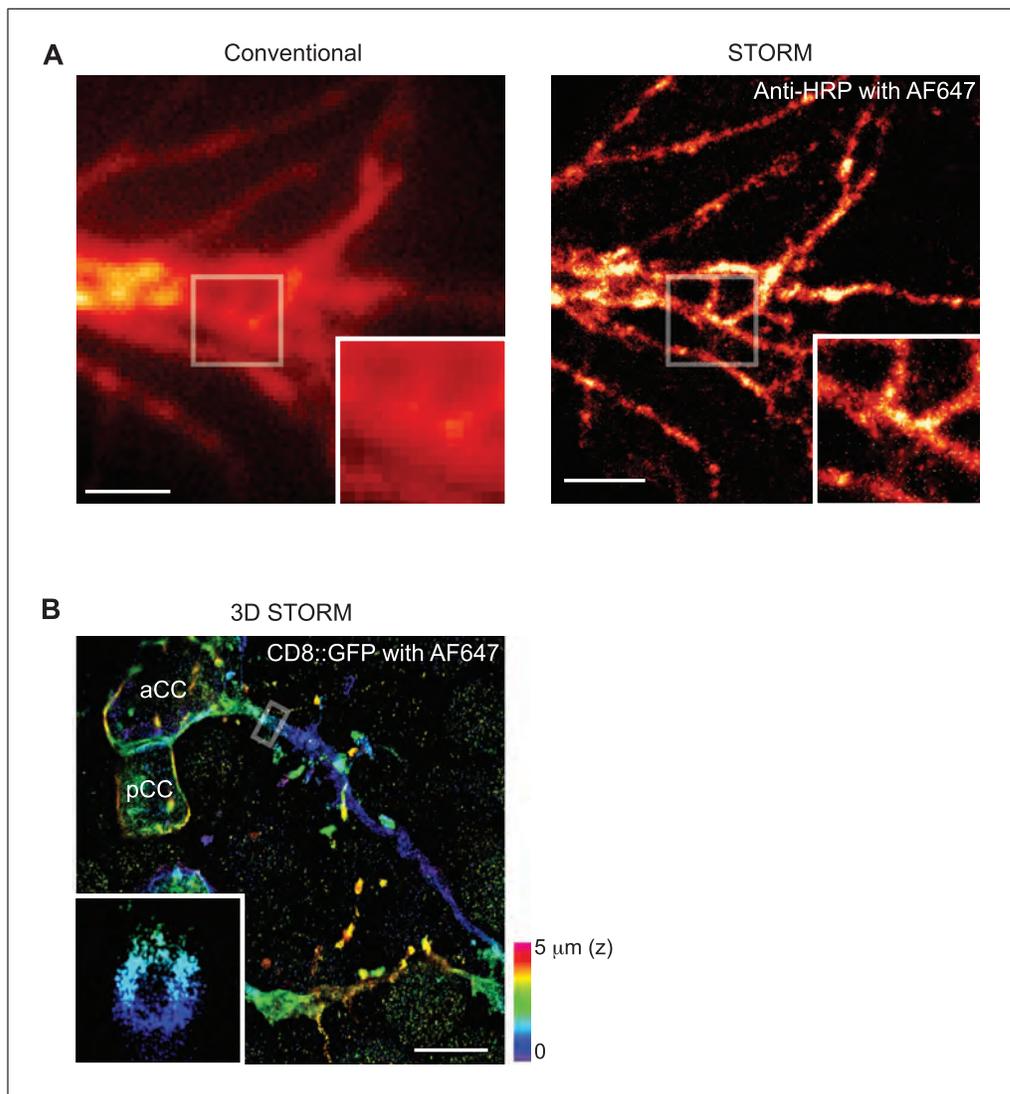


Figure 8 Reconstructed super-resolution images of neuronal membranes in dissected embryos. **(A)** Conventional wide-field (left) and super-resolution STORM (right) images of motor neuron RP5 in anti-HRP labeled embryos immunostained with AF647. The insets show the magnified regions of the white boxes. **(B)** An example of 3D STORM image. 3D reconstructed super-resolution image of aCC/pCC motoneurons labeled with GFP and immunostained with AF647. The image is reconstructed from six z-slices that cover a total of 5 μm . The inset shows a hollow cross-section of the axon from the boxed region. Scale bars: **(A)** 2 μm , **(B)** 3 μm . Genotypes: **(A)** *UAS-CD4-tdGFP, 2702-GAL4/TM3* and **(B)** *UAS-CD8-GFP, eve-GAL4^{RN2}*.

individual blinking events to achieve the highest resolution without compromising the quality of the reconstructed image. In Basic Protocol 4 (see also Figs. 3 and 4), we show our workflow for identifying the optimal parameters for our samples. These parameters can vary between setups, sample types (cultured neuron vs. in vivo neuron), and even individual acquisition samples. Although there is no one-size-fits-all approach, the variability among acquisition samples is not so significant as to require testing many different parameters each time. Therefore, the results of reconstruction should be easily reproducible among multiple samples.

STORM imaging of in vitro and in vivo neurons

We show a conventional wide-field image of a neuron in culture (Fig. 6) and the reconstruction of the same neuron using STORM imaging (Fig. 7). With the protocol we describe here, we can image not only the membranes of the neuronal cells in culture but also the cytoskeletal architecture of them. Figure 7A presents images of the microtubules acquired from primary neuronal cultures. As the inset indicates, each microtubule bundle can be clearly seen, compared to the inset of the wide-field image. Figure 7B and 7C show the results of different approaches for

labeling the neuronal membrane: using anti-GFP (expressed on the cell membrane through *UAS-GAL4* system) or anti-HRP (a pan-neuronal membrane marker) antibodies. Finally, Figure 7D demonstrates the use of photoconvertible fluorescent proteins, in this case tdEos, as an alternative means to image the membrane structures of the neurons. Using similar labeling approaches, we also apply these tools to in vivo neuronal imaging. Figure 8A and 8B show, respectively, neurons labeled with anti-HRP antibody and neurons labeled with the *UAS-GAL4* system and immunostained against GFP with AF647 dye.

Although we demonstrate a limited set of examples for using these tools, other proteins can also be imaged with this method using STORM to individually localize and elucidate their cellular roles at resolutions of ~20 nm. Additionally, other photo-switchable dyes or fluorescent proteins can be used to look at cellular or molecular interactions in multicolor STORM imaging.

Time Considerations

Flies need to be trained for 2 days before primary culture and embryonic sample preparation. The culturing step for primary neurons requires an additional ~2 days to allow neuronal morphology to differentiate before immunostaining. However, the embryos can be collected immediately after the 2-day training period and dissected. Dissection of embryos can take as little as 5 min for 10 embryos for an experienced individual, although a beginner may at first take 5 min or longer to dissect a single embryo. Depending on the efficiency of the individual, the number of embryos to be dissected can be modified, especially for developmentally time-sensitive experiments. After the fixation step, which takes 5 min, permeabilization and blocking take ~1 hr and 15 min. This is a good point at which to pause, if necessary. It is also a good time to prepare dye-conjugated antibodies, which can be stored for subsequent experiments. For indirect labeling, primary antibody incubation takes place overnight and secondary antibody incubation takes 2 hr. In case of direct labeling, incubation takes a maximum of 2 hr at room temperature. The subsequent washing steps and post-fixation should take ~25 min.

Image acquisition is controlled by Manager running on a Dell workstation (Intel Xeon CPU ES-1603 v4, 2.80 GHz, 16 GB RAM) with a 500-GB solid-state hard drive. Acquiring 40,000 raw frames with a 15-ms exposure

time takes ~15 min. On the same computer, the analysis can take up to 20 min, depending on the number of localizations that are found and the analysis steps that are used. Opening the acquired data takes 5 min. The image stack is then analyzed as described in Basic Protocol 4. Running the analysis in ThunderSTORM takes 3-4 min. Running the density filter takes 3-4 min, and if drift correction is performed, that will take an additional 5 min. The post-processing steps must typically be run a few times to optimize the parameters in order to generate an optimal final image.

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Author Contributions

Daichi Kamiyama: Conceptualization, funding acquisition, project administration, supervision, writing–review and editing; **Melissa Inal:** Formal analysis, investigation, methodology, resources, validation, visualization, writing–original draft, writing–review and editing; **Kathy Bui:** Formal analysis, investigation, methodology, resources, validation, visualization, writing–original draft; **Abhijit Marar:** Formal analysis, investigation, methodology, software, validation, visualization, writing–original draft; **Shaoheng Li:** Formal analysis, investigation, methodology, software, validation, visualization; **Peter Kner:** Conceptualization, funding acquisition, project administration, supervision, writing–review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

No new data were produced during this project.

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