Single-Molecule Localization Super-Resolution Microscopy of Synaptic Proteins

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Abstract

Recent years have witnessed huge progress in the field of light microscopy with the development and implementation of new approaches leading to dramatic improvements in the spatial and temporal resolution of this form of imaging, most particularly in its biological applications. The limitations in spatial resolution imposed by the diffraction of light have been circumvented by resorting to different strategies, which are briefly outlined in the Introduction. These protocols are intended to provide practical guidelines for the imaging of synaptic proteins using one such strategy, namely, single-molecule stochastic localization super-resolution microscopy.

The protocols use neuronal cells from the hippocampus of rodent embryos as the experimental paradigm and outline the steps for obtaining dissociated neurons and establishing primary cultures for in vitro studies. The techniques can be adapted to the culture of neurons from other brain regions. Procedures for handling fixed and live specimens are described, as well as the use of extrinsic fluorescent probes and fluorescent proteins, mounting media, examples of hardware configurations, software for image analysis, and some hints for the implementation of minimalist approaches to single-molecule localization nanoscopy.

Keywords Nanoscopy, Neuronal cell culture, Sample preparation, Single-molecule imaging, Staining, Super-resolution microscopy

1 Introduction

Optical ("light," "wide-field," "far-field") microscopy is undoubtedly the most popular technique for imaging cells and tissues, and the successful combinations of appropriate probes (organic dyes, fluorescent proteins, and inorganic nanoparticles, e.g., quantum dots), new light sources (solid-state lasers, light-emitting diodes), and improved detectors (CCD cameras, avalanche photodiodes) have made fluorescence microscopy the method of choice, essentially because of the unparalleled selectivity and sensitivity achieved in biological applications, pervading practically all realms of biology. Moreover, recent years have witnessed a revolution in fluorescence microscopy: using conventional lenses and visible light it has been possible to circumvent the century-old limit dictated by the

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diffraction barrier, a fundamental law formulated by the German physicist Ernst Abbe in 1873. This limitation in the resolution of optical imaging instruments imposed by the diffraction of light was solved by independent and complementary methodologies which were acknowledged by the 2014 Nobel Prize in Chemistry, jointly awarded to Stefan W. Hell, William E. Moerner, and Eric Betzig. These advances were materialized in the techniques of stimulated emission depletion (STED) microscopy, conceived [1, 2] and implemented [3] by Stefan Hell and his group in Germany, and single-molecule localization microscopies, the methodologies developed by Betzig, Moerner, and Zhuang and their groups in the USA [4–11].

A third approach is structured illumination microscopy (SIM), which excites the sample with a series of spatially structured patterns in a wide-field configuration and resolves otherwise inaccessible high-resolution information in the observed image in the form of Moiré fringes. Multiple images of the emitted fluorescence are recorded in the high-frequency domain at lower spatial frequencies and computationally separated to extract low- and high-frequency data. This results in about a twofold gain in resolution in the *x*-, *y*-, and *z*-axes [12, 13]. A main feature of SIM is that unlike a confocal microscope, it utilizes full amplitude of the emitted fluorescence, thus producing brighter images which require relatively shorter times to acquire. It is a relatively fast super-resolution technique, currently available commercially (Zeiss Elyra, Nikon N-SIM, GE Healthcare DeltaVision).

In the STED "deterministic" point-by-point targeted nanoscopy mode, the physical tools employed to interrogate fluorescent molecules are (1) visible light and (2) regular lenses, much like the manner used in a conventional diffraction-limited wide-field laser scanning microscope. It is interesting to note that the crucial improvement in spatial resolution afforded by STED microscopy also relies on a purely physical effect: reducing the effective volume from which fluorescence emission is collected. How is this accomplished? In simple terms, the specimen is scanned with two concentrically focused laser beams: the central, interrogating laser beam is surrounded by a red-shifted doughnut-shaped laser beam depleting the emission of fluorescent molecules at the periphery of the former. An effective point-spread function (PSF) is created in which fluorescence is confined to the immediate vicinity of the central intensity zero; the volume of this region is no longer limited by diffraction. The resolution of the STED microscope can be adjusted by varying the intensity of the depletion peripheral laser beam.

The concept of targeted deterministic switching in the vicinity of an intensity zero originally resulting in the development of STED microscopy has been generalized to include any reversible and saturable optical transition in the fluorescence mode (e.g., in the so-called reversible saturable optical linear fluorescence transition ("RESOLFT") microscopy technique [14, 15]), but interestingly, this concept is not limited to fluorescence: any other on–off

spectroscopic transition in the state of a molecule (e.g., absorption) can also be exploited; the "off" state need not be dark [3, 16]. Commercial versions of STED microscopes are available (Leica TCS STED, Abberior Instruments in various models ranging from the one-color pulsed STED, the easy3D STED, and RESCue 3D STED to the correlative STED nanoscope combined with atomic force microscopy module). In the case of the nervous system, STED has been successfully applied to the study of synaptic vesicles, dendritic spines, and other subcellular aspects of the synapse (reviewed in [17–19]). It was also the technique employed for the first imaging of supramolecular aggregates of a neurotransmitter receptor [20], live-cell imaging of dendritic spines [21], actin dynamics in synapses in brain slices [22], and most dramatically, the imaging of dendritic spines in the intact brain of a living mouse [23], among other applications. A new technique, known as mirror-enhanced, axial narrowing, super-resolution (MEANS) microscopy, is a spin-off of STED nanoscopy in which cells to be studied are grown on custom-made tiny mirrors instead of transparent glass coverslips [24]. The MEANS technique is claimed to improve axial resolution sixfold and lateral resolution twofold.

In contrast, in single-molecule methods such as photoactivated localization microscopy (PALM) [4, 25] and stochastic optical reconstruction microscopy (STORM) [8], super resolution is accomplished by cumulative spatial localization of fluorescent molecules resulting from sequential imaging of sparse, stochastic subsets of fluorescence emitters, i.e., by interrogating the *on*-state ensemble molecule by molecule and recording these individual emitters on a grid detector, e.g., a CCD camera. Thousands of frames are imaged in a stream of individual pictures that include only a few, preferably well-separated molecules, and the coordinates of the *on*-state individual molecules are subsequently established by off-line localization analysis. In other words, in single-molecule localization nanoscopy, the *temporal* separation enables the *spatial* distinction of individual fluorescence emitters. The difference between the two aspects of the same principle is that in PALM, imaging is performed on bioengineered fluorescent proteins, whereas in STORM much smaller molecules, i.e., organic fluorescent dyes, are interrogated [26–33]. The basic principles behind the stochastic nanoscopy techniques are the same for STORM and PALM: they rely on the fact that a single fluorescent source (e.g., a fluorescent molecule) can be localized with nanometer precision by fitting an appropriate function (e.g., a two-dimensional Gaussian function) to determine the center of mass – the centroid – of the diffraction-limited, blurred wide-field image formed by the compound optical microscope. The super-resolved final image is a reconstructed image of the centroids of all validated fluorescent molecules in the individual images. The precision with which the centroid can be determined depends on the number of photons collected; in practice, this can be a few tens of nanometers or better.

STORM and PALM techniques currently provide the best spatial resolution in nanoscopy but have relatively less time resolution than parallelized scanning in the RESOLFT mode, which involves the use of many thousands of doughnuts [34]. STORM was one of the first applications of super-resolution techniques for imaging proteins in brain synapses with nanometric precision [35].

As with the other modalities of super-resolution microscopy, commercial firms have released various microscopes suitable for single-molecule stochastic localization nanoscopy: N-STORM from Nikon, GSD from Leica, Zeiss Elyra P1 from Zeiss, or Vutara 350 from Bruker. I should like to add that building a STED or a RESOLFT nanoscope requires considerable skill and is relatively expensive because of the type of lasers needed; constructing a SIM is not trivial, but building a PALM or STORM instrument on the basis of an existing commercial epifluorescence microscope is an accessible project for the biologist wishing to improve the resolution of the instrument beyond the diffraction limit.

In addition to the stochastic photoswitching single-molecule localization techniques like PALM, STORM, and GSDIM, the method termed "point accumulation imaging in the nanoscale topography" (PAINT) is based on the binding of a fluorescent-tagged ligand to the protein or other molecule to be identified [36–38]. Free ligands do not contribute to the image because of the combined effects of oblique illumination and their very fast diffusion in 3-D, resulting in their inefficient excitation. The PAINT technique was initially applied to the study of lipid bilayers and subsequently evolved to imaging proteins in living cells, giving rise to the "universal PAINT" (uPAINT) improved method [39]. This technique has found very interesting applications in the study of the dynamics of the synapse [40–42].

Here I would like to share various protocols adopted (and many of them adapted to particular applications; very few are developed by ourselves) in our laboratory for the imaging of synaptic proteins in primary cultures of neuronal cells. These protocols are by no means intended to cover in a comprehensive manner the current state of affairs in the field. Only one super-resolution modality is discussed; approaches based on quantum-dot technology will not be dealt with. The protocols are intended to provide useful hints and tips and refer the reader to the original sources in the use of one particular super-resolution technique, namely, single-molecule stochastic nanoscopy, as applied to the study of proteins in a specialized substructure of the neuron, the synapse. Understanding synapse function will require detailed knowledge of the spatiotemporal organization of its constituent molecules, and neurotransmitter receptors, transporters, enzymes, and scaffolding proteins alike – outnumbered by a considerable variety of lipid species – are major actors in the synaptic scenario.

A substantial section of the protocols is devoted to the preparation of dissociated neuronal cell cultures. As any model system, this preparation has its pros and cons. It is a compromise between the complexity of the 3-D architecture of the brain and the apparent simplicity of isolated molecules. The 2-D array of living neuronal cells adhered to a thin cover glass offers the possibility of addressing, with unprecedented time and space resolution, rather sophisticated questions on the changes occurring in the synapse at the molecular level. Physiological and pathological conditions can be met at the Petri dish and subjected to pharmacological intervention under controlled conditions. A step further in nanoscopy of synaptic structures in a living animal was taken by the STED imaging of dendritic spines in an intact live mouse brain [23]. In parallel, another leading-edge technology, optogenetics [43, 44] – which shares with nanoscopy the temporally and spatially resolved use of light as its major tool – is increasingly delivering information on the function of neuronal networks, including complex circuit behavior in the whole animal. It will not be long before the super-resolution imaging techniques are combined with optogenetic techniques, in real time, to correlate vis-à-vis structure and function at cellular, subcellular, and multicellular (neuronal network) levels in a living organism.

2 Materials

2.1 For Neuronal Primary Cultures

Pregnant rodent carrying embryonic stage 18 (E18) pups (preferably rat for hippocampal neuronal cultures) or E16 from mouse (more difficult to dissect, for mixed neuronal–glial cultures)

Distilled, deionized, sterile, filtered water

Methanol, spectroscopic grade

Ethanol, spectroscopic grade

Binocular stereoscopic microscope for the dissection of brains and hippocampi

Cell culture incubator at 35.5°C, preferably triple-gas, with automatic humidity control and automatic self-cleaning cycles

CO_2 cylinder

Water bath, 37°C

Compressed butane or natural gas burner

Chemical fume hood for preparing glass coverslip cleaning solution and undertaking cleaning

Corrosion-resistant ceramic staining rack for holding coverslips (Thomas Scientific, cat. no. 8542E40)

Hemocytometer for counting cells

Tabletop centrifuge

Freezing vials (Nunc CryoTubes 352350)

Sterile plasticware:

Large-volume filter units (Stericup-GP filter, Millipore SCGPU02RE)

Small-volume filter units for syringes (Steriflip-GP, Millipore SCGP00525)

5-, 10-, and 25-mL serological pipettes

60-mm tissue culture dishes

25-mL tissue culture flasks (Primaria, BD Biosciences 353824)

15- and 50-mL conical centrifuge tubes

Trypan blue, 0.4% solution

Sodium dodecyl sulfate, 0.1% solution

10 mg/mL DNase in CMF-HBSS (Roche Applied Science 10104159)

Trypsin (0.5%)/0.2% EDTA (Sigma-Aldrich 59418C; Invitrogen 1525300-054)

Alternatively, trypsin (2.5% wt/v) (Invitrogen 15090-046) stock kept at −20°C

Trypsin inhibitor

Polylysine (30,000–70,000 MW, Sigma-Aldrich P2636 or P7405), 1 mg/mL solution in borate buffer

Borate buffer (boric acid + tetraborate) 0.1 M, pH 8.5

Sorensen's phosphate buffer (133 mM)

0.133 M Na_2HPO_4

0.133 M KH_2PO_4

Mix 71.5 mL of Na_2HPO_4 and 28.5 mL of KH_2PO_4 to obtain pH 7.2

CMF-HBSS medium (Ca2^{+}, Mg^{2+}, and bicarbonate-free Hank's balanced salt solution [BSS] buffered with 10 mM HEPES, pH 7.3)

10× Hanks' BSS (Invitrogen 14185-052)

1 M HEPES buffer, pH 7.3 (Invitrogen 15630-080)

Hanks' Plus (HBSS+) medium with antibiotic

HBSS (without Ca^{2+} and Mg^{2+}).

HEPES (Sigma-Aldrich) 10 mM.

Glucose 33.3 mM.

Gentamicin (Sigma-Aldrich), 5 µg/mL.

Store up to 1 month at 4°C.

Neuronal dissociation medium

Hanks' balanced salt solution plus (HBSS, GIBCO BRL 24020-091)

0.3% (w/v) BSA (Sigma-Aldrich or Thermo Scientific)

0.25% trypsin (Gibco, Invitrogen Life Science, Carlsbad; Sigma T-9253)

Alternatively, trypsin/EDTA premixed solution (Biochrom L2143)

Trypsin inhibitor solution

25 mg albumin (bovine: Sigma A-4503)

25 mg trypsin inhibitor (Sigma T-9253)

10 mL 10% FCS medium (5% FCS medium), warmed to 37°C

Plating medium

MEM supplemented with glucose (0.6% wt/vol) and containing 10% (vol/vol) horse serum or 5% (vol/vol) fetal bovine serum:

D-glucose (Sigma-Aldrich G8769)

MEM with Earle's salts and L-glutamine (Invitrogen Life Science 11095-080)

5% (v/v) bovine fetal serum (Invitrogen 16000) or 10% horse serum (Thermo Scientific)

Neuronal maintenance N2 medium [45]

MEM containing the N2 supplement. It is prepared by combining nine parts MEM supplemented with:

Glucose (0.6% wt/vol) and one part 10× N2 supplement

10× N2 supplement contains the following ingredients prepared in MEM:

10 mM sodium pyruvate (Sigma P2256)

1 mM putrescine (Sigma P5780)

0.2 mM progesterone (Sigma P8783)

0.3 mM selenium dioxide (Sigma 200107)

1 mg/mL bovine transferrin (Sigma T1428)

50 mg/mL insulin (Sigma I5500)

Alternatively,
Neurobasal medium for hippocampal neuronal culture [45]

Neurobasal medium (GIBCO BRL 10888-022; Invitrogen 21103-049) supplemented with:

2% (v/v) B27 serum-free supplement (Invitrogen 17504-044; GIBCO BRL 17504-044)

GlutaMAX-I supplement (Invitrogen 35050-061)

100 U/mL penicillin

100 μg/mL streptomycin

1 mg/mL ovalbumin

Microsurgical tools (dry-heat sterilize in a metallic instrument sterilization container with lid)

10-cm diameter glass Petri dishes to dissect embryos

Sharp-ended scissors for caesarean section of adult rodent

100-mm straight Graefe tissue forceps with teeth for caesarean section

No. 2 Dumont tweezers 0.3 × 0.13 tip (Agar Sci. or Electron Microscopy Sciences)

No. 5 Dumont 45° tip forceps (F.S.T., Fine Science Tools)

Ophthalmological straight scissors (e.g., McPherson-Vannas scissors (Asico)) or 105-mm Castroviejo corneal scissors (Katalyst Surgical)

Rounded-end micro-spatula

Stainless steel instrument sterilization container with lid (MedicalExpo)

15-mL tubes

Pasteur pipettes, sterile (glass or disposable plastic ones)

Glass coverslips and material for cleaning them

Carefully select coverslips one by one, discarding those with scratches or digs (which generate diffraction and stray light), and submit them to the cleaning procedure (to avoid dirt and/or fluorescent background):

Glass coverslips (e.g., 18-mm round No. 1.5 coverslips, Warner Instruments CS18-R CS-18R15)

Toxic gas fume hood

N_2 cylinder or central air/air compressor system with filter

Magnetic/hot stir plate and magnetic bar

Coverslip-staining jars with lid

Ceramic coverslip-staining rack (e.g., Thomas Scientific 8542E40)

12-well cell culture plates

Metal plastic-coated tweezers or plastic tongs

250-mL tall beaker

250-mL flat bottom wide beaker ("crystallization-type")

Plastic squirting bottle with methanol

Millipore-filtered, deionized, and distilled water

Ammonium hydroxide

Hydrogen peroxide

2.2 Plasmids and Transfection Material

As its name indicates, PALM localization super-resolution microscopy is based on the use of photoactivatable fluorescent proteins (see review in [46]). If this technique is to be employed for the study of synaptic proteins, a variety of probes are already available. Several commercial firms have a wide selection of such plasmids covering the visible excitation spectrum (e.g., Addgene, https://www.addgene.org/fluorescent-proteins/ containing 3,300 plasmids from Michael Davidson's collection, Florida State University; BioVision, http://www.biovision.com/) and specific target organelles, cytoskeletal proteins, or subcellular structures (Golgi apparatus, mitochondria, centrioles, actin, F-actin, centrosomes, lysosomes, etc.).

Lipid carrier transfection techniques applied to primary neuronal cell cultures have relatively low efficiency. On average, only 10–20 neurons can be identified in a coverslip. For most purposes, this is sufficient to image the relevant areas in super-resolution mode.

Viral vectors, and in particular lentivirus and adenovirus, yield higher efficiencies. Techniques for constructing, packing, and delivering adeno-associated viral vectors and lentivirus are outside the scope of these protocols. The reader is referred to Ref. [47] for a review on the application of these techniques in primary neuronal cultures with 85–90% efficiency.

Self-labeling of proteins using Halo tags, SNAP, dihydrofolate reductase (DHFR) targeting with trimethoprim conjugates, or CLIP tags is a recent alternative in super-resolution studies (e.g., [48]). A variety of fluorescent dyes can be used in single-molecule super-resolution microscopy utilizing the SNAP-tag labeling system, as successfully reported for live-cell STORM nanoscopy of histone H2B proteins using rhodamine and green and tetramethylrhodamine dyes [49]. Dy549 and CF640 have been recommended as the best choices for single-molecule tracking studies. The possibility of dual-color single-molecule imaging of SNAP-tagged fusion proteins is also analyzed in a recent study [50].

The conventional fluorescent protein heterologous expression requires a few components:

1. Plasmid expressing the protein of interest engineered in a construct with the photoconvertible fluorescent protein (e.g., BFP, CFP, GFP, EGFP, YFP, mCherry, mTurquoise2, tdEos,

tdTomato, mRuby, mEmerald, DRONPA, LifeAct, mTurquoise2)

2. Opti-MEM I reduced serum medium (Invitrogen)
3. Lipofectamine 2000 reagent (Invitrogen 11668) for transfection with the plasmid of choice

2.3 Extrinsic Fluorescent Probes

Appropriate extrinsic fluorescent probes and fluorescent-labeled antibodies for tagging the protein(s) of interest when using STORM/GSDIM techniques are also available from various sources. Small organic dyes have much higher photon outputs than fluorescent proteins (e.g., 6,000 photons emitted by a Cy5 dye in contrast to 490 photons detected from a mEos protein [51]). An important advancement in this topic was the appreciation that conventional organic fluorophores could be used for super-resolution imaging in cell biology [27, 30].

1. ATTO-TEC GmbH, Siegen, Germany (http://www.atto-tec.com/), has a collection of fluorescent probes covering the 350–750-nm excitation range and fluorescent-labeled lipids (DPPE, DOPE, PPE, DMPE) conjugated to ATTO dyes, which exhibit high quantum yield and photostability for localization super-resolution microscopy, some of them with a large Stokes shift, as well as "quenching labels," designed as energy transfer acceptors in experiments using Förster's resonance energy transfer (FRET). The ATTO dyes for the red region of the excitation spectrum are particularly advantageous in localization nanoscopy (e.g., ATTO-647N exhibits fluorescence emission intensity more than twice the strength of Cy5).
2. The ATTO dyes are also commercialized by various other sources (Sigma-Aldrich, Alomone Labs, Abberior, Invitrogen Life Sciences, Miltenyi Biotec).
3. Synaptic Systems (https://www.sysy.com/) has a large collection of purified antibodies against a wide variety of synaptic proteins (Ca^{2+} binding proteins, neurotransmitter transporters, neurotransmitter receptors, SNARE proteins, synaptic adhesion proteins, glial proteins, snRNPs, postsynaptic proteins like gephyrin, Homer, PSDs).
4. Alomone Labs (http://www.alomone.com/) specializes in fluorescent-labeled antibodies against a wide selection of synaptic proteins, in particular ion channels and neurotransmitter receptors/transporters, GPCRs, pumps, and proteins involved in Ca^{2+} homeostasis.
5. Secondary antibodies labeled with Alexa Fluor or ATTO fluorophores, from the same sources quoted in **steps 1–4** above. Miltenyi Biotec provides a custom service to derivatize antibodies with ATTO STAR dyes from Abberior.

2.4 Solutions for Fixation of Cultured Neurons

1. Phosphate buffer saline (PBS, Sörensen buffer), pH 7.3–7.4.
2. Fixative solution: 4% paraformaldehyde (PFA) (e.g., USB Corp. 19943) in PBS, pH 7.4, containing 4% sucrose.
3. Blocking solution: 10% normal serum containing 0.1% Triton X-100/PBS (normal serum from the host species of the secondary antibodies is recommended for blocking).
4. Incubation solution: 3% serum/0.1% Triton X-100/PBS (Triton may be omitted; normal serum from the host species of the secondary antibodies is recommended for blocking).

2.5 Mounting Media

2.5.1 Fixed Specimens

1. Polyvinyl alcohol (PVA) (MW 25,000) (9002-89-5, www.polysciences.com), a low-toxicity resin soluble in water at 96°C.
2. The commercial mounting medium VECTASHIELD (H-1000, Vectorlab) has been recommended for specimens labeled with Alexa Fluor*647* (Life Technologies), especially for 3-D imaging in STORM [52]. Olivier and coworkers also used a combination of VECTASHIELD and 95% glycerol-50 mM Tris buffer, pH 8.
3. The commercial mounting medium ProLong® Gold Antifade Mountant (Thermo Fisher Sci. P10144) is a curing medium with a refractive index of 1.47 which allows long-term storage of the sample. It is available with or without DAPI nuclear stain. It is not recommended for imaging fluorescent proteins, in which case ProLong® Diamond is suggested by the manufacturers. The use of the former mounting medium has been reported in STED intracellular imaging of pneumococcal autolysin LytA-infected cells [53], but to my knowledge it has not been used in imaging of the synapse.

2.5.2 Live Cells

There have been important developments on this front. Some were inherited from FRET know-how, some newly introduced to adapt to the demands of super-resolution microscopy, especially in relation to the control of photobleaching transitions, blinking frequency, and ultimately on–off transitions. In STORM-type nanoscopy, initial studies were performed with fluorescent probes capable of undergoing photoswitching transitions which arrested them in the off state. This was the case with cyanine dyes which can be induced to undergo transitions in the presence of a second fluorophore [8]. An important development in the field was the recognition that conventional fluorophores could also undergo photoswitching [27, 28, 30], leading to the development of a simplified and more universal single-molecule imaging procedure, direct STORM (dSTORM) [27]. This called for appropriate buffer systems [26]. There are excellent reviews on the use and pros and cons of different buffers and reducing–oxidizing additives [51]. In

all cases: filter through a 0.22-μm filter to reduce fluorescent contaminations. Some media can be stored at –20°C, but those containing oxidative/reducing enzyme are better prepared fresh. The pH of the STORM buffers is critical and should be carefully controlled.

Standard buffer I

120 mM NaCl

3 mM KCl

2 mM $CaCl_2$

2 mM $MgCl_2$

10 mM glucose (Sigma-Aldrich)

10 mM HEPES (Sigma-Aldrich)

Adjust pH to 7.35

This imaging buffer can be stored up to 1 month at 4°C.

STORM buffer I, an *oxygen*-scavenging buffer on the basis of a PBS buffer, pH 7.4, containing:

5% glucose

10 U mL^{-1} glucose oxidase

200 U mL^{-1} catalase

100 MEA

Buffer recommended for Alexa Fluor^{647}

Same buffer, containing:

5% glucose

4 U mL^{-1} glucose oxidase

80 U mL^{-1} catalase

100 mM glutathione

Oxygen-scavenging medium for improving dye stability in single-molecule experiments [54]

Protocatechuic acid (PCA) (Sigma-Aldrich).

Protocatechuate 3,4-dioxygenase (PCD) (Sigma-Aldrich).

Trolox (97%).

COT (98%).

NBA (99%) (Sigma-Aldrich).

Resuspend in DMSO and add to Tris-polymix buffer to a final concentration of 2 mM.

Simple PBS containing 10 mM β-mercaptoethylamine (MEA) adjusted to pH 7.4

This oxygen-scavenging buffer should be prepared fresh before use. Dissolve the MEA (cysteamine, Sigma-Aldrich 30070) in PBS and adjust to pH 7.4. MEA concentration can be varied between 10 and 100 mM.

3 Super-Resolution Microscopy Setup

1. Low-profile quick-change chamber (e.g., Warner Instruments model W4 64-0368 RC-41LP for 18-mm round coverslips); other models exist for 12-, 15-, and 25-mm round coverslips. ("Low-profile" design is intended to facilitate low entry angle for microelectrodes in electrophysiological recordings.)
2. Alternatively, plastic sample chambers having the size of a microscope slide with glass coverslip bottoms (Labtek, Nalge Nunc International Corp) can be used. They come in different sizes; we find the eight-chamber model (Labtek Prod. No. 155411) to be the most convenient. We prefer the quick chamber (alternative 1 above), because the flat chambers allow perfusion and rapid change of medium of the cells; they are apt for electrophysiological recording, are easier to keep at a given temperature, and can be conveniently cleaned and thus used indefinitely.
3. Vibration isolation system connected to a nitrogen gas tank or air compressor (we use the 780 Performance Series CleanTop Optical tabletop model 781-432-02R mounted on a Micro-G 4-Post Gimbal Piston isolator system with safety tie bars from TMC vibrational control).
4. Inverted fluorescence microscope (we use a Nikon TE-2000 stand with multiple optical bench/optical/laser additional components).
5. Objective lens(es). Minimally, one high-quality objective lens. TIRF plan-apochromatic oil immersion objective 60×/1.49 N.A. or 100×/1.49 N.A. will produce excellent results. Oil immersion objectives provide higher N.A., but water-immersion objectives, with lower N.A., reduce spherical aberrations resulting from refractive index mismatches of living cells in aqueous media.
6. Stage. Having tried various sophisticated (and particularly expensive) piezoelectric-driven stages as replacement for the original X,Y stage of the epifluorescence microscope, we settled for a purely mechanical, highly stable stage driven by very sturdy micrometer screws (Physik Instrumente GmbH, Karlsruhe, Germany, model MN-545.2MN). This is an important element for minimizing drift.

7. Epifluorescence light source (we use a LED system with four different excitation wavelengths, from the firm Tolket, Argentina) for initial wide-field microscopy. Of course a conventional Hg arc source suffices.
8. Activation laser. A 405-nm UV laser, or a powerful LED or Hg lamp source, for activation in case of PALM super-resolution microscopy (in our microscope setup, the laser excitation sources are via the TIRF port simultaneously with the LED or Hg arc excitation from wide-field illumination port).
9. A 532-nm DPSS laser (our main excitation source for red emission STORM super-resolution microscopy, 300 mW). Excitation source for various rhodamine-based fluorochromes, tetramethyl-rhodamine, Cy3, Alexa Fluor^{532}, Alexa Fluor^{546}, Alexa Fluor^{555}, Alexa Fluor^{568}, Texas Red, HcRed, Cherry and related fluorescent proteins, etc. There are various commercial sources for this type of DPSS laser: Coherent, RGB, and Quantum Laser.
10. A 561-nm diode-pumped solid-state (DPSS) laser (alternative excitation source for tdEos fluorescent protein, Ds Red fluorescent protein, Alexa Fluor^{568}, phycoerythrins, etc. Commercial sources of this laser: Cobolt Jive, RGB Laser, and GEM 561 from Quantum Laser).
11. A 660-nm DPSS laser. Excitation source for Cy5, Cy5.5, Alexa Fluor^{660}, DyLight^{649}, FluoSpheres Dark Red fluorescent microspheres, etc.
12. Filter cubes matching the wavelength characteristics of the above illumination sources.
13. Sensitive electron-multiplying charge-coupled-device (CCD) camera. We use the iXon + 897 EM-CCD camera (Andor Technology, Belfast, UK) and the QEM512SC Photometrics (Tucson, AZ) CCD camera. Both have the same chip with a pixel size of 16 μm and a high quantum efficiency (>90% peak QE).
14. In the last few years, the fast complementary metal-oxide semiconductor (sCMOS) camera sensors and ancillary technology (most importantly specific software development) have found a niche for super-resolution microscopy applications [55]. High-throughput applications requiring fast acquisition rates benefit from the use of these cameras.
15. For co-localization studies, a dual-viewer instrument (e.g., DV2 splitter, Photometrics) positioned on the emission side before the CCD camera enables one to simultaneously project the emission of two different channels onto the same CCD camera (see, e.g., [56]), thus enabling one to acquire two spatially identical but spectrally different images simultaneously. We

have a similar instrument built at the workshop of the Max Planck Institute for Biophysical Chemistry in Göttingen.

16. Computer. Super-resolution image acquisition and storage in the single-molecule localization modalities (PALM, STORM/GSDIM) is computer CPU and storage-space demanding. Acquiring 10,000–50,000 images at the highest CCD camera frame rates (few milliseconds/frame) is preferably done by streaming the image data directly to memory. It is thus best to have a fast CPU, at least 16 GB of storage memory, a fast and robust hard disk (2–4 TB), and a high-resolution monitor to focus the specimen and for image inspection and subsequent analysis.

4 Software

1. Camera control and image acquisition: In the case of the Andor cameras, the software packages Solis and the IQ CCD camera control software from Andor Technology are simple, convenient tools developed by the manufacturer. We control this camera with the image acquisition and hardware control software SlideBook (Intelligent Imaging Innovations, Boulder, CO) (www.intelligent-imaging.com).
2. In the case of the Photometrics QuantEM:512SC back-illuminated EMCCD camera, we also use SlideBook to drive the acquisition.
3. The open-source image processing package ImageJ (https://imagej.nih.gov/ij/), developed by Dr. Wayne S. Rasband at the National Institutes of Health, is a very convenient software package with several possibilities for analyzing super-resolution microscopy images.
4. One such possibility, developed specifically for PALM stochastic localization microscopy, is QuickPALM developed by Dr. Ricardo Henriques, currently at the MRC in University College London (https://code.google.com/p/quickpalm/).
5. MATLAB software (Image Processing Toolbox, MathWorks) is a very comprehensive and versatile software package which can be adapted to essentially all aspects of image analysis (localization, tracking, statistics, etc.)
6. The École Polytechnique Fedérale de Lausanne (EPFL) has compiled and maintains a catalog of available software for stochastic localization super-resolution microscopy and deconvolution. At the time of writing this protocol, there were 54 different software packages listed under http://bigwww.epfl.ch/smlm/software/.

7. The software package Localizer https://bitbucket.org/pdedecker/localizer, developed and regularly updated by Prof. Dr. Peter Dedecker and colleagues at the Department of Chemistry of the University of Leuven, Belgium, is a modular and very comprehensive software package for both static localization and single-particle tracking (SPT) running under Igor Pro (WaveMetrics, Inc.).
8. Google Inc. and Drs. Stefan Bollmann and Felix Rüdinger (Univ. Aachen and Biozentrum at the Departments of Biotechnology and Biophysics of the University of Würzburg, Germany) have developed rapidSTORM, a project containing a software package for the analysis of stochastic localization microscopy images and with capabilities for hardware control (http://www.super-resolution.biozentrum.uni-wuerzburg.de/research_topics/rapidstorm/).
9. Ovésny and coworkers from the Charles University in Prague, Czech Republic, have developed ThunderSTORM, a software package for PALM and STORM nanoscopies with Bayesian statistical analysis [57]. http://code.google.com/p/thunder-storm/.
10. For SPT analysis of molecular motions, a MATLAB-based single-particle tracking package analysis is freely available from http://physics.georgetown.edu/matlab/.

5 Methods

5.1 Cell Culture

5.1.1 Before Use of the Cell Culture Hood

1. If UV lamp is routinely used overnight, turn it off. Turn on hood lights and air filter.
2. Wear gloves. Clean them with 70% ethanol. Do the same with working surfaces, tube and pipette holders, and other materials to be introduced under the hood.

5.1.2 During Use of the Cell Culture Hood

1. Do not interpose hands or arms interrupting clean air flow on sterile material.
2. Avoid touching working surfaces with your hands or arms. If you inadvertently do so, clean surface with ethanol.
3. Follow safety rules and throw waste in appropriate container as you follow the procedures.
4. Return Petri dishes/flasks to the CO_2 incubator. Check gas and water levels and tightness of doors.

5.1.3 After Using the Cell Culture Hood

1. If you are using glass pipettes, immerse the used ones in the storage/wash cylinder, cotton-filled side up.
2. Turn off Bunsen or alcohol burner if you used one.

3. Wipe working surface of the hood/tube racks, etc., with 0.1% SDS followed by 70% ethanol.
4. Check the occupancy of waste material and dispose of container when 80% of its volume is full.
5. Turn off gas valve if you used a gas Bunsen burner.
6. Close hood.
7. Turn off air filter.
8. Turn on UV lamp.

6 Cleaning and Poly-L-Lysine Coating of the Coverslips for Neuronal Cell Primary Culture

Coverslips are a most important but often neglected component in light microscopy. One should bear in mind that they sit immediately adjacent to the main optical element of the light microscope – the objective lens – and as such they are not only the physical support for the adhered cells but also part of the imaging system itself. Two main considerations for optimally contributing to the image quality are (1) their physical and chemical composition (high-performance thin borosilicate glass) and (2) the uniformity of their thickness (e.g., 0.17 mm for No. 1.5 glass coverslips). If the coverslip departs too much from the optimal thickness of 170 nm, axial shift of the best focus may result. This affects spatial resolution (particularly in the *z*-axis) and reduces the fluorescence intensity. The rather painstaking cleaning procedure – not without risk – is worth the while for obtaining aberration-free, good-quality super-resolution images.

6.1 Simplified Procedure for Cleaning Glass Coverslips for Routine Experiments

1. Lay glass coverslips in a flat bottom crystallizer flask and cover them with a small volume of 1 N HCl. Leave overnight in acid.
2. On the next day, rinse abundantly with distilled water followed by 70% EtOH.
3. Coverslips may be stored in 95% EtOH and sterilized by passing them briefly over the flame of a Bunsen burner before coating them with poly-L-lysine.

6.2 Cleaning Coverslips for Critical Super-Resolution Microscopy and Poly-L-Lysine Coating

1. Turn on the air extraction of the chemical fume hood. Wear gloves, goggles, and lab coat. Avoid exposure of skin and most importantly eyes to the highly corrosive glass cleaning solution used in **steps 4** or **5**.
2. Put the coverslips in a ceramic staining rack, immerse into the glass coverslip-staining jars, and fill the jars with acetone. Incubate for 1 h, drain the acetone, and wash ten times with deionized water.

3. Prepare the etch solution by mixing 125 mL Millipore-filtered, distilled, and deionized water, 25 mL ammonium hydroxide, and 25 mL hydrogen peroxide in a 250-mL *tall* cylindrical beaker sitting on top of the stirring hot plate. Heat the mixture to 80°C. Place the staining rack with coverslips into the etch solution with metal plastic-coated tweezers or plastic tongs. Leave the coverslips overnight with gentle stirring to avoid spilling.
4. Lift the rack carefully with the metal plastic-coated tweezers or plastic tongs and transfer it into a flat bottom ("crystallizer-type") beaker filled with Millipore-filtered, deionized, and distilled water. Rinse the rack profusely, replacing the water several times.
5. An alternative to the etch solution is the "piranha" solution, requiring shorter incubation times: Put 60 mL of hydrogen peroxide solution (30% in water) and slowly add 100 mL of sulfuric acid (final ratio of acid to hydrogen peroxide solution is 5:3). The mixture will heat up.
6. When using the "piranha" solution, it is convenient to carry out the operation in glass coverslip-staining jars with lids. Immerse the ceramic coverslip rack with the coverslips into the jars, close the lids, and place the jar on the hot stirring plate at 80°C for about 1 h. Wash coverslips as in **step 4**.
7. Neutralize residual acid remaining on the coverslips after "piranha" treatment by filling the coverslip-staining jars with 0.1 M KOH, incubate 10 min, and wash ten times with Millipore-filtered, deionized, and distilled water.
8. Carefully hold and remove the cleaned coverslips from the rack with plastic forceps and rinse both sides by squirting methanol from a squeeze plastic bottle.
9. Blow excess methanol by gently blowing N_2 or filtered air from a plastic pipette tip connected via rubber tubing to either a N_2 cylinder or a central air/air compressor system with filter, respectively.
10. A pipette tip can be connected with tubing to the air supply and used as an aid in blowing away the methanol.
11. Connect the burner to the butane (or natural gas) and light. Pass the coverslip quickly through the topmost portion of the flame, and repeat this process a couple of times for a few seconds passage under the flame to avoid cracking.
12. An even shorter cleaning procedure is to use a plasma cleaner set at 30 W for 15 min. Pressure inside the cleaning chamber is set at 100–200 mTorr. Both atmospheric and compressed oxygen can be used.

13. Store the cleaned coverslips preferably in vertical position in a staining rack kept in a clean vessel with lid until use.
14. Move the necessary amount of racks to suit your experiments to the cell culture hood. As an additional precaution, if coverslips have been stored for a prolonged period, dip them in spectroscopic-grade methanol, flame briefly to dry, let cool for a few seconds, and then place them in Petri dishes or 6-well culture plates.
15. Immediately before use, prepare the poly-L-lysine (e.g., Sigma P7405) solution at a final concentration of 10 mg/mL in 0.1 M borate buffer, pH 8.5, and sterilize by filtration. Add a few drops of this solution spreading with a sterile tip or swirling the plate to cover the surface of the coverslip. Incubate for 1 h at 37°C in the cell culture incubator or leave them covered in the hood with the air blower off at room temperature for 12–24 h.
16. Wash coverslips thrice with sterile water, for a total of 3 h. Leave the coverslips in plating medium in the incubator until ready to plate cells.
17. Remove the water and add neuronal plating medium. Place the coverslips in the incubator to reach temperature.

7 The Hippocampal Embryonic Neuronal Cell Culture

The hippocampal neuronal cell cultures constitute a useful in vitro model system to study physiological and structurally relevant issues in neurobiology, from neuronal development, the birth of the axon – the "symmetry-breaking event" [58] in the establishment of neuronal polarity, synaptogenesis, spinogenesis, localization, and trafficking of synaptic proteins (the purpose of these protocols) and organelles – to changes in dendritic spine morphology associated with long-term potentiation, purportedly linked to learning, memory, and several other physiologically relevant processes. The procedure outlined in these protocols is circumscribed to the culture of neuronal cells obtained from embryonic day 18 (E18) rat hippocampi, the stage at which pyramidal neurons have already developed, following a procedure involving caesarean section of the parental rodent, extraction and sacrifice of the pups, enzymic-mechanical dissociation of the nervous tissue, and establishment of the neuronal cell only or mixed neuronal–glial cell cultures in a Petri dish. The original procedure developed by Gary Banker and colleagues [59] and its subsequent improvements (see, e.g., [45]) have been field-tested over the course of the last few decades and by now are well established, having generated abundant literature on the subject, thus facilitating comparisons

between laboratories. The procedure is essentially the same for cerebral cortex neuronal cell cultures, except that the cortex is much easier and faster to dissect. In this sense, rat hippocampi are easier to dissect than mouse hippocampi, but this difficulty is counterbalanced by the fact that mice open the door to studying knockout animals. There is abundant literature raising relevant questions on various neurobiological topics addressed to the neuronal cell model using embryonic stage 18 (E18) rodent pups. There are also procedures for the cell culture of adult brain tissue and for cells cultured on other simple substrates (plastic, meshes, with or without special coatings) or even patterned (in the form of microdots, meshes, channels) substrates. Here I describe a simple procedure to establish high-density hippocampal primary neuronal cell cultures on poly-L-lysine-coated glass coverslips. The reader is encouraged to read the classical original work [59] and/or its well-illustrated updated protocol [45]. Comprehensive protocols on this and related subjects have appeared more recently, e.g., Cold Spring Harbor Protocols (http://cshprotocols.cshlp.org/) (see also [60–62]), especially for implementing the hybrid Banker's sandwich cultures of low-density neuronal cells growing on a feeder layer of astroglial cells in an adjacent coverslip, providing nutrients and growth factors that diffuse through the medium to reach the neuronal layer. The sandwich cultures provide the best results for super-resolution imaging because the low-density neuronal cells and especially their neurites, lie flat on a single plane with very low contamination from glial cells: however, these cultures are more involved, requiring the preparation of glial feed cultures 2 weeks in advance. Also, the preparation of the coverslips is lengthier and more elaborate.

8 Dissection of Brain Tissue for Primary Embryonic Neuronal Cell Culture

1. Prepare all necessary materials, including stereomicroscope, sterile surgical instrumentation, media, 70% ethanol in plastic squirting bottle, ice, etc., on clean labtop area for the first part of the procedure and under the laminar flow hood for the second half.
2. Place a 60-mm Petri dish on the stereomicroscope stage and fill up to 3/4 of its volume with ice-cold medium for dissecting embryos' heads (CMF-HBSS or HBSS+ medium with antibiotic).
3. Anesthetize an E18-period pregnant rodent with CO_2 and euthanize following the protocol authorized in your research establishment (e.g., head dislocation).
4. Perform caesarean section by opening the abdominal wall with sharp-end scissors, with an incision from anus to diaphragm

region. Remove embryos and place in a 100-mm glass dish filled with ice-cold CMF-HBSS. The remainder of the operation should be conducted in a sterile environment, ideally in a laminar flow hood. For this purpose, transfer the Petri dish containing embryos to the hood.

5. The second half of the procedure is performed under sterile conditions. Wash embryos in sterile cold dissection medium and transfer to clean glass Petri dish. Decapitate embryos by cutting the head behind the ears, and proceed to remove their brains. To do this, hold the head of an embryo with forceps at the level of the brainstem, and cut the skin and skull through the midline with fine scissors under the stereomicroscope. Lift the skin and skull to expose the brain. Transfer the brain into a 60-mm dish containing ice-cold dissection medium or CMF-HBSS using a micro-spatula. Using eye-surgical (iridectomy) fine spring scissors, proceed to separate hemispheres. The hippocampus can be recognized on the medial internal surface by its characteristic hippocampal fissure, with blood vessels marking the separation with adjacent entorhinal cortex.
6. Dissect meninges and adherent choroid plexus to expose the hippocampus using fine shanks forceps. Flip the brain upside down such that the hypothalamus is facing up. Dissect the hippocampus from adjoining entorhinal cortex, cutting first parallel to the hippocampal fissure and secondly using transverse cuts at its rostral and caudal ends using the iridectomy fine spring scissors while holding the rest of the tissue with fine Dumont forceps [59]. Always maintain the dissected hippocampi under cold medium.

9 Enzymic Digestion and Mechanical Dissociation of Hippocampal Embryonic Tissue

9.1 According to MacGillavry and Blanpied [63]

1. Chop embryonic day 18 (E18) hippocampal tissue into pieces smaller than the opening of a 200-μL pipette tip, and pipette the small pieces of hippocampal tissue into a 15-mL sterile plastic tube containing 4 mL ice-cold HBSS dissociation medium.
2. Rinse tissue with 10 mL ice-cold dissection medium.
3. Centrifuge tissue for 30 s at 120 × *g*, at RT, and remove supernatant with a Pasteur pipette.
4. Resuspend hippocampal tissue in 4 mL of trypsin/EDTA solution (prewarmed to 37°C) and digest hippocampal tissue 5–8 min at 37°C, slowly agitating from time to time. During incubation, dissolve 3 mg DNase in 3 mL trypsin/EDTA solution.

5. Centrifuge cells for 30 s at 120 × *g*, at RT, and remove supernatant with a Pasteur pipette.
6. Incubate with filter-sterilized, prewarmed trypsin inhibitor solution for 3–5 min at 37°C.
7. Centrifuge for 1 min at 120 × *g* at room temperature, and aspirate supernatant with a Pasteur pipette. If cells are still stringy with DNA and the supernatant cannot be removed, add a more concentrated, filter-sterilized solution of DNase (e.g., 2 mg in 500 μL for 4 mL tissue), and incubate until supernatant can be removed (1–2 min).
8. Gently resuspend tissue on ice in 2 mL dissection medium, avoiding air bubbles. First, use a fire-polished glass Pasteur pipette, and then use a fire-polished Pasteur pipette with a smaller tip diameter. Finally, use a third pipette with an even smaller diameter.

9.2 According to Kaech and Banker [45]

1. When all of the hippocampi have been removed, place them in a 15-mL conical centrifuge tube and bring the volume to 4.5 mL with CMF-HBSS. Add 0.5 mL of 2.5% trypsin and incubate for 15 min in a water bath at 37°C.
2. Gently remove trypsin solution, leaving the hippocampi at the bottom of the tube; add 5 mL of CMF-HBSS and let stand for 5 min at room temperature. Repeat this twice to allow residual trypsin to diffuse from the tissue. Bring the final volume to 2–3 mL.
3. Dissociate the hippocampi by repeatedly pipetting them up and down in a Pasteur pipette. Begin with five to ten passes through a regular pipette and then continue with five to ten passes through a Pasteur pipette that has been flame-polished so that its tip diameter is narrowed by half. Expel the suspension forcefully against the wall of the tube to minimize foaming. By the end, there should be no chunks of tissue left. The diameter of the flame-polished pipette is important. A too narrow tip will damage the cells; a too wide tip will not fully dissociate the tissue. Pipette only as many times as is necessary to obtain a homogeneous solution with no tissue pieces.
4. Determine the cell density by adding a drop of the cell suspension to a hemocytometer. Also determine the total yield, which should be 400,000–500,000 cells per hippocampus. The viability, according to exclusion of trypan blue, should be 85–90%. Adjust the cell density to about 1–1.5 million cells/mL.
5. Using a micropipette, add the desired number of cells to each of the dishes containing the poly-L-lysine-treated coverslips in neuronal plating medium.

6. After 3–4 h, examine the dishes to ensure that most of the cells have attached and then establish the mixed neuron–glial hybrid cultures as described under procedure 12 below.

10 Preparation of Astroglial Feeder Cells [45]

1. Kill three or four postnatal rat pups following the procedure described above.
2. Remove the brains and place them in a dish containing CMF-HBSS. The tissue needs to remain submerged at all times.
3. Remove the cerebral hemispheres and carefully strip away the meninges with fine forceps. Make sure to remove meningeal tissue penetrating deep in cerebral fissures, which contains fibroblasts that can overgrow astroglial cultures.
4. Transfer the hemispheres to a drop of CMF-HBSS and chop the tissue as finely as possible with scissors.
5. Transfer the tissue pieces to a 50-mL conical centrifuge tube in a final volume of 12 mL CMF-HBSS and add 1.5 mL each of 2.5% trypsin and 1% (wt/vol) DNase. Incubate in a 37°C water bath for 5 min, swirling the tube occasionally.
6. Triturate by passing the solution up and down 10–15 times in a 10-mL pipette. Return to the water bath for another 10 min, swirling occasionally. Triturate another 10–15 times with a 5-mL pipette until most chunks disappear.
7. Pass the cell suspension through a cell strainer to remove chunks of undissociated tissue and collect in a 50-mL conical tube containing 15 mL glial medium.
8. Centrifuge the cells for 5–10 min at $120 \times g$ to remove enzymes and lysed cells. Discard the supernatant and resuspend the cell pellet in 15–20 mL glial medium. Count cells using a hemocytometer – the yield should be in the order of 10^7 cells per brain.
9. Plate 7.5×10^6 cells per 75-mL flask and add glial medium to a final volume of 15–20 mL.
10. After 1 day, feed cultures with glial medium. First, swirl the flask to remove loosely attached cells, then aspirate off the medium, and replace with fresh medium.
11. Feed the culture every 2–3 days. Before removing the medium, slap the flask 5–10 times against your hand to dislodge loosely attached cells.
12. Contaminating cells such as O2A progenitors and microglia (macrophages) sometimes grow on the surface of the astrocyte monolayer. Removing loosely attached cells at this step helps to

minimize their number. Microglia (recognizable as round, phase-bright, loosely attached cells) release cytokines that are highly neurotoxic. Preparations with high numbers of microglia should be discarded.

13. Harvest the astroglia when the cells are near confluence (usually within 7–10 days). Rinse each flask briefly with 3–5 mL of trypsin/EDTA, then add 2–3 mL of trypsin/EDTA, and incubate at 37°C until the first cells just begin to lift off (usually in less than 2 min). Add 5 mL glial medium to stop the trypsin enzymic digestion, release cells by repetitive pipetting, transfer the cell suspension to a conical centrifuge tube, and pellet the cells at 120 × *g* for 7 min. Resuspend in glial medium and passage into 60-mm dishes (10–30 dishes per flask).
14. Extra cells can be resuspended in cell-freezing medium at ~2×10^6/mL, frozen in cryotubes and stored in liquid nitrogen, and then thawed and plated as needed.
15. One to two days before seeding the neuronal cells, remove the medium from the glial feeder cultures and add 6 mL neuronal maintenance medium, either N2 or Neurobasal/B27, for preconditioning.

11 Counting the Number of Neuronal Cells

1. Remove a 10 μL sample of cells and dilute into 90 μL of 0.4% trypan blue stain. Mix.
2. Take a 10 μL aliquot of the cell suspension and pipette into a hemocytometer, determining the number of cells per unit volume.

12 Plating the Cells

12.1 Neuronal Cell-Only Cultures

1. Plate 50,000–70,000 cells/mL of the initial plating medium in each well (or ~150,000 cells/well for low-density cultures suitable for imaging). Return multiwell to the incubator and let the cells plate for 2 h at 37°C in *plating medium*.
2. After 2 h, aspirate the medium and replace with *medium for culturing hippocampal neurons* (Neurobasal medium containing B27 serum-free supplement) and incubate cells at 37°C.
3. Aspirate half the volume of medium twice a week, replacing the volume with fresh medium. Some researchers do not carry out this step, maintaining the original medium.

12.2 Neuronal–Glial Cell Hybrid Sandwich Cultures

1. As stated in procedure 10 above, 1–2 days before establishing the hybrid culture, the medium from the glial feeder culture should be removed and replaced by neuronal maintenance medium, either N2 or Neurobasal/B27 for preconditioning.
2. Follow the same steps as in Sect. 12.1 above, except that glial cell feeder coverslips with spacers are transferred to the plastic multiwell (or Petri dish) containing the neuronal culture and placed upside down on their spacer feet. Make sure the upper glial coverslips are submersed in the N2 or Neurobasal/B27 medium.
3. Three days after plating, add cytosine arabinoside (1-b-D-arabinofuranosylcytosine) to a final concentration of 5 mM to inhibit glial proliferation.

13 Transfection of Dissociated Hippocampal Cultured Neurons (Modified from Ref. [63])

The predominant type of cell in the hippocampus is the pyramidal neuron. Interneurons are fewer in number and can be distinguished on a morphological basis from the pyramidal cells. Of further aid in the distinction between different neuronal subtypes are a few specific markers for some of the interneurons.

1. Transfect neuronal cell cultures 1–3 days before imaging using Lipofectamine 2000. This reagent has a low transfection efficiency (<1%), but the apparent drawback may prove convenient for very dense cultures.
2. Dilute 0.5–2.0 μg plasmid DNA in 25 μL of serum-free medium or Opti-MEM I reduced serum medium.
3. Dilute 1.0 μL Lipofectamine 2000 in 25 μL serum-free medium or Opti-MEM I reduced serum medium. Allow the tubes to stand for 5 min at room temperature.
4. Combine the contents of the two tubes containing the suspensions (Lipofectamine and DNA), mix them vigorously by pipetting with a micropipette, and leave them for 20–30 min at room temperature to allow the DNA–Lipofectamine complexes to form.
5. Retrieve the multiwell containing the coverslips with the cells from the incubator, and add the DNA–Lipofectamine suspension dropwise (about 50 μL per well). Mix by gently swirling the plate by hand or sitting it on a rotary shaker. Return the multiwell plate to the incubator and let it stand for 90–120 min.
6. Retrieve the multiwell from the incubator, remove coverslips, and place them in a new multiwell plate. If a mixed coculture with glial cells is used [62], place the coverslips with the cultured neuronal cells facing the glial monolayer below

them. Return to the incubator and retrieve for microscopy 24–48 h later.

7. The use of viral vectors and of adenovirus and lentivirus in particular is very advantageous for introducing foreign constructs in neuronal cells in culture. The reader is referred to recent protocols for the preparation of the virus vectors [47] and transfection of neuronal cells [64].
8. Similarly the "self-labeling techniques" (Halo tag, SNAP tags, etc.) mentioned under Materials are gaining momentum and could find wider application in the labeling of synaptic proteins.

14 Fixation and Immunostaining

Fixatives are carcinogenic. Wear gloves and other appropriate protective equipment and avoid contact with skin or eyes.

Strong and/or prolonged fixation is detrimental for fluorescence microscopy. Inclusion of glutaraldehyde in a cocktail with paraformaldehyde, as is customary in electron microscopy, is particularly critical; glutaraldehyde concentrations >1% practically abolish emission from fluorescent proteins.

Fix cells in 4% (wt/vol) paraformaldehyde (electron microscopy grade is recommended, e.g., from Electron Microscopy Sciences) fixative solution following the protocol below.

14.1 Method A

1. Wash coverslips twice with 0.1 M (Sörensen's) phosphate buffer saline (PBS, Invitrogen), pH 7.3–7.4.
2. Fix cells with 3 mL/well paraformaldehyde solution for 15–20 min at RT or 4°C (on ice).
3. Wash three times with PBS for 10–20 min.
4. Incubate for 30 min with blocking solution (4% goat normal serum, NGS, Jackson ImmunoResearch Laboratories, West Grove).
5. Put a piece of Parafilm on a wet Whatman paper inside a Petri dish and apply 200 μL of primary antibody solution in incubation solution (appropriate dilution must be determined experimentally). Lay coverslips upside down on antibody-containing solution and incubate for 1–2 h at RT.
6. Transfer coverslips to multiwell plate and wash three times with PBS for 10 min.
7. Repeat **steps 4–7** with secondary antibody (optimal dilution must be determined experimentally).

14.2 Method B. Fixation-Permeabilization for Immunostaining Intracellular Proteins

1. Fix cells as in **steps 1–3** above.
2. Incubate for 30 min with blocking solution (4% goat normal serum) containing 0.1% Triton X-100 (Sigma) in PBS for 15 min.
3. Wash with PBS for 15 min.

14.3 Method C

1. Fix cells with 3 mL/well paraformaldehyde solution at RT.
2. Immediately transfer to freezer and leave at −20°C for 5 min.
3. Without washing, transfer coverslip to spectroscopic-grade methanol at −20°C and leave for 15 min in the freezer at this temperature.
4. Wash thrice with PBS.
5. Permeabilize with 0.2% Triton X-100 solution in PBS for 10 min.
6. Block in 10% goat serum in PBS for 1 h.
7. Dilute primary antibody in blocking solution. Incubate sample with primary antibody at final concentrations which vary according to antibody (e.g., 1:200 for anti-glutamate receptor antibody, 1:1.000 for myc, etc.). The optimal dilution should be found by the combination of manufacturer's recommendation + one's own experience.
8. Wash thrice with PBS.
9. Incubate with fluorescent-labeled secondary antibody for 45 min–1 h at room temperature. Dilutions also vary (e.g., 1:500 for Alexa Fluor^{488}, 1:1,000 for Alexa Fluor^{647}).
10. Wash thrice with PBS buffer and mount for microscopy as described in Sect. 16.
11. In the case of fluorescent proteins, if the neuronal specimens are to be imaged after fixation, bear in mind that neurons transfected with Venus-FP or Tomato-FP withstand fixation better than GFP or EGFP.
12. It is advisable to run a "control" experiment using a marker of the glutamatergic nerve terminals (e.g., vesicular glutamate transporter 1, VGLUT1) and a dendritic marker (e.g., microtubule-associated protein 2 [MAP2]) to ensure the quality of the specimen and the entire fixation/immunostaining procedure.
13. Incubate coverslips with a polyclonal, guinea-pig anti-VGLUT1 antibody (e.g., AB5905, Chemicon-Millipore, Billerica, MA) (1:2,000 dilution) and a polyclonal, rabbit anti-MAP2 antibody (AB5622, Chemicon-Millipore) (1:2,000 dilution) in PBS with 4% NGS (blocking solution) for 3 h.
14. Wash with PBS for 15 min.

15. Incubate coverslips with goat anti-guinea-pig IgG (H + L) antibody conjugated with, e.g., Alexa Fluor^{594} (A-11076, Invitrogen) (1:1,000 dilution in blocking solution) and goat anti-rabbit IgG (H + L) antibody conjugated with Alexa Fluor^{488} (1:1,000 dilution) for 60 min.
16. Rinse thrice with PBS for a total of 30 min.

14.4 Staining Specific Synaptic Proteins and Counterstaining Whole Neurons, Axonal, and Dendritic Arborizations

1. A variety of presynaptic protein markers is available: anti-vGlut1 (Antibodies Inc., NeuroMab) labels glutamatergic terminals and antibodies against Vamp, synapsin 1, synaptotagmin, synaptophysin (Abcam), synapsin (Synaptic Systems), synaptopodin, Bassoon, Munc18, etc.
2. Similarly, various antibodies against postsynaptic proteins produce very strong signals, like anti-PSD-95 (Synaptic Systems, NeuroMab), anti-extracellular epitopes of various subunits of the NMDA receptor (Alomone Labs), GluN1 and GluNR2 obligatory pore-forming subunits (Chemicon mAb397), nicotinic acetylcholine receptor subunits (Alomone Labs), etc.
3. Various synaptic proteins can be visualized using fluorescent peptides (e.g., for actin, F-actin-binding peptides, F-tractin from Clontech, phalloidin conjugated to various fluorophores).
4. In addition to specific neuronal protein markers, it is convenient to counterstain with antibodies against MAP-2 (a neuronal dendritic marker, Novus Biological) and the tau protein to identify each type of neurite.
5. To differentiate between inhibitory and excitatory synapses, one can employ antibodies against vGAT (presynaptic)/gephyrin (postsynaptic) for inhibitory synapses, occurring predominantly on dendritic shafts, and vGlut1 (pre)/PSD-95 (postsynaptic) for excitatory synapses, mostly present on dendritic spine heads.
6. For quantifying dendritic spines, the developmental protein drebrin can be used as a general marker (1:500 anti-drebrin from Med. Biol. Labs followed by a 1:500 dilution of a fluorescent anti-mouse IgG antibody).

15 Excitation Modalities in Super-Resolution Microscopy

In order to efficiently detect single molecules in the focal imaging plane, one must suppress fluorescence emission from those emitters that are out of plane. The illumination modality plays a key role in accomplishing this task. The position at which the laser beam enters the back aperture of the objective lens determines the angle at which the light will excite the sample. In conventional wide-field

epifluorescence microscopy, the laser beam is symmetrically centered and parallel to the *z*-axis of the objective lens (and the microscope); the resulting illuminated field is an inverted cone-shaped volume of the cell above and below the focus. Displacement of the laser beam off the central axis of the objective lens will result in oblique illumination, a condition which reduces the volume of the cone of light exciting fluorophores and scattering in the cell above the focus. If the incident excitation laser beam is positioned at an angle larger than the critical angle $\alpha = 61°$, where $\sin(\alpha) = \eta_w/\eta_g$, the ratio of the refractive indices of water (the medium in which the cells are embedded and that of the cells proper) and immersion oil/cover glass (the two media that the light must transverse before hitting the cell), respectively, we meet the condition of another wide-field technique, total internal reflection fluorescence (TIRF) microscopy (see, e.g., [65]). In this mode of illumination, an evanescent electromagnetic field is used to selectively illuminate and excite fluorophores in a restricted volume of the specimen, immediately adjacent to the glass–water interface. The incident light is reflected back into the high refractive index medium at the interface, whereas a non-propagating evanescent wave is used to excite the fluorophores.

The good and the bad thing about TIRF illumination is that the evanescent wave, decaying exponentially from the interface, penetrates to depths between 80 and 200 nm (depending on the incident angle) into the low-refractive-index aqueous medium containing the specimen. Thus only fluorescent molecules in a narrow disk of about 100-nm height are excited. When using coverslip-adhered cells and epi-illumination in an inverted fluorescence microscope, this is undoubtedly the best choice for interrogating fluorescent molecules in the ventral, glass-attached cell membrane, rendering an improvement in contrast of about fourfold over conventional wide-field illumination, and considerably reducing the background fluorescence. In the case of primary cultures of neuronal cells, TIRF microscopy is best for imaging molecules in the ventral surface of the soma, the growth cones at early culture stages (D2–D5), and fully developed dendritic spines at culture stages D15–D25. TIRF illumination in the form of a standing wave can further produce a marked improvement in spatial resolution [66, 67]. The most dramatic improvement in axial resolution has been recently reported by George Patterson and colleagues at the NIH: TIRF illumination at varying penetration depths, produced by tuning the angle of the TIRF beam in combination with photobleaching. The latter preferentially affected the fluorescence from the shallow layers, allowing observation of deeper layers. Images constructed by sequential imaging and photobleaching the fluorescent molecules rendered, upon reconstruction, an axial resolution of ~20 nm [68]. Although primarily intended for illumination of

cell-surface components, some illumination modalities such as the so-called "highly inclined and laminated optical" (HILO) sheet microscopy [69] or "selective-plane illumination" microscopy (SPIM) [70] have also been specifically developed for single-molecule imaging deeper inside cells, achieving improved signal-to-noise ratio. HILO illumination is also at the root of the so-called uPAINT super-resolution technique, which makes use of oblique illumination from a slightly below the critical angle laser beam to reject background fluorescence of unbound fluorescent ligands. These techniques are ideal for studying plasma membrane-resident proteins, cell-surface neurotransmitter receptors being ideal targets among synaptic proteins. The uPAINT technique is particularly attractive for dynamic measurements, such as following the trajectories of ligands bound to surface proteins, degree of confinement, and organization of the target proteins in clusters [40, 41].

16 Preparation of the Samples for Imaging

Super-resolution microscopy shares with conventional microscopy many of the requirements for identifying structures illuminated with visible light and interrogated with fluorescence. The first common requisite with wide-field microscopy is to make objects visible. This can be accomplished by using extrinsic fluorescent probes that tag proteins or subcellular structures or by constructing chimeras of, e.g., the protein of interest with a foreign fluorescent protein, introducing the construct into the cell via the resulting plasmid, and letting the cell express the fluorescent product. The technique for labeling proteins, lipids, and other cellular chemical components have been worked out in great detail, and the use of fluorescent proteins in fluorescence microscopy, a younger field, has also reached a stage of maturity. In addition to these and other aspects common with conventional light microscopy, super-resolution techniques have demanded new developments in sample preparation, coverslip handling, protein tagging, embedding media, oxygen-scavenging requirements, mounting of the specimens, etc. Sample preparation is an important step for successful nanoscopy.

The commonly used cyanine and Alexa Fluor dyes are known to undergo dark state transitions over a broad range of time scales. Fluorophore dark states have been attributed to *cis–trans* isomerization within the conjugated polyene cores, the buildup of triplet states, charge-relay mechanisms, photo-ionization, photooxidation, and the absorbance of short-wavelength photons from the first excited singlet state [71]. Several procedures have been

developed to curb this phenomenon, including the use of oxygen-scavenging imaging buffers containing oxygen-scavenging enzymes for wet live specimens or dry coatings for fixed specimens. Other requirements in some super-resolution experiments arise from the need to match the high numerical aperture of the immersion lenses with the aqueous mounting media, calling for nontoxic embedding media capable of suppressing the refractive index mismatch. One such mounting medium is based on the use of 2,2′-thiodiethanol, which, by being miscible with water at any ratio, allows fine adjustment of the average refractive index of the sample from that of water (1.33) to that of immersion oil (1.52). The thiol compound thus enables super-resolution imaging deep inside fixed specimens with objective lenses of the highest available aperture angles [72].

16.1 Fixed Specimens

1. Coat the coverslip with the fixed neuronal cells with a few microliter (this will depend on the diameter of the coverslip) of a 1% aqueous solution of polyvinyl alcohol in Millipore-filtered distilled water (25,000 MW PVA, Sigma-Aldrich 184632; Polysciences 02975-00).
2. Spin the coverslip in a spin coater (e.g., SCS G3 spin coater, Kisco; spin coater model WS-400 from Laurell Technologies).
3. A simple procedure developed in our laboratory replaces a commercial spin coater with a tabletop centrifuge. Remove the rotor from the tabletop centrifuge. Place a strip of Parafilm on the shaft of the tabletop centrifuge and adhere firmly. Place the round coverslip with the wet side up on the Parafilm, adhere gently but firmly, close the lid of the centrifuge, and spin for 30 s–1 min. The PVA film will form a uniform dry thin film. Let dry for about five additional minutes before imaging. The thin PVA film suffices to prevent oxygen quenching to the biological sample. Dried samples in the dark have been successfully imaged after several months of storage in the dark.
4. The commercial mounting medium VECTASHEILD has been recommended for STORM super-resolution imaging with Alexa Fluor dyes, with optimal results with Alexa Fluor^{647} [52]. The authors state that this medium yields images comparable or superior to those obtained with more complex buffers, especially for 3-D super-resolution imaging. VECTASHIELD has a refraction index of ca. 1.45 measured at 590 nm. A mixture of 25% VECTASHIELD–75% Tris-glycerol buffer was also suggested by these authors.

16.2 Live Specimens

1. Wash the coverslip containing the neuronal cells with extracellular imaging buffer and place in coverslip chamber containing imaging buffer to cover the cells (a few microliter, depending

on coverslip diameter and model of chamber but sufficient to keep the specimen wet even after a long imaging session).

2. If available, maintain the temperature of the specimen relatively constant. One of our setups combines the super-resolution microscope with a Peltier-element thermostated holder for temperature-controlled patch-clamp recordings (Luigs & Neumann, Ratingen, Germany).

17 Single-Molecule Stochastic Localization Microscopy

Since the publication of the first step-by-step protocols on stochastic localization microscopy only a few years ago [73], several methodological advances have occurred. Among them are the explosion in the number of available fluorescent proteins for PALM microscopy and the recognition that most fluorophores undergo off–on dark–light transitions, thus enormously broadening the possible probes that can be used in nanoscopy. In this section, a formula-less practical protocol is provided for the implementation of stochastic localization super-resolution microscopy of synaptic components in the PALM or STORM/GSDIM modalities. More detailed protocols can be found in the literature (e.g., [63, 74]).

17.1 Image Acquisition

1. Set the image acquisition conditions to be used in the actual experiment using a standard "sea of fluorescence" specimen and set the incidence angle of the TIRF beam for oblique illumination of the sample.
2. Knowledge of the number of photons detected by the camera after background subtraction is necessary to calibrate both the latter and the precision with which a single molecule is localized. Calibrate camera photoelectron conversion – the number of photons per image pixel intensity units – a parameter that is required to calculate the number of photons emitted per molecule and thus estimate the localization precision of the recorded single molecules. Calibration can be obtained as follows: (1) record images under uniform Koehler illumination at several different intensities, covering the dynamic linear range of the camera, at a given CCD camera gain setting; (2) calculate the mean pixel value and variance of the uniformly illuminated region at each intensity; (3) plot variance as a function of mean pixel value; and (4) the slope of the variance as a function of mean pixel value is a good approximation of the ratio of recorded pixel value per detected photon [75].
3. Switch to the actual specimen and turn on transmitted light, coarse focusing the specimen. At this stage, one may take a snapshot image in a contrast-enhancing mode (phase contrast,

DIC interference contrast, Hoffmann contrast microscopy) to record the fine overall morphology of the cells.

4. Switch to fluorescence epi-illumination, turning on a standard light source (Hg arc, LED). Use the minimal excitation intensity compatible with localizing a region of interest (ROI) through the eyepiece or preferably use the CCD camera in your imaging software to carry out the procedure digitally using the focusing mode at a reasonably high camera gain.
5. In the case of PALM localization microscopy, carry out these preliminary steps imaging the unconverted state of the fluorescent protein using the appropriate filter combination. As MacGillavry and Blanpied [63] warn, one should avoid long exposures, since photoactivatable fluorescent proteins are already activated and partially depleted at wavelengths used for wide-field epifluorescence visualization. For visualizing unconverted green Eos, a standard EGFP filter set is recommended. For photoactivation and imaging of converted red Eos, use a filter cube containing a dual-band excitation filter (405 and 561 nm), a dual-band dichroic to reflect these laser lines, and a red-pass emission filter [76].
6. Set out all acquisition parameters (camera gain, frame rate) to maximize efficient performance and check microscope hardware conditions (filter combinations, projection lens resulting in adequate camera pixel size, etc.) and switch to camera acquisition mode. Select a region of interest (ROI), minimizing the void areas outside the cell(s), thus maximizing acquisition rates and, correspondingly, minimizing off-line analysis time. The actual pixel size of our Andor iXon-897 or Photometrics CCD cameras is 16 μm. We use the 1.5× projection lens of the Nikon TE-2000 to obtain a 160-nm pixel size in the image.
7. Switch off LED or Hg lamp and turn on laser(s). After the initial burst of fluorescence, individual blinking events will appear stochastically, and their number should be modified by varying the intensities of the excitation and activation lasers. We do this manually with precision potentiometers to reach a number of blinking events which are adequate for off-line analysis. Since the acquisition rate is about 10 ms/frame, and the refreshing rate of the visualization mode is much slower than this, it is a matter of experience acquired by trial and error to set the relative intensities of the two lasers (essential for PALM imaging) or the single laser (in STORM/GSDIM mode) to acquire the series of several thousand individual frames that will make the "movie." In the SlideBook software package, we set the "streaming mode" to record the individual images and store them directly in memory. A standard "movie" consists of a stack of 5,000–30,000 individual frames

depending on the size of the chosen ROI and the brightness and stability of the specimen.

8. In SlideBook, the compound image is recorded as a *.sld file, which can be read directly by ImageJ Fiji or converted and exported as a TIF or MATLAB file for subsequent image analysis.

17.2 Image Analysis

At the time of writing this protocol, there are a few dozen software packages for analyzing super-resolution single-molecule localization microscopy images, as listed in Sect. 4 above. It is difficult to recommend a given one because the applications vary, and the know-how of the microscopists differs. It is advisable to read the literature on nanoscopy applied to specific problems and select approaches similar to those that the experimentalist is tackling, trying a few. Some software packages are more user-friendly and demand less knowledge of computer programming than others. Some software packages have been conceived as "key in hand" and require little effort from the experimentalist. There is always a compromise between the size of the interrogated area, speed, computer "muscle" devoted to the analysis, accuracy required to identify and localize single molecules, and whether it is a still, fixed specimen or living cell.

17.2.1 Fixed Specimens

If one inspects the stream of images collected in the acquisition step in a typical stochastic localization experiment, each frame should contain a sparse number of blurred spots stochastically appearing and disappearing in successive frames. This results from the turning on and off of the individual molecules undergoing transitions between light-emitting and dark states. The movie is, however, a series of wide-field photomicrographs with diffraction-limited spots from which one must extract x and y positions at the molecular scale. In other words, the main task of the stochastic super-resolution microscopy analysis is to extract with sub-diffraction, nanometer precision the centroid of the photon-emitting particle (the molecule) from the blurred, diffraction-limited intensity profile of the spots recorded by the CCD. This is accomplished by fitting the PSF of the intensity distribution of each spot with an appropriate function (Gaussian, 2-D Gaussian, elliptical Gaussian, etc.). This will result in a 2-D map of all localizations in the captured stream of data. Most software analysis packages produce a table of x and y coordinates of all centroids and provide ancillary modules to eliminate uncertain localizations, correct for drift, etc.

1. Select a ROI size in your streamed stack of image frames in the file format suitable for the next image analysis program.
2. Run the initial analysis of the raw data files using a software package of your choice, e.g., QuickPALM [77] in ImageJ

(NIH, USA, http://rsb.info.nih.gov/ij/). QuickPALM produces immediate graphic outputs which enable one to assess the quality of the experimental data and obtain preliminary parameters. Localizer (BitBucket Localizer), written by Dedecker and coworkers [78, 79]: http://www.igorexchange.com/project/Localizer, implemented in Igor Pro (http://www.wavemetrics.com), also offers the possibility of rapidly evaluating the quality of the experimental data. The first output is a list of all sub-pixel localizations generated through a least-square fitting of the PFSs, resulting from application of a generalized likelihood ratio test algorithm specifically designed to identify PSF-shaped, that is, Gaussian-like, spots. There is a choice between symmetrical (round-shaped) and asymmetrical-shaped (elliptical) 2-D Gaussian functions describing the PSFs. Calculate the standard deviation of the PSFs.

3. If, e.g., ThunderSTORM (GitHub thunderstorm) (https://code.google.com/p/thunder-storm/) [57] was employed in the ImageJ environment, choose an appropriate filter (e.g., Wavelet, B-spline) for image filtering and perform approximate localization of molecules using the modules local maximum and PSF:integrated Gaussian for sub-pixel localization of molecules. ThunderSTORM has provision for performing lateral drift correction using cross correlation.

4. Estimate the drift. Hardware measures preventing drift (thermostated room, high-quality vibrational isolation system, stable stage) or correcting it online also via hardware (automatic focal drift correction) in combination with short acquisition times are the best alternatives. Inclusion of fiduciary markers (e.g., 50 nm gold, nanoshell particles or fluorescent beads) in the specimen is also helpful. Most super-resolution software packages include a drift correction algorithm, which may or may not require fiduciary markers. In Localizer [78] the consecutive drift estimations are interpolated using a polynomial fit and used to determine the approximate position of the sample relative to the starting point at each time point. The resulting offset is subtracted from the fluorophore positions to obtain the drift-corrected positions. Other methods of drift correction for single-molecule localization microscopy include sub-pixel cross correlation of 2-D histograms binned for single-molecule localizations every n number of frames [10, 80] and also see a comprehensive treatment of drift correction for various super-resolution techniques in [81].

5. Estimate the precision of the single-molecule localizations. The accuracy of the localizations, the result of model fitting to the emission pattern, is a measure of the confidence in the estimated position of the single-molecule localizations. It scales inversely with the square root of the number of photons

collected per molecule, N, and increases with the amount of background noise, given by the standard deviation, σ, of the spatial response function of the optical system to an infinitely small object (i.e., the point-spread function, PSF). This can be approximated by $\sigma/N^{1/2}$ in the absence of negligible background [82].

6. Exclude poorly localized molecules by selecting only localizations that have a high photon count (e.g., >100) and acceptable localization precision (e.g., <30 nm).
7. Produce a rendering of the spatial distribution of the collection of localized molecules. This can be done in multiple ways, ranging from the simple x and y point distribution of all centroids superimposed on a black and white depiction of the wide-field image to color-coded distributions depicting density, time of appearance, or any other property comparing representative samples under different experimental conditions.
8. Perform statistical analysis of the distribution of single molecules. Even an elementary property such as the number of particles per unit area may provide information on the functional arrangement or other relevant properties of proteins in the synapse. Knowledge of the photophysical properties of the fluorophores is important in determining the actual number of proteins in quantitative PALM super-resolution experiments [83].
9. Various other analytical tools can be employed to extract relevant information from images of synaptic proteins in fixed neuronal cultures. We are interested in the supramolecular organization of neurotransmitter receptors in the cell-surface membrane, a property which can be interrogated with various statistical analytical tools like Ripley's K function [84, 85] and other tests of two-dimensional spatial point pattern distribution [86, 87]. We have used Ripley's K function to analyze the nanocluster organization of adult muscle-type nicotinic acetylcholine receptors [20]. The use of Ripley-like functions has improved considerably and is successfully applied to the analysis of protein nanoclusters in the immunological synapse imaged with PALM and STORM techniques [88]. The pair correlation or cross correlation function $G(r)$ estimates the probability of finding another particle at a distance r and compares the data to the expected values for a random distribution of particles. $Gs(r) > 1$ correlations can be attributed to clustering of labeled molecules [89–91] and hence can be employed to determine the length scale of the clustering.

17.2.2 Live Specimens

The basic questions in a live-cell experiment concern the time-dependent evolution of some physiologically relevant dynamic phenomenon. In single-molecule super-resolution experiments, this immediately refers to the displacement of the molecule(s) in a functionally adequate time window, to establish molecular trajectories by single-particle tracking (SPT) analysis, determine the speed of the motions (the mean square displacement [MSD]), and derive the apparent diffusion coefficient D of the molecules.

1. In addition to particle detection **steps 1–3** as in fixed specimens, the single-molecule analysis in a dynamic time-lapse sequence is more complex, since in addition to the detection of the centroid of connected components of segmented objects, the analysis should include application of modules which build the trajectories of the connected components, perform additional steps to reject potentially overlapping false maxima in motion, and validate the trajectories using physically meaningful models that predict the particle positions in a frame based on the positions in the past.
2. The MATLAB-based SPT analysis from http://physics.georgetown.edu/matlab/, the equivalent module in Localizer, or the software package ThunderSTORM can be applied to track individual molecules that appear in consecutive frames and assemble them into a molecular trajectories. In brief, the essential requirement in this step is to set an a priori maximum allowable frame-to-frame displacement, i.e., the tracking radius of the molecule along its trajectory, on the basis of the expected diffusion coefficient D of the given molecule.
3. Calculate the MSD of the population(s) of molecules.
4. Calculate the mean diffusion coefficient for each track. In two dimensions, the relationship between the MSD and the effective diffusion coefficient (D_{eff}) is given by MSD $= 4D_{\mathrm{eff}}\,\Delta t$, where Δt is the interval between observations. For freely diffusing molecules, the MSD increases linearly with time, whereas for confined diffusion (as found when molecules are confined within a cytoskeletal corral of filamentous actin, for instance), this relationship plateaus after an initial linear rise. In such cases, one can calculate the instantaneous D_{eff} from the slope of the first few time points of the linear fit of the MSD versus time curve at $2\Delta t$, $3\Delta t$, and $4\Delta t$, referred to as short-range diffusion coefficient or $D_{2\text{–}4}$ [92]. $D_{2\text{–}4}$ is convenient because it can be determined independently of the motional modes. On average, $D_{2\text{–}4}$ is larger than the values of D determined using Eqs. 5–12 of Kusumi and coworkers [92–94] by a factor of 1.2 in the case of constrained or restricted diffusion.
5. Produce graphical displays of single-molecule trajectories. For very high molecular densities, the complete set of trajectories

may produce overcrowded and uninformative graphical representations, and it may be advisable to select identical intervals under different experimental conditions for comparison. Color-coded individual molecular trajectories on the basis of frame intervals can help to identify changes in the behavior of molecular mobility and can also help to detect artifactual lateral drift.

6. It is useful to construct a "probability map" in the form of a 2-D Gaussian with standard deviation σ_{xy} with pixel values proportional to the probability that a molecule can be found at that location [95].

18 Summing Up

These protocols are intended to provide practical guidelines to perform super-resolution imaging of single molecules in synapses using the most inexpensive of currently available nanoscopy techniques, namely, the stochastic single-molecule localization approach. The methodology is relatively easy to implement using an existing fluorescence microscope as the basic body on which to mount the additional building blocks, i.e., the laser lines, CCD camera, filters, and other optical components. As stated in Sect. 3, the objective lens – and its quality – is the most vital individual piece of equipment. The mechanical stability of the optical system is also critical, and investing in a good optical isolation table in combination with a purely mechanical stage pays off good dividends. The other actor in the play is, of course, the specimen. I have reserved these final lines to confess that (at least in our hands) primary neuronal cells from the hippocampus are not easy to maintain in culture, but they provide a unique middle ground between slices and biochemically isolated molecules for in vitro studies of the latter in situ. The field is developing at a fast pace, and the techniques are likely to need frequent update.

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