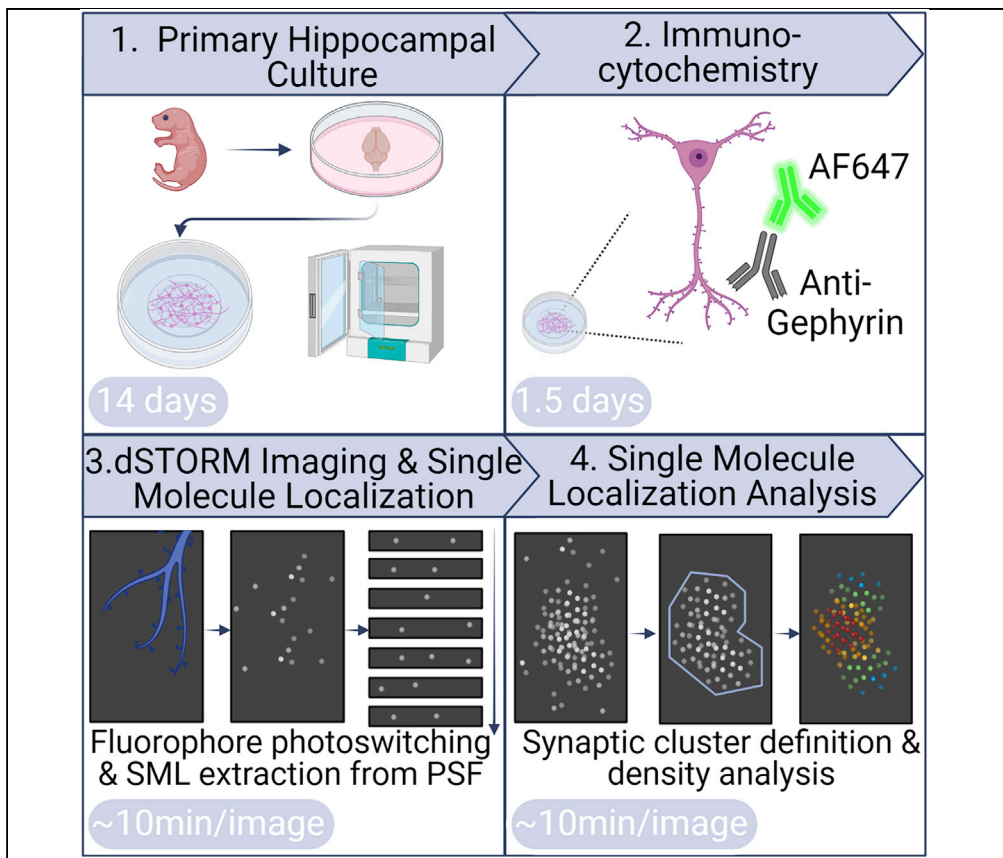


Protocol

Super-resolution imaging of synaptic scaffold proteins in rat hippocampal neurons



Visualizing the nano-organization of the synapse is fundamental to elucidating the structure-function relationship of the nervous system. The advent of super-resolution microscopy provides a tool to assess and quantify the dynamic organization of numerous proteins at the synapse. Here we present a protocol assessing inhibitory synapse scaffold protein, gephyrin, in rat primary hippocampal cultures using dSTORM microscopy. We delineate the steps for artemisinin treatment, immunocytochemistry, dSTORM image acquisition, single-molecule localization, and the analysis of synaptic scaffold protein dynamics.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol to assess inhibitory synapse scaffold protein gephyrin with dSTORM microscopy

Immunocytochemistry steps for staining primary hippocampal cultures

dSTORM imaging and single-molecule localization of pre- and post-synaptic proteins

Image analysis to investigate synaptic clusters and their spatial distribution

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Protocol

Super-resolution imaging of synaptic scaffold proteins in rat hippocampal neurons

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SUMMARY

Visualizing the nano-organization of the synapse is fundamental to elucidating the structure-function relationship of the nervous system. The advent of super-resolution microscopy provides a tool to assess and quantify the dynamic organization of numerous proteins at the synapse. Here we present a protocol assessing inhibitory synapse scaffold protein, gephyrin, in rat primary hippocampal cultures using dSTORM microscopy. We delineate the steps for artemisinin treatment, immunocytochemistry, dSTORM image acquisition, single-molecule localization, and the analysis of synaptic scaffold protein dynamics. For complete details on the use and execution of this protocol, please refer to Guzikowski and Kavalali (2022).¹

BEFORE YOU BEGIN

The protocol detailed below describes the specific steps for staining and dSTORM imaging in primary hippocampal cultures. Parental Artemisinin and its' two semisynthetic derivatives (Artemether and Artesunate) bind in the gephyrin universal receptor binding pocket, disrupting GABA_AR-gephyrin binding at central inhibitory synapses.² In Guzikowski and Kavalali (2022)¹ this protocol was used to understand how Artemisinin treatment disrupted the structural organization of the synapse and how that corresponded to subsequent synaptic signaling deficits with electrophysiology. The pharmacological manipulation of inhibitory synapses with Artemisinin can be expanded to probe other nano-organizational, functional, and signaling pathways downstream of GABA_AR mediated neurotransmission. In addition, the staining, image acquisition, and image analysis portion of this protocol has also been used to investigate excitatory synapses in hippocampal culture and slices including staining for vGluT, Homer, Bassoon, GluA1, and GluA2.

Institutional permissions

All animal procedures were performed in accordance with the guide for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at Vanderbilt University. If you are conducting this protocol institutional permissions from relevant bodies are necessary for the use of laboratory animals.

Prepare rat hippocampal cultures

⌚ Timing: 2 weeks

1. Prepare primary hippocampal cultures on #1.5 thickness 12 mm diameter coverslips.



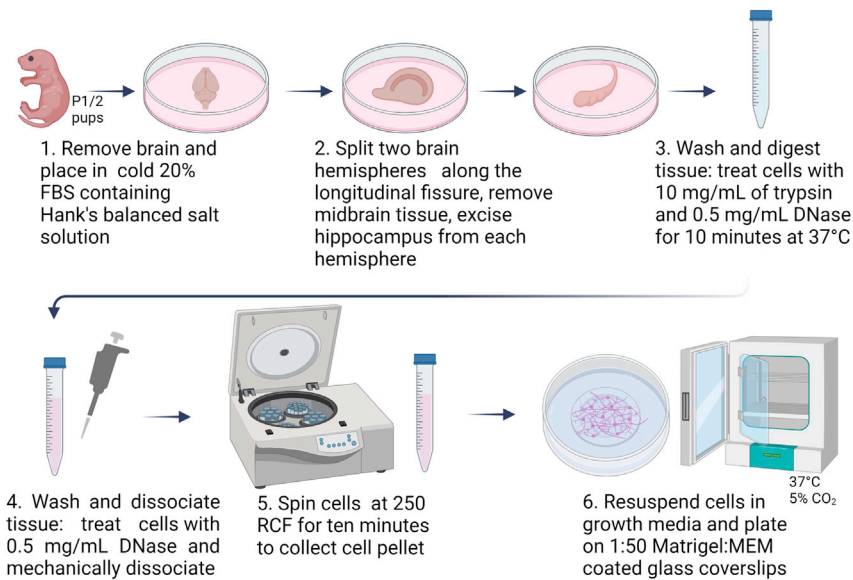


Figure 1. Hippocampal dissection and preparation of primary cultures

Steps to isolate hippocampi from P1/2 pups and prepare primary cultures.

- a. To generate dissociated hippocampal cultures, postnatal pups (P1-2 rats) are decapitated at the head/neck junction with surgical scissors. The entire brain is then removed from the skull using a surgical scalpel and put into cold 20% FBS containing Hank's balanced salt solution. With a dissection microscope the cerebellum is removed, the two brain hemispheres are split along the longitudinal fissure, and midbrain tissue is removed. The hippocampus is subsequently removed from each hemisphere and placed in cold 20% FBS containing Hank's balanced salt solution. Tissue is then washed and treated with 10 mg/mL of trypsin and 0.5 mg/mL DNase for 10 min at 37°C. Following trypsinization tissue is washed again and mechanically dissociated. Cells are then plated on 1:50 Matrigel:MEM coated glass coverslips. Hippocampal tissue from one rat pup is used to plate 10 coverslips. Neurons are incubated for the first 24 h in media containing: MEM (no phenol red), 5 g/l D-glucose, 0.2 g/l NaHCO₃, 0.1 g/l transferrin, 10% FBS, 2 mM L-glutamine and 20 mg/l insulin. On DIV 1 media is changed to growth media with 5% FBS, 0.5 mM L-glutamine, no insulin, and the addition of B27 supplement and 4 μM cytosine arabinoside. On DIV 4 growth media is changed to contain only 2 μM cytosine arabinoside. Cells are maintained at 37°C in a 5% CO₂ atmosphere until DIV 14–18 for experiments³ (Figure 1).

Note: We have found 35 mm MatTek dishes with #1.5 thickness 12 mm coverslips are optimal for all three major steps of the protocol: culturing, staining, and imaging.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Gephyrin (mouse monoclonal)	Synaptic Systems	Cat# 147 021; RRID: AB_2232546
Anti-vGAT (rabbit monoclonal)	Synaptic Systems	Cat# 131 008; RRID: AB_2800534
Anti-MAP2 (guinea pig polyclonal)	Synaptic Systems	Cat# 188-004 RRID: AB_2138181

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
AF488	Invitrogen	A11073
AF568	Invitrogen	A10042
AF647	Invitrogen	A21236
Chemicals, peptides, and recombinant proteins		
Glucose oxidase from <i>Aspergillus niger</i>	Sigma-Aldrich	Cat# G2133
Cysteamine	Sigma-Aldrich	Cat#30070
Catalase from bovine liver	Sigma-Aldrich	Cat#C40
Artemisinin	Sigma-Aldrich	Cat# 361593
Artemether	Sigma-Aldrich	Cat# A9361
Artesunate	Sigma-Aldrich	Cat# A3731
Paraformaldehyde	Sigma-Aldrich	Cat# P6148
NaOH	Sigma-Aldrich	Cat# S8045
PBS10x	Thermo Fisher	Cat# 71448-16
Sucrose	Sigma-Aldrich	Cat# S0389
Triton X-100	Sigma-Aldrich	Cat# T9284
Normal goat serum	Vector Laboratories	Cat# S-1000
Bovine serum albumin	Sigma-Aldrich	Cat# A9647
Sodium azide, 5% (w/v) aqueous solution	Thermo Fisher	Cat# 71448-16
1 mM Tris-HCl, pH 8.0	Corning	Cat# 46-031-CM
NaCl	Thermo Fisher	Cat# BP358-1
Glucose	Sigma-Aldrich	Cat# G7528
Hydrochloric acid	Fisher Chemical	Cat# 7647-01-0
Poly-D-lysine	Thermo Fisher	Cat# A3890401
Experimental models: Organisms/strains		
Sprague-Dawley rat pups (P1–P2)	Charles River	Strain code: 400
Software and algorithms		
Prism 8	GraphPad	http://www.graphpad.com
Vutara VXL software	Bruker	N/A
Other		
Vutara Comprehensive workstation	Bruker	N/A
MatTek glass bottom dishes #1.5	MatTek	Cat# P35G-1.5-14-C
pH strips	Fisher Scientific	Cat# 13-640-516
Tetraspeck beads	Thermo Fisher	T7279
Silicone immersion oil	Olympus	N/A

MATERIALS AND EQUIPMENT

Equipment set-up:

Vutara VXL Comprehensive Workstation (microscope and software):

Image acquisition, single molecule localization, and analysis were completed on a Vutara comprehensive workstation. Which includes a VXL Single Molecule Localization (SML) Super-Resolution Microscope outfitted for 3D direct stochastic optical reconstruction microscopy (dSTORM) with 405 nm, 488 nm, 555 nm, and 640 nm excitation lasers with biplane illumination, a 60× objective, a sCMOS (scientific Complementary metal–oxide–semiconductor) detector, and SRX analysis software.

Artemisinins			
Reagent	Stock concentration	Working concentration	Final concentration
Artemisinin	175 mM	10 mM	50 μM
Artemether	50 mM	10 mM	50 μM
Artesunate	65 mM	10 mM	50 μM

Store stock solution at RT for up to one month or –20°C for up to 3 months.

Preparation: Dissolve appropriate amount of Artemisinins based on molecular weight in DMSO to reach desired stock concentration. Prepare working concentration day of experiments.

4% paraformaldehyde buffer

Reagent	Final concentration	Amount
Paraformaldehyde (PFA)	4%	5 g
NaOH	4%	5 g
PBS 10x	10%	12.5 mL
Sucrose	4%	5 g
ddH ₂ O	N/A	112.5 mL
Total	N/A	125 mL

Store solution at 4°C for up to one month.

Preparation: Dissolve PFA and NaOH in ddH₂O over medium heat in the hood. When the PFA is dissolved the solution will go from milky to clear. Adjust the pH to 7. Then add the PBS and sucrose. Filter the final solution.

△ CRITICAL: Paraformaldehyde is toxic and a potential carcinogen and therefore should be prepared in the hood with appropriate PPE (gloves, lab coat, safety glasses, and mask). We preferably use pH strips to test pH, in order to prevent pH meter contamination (an approximate pH is sufficient for this buffer).

0.2% Triton X-100 buffer

Reagent	Final concentration	Amount
PBS 1x	N/A	29.94 mL
Triton X-100	0.2%	60 µL
Total	N/A	30 mL

Store solution at 4°C in well-sealed for up to six months.

Preparation: Dilute PBS10x (listed in [key resources table](#)) to 1x with ddH₂O.

Note: Prepare 10% stock solution and before the experiment dilute to 0.2% with PBS. Due to the viscosity of Triton X it is easier to prepare a higher concentration stock solution to work from.

Blocking solution

Reagent	Final concentration	Amount
Normal goat serum	2%	400 µL
BSA	1%	0.2 g
PBS 1x	X	20 mL
Total	N/A	20 mL

Make fresh before use.

Alternatives: If secondary antibodies are from donkey and goat, prepare blocking solution that contains 2% of each species serum.

Sample storage buffer

Reagent	Final concentration	Amount
4% PFA Buffer	0.5%	2.5 mL
Sodium azide (5%)	0.02%	80 µL

(Continued on next page)

Continued

Reagent	Final concentration	Amount
PBS 1x	N/A	17.42 mL
Total	N/A	20 mL

Store solution at 4°C for up to one month.

Dilution buffer

Reagent	Final concentration	Amount
1 M Tris, pH 8.0	10 mM	0.1 mL
1 M NaCl solution	50 mM	0.5 mL
PBS 1x	N/A	9.4 mL
Total	N/A	10 mL

Store solution at room temperature for up to 6 months.

Imaging buffer base

Reagent	Final concentration	Amount
Glucose	10%	1 g
1 M Tris, pH 8.0	50 mM	0.5 mL
1 mM NaCl solution	10 mM	0.1 mL
Total	N/A	10 mL

Store solution at room temperature for up to 6 months.

Enzyme stock solution (oxygen scavengers)

Reagent	Final concentration	Amount
Glucose Oxidase	N/A	14 mg
Dilution Buffer (ingredients detailed above)	N/A	200 μ L
Catalase [20 mg/mL]	N/A	50 μ L
Total	N/A	250 μL

Store solution at 4°C for up to 2 weeks.

Preparation: Dissolve glucose oxidase in 200 μ L of dilution buffer. Dilute 20 mg of catalase in 1 mL of dilution buffer separately. Add 50 μ L of catalase solution to the glucose oxidase solution.⁴

Cysteamine (MEA) solution

Reagent	Final concentration	Amount
Cysteamine	1 M	77 mg
360 mM Hydrochloric acid	N/A	1 mL
Total	N/A	1 mL

Store solution at 4°C for up to 1 month.⁴

△ CRITICAL: Store solid MEA at 4°C in desiccating conditions. Remove MEA from the fridge and let warm to room temperature before weighing.

dSTORM imaging buffer

Reagent	Final concentration	Amount
Imaging buffer base	N/A	890 μ L
Enzyme Stock solution	1x	10 μ L

(Continued on next page)

Continued

Reagent	Final concentration	Amount
MEA solution	100 mM	100 μ L
Total	N/A	1 mL

Make solution fresh before imaging and use for up to 2 h.⁴

△ **CRITICAL:** Prepare solution immediately before use on ice. The buffer lasts for about two hours in an open room temperature environment, efficacy of fluorophore photo-switching will be determined by buffer conditions. Prepare fresh buffer at least every 2 h.

Alternatives: dSTORM imaging buffer with BME

dSTORM imaging buffer with β ME

Reagent	Final concentration	Amount
Imaging buffer base	N/A	980 μ L
Enzyme Stock solution	1 \times	10 μ L
β ME solution	1 \times	10 μ L
Total	N/A	1 mL

Make solution fresh before imaging and use for up to 2 h.⁴

△ **CRITICAL:** Ensure to prepare fresh β ME.

△ **CRITICAL:** Prepare solution immediately before use on ice. The buffer lasts for about two hours in an open room temperature environment, efficacy of fluorophore photo-switching will be determined by buffer conditions. Prepare fresh buffer at least every 2 h.

STEP-BY-STEP METHOD DETAILS

Artemisinins treatment

⌚ **Timing:** ~1 h

This section explains the steps to treat hippocampal neurons with Artemisinins. In Guzikowski and Kavalali (2022)¹ this treatment scheme was used to understand how pharmacological manipulation with parental Artemisinin and its' two semisynthetic derivatives (Artemether and Artesunate) disrupts GABAergic synapse nano-organization. Therefore, the first section of this protocol is to treat cells with the Artemisinins. All three Artemisinins were used as their orientation in the Gephyrin (GephE domain) universal receptor binding pocket is different.² An acute 1-h treatment was used to rapidly probe the functional and structural effects of distributed GABA_AR-gephyrin binding however treatment length can be extended.

1. The day of experiments prepare working solutions of 10 mM Artemisinin, Artemether, and Artesunate.
2. Treat primary hippocampal cultures with 50 μ M of Artemether, Artemisinin, and Artesunate for 1 h by directly adding Artemisinins to growth media. Treat control samples with the equivalent volume of DMSO.
 - a. Recommended to have volume of DMSO < 1% of total volume.

Note: Immediately move to step 3 following 1 h incubation to begin fixation.

Note: The above culturing and Artemisinins treatment protocol can also be used for electrophysiology whole cell voltage and current clamp recordings. It is important to also include Artemisinins in bath solution while recording.

3-color immunocytochemistry

⌚ Timing: ~1.5 days

This section explains the steps to stain primary hippocampal cultures with desired primary and secondary antibodies to visualize protein location within the neuron. In Guzikowski and Kavalali (2022)¹ the main protein of interest was post-synaptic protein, gephyrin. In addition to gephyrin staining vesicular GABA transporter (vGAT) was stained for as a pre-synaptic marker and Microtubule-associated protein 2 (MAP2) as a dendritic marker.

Day 1.

3. Remove growth media from cell culture MatTek dishes and immediately replace with 4% PFA solution for 20 min at room temperature (RT) (20°C–22°C). Following fixation wash cells 3 × 5 min with PBS 1 ×.
4. Remove PBS and permeabilize with 0.2% Triton X-100 for 30 min at RT (20°C–22°C). Following permeabilization wash cells 3 × 5 min with PBS 1 ×.
5. Block with 1% BSA and 2% normal goat serum diluted in PBS for 2 h at RT (20°C–22°C).
6. Prepare primary antibodies in blocking buffer.
 - a. Prepare 120 μL of antibody solution per coverslip (optimize antibody solution volume depending on coverslip diameter, just need to cover). To investigate gephyrin, stain inhibitory presynaptic marker, vesicular GABA transporter (vGAT) (1:500), inhibitory postsynaptic scaffold protein gephyrin (1:150) and dendritic marker MAP2 (1:1000) (Refer to [Table 1](#) for antibody dilution and concentration information).
7. Incubate cells in primary antibody diluted in blocking buffer overnight (15–20 h) at 4°C. Place dishes into a humid chamber for incubation to ensure primary antibody does not evaporate.

Note: Humid chambers can be made simply from supplies around the lab – an empty tip box padded with a layer of moist tissues will ensure the primary antibody solution does not evaporate during overnight incubation inside the tip box.

Note: For optimization of primary antibody concentration start at a 1:500 dilution or concentration recommended by the supplier (Refer to troubleshooting [problems 1](#) and [2](#)). In tissue increasing staining incubation length can increase antibody penetrance however for staining in cell culture, concentration should initially be adjusted before incubation length.

Note: Always vortex and spin down antibodies before use.

Day 2.

8. Wash cells 3 × 5 min with PBS 1 ×.
9. Prepare secondary antibodies in blocking buffer.
 - a. Prepare 120 μL of antibody solution per coverslip (optimize antibody solution volume depending on coverslip diameter, just need to cover). To tag the primary antibodies listed above use AF647 (1:350), AF568 (1:1000), and AF488 (1:500). Gephyrin is the primary protein of interest and was therefore tagged with the AF647 antibody and vGAT with AF568.

Note: AF647 is an excellent fluorophore for dSTORM as it is extremely bright (high photon yield per switching event) and has a low on-off duty cycle. Therefore, if performing single color

Table 1. Antibody concentrations and dilutions

Antibody	Stock concentration	Dilution
Anti-Gephyrin (mouse monoclonal)	1 μ L/ μ L Hybridoma supernatant	1:150
Anti-vGAT (rabbit monoclonal)	1 mg/mL	1:500
Anti-MAP2 (guinea pig polyclonal)	1 μ L/ μ L Antiserum	1:1000
AF488	2 mg/mL	1:500
AF568	2 mg/mL	1:1000
AF647	2 mg/mL	1:350

Manufactures instructions were followed for all antibody dilutions.

dSTORM, AF647 should be used. The quality of super resolution images is directly dependent on the photo-switchable probe's photon number, duty cycle, survival fraction, and the number of switching cycles,⁵ therefore the selection of fluorophores is of utmost importance. Although in our imaging we used AF568, CF568 is a better fluorophore in the orange channel. Additionally, MAP2 was only used as a reference under widefield and therefore a dSTORM compatible antibody was not used. If you would like to conduct 3-color dSTORM use Atto488.

Note: For optimization of primary antibody concentration start at a 1:500 dilution or concentration recommended by the supplier (Refer to troubleshooting [problems 1](#) and [2](#)).

Note: Always vortex and spin down antibodies before use.

- Incubate cells in secondary antibody diluted in blocking buffer for 90 min at RT (20°C–22°C). Place dishes back into the humid chamber for incubation to ensure secondary antibody does not evaporate. Cover humid chamber in foil to prevent photobleaching of secondary antibodies.
- Complete a post staining fixation by incubating cells in 4% PFA buffer for 20 min at RT (20°C–22°C).
- Store samples in storage buffer in dark at 4°C.

Note: Fixation time can be optimized to mitigate potential side effects.

▣▣ Pause point: Samples can be stored for at least one month before imaging (have not used samples beyond that timepoint).

dSTORM image acquisition and SML localization

⌚ Timing: Typically, it takes ~10 min per image (50 fps), requiring multiple hours-days to image an entire experiment

This section describes the steps to acquire images and localize single molecules of inhibitory presynaptic protein vGAT and post-synaptic scaffold protein gephyrin. The imaging acquisition and analysis portion of this protocol can be expanded to a variety of other synaptic proteins to address the same fundamental question - how do different proteins cluster and organize at the synapse. This protocol is robust and applicable to other synaptic proteins with basic adjustments in antibody concentrations and the optimization of image acquisition settings (please see [troubleshooting](#) section). In our laboratory this protocol has been used to investigate excitatory synapses in primary hippocampal culture, visualizing vGlut, PSD-95, Bassoon and in hippocampal slices visualizing Homer, Bassoon, GluA1, and GluA2.

Note: The microscopy set-up used had these essential components: bipplane illumination, lasers: 405 nm, 488 nm, 555 nm, and 640 nm, sCMOS detector, multi-band dichroic mirror,

pentaband emission filter, and standard emission single bandpass filters (405, 488, 555, 640). Lasers that are compatible with 647, 568, and 488 (red, orange, and green respectively) fluorophores must be used.

13. Optical Calibration: For accurate single molecule localization (SML) the focal planes and color channels of the optical system need to be aligned. Furthermore, to generate 3D measured point spread functions for sub-diffraction limit localization the point spread function (PSF) of a point source must be determined. 100 nm Tetraspeck bead samples that emit at blue, green, orange, and red were prepared on poly-d-lysine coated MatTek dishes [1:500] to calibrate the channels and planes. Resolution: X-Y: 20 nm, Z: 50 nm.
14. Take dSTORM images with a 60× objective (1.3 NA, silicone oil).
15. Apply one drop of silicone immersion oil to the center of 60× objective (ensure no air bubbles during application).
16. Mount MatTek dish in chamber and replace storage solution with imaging buffer (~200 μL to only cover the coverslip). Note the time to ensure image acquisition is within the buffer's lifetime.
17. Mount the chamber into the stage-holder.
18. Using the 488 channel (exciting fluorophores tagged to MAP2) find your region of interest. Conduct search on a low laser power to prevent photo-switching and photobleaching. Ideal region is not too dense to allow for the isolation of pre and post synaptic localizations.
19. Perform sequential imaging of the 640 channel and then the 555 channel. Begin image acquisition with constant illumination with the 640 nm laser at 463.4 mW while gradually increasing the 405 laser power from 1–10% of max power (49.7 mW) over imaging period. Take 20,000 frames of the 647 channel. Then illuminate the 555 nm laser at constant 555 mW for 10,000 frames. Generally, 3 regions of interest were imaged per coverslip.
 - a. Take single z-plane images (z-distance = 2 μm due to biplane illumination).
 - b. Exposure time: 20 ms, 50 Frames per second.
 - c. Widefield field of view of 200 μm × 200 μm and super resolution field of view 50 μm × 50 μm.

Note: Upon excitation of the sample there should be robust photo-switching of the fluorophore, appearing as blinking. Following initiation, photo-switching should reach a steady state level. Adequate blinking is fundamental to SML therefore antibody concentrations, buffer conditions, and laser power should be optimized for experiments (please refer to troubleshooting [problems 1, 2, and 3](#)).

Note: Silicone oil closely matches the refractive index of cells decreasing spherical aberration while imaging and therefore increasing the quality of super resolution images; neurons ($n = \sim 1.38$), silicone oil ($n = 1.406$).⁶

20. Identify individual molecules by their brightness above the background in a frame-by-frame process ([Figure 2](#)). Generate a molecule's coordinates in space by fitting the imaged PSF with the 3D measured PSF (step 13). As the raw data comes from the camera the SRX software works in parallel to conduct single molecule localizations. Images are filtered, particles are defined, and fluorophores localized. Analysis and filtering settings⁷:
 - a. Particle Detection: Parameters for PSF identification and extraction (filtering) to define a particle:
 - i. Cutout width: 15 px (1px = 100 nm) - Size around an ROI's brightest pixel that is used for localization routines.
 - ii. ROI border: 0 px - How large of a region around the field of view border is discarded.
 - iii. Max frame accumulation: 1 frame - Depending how long a fluorophore is in its "on" state (fluorescing) will determine how many frames it is visible. If a fluorophore is active for a longer period of time max frame accumulation should be increased so the same fluorophore will not be detected as multiple blinking events.

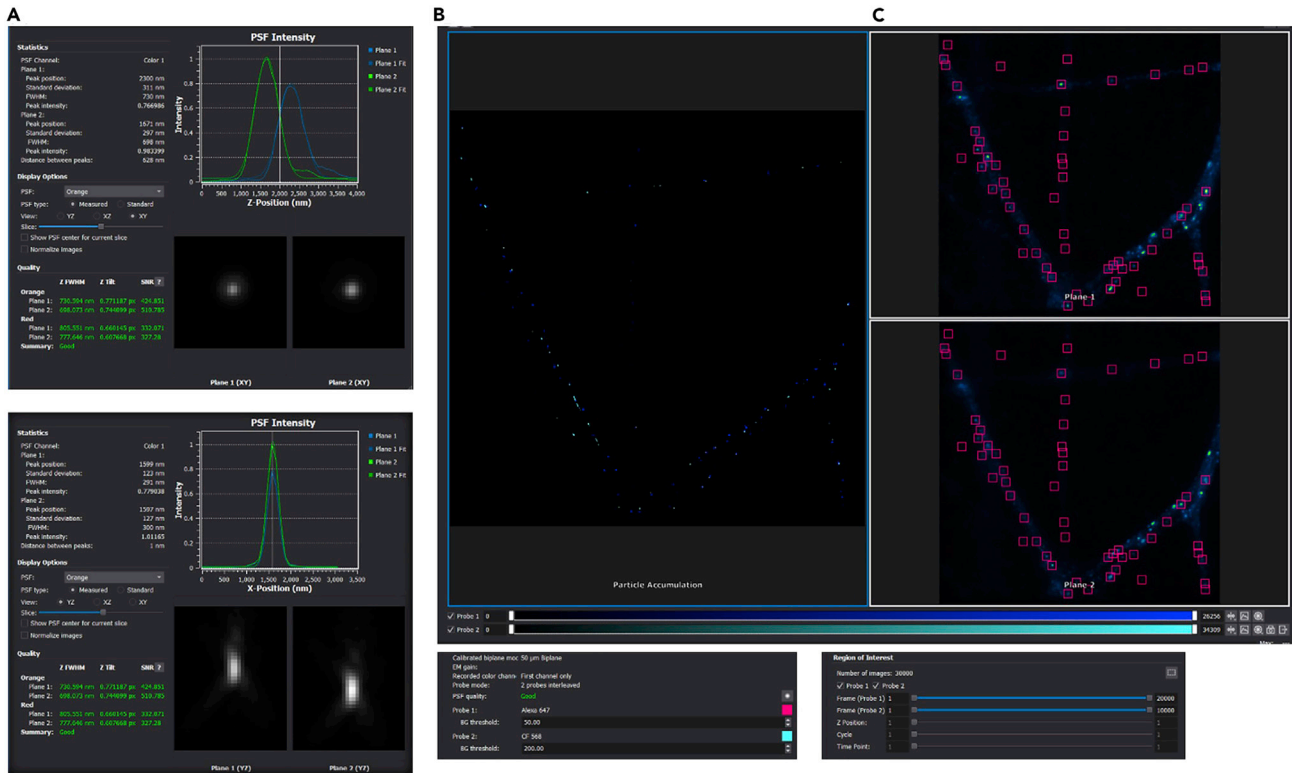


Figure 2. Representative images during particle detection and fluorophore localization

(A) Representative images of the experimental point spread function (PSF) in the XY and YZ views for the two imaging colors (red 647, orange 568) collected in step 13.

(B) Particle accumulation of single molecule localizations summated from multiple frames of a neuronal projection.

(C) Representative image of a single frame during particle detection with detected particles in pink boxes.

- iv. Max accumulation offset: 1px (100 nm) - Degree of fluorophore shift across sequential frames to be considered the same fluorophore.
- v. Max particles per frame: no max - Number of particles to be localized per frame.
- vi. Background slope removal threshold: 20 - The threshold of heterogeneity across the background of fluorophore's cutout that is admissible for localization.
- vii. Cutout/background removal threshold: 0.2 - A signal to noise threshold ((total photon count of cutout – background photon count)/background photon count) that determines if a fluorophore is included for localization.
- viii. Detection bandpass low frequency cutoff: 7.07px, Detection bandpass high frequency cutoff: 1.41px - 1st step of localization is the application of a smoothing filter to reduce noise for fluorophore identification.
- b. Parameters for fluorophore localization:
 - i. 3D measured point spread functions are used for localization (experimental PSF is collected from bead calibration step 13). This is the optimal routine to use as calibration is specific to your system and imaging set-up.
 - ii. Max iterations: 40 – maximum number of iterations for one candidate particle.
 - iii. PSF Interpolation Spline method: B-Spline – method for PSF fitting for localization.
 - iv. Temporal Median Filter: 0 frames – method for the estimation of background signal.⁸
 - v. Background threshold 647 channel: 50, 568 channel: 200 - Fluorescence values above the mean background that the software will treat as a localization.

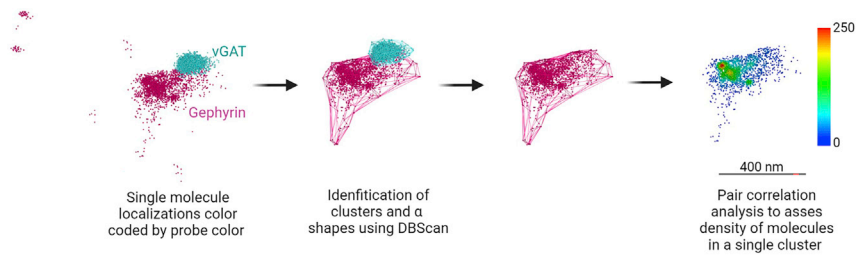


Figure 3. Image analysis pipeline

Single molecule localizations of the same probe are clustered according to the DBScan algorithm. Molecules not localized to a cluster are eliminated from further analysis. Cluster α shapes are determined and further analysis is conducted on gephyrin volume, vGAT-gephyrin centroid distance, and density. (Representative clusters obtained from data generated in Guzikowski and Kavalali,¹ Images have been manipulated to represent potential outcomes of this protocol).

Note: Refer to troubleshooting [problem 4](#) for suggestions on how to optimize localization parameters.

Note: Single molecule localizations can generate large data sets, ensure your computer or external hard drive have adequate storage space (TB range).

dSTORM image analysis

⌚ **Timing:** Typically, it takes ~ 10 min per image, requiring multiple hours to days to analyze an entire experiment

This section describes the steps to analyze single molecule localizations of inhibitory presynaptic marker vGAT and post-synaptic scaffold protein gephyrin. After single molecules have been localized to a X,Y, and Z position in space based on their PSF, they are then grouped and clustered; single molecules are localized by their PSFs and clusters defined based on their spatial proximity to other molecules. To define synaptic clusters a clustering algorithm Density-Based Spatial Clustering of Applications with Noise (DBScan) is used as it can define arbitrarily shaped clusters from a spatial database.⁹ Stochastic optical reconstruction microscopy-based relative localization analysis (Storm-RLA) is used to define a pre- and post-synaptic pair.

Synaptic protein cluster analysis

The goal of this analysis is to understand the clustering dynamics of vGAT and gephyrin single molecule localizations using a proximity- and density-based analysis.

21. Conduct Density-Based Spatial Clustering of Applications with Noise (DBScan) to define clusters of SMLs of the same probe based on proximity ([Figures 3](#) and [4](#)). Parameters:
 - a. Maximum Particle distance: $0.1 \mu\text{m}$ - threshold distance to determine if a particle is in one cluster or not clustered.
 - b. Cluster minimum particle count: 6 particles - need at least 6 particles within the defined distance to be labeled a cluster.
 - c. Surface hulls α shape radius: $0.15 \mu\text{m}$ ¹⁰ - To determine cluster shape, point patterns of clusters were fit with an α shape, a bounding form that contains a clusters' individual points to then extract cluster information i.e., volume. Ideal for clustering 3D shapes ([Figure 3](#)).

Note: Cluster characteristics of the least dense cluster were used as global parameters to set threshold values.⁹ Due to Artemisinins treatment disrupting gephyrin clustering, the cluster minimum particle count value was quite low and should be optimized based on experimental question.

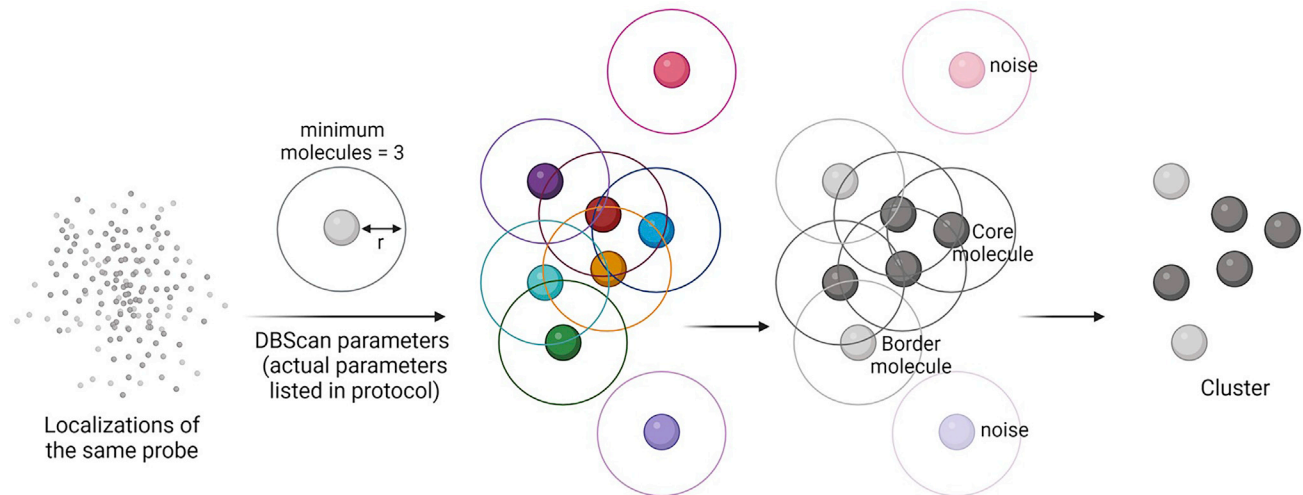


Figure 4. DBScan clustering schematic

r is the distance around one molecule that is searched for another molecule of the same probe. For a molecule to form a cluster it must have 2 molecules within the distance r . This molecule is considered a core molecule. The same routine is conducted for the 2 new molecules and subsequent localizations in their radii are considered part of the original cluster. If a molecule is considered part of a cluster but does not meet the density parameter, it is considered a boundary molecule. A molecule that does not meet the density or distance parameters is considered noise.⁹

22. Following the determination of clusters, localizations not grouped were eliminated from analysis and considered noise (Figure 4).
23. From α shapes calculate cluster volume.

Pre-synaptic vGAT and post-synaptic gephyrin cluster spatial proximity

The spatial distribution of vGAT and gephyrin clusters were mapped to define which clusters composed a pre- and post-synaptic juxtaposed synapse using stochastic optical reconstruction microscopy-based relative localization analysis (Storm-RLA). Only vGAT and gephyrin clusters that were categorized as a paired pre- and post-synapse using this relative localization analysis (details in step 24) were included in further analysis.

Note: With conventional confocal microscopy often pre-post-synapse colocalization analysis is done to define synaptic puncta. However, with super resolution microscopy x-y resolution is 20 nm, therefore pre- and post-markers can be differentiated in space and a spatial proximity analysis is performed (however there can be overlap in localizations from two different probes).

24. Investigate the spatial organization of vGAT and gephyrin clusters using stochastic optical reconstruction microscopy-based relative localization analysis (Storm-RLA). Using the Storm—RLA algorithm cluster α shapes generated in step 21 are used to define a pre- and post-synaptic pair based on their position in space. From this eliminate non-paired clusters and calculate vGAT and gephyrin cluster centroid distance (Figure 3).¹¹ Parameters:
 - a. Max cluster distance: 1 μm (generous threshold however the matrix created allowed the selection of the closest vGAT and gephyrin clusters as a synapse).

Cluster density analysis

To understand the spatial distribution of gephyrin molecules.

25. Asses individual gephyrin clusters in 2D by condensing localizations to the X-Y plane. For each individual cluster determine a theoretical pair correlation function (PCF). A theoretical fit accounts for the density and shape of the cluster and generates a function as if the molecules

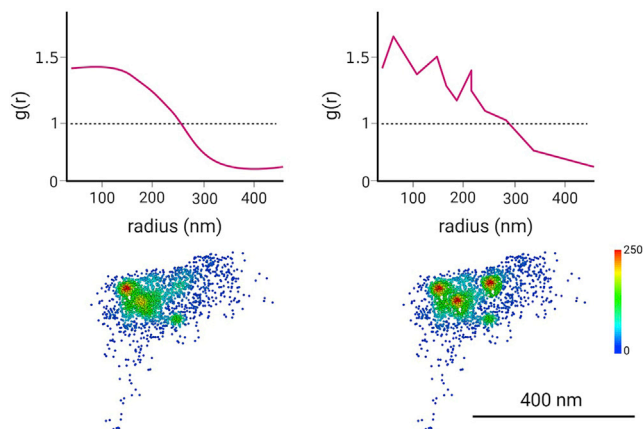


Figure 5. Pair correlation function density analysis

Example pair correlation functions with corresponding gephyrin clusters colored coded by heat map. The function quantifies the probability of another molecule being within a given distance, $g(r)$, of a given molecule. With a $g(r) > 1$ representing a heterogeneous organization (i.e., clustering), $g(r) = 1$ a uniform random distribution and $g(r) < 1$ a molecularly de-enriched area. The peaks in the function when $g(r) > 1$ demonstrate sub-synaptic clustering within a single cluster (Images have been manipulated to represent potential outcomes of this protocol).

were randomly organized. To compare density distributions across groups normalize individual PCFs of each individual cluster to their theoretical fit to account for shape, volume, and density.^{12,13} Parameters:

- Search radius from center: 500 nm, 100 bins.
- The output function will be a probability of how likely you are to find another molecule within a given distance, $g(r)$. With a $g(r) > 1$ representing a heterogeneous organization (i.e., clustering), $g(r) = 1$ a uniform random distribution and $g(r) < 1$ a molecularly de-enriched area¹⁰ (Figure 5).

Note: The pair correlation function generated represents the point pattern of individual gephyrin molecules within a single cluster; $g(r)$ as a function of distance from reference particle. Ultimately providing insight into density variations and nanoclustering within a defined cluster.

EXPECTED OUTCOMES

Synaptic nanostructure is fundamental in directing different modes of neurotransmission and their autonomous downstream roles.^{1,10,14–16} Understanding basal synaptic structural dynamics in addition to how pharmacological or genetic manipulations disrupt synaptic architecture provides insight into how an individual protein's organization facilitates cell signaling. Since dSTORM microscopy provides single synapse level information this staining protocol in parallel to electrophysiology is a very powerful tool; allowing you to delineate how structural changes in scaffolding proteins might lend to changes in evoked and spontaneous neurotransmission. We have demonstrated that gephyrin clusters in rat hippocampal neurons are mostly uni-domain with the center having a higher density of molecules than the periphery. Furthermore, using Artemisinins as a tool to disrupt GABA_AR binding to gephyrin we establish a center surround organization of evoked and spontaneous GABAergic neurotransmission at central synapses, with the periphery of gephyrin clusters fundamental in maintaining spontaneous neurotransmission.¹

QUANTIFICATION AND STATISTICAL ANALYSIS

The image analysis steps detailed above will produce individual data points for cluster volume, vGAT-gephyrin centroid distance, and pair correlation functions for each gephyrin cluster. For

statistical analysis *GraphPad Prism* was used to compare groups pre and post pharmacological manipulation.¹ For statistical analysis experimental replicates were pooled together and an equal number of points were selected to compare across groups, a test for normality was run, and the appropriate statistical test conducted. To analyze pair correlation functions the $y = 1$ intercepts of cluster functions were compared.

LIMITATIONS

The super-resolution optical analysis of synaptic scaffold proteins is a valuable methodology to visualize the synapse at the nanometer level. However, it should be noted that the use of this protocol requires multiple points of optimization for your microscopy set-up. First, sample preparation and antibody concentrations must be optimized for protein density and synapse density. Second, due to variation in labeling density and fluorophore brightness PSF identification, extraction and single molecule localization criteria need to be fine-tuned to detect signal while preventing the inclusion of noise. The first parameters to consider are max frame accumulation and background removal thresholds. Furthermore, this analysis does not account for neuronal morphology i.e., where post-synaptic clusters are located on the dendrite, spine vs. shaft. If neuronal morphology is of interest other super-resolution approaches (e.g., STED nanoscopy) or conventional light microscopy methods will likely be better suited (cell filling probes can be used to visualize morphology).

TROUBLESHOOTING

Problem 1

Weak Staining (step 7).

Potential solution

The immunoreactivity of different primary antibodies can cause weak staining, while the recommended gephyrin concentration is high, 1:150, this can be increased to boost immunoreactivity. Furthermore, to optimize antibody concentrations the dilution can be adjusted while all other variables are maintained (buffer and incubation length).

Problem 2

High background (steps 7, 10).

Potential solution

Primary or secondary antibody concentration is potentially too high. To combat this antibody concentrations can be decreased, or secondary incubation time can be decreased to 1 h.

Problem 3

Low or absent photo-switching (step 19).

Potential solution

One of the most sensitive portions of the protocol is the imaging buffer. Quality photo-switching is directly dependent on buffer quality ultimately determining the success of the experiment.

Prepare fresh solutions of Glucose Oxidase, Catalase and MEA, if any have degraded, fluorophores will not be able to photo-switch well. Ensure you are using imaging buffer for no more than 2 h at room-temperature and are keeping all stock solutions on ice. To increase the longevity of the buffer you can seal your sample. Double check the pH of your buffer solution is pH 8.

Buffer components can also be optimized for your sample and the number of colors being imaged. For single color imaging the 647 channel and a β ME based imaging buffer should be used. A β ME based buffer will produce a higher photon count relative to MEA. However, if your sample is densely labeled, you are labeling highly abundant proteins, or using more than one color a MEA based buffer is optimal.

Laser power must be strong enough to cause fluorophores to photo-switch. Low laser power should be used to find regions of interest (view as an epifluorescence image) and increased for image acquisition. Laser power should be optimized for photo-switching behavior.

Problem 4

Poor single molecule localization (step 20).

Potential solution

The first thing to check is that image acquisition is working correctly, and fluorophore photo-switching is robust. Following this, parameters for PSF identification, extraction and molecule localization can be adjusted to ensure the adequate detection of signal. Parameter optimization suggestions⁷:

- Cutout width: If samples are densely labeled the cutout width should be lowered to decrease the probability that light from another particle is included in localization.
- Max frame accumulation: It is important to tune this setting to the blinking behavior of individual fluorophores. Depending how long a fluorophore is in its “on” state (fluorescing) will determine how many frames it is visible. If a fluorophore is active for a longer period of time max frame accumulation should be increased so the same fluorophore will not be detected as multiple blinking events. If there is a high degree of noise adjust max frame accumulation to eliminate bright spots that aren’t blinking from your localization acquisition.
- Max particles per frame: Can be optimized to eliminate dim candidate events from localization routines. If you want to only localize a subset of the brightest fluorophores per frame the max number of particles per frame can be set.
- Back slope removal threshold: Adjusted based on background homogeneity of fluorophore cutouts of your sample. If your background is heterogenous the background slope threshold can be increased. For brain slices this value can be increased due to the decreased homogeneity of the imaging area relative to culture.
- Cutout/background removal threshold: Adjusted based on signal to noise ratio, i.e., if the background is high this number can be lowered. For brain slices this value can be decreased due to high background signal.
- Background threshold: If your signal is dim decrease background thresholds, typically CF568 will not be as bright as AF647. For brain slices these values were decreased relative to cell culture due to weaker signal in tissue.

Problem 5

Discrepancies in protein organization analysis compared to what is in the literature (steps 20, 21, 25).

Potential solution

Particle detection and fluorophore localization threshold settings can cause discrepancies in results across research groups. Furthermore, parameters set for DBScan strongly effect cluster size and density measurements. The normalization of data within your experimental set-up and treatment group will greatly augment the applicability of results across research groups, ultimately aiding the consensus of how different manipulations alter structure.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ege T. Kavalali (ege.kavalali@vanderbilt.edu).

Materials availability

This study did not generate new reagents.

Data and code availability

No original code has been generated in this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, discussion, writing, and editing: N.J.G., E.T.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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