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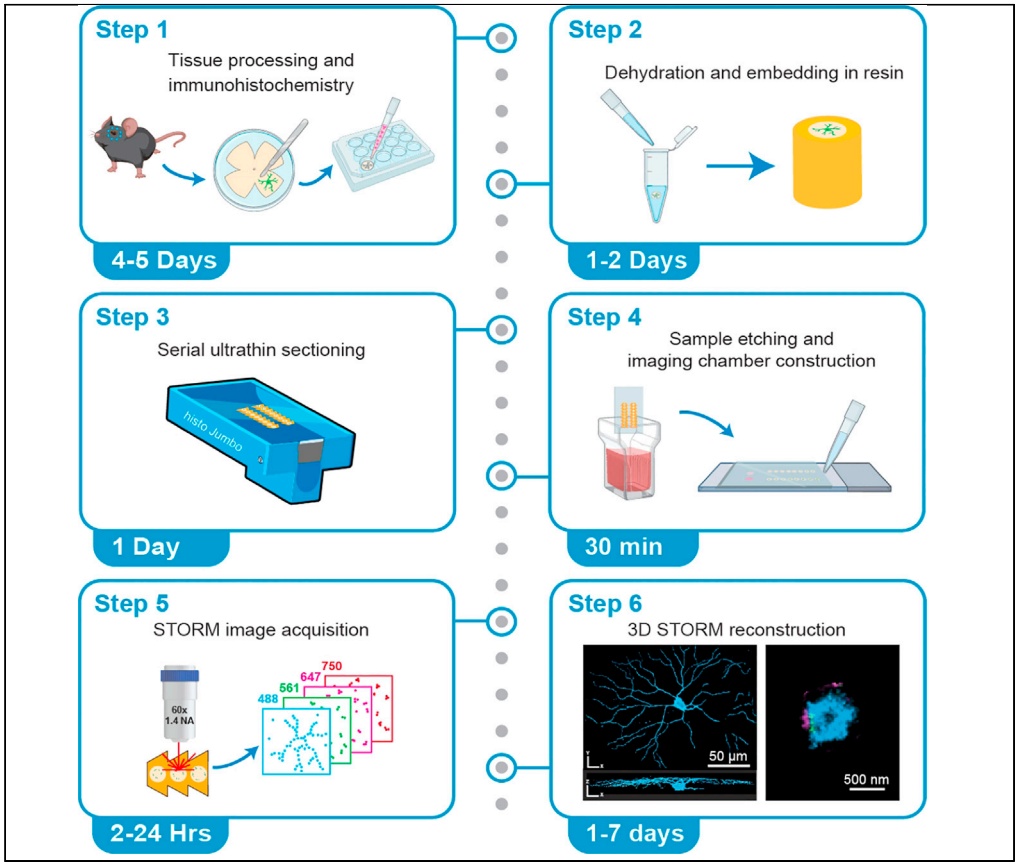
Protocol

Volumetric super-resolution imaging by serial ultrasectioning and stochastic optical reconstruction microscopy in mouse neural tissue



Here, we present a protocol for collecting large-volume, four-color, single-molecule localization imaging data from neural tissue. We have applied this technique to map the location and identities of chemical synapses across whole cells in mouse retinae. Our sample preparation approach improves 3D STORM image quality by reducing tissue scattering, photobleaching, and optical distortions associated with deep imaging. This approach can be extended for use on other tissue types enabling life scientists to perform volumetric super-resolution imaging in diverse biological models.

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Highlights

Optimized sample preparation protocol for serial-section super-resolution microscopy

Four-color super-resolution imaging in tissue volumes

Automated data acquisition and processing platform for 3D reconstructions

Whole-neuron reconstruction with nanoscale resolution

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Volumetric super-resolution imaging by serial ultrasectioning and stochastic optical reconstruction microscopy in mouse neural tissue

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SUMMARY

Here, we present a protocol for collecting large-volume, four-color, single-molecule localization imaging data from neural tissue. We have applied this technique to map the location and identities of chemical synapses across whole cells in mouse retinae. Our sample preparation approach improves 3D STORM image quality by reducing tissue scattering, photobleaching, and optical distortions associated with deep imaging. This approach can be extended for use on other tissue types enabling life scientists to perform volumetric super-resolution imaging in diverse biological models.

For complete details on the use and execution of this protocol, please refer to Sigal et al. (2015).

BEFORE YOU BEGIN

This protocol is written for researchers with a working understanding of immunohistochemical (IHC) labeling approaches and fluorescence microscopy. Before implementing this protocol, users should optimize labeling specificity and signal-to-noise ratios for the immunohistochemical assay (Buchwalow and Böcker, 2010; Im et al., 2019; Schneider Gasser et al., 2006). Furthermore, this protocol requires technical skills in histology and ultrasectioning (Harris et al., 2006; Micheva and Smith, 2007). Additionally, users should familiarize themselves with principles of single-molecule localization microscopy (SMLM) (Lelek et al., 2021) and operation of available commercial instrumentation (Albrecht et al., 2021; Chang, 2015; Tröger et al., 2020).

Animal work in the current study was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Maryland. Male and female Thy1-YFP-H and C57BL/6J mice (The Jackson Laboratory), aged 4–40 days, were used interchangeably in our experiments.

Prepare fluorescent dye conjugates for IHC

Timing: 2 h

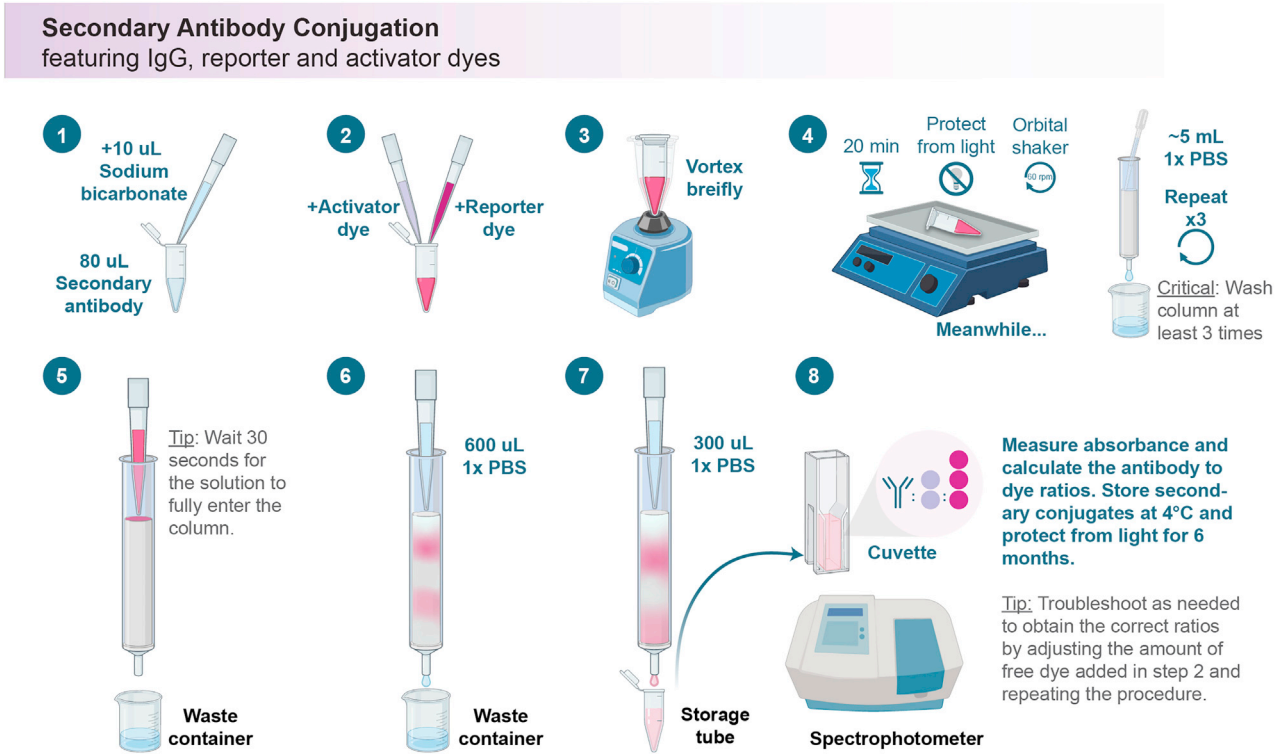


Figure 1. Secondary antibody conjugation workflow

This protocol uses indirect immunohistochemistry (IHC) to label proteins of interest in tissue. To achieve faster stochastic optical reconstruction microscopy (STORM) imaging, we produce custom secondary antibodies conjugated with both reporter and activator dyes to increase the photoswitching rate of certain probes. Strong laser activation (1–3 kW/cm^2 at the sample) near the peak absorbance wavelength of the reporter dye drives photoswitching (Bates et al., 2007; Dempsey et al., 2009; Rust et al., 2006) and weak stimulation of the activator dye drives reactivation (fluorescence emission) of the reporter dye (Bates et al., 2007). Ultimately, the reporter dye emission defines single molecule positions. In addition to secondary antibodies, we also custom-conjugate lectins such as wheat germ agglutinin (WGA), which we use as a global neuropil stain to facilitate image registration in our experiments (example images shown in Figure 8). This custom conjugation protocol can be used for both probes (IgG and WGA). See Figure 1 for a workflow illustration.

Dye combinations used in this paper:

Reporter dye	Activator dye
DY-749P1-NHS	Alexa 405
Alexa 647-NHS	Alexa 405
Cy3B-NHS	N/A
Atto 488-NHS	N/A

Note: A full list of dyes compatible with STORM imaging can be found in the key resources table (see also Dempsey et al., 2011; Li and Vaughan, 2018). Additional considerations when selecting dyes for multicolor imaging are described in Figure 12.

Prepare the following reagents before you begin

Sodium Bicarbonate Solution (1M $NaHCO_3$)		
Reagent	Final concentration	Amount
$NaHCO_3$	1 M	84 mg
dH_2O	n/a	1 mL
Total	**n/a**	**1 mL**
Make fresh before use and keep ice cold. Vortex prior to pipetting.		

WGA Stock (10 mg/mL)		
Reagent	Final concentration	Amount
WGA, unconjugated, lyophilized	10 mg/mL	10 mg
1× PBS	n/a	1 mL
Total	**n/a**	**1 mL**
One conjugation reaction will use 1/10 of this stock volume. Freeze the remaining WGA stock volume in 100 µL aliquots at −80°C for 6 months.		

Alternatives: We have tested other lectin probes (e.g., Concanavalin A, *Wisteria floribunda*, others) and these work well (data not shown). Custom NHS probe conjugates should be tested empirically in user applications.

Fluorescent Dye Stocks (Alexa 405 NHS-ester, Atto 488 NHS-ester, Alexa 647 NHS-ester, and DY-749P1 NHS-ester)		
Reagent	Final concentration	Amount
Fluorescent Dye	2 mg/mL	2 mg
DMSO	n/a	1 mL
Total	**n/a**	**1 mL**
Create 10 µL aliquots to avoid multiple freeze-thaw cycles, which can decrease the efficacy of the NHS linkage in this conjugation reaction. Store aliquots at −80°C for 9–12 months. Keep aliquots protected from light at all times.		

Cy-3B Mono NHS-ester		
Reagent	Final concentration	Amount
Cy-3B	50 mg/mL	50 mg
DMSO	n/a	1 mL
Total	**n/a**	**1 mL**
Create 10 µL aliquots to avoid multiple freeze-thaw cycles, which can decrease the efficacy of the NHS linkage in this conjugation reaction. Store aliquots at −80°C for 9–12 months. Keep aliquots protected from light at all times.		

1. Prepare fresh 1M $NaHCO_3$ and keep solution ice cold. Mix 80 µL of secondary antibody (stock concentration 1 mg/mL) or WGA with 10 µL 1M $NaHCO_3$.

See Figure 1.

2. Add 1.8 µL reporter dye and 1.2 µL activator dye (if applicable) to the solution.

Note: Dual labeled antibodies are used to increase reporter dye photoswitching (DY-749P1/Alexa 647) through UV illumination of the activator dye (Alexa 405). Cy3B and Atto 488

reporter photoswitching is not increased in the presence of activator dyes therefore no Alexa 405 is added to those conjugation reactions.

3. Vortex the solution briefly.
4. Incubate the solution at room temperature (20°C–25°C) for 20 min with constant agitation and protect from light. Meanwhile, secure the NAP-5 column in a test tube holder and allow storage buffer to drain by gravity into a waste receptacle. Fill the empty NAP-5 column with 1× PBS and allow the waste to drain. Repeat washing two more times.

⚠ CRITICAL: Rinse the NAP-5 column at least three times (troubleshooting 1).

5. Pipette the conjugate solution onto the surface of the NAP-5 column and allow solution to sink into the column surface for ~30 s.
6. Add 600 µL 1× PBS to the column. Allow the clear PBS fraction to drain from the column into a waste receptacle.

Note: Free dyes will separate from conjugates creating two bands that may be visible depending on fluorophore wavelength (Cy3B is visible, Atto 488 and Alexa 647 are weakly visible and DY-749P1 is invisible).

7. Add 300 µL 1× PBS and collect the flowthrough into the final storage tube. This 300 µL of flowthrough is the final product and contains the conjugated probes (IgG- or WGA-dye conjugates). Discard the column.
8. Measure conjugate concentration and dye ratios per antibody/WGA molecule:
   a. Blank the spectrophotometer using the same 1× PBS as above.
   b. Measure the absorbance profile of the conjugate and record the peak absorbance values of the antibody (~280 nm) and the dye molecules (~405–750 nm depending on dyes used). Save absorbance profile for future reference.
   c. Using the concentration values, determine the number of dye molecules per antibody molecule. A calculator used to determine the molecular ratios is provided in an Excel spreadsheet (https://github.com/SpeerLab/Antibody-Conjugation).

Note: Ideally, each labeling protein molecule (IgG or WGA) will be conjugated to 3–4 reporter dye molecules and 1–2 activator dye molecules. If no activator is used, 3–4 reporter dye molecules should still be conjugated.

⚠ CRITICAL: If ratios are not ideal, repeat the procedure and adjust dye volumes added to solution in step 2 to achieve molecular ratios within the appropriate bounds. If more than 4 µl of dye is needed in step 2 in order to achieve correct ratios, then dye stocks may need to be remade (troubleshooting 1).

   d. Recollect conjugate from cuvette and store in the dark at 4°C for up to 6 months.
   e. To confirm conjugate quality after a period of storage, remeasure and compare absorbance values to originals.

Coat glass coverslips

Timing: 2 h

The following steps are used to coat glass coverslips with gelatin, which will cause ultrathin sections to adhere when they are collected after ultrasectioning (Micheva and Smith, 2007). While performing all steps, avoid allowing dust to collect onto the surface of the coverslips. Work in an area free from heavy airflow and filter all solutions. Refer to Figure 2 for a workflow illustration.

Potassium Hydroxide Solution (1M KOH)		
Reagent	Final concentration	Amount
KOH	1 M	56.11 g
dH_2O	n/a	1 L
Total	**n/a**	**1 L**
Dissolve and filter. Store at room temperature for up to 12 months.		

Ethanol Solution (95% EtOH)		
Reagent	Final concentration	Amount
EtOH (100%)	95% v/v	950 mL
dH_2O	n/a	50 mL
Total	**n/a**	**1 L**
Store at room temperature for up to 12 months.		

Gelatin Solution		
Reagent	Final concentration	Amount
Gelatin from bovine or porcine skin	0.5 % w/v	2.5 g
Chromium Potassium Sulfate	0.05% w/v	0.25 g
dH_2O	n/a	500 mL
Total	**n/a**	**500 mL**
Dissolve with heat while stirring. Filter once solution is clear of particulates. Store full volume at 4°C. Gelatin solution should be reheated from a semisolid gel into a liquid before repeated use.		

⚠ CRITICAL: We do not recommend long term storage of gelatin solution (>1 week) because of potential ultrathin section loss with poorly coated coverslips (see troubleshooting 3).

See Figure 2.

9. Prepare 1M KOH and 95% EtOH. Separate coverslips in a rack, completely submerge into 1M KOH solution and sonicate with heat (~40°C–60°C) for 15 min.

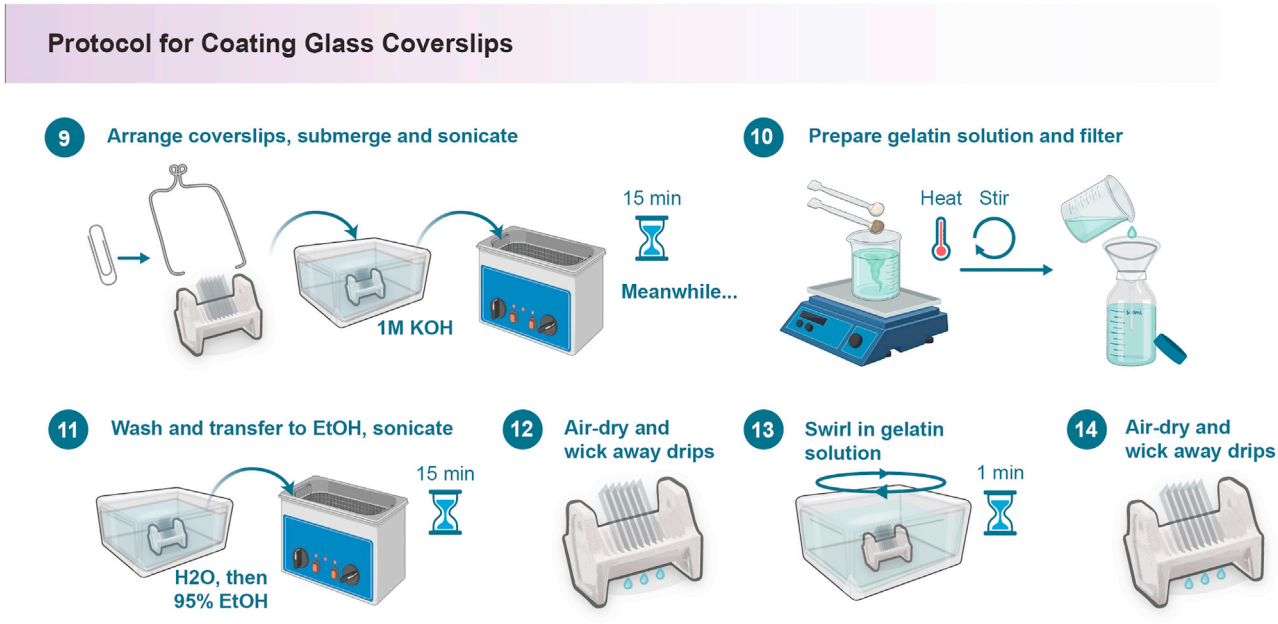


Figure 2. Protocol for coating glass coverslips

10. Prepare gelatin solution. Heat to dissolve particulates, then filter the solution.
11. Rinse coverslips in filtered H_2O, completely submerge them in 95% EtOH and sonicate with heat (~40°C–60°C) for 15 min.
12. Remove coverslip racks from 95% EtOH and allow them to air dry. Wick away excess ethanol.

Alternatives: Place racks in a 50°C oven until dry.

⚠ CRITICAL: Examine coverslips at this stage to check for clarity. If coverslips are not completely clean, check that 1M KOH and 95% EtOH are clean and properly prepared, then start procedure again at step 9.

13. Completely submerge clean, dry coverslips in gelatin solution and swirl for 1 min. The gelatin solution temperature at this stage can range from ~25°C–80°C.
14. Remove coverslip racks from gelatin solution and allow to air dry completely. Wick away excess solution from troughs of the coverslip rack.

Note: Moving coverslips to a clean, dry rack can help eliminate gelatin pooling at the lower edges of the coverslips. Allow coverslips to dry completely before use or storage. Storage for 3–6 months in a closed container at room temperature is recommended. During storage it is critical that the coverslips remain free from dust/debris prior to use. Visibly dirty coverslips should be discarded.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Chicken polyclonal ani-GFP (1:100)	Invitrogen	Cat#A10262
Donkey anti-Guinea pig IgG unconjugated (1:100)	Jackson ImmunoResearch	Cat#706-005-148
Donkey anti-Chicken IgY unconjugated (1:100)	Jackson ImmunoResearch	Cat#703-005-155
Donkey anti-Mouse IgG unconjugated (1:100)	Jackson ImmunoResearch	Cat#715-005-150
Donkey anti-Rabbit IgG unconjugated (1:100)	Jackson ImmunoResearch	Cat#711-005-152
Guinea pig polyclonal anti-Bassoon (1:100)	Synaptic Systems	Cat#141 004
Guinea pig polyclonal anti-Calbindin (1:100)	Synaptic Systems	Cat#214 005
Guinea pig polyclonal anti-VGluT2 (1:100)	Synaptic Systems	Cat#135 404
Mouse monoclonal anti-Bassoon (1:100)	Abcam	Ab82958
Mouse monoclonal anti-Gephyrin (1:100)	Synaptic Systems	Cat#147 011
Rabbit polyclonal anti-ERC 1b/2 (1:100)	Synaptic Systems	Cat#143 003
Rabbit polyclonal anti-Homer1 (1:100)	Synaptic Systems	Cat#160 103
Rabbit polyclonal anti-Munc 13-1 (1:100)	Synaptic Systems	Cat#126 103
Rabbit polyclonal anti-Piccolo (1:100)	Synaptic Systems	Cat#142 003
Rabbit polyclonal anti-Ribeye B-domain (1:100)	Synaptic Systems	Cat#192 103
Chemicals, peptides, and recombinant proteins		
Alexa Fluor 405 NHS-ester	Thermo Fisher Scientific	Cat#A30000
Alexa Fluor 647 NHS-ester	Thermo Fisher Scientific	Cat#A20006
Atto 488 NHS-ester	ATTO-TEC GmbH	AD 488-31
Catalase from bovine liver	Sigma-Aldrich	C1345
Chloroform	Sigma-Aldrich	Cat#288306
Chromium potassium sulfate	Sigma-Aldrich	Cat#243361
Cy-3B Mono NHS-ester	Cytiva	PA63101
Cysteamine	Sigma-Aldrich	Cat#30070
DY-749P1 NHS-ester	Dyomics GmbH	Cat#749P1-01
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich	D8662
Ethanol	Pharmco	Cat#111000200C1GL

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
FluroSpheres Infrared (715/755)	Invitrogen	Cat#F8799
FluroSpheres Orange (540/560)	Invitrogen	Cat#F8809
Gelatin from bovine or porcine skin	Sigma-Aldrich	G9391 or G1890
Glucose Oxidase	Sigma-Aldrich	G2133
Glutaraldehyde 70%, EM grade	Electron Microscopy Sciences	Cat#16360
Immersion Oil Type HF	Cargille	Cat#16245
Methanol	Sigma-Aldrich	Cat#322415-100ML
Normal Donkey Serum	Jackson ImmunoResearch	Cat#017-000-121
Paraformaldehyde 16%, EM grade	Electron Microscopy Sciences	Cat#15710
Potassium hydroxide	Sigma-Aldrich	Cat#221473
Sodium azide	Sigma-Aldrich	S2002
Sodium bicarbonate	Fisher Scientific	Cat#S233
Sodium chloride	Sigma-Aldrich	S9888
Sodium hydroxide pellets	Sigma-Aldrich	Cat#567530
Tris-base (Trizma-base)	Sigma-Aldrich	T8524
Triton X-100	Sigma-Aldrich	Cat#11332481001
Wheat Germ Agglutinin unconjugated	Vector Labs	L-1020-10
Critical commercial assays		
UltraBed Kit	Electron Microscopy Sciences	Cat#14310
Experimental models: Organisms/strains		
Mouse: C57BL/6J Age: 4-40 days; Sex: M/F	The Jackson Laboratory	Cat#000664
Mouse: Thy1-YFP-H Age: 28-40 days; Sex: M/F	The Jackson Laboratory	Cat#003782
Software and algorithms		
Antibody ratio calculator	This paper	https://github.com/SpeerLab/Antibody-Conjugation
Directory of SMLM fitting software	Biomedical Imaging Group, Ecole Polytechnique Fédérale de Lausanne (EPFL)	http://bigwww.epfl.ch/smlm/software/
Excel	Microsoft	http://www.microsoft.com
Fiji (ImageJ)	Schindelin et al., 2012	https://fiji.sc/
"STORMpro" custom GUI for volumetric STORM image processing	This paper	https://github.com/SpeerLab/STORM-UI
MATLAB	MathWorks	https://mathworks.com
Python3	Python	https://www.python.org
STORM acquisition control code (packages include hal4000.py, steve.py and dave.py); version V2019.06.28	Zhuang Lab, Harvard University	https://github.com/ZhuangLab/storm-control
3D-DAOSTORM analysis (single-molecule localization fitting code); version 2.1	Babcock et al., 2012	https://storm-analysis.readthedocs.io/en/latest/install.html
Other		
1.5mL Microcentrifuge tubes	Fisher Scientific	Cat#05-408-129
1" Optical post	THOR Labs	RS12
$^1/_2$" Optical posts (x2)	THOR Labs	TR075
24-well plates	Corning	Cat#351147
5-min Epoxy in DevTube	Jenson Tools	Cat#14250
Analytical Balance	Sigma-Aldrich	Z760420-1EA
Antistatic plastic flat end tweezers	Uxcell	Cat#99301
BEEM embedding capsules	Electron Microscopy Sciences	Cat#70020-B
Bench-top oven	Thermo Fisher Scientific	Cat#51028112
Blunt ended needle 25G/0.52mm	C-U Innovations	N/A
Centrifuge	Sigma-Aldrich	EPO226620509
Custom-built STORM microscope	Babcock, 2018; Zhang and Speer, 2021	For more information on our build, contact technical lead and see note below.
Cover glass staining rack	Electron Microscopy Sciences	Cat#72238
Coverslip No. 1.5 (24 × 30)	VWR	Cat#48404-467

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Coverslip staining rack	VWR	Cat#16004-422
DiskStation 8-bay Network attached storage	Synology	DS1815+
Double sided adhesive tape	Scotch	N/A
Eyelash manipulator	Electron Microscopy Sciences	Cat#71182
Falcon 15 mL conical tubes	Fisher Scientific	Cat#05-527-90
Falcon 50 mL conical tubes	Fisher Scientific	Cat#14-959-49A
Filter paper	VWR	Cat#28320
Flexure clamping mechanism	THOR Labs	BA2F
Glass Coplin staining jar	Electron Microscopy Sciences	Cat#70315
Glass staining dish	Electron Microscopy Sciences	Cat#71420
Histo Jumbo diamond knife	DiATOME	HistoJumbo
Lens paper	THOR Labs	Cat#MC-5
Microscope slides	VWR	Cat#16004-422
Movement stage	Edmund Optics	Cat#53-384
NAP-5 Columns	VWR	Cat#95017-009
Nitrocellulose membranes	ThermoFisher Scientific	Cat#LC2001
Orbital shaker	VWR	Cat#12620-938
Petri dishes	Fisher Scientific	Cat#FB0875713
Post clamp	THOR Labs	RM1C
RackStation Storage System	Synology	RS3617RPxs
Right angle clamp	THOR Labs	RA90
Roto-Shake Genie	Scientific Industries	SI-1100
Single edge razor blades	Garvey	Cat#40475
Spectrophotometer	ThermoFisher Scientific	Cat#840-210800
Stainless steel cap screws (x2)	THOR Labs	SH6MS16
Stereomicroscope	Nikon	SMZ18
Stirring hotplate	ThermoFisher Scientific	Cat#SP88854100
Switchable magnetic base	THOR Labs	MB175
Three-prong extension clamp	Fisher Scientific	05-769-8Q
Transfer pipettes	Fisher Scientific	Cat#13-680-50
TrimTool 90°	DiATOME	TrimTool 90°
Ultramicrotome	Leica Microsystems	UC7
Ultrasonic cleaner	Branson	CPX-952-218R
Vortex	Fisher Scientific	Cat#50-728-002
Weighing boat	VWR	Cat#10770

Note: The instrument used to acquire data presented in this paper was custom built by C.M.S. Resources for building SMLM instruments have been published (Bates et al., 2013; Dempsey, 2013; Ma et al., 2017). Commercially available instruments for SMLM are also available (Albrecht et al., 2021; Chang, 2015; Tröger et al., 2020).

MATERIALS AND EQUIPMENT

Collect and fix tissue sample

4% Paraformaldehyde (4% PFA)		
Reagent	Final concentration	Amount
16% PFA	4%	10 mL
1× PBS	n/a	30 mL
Total	**n/a**	**40 mL**

Store as recommended by manufacturer.

⚠ CRITICAL: Paraformaldehyde is hazardous; wear proper protective equipment and use only in a fume hood. Paraformaldehyde waste needs to be collected and disposed of according to institute regulations.

Alternatives: Aldehyde based chemical fixatives may cause tissue distortions. Preserving cytoarchitecture may require adjusting the fixative solution osmolarity. Users may consider supplementing the above 4% PFA solution with sucrose (200 mM) (Stradleigh et al., 2015).

Immunohistochemistry

Triton-X 100 Stock		
Reagent	Final concentration	Amount
Triton X-100	3% v/v	3 mL
1× PBS	n/a	97 mL
Total	**n/a**	**100 mL**
Store full volume at 4°C for 9–12 months.		

Sodium Azide Stock		
Reagent	Final concentration	Amount
Sodium Azide	2% w/v	1 g
1× PBS	n/a	50 mL
Total	**n/a**	**50 mL**
Store full volume at 4°C for 9–12 months.		

⚠ CRITICAL: Sodium Azide is highly toxic; wear proper protective equipment and use only in a fume hood. Sodium Azide waste needs to be collected and disposed of according to institute regulations.

Blocking Buffer		
Reagent	Final concentration	Amount
Donkey serum	10%	500 µL
Triton-X 100 Stock	0.3%	500 µL
Sodium Azide Stock	0.02%	50 µL
1× PBS	n/a	3.95 mL
Total	**n/a**	**5 mL**
Store 1 mL aliquots at −20°C for up to 6 months and defrost before use. Avoid freeze-thaw cycles.		

Wash Buffer		
Reagent	Final concentration	Amount
Blocking Buffer	20% v/v	3 mL
1× PBS	n/a	12 mL
Total	**n/a**	**15 mL**
Store full volume at 4°C for up to 1 week.		

Post Fixative (3% PFA, 0.1% glutaraldehyde (GA))		
Reagent	Final concentration	Amount
16% PFA	3%	1.88 mL
70% GA	0.1%	14.3 µL
1× PBS	n/a	8.11 mL
Total	**n/a**	**10 mL**
Store 1 mL aliquots at −20°C for 9–12 months.		

⚠ CRITICAL: Paraformaldehyde is hazardous; wear proper protective equipment and use only in a fume hood. Paraformaldehyde waste needs to be collected and disposed of according to institute regulations.

Dehydrate and embed samples in resin

UltraBed Resin		
Reagent	Final concentration	Amount
Component A	50% w/w	5 g (approx. 6 mL)
Component B	50% w/w	5 g (approx. 6 mL)
Total	**n/a**	**10 g (approx. 12 mL)**

⚠ CRITICAL: Ensure 1:1 mixture of components A and B by weight. Make several hours (~4) before needed and place on rotator to ensure complete mixing. If resin is not fully mixed, it will not polymerize properly (troubleshooting 2).

Etch away resin and assemble imaging chamber

Sodium Ethoxide		
Reagent	Final concentration	Amount
NaOH	10% w/v	20 g
100% EtOH	n/a	Up to 200 mL
Total	**n/a**	**200 mL**

Stir solution at room temperature overnight (18–24 h). Store full volume at room temperature for 1 month. Solution will turn dark brown.

⚠ CRITICAL: Solution must remain free from water contamination to avoid damaging tissue sections (troubleshooting 3 and 5).

Bead Stock		
Reagent	Final concentration	Amount
Orange Invitrogen FluroSphere	90% v/v	9 µL
Infrared Invitrogen FluroSphere	10% v/v	1 µL
Total	**n/a**	**10 µL**

Store full volume at 4°C for 4–6 weeks.

Tris Base Stock		
Reagent	Final concentration	Amount
Tris Base	1 M	6.06 g
dH_2O	n/a	Up to 50 mL
Total	**n/a**	**50 mL**

Adjust the pH to 7.5 using HCl. Store full volume at room temperature for 9–12 months.

Imaging Buffer		
Reagent	Final concentration	Amount
NaCl	10 mM	29.22 mg
Tris Base Stock	200 mM	10 mL
1× PBS	n/a	40 mL
Total	**n/a**	**50 mL**

Store 150 µL aliquots at −20°C for 9–12 months.

Dilution Buffer		
Reagent	Final concentration	Amount
NaCl	50 mM	146.1 mg
Tris Base Stock	10 mM	500 µL
10× PBS	n/a	5 mL
dH_2O	n/a	44.5 mL
Total	**n/a**	**50 mL**
Store 1 mL aliquots at −20°C for 9–12 months.		

HCl Stock		
Reagent	Final concentration	Amount
HCl	360 mM	656.28 mg
dH_2O	n/a	50 mL
Total	**n/a**	**50 mL**
Store full volume at room temperature for 9–12 months.		

MEA Stock		
Reagent	Final concentration	Amount
Cysteamine	1 M	77 mg
HCl Stock	n/a	1 mL
Total	**n/a**	**1 mL**
Store 10 µL aliquots at −20°C for 2–3 months.		

Glucose Stock		
Reagent	Final concentration	Amount
Glucose	50% w/v	500 mg
Imaging Buffer	n/a	1 mL
Total	**n/a**	**1 mL**
Warm to dissolve. Store 50 µL aliquots at −20°C for 9–12 months.		

Catalase Stock		
Reagent	Final concentration	Amount
Catalase from bovine liver	1.7% w/v	17 mg
dH_2O	n/a	1 mL
Total	**n/a**	**1 mL**
Store 30 µL aliquots at −20°C for 9–12 months.		

Glucose Oxidase (GLOX) Stock		
Reagent	Final concentration	Amount
Glucose Oxidase	350 µM	7 mg
Dilution Buffer	n/a	100 µL
Catalase Stock	n/a	25 µL
Total	**n/a**	**125 µL**
Centrifuge at 14,000 rpm (20,817 x g) for 1 min and store 15 µL aliquots of the supernatant at −20°C for 2–3 months.		

STORM Buffer		
Reagent	Final concentration	Amount
Imaging Buffer	50 mM	148 μL
Glucose Stock	10%	40 μL
MEA Stock	10 mM	2 μL
GLOX Stock	0.0175 mM	10 μL
Total	n/a	200 μL

⚠ CRITICAL: Prepare STORM buffer immediately prior to imaging chamber assembly. Do not vortex. Pipette gently to mix. DO NOT use old or stored STORM buffer (trouble-shooting 5).

STEP-BY-STEP METHOD DETAILS

Collect and fix the tissue sample

Timing: 4–5 h

During the following steps, tissue will be dissected and prepared for IHC. Importantly, the region of interest is punched out creating a small disk of tissue which is easy to maneuver while minimizing mechanical damage. The small disk improves antibody penetration and allows the tissue to lay flat for embedding. Fixative solution and blocking buffer should be prepared before you begin.

See Figure 3.

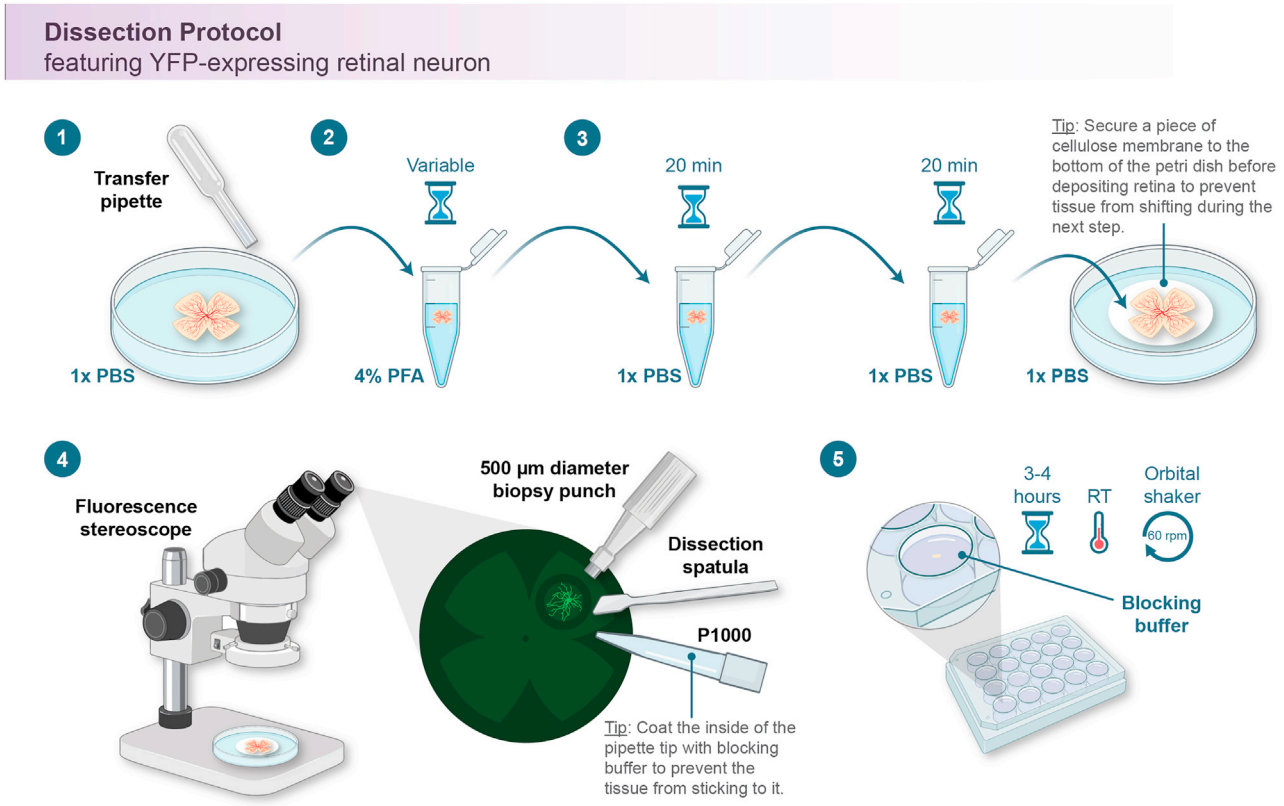


Figure 3. Dissection protocol featuring YFP-expressing retinal neuron

1. Euthanize mouse, enucleate eyes, and dissect retinae in room temperature 1× PBS (see videos in Claybon and Bishop, 2011; Sondereker et al., 2018).

   Note: These steps describe processing retinal tissue, but brain sections can also be used.

2. Fix tissue in room temperature 4% PFA for 10 min to 1 h.

   Note: Users should perform time-series fixation control experiments to empirically optimize their immunohistochemical labeling for conventional fluorescence imaging prior to performing STORM experiments (Buchwalow and Böcker, 2010; Im et al., 2019; Schneider Gasser et al., 2006). The example data presented in Figure 10 were collected from retinal tissue fixed for 30 min at room temperature.

3. Wash retinae in 1× PBS for 2× 20 min, then place it in a clean petri dish filled with room temperature 1× PBS.
4. Punch out a 500 μm diameter region of interest using a 25G blunt ended needle under stereoscope guidance.
5. Transfer punch to a 24-well plate and incubate in blocking buffer for 3–4 h at room temperature with constant agitation. Continue on to IHC.

Immunohistochemistry

Timing: 4–5 days

The following steps are performed to label antigen targets with primary and secondary antibodies. Target-specific staining is followed by a global neuropil stain using a WGA conjugate, which functions as an image alignment tool in later STORM data processing steps. Lastly, an aldehyde fixation establishes crosslinking to stabilize all molecular labels. Numbered steps are illustrated in Figure 4.

See Figure 4.

6. Prepare primary antibody solution by diluting 1 mg/mL antibody stocks to 1:100 in blocking buffer. Primary antibody dilution can be adjusted based on empirical testing. Transfer tissue

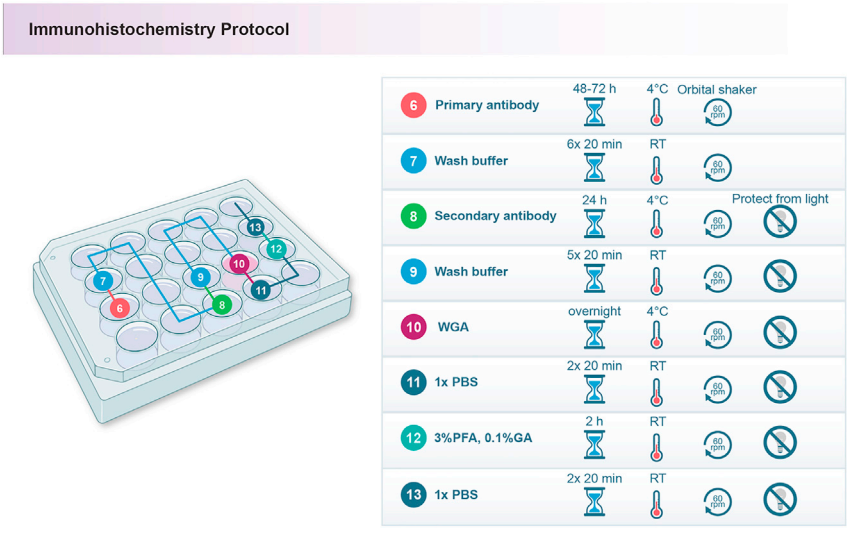


Figure 4. Immunohistochemistry protocol

punches to a new well containing primary antibody solution and incubate for 48–72 h at 4°C with constant agitation.

7. Transfer tissue to a new well containing wash buffer and incubate at room temperature for 6 × 20 min washes with constant agitation.
8. Prepare secondary antibody solution by diluting secondary antibody conjugate stocks to 1:100 in blocking buffer. Secondary antibody dilution can be adjusted based on empirical testing. Secondary antibodies should be custom conjugated to photoswitchable dyes compatible with STORM imaging ahead of this step (see section: prepare fluorescent dye conjugates for IHC). Transfer tissue punches to a new well containing secondary antibody solution and incubate for 24 h at 4°C with constant agitation. Protect samples from light.
9. Transfer tissue to a new well containing wash buffer and incubate at room temperature for 5 × 20 min washes with constant agitation. Protect samples from light.
10. Prepare wheat germ agglutinin (WGA) solution by diluting WGA conjugate stock 1:10 in 1 × PBS. WGA should be custom conjugated to photoswitchable dyes compatible with STORM imaging ahead of this step (see section: prepare fluorescent dye conjugates for IHC). Transfer tissue to a new well containing WGA solution and incubate at room temperature for 12–16 h (overnight) with constant agitation. Protect samples from light.
11. Transfer tissue to a new well containing 1 × PBS and incubate at room temperature for 2 × 20 min washes with constant agitation. Protect samples from light.

⚠ CRITICAL: Tissue punches may stick to the inside of the pipette tip here. To avoid this, coat the inside of the pipette tip with blocking solution first, then transfer the tissue punches.

12. Transfer tissue to a new well containing post-fixation solution (3% PFA, 0.1% GA) and incubate for 2 h with constant agitation. Protect samples from light.
13. Transfer tissue to a new well containing 1 × PBS and incubate at room temperature for 2 × 20 min with constant agitation. Protect samples from light. Continue on to dehydration.

Dehydrate and embed samples in resin

Timing: 1–2 days

The following steps plasticize the tissue punches to make them suitable for ultrasectioning. Refer to Figure 5 for a workflow illustration.

See Figure 5.

14. Perform a graded alcohol dehydration:
    a. Transfer tissue into a clean Eppendorf tube and immerse in a 50% EtOH solution (100% EtOH diluted in ddH_2O). Incubate at room temperature for 20 min with constant agitation and protect sample from light.
    b. Repeat with 70%, 90%, 100% and 100% EtOH.

⚠ CRITICAL: If tissue is not thoroughly dehydrated, the resin will not polymerize properly and crack or flake when taken out of the mold (troubleshooting 2).

15. Perform a graded resin infusion:
    a. Transfer tissue into a clean Eppendorf tube and immerse in a solution containing 2 parts 100% EtOH to 1 part resin. Incubate at room temperature for 2 h with constant agitation and protect sample from light.
    b. Repeat with 1:1 EtOH: resin, 1:2 EtOH: resin, 100% resin and 100% resin.

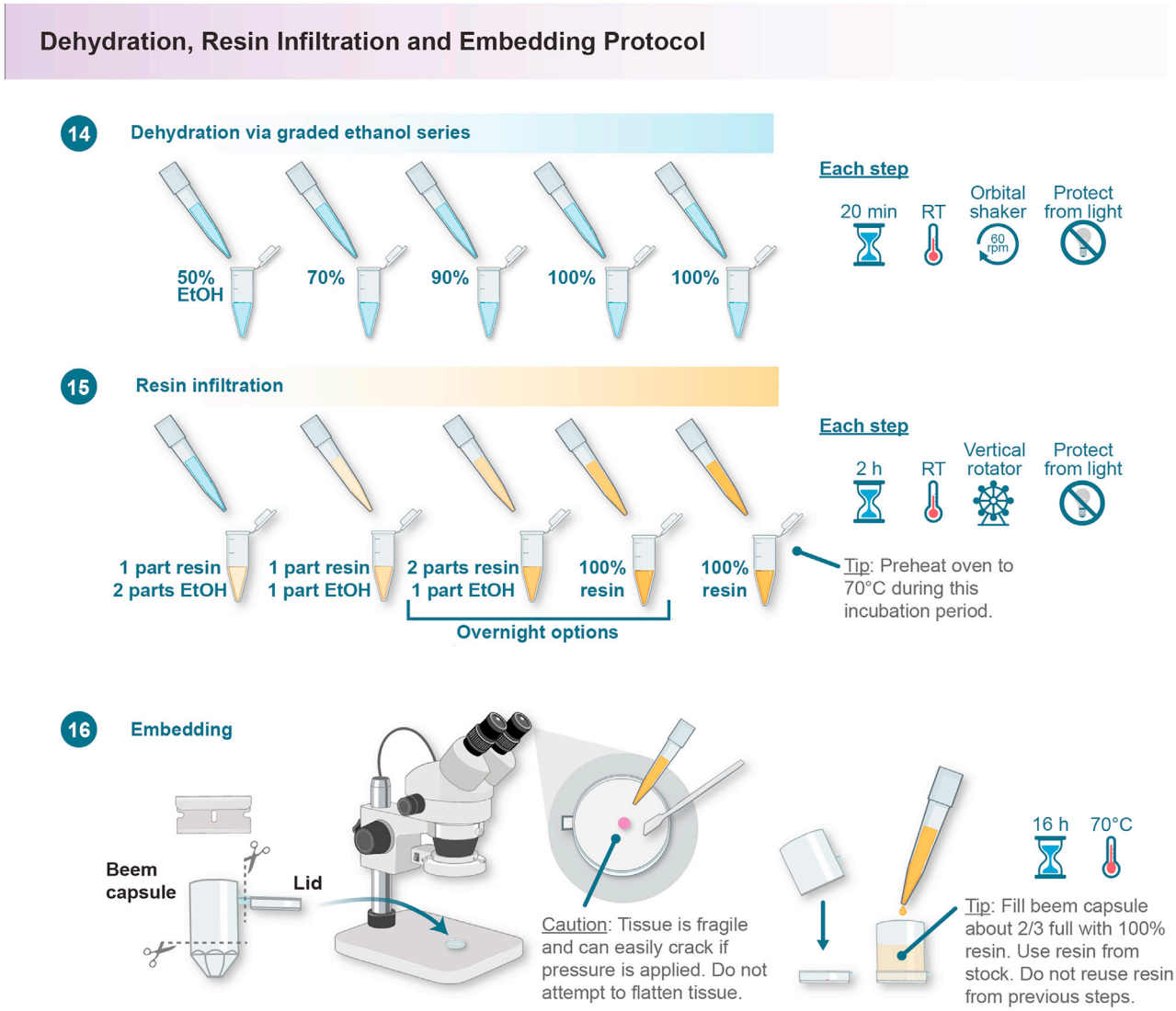


Figure 5. Dehydration, resin infiltration, and embedding workflow

Pause point: If needed, tissue punches can be left in 1:2 EtOH: resin or 100% resin overnight (12–18 h).

16. Transfer individual tissue punches into lids of embedding (Beem) capsules and gently center them under stereoscope guidance. Fit capsule body onto the lid and fill with 100% resin. Incubate samples at 70°C for 16 h.

Pause point: Polymerized tissue punches have long-term stability. Store them in their molds in a dark, dry place at room temperature and protect them from dust.

Ultrasectioning

Timing: 1 day

The following steps describe the ultrasectioning and collection of serial ultrasections onto glass coverslips (Franke and Kolotuev, 2021; Wacker et al., 2018). For further technical troubleshooting and tips,

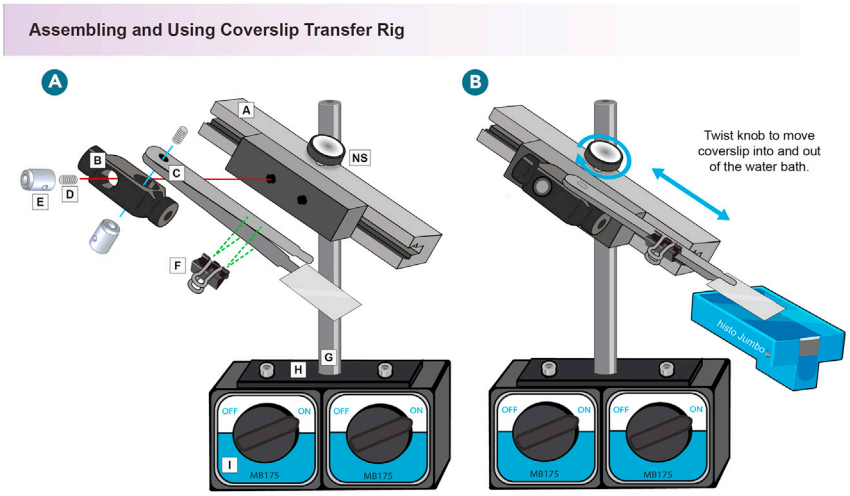


Figure 6. Assembling and using coverslip transfer rig

This instrument is designed to stabilize a coverslip in the bath of an ultrasectioning knife. The movement stage/micromanipulator allows the user to insert and retract the coverslip in a single, smooth motion.

(A–I) Parts list: (A) Movement stage, Edmund Optics (53–384); (B) Right angle clamp, THOR Labs (RA90); (C) Antistatic, plastic, flat-end tweezers, Uxcell (99301); (D) $^{1}/_{4}$"-20 set screws (2), THOR Labs (SH6MS16); (E) $^{1}/_{2}$" optical posts (2), THOR Labs (TR075); (F) Binder clip; (G) 1" optical post, THOR Labs (RS12); (H) Post clamp, THOR Labs (RM1C); (I) Switchable magnetic base, THOR Labs (MB175); NS, not shown) Flexure clamping base, THOR Labs (BA2F).

see publications on array tomography and serial sectioning for electron microscopy (Harris et al., 2006; Micheva and Smith, 2007). Figure 6 provides an illustration supporting this workflow description.

17. Trim and cut resin to produce 70 nm-thick serial section ribbons. Expand sections using chloroform vapor.
18. Secure gelatin coated coverslip in plastic forceps and guide it into the histo Jumbo diamond knife water bath (as illustrated in Figure 6).
19. Using an eyelash tool, guide sections onto the middle of the coverslip and slowly retract the coverslip from the water bath by turning the knob on the micromanipulator (as illustrated in Figure 6).

Caution: Leave sufficient space in the margins of the coverslip to accommodate the imaging objective.

20. Warm coverslips with sections facing upward on a hot plate set to 60°C for 20 min.

Pause point: Coverslips can be stored long-term prior to imaging. Store them in a dark, dry place at room temperature and protect them from dust.

Etch away resin and assemble imaging chamber

Timing: 30 min

The following steps prepare tissue sections for optimal photoswitching during STORM imaging. Here, the resin surrounding individual sections is removed allowing fluorophore exposure to the thiol-containing STORM imaging buffer. Fluorescent beads are spotted on the coverslips which will serve later as fiducial markers. The imaging chamber is assembled, filled with STORM imaging buffer, and sealed with epoxy. Prepare 10 % sodium ethoxide before you begin; sodium ethoxide solution can be reused for multiple experiments but should be remade after 1 month. Refer to Figure 7 for a workflow illustration.

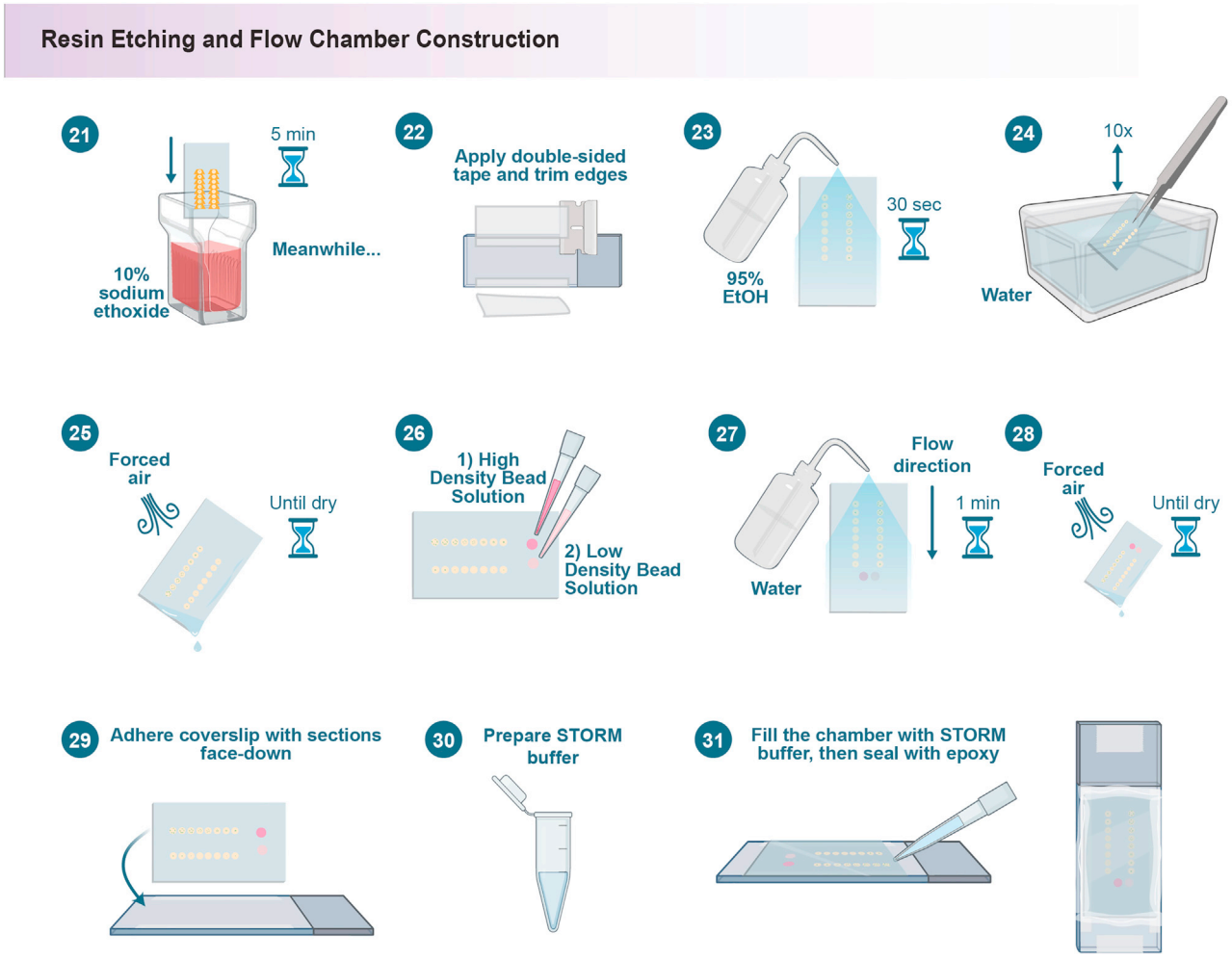


Figure 7. Resin etching and imaging chamber construction

See Figure 7.

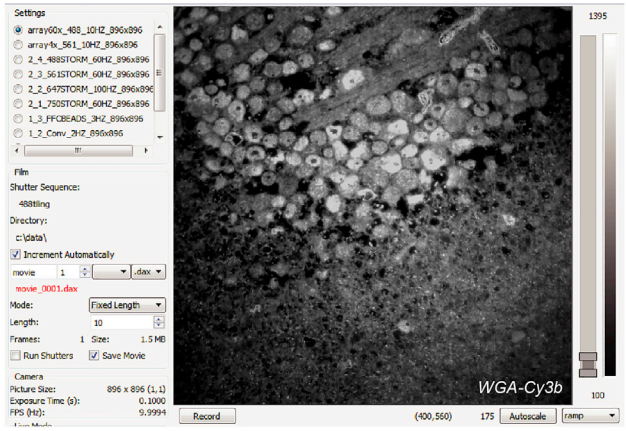
21. Immerse the coverslip in 10% sodium ethoxide for 5 min.

⚠ CRITICAL: Following step 21, samples will be difficult to see. Note sample orientation on the coverslip.

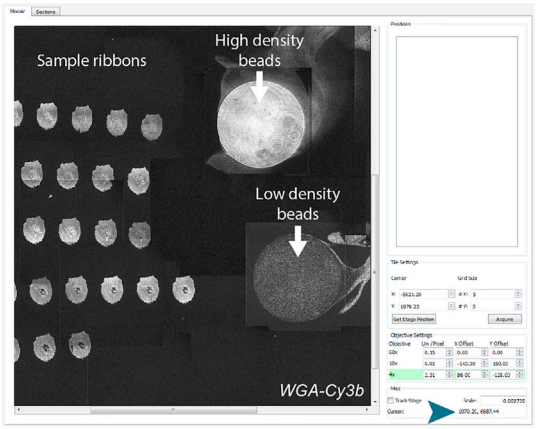
22. Meanwhile, clean a glass slide with EtOH and allow to dry completely, then apply two layers of double-sided tape to the long sides of the slide. Only cover ~5 mm of the slide edge and shave off the overhanging tape using a razor blade.
23. Remove the coverslip using forceps and immediately wash with 95% EtOH ~30 s.
24. Wash coverslip by immersing in filtered H_2O.
25. Dry coverslip using compressed/forced air.
26. Spot beads onto coverslip:
    a. Make high density bead solution by mixing 1 μL bead stock to 9 μL 1× Dulbecco's PBS (DPBS). Vortex and spin down. Apply a small droplet (~1 μL) to coverslip.
    b. Make low density bead solution by adding 750 μL DPBS to dense bead working stock. Vortex. Apply a small droplet (~1 μL) to coverslip.

Automated Image Acquisition

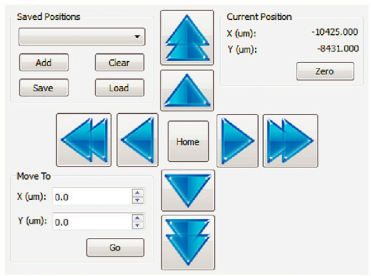
A Hal interface



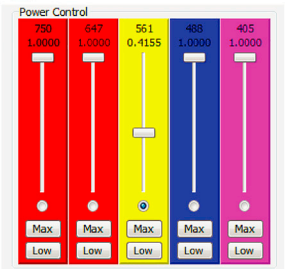
B Steve interface



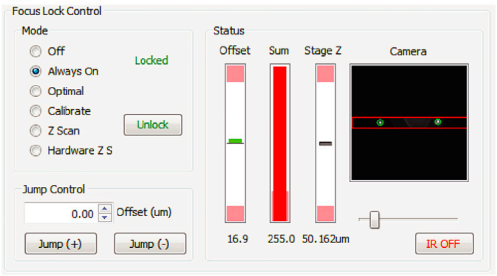
C Stage control



D Illumination control

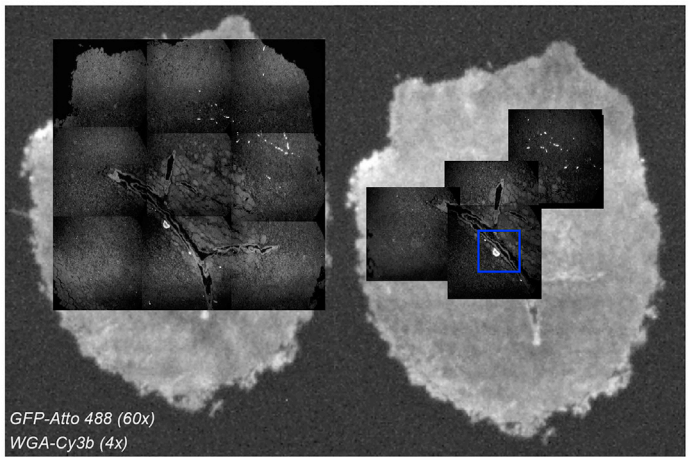


E Focus lock control



F Sections tiled under high magnification (60X)

Automated tiling *Manual tiling with ROI*



G Automated image acquisition

Dave interface

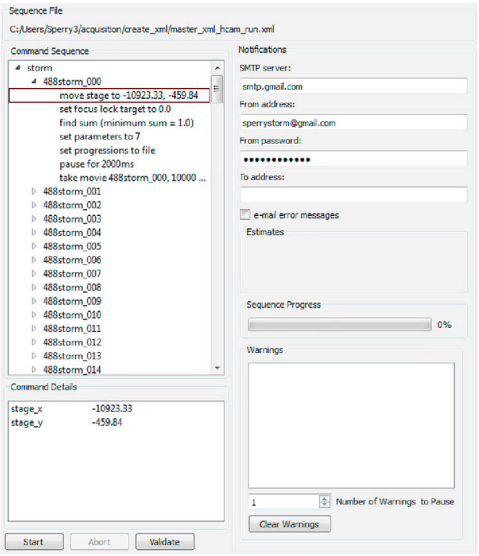


Figure 8. Automated image acquisition

(A) Screenshot of the hal4000.py user interface showing a ROI in the 561 nm channel (WGA) within a single ultrathin section of mouse retina.

(B) Screenshot of the steve.py user interface showing low magnification (4×) tiling of sample ribbons and high/low density bead spots in the 561 nm channel.

(C) Hal stage control panel.

(D) Hal illumination control panel showing the 561 nm laser engaged.

(E) Hal focus lock control panel.

(F) Two consecutive ultrathin retina sections (from B) shown tiled at high magnification (60×) in the 488 nm channel (GFP). Tiling may be automated (3×3 array, left image) or performed manually (right image) prior to within-section ROIs selection (blue box in right image).

(G) Screenshot of the dave.py user interface with a 'master_run.xml' file loaded.

⚠ CRITICAL: Dulbecco's PBS must be used to prevent bead aggregation.

⚠ CRITICAL: High/low density bead spots MUST be placed away from the serial sections.

27. Immediately wash coverslip using filtered H_2O for ~1 min.

⚠ CRITICAL: Allow water to flow in the direction away from sample so that beads do not wash into the sample.

28. Dry coverslip using forced air.
29. Place the dry coverslip tissue-side-down onto the slide with double sided tape. Use a pipette tip to apply pressure on the tape sides to seal the coverslip to the adhesive.
30. Prepare STORM imaging buffer and mix by gentle pipetting. DO NOT vortex. Avoid forcing air into the solution.
31. Draw up 160 μL of the STORM buffer into a pipette and slowly release it in the space between the coverslip and slide. Avoid creating bubbles by applying gentle pressure to the coverslip with a blunt object. Avoid cracking the coverslip when doing this.
32. Lay a piece of foil on the benchtop and push some epoxy out of the double-barrel syringe onto the foil. Mix with a pipette tip.
33. Apply a strip of epoxy to all sides of the coverslip (short sides first) to seal the imaging chamber. Place in a dark place to dry for ~5 min.
34. Test that the epoxy created a seal around the imaging chamber by applying gentle pressure with a pipette tip to the surface of the coverslip. If no buffer leaks, then the seal is good. If some buffer leaks, then patch the hole using more, freshly mixed epoxy.

⚠ CRITICAL: Be careful to not overapply epoxy and encroach on the sample area.

35. Apply a single strip of double-sided tape onto the short sides of the slide and trim off overhangs with razor blade. This is useful when securing slide to the STORM microscope. The preparation is ready for imaging.

Acquire STORM and conventional imaging data

Timing: 1–3 h to set up, 1–3 min per STORM movie (automated acquisition)

The protocol below describes the use of open-source software ("storm_control" v2019.06.28) for the collection of multi-color STORM images (https://github.com/ZhuangLab/storm-control). This software includes specific programs (hal4000.py, steve.py, dave.py) with graphical user interfaces (GUIs) for automated image acquisition (Figure 8). The software should be installed on a local acquisition computer that controls the microscope instrumentation. All three packages are operated by the user simultaneously while setting up an imaging experiment. Briefly, hal4000.py is the main acquisition controller for the microscope stage, laser illumination, focus lock, emission filter wheel, and sCMOS camera (Figures 8A and 8C–8E). Steve.py is an interface for image tiling and ROI

selection for automated imaging (Figure 8B). Dave.py is master control software (Figure 8H) that passes automated image acquisition instructions to hal4000.py based on user-determined ROIs set using steve.py. Following the STORM and conventional image acquisition users should move on to the final section of this protocol paper (Process images to generate 3D volumes).

Note: The STORM acquisition software provided here is available as an open-source resource under the MIT license.

Alternatives: Commercial instrumentation and software may be used in lieu of custom instrumentation and specific codebases we reference here (Albrecht et al., 2021; Chang, 2015; Tröger et al., 2020).

In the protocol below, we describe our approach for collecting three main types of imaging data: 1) serial section conventional, wide-field (diffraction-limited) 2) STORM data (TIRF configuration), and 3) fiducial marker images for correcting chromatic aberration and uneven illumination of the sample. The key steps are to 1) collect conventional images of all color channels in rapid succession for the ROI(s) in each physical section of the sample. This limits sample drift between color channels for conventional images in each ROI. During subsequent image postprocessing/alignment, STORM images will be correlated to their conventional image counterparts to correct for sample drift. 2) Collect STORM movies for ROI(s) across all physical sections. For each ROI, the red emitters (DY-749P1/Alexa647) are imaged first (sequentially within each ROI across the entire sample) as these dyes are susceptible to STORM buffer acidification (Dempsey et al., 2011). Then, Cy3B and Atto488 emitters are imaged sequentially for each ROI in a second image pass across the sample. Sample drift that occurs during each STORM movie acquisition is corrected as part of the single-molecule fitting analysis by back correlating binned movie frames to the start of the movie. 3) Collect fiducial marker images that will later be used to correct chromatic aberration in conventional and STORM images. Once all acquisition processes are complete (time varies depending mainly on number of sections and colors imaged), we begin processing images for 3D reconstruction (steps for which are outlined in the final section of this protocol).

See Figure 8.

36. Power on the microscope and lasers.
37. Prepare objectives for imaging:
    a. Clean the oil immersion objective. Apply 2–3 drops of 100% methanol to a folded piece of lens paper and gently wipe the surface of the objective.
    b. Rotate the turret to position the 4× objective in the light path. The 4× objective will be used first to collect a low-magnification tiled mosaic of the sample.
38. Secure the imaging chamber in the stage holder.

⚠ CRITICAL: Sample should be immobile once loaded in the stage. Use a top-side clamping stage insert or double-sided tape applied to the glass slide (Figure 7) to secure the specimen in the stage as described (previous section "Etch away resin and assemble imaging chamber", step 35).

39. Launch hal4000.py acquisition software (hereafter referred to as Hal).
40. Load settings .xml files by dragging and dropping settings files into the main Hal interface (Figure 8A) or selecting 'File/load/settings' and then selecting desired settings files.
41. Engage the low magnification array setting in Hal.
42. Use the 'Illumination' control panel (Figure 8D) to engage the 561 nm laser, locate the bead fields on the coverslip and focus the objective.
43. Use the 'Stage Control' panel (Figure 8C) to zero the stage position.
44. Create a low-magnification mosaic of the bead fields and sample (serial sections):

a. Launch 'Steve.py' software (hereafter referred to as 'Steve') (Figure 8B) and place cursor at or near the 0, 0 coordinates and press the spacebar to take an image. Cursor coordinates appear on the lower, right-hand side of the Steve interface (Figure 8B, arrowhead).
b. Continue taking images of the field in manual or semi-automated modes (Figure 8F):
   i. manual imaging mode: click on an ROI and press the spacebar to take an image.
   ii. semi-automated mode: click on an ROI and press keypad #3 or #9 for automated 3×3 or 9×9 image tiling, respectively. Press the spacebar to interrupt the sequence.

Note: Adjust laser output power empirically to maximize the image intensity without saturating the image.

Note: User may navigate the sample view in Steve.py by right-clicking on an ROI and selecting "Go to Position".

c. Finish mosaic and save. Adjust the stage position back to the sparse bead field.

45. Create a high-magnification mosaic of the bead fields and sample (serial sections):
    a. Lower the 4× objective away from the sample by turning the focus knob on the microscope.
    b. Rotate the turret to position the oil-immersion objective in the light path. The oil-immersion objective will be used throughout the rest of the image acquisition.
    c. Place a droplet of immersion oil on the objective.
    d. Focus on the sparse beads:
       i. Engage the high magnification array setting in Hal.
       ii. Using the illumination control panel in Hal, turn on a visible imaging laser (488 nm or 561 nm) and bring the sample into focus.
       iii. Use the 'Focus lock' control panel (Figure 8E) to lock the focal plane.
    e. Set TIRF angle to maximize signal-to-noise at low illumination power.
    f. Reimage selected regions (both sample and beads) of the low magnification mosaic at high magnification.

Note: Steve allows users to correct offsets between low and high magnification by adjusting the mosaic positions within the 'Objective Settings' panel.

46. Select sample ROIs for automated imaging (manual) (Figure 8F):
    a. Use mouse cursor to hover over an ROI in first physical section.
    b. Right click and select 'Record position'.
    c. Repeat for each ROI across all serial sections.
    d. When all positions are selected, click 'File' and 'Save positions' to save a 'positions.txt' file.
    e. Delete all recorded positions in Steve.

Note: Select ROIs in sequential order of physical sectioning to simplify downstream alignment processing. To prevent unwanted bleaching during acquisition of serial section images, select the order of ROIs to begin imaging the end of the sample ribbon at the furthest end of the coverslip in the direction of the TIRF beam.

47. Select bead ROIs for automated imaging (manual):
    a. In Steve, use the mouse cursor to hover over ROIs in bead fields.
    b. Starting in the sparse bead field, right click within a region away from the edge of the bead spot and select 'Record position'.
    c. Move cursor to new position within the bead spot (away from the edge and the first position), right click and select 'Record position'.
    d. Move cursor to center region of the dense bead field, right click and select 'Record position'.
    e. When positions are selected, click 'File' and 'Save positions' to save a 'bead_positions.txt' file.

48. Prepare illumination power settings for conventional image acquisition to ensure that images are not dim/saturated:
    a. Engage the 'Conv' setting in Hal.
    b. Focus on a representative ROI within the sample.
    c. Adjust laser powers using the illumination control panel for each channel to be imaged.

⚠ CRITICAL: Avoid saturating dense bead images. Ensure sufficient illumination intensity to use the full dynamic range of the camera.

49. Prepare illumination power settings for sparse bead field image acquisition to ensure that images are not dim/saturated:
    a. Engage the 'Regbeads' setting in Hal.
    b. Focus on a representative ROI within the sparse bead field.
    c. Adjust 750 nm laser power using the illumination control panel to visualize IR beads in 750 and 647 channels.
    d. Adjust 488 nm laser power using the illumination control panel to visualize visible beads in the 647, 561, and 488 nm channels.

⚠ CRITICAL: Avoid saturating sparse bead images.

50. Prepare illumination power settings for dense bead field image acquisition to ensure that images are not dim/saturated:
    a. Engage the 'FFCbeads' setting in Hal.
    b. Focus on a representative ROI within the dense bead field.
    c. Adjust 750/647/561/488 nm imaging laser powers using the illumination control panel to visualize dense beads in 750/647/561/488 nm channels, respectively.

⚠ CRITICAL: Avoid saturating dense bead images. Ensure sufficient illumination intensity to use the full dynamic range of the camera.

51. Prepare illumination power settings for STORM image acquisition to optimize photoswitching and minimize movie length:
    a. Engage the '750STORM' setting in Hal.
    b. Focus on a representative ROI within the sample.
    c. Using the illumination control panel, adjust the 750 nm laser power to maximum and the 405 nm laser to minimum.
    d. In Hal, change the directory filename to '750storm' and set the working directory to 'Acquisition\create_xml\powertest\'.
    e. Set 'Mode' to 'Run_til_abort'.
    f. Click 'Record' to begin manual STORM movie acquisition.

    Note: Each wavelength specific .xml file calls a preset shutter file that engages the appropriate imaging (750/647/561/488 nm) and activation (405 nm) lasers.

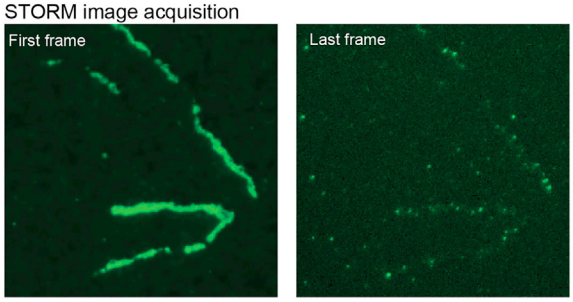
    g. Manually ramp 405 nm laser power using the illumination control panel to reactivate fluorophore switching to maintain optimal emitter density.
    h. Pressing 'Stop' ends the movie recording and saves the power progression file.
    i. Repeat steps 16 (a-h) for 647 nm, 561 nm, and 488 nm STORM movies. Change the directory filenames for each movie according to wavelength using the format specified above.

    Note: Cy3B (561 nm) and Atto488 (488 nm) probes do not require 405 nm illumination for reactivation.

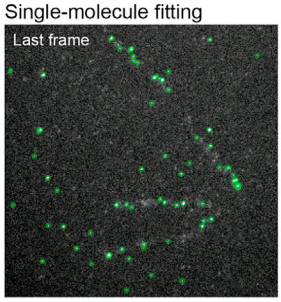
Volumetric STORM Data Processing to Generate 3D Volumes

A STORM image generation

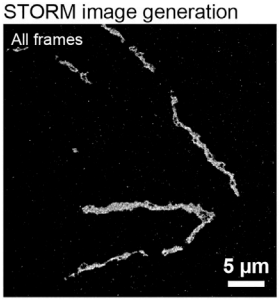
STORM image acquisition



Single-molecule fitting



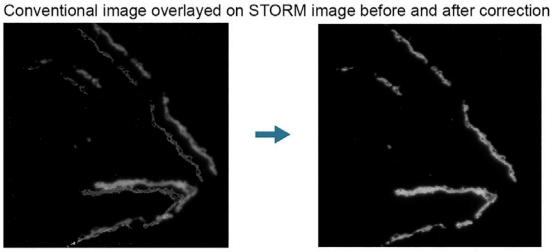
STORM image generation



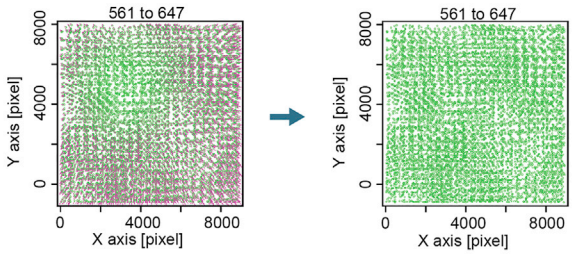
B Single-section drift and chromatic correction

Drift correction from STORM to conventional

Conventional image overlayed on STORM image before and after correction

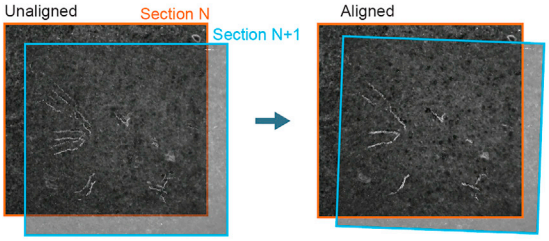


Offsets before and after fiducial-guided chromatic abberation correction

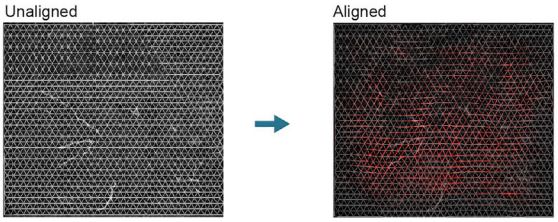


C Serial-section z-alignment

Rigid XY-alignment



Elastic Z-alignment



D Automated graphical user interface for STORM Data Processing

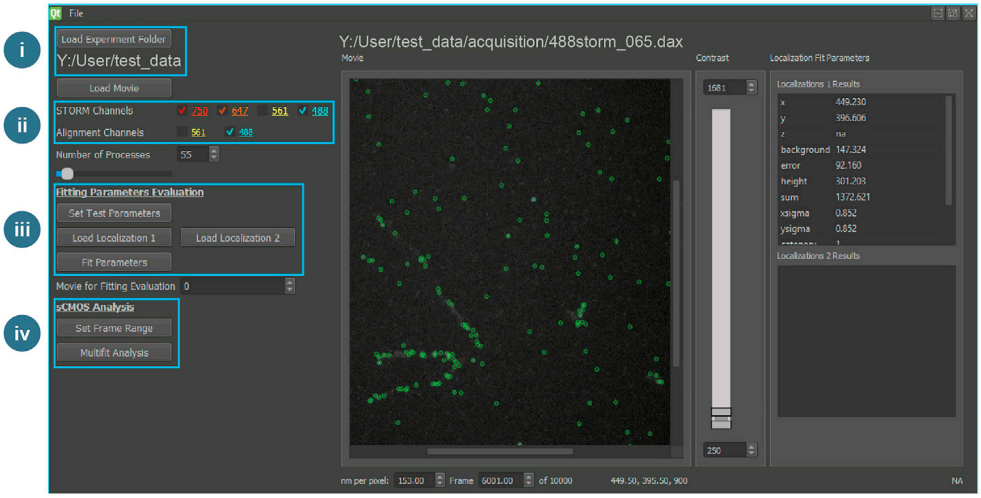


Figure 9. Volumetric STORM data processing to generate 3D volumes

(A) The workflow for STORM image generation is shown for a single ROI containing YFP-expressing retinal ganglion cell neurites imaged in the 488 nm channel. Single molecules in STORM movies are localized and drift corrected across each movie and localization information for all molecules is exported to a final reconstructed image file. Left to right: first frame of STORM movie, last frame of STORM movie, 3D-DAOSTORM SMLM fitting result for last frame of STORM movie, and the final STORM image.

(B) ROI (from A) shown before and after STORM-conventional drift correction (left panel) and chromatic aberration correction (example shown for fiducial bead 561 nm emitters mapped to the 647 nm channel, right panel).

(C) ROI (from A) shown before/after rigid (left panel) and elastic alignment (right panel) to an adjacent serial section.

(D) Screenshot of "STORMpro", our open-source graphical user interface (GUI) for automated volumetric STORM data processing. Operation of the interface (steps i-iv) is described within the text and a readme.txt file accompanying the GUI (see key resources table).

Alternatives: The above steps 51 (a-i) describe the setting of custom 'power progression' files to control illumination settings during STORM movie acquisition. Hal also allows users to engage linear or exponential power ramping using the 'Progressions' control panel.

52. Begin automated image acquisition of all conventional, bead, and STORM files:
    a. In main Hal interface, set working directory to a local storage drive.

⚠ CRITICAL: Ensure local storage drive has sufficient capacity for image acquisition. Alternatively, users should set up automated transfer routine to relocate files following acquisition. Overfilling the drive will cause the acquisition control software to crash. See "limitations" section below for a discussion on raw data sizes.

    b. Adjust desired imaging parameters (e.g., number of frames, frame rate, focus lock position, illumination control, etc.) for multi-channel acquisition in a 'master.xml' file.
    c. Launch 'Master_xml_generator.py'.
    d. Select the desired 'master.xml' file from Step B above (.xml format) containing imaging parameters.
    e. Change file name and save out final 'master_run.xml' file.
    f. Launch 'Dave.py' software (Figure 8G).
    g. Click 'File/load' and select the 'master_run.xml' file for automated imaging.
    h. Click 'Validate' to confirm the proper function of the automated image acquisition.
    i. Select 'Start' to begin automated imaging.
    j. Output includes .dax files, which will be used in step 57 below.

Alternatives: Use commercial instrument with automated image acquisition software to set imaging parameters for selected ROIs. Alternative open-source software may also be used.

Note: Adjust the total laser output power as needed to ensure optimal image quality during image acquisition. For conventional image acquisition this may require reducing laser output while for STORM movie acquisition power output should be increased to achieve sufficient density at the sample plane (1–3 kW/cm^2).

Process images to generate 3D volumes

Timing: 1–7 days (largely automated)

The following section discusses the transformation of serial-section 2D STORM and conventional imaging data into aligned 3D imaging volumes (Figure 9). The first step in the pipeline is to identify single molecule positions within STORM movies using single-molecule fitting software (Figure 9A). The data generated in this protocol were fit using 3D-DAOSTORM (Version 2.1, Key Resources; Babcock et al., 2012), which is one of many open-source solutions for single-molecule detection (Sage et al., 2019). Once molecules have been identified and corrected for drift that occurs during STORM image acquisition, single molecule positions and emitter PSF information are used to generate STORM image files.

During serial-section imaging there is a temporal offset between the collection of STORM movies that introduces XY drift between images collected in different color channels for the same ROI. To correct for this lateral movement, conventional images of all colors are acquired in rapid succession for each ROI to eliminate the impact of drift (see section acquire STORM and conventional imaging data) and image correlation is used to align each STORM image to its corresponding conventional image (Figure 9B).

A separate imaging distortion, chromatic aberration, creates nanoscale offsets between the color channels that must be corrected in multi-color super-resolution imaging. Here, images of fiducial beads (fluorescent nanospheres) acquired across all color channels are used to create polynomial warping transforms that map all color channels to a shared coordinate system (Figure 9B). These corrections are applied to all conventional and STORM images for each ROI.

Following drift and chromatic aberration corrections, images of physical sections must be aligned in three dimensions. This is accomplished by matching image features between adjacent ROIs and applying rigid/elastic registration algorithms to shift/warp sections into alignment across the image series (Figure 9C).

To help users accomplish these tasks, we have packaged these steps into an open-source graphical user interface (GUI) (Figure 9D) named "STORMpro" that automates the entire pipeline in Python (key resources table). Below, we describe the use of the interface, which is accompanied by documentation to assist users. The underlying source code is also available to users who may wish to modify aspects of the pipeline for custom processing. Links to repositories hosting all analysis code are listed in the 'Software and algorithms' section of the key resources table.

See Figure 9.

53. Download and install "STORMpro" custom GUI for volumetric STORM image processing (see key resources table).
54. Download and Install FIJI (Schindelin et al., 2012).
55. Download and install FIJI Z-align macros (https://github.com/SpeerLab/STORM-UI/tree/main/analysis_scripts/macros) and XML files (https://github.com/SpeerLab/STORM-UI/tree/main/ex_pipeline/XMLs).
56. Open STORMpro and load the path to the data for analysis (Figure 9i).
57. Load any chosen .dax STORM file into the visualizer. This enables interactive visualization of the raw STORM data using keyboard entries and mouse control to scroll through the movie. Full instructions are specified in the Readme.txt file accompanying the Storm-Analysis software.

    Note: .dax files may be dragged/dropped into the visualizer to load.

58. Select all appropriate "STORM channels" corresponding to the data collected (Figure 9ii).
59. Select the appropriate "Alignment channel", which is the imaging channel to be used for Z-section alignment (Figure 9ii).
60. Select the appropriate "Number of processes" to run concurrently during the STORM fitting analysis. This number is limited by the total threads available for computation on the analysis machine.
61. Set 3D-DAOSTORM fitting parameters and test the fit quality (Figure 9iii):
    a. Select "Set test parameters" to load the multichannel XML files specified fitting parameters.
    b. Adjust start/end frames in the corresponding channel of the selected .dax file to analyze a small sample portion of the selected STORM movie. Typically, 10–20 frames are selected in the middle or near the end of the .dax STORM movie.
    c. Adjust 3D-DAOSTORM SMLM fitting parameters (e.g., frame range, background, sigma, drift correction, etc.) in .xml files by manually inputting desired values. Guidance for

determining these parameters is available (https://storm-analysis.readthedocs.io/en/latest/parameters.html).

d. Select "Save XMLs" to save the selected parameters to the XML directory.

e. Set "Movie for fitting evaluation" to desired .dax file for analysis.

62. Select "Fit Parameters" to run test fits on the chosen .dax movie.

63. Load the output .hdf5 single-molecule localization list by dragging/dropping into the visualizer or selecting "Load localization 1" to select the desired .hdf5 file.

64. Inspect the fit quality by visualizing the fit beacons (green circles in Figures 9A and 9D) to determine if single-molecules are over-fit (fits with no single-molecule PSFs present) or under-fit (raw PSFs present with no corresponding fits).

65. Based on the fit quality determined in step 64, repeat steps 61–64 to optimize the fitting parameters.

66. Inspect raw .dax STORM movies in renderer software to determine the frame range for full .dax file single molecule fitting. Select "Set Frame Range" and enter the start and end frames for full STORM movie analysis (Figure 9iv).

Note: The selected start frame for full STORM analysis should be set to a number where the majority of single molecules have begun to photoswitch.

67. Select "Multifit Analysis" to process the entire 2D dataset to a 3D volume (Figure 9iv):

a. When the first FIJI subprocess launches, input rigid registration parameters to continue the alignment. Information on rigid alignment in FIJI is found here: https://imagej.net/imaging/registration.

b. A Python subprocess opens displaying the compressed final image field. Drag cursor within the displayed image to set a sample ROI for cropping the image stack. Release the cursor to identify the ROI. Close the window and press "Escape" on the keyboard to crop the dataset.

c. When the second FIJI subprocess launches, input elastic registration parameters to continue the alignment. Elastic alignment is implemented in TrakEM2 based on the original publication (Saalfeld et al., 2012). Information on elastic alignment parameters is found here: https://imagej.net/plugins/elastic-alignment-and-montage.

Alternatives: Single molecule fitting – a list of available renderers and single-molecule fitting algorithms is referenced in the 'Software and algorithms' section of the 'key resources table'. Use commercial or preferred open-source software to interact with raw SMLM data, generate SMLM localization lists, and convert these to image files.

Alternatives: Drift/aberration correction – use commercial or other open-source software to correct sample drift for each ROI by cross-correlating each individual STORM image to its conventional image counterpart. Use fiducial bead imaging data to generate a non-linear warping transform that registers beads across color channels to correct chromatic aberration. Apply the resultant transform to all conventional and STORM images for each ROI.

Alternatives: Serial stack alignment – use alternative commercial or open-source software to perform 3D serial-section alignment.

Note: We continue to develop this software to improve functionality, ease of use, and open-source accessibility. Updates will be made available on GitHub (see 'Software and algorithms' section of the key resources table).

EXPECTED OUTCOMES

By following this protocol, users can expect to collect volumetrically aligned, multi-color STORM and conventional (diffraction limited) image stacks. In Figure 10, we show maximum projection images of

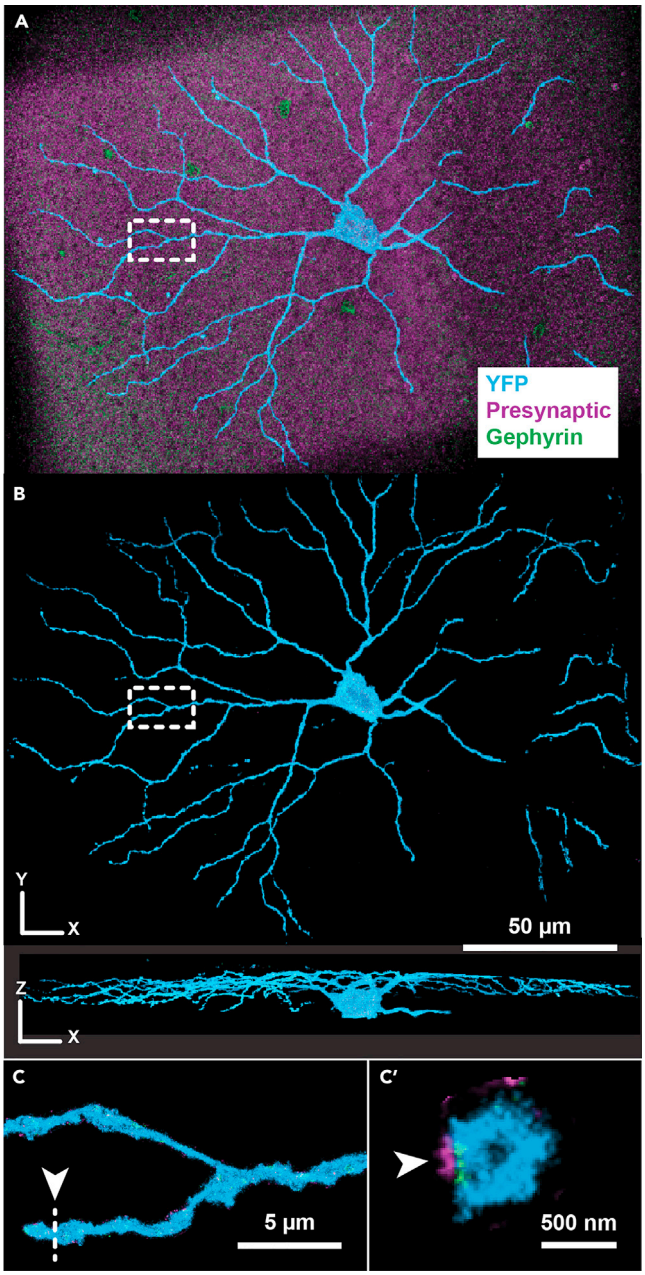


Figure 10. Volumetric super-resolution imaging by serial ultrasectioning and STORM captures whole cells with synaptic resolution

(A) Maximum projection image of a YFP-expressing retinal ganglion cell with presynaptic and postsynaptic (gephyrin) labeling.

(B and C) Segmented images (top: en face; bottom: side view) and insets (C, C′) highlight synaptic inputs (arrowhead).

a YFP-expressing retinal ganglion cell with presynaptic active-zone protein and postsynaptic gephyrin labeling, demonstrating volume and resolution capabilities of the approach. Entire neurons and surrounding synaptic fields can be imaged with nanoscale resolution and molecular specificity based on target IHC labeling. In Figure 11, a separate example, we show a maximum projection transverse section image across the retina highlighting the subsynaptic molecular organization of photoreceptor terminals. The image volume captures nanoscale molecular details for thousands of individual synapses in the outer and inner plexiform layers.

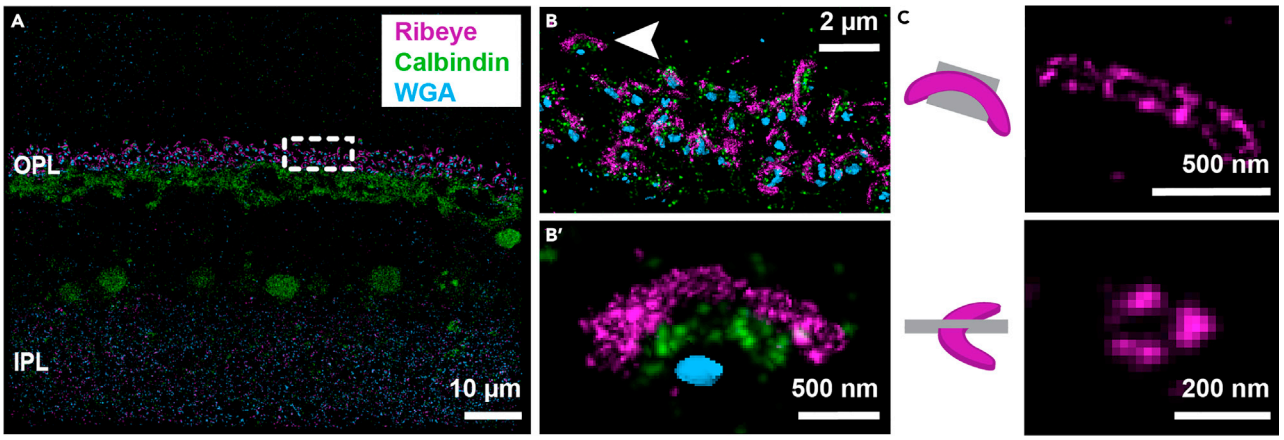


Figure 11. STORM images of ribbon synapses in the mouse retina

(A) Maximum projection image of the mouse retina in cross section. OPL, outer plexiform layer; IPL, inner plexiform layer.

(B) Magnified view (~8×) of the OPL in (A). (B′) Each photoreceptor synaptic terminal is seen as clustering of ribeye (ribbon synapse protein), calbindin (horizontal cell label), and WGA (bipolar cell dendrite label).

(C) Orthogonal cross section views (top, bottom) through a ribbon synapse in the OPL.

In STORM imaging experiments, differences in dye properties impact the final image resolution (Dempsey et al., 2011). Users should consider wavelength-dependent differences that influence single-molecule localization precision and biological measurements. In Figure 12, we illustrate this point in the context of synapse imaging in mouse brain tissue. We immunolabeled synapses in the dorsal lateral geniculate nucleus (dLGN) of the mouse thalamus with primary antibodies against presynaptic (bassoon and vesicular glutamate transporter 2 [VGlut2]) and postsynaptic targets (Homer1). We tested two alternative secondary staining conditions that flipped the dyes, DY-749P1 or Alexa647, used to label the synaptic proteins Homer1 and Bassoon (Figure 12A). We then collected STORM images and measured the synaptic properties (density, volume, signal intensity) for thousands of synapses across ~ 40,000 cubic microns of dLGN tissue for both conditions (Figures 12B–12D). Results indicate the measured synaptic densities are independent of dye selection, while synaptic cluster volume and total within-cluster signal intensity is greater when imaging with DY-749P1 compared with Alexa647 (Figures 12C and 12D). This is consistent with an increase in the size of the point spread function and decrease in photon emission of red-shifted probes compared to Alexa647 dyes (Dempsey et al., 2011).

QUANTIFICATION AND STATISTICAL ANALYSIS

For the quantitative comparisons shown in Figure 12, synaptic clusters were identified using MATLAB 'conncomp' function and filtered based on signal intensity, cluster volume, and apposition with other channels. Volume and total signal intensity were given by MATLAB 'regionprops' function. Data are from a single biological replicate for each staining condition.

LIMITATIONS

Our approach is limited to fixed specimens and is incompatible with live imaging. The method requires access to an ultratome and a STORM (or related) super-resolution microscope. Skilled serial-ultrasectioning is necessary for sample preparation. The final axial image resolution is dependent on the physical section thickness.

The file size of raw SMLM data files is large and requires dedicated hard drives or server storage space for volumetric data collection. Briefly, STORM "movies" are data files containing individual camera image frames collected over time. The final size of each individual STORM movie depends on the camera field size multiplied by the number of frames collected. Based on this, the final size of

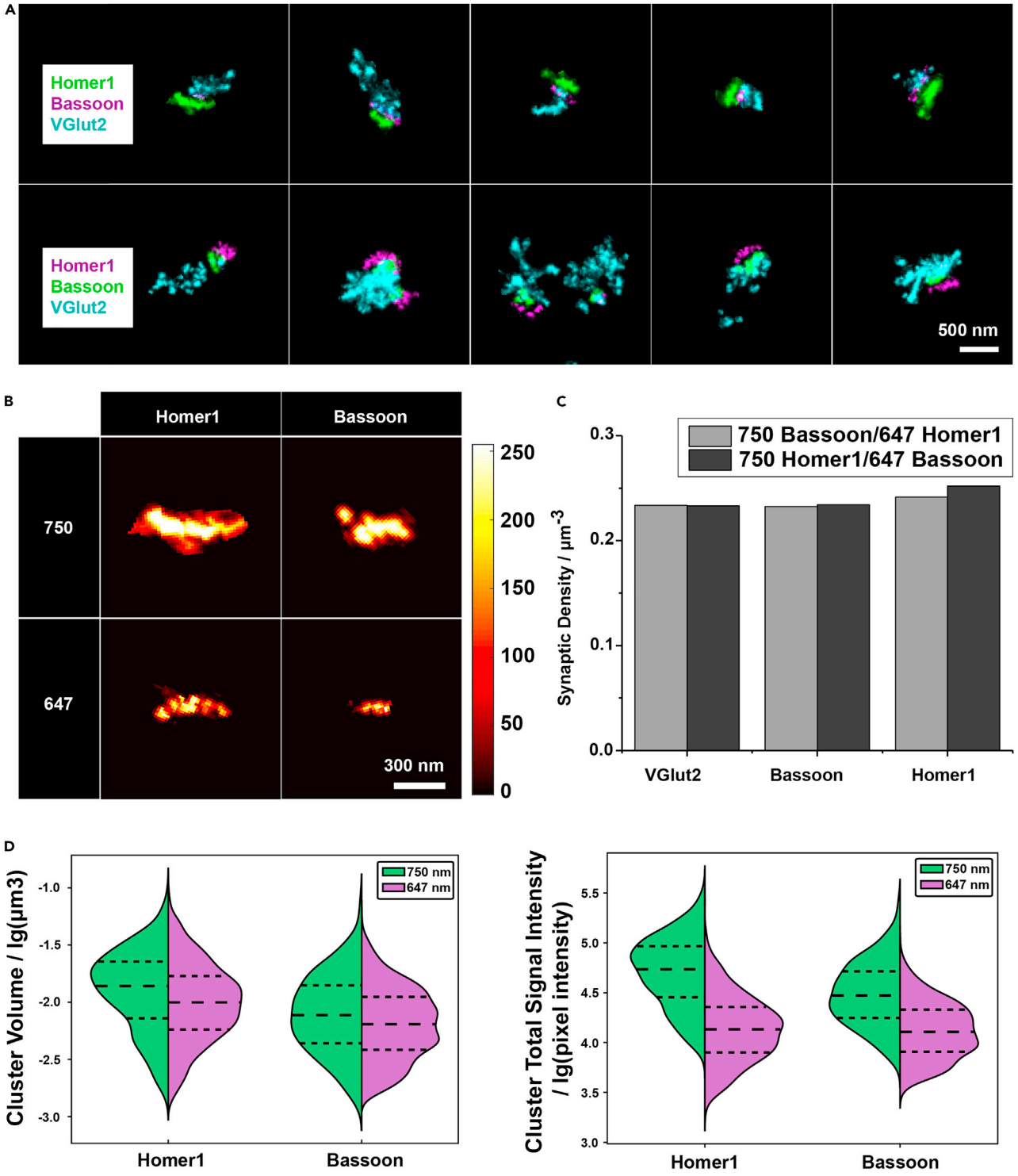


Figure 12. Dye properties impact image quality

(A) Synapses in the mouse dLGN are labeled with presynaptic (Bassoon, VGluT2) and postsynaptic (Homer1). Two labeling results are shown with flipped secondary staining conditions (top panel: Homer1-DY-749P1, Bassoon-Alexa647; bottom panel: Bassoon-DY-749P1, Homer1-Alexa647).

(B) Maximum projection images of representative synaptic proteins imaged with DY-749P1 or Alexa647. Scale = 8-bit voxel intensity values (0–255).

(C) Measured synaptic protein cluster density all imaged synaptic markers across the two labeling conditions.

(D) Measured synaptic protein cluster volume and signal intensity distributions for Homer1 and Bassoon across the two labeling conditions.

a volumetric serial-section reconstruction is a product of the number of STORM acquisition channels (colors imaged) multiplied by the number of ROIs imaged.

In our experiments, a typical number of ROIs imaged in a single session ranges from 10–250. Typical movie lengths for individual color channels are between 3–10K frames each. We routinely perform 4 color imaging experiments with ~100–200 ROIs, which generate ~5–10 TB of raw data in a single automated imaging run. For data storage, we recommend that users adopt a scalable Network Attached Storage (NAS) solution, examples of which can be found in the key resources table.

During STORM single-molecule fitting, the provided 3D-DAOSTORM fitting software runs parallel Python jobs on separate computer cores and the total processing time decreases with the number of cores used. Additionally, loading large volumetric STORM image data sets for visualization or image analysis requires significant random-access memory (RAM, >250GB). The data presented in this protocol were analyzed using a dual Intel chip machine with 28 total cores and 8 x 64 GB of DDR4 RAM (512 GB total).

TROUBLESHOOTING

Problem 1

No or low absorbance at the expected wavelengths on the spectrophotometer in the secondary antibody conjugation step. This suggests that the conjugation reaction did not occur as expected and dye molecules were not properly linked to antibody/WGA probes. To rule out the possibility that an improperly washed NAP-5 column and/or an improperly blanked spectrophotometer interfered with the detection of eluted antibody, ensure that columns are thoroughly washed, and the spectrophotometer is properly calibrated/blanked (see section on prepare fluorescent dye conjugates for IHC).

Potential solution 1

Make fresh sodium bicarbonate and repeat the conjugation procedure. Ensure the NAP-5 columns are thoroughly washed (3 full washes with 1× PBS). Make sure the spectrophotometer is set up correctly and blanked with antibody dilution buffer (PBS), and the cuvette is properly oriented.

Potential solution 2

Reconstitute secondary antibodies and store 80 μL aliquots at −80°C. Avoid freeze-thaw cycles. Reconstitute dyes and store 10 μL aliquots at −80°C. Avoid repetitive freeze-thaw cycles as these degrade the NHS-ester reaction efficacy over time.

Problem 2

Polymerized samples are still liquid after baking *OR* brittle and crack/flake during ultrasectioning (associated with Dehydrate and embed samples in resin).

Potential solution

Problems with uncured or poorly cured blocks could be due to improperly mixed resin. Prepare resin per manufacturer's recommendations several hours (~4) before needed and keep resin on a rotator to ensure complete mixing. Problems with brittle samples could be caused by residual water in the sample during the resin embedding step. Make sure the tissue is taken through the recommended series of ethanol and is fully dehydrated.

Problem 3

Sections not adhering to the coverslip or damaged sections (associated with Coat glass coverslips and/or Etch away resin and assemble imaging chamber steps 21–25)

Potential solution 1

Section loss may be due to poorly coated coverslips. Remake coverslips with fresh gelatin coating solution.

Potential solution 2

Lost/damaged sections could be caused by the presence of water in the sodium ethoxide solution. Remake fresh etching solution periodically (~4–6 weeks) when stored at room temperature.

Problem 4

Loss of auto-focus from section to section during image acquisition (associated with Acquire STORM and conventional imaging data step 45 and/or Ultrasectioning).

Potential solution 1

For extended imaging (8–24 h), ensure that sufficient immersion oil is added to the objective lens to prevent oil drying or thinning when scanning across the specimen over time. Ensure the external surface of the coverslip and the objectives are clean.

Potential solution 2

Loss of optimal focus in some sections could be due to variable section thickness. Refer to standard ultrasectioning troubleshooting to ensure uniform section thickness.

Problem 5

Photoswitching of single fluorescent molecules is slow or does not occur at all (associated with STORM buffer solution, Etch away resin and assemble imaging chamber steps 30–31, and/or laser power density steps 48–51).

Potential solution 1

Poor photoswitching is often caused by the use of old STORM buffer solution. Remake fresh STORM buffer solution immediately prior to each imaging experiment. Do not vortex. Pipette gently to mix and avoid introducing oxygen into the solution.

Potential solution 2

The chemical etching process exposes the fluorophores to the thiol-containing imaging buffer for STORM imaging. Without proper etching, the dyes will not be exposed to the imaging buffer and photoswitching could be slow or absent. Remake the sodium ethoxide solution and ensure proper etching time (5 min). It is crucial that the sodium ethoxide solution is not contaminated with water (see problem 3).

Potential solution 3

SMLM methods require high-power excitation to drive optimal single molecule photoswitching. Ensure the imaging system achieves sufficient power density (1–3 kw/cm^2) at the sample plane for each imaging wavelength.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Colenso M. Speer (cspeer@umd.edu).

Materials availability

No new materials were generated in this study.

Data and code availability

The datasets in the current study have not been deposited in a public repository because of file size limitations but are available from the corresponding author on request. The code generated during this study is available on GitHub (https://github.com/SpeerLab/STORM-UI).

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AUTHOR CONTRIBUTIONS

T.V. and J.A.M. co-wrote the manuscript, created the figures, and contributed STORM data. C.Z. contributed STORM data and contributed writing for the manuscript. V.A. developed the STORM analysis graphical user interface and J.Y. contributed Python code for image processing. C.M.S. designed the protocol, contributed STORM data, and co-wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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