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Ultrastructural detection of neuronal markers, receptors, and vesicular transporters

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Abstract

At the ultrastructural level, axon terminals containing synaptic vesicles are clearly observed. These axon terminals (presynaptic component of a synapse) may be seen establishing contacts (synapses) with cell bodies, axons or dendrites (postsynaptic component of a synapse). By a combination of ultrastructural analysis and immunodetection of molecules, it is possible to determine the subcellular distribution of specific cellular markers (i.e., enzymes), neurotransmitters (within synaptic vesicles), vesicular transporters (in association with vesicles), and receptors (within the presynaptic or postsynaptic component of a synapse). Here we will provide detailed protocols that facilitate the ultrastructural detection of cellular markers, receptors, and vesicular transporters. These protocols include brain ultrastructural immunodetection of one, two or three different types of molecules prior to brain tissue processing for ultrastructural analysis (pre-embedding immunolabeling), brain molecular immunodetection after tissue processing for ultrastructural analysis (post-embedding immunolabeling), or molecular immunodetection in purified synaptic vesicles.

Keywords

ultrastructure; synaptic vesicles; neurotransmitter; receptor; vesicular transporter; synapse

Introduction:

Brain analysis by electron microscopy has provided fundamental information on ultrastructural changes that occur in development, brain disorders (i.e., Alzheimer's disease) or in response to external agents (virus, toxins, drugs of abuse, etc.). Electron microscopy analysis has also been fundamental in advancing our knowledge on connectivity among different neurons, and essential for advancing our understanding on brain function. By a

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Conflict of Interest

The authors declare that they do not have any conflicts of interest (financial or otherwise) related to the data presented in this manuscript.

combination of ultrastructural analysis and immunodetection of molecules, it is possible to determine the subcellular distribution of specific cellular markers (i.e., enzymes), neurotransmitters (within synaptic vesicles), vesicular transporters (in association with vesicles), and receptors (within the presynaptic or postsynaptic component of a synapse).

In this unit, we provide four detailed protocols that facilitate the ultrastructural detection of cellular markers, receptors, and vesicular transporters. Basic Protocol 1 allows the ultrastructural immuno detection of a single type of molecule (single immunolabeling technique) prior to brain tissue processing (pre-embedding) for subsequent electron microscopy analysis. In this pre-embedding immunolabeling protocol, we describe the necessary steps for immunodetection of molecules by using specific antibodies against the molecule of interest (antigen), and further detailed two methods for the detection of antigen-antibody complexes, one method relies on the detection of electron dense product resulting from peroxidase enzymatic reaction (immunoperoxidase labeling) and the other method relies on the detection of colloidal gold particles after their intensification with silver exposure (immunogold – silver intensification labeling). Basic Protocol 2 is also a pre-embedding immunolabeling technique, but allows the ultrastructural immuno detection of two to three different molecules (double or triple immuno-labeling) by using a combination of peroxidase immunolabeling and gold-silver immunolabeling. We expand on the application of both double and triple immuno-labeling techniques for ultrastructural detection of molecules in both wild type rats and cre transgenic mouse lines. These transgenic mice are endowed with the capability to induce cell specific expression of reporter molecules (i.e., mCherry) within the entire neuron, including axon terminals. Basic Protocol 3 allows brain ultrastructural immunodetection of cellular marker on brain ultrathin sections collected after tissue processing (post-embedding) for subsequent electron microscopy analysis. Basic Protocol 4 allows immuno-gold detection of molecular markers within purified organelles. In addition of identifying the subcellular neuronal distribution of transmitters, transporters or receptors in brain tissue, another application of transmission electron microscopy is to evaluate the purity and quality of isolated particles, organelles, synaptic vesicles, exosome, etc.

Basic Protocols 1: Brain ultrastructural immunodetection by pre-embedding single immuno-labeling

Immuno-labeling combined with electron microscopy analysis (immuno-electron microscopy) is so far the most reliable approach towards identifying and observing the subcellular distribution of molecules involved in neurotransmission. Immuno-electron microscopy also allows identification of the specific ultrastructural and molecular characteristics of synapses, the presynaptic and postsynaptic components of a synapse, and defining synaptic connectivity within specific neuronal circuitry. Immuno-electron microscopy techniques are broadly classified into those in which molecules are detected prior to tissue processing for ultrastructural analysis (pre-embedding immunolabeling techniques), and those in which molecules are detected after tissue processing (post-embedding immunolabeling techniques). In this unit, we describe the protocol for pre-embedding immunolabeling for the detection of a single type of molecules (“single immuno-

labeling techniques”) by using specific antibodies (primary antibodies) against these molecules (antigens). We will also provide steps for two methods of detection of antigen-antibody complexes, one method relies on the detection of electron dense product resulting from peroxidase enzymatic reaction (immunoperoxidase labeling) and the other method relies on detection of colloidal gold particles after their intensification by silver exposure (immunogold – silver intensification labeling).

As indicated above, the pre-embedding immunolabeling techniques involve the use of specific antibodies for the detection of cellular marker prior to “Tissue processing for ultrastructural analysis” (including tissue dehydration, resin embedding and resin polymerization). To allow cellular antibody penetration, the tissue sections are permeabilized, which may compromise ultrastructural quality due to the disruption of membranes from cells or organelles. Despite tissue permeabilization, the antibody penetration is restricted to the outer layers of the tissue sections.

Materials

Animal perfusion

Wild type adult Sprague–Dawley male rats (9 weeks).

Sodium phosphate monobasic monohydrate (S9638–500G; MilliporeSigma, St. Louis, MO).

Sodium hydroxide (S318; Fisher Scientific, Pittsburgh, PA).

0.1 M phosphate buffer (PB), pH 7.3.

Chloral hydrate (C8383; MilliporeSigma, St. Louis, MO).

Sodium chloride (S7653; MilliporeSigma, St. Louis, MO).

Heparin (H3393–1MU; MilliporeSigma, St. Louis, MO).

Glutaraldehyde (18426; Ted Pella, Inc., Redding, CA).

Picric acid solution (P6744–1GA; MilliporeSigma, St. Louis, MO).

Isoflurane solution (1169567761; Henry Schein, Dublin, OH).

Fixative solution.

Paraformaldehyde (PFA, 19210; Electron Microscopy Sciences, Hatfield, PA).

Standard hot plate stirrers (97042–598; VWR, Radnor, PA).

Micro spatula (13507; Ted Pella, Inc., Redding, CA).

1 mL BD slip-tip disposable syringe (309659; Becton Dickinson, Franklin Lakes, NJ).

5 mL BD slip-tip disposable syringe (309603; Becton Dickinson, Franklin Lakes, NJ).

BD precisionglide 26G $\times$ 1/2" hypodermic needles (305111; Becton Dickinson, Franklin Lakes, NJ).

BD vacutainer safety-Lok blood collection set (367281; Becton Dickinson, Franklin Lakes, NJ).

Cole-Parmer elements Erlenmeyer flask, glass, 2000 mL (UX-34502-63; Cole-Parmer, Vernon Hills, IL).

50 mL centrifuge tubes with printed graduation & plug (89039-660; VWR, Radnor, PA).

Doyen Abdominal Scissors 7" Straight (RS-6930; Roboz Surgical Instrument, Gaithersburg, MD).

Operating Scissors; Straight; Sharp-Sharp; 5" Length (RS-6808; Roboz Surgical Instrument, Gaithersburg, MD).

Micro Dissecting Scissors 3.5" Straight Sharp/Sharp (RS-5910; Roboz Surgical Instrument, Gaithersburg, MD).

Hemostatic Forceps (RS-7130; Roboz Surgical Instrument, Gaithersburg, MD).

Hartman Mosquito Forceps 4" Straight (RS-7100; Roboz Surgical Instrument, Gaithersburg, MD).

Fine Hemostats (13006-12; Fine Science Tools, Foster City, CA).

Luer Rongeur (16001-15; Fine Science Tools, Foster City, CA).

Razor blades (55411-050; VWR, Radnor, PA).

Beakers (VWR, Radnor, PA).

Tips LTS 1000 μ l (17007083; Mettler Toledo, Columbus, OH).

1000 mL vacuum filter/storage bottle system (430517; Corning, Corning, NY).

Balance (PR503; Mettler Toledo, Columbus, OH).

Masterflex piston pump fixed speed drive (UX-07108-18; Cole-Parmer, Vernon Hills, IL).

Vibratome sectioning for single immunoperoxidase labeling

0.1 M phosphate buffer (PB), pH 7.3.

70% ethanol.

Sucrose (S0389-5KG; MilliporeSigma, St. Louis, MO).

Glycerol (G5516-1L; MilliporeSigma, St. Louis, MO).

Sodium azide (NaN_3 , S2002-100G; MilliporeSigma, St. Louis, MO).

Storage solution for vibratome sections.

50 mL centrifuge tubes with printed graduation & plug (89039–660; VWR, Radnor, PA).

Razor blades (55411–050; VWR, Radnor, PA).

Injector blade in a dispenser (71991; Electron Microscopy Sciences, Hatfield, PA).

Beakers (VWR, Radnor, PA).

Tips LTS 1000 µl (17007083; Mettler Toledo, Columbus, OH).

Plate, 12 well (UX-01959–42; Cole-Parmer, Vernon Hills, IL).

Plate, 6-well (UX-01927–70; Cole-Parmer, Vernon Hills, IL).

Micro plate shaker (12620–938; VWR, Radnor, PA).

Corning external thread cryogenic vial (430659; Corning, Corning, NY).

Loctite 401 prism surface insensitive, 3-gm (40104; Loctite Corporation, Rocky Hill, CT).

Rodent brain matrix – rat, adult 125–180g, coronal sections (RBM-3000C; ASI Instruments, Warren, MI).

Rodent brain matrix – rat, adult 200–400g, coronal sections (RBM-4000C; ASI Instruments, Warren, MI).

VT1000 S vibratome (Leica Microsystems Inc., Buffalo Grove, IL).

Pre-embedding single immunoperoxidase labeling

Primary antibodies: guinea pig anti-VGluT3 (VGluT3-GP-Af300; RRID: AB_2571855; 1:500; Frontier Institute Co., Ltd, Japan).

VECTASTAIN ABC HRP kit (PK-4007; RRID: AB_2336816; 1:200; Vector Laboratories, Burlingame, CA).

0.1 M PB (pH 7.3).

Sodium borohydride (452882; MilliporeSigma, St. Louis, MO).

Glutaraldehyde (18426; Ted Pella, Inc., Redding, CA).

Blocking solution.

Normal goat serum (NGS, S-1000; Vector Laboratories, Burlingame, CA).

Bovine serum albumin (BSA, A9647–110G; MilliporeSigma, St. Louis, MO).

Saponin (47036–50G-F; MilliporeSigma, St. Louis, MO).

3,3-diaminobenzidine-4 HCl (DAB, 13080; Electron Microscopy Sciences, Hatfield, PA).

Hydrogen peroxide (H₂O₂, 16790; Electron Microscopy Sciences, Hatfield, PA).

Ammonium chloride (A9434–500G; MilliporeSigma, St. Louis, MO).

Ammonium nickel (II) sulfate hexahydrate (A1827–500G; MilliporeSigma, St. Louis, MO).

Glucose (G7528–250G; MilliporeSigma, St. Louis, MO).

Glucose oxidase (G2133–10KU; MilliporeSigma, St. Louis, MO).

Plate, 6 well (UX-01927–70; Cole-Parmer, Vernon Hills, IL).

Plate, 12 well (UX-01959–42; Cole-Parmer, Vernon Hills, IL).

Plate, 24 well (UX-01959–35; Cole-Parmer, Vernon Hills, IL).

Red sable brushes (11806; Ted Pella, Inc., Redding, CA).

Micro spatula (13507; Ted Pella, Inc., Redding, CA).

Micro plate shaker (12620–938; VWR, Radnor, PA).

CPA series analytical balances, Sartorius (97109–322; VWR, Radnor, PA).

Tissue processing for ultrastructural analysis

4% osmium tetroxide (18459; Ted Pella, Inc., Redding, CA).

Uranyl acetate (19481; Ted Pella, Inc., Redding, CA).

A series of graded ethanol: 30%, 50%, 70%, 90%, 100%.

Propylene oxide (18601; Ted Pella, Inc., Redding, CA).

Durcupan ACM, Epoxy Resin (14040; Electron Microscopy Sciences, Hatfield, PA).

Sato's lead (Sato, 1968).

Lead nitrate (17900; Electron Microscopy Sciences, Hatfield, PA).

Lead citrate (19312; Ted Pella, Inc., Redding, CA).

Lead acetate (17600–25; Electron Microscopy Sciences, Hatfield, PA).

Sodium citrate (C3434–250g; MilliporeSigma, St. Louis, MO).

Sodium hydroxide (S318; Fisher Scientific, Pittsburgh, PA).

Formvar solution in ethylene dichloride (15830–25; Electron Microscopy Sciences, Hatfield, PA).

Whatman 1004–125 filter paper (UX-06648–04; Cole-Parmer, Vernon Hills, IL).

PELCO® Slot Grids, 1 × 2mm, 3.0mm O.D., Copper (1GC12H; Ted Pella, Inc., Redding, CA).

Corning 15-ml centrifuge tubes (CLS430791–500EA; MilliporeSigma, St. Louis, MO).

Argos technologies sterile tissue culture 6-well plate (UX-01927–70; Cole-Parmer, Vernon Hills, IL).

Plate, 6 well (UX-01927–70; Cole-Parmer, Vernon Hills, IL).

Plate, 12 well (UX-01959–42; Cole-Parmer, Vernon Hills, IL).

Plate, 24 well (UX-01959–35; Cole-Parmer, Vernon Hills, IL).

Red sable brushes (11806; Ted Pella, Inc., Redding, CA).

Micro plate shaker (12620–938; VWR, Radnor, PA).

Micro spatula (13507; Ted Pella, Inc., Redding, CA).

Scintillation vials (66020–326; VWR, Radnor, PA).

PYREX petri dishes (25354–069; VWR, Radnor, PA).

Weigh dish (UX-01018–12; Cole-Parmer, Vernon Hills, IL).

Millex-GV syringe filter unit, 0.22 μ m (SLGV033RS; MilliporeSigma, St. Louis, MO).

Plastic non-sterile Luer-Lok tip syringe, 60 mL capacity (301035; Becton Dickinson, Franklin Lakes, NJ).

Filtered ddH₂O (60 ml BD syringe attached with Millex-GV syringe filter unit).

Microscope slides (125447; Fisher Scientific, Waltham, MA).

ACLAR embedding film (50425–25; Electron Microscopy Sciences, Hatfield, PA).

Binder clips (103549; Staples).

Nail polish (72180; Electron Microscopy Sciences, Hatfield, PA).

Glass knife boats (71008; Electron Microscopy Sciences, Hatfield, PA).

Scalpel handle #3 (72040–03; Electron Microscopy Sciences, Hatfield, PA).

Scalpel blades feather #11 (72044–11; Electron Microscopy Sciences, Hatfield, PA).

Flat bottom embedding capsules (70021; Electron Microscopy Sciences, Hatfield, PA).

TEM razor blades (7520; Tousimis, Rockville, MD).

Pipetman kits (F167350 and F167550; Gilson Inc, Middleton, WI).

Diamond knife (Diatome, Hatfield, PA).

LKB 100-grid storage box (71147–12; Electron Microscopy Sciences, Hatfield, PA).

Chrome nozzle only (70702; Electron Microscopy Sciences, Hatfield, PA).

Dust-Off FGSA refill 8oz can (70707; Electron Microscopy Sciences, Hatfield, PA).

No. 1 superfine eyelash (113; Ted Pella, Inc., Redding, CA).

#5 Dumoxel tweezers (72700-D; Electron Microscopy Sciences, Hatfield, PA).

Complete film casting device (71305–01; Electron Microscopy Sciences, Hatfield, PA).

CPA series analytical balances, Sartorius (97109–322; VWR, Radnor, PA).

Blue M oven (Thermo Fisher Scientific, Waltham, MA).

MVX10 microscope (Olympus Life Science, Center Valley, PA).

Glass knife maker (EM KMR3; Leica Microsystems Inc., Buffalo Grove, IL).

UC-7 ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL).

Tecnai G² 12 transmission electron microscope (TEM) (FEI, Hillsboro, OR) equipped with a digital OneView camera (Gatan, Pleasanton, CA).

Protocol steps

Animal perfusion

1. Animals are deeply anesthetized with 80 mg/ml chloral hydrate by intraperitoneal injection (0.2 ml for mice and 2 ml for rats). Use 1 ml BD syringe and 26G × 1/2 BD needle for mice, 5 ml BD syringe for rats and 26G × 1/2 BD needle for rats to do intraperitoneal injection. If chloral hydrate is not allowed, exposure to isoflurane in an induction chamber is an alternative.
2. Perfuse animals first with heparin solution and follow by fixative solution. Follow the procedures of transcardial perfusion. Try to start perfusion within 30 seconds after cutting the diaphragm. Otherwise the postsynaptic density and mitochondria won't be well preserved. Usually apply 50 ml of heparin, 200 ml of fixative solution per mouse and 150 ml of heparin, 500 ml of fixative solution per rat.
3. Leave brains in the fixative solution at 4°C for 2 h. Replace the fixative solution with 2% PFA and postfix the brains at 4°C overnight.
4. Rinse brains with 0.1 M PB.

Vibratome sectioning for single immunoperoxidase labeling

5. Place brains in the rodent brain matrix and coronally cut the brain into half in the bregma 0.14 mm for mice and −0.12 mm for rats.
6. Mount the brain on the bottom of container in the VT1000 S vibratome using Loctite 401 glue. The cut surface faces down onto the glue. Immediately add 0.1 M PB into the container to completely cover the brain.
7. Clean the injector blade with 70% ethanol. Place the blade in the vibratome and tighten it.
8. Cut brains into coronal serial sections (50 µm thick for rats, 40 µm thick for mice). Collect series of brain sections in a 6-well plate. Each well has 1 section of 6 series.
9. Transfer coronal serial sections to the storage solution and leave the 6-well plate on the shaker overnight at 4°C.

10. Transfer coronal serial sections with brushes to the corning external thread cryogenic vials containing the new storage solution.
11. Leave the transferred sections in the cryogenic vials on the shaker at room temperature for 1 h.
12. Transfer the cryogenic vials containing sections to liquid nitrogen and stay for 1 min for fast freezing. Store the sections at -80°C .

Pre-embedding single immunoperoxidase labeling

13. Take the cryogenic vials containing sections from -80°C freezer. Thaw the sections at room temperature.
14. Rinse vibratome brain sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
15. Incubate sections with 1% sodium borohydride in PB for 30 min to inactivate free aldehyde groups.

Sodium borohydride solution needs to be prepared just before use. Gas bubbles forms when sodium borohydride reacts with water. Don't add too much sodium borohydride solution to avoid the sections to stick with bubbles on the top wall of well. Keep shaking slowly.
16. Rinse sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
17. Incubate sections with the blocking solution on the shaker at room temperature for 30 min.
18. Incubate sections with the primary antibody (guinea pig anti-VGluT3 antibody) in the blocking solution overnight on the shaker at 4°C
19. Rinse sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
20. Incubate sections in the biotinylated goat-anti-guinea pig antibody in the blocking solution overnight on the shaker at 4°C .
21. Rinse sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
22. Process sections with an ABC kit on the shaker at room temperature for 1–2 h. Add 2 drops of A in 5 ml of 0.1 M PB and mix, then add 2 drops of B and keep rotating for 30 min before use.
23. Rinse sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
24. Postfix sections with 1.5% glutaraldehyde on the shaker at room temperature for 10 min.
25. Rinse sections with 0.1 M PB on the shaker at room temperature for 2×10 min.
26. **Option 1:** for regular peroxidase reaction

Incubate samples 5–10 min in fresh solution containing 0.025% DAB and 0.003% hydrogen peroxide (H_2O_2) on the shaker at room temperature. For 10 ml

of substrate, dissolve 2.5 mg of DAB in 10 ml of 0.1 M PB, then add 100 μ l of 0.3% H_2O_2 .

27. Option 2: for NiDAB reaction

Incubate samples in fresh solution:

- a. mix 50 mg DAB and 40 mg NH_4Cl in 100 ml 0.1 M PB;
- b. after DAB is dissolved, mix with 0.4 mg glucose oxidase;
- c. add 10 ml DAB mixture to the wells in a 6-well dish;
- d. add 200 μ l of 0.05 M stock nickel-ammonium sulfate solution to each 10 ml well and stir. The final concentration will be 0.001 M;
- e. add tissue sections to the appropriate wells and incubate 5 min;
- f. after 5 min start the reaction by adding 200 μ l 10% of glucose;
- g. incubate sections as long as necessary up to approximately 1 hour on the shaker at room temperature.

28. Rinse sections with 0.1 M PB on the shaker at room temperature for 4 $\times$ 10 min.

Tissue processing for ultrastructural analysis

- 29.** Postfix sections with 0.5% osmium tetroxide in the fume hood on the shaker at room temperature for 25 min.
- 30.** Rinse sections with ddH₂O in the fume hood on the shaker at room temperature for 2 $\times$ 10 min.
- 31.** Transfer sections into scintillation vials with ddH₂O.
- 32.** Dehydrate sections with a series of graded ethanol (30%, 50%, 70%, 90%, 100%, 100%, and 100%) for 10 min each.

It is okay to leave the sections in 70% ethanol overnight. Usually we leave the sections in 70% ethanol containing 1% uranyl acetate for 1 h. Use a new unopened bottle of 100% ethanol for each experiment. If some water remains after dehydration, the resin will not polymerize properly, and sectioning of the embedded samples won't be possible.

- 33.** Rinse sections with propylene oxide for 2 $\times$ 10 min to remove the residual ethanol.
- 34.** Infiltrate sections with Durcupan ACM epoxy resin for overnight.
For preparation of Durcupan ACM epoxy resin, use pipettes to measure 10 ml of A, 10 ml of B. Mix on the nutator for 10 min. Then add 0.3 ml of C and mix on the nutator for 10 min. Add 0.2 ml of D and mix on the nutator for 30 min. it will be ready to use.
- 35.** Transfer the sections onto a plastic sheet (cut from the ACLAR embedding film) and drop the fresh Durcupan ACM epoxy resin on the sections and cover with

another plastic sheet (we called plastic sandwich). Put the plastic sandwich between two glass slides and hold to make it flat using two binder clips.

36. To polymerize the resin, bake the plastic sandwich units in a 60°C oven for 48 h.
37. Remove the top plastic sheet and use the scalpel blades to cut the small pieces of target brain areas. Transfer each of them to the cap of flat embedding capsule. Stick each piece to the prepared resin holder by using the freshly made resin.
38. Bake the resin holder together with samples in a 60°C Blue M oven for 24–48 h.
39. Use a glass knife maker (Leica EM KMR3) to make glass knives following the manual. Use nail polish to seal the glass knife boat to the glass knife. Make sure that there is no leakage.
40. Section the sample block using a UC-7 ultramicrotome.

Perform coarse sectioning with the glass knife until you visualize tissue embedded in the plastic coarse section, which will be floating on the water contained in the glass knife boat. Switch to a diamond knife to obtain ultrathin serial sections. Collect 100–150 serial sections onto slots grids with carbon-formvar supporting film. Store the grids in a grid storage box.
41. Line the bottom of a glass petri dish with parafilm. Add drops of 5% filtered uranyl acetate onto the parafilm. Stain the grid for 10–15 min with 5% filtered uranyl acetate.
42. Carefully rinse the grid with filtered ddH₂O, dry it with the filter paper and place it back into the grid storage box.
43. Stain with Sato's lead as described (Sato, 1968, see Reagents and Solutions) for 3 min. This is done in a covered glass petri dish in the presence of sodium hydroxide pellets. Do not stain more than 5 grids at one time.
44. Carefully rinse the grid with filtered ddH₂O, dry it with the filter paper and place it back into the grid storage box.
45. Screen sections under a transmission electron microscope.

Get properly trained before using the transmission electron microscope. Take care not to burn the supporting film by abruptly increasing the voltage of the filament or switching from high to low magnifications without decondensing the electron beam.
46. Collect the TEM images and analyze the data (Figure 1A).

Alternate Protocol 1: Pre-embedding single immunogold – silver intensification labeling

For pre-embedding single immunolabeling techniques, one method relies on the detection of electron dense product resulting from peroxidase enzymatic reaction (immunoperoxidase

labeling). As an alternate method, we can detect colloidal gold particles after their intensification by silver exposure (immunogold – silver intensification labeling).

Additional Materials to Basic Protocol 1

Nanogold-Fab' goat anti-guinea pig IgG antibody (2054; Nanoprobes, Yaphank, NY).

HQ Silver (2012–45 mL; Nanoprobes, Yaphank, NY).

Protocol steps

Animal perfusion

Follow Basic Protocol 1 protocol steps 1–4.

Vibratome sectioning for single immunogold – silver intensification labeling

Follow Basic Protocol 1 protocol steps 5–12.

Pre-embedding single immunogold – silver intensification labeling

1. Take the cryogenic vials containing sections from -80°C freezer. Thaw the sections at room temperature.
2. Rinse vibratome brain sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
3. Incubate sections with 1% sodium borohydride in PB for 30 min to inactivate free aldehyde groups.

Sodium borohydride solution needs to be prepared just before use. Gas bubbles forms when sodium borohydride reacts with water. Don't add too much sodium borohydride solution to avoid the sections to stick with bubbles on the top wall of well. Keep shaking slowly.
4. Rinse sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
5. Incubate sections with the blocking solution on the shaker at room temperature for 30 min.
6. Incubate sections with the primary antibody (guinea pig anti-VGluT3 antibody) in the blocking solution overnight on the shaker at 4°C
7. Rinse sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
8. Incubate the 1.4 nm nanogold conjugates secondary anti-guinea pig-IgG Fab' fragment antibody overnight at 4°C .
9. Rinse sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
10. Postfix sections with 1.5% glutaraldehyde for 10 min on the shaker at room temperature to keep gold particles intact.
11. Rinse sections with 0.1 M PB on the shaker at room temperature for 2×10 min.

12. Transfer sections to 24-well plate. Keep one section per well. Rinse the sections with ddH₂O on the shaker at room temperature for 5×1 min.
13. Process sections by silver enhancement of the gold particles with the Nanoprobes silver kit for 7 min at room temperature.
14. Rinse sections with ddH₂O for 5×1 min.
15. Rinse sections with 0.1 M PB on the shaker at room temperature for 4×10 min.

Tissue processing for ultrastructural analysis

Follow Basic Protocol 1 protocol steps 29–45.

16. Collect the TEM images and analyze the data (Figure 1B).

Basic Protocol 2: Brain ultrastructural immunodetection by pre-embedding double or triple immuno-labeling

To identify either two or three different cellular markers, we had been combining peroxidase immunolabeling and gold-silver immunolabeling. As detailed above, the product from peroxidase immunolabeling is seen as scattered dark material, whereas the silver-enhanced gold particles immunolabeling is seen as electrodense particulates. In this unit, we describe pre-embedding double or triple immuno-labeling by using a combination of peroxidase immunolabeling and gold-silver immunolabeling. We also describe the application of these immuno-labeling methods for the molecular detection in both wild type rats and cre transgenic mouse lines. These transgenic mice are endowed with the capability to induce cell specific expression of reporter molecules (i.e., mCherry) within the entire neuron, including axon terminals.

Materials

Animals and surgical procedures for double or triple immuno-labeling

Adult Sprague–Dawley male rats (9 weeks).

VGluT3::Cre transgenic mice (9 weeks).

Isoflurane solution (1169567761; Henry Schein, Dublin, OH).

Chamber for anesthetizing animals.

The AAV5-DIO-ChR2-mCherry viral vector (AAV-EF1a-DIO-hChR2(H134R)-mCherry-WPRE-pA, 3×10^{12} genomes per ml) from the UNC Vector Core Facility.

Sodium cacodylate (12310; Electron Microscopy Sciences, Hatfield, PA).

The retrograde tracer Fluoro-Gold (FG, Fluorochrome, Denver, CO).

Mini clipper with no. 0000 blade (BMR Supply, Hudson, OH).

Butler Schein Animal Health 4–0 VLT.BRAIDED SUT. 3/8 36/PK (011341; Fisher Scientific, Pittsburgh, PA).

Scalpel blades feather #11 (72044–11; Electron Microscopy Sciences, Hatfield, PA).

Micro Dissecting Scissors 3.5” Straight Sharp/Sharp (RS-5910; Roboz Surgical Instrument, Gaithersburg, MD).

Gauze pads (2”x2”).

Cotton-tipped applicators.

50 ml centrifuge tubes with printed graduation & plug (89039–660; VWR, Radnor, PA).

70% alcohol.

Adhesive tape (1–2” wide)

NanoFil syringe with 35-gauge needle (World Precision Instruments, Sarasota, FL).

Glass micropipette (20 µm).

Hot bead sterilizer (18000–50; Fine Science Tools, Foster City, CA).

Picospritzer III (Parker-Hannifin Filtration, Halethorpe, MD).

Stereotaxic apparatus (David Kopf Instruments, Tujunga, CA).

Animal perfusion

Adult Sprague–Dawley male rats (9 weeks).

VGluT3::Cre transgenic mice (9 weeks).

Sodium phosphate monobasic monohydrate (S9638–500G; MilliporeSigma, St. Louis, MO).

Sodium hydroxide (S318; Fisher Scientific, Pittsburgh, PA).

0.1 M phosphate buffer (PB), pH 7.3.

Chloral hydrate (C8383; MilliporeSigma, St. Louis, MO).

Sodium chloride (S7653; MilliporeSigma, St. Louis, MO).

Heparin (H3393–1MU; MilliporeSigma, St. Louis, MO).

Glutaraldehyde (18426; Ted Pella, Inc., Redding, CA).

Picric acid solution (P6744–1GA; MilliporeSigma, St. Louis, MO).

Isoflurane solution (1169567761; Henry Schein, Dublin, OH).

Fixative solution.

Paraformaldehyde (PFA, 19210; Electron Microscopy Sciences, Hatfield, PA).

Standard hot plate stirrers (97042–598; VWR, Radnor, PA).

Micro spatula (13507; Ted Pella, Inc., Redding, CA).

1 mL BD slip-tip disposable syringe (309659; Becton Dickinson, Franklin Lakes, NJ).

5 mL BD slip-tip disposable syringe (309603; Becton Dickinson, Franklin Lakes, NJ).

BD precisionglide 26 G x1/2" hypodermic needles (305111; Becton Dickinson, Franklin Lakes, NJ).

BD vacutainer safety-Lok blood collection set (367281; Becton Dickinson, Franklin Lakes, NJ).

Cole-Parmer elements Erlenmeyer flask, glass, 2000 mL (UX-34502-63; Cole-Parmer, Vernon Hills, IL).

50 mL centrifuge tubes with printed graduation & plug (89039-660; VWR, Radnor, PA).

Doyen Abdominal Scissors 7" Straight (RS-6930; Roboz Surgical Instrument, Gaithersburg, MD).

Operating Scissors; Straight; Sharp-Sharp; 5" Length (RS-6808; Roboz Surgical Instrument, Gaithersburg, MD).

Micro Dissecting Scissors 3.5" Straight Sharp/Sharp (RS-5910; Roboz Surgical Instrument, Gaithersburg, MD).

Hemostatic Forceps (RS-7130; Roboz Surgical Instrument, Gaithersburg, MD).

Hartman Mosquito Forceps 4" Straight (RS-7100; Roboz Surgical Instrument, Gaithersburg, MD).

Fine Hemostats (13006-12; Fine Science Tools, Foster City, CA).

Luer Rongeur (16001-15; Fine Science Tools, Foster City, CA).

Razor blades (55411-050; VWR, Radnor, PA).

Beakers (VWR, Radnor, PA).

Tips LTS 1000 µl (17007083; Mettler Toledo, Columbus, OH).

1000 mL vacuum filter/storage bottle system (430517; Corning, Corning, NY).

Balance (PR503; Mettler Toledo, Columbus, OH).

Masterflex piston pump fixed speed drive (UX-07108-18; Cole-Parmer, Vernon Hills, IL).

Vibratome sectioning for double or triple immuno-labeling

0.1 M phosphate buffer (PB), pH 7.3.

70% ethanol.

Sucrose (S0389-5KG; MilliporeSigma, St. Louis, MO).

Glycerol (G5516-1L; MilliporeSigma, St. Louis, MO).

Sodium azide (NaN₃, S2002-100G; MilliporeSigma, St. Louis, MO).

Storage solution for vibratome sections.

50 mL centrifuge tubes with printed graduation & plug (89039–660; VWR, Radnor, PA).

Razor blades (55411–050; VWR, Radnor, PA).

Injector blade in a dispenser (71991; Electron Microscopy Sciences, Hatfield, PA).

Beakers (VWR, Radnor, PA).

Tips LTS 1000 µl (17007083; Mettler Toledo, Columbus, OH).

Plate, 12 well (UX-01959–42; Cole-Parmer, Vernon Hills, IL).

Plate, 6-well (UX-01927–70; Cole-Parmer, Vernon Hills, IL).

Micro plate shaker (12620–938; VWR, Radnor, PA).

Corning external thread cryogenic vial (430659; Corning, Corning, NY).

Loctite 401 prism surface insensitive, 3-gm (40104; Loctite Corporation, Rocky Hill, CT).

Rodent brain matrix – mouse, adult 30g, coronal sections (RBM-2000C; ASI Instruments, Warren, MI).

Rodent brain matrix – rat, adult 125–180g, coronal sections (RBM-3000C; ASI Instruments, Warren, MI).

Rodent brain matrix – rat, adult 200–400g, coronal sections (RBM-4000C; ASI Instruments, Warren, MI).

VT1000 S vibratome (Leica Microsystems Inc., Buffalo Grove, IL).

Pre-embedding double or triple immuno-labeling

Guinea pig anti-VGluT3 antibody (VGluT3-GP-Af300; RRID: AB_2571855; 1:500; Frontier Institute Co., Ltd, Japan).

Guinea pig anti-VGluT3 antibody (135204; RRID: AB_2619825; 1:500; Synaptic Systems, Germany).

Rabbit anti-GluA1 antibody (GluA1-Rb-Af690; RRID: AB_2571752; 1:200; Frontier Institute Co., Ltd, Japan).

Mouse anti-mCherry antibody (632543; RRID: AB_2307319; 1:1000; Clontech Laboratories, Mountain View, CA).

Mouse anti-TH antibody (MAB318; RRID: AB_2201528; 1:1000; MilliporeSigma, Burlington, MA).

Rabbit anti-Fluoro-Gold antibody (AB153; RRID: AB_90738; 1:2000; MilliporeSigma, Burlington, MA).

VECTASTAIN ABC HRP kit (peroxidase, guinea pig IgG; PK-4007; RRID: AB_2336816; 1:200; Vector Laboratories, Burlingame, CA).

VECTASTAIN ABC HRP kit (peroxidase, mouse IgG; PK-4002; RRID: AB_2336811; 1:200; Vector Laboratories, Burlingame, CA).

Anti-guinea pig-IgG Fab' fragment coupled to 1.4-nm gold (2055; 1:100; Nanoprobes, Yaphank, NY).

Anti-rabbit-IgG IgG coupled to 1.4-nm gold (2003; 1:100; Nanoprobes, Yaphank, NY).

Anti-mouse-IgG IgG coupled to 1.4-nm gold (2001; 1:100; Nanoprobes, Yaphank, NY).

0.1 M PB (pH 7.3).

Sodium borohydride (452882; MilliporeSigma, St. Louis, MO).

Sodium hydroxide (S318; Fisher Scientific, Pittsburgh, PA).

Glutaraldehyde (18426; Ted Pella, Inc., Redding, CA).

Blocking solution.

Normal goat serum (NGS, S-1000; Vector Laboratories, Burlingame, CA).

Bovine serum albumin (BSA, A9647-110G; MilliporeSigma, St. Louis, MO).

Saponin (47036-50G-F; MilliporeSigma, St. Louis, MO).

3,3-diaminobenzidine-4 HCl (DAB, 13080; Electron Microscopy Sciences, Hatfield, PA).

Hydrogen peroxide (H₂O₂, 16790; Electron Microscopy Sciences, Hatfield, PA).

Ammonium chloride (A9434-500G; MilliporeSigma, St. Louis, MO).

Ammonium nickel (II) sulfate hexahydrate (A1827-500G; MilliporeSigma, St. Louis, MO).

Glucose (G7528-250G; MilliporeSigma, St. Louis, MO).

Glucose oxidase (G2133-10KU; MilliporeSigma, St. Louis, MO).

HQ silver enhancement kit (2012; Nanoprobes, Yaphank, NY).

4% osmium tetroxide (18459; Ted Pella, Inc., Redding, CA).

Uranyl acetate (19481; Ted Pella, Inc., Redding, CA).

A series of graded ethanol: 30%, 50%, 70%, 90%, 100%.

Propylene oxide (18601; Ted Pella, Inc., Redding, CA).

Durcupan ACM, Epoxy Resin (14040; Electron Microscopy Sciences, Hatfield, PA).

Sato's lead (Sato, 1968).

Lead nitrate (17900; Electron Microscopy Sciences, Hatfield, PA).

Lead citrate (19312; Ted Pella, Inc., Redding, CA).

Lead acetate (17600–25; Electron Microscopy Sciences, Hatfield, PA).

Sodium citrate (C3434–250g; MilliporeSigma, St. Louis, MO).

Formvar solution in ethylene dichloride (15830–25; Electron Microscopy Sciences, Hatfield, PA).

Whatman 1004–125 filter paper (UX-06648–04; Cole-Parmer, Vernon Hills, IL).

PELCO® Slot Grids, 1 × 2mm, 3.0mm O.D., Copper (1GC12H; Ted Pella, Inc., Redding, CA).

Corning 15-ml centrifuge tubes (CLS430791–500EA; MilliporeSigma, St. Louis, MO).

Argos technologies sterile tissue culture 6-well plate (UX-01927–70; Cole-Parmer, Vernon Hills, IL).

Plate, 6 well (UX-01927–70; Cole-Parmer, Vernon Hills, IL).

Plate, 12 well (UX-01959–42; Cole-Parmer, Vernon Hills, IL).

Plate, 24 well (UX-01959–35; Cole-Parmer, Vernon Hills, IL).

Red sable brushes (11806; Ted Pella, Inc., Redding, CA).

Micro plate shaker (12620–938; VWR, Radnor, PA).

Micro spatula (13507; Ted Pella, Inc., Redding, CA).

Scintillation vials (66020–326; VWR, Radnor, PA).

Flat bottom embedding capsules (70021; Electron Microscopy Sciences, Hatfield, PA).

PYREX petri dishes (25354–069; VWR, Radnor, PA).

Weigh dish (UX-01018–12; Cole-Parmer, Vernon Hills, IL).

Millex-GV syringe filter unit, 0.22 µm (SLGV033RS; MilliporeSigma, St. Louis, MO).

Plastic non-sterile Luer-Lok tip syringe, 60 ml capacity (301035; Becton Dickinson, Franklin Lakes, NJ).

Filtered ddH₂O (60 ml BD syringe attached with Millex-GV syringe filter unit).

Microscope slides (125447; Fisher Scientific, Waltham, MA).

ACLAR embedding film (50425–25; Electron Microscopy Sciences, Hatfield, PA).

Binder clips (103549; Staples).

Nail polish (72180; Electron Microscopy Sciences, Hatfield, PA).

Glass knife boats (71008; Electron Microscopy Sciences, Hatfield, PA).

Scalpel handle #3 (72040–03; Electron Microscopy Sciences, Hatfield, PA).

Scalpel blades feather #11 (72044–11; Electron Microscopy Sciences, Hatfield, PA).

TEM razor blades (7520; Tousimis, Rockville, MD).

Pipetman kits (F167350 and F167550; Gilson Inc, Middleton, WI).

Diamond knife (Diatome, Hatfield, PA).

LKB 100-grid storage box (71147–12; Electron Microscopy Sciences, Hatfield, PA).

Chrome nozzle only (70702; Electron Microscopy Sciences, Hatfield, PA).

Dust-Off FGSA refill 8oz can (70707; Electron Microscopy Sciences, Hatfield, PA).

No. 1 superfine eyelash (113; Ted Pella, Inc., Redding, CA).

#5 Dumoxel tweezers (72700-D; Electron Microscopy Sciences, Hatfield, PA)

Complete film casting device (71305–01; Electron Microscopy Sciences, Hatfield, PA).

CPA series analytical balances, Sartorius (97109–322; VWR, Radnor, PA).

Blue M oven (Thermo Fisher Scientific, Waltham, MA).

MVX10 microscope (Olympus Life Science, Center Valley, PA).

Glass knife maker (EM KMR3; Leica Microsystems Inc., Buffalo Grove, IL).

UC-7 ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL).

Tecnai G² 12 transmission electron microscope (TEM) (FEI, Hillsboro, OR) equipped with a digital OneView camera (Gatan, Pleasanton, CA).

Protocol steps

Animals and surgical procedures for double or triple immuno-labeling in combination with neuronal track tracing

1. Deeply anesthetize transgenic mice with isoflurane. The detailed procedures include:
 - A. Check system to ensure adequate amounts of supply gas and isoflurane for during of the procedure.
 - B. Turn on the gas. Place animal in induction chamber and seal top.
 - C. Turn on vaporizer to 5%.
 - D. Monitor animal until recumbent.
 - E. Switch system to flow to nosecone.
 - F. Remove animal from chamber and position in nosecone.
 - G. Restart gas flow with flowmeter at 100–200 ml/min and vaporizer at 2–3%. If animal has started responding, gently restrain in nosecone until fully anesthetized again.
2. Using the clipper remove the hair from the surgical site.

3. Dab the clipped area with a piece of adhesive tape or moistened gauze to pick up loose hair that could otherwise migrate into the incision.
4. Fix animals in a stereotaxic apparatus for viral injections.
5. Inject male VGluT3::Cre mice with 0.5 μ l (0.1 μ l min⁻¹) of a Cre-inducible recombinant AAV encoding ChR2 tethered to mCherry (VGluT3-ChR2-mCherry mice).

The virus is injected using a NanoFil syringe (with 35-gauge needle) into the dorsal raphe, DR (bregma AP -4.3, ML $\pm$ 0, DV -3.3). This allows expression of mCherry within the DR VGluT3 neurons.

6. Close the surgical wound with a suture and move animal to a new cage for recovery.
7. After 6 weeks, VGluT3-ChR2-mCherry mice are anaesthetized with 2–3 % isoflurane.
8. Using the clipper remove the hair from the surgical site.
9. Dab the clipped area with a piece of adhesive tape or moistened gauze to pick up loose hair that could otherwise migrate into the incision.
10. Fix animals in a stereotaxic apparatus for FG injections.
11. The retrograde tracer FG is delivered unilaterally into the VTA (coordinates in mm: AP -3.3, ML +0.2, DV -4.3).

FG is delivered iontophoretically through glass micropipette (20 μ m) by applying 1 μ A current in 5 s pulses at 10 s intervals for 10 min. Leave the micropipette in place for an additional 10 min after each injection. The additional at least 10 min is to prevent backflow of tracer up the injection track.

12. Close the surgical wound with a suture and move animal to a new cage for recovery.
13. All mice are housed in groups of up to four animals per cage in the animal rooms at 22°C under a 12-h light/dark cycle (light on at 7 a.m.), with *ad libitum* access to food and water. All animal procedures need to be approved by the local Animal Care and Use Committee.

Animal perfusion

14. Animals (rats or VGluT3-ChR2-mCherry mice) are deeply anesthetized with 80 mg/ml chloral hydrate (0.2 ml for mice and 2 ml for rats). If chloral hydrate is not allowed, exposure to isoflurane in an induction chamber is an alternative.
15. Perfuse animals first with heparin solution and follow by fixative solution. Follow the procedures of transcardial perfusion.

Try to start perfusion within 30 seconds after cutting the diaphragm. Otherwise the postsynaptic density and mitochondria won't be well preserved. Usually

apply 50 ml of heparin, 200 ml of fixative solution per mouse and 150 ml of heparin, 500 ml of fixative solution per rat.

16. Leave brains in the fixative solution at 4°C for 2 h. Replace the fixative solution with 2% PFA and postfix the brains at 4°C overnight.
17. Rinse brains with 0.1 M PB.

Vibratome sectioning for double or triple immuno-labeling

18. Place brains in the rodent brain matrix and coronally cut the brain into half in the bregma 0.14 mm for mice and -0.12 mm for rats.
19. Mount the brain on the bottom of container in the VT1000 S vibratome using Loctite 401 glue. The cut surface faces down onto the glue. Immediately add 0.1 M PB into the container to completely cover the brain.
20. Clean the injector blade with 70% ethanol. Place the blade in the vibratome and tighten it.
21. Cut brains into coronal serial sections (50 µm thick for rats, 40 µm thick for mice). Collect series of brain sections in a 6-well plate. Each well has 1 section of 6 series.
22. Transfer coronal serial sections to the storage solution and leave the 6-well plate on the shaker overnight at 4°C.
23. Transfer coronal serial sections with brushes to the corning external thread cryogenic vials containing the new storage solution.
24. Leave the transferred sections in the cryogenic vials on the shaker at room temperature for 1 h.
25. Transfer the cryogenic vials containing sections to liquid nitrogen and stay for 1 min for fast freezing. Store the sections at -80°C.

Pre-embedding double or triple immuno-labeling

26. Rinse vibratome brain sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
27. Incubate sections with 1% sodium borohydride in PB for 30 min on the shaker at room temperature to inactivate free aldehyde groups.
28. Rinse sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
29. Incubate sections with the blocking solution on the shaker at room temperature for 30 min.
30. Incubate sections with the primary antibodies:
 - A. guinea pig anti-VGluT3 (VGluT3-GP-Af300) + mouse anti-TH for rat VTA sections;

- B. guinea pig anti-VGluT3 (VGluT3-GP-Af300) + rabbit anti-GluR1 for rat VTA sections;
 - C. mouse anti-mCherry + guinea pig VGluT3 (135204) + rabbit anti-FG for VGluT3-ChR2-mCherry mice
 - D. in the blocking solution on the shaker at 4°C overnight.
31. Rinse sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
 32. Incubate the corresponding cocktails of biotinylated and 1.4 nm nanogold conjugates secondary antibodies in the blocking buffer on the shaker at 4°C overnight.
 - A. Biotinylated anti-guinea pig (PK-4007) + 1.4 nm nanogold conjugated anti-mouse secondary antibody (2001);
 - B. Biotinylated anti-guinea pig (PK-4007) + 1.4 nm nanogold conjugated anti-rabbit secondary antibody (2003);
 - C. Biotinylated anti-mouse (PK-4002) + 1.4 nm nanogold conjugated anti-rabbit secondary antibody (2003) + 1.4 nm nanogold conjugated anti-guinea pig secondary antibody (2055).
 33. Rinse sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
 34. Process sections with an ABC kit on the shaker at room temperature for 1–2 hours. Add 2 drops of A in 5 ml of 0.1 M PB and mix, then add 2 drops of B and keep rotating for 30 min before use.
 35. Rinse sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
 36. Postfix sections with 1.5% glutaraldehyde on the shaker at room temperature for 10 min keep gold particles intact.
 37. Rinse sections with 0.1 M PB on the shaker at room temperature for 2×10 min.
 38. Transfer sections to 24-well plate. Keep one section per well. Rinse the sections with ddH₂O on the shaker at room temperature for 5×1 min.
 39. Process sections by silver enhancement of the gold particles with the Nanoprobes silver kit for 7 min on the shaker at room temperature.
 40. Rinse sections with ddH₂O on the shaker at room temperature for 5 min.
 41. Rinse sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
 42. Incubate samples on the shaker at room temperature 5–10 min in fresh solution containing 0.025% DAB and 0.003% hydrogen peroxide (H₂O₂). For 10 mL of substrate, dissolve 2.5 mg of DAB in 10 ml of 0.1 M PB, then add 100 µl of 0.3% H₂O₂.
 43. Rinse sections with 0.1 M PB on the shaker at room temperature for 4×10 min.
 44. Postfix sections with 0.5% osmium tetroxide in the fume hood on the shaker at room temperature for 25 min.

45. Rinse sections with ddH₂O on the shaker at room temperature for 2×10 min.
46. Transfer sections into scintillation vials with ddH₂O.
47. Dehydrate sections with a series of graded ethanol (30%, 50%, 70%, 90%, 100%, 100%, and 100%) for 10 min each.

It is okay to leave the sections in 70% ethanol overnight. Usually we leave the sections in 70% ethanol containing 1% uranyl acetate for 1 h. Use a new unopened bottle of 100% ethanol for each experiment. If some water remains after dehydration, the resin will not polymerize properly, and sectioning of the embedded samples won't be possible.

48. Rinse sections with propylene oxide for 2×10 min to remove the residual ethanol.
49. Infiltrate sections with Durcupan ACM epoxy resin for overnight.

For preparation of Durcupan ACM epoxy resin, use pipettes to measure 10 ml of A, 10 ml of B. Mix on the nutator for 10 min. Then add 0.3 ml of C and mix on the nutator for 10 min. Add 0.2 ml of D and mix on the nutator for 30 min. it will be ready to use.
50. Transfer the sections onto a plastic sheet (cut from the ACLAR embedding film) and drop the fresh Durcupan ACM epoxy resin on the sections and cover with another plastic sheet (we called plastic sandwich). Put the plastic sandwich between two glass slides and hold to make it flat using two binder clips.
51. To polymerize the resin, bake the plastic sandwich units in a 60°C oven for 48 h.
52. Remove the top plastic sheet and use the scalpel blades to cut the small pieces of target brain areas. Transfer each of them to the cap of flat embedding capsule. Stick each piece to the prepared resin holder by using the freshly made resin.
53. Bake the resin holder together with samples in a 60°C oven for 24–48 h.
54. Use a glass knife maker (Leica EM KMR3) to make glass knives following the manual. Use nail polish to seal the glass knife boat to the glass knife. Make sure that there is no leakage.
55. Sectioning using a UC-7 ultramicrotome.

Perform coarse sectioning with the glass knife until you visualize tissue embedded in the plastic coarse section, which will be floating on the water contained in the glass knife boat. Switch to a diamond knife to obtain ultrathin serial sections. Collect 100–150 serial sections onto slots grids with carbon-formvar supporting film. Store the grids in a grid storage box.
56. Line the bottom of a glass petri dish with parafilm. Add drops of 5% filtered uranyl acetate onto the parafilm. Stain the grid for 10–15 min with 5% filtered uranyl acetate.
57. Carefully rinse the grid with filtered ddH₂O, dry it with the filter paper and place it back into the grid storage box.

58. Stain with Sato's lead as described (Sato, 1968, See Reagents and Solutions 19) for 3 min. This is done in a covered glass petri dish in the presence of sodium hydroxide pellets. Do not stain more than 5 grids at one time.
59. Carefully rinse the grid with filtered ddH₂O, dry it with filter paper and place it back into the grid storage box.

Screen sections under a transmission electron microscope. Get properly trained before using the transmission electron microscope. Take care not to burn the supporting film by abruptly increasing the voltage of the filament or switching from high to low magnifications without decondensing the electron beam.
60. Collect the TEM images and analyze the data (Figure 2).

Basic Protocol 3. Brain ultrastructural immunodetection by post-embedding immuno-labeling

In post-embedding immunolabeling, in contrast to pre-embedding, the detection of cellular marker is done on ultrathin sections (instead of vibratome sections), obtained after tissue processing for ultrastructural analysis, and collected on a grid. The ultrastructural preservation is not optimal due to mild tissue fixation, such as low concentration of glutaraldehyde and omission of osmium tetroxide. While post-embedding labeling do not require tissue permeabilization, it requires that ultrathin section be treated with agents to allow exposure of antigenic sites, which may become inaccessible after fixation or resin polymerization.

Materials

Animal perfusion

Wild type adult Sprague–Dawley male rats (9 weeks).

Sodium phosphate monobasic monohydrate (S9638–500G; MilliporeSigma, St. Louis, MO).

Sodium hydroxide (S318; Fisher Scientific, Pittsburgh, PA).

0.1 M phosphate buffer (PB), pH 7.3.

Chloral hydrate (C8383; MilliporeSigma, St. Louis, MO).

Sodium chloride (S7653; MilliporeSigma, St. Louis, MO).

Heparin (H3393–1MU; MilliporeSigma, St. Louis, MO).

Glutaraldehyde (18426; Ted Pella, Inc., Redding, CA).

Picric acid solution (P6744–1GA; MilliporeSigma, St. Louis, MO).

Isoflurane solution (1169567761; Henry Schein, Dublin, OH).

Fixative solution.

Paraformaldehyde (PFA, 19210; Electron Microscopy Sciences, Hatfield, PA).

Standard hot plate stirrers (97042–598; VWR, Radnor, PA).

Micro spatula (13507; Ted Pella, Inc., Redding, CA).

1 mL BD slip-tip disposable syringe (309659; Becton Dickinson, Franklin Lakes, NJ).

5 mL BD slip-tip disposable syringe (309603; Becton Dickinson, Franklin Lakes, NJ).

BD precisionglide 26G × 1/2" hypodermic needles (305111; Becton Dickinson, Franklin Lakes, NJ).

BD vacutainer safety-Lok blood collection set (367281; Becton Dickinson, Franklin Lakes, NJ).

Cole-Parmer elements Erlenmeyer flask, glass, 2000 mL (UX-34502–63; Cole-Parmer, Vernon Hills, IL).

50ml centrifuge tubes with printed graduation & plug (89039–660; VWR, Radnor, PA).

Doyen Abdominal Scissors 7" Straight (RS-6930; Roboz Surgical Instrument, Gaithersburg, MD).

Operating Scissors; Straight; Sharp-Sharp; 5" Length (RS-6808; Roboz Surgical Instrument, Gaithersburg, MD).

Micro Dissecting Scissors 3.5" Straight Sharp/Sharp (RS-5910; Roboz Surgical Instrument, Gaithersburg, MD).

Hemostatic Forceps (RS-7130; Roboz Surgical Instrument, Gaithersburg, MD).

Hartman Mosquito Forceps 4" Straight (RS-7100; Roboz Surgical Instrument, Gaithersburg, MD).

Fine Hemostats (13006–12; Fine Science Tools, Foster City, CA).

Luer Rongeur (16001–15; Fine Science Tools, Foster City, CA).

Razor blades (55411–050; VWR, Radnor, PA).

Beakers (VWR, Radnor, PA).

Tips LTS 1000 µl (17007083; Mettler Toledo, Columbus, OH).

1000ml vacuum filter/storage bottle system (430517; Corning, Corning, NY).

Balance (PR503; Mettler Toledo, Columbus, OH).

Masterflex piston pump fixed speed drive (UX-07108–18; Cole-Parmer, Vernon Hills, IL).

Vibratome sectioning for post-embedding immunolabeling

0.1 M phosphate buffer (PB), pH 7.3.

Sucrose (S0389–5KG; MilliporeSigma, St. Louis, MO).

Glycerol (G5516–1L; MilliporeSigma, St. Louis, MO).

Sodium azide (NaN₃, S2002–100G; MilliporeSigma, St. Louis, MO).

Storage solution for vibratome sections.

50ml centrifuge tubes with printed graduation & plug (89039–660; VWR, Radnor, PA).

Razor blades (55411–050; VWR, Radnor, PA).

Rodent brain matrix coronal sections (RBM-2000C; ASI Instruments, Warren, MI).

Injector blade in a dispenser (71991; Electron Microscopy Sciences, Hatfield, PA).

Beakers (VWR, Radnor, PA).

Tips LTS 1000 µl (17007083; Mettler Toledo, Columbus, OH).

Plate, 12 well (UX-01959–42; Cole-Parmer, Vernon Hills, IL).

Plate, 6-well (UX-01927–70; Cole-Parmer, Vernon Hills, IL).

Micro plate shaker (12620–938; VWR, Radnor, PA).

Corning external thread cryogenic vial (430659; Corning, Corning, NY).

VT1000 S vibratome (Leica Microsystems Inc., Buffalo Grove, IL).

Post-embedding immunolabeling

Primary antibody guinea pig-anti-VGluT2 (VGluT2-GP-Af240–1; RRID: AB_2571621; 1:20; Frontier Institute Co., Ltd, Japan).

Primary antibody goat-anti-VMAT2 (EB06558; RRID: AB_2187855; 1:25; Everest Biotech Ltd., United Kingdom).

Secondary antibody donkey-anti-goat 12 nm colloidal gold (705-205-147; RRID: AB_2340418; 1:20; Jackson ImmunoResearch Laboratories, West Grove, PA).

Secondary antibody donkey-anti-guinea pig 18 nm colloidal gold (706-215-148; RRID: AB_2340466; 1:10; Jackson ImmunoResearch Laboratories, West Grove, PA).

0.1 M PB (pH 7.3).

Tannic acid (21700; Electron Microscopy Sciences, Hatfield, PA).

Uranyl acetate (19481; Ted Pella, Inc., Redding, CA).

Tween 20 (P9416; MilliporeSigma, St. Louis, MO).

Glycine (G7126; MilliporeSigma, St. Louis, MO).

A series of graded ethanol: 30%, 50%, 70%, 90%, 100%.

LR white embedding medium (14380; Electron Microscopy Sciences, Hatfield, PA).

Blocking solution.

Normal goat serum (NGS, S-1000; Vector Laboratories, Burlingame, CA).

Bovine serum albumin (BSA, A9647–110G; MilliporeSigma, St. Louis, MO).

Phosphate buffered saline (PBS, 10010031; Thermo Fisher Scientific, Waltham, MA).

Glutaraldehyde (18426; Ted Pella, Inc., Redding, CA).

Uranyl acetate (19481; Ted Pella, Inc., Redding, CA).

Lead nitrate (17900; Electron Microscopy Sciences, Hatfield, PA).

Lead citrate (19312; Ted Pella, Inc., Redding, CA).

Lead acetate (17600–25; Electron Microscopy Sciences, Hatfield, PA).

Sodium citrate (C3434–250g; MilliporeSigma, St. Louis, MO).

TEM razor blades (7520; Tousimis, Rockville, MD).

Ni-mesh 200 with carbon-formvar supporting film (FCF200-Ni; Electron Microscopy Sciences, Hatfield, PA).

PYREX petri dishes (25354–069; VWR, Radnor, PA).

Sato's lead.

Scalpel handle #3 (72040–03; Electron Microscopy Sciences, Hatfield, PA).

Scalpel blades feather #11 (72044–11; Electron Microscopy Sciences, Hatfield, PA).

Plastic non-sterile Luer-Lok tip syringe, 60mL capacity (301035; Becton Dickinson, Franklin Lakes, NJ).

Plate, 6 well (UX-01927–70; Cole-Parmer, Vernon Hills, IL).

Flat bottom capsules (133-P; Ted Pella, Inc., Redding, CA).

Millex-GV syringe filter unit, 0.22 μm (SLGV033RS; MilliporeSigma, St. Louis, MO).

Whatman 1004–125 filter paper (UX-06648–04; Cole-Parmer, Vernon Hills, IL).

Nail polish (72180; Electron Microscopy Sciences, Hatfield, PA).

Pipetman kits (F167350 and F167550; Gilson Inc, Middleton, WI).

Micro plate shaker (12620–938; VWR, Radnor, PA).

Diamond knife (Diatome, Hatfield, PA).

LKB 100-grid storage box (71147–12; Electron Microscopy Sciences, Hatfield, PA).

Chrome nozzle only (70702; Electron Microscopy Sciences, Hatfield, PA).

Dust-Off FGSA refill 8oz can (70707; Electron Microscopy Sciences, Hatfield, PA).

No. 1 superfine eyelash (113; Ted Pella, Inc., Redding, CA).

#5 Dumoxel tweezers (72700-D; Electron Microscopy Sciences, Hatfield, PA).

CPA series analytical balances, Sartorius (97109–322; VWR, Radnor, PA).

Lindberg Blue M oven (Thermo Fisher Scientific, Waltham, MA); MVX10 microscope (Olympus Life Science, Center Valley, PA Blue M oven (Thermo Fisher Scientific, Waltham, MA).

Glass knife maker (EM KMR3; Leica Microsystems Inc., Buffalo Grove, IL).

MVX10 microscope (Olympus Life Science, Center Valley, PA).

UC-7 ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL).

Tecnai G2 12 transmission electron microscope (TEM) (FEI, Hillsboro, OR) equipped with a digital OneView camera (Gatan, Pleasanton, CA).

Protocol steps

Animal perfusion

1. Animals are deeply anesthetized with 80 mg/ml chloral hydrate by intraperitoneal injection (0.2 ml for mice and 2 ml for rats). Use 1 ml BD syringe and 26G $\times$ 1/2 BD needle for mice, 5 ml BD syringe for rats and 26G $\times$ 1/2 BD needle for rats to do intraperitoneal injection. If chloral hydrate is not allowed, exposure to isoflurane in an induction chamber is an alternative.
2. Perfuse animals first with heparin solution and follow by fixative solution.
3. *Follow the procedures of transcardial perfusion. Try to start perfusion within 30 seconds after cutting the diaphragm. Otherwise the postsynaptic density and mitochondria won't be well preserved. Usually apply 50 ml of heparin, 200 ml of fixative solution per mouse and 150 ml of heparin, 500 ml of fixative solution per rat.*
4. Leave brains in the fixative solution at 4°C for 2 h. Replace the fixative solution with 2% PFA and postfix the brains at 4°C overnight.
5. Rinse brains with 0.1 M PB.

Vibratome sectioning for post-embedding labeling

6. Place brains in the rodent brain matrix and coronally cut the brain into half in the bregma 0.14 mm for mice and -0.12 mm for rats.
7. Mount the brain on the bottom of container in the VT1000 S vibratome using Loctite 401 glue. The cut surface faces down onto the glue. Immediately add 0.1 M PB into the container to completely cover the brain.
8. Clean the injector blade with 70% ethanol. Place the blade in the vibratome and tighten it.
9. Cut brains into coronal serial sections (50 μ m thick for rats, 40 μ m thick for mice). Collect series of brain sections in a 6-well plate. Each well has 1 section of 6 series.
10. Transfer coronal serial sections to the storage solution and leave the 6-well plate on the shaker overnight at 4°C.

11. Transfer coronal serial sections with brushes to the corning external thread cryogenic vials containing the new storage solution.
12. Leave the transferred sections in the cryogenic vials on the shaker at room temperature for 1 h.
13. Transfer the cryogenic vials containing sections to liquid nitrogen and stay for 1 min for fast freezing. Store the sections at -80°C .

Post-embedding immunolabeling

14. Transfer the vibratome brain sections into the petri dish containing $0.1 \times \text{PB}$. Use a scalpel blade to cut small pieces in the target brain areas under a dissecting microscopy (MVX10 microscope). Rinse them with 0.1 M PB for 3×10 min.
15. Rinse sections with ddH₂O for 3×5 min.
16. Incubate sections with 0.25% tannic acid in ddH₂O for 5 min.
17. Rinse sections with ddH₂O for 3×5 min.
18. Incubate sections with 2% uranyl acetate in ddH₂O for 30 min.
19. Dehydrate sections with a series of graded ethanol (30%, 50%, 70%, 90%, 100%, 100%, and 100%) for 10 min each.
20. Infiltrate sections in LR white/100% ethanol (1:1) for 1–2 h.
21. Aliquot LR white and leave at room temperature to warm up at least 30 min before use.
22. Infiltrate sections in LR white/100% ethanol (2:1) for 1–2 h.
23. Infiltrate sections in 100% LR white for overnight.
24. Change sections into flat capsule containing new LR white. Close the capsule cap and avoid air bubbles. Bake the sections in a 60°C vacuum oven for 48 h.
25. Perform coarse sectioning with the glass knife until you visualize tissue embedded in the plastic coarse section, which will be floating on the water contained in the glass knife boat. Switch to a diamond knife to obtain ultrathin serial sections. Collect serial sections onto Ni-mesh 200 with carbon-formvar supporting film. Leave overnight for air dry.
26. Rinse the Ni-mesh containing sections with PBST for 2×2 min.
27. Incubate sections with 0.05 M glycine in PBS buffer for 15 min to inactivate residual aldehyde groups present after aldehyde fixation.
28. Incubate sections in blocking buffer for 1h.
29. Incubate sections with goat anti-VMAT2 primary antibodies overnight at 4°C .
30. Rinse sections with PBST for 5×5 min.
31. Incubate sections in donkey-anti-goat 12 nm colloidal gold secondary antibody at RT for 1 h.

32. Rinse sections with PBST for 3×5 min.
33. Postfix sections with 2% glutaraldehyde in PBST for 5 min.
34. Rinse sections with PBST for 3×5 min.
35. Incubate sections in blocking buffer for 1h.
36. Incubate sections with guinea pig-anti-VGluT2 primary antibodies overnight at 4°C.
37. Rinse sections with PBST for 5×5 min.
38. Incubate sections in donkey-anti-guinea pig 18 nm colloidal gold secondary antibody at RT for 1 h.
39. Rinse sections with PBST for 3×5 min.
40. Postfix sections with 2% glutaraldehyde in PBST for 5 min.
41. Rinse sections with PBST for 3×5 min.
42. Rinse sections with ddH₂O for 5×2 min.
43. Counterstain sections with 5% uranyl acetate for 3 min and Sato's lead as described by Sato (See Reagents and Solutions 19) for 1 min. This is done in a covered glass petri dish in the presence of sodium hydroxide pellets. Do not stain more than 5 grids at one time.
44. Screen sections under a transmission electron microscope.
45. Collect the TEM images and analyze the data (Figure 3).

Basic Protocol 4. Immuno-gold detection within purified synaptic vesicles

In addition of identifying the subcellular neuronal distribution of transmitters, transporters or receptors in brain tissue, another application of transmission electron microscopy is to evaluate the purity and quality of isolated particles, organelles, synaptic vesicles, exosome, etc. Moreover, immuno-gold detection of specific markers within isolated organelles allows evaluation of distribution of specific molecular markers within subpopulations of specific organelles, such as distribution of vesicular transporters within same or separate synaptic vesicles. In this unit, we describe immuno-detection of molecular markers within purified vesicles.

Materials

Synaptic vesicle purification

Male Sprague-Dawley rats (6 weeks of age).

Chloral hydrate (C8383; MilliporeSigma, St. Louis, MO).

Isoflurane solution (1169567761; Henry Schein, Dublin, OH).

Sigmacote (SL2–100 ml; MilliporeSigma, St. Louis, MO).

Potassium hydroxide (P5958–250G; MilliporeSigma, St. Louis, MO).

Tartaric acid (25138–500G; MilliporeSigma, St. Louis, MO).

HEPES buffer (H3375–1KG; MilliporeSigma, St. Louis, MO).

Phenylmethylsulfonyl fluoride (PMSF, P7626–25G; MilliporeSigma, St. Louis, MO).

100% ethanol or 2-propanol (MilliporeSigma, St. Louis, MO).

Sucrose (S0389–5KG; MilliporeSigma, St. Louis, MO).

Protein inhibitor (11697498001; Roche Diagnostics Corporation, Indianapolis, IN).

Neutral L-(+)-tartaric acid dipotassium (P313–500; Fisher Scientific, Pittsburgh, PA).

Magnesium sulfate (M7506–500G; MilliporeSigma, St. Louis, MO).

PYREX petri dishes (25354–069; VWR, Radnor, PA).

Pipetman kits (F167350 and F167550; Gilson Inc, Middleton, WI).

Scalpel handle #3 (72040–03; Electron Microscopy Sciences, Hatfield, PA).

Scalpel blades feather #11 (72044–11; Electron Microscopy Sciences, Hatfield, PA).

Razor blades (55411–050; VWR, Radnor, PA).

50ml centrifuge tubes with printed graduation & plug (89039–660; VWR, Radnor, PA).

Tips LTS 1000 µl (17007083; Mettler Toledo, Columbus, OH).

Wilkinson sword double edge razor blades (West Coast Shaving, Chino, CA).

Rodent brain matrix coronal sections (RBM-2000C; ASI Instruments, Warren, MI).

Curved dissecting forceps (5431; Ted Pella, Inc., Redding, CA).

Flat tip tweezer (72972-AP; Electron Microscopy Sciences, Hatfield, PA).

Parafilm.

Micro plate shaker (12620–938; VWR, Radnor, PA).

Centrifuge tubes for Optima MAX-XP ultracentrifuge (347357; Beckman Coulter Inc., Brea, CA).

Overhead stirrers/Homogenizer (Wheaton, Millville, NJ).

Beckman Coulter Avanti J-25 centrifuge (Beckman Coulter Inc., Brea, CA).

Beckman Coulter Optima MAX-XP ultracentrifuge (Beckman Coulter Inc., Brea, CA).

Rodent and small animal guillotine (World Precision Instruments, Sarasota, FL)

Overhead stirrers/Homogenizer (Wheaton, Millville, NJ).

Beckman Coulter Avanti J-25 centrifuge (Beckman Coulter Inc., Brea, CA).

Beckman Coulter Optima MAX-XP ultracentrifuge (Beckman Coulter Inc., Brea, CA).

Immuno-gold detection within purified synaptic vesicles

Osmium tetroxide (18459; Ted Pella, Inc., Redding, CA).

Paraformaldehyde (PFA, 19210; Electron Microscopy Sciences, Hatfield, PA).

Uranyl acetate (19481; Ted Pella, Inc., Redding, CA).

A series of graded ethanol: 30%, 50%, 70%, 90%, 100%.

Propylene oxide (18601; Ted Pella, Inc., Redding, CA).

Durcupan ACM, Epoxy Resin (14040; Electron Microscopy Sciences, Hatfield, PA)

Lead nitrate (17900; Electron Microscopy Sciences, Hatfield, PA).

Lead citrate (19312; Ted Pella, Inc., Redding, CA).

Lead acetate (17600–25; Electron Microscopy Sciences, Hatfield, PA).

Sodium citrate (C3434–250g; MilliporeSigma, St. Louis, MO).

Sato's lead.

EDTA (E9884; MilliporeSigma, St. Louis, MO).

Ethanolamine-HCl (E6133–100G; MilliporeSigma, St. Louis, MO).

Tris/HCl (221–228; Crystalgen, Commack, NY)

TBS buffer.

Sodium chloride (S7653; MilliporeSigma, St. Louis, MO).

Blocking buffer.

Newborn calf serum (NCS, 4762; MilliporeSigma, St. Louis, MO).

Primary antibody guinea pig-anti-VGluT2 (VGluT2-GP-Af240–1; RRID: AB_2571621; 1:20; Frontier Institute Co., Ltd, Japan).

Primary antibody goat-anti-VMAT2 (EB06558; RRID: AB_2187855; 1:25; Everest Biotech Ltd., United Kingdom).

Secondary antibody donkey-anti-goat 12 nm colloidal gold (705-205-147; RRID: AB_2340418; 1:20; Jackson ImmunoResearch Laboratories, West Grove, PA).

Secondary antibody donkey-anti-guinea pig 18 nm colloidal gold (706-215-148; RRID: AB_2340466; 1:10; Jackson ImmunoResearch Laboratories, West Grove, PA).

Polyethylene glycol (PEG, P3015–250G; MilliporeSigma, St. Louis, MO).

0.1 M PB (pH 7.3).

Glutaraldehyde (18426; Ted Pella, Inc., Redding, CA).

Durcupan ACM, Epoxy Resin (14040; Electron Microscopy Sciences, Hatfield, PA).

Nail polish (72180; Electron Microscopy Sciences, Hatfield, PA).

TEM razor blades (Tousimis, Rockville, MD).

Pipetman kits (F167350 and F167550; Gilson Inc, Middleton, WI).

Diamond knife (Diatome, Hatfield, PA).

LKB 100-grid storage box (71147–12; Electron Microscopy Sciences, Hatfield, PA).

Chrome nozzle only (70702; Electron Microscopy Sciences, Hatfield, PA).

Dust-Off FGSA refill 8oz can (70707; Electron Microscopy Sciences, Hatfield, PA).

No. 1 superfine eyelash (113; Ted Pella, Inc., Redding, CA).

#5 Dumoxel tweezers (72700-D; Electron Microscopy Sciences, Hatfield, PA).

Micro plate shaker (12620–938; VWR, Radnor, PA).

Blue M oven (Thermo Fisher Scientific, Waltham, MA).

MVX10 microscope (Olympus Life Science, Center Valley, PA).

UC-7 ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL).

Tecnai G² 12 transmission electron microscope (FEI, Hillsboro, OR) equipped with a digital OneView camera (Gatan, Pleasanton, CA).

Blue M oven (Thermo Fisher Scientific, Waltham, MA).

MVX10 microscope (Olympus Life Science, Center Valley, PA).

UC-7 ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL).

Tecnai G² 12 transmission electron microscope (FEI, Hillsboro, OR) equipped with a digital OneView camera (Gatan, Pleasanton, CA).

Protocol steps

Synaptic vesicle purification

1. Rats are deeply anesthetized with 2 ml of 80 mg/ml chloral hydrate by intraperitoneal injection. Use 5 ml BD syringe for rats and 26G × 1/2 BD needle to do intraperitoneal injection. If chloral hydrate is not allowed, exposure to isoflurane in an induction chamber is an alternative
2. Decapitate rats using a guillotine and dissect out brains and quickly place them in ice-cold SB320 buffer. Drain off the SB320 buffer and dissect the slices containing the nAcc. Bring the slices to the ice-cold SB320I.
3. Dissect the nAcc out from the slices and transfer the nAcc materials in 8 ml ice-cold SB320I to a homogenizer and homogenize with 12 strokes.
4. Centrifuge the homogenate at 2000 g for 10 min.
5. Collect the supernatant and centrifuge at 10,000 for 30 min. The pellet contains the enriched synaptosomal fraction.

6. Osmotic shock for 5 min after adding 7 ml of ddH₂O containing 1× protease inhibitor.
7. Homogenize with 5 strokes on ice.
8. Readjust the osmolarity by adding 0.25 M potassium HEPES (pH 6.5) and neutral 1.0 M potassium tartrate (pH 7.5) in 1/10 volume (if add 7 ml of ddH₂O containing 1× protease inhibitor to 2 ml of synaptosome, add 900 μL of 0.25 M potassium HEPES (pH 6.5) and 900 μL of neutral 1.0 M potassium tartrate (pH 7.5)).
9. Centrifuge the preparation at 20,000 g for 20 min to remove the mitochondria.
10. For immuno-gold labeling and electron microscopy analysis, collect the supernatant and dilute with 1M HEPES/K+ tartrate to a final concentration of 0.1 M and process for immuno-gold EM.
11. For co-immunoprecipitation and western blot experiments, collect the supernatant and centrifuge at 55,000g for 60 min.
12. Collect the supernatant and add MgSO₄ buffer (final concentration is 1 mM), centrifuge at 100,000g for 45 min.
13. Resuspend the pellets containing the isolated synaptic vesicles with vesicle assay buffer containing 5 mM HEPES, 0.32 M sucrose, and protease inhibitors. Freeze the synaptic vesicles in liquid nitrogen and store at −80°C until use. Male Sprague-Dawley rats (6 weeks of age).

Immuno-gold detection in preparations of purified synaptic vesicles

1. To determine the purity of the synaptic vesicle preparation, pellets harvested in the last step of synaptic purification procedure is fixed with 2% osmium tetroxide for 1 h.
2. Rinse pellets with ddH₂O and postfix with 12% glutaraldehyde in ddH₂O overnight at 4°C.
3. Rinse pellets with ddH₂O and stain with 4% uranyl acetate for 30 min.
4. Rinse pellets with ddH₂O and dehydrate pellets with a series of graded ethanol (30%, 50%, 70%, 90%, 100%, 100%, and 100%) for 10 min each.
5. Infiltrate pellets with Durcupan ACM epoxy resin for overnight.

For preparation of Durcupan ACM epoxy resin, use pipettes to measure 10 ml of A, 10 ml of B. Mix on the nutator for 10 min. Then add 0.3 ml of C and mix on the nutator for 10 min. Add 0.2 ml of D and mix on the nutator for 30 min. it will be ready to use.
6. Exchange with freshly made Durcupan ACM epoxy resin. To polymerize the resin, bake in a 60°C Blue M oven for 48 h.

7. Use a glass knife maker (Leica EM KMR3) to make glass knives following the manual. Use nail polish to seal the glass knife boat to the glass knife. Make sure that there is no leakage.
8. Section the sample block at 60 nm thickness using a UC-7 ultramicrotome.
9. Line the bottom of a glass petri dish with parafilm. Add drops of 5% filtered uranyl acetate onto the parafilm. Stain the grid for 10–15 min with 5% filtered uranyl acetate.
10. Carefully rinse the grid with filtered ddH₂O, dry it with the filter paper and place it back into the grid storage box.
11. Stain with Sato's lead as described (Sato, 1968, see Reagents and Solutions 19) for 3 min. This is done in a covered glass petri dish in the presence of Sodium hydroxide pellets. Do not stain more than 5 grids at one time.
12. Carefully rinse the grid with filtered ddH₂O, dry it with the filter paper and place it back into the grid storage box.
13. Screen sections under a transmission electron microscope.
14. Thaw the vesicle aliquot from step 12.
15. Dilute vesicles with 1× K⁺-Tartrate/EDTA (1/2, 1/10, 1/20).
16. Put the diluted vesicles onto formvar coated grids (10 or 20 µL).
17. Wait until most of the fluid has evaporated, but do not allow grids to dry. Usually need 1 h.
18. Fix vesicles with 4 % PFA in a set of glass petri dish overnight at 4°C.
19. Rinse 3 times with 0.1 M PB.
20. Incubate in ethanolamine-HCl for 10 min.
21. Rinse 3 times with 0.1 M PB.
22. Incubate in the blocking buffer 10% NCS/TBS for 1 h.
23. Incubate with the primary antibody goat-anti-VMAT2 in 1% NCS/TBS overnight at 4°C.
24. Rinse with 1% NCS/TBS.
25. Incubate with the secondary antibody donkey-anti-goat 12 nm colloidal gold in 1% NCS/TBS containing 5 mg/10 ml PEG at RT for 2 h.
26. Rinse 3 times with TBS.
27. Postfix with 2.5% glutaraldehyde in 0.1 PB for 10 min.
28. Rinse 3 times with 0.1 M PB.
29. Incubate in the blocking buffer 10% NCS/TBS for 1 h.

30. Incubate with the primary antibody guinea pig-anti-VGluT2 in 1% NCS/TBS overnight at 4°C.
31. Rinse with 1% NCS/TBS.
32. Incubate with the secondary antibody donkey-anti-guinea pig 18 nm colloidal gold in 1% NCS/TBS containing 5 mg/10 ml PEG at RT for 2 h.
33. Rinse 3 times with TBS.
34. Postfix with 2.5% glutaraldehyde in 0.1 M PB for 10 min.
35. Rinse 3 times with ddH₂O.
36. Postfix with 1% osmium tetroxide in 0.1 M PB for 30 min.
37. Rinse 3 times with ddH₂O.
38. Rinse with 3 drops of 1% uranyl acetate and immediately dry on filter paper.
39. Screen the samples under a transmission electron microscope.
40. Collect the TEM images and analyze the data (Figure 4).

Reagents and Solutions

Animal perfusion

1. 0.9% saline solution.
2. 80 mg/ml chloral hydrate in 0.9% saline solution.
3. Fixative solution: 4% PFA + 0.15% glutaraldehyde + 15% picric acid solution in 0.1 M PB.

The role of the picric acid in this solution is to slowly penetrate into the tissue and cause coagulation of proteins by forming salts with basic proteins. Prepare 1 L of 8% PFA in 2× PB, filter the solution and bring it to final volume of 2 L (to obtain 4% PFA in 1× PB) by adding 300 ml of picric acid, 12 ml of 25% glutaraldehyde and 688 ml of ddH₂O. In details, for 1 L of fixative, heat 800 ml of ddH₂O and add 8.0 g of NaOH, stir until dissolved. Add 80 g of PFA, stir until dissolved. Add 33.66 g of NaH₂PO₄, stir until dissolved. Cool to room temperature. Filter the solution. Bring to a final volume to 1 L. For working solution, dilute 1 L of 2 × solution (8% PFA in 2 × PB buffer) with 1 L of ddH₂O to obtain 4% PFA in 0.1 M PB. To obtain an optimal tissue preservation, 4% PFA must be freshly prepared within a few hours prior to the perfusion.

4. 2% paraformaldehyde in 0.1 M PB.
5. 1,000 U/ml of heparin in saline solution: add 550 mg of heparin per 100 ml of 0.9% saline solution.

Vibratome sectioning

6. 0.1 M phosphate buffer (PB), pH 7.3: dissolve 33.67 g Sodium phosphate monobasic monohydrate and 7.7 g Sodium hydroxide to 1L ddH₂O. The pH is

7.3–7.4. It is not necessary to use pH meter to adjust pH for this recipe. You can also make $2 \times$ PB as a stock solution, dilute to $1 \times$ PB with ddH₂O before use. The recipe is different from the classic sodium phosphate buffer, but it is convenient to make this buffer and the buffer works well for immuno-electron microscopic studies.

7. Storage solution for vibratome sections: 25% sucrose and 10% glycerol in ddH₂O with 2mM sodium azide.

Pre-embedding single immunoperoxidase labeling or immunogold – silver intensification labeling

8. 0.1 M PB (pH 7.3) (See Reagents and Solutions 6).
9. 1% sodium borohydride in 0.1 M PB.
10. 1.5% glutaraldehyde in 0.1 M PB.
11. Blocking solution: 1% normal goat serum and 4% bovine serum albumin in 0.1 M PB supplemented with 0.02% saponin.
12. 0.003% hydrogen peroxide.
13. 4% sodium hydroxide.
14. Make aliquots for A, B, and C in the silver enhancement kit to centrifuge tubes. Cover tubes with foil and keep in -20°C . Take the aliquots out of the freezer and put them at room temperature before use. Aliquots should be frozen and thawed once.
15. 0.5% osmium tetroxide in 0.1 M PB.
16. Osmium tetroxide is highly toxic and is a rapid oxidizer. Exposure to the vapor can cause severe chemical burns to the eyes, skin, and respiratory tract. Wear nitrile gloves and eye protection. Do not open any vials of osmium tetroxide outside of the fume hood.
17. A series of graded ethanol: 30%, 50%, 70%, 90%, 100%.
18. 1% uranyl acetate in 70% ethanol. Uranyl acetate is both radioactive and toxic. For better staining, filter the uranyl acetate with a $0.22\ \mu\text{m}$ filter before use. Uranyl acetate helps to increase membrane contrast.
19. 5% uranyl acetate in ddH₂O.
20. Sato's lead (Sato, 1968): Weigh out 0.1 g of lead nitrate, 0.1 g of lead citrate, 0.1 g of lead acetate, and 0.2 g of sodium citrate. Add 8.2 ml of degassed distilled water to the above mixture of chemicals in a 15-ml Falcon tube and shake vigorously for 1 minute. The solution looks very milky. Add 1.8 ml of freshly made 4% sodium hydroxide. The solution becomes clear except for some large white grains at the bottom of the tube. Filter the solution with a $0.22\text{-}\mu\text{m}$ syringe driven filter unit. It is ready for use (Sato, 1968). Usually, the staining solution is

ineffective after 3 days. For good staining, always use freshly prepared lead staining solution in each experiment.

Animals and surgical procedures

21. Isoflurane solution (1169567761; Henry Schein, Dublin, OH).
22. The AAV5-DIO-ChR2-mCherry viral vector (AAV-EF1a-DIO-hChR2(H134R)-mCherry-WPRE-pA, 3×10^{12} genomes per ml) from the UNC Vector Core Facility.
23. 1% retrograde tracer Fluoro-Gold in sodium cacodylate buffer, pH 7.5.

Post-embedding immunolabeling

24. 0.1 M PB (pH 7.3) (See Reagents and Solutions 6).
25. 0.25% tannic acid in ddH₂O. Tannic acid fixation can improve the resolution of the ultrastructure.
26. 2% uranyl acetate in ddH₂O.
27. 1× PBS-Tween 20 buffer.
28. PBST buffer.
29. 0.05 M glycine in 1× PBS buffer.
30. Blocking solution: 2% normal goat serum (NGS) and 2% BSA in 1× PBS-Tween 20 buffer.
31. 2% glutaraldehyde in 1× PBS-Tween 20 buffer.
32. 5% uranyl acetate in ddH₂O.
33. Sato's lead (see Reagents and Solutions 19).

Synaptic vesicle purification

34. 0.9% saline solution.
35. 80 mg/ml chloral hydrate in 0.9% saline solution.
36. Coat the centrifuge tubes and pipettes with sigmacote. Glass or plastic ware should be siliconized with sigmacote. In a fume hood, evenly cover the plastic or glass surface and recover to solution to apply to the next piece of glassware or plasticware. Let the treated pieces stand overnight in the fume hood.
37. 1 M potassium hydroxide solution.
38. 1 M tartaric acid.
39. 5 mM HEPES buffer (pH 7.4). The pH is adjusted with potassium hydroxide solution or tartaric acid.

40. 0.2 M phenylmethylsulfonyl fluoride (PMSF) in 100% ethanol or 2 propanol (500× stock solution). Final working solution is 0.1 mM. Keep in −20°C for 2 months.
41. SB 320 buffer (0.32 M sucrose in 5 mM HEPES buffer, pH 7.4).
42. SBI: SB 320 buffer containing 1× protein inhibitor and PMSF. Every 50 ml SB 320 buffer, dissolve 1 tablet protein inhibitor and add 0.2 M PMSF 100 µL before use.
43. Osmotic shock solution: ddH₂O containing 1× protein inhibitor and PMSF.
44. 0.25 M potassium HEPES (pH 6.5); 1 M neutral L-(+)-tartaric acid dipotassium salt buffer (pH 7.5).
45. 1 M magnesium sulfate (1000× stock solution).

Immuno-gold detection within purified synaptic vesicles

46. 0.1 M PB (pH 7.3) (See Reagents and Solutions 6).
47. 2% osmium tetroxide in 0.1 M PB.
48. Freshly made 4% PFA in ddH₂O.
49. 1%, 4% and 5% uranyl acetate in ddH₂O.
50. A series of graded ethanol: 30%, 50%, 70%, 90%, 100%.
51. Sato's lead (see Reagents and Solutions 19).
52. 1 M K+-tartrate/EDTA. To make 20 ml of 1 M K+-tartrate/EDTA buffer, to dissolve 1.19 g HEPES in 16 ml, adjust pH with KOH or tartaric acid to 7.4. Add 4.7 g K+-tartrate and 0.03722 g EDTA.
53. Freshly made 4% PFA in 0.1 M PB.
54. 1 M ethanolamine-HCl.
55. TBS buffer: 0.1 M Tris/HCl (pH 7.4) containing 0.3 M NaCl.
56. Blocking buffer: 10% newborn calf serum (NCS) in TBS buffer.
57. Primary antibodies in 1% NCS/TBS buffer:
58. Secondary antibodies in 1% NCS/TBS buffer containing 5 mg/10 ml polyethylene glycol (PEG):
59. 2.5% glutaraldehyde in 0.1 M PB.
60. 1% osmium tetroxide in 0.1 M PB.

Commentary

Background Information

The maximum resolution of light microscopy (200 nm) far exceeds the thickness of cell membrane (3–10 nm). Electron microscopy utilizes an electron beam with a far smaller

wavelength than light. As a result, the resolution of a standard transmission electron microscope is about 0.2 nm. Brain analysis by electron microscopy has provided fundamental information on ultrastructural changes that occur in development, brain disorders (i.e., Alzheimer's disease) or in response to external agents (virus, toxins, drugs of abuse, etc.). Electron microscopy analysis has also been fundamental in advancing our knowledge on neuronal connectivity among different neurons, essential for advancing our understanding on brain function. While recently developed tools allow analysis and manipulation of specific neurons, combination of these tools with electron microscopy analysis provides fundamental information on molecular and ultrastructural characteristics of synapses within specific brain circuitry. It can also provide information on the subcellular presence and distribution of molecules involved in neurotransmission (i.e., neurotransmitters, receptors, and vesicular transporters), to determine mechanisms of neuronal signaling.

At the ultrastructural level, axon terminals containing synaptic vesicles are clearly seen, these axon terminals (presynaptic component of a synapse) may establish contacts (synapses) with cell bodies, axons or dendrites (postsynaptic component of a synapse). Synapses are normally classified as either asymmetric (excitatory) or symmetric (inhibitory) with a fine synaptic cleft (~20 nm) separating the pre- and postsynaptic components (Peters, Palay & Webster, 1991) (Stewart et al., 2014).

Immuno-electron microscopy is a powerful tool to analyze the presence and distribution of molecules at the subcellular level in biological material. The immuno-electron microscopy techniques are broadly divided into pre-embedding and post-embedding techniques. Here, we detail protocols of two types of pre-embedding immunolabeling techniques: immunoperoxidase and immunogold-silver labeling, and provide examples comparing results obtained with each of these protocols (Figure 1). This comparison illustrates the high sensitivity of antigen detection provided by immunoperoxidase immunolabeling and the localized antigen detection provided by immunogold-silver labeling. In post-embedding immunolabeling, in contrast to pre-embedding, the detection of cellular marker is done on ultrathin sections (instead of vibratome sections), obtained after tissue processing for ultrastructural analysis, and collected on a grid. The ultrastructural preservation is not optimal due to mild tissue fixation, such as low concentration of glutaraldehyde and omission of osmium tetroxide. While post-embedding labeling does not require tissue permeabilization, it requires that ultrathin section be treated with agents to allow exposure of antigenic sites, which may be crosslinked during fixation or resin polymerization. Over the years, a number of modifications have improved the post-embedding methods to enhance immunoreactivity and to preserve ultrastructure (Akagi et al., 2006; Berryman, Porter, Rodewald, & Hubbard, 1992; Phend, Weinberg, & Rustioni, 1992; Zhong, Brown, Wells, & Gerges, 2013).

In addition to determine the localization of molecular markers in brain tissue, transmission electron microscopy analysis also allows evaluation of isolated cells, organelles, synaptic vesicles, exosome, etc. (Kadota & Kadota, 1973). Moreover, immuno-gold detection of specific molecules within the purified samples provides crucial information on their

molecular composition (Boulland et al., 2009; Erickson, Masserano, Barnes, Ruth, & Weiner, 1990; Teng, Crooks, & Dwoskin, 1998).

Critical Parameters and Troubleshooting

1. Animal perfusion

The ideal fixation is to rapidly and uniformly preserve tissue in a life-like state. By fixative perfusing through the circulatory system, chemicals quickly reach every corner of the organism using the natural vascular network. For electron microscopic studies, we add picric acid to the 4% paraformaldehyde fixative solution, which slowly penetrates into the tissue and causes coagulation of proteins by forming salts with basic proteins. To preserve the integrity of postsynaptic densities and mitochondria, we perfuse animals within a period of 30 seconds after cutting the diaphragm. To remove blood from the brain, we perfuse 50 ml of heparin prior to 200 ml of fixative solution per mouse or 150 ml of heparin prior to 500 ml of fixative solution per rat.

2. Specificity of antibodies

Prior to the use of antibodies for immuno-electron microscopy analysis, their specificity has to be established. In addition, we recommend to test different antibody concentrations by bright microscopy to have an idea of the optimal concentration for ultrastructural studies.

3. Silver enhancement

Although 1.4 nm-Nanogold particles (Nanoprobes Yaphank, NY) can be detected by transmission electron microscopy without silver enhancement, commonly used heavy metals for counterstaining (such as osmium tetroxide and lead citrate) may obscure the detection of nanogold particles. To enhance the detection of nanogold particles, we enlarge their size to 10–20 nm by a silver enhancement procedure using the Nanoprobes HQ silver kit.

4. Peroxidase reaction

Immunoperoxidase labeling involves localizing of the product from horseradish peroxidase enzymatic reaction. The enzyme horseradish peroxidase is easily conjugated to antibodies, and because it is a relatively a small molecule, it can readily penetrate membranes of cells fixed with aldehydes. When the peroxidase molecule is exposed to a hydrogen peroxide substrate and the capturing agent DAB, the oxidation products of the DAB will form a brown reaction product. This insoluble product becomes electron dense following chelation by osmium tetroxide. The timing of incubation is critical for an optimal level of scattered dark material under the electron microscope.

5. Dehydration and infiltration

It is critical to avoid that the samples dry during the exchange of series of graded ethanol. Use a new unopened bottle of 100% ethanol for each experiment. If

some water remains after dehydration, the resin will not polymerize properly, and sectioning of the embedded samples won't be possible.

6. Ultrathin sectioning

For optimal ultrastructural examination, high quality ultrathin sections are needed with thickness ranging between 20 to 150 nm, which requires several months of practice.

7. Ultrastructural analysis

Synaptic contacts are classified according to their morphology and immunolabel, and photographed at a magnification of 6,800–13,000 $\times$. As detailed above, asymmetric synapses (also referred as Type I synapses) are defined by the presence of contiguous synaptic vesicles within the presynaptic axon terminal and a thick postsynaptic density (PSD) greater than 40 nm in thickness. In contrast, the symmetric synapses (also referred as Type II synapses) are defined by the presence of a thin PSD (Peters, Palay & Webster, 1991). Serial sections are necessary to determine if a synapse is asymmetric (excitatory) or symmetric (inhibitory). Regarding results with immunogold labeling, we consider a structure to be immuno positive when it contains more than 5 immunogold particles in serial sections.

Anticipated Results

In Unit 1, we describe the protocol for pre-embedding immunolabeling for the detection of a single type of molecule ("single immuno-labeling techniques") by using specific antibodies (primary antibodies) against these molecules (antigen). We also provide steps for two methods of detection of antigen-primary antibodies complexes, one method relies on the detection of an electron dense product resulting from peroxidase enzymatic reaction (immunoperoxidase labeling) and the other method relies on detection of colloidal gold particles after their intensification by silver exposure (immunogold – silver intensification labeling). Figure 1 shows the ultrastructural distribution of VGluT3 protein detected with primary antibodies against VGluT3, and further detection of the VGluT3 protein- primary antibodies complexes by immunoperoxidase reaction (**A**) or immunogold-silver intensification (**B**).

We had been combining peroxidase immunolabeling and gold-silver immunolabeling to identify either two or three different cellular markers. As shown above, the immunoperoxidase reaction product is seen as scattered dark material, whereas the silver-enhanced gold particles are seen as electrodense particulates. In Unit 2, we describe pre-embedding double or triple immuno-labeling by using a combination of peroxidase immunolabeling and gold-silver immunolabeling. We also describe the application of these immuno-labeling methods for the molecular detection in both wild type rats and cre transgenic mouse lines. These transgenic mice are endowed with the capability to induce cell specific expression of reporter molecules (i.e., mCherry) within the entire neuron, including axon terminals. In our laboratory, by the immunoperoxidase detection of VGluT3 and immunogold-silver detection of TH, we found that VGluT3 terminals establish asymmetric

synapses mostly on TH-positive ($62.51 \pm 0.44\%$; 291 terminals out of 466, Figure 2A). We detected GluR1 (by immuno-gold silver labeling) along the postsynaptic membrane of asymmetric synapses established by VGluT3 axon terminals (detected by immunoperoxidase reaction) in rats (Figure 2B). By triple immuno-electron microscopy, we found that dorsal raphe VGluT3 axon terminals containing mCherry (expressed in vivo in VGluT3 neurons by viral vector targeting) formed asymmetric synapses on FG-positive mesoaccumbens neurons in mice (Figure 2C). Together these findings suggest that dopamine mesoaccumbens neurons are targeted by glutamate inputs from the dorsal raphe (Qi, et al., 2014).

In Unit 3, we describe the post-embedding immunolabeling procedure. We compare the pre-embedding and post-embedding immunolabeling procedure (Figure 3). By pre-embedding immunolabeling, we observed VMAT2 (detected by immunoperoxidase reaction, seen as scattered dark material) in the axon segment proximal to a VGluT2 axon terminal (detected as gold particles). This VGluT2 axon terminal makes an asymmetric synapse on the head of a dendritic spine (Figure 3A). In the post-embedding immunolabeling procedure, ultrathin sections of the nucleus accumbens shell were probed with antibodies against VGluT2 or VMAT2 and detected with secondary antibodies bound to colloidal gold of two different sizes (18 nm and 12 nm). In keeping with results from pre-embedding labeling, we found with post-embedding labeling that when present in the same axon, VMAT2 was found in the axon segment proximal to a VGluT2-IR terminal making an asymmetric synapse on the head of a dendritic spine (Figure 3B). We determined that $85.48 \pm 3.03\%$ of the labeled ATs had VGluT2; $13.37 \pm 2.78\%$ had VMAT2, and $1.15 \pm 0.40\%$ appeared to co-express VGluT2 and VMAT2 (Figure 3C). From these findings, we suggest that VGluT2-TH neurons have the capability to store glutamate (via VGluT2) and dopamine (via VMAT2) in distinct vesicular populations that are differentially segregated in subcellular compartments within the same axon (Zhang, et al., 2015).

The ultrastructural findings detailed above provide evidence that in the nucleus accumbens the storage of dopamine and glutamate takes place in distinct vesicular pools enriched in different axonal micro-domains. However, to discard that our ultrastructural techniques lacked the sensitivity for co-detection of VGluT2 and VMAT2, we next explored the possibility of coexistence of VGluT2 and VMAT2 at the vesicular level by immunolabeling of VGluT2 and VMAT2 in purified vesicles from rat nucleus accumbens synaptomes. After testing several conditions of isolation for synaptic vesicles, we achieved the experimental conditions (see Unit 4) that allowed the purification of a homogeneous population of synaptic vesicles, the quality and purity of which were confirmed by electron microscopy analysis (Figure 4A). By immunolabeling of these isolated synaptic vesicles, we detected two distinct pools of vesicles, those containing VGluT2-IR and those containing VMAT2-IR (Figure 4B–D) (Zhang, et al., 2015).

Time Considerations

Procedures for immunolabeling and ultrastructural studies are very time consuming. While it may take about 3 weeks from the time of animal perfusion until the first evaluation of ultrastructural immunolabeling, the optimization of immunolabeling conditions (antibody

concentrations, detection of antigen-antibody complexes, etc), image collection and sampling for quantitative analysis (requiring several sections from tissue samples from more than 3 animals) may take 6 months to a year.

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Significance statement

Brain analysis by electron microscopy has provided fundamental information on ultrastructural changes that occur in development, brain disorders (i.e., Alzheimer's disease) or in response to external agents (virus, toxins, drugs of abuse, etc.). Electron microscopy analysis has also been fundamental in advancing our knowledge on neuronal connectivity among different neurons, essential for advancing our understanding on brain function. While recently developed tools allow analysis and manipulation of specific neurons, combination of these tools with electron microscopy analysis provides fundamental information on molecular and ultrastructural characteristics of synapses within specific brain circuitry. It can also provide information on the subcellular presence and distribution of molecules involved in neurotransmission (i.e., neurotransmitters, receptors, and vesicular transporters) to determine mechanisms of neuronal signaling.

Figure 1. Ultrastructural detection of the vesicular glutamate transporter 3 (VGLUT3) in the ventral tegmental area (VTA) of wild type adult Sprague Dawley rat.

A-B. The ultrastructural distribution of VGLUT3 protein was detected with primary antibodies against VGLUT3, and the complexes established between the VGLUT3 protein and the specific primary antibodies against this protein were further demonstrated by immunoperoxidase reaction (A) or immunogold-silver intensification (B).

A. Immunoperoxidase detection of VGLUT3 within an axon terminal (AT; pink outline) is seen as scattered dark material. This AT is making an asymmetric synapse (green arrow) on a dendritic spine.

B. Immunogold-silver detection of VGluT3 within an AT (pink outline) is seen as gold particles (arrow head). This AT is making an asymmetric synapse (green arrow) on a dendrite. Note visualization of synaptic vesicles within the AT.

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Figure 2. Ultrastructural detection of VGlut3, TH, AMPA receptor subunit GluR1 in the VTA of wild type adult Sprague Dawley rat and ultrastructural detection of the VGlut3, mCherry, and Fluoro-Gold (FG) in VTA of VGlut3-ChR2-mCherry mice.

A. VTA immunoperoxidase detection of VGlut3 within an axon terminal (AT; pink outline) is seen as scattered dark material. Immunogold-silver detection of tyrosine hydroxylase (TH) within a dendrite (blue outline) is seen as gold particles (blue arrowhead). This AT containing VGlut3 is making an asymmetric synapse (green arrow) with a TH dendrite. Wild type adult Sprague Dawley rats.

B. VTA immunoperoxidase detection of VGlut3 within an axon terminal (AT; pink outline) is seen as scattered dark material. Immunogold-silver detection of AMPA receptor subunit GluR1 detection along the postsynaptic membrane of two dendrites (blue outline) is seen as gold particles (green arrowhead). GluR1 signal is detected along the postsynaptic membrane of two dendrites making asymmetric synapses (green arrows) with a single AT containing VGlut3. Wild type adult Sprague Dawley rats.

C. VTA immunoperoxidase detection of mCherry and immunogold-silver detection for both VGlut3 and FG in the VGlut3-ChR2-mCherry mice. DR VGlut3 neurons were infected by injections of AAV5-DIO-ChR2-mCherry into the DR of VGlut3::Cre mice. The same mice were injected with the retrograde tracer FG into the nucleus accumbens to label VTA

neurons that innervate the nucleus accumbens and establish synapses with axon terminals from DR-VGluT3 neurons. The mCherry signal is seen as scattered dark material and VGluT3 signal is seen as gold particles (pink arrowhead) within an axon terminal (AT1; pink outline). FG signal is seen as gold particles (blue arrowhead) in a dendrite (blue outline). This AT1 containing mCherry and VGluT3 is making an asymmetric synapse (green arrow) with an FG dendrite. AT2 is a mCherry-VGluT3 negative axon terminal. Modified from Qi et al. (Qi et al., 2014).

Figure 3. Comparison of ultrastructural preservation and detection of vesicular transporters (VGluT2 and VMAT2) obtained by pre-embedding or post-embedding immunolabeling (nucleus accumbens of wild type adult Sprague Dawley rat).

A. Pre-embedding detection of VGluT2 in axon terminals lacking VMAT2, which establish asymmetric synapses (green arrows) on dendritic spines (orange outlines). The VGluT2 detected by immunogold-silver labeling seen as gold particles is confined to the axon terminals. The contiguous axon segments to this VGluT2 terminal contains VMAT2 detected by immunoperoxidase reaction seen as scattered dark material.

B. Post-embedding detection of VGluT2 (18 nm gold particles, black arrows) in an axon terminal lacking VMAT2 and establishing an asymmetric synapse (green arrow) on a dendritic spine (orange outline). The contiguous axon segment to this VGluT2 terminal contains VMAT2 (12 nm gold particles, black arrowheads).

C. Bars indicating the frequency (mean + s.e.m.) of axon terminals (ATs) containing VGluT2-immunoreactivity (IR) or VMAT2-IR from a total of 257 ATs. Out of these ATs, $85.48 \pm 3.03\%$ have VGluT2-IR; $13.37 \pm 2.78\%$ have VMAT2-IR and $1.15 \pm 0.40\%$ appear to co-express VGluT2-IR and VMAT2-IR (paired t-test, $t(3) = 12.42$, $p = 0.0011$). ATs were quantified from the nucleus accumbens of rats. Modified from Zhang et al. (Zhang et al., 2015).

Figure 4. Purity and integrity of isolated synaptic vesicles and immuno-gold detection of VGluT2 and VMAT2.

- A. Electron micrograph showing the purity and integrity of nucleus accumbens isolated synaptic vesicles used for dual detection of VGluT2-IR and VMAT2-IR (B-D).
- B. Detection of VGluT2-IR (arrows; 18 nm gold particles) or VMAT2-IR (arrowheads; 12 nm gold particles) associated to purified synaptic vesicles.
- C. High magnification of VGluT2 associated to purified synaptic vesicle detected by 18 nm gold particle.
- D. High magnification of VMAT2 associated to purified synaptic vesicle detected by 12 nm gold particle. Modified from Zhang et al. (Zhang et al., 2015).