Ultrastructural readout of functional synaptic vesicle pools in hippocampal slices based on FM dye labeling and photoconversion

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Fast activity-driven turnover of neurotransmitter-filled vesicles at presynaptic terminals is a crucial step in information transfer in the CNS. Characterization of the relationship between the nanoscale organization of synaptic vesicles and their functional properties during transmission is currently of interest. Here we outline a procedure for ultrastructural investigation of functional vesicles in synapses from native mammalian brain tissue. FM dye is injected into the target region of a brain slice and upstream axons are electrically activated to stimulate vesicle turnover and dye uptake. In the presence of diaminobenzidine (DAB), photoactivation of dye-filled vesicles yields an osmiophilic precipitate that is visible in electron micrographs. When combined with serial-section electron microscopy, fundamental ultrastructure-function relationships of presynaptic terminals in native circuits are revealed. We outline the utility of this protocol for the 3D reconstruction of a recycling vesicle pool in CA3–CA1 synapses from an acute hippocampal slice and for the characterization of its anatomically defined docked pool. This protocol requires 6–7 d.

INTRODUCTION

In the CNS, fast neuron-neuron information transfer primarily takes place at chemical synapses, which are specialized and ultrastructurally distinct junction points at which presynaptic and postsynaptic structures lie closely apposed[1]. The presynaptic terminal is characterized by a cluster of neurotransmitter-containing synaptic vesicles, and transmission proceeds with activity-driven vesicle fusion leading to the discharge of chemical transmitter toward postsynaptic receptors. Although vesicles appear morphologically equivalent, they can be subdivided into pools on the basis of their functional behavior, including a recycling pool[2,3] readily releasable pool[4], spontaneous pool[5,6] and superpool[7,8]. Understanding the properties of these pools has become increasingly important with the realization that they are potentially important substrates in setting synaptic strength[9,10], and they represent modifiable targets on which forms of plasticity[9,11–15] or disease-like conditions[16–18] might act to modulate or disrupt information flow.

Labeling and visualization of functional vesicles

Extensive research has focused on the kinetic characterization of vesicle turnover in specific pools[19], but there is also substantial interest in approaches that can link such functional properties with information about the physical organization of vesicles within synaptic compartments. This presents a challenge, because synapses are small with complex nanoscale morphology that is not readily resolvable by using conventional light microscopy. Elegant methods relying on nanoparticles such as quantum dots[20,21] and super-resolution fluorescence imaging[8] are important strategies to address this challenge, but they still provide limited spatial information and ultrastructural context. An alternative approach is to combine a functional measure of vesicle pools with subsequent ultrastructural investigation. One of the most widely used methods exploits FM dyes, which are fluorescent reporters that readily bind to the lipid membrane and are taken up into recycling vesicles during endocytosis to provide a functional readout of vesicle turnover[22–24]. One dye variant FM1-43, and its fixable form FM1-43FX, also efficiently drives the polymerization of DAB when it is photoactivated, leading to the formation of an osmiophilic precipitate[25–32]. In this way, functional vesicle pools that were previously labeled with FM dye can be directly identified in electron micrographs. This goes substantially beyond the information offered by fluorescence-based assessment of FM dye signal, and it provides an opportunity to assess the arrangement of recycled vesicles in the context of their local ultrastructure, for example, with respect to defined synaptic structures (e.g., the active zone) and other vesicle pools. An established alternative method to the use of FM dye staining/photoconversion relies on the labeling of endocytosing vesicles with horseradish peroxidase, which can catalyze the conversion of DAB to an electron-dense form. This approach has substantial merit, but the efficiency of horseradish peroxidase labeling of endocytosing vesicles is much lower than with FM dyes, which is perhaps attributable to its higher molecular weight and low solubility[32]. As such, its usefulness for detailed characterization of vesicle pools is more limited.

Ultrastructural analysis based on FM dyes has been used extensively and highly successfully in cultured neurons[7,10,25,29,31,33–36], a number of large, peripheral terminals[27–30,32,37–39] and large central release sites such as calyx of Held[26], which revealed important information about organizational principles of vesicle pools. However, only recently[40] has this method been successfully applied to small central synapses in native brain tissue, where neurons are retained in relevant circuits with defined cytoarchitecture. This reflects the fact that the thick tissue of a brain slice presents a number of technical challenges compared with, for example, cultured neurons. These include the need to devise ways to provide good access for FM dye to the target region, the requirement for fast fixation with excellent ultrastructural preservation, the accurate calibration

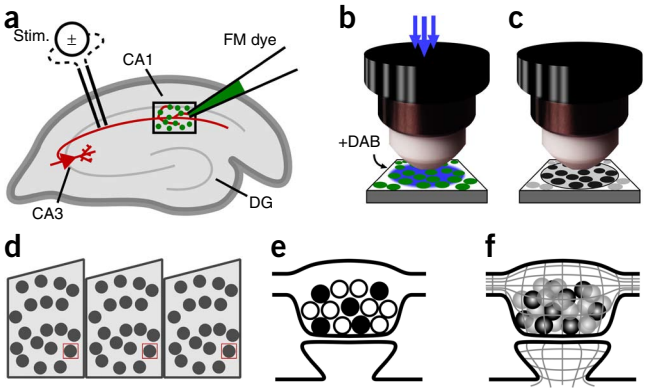

Figure 1 | Overview of the experimental protocol. (**a**) Schematic of an approach for fixable FM dye-labeling of terminals in the stratum radiatum of acute hippocampal slice. A bipolar stimulating electrode is placed on Schaffer collaterals and an FM dye–filled pipette is positioned in CA1. DG, dentate gyrus. The dye is pressure-ejected while stimulation (1,200 action potentials (APs), 10 Hz) is applied. (**b**) Labeled terminals are photoilluminated with blue light in the presence of DAB. (**c**) After photoconversion, synapses appear dark owing to the formation of osmiophilic precipitate. (**d**) The target region is embedded in resin and serially sectioned. (**e**) In electron micrographs, presynaptic terminals contain photoconverted (PC+) vesicles with dark lumen and nonphotoconverted (PC–) vesicles. (**f**) 3D models of synaptic terminals can be constructed from serial sections. Parts of this figure have been adapted with permission from Marra *et al.*[40].

of the photoconversion reaction to achieve the correct formation of an electron-dense precipitate and the nanoscale relocation of the target region in embedded tissue. The current protocol outlines an approach that addresses these issues to allow detailed analysis of functional vesicle pools in synaptic terminals in the stratum radiatum of CA1 in acute hippocampal slices (**Fig. 1**). The principal dye-labeling step is based on an established approach adopted in a number of previous studies[7,13,35,40–44], and we outline key strategies to validate its success. Subsequently, detailed methodology for rapid microwave-enhanced fixation[45] and calibrated photoconversion of the target region are provided. By using serial-section and 3D reconstruction methods, we demonstrate the utility of this approach for the characterization of a functional vesicle pool in a target synapse.

Applications and future adaptations

The protocol lends itself to an array of applications and future adaptations. As it makes use of a standard and widely used acute brain slice preparation, it can be readily combined with conventional methodologies to provide an ultrastructural extension to established experimental approaches, for example, electrophysiological investigation, fluorescence imaging or pharmacological studies. Key applications could include the investigation of presynaptic changes occurring in long-term plasticity or in elucidating presynaptic changes in genetic mouse models. With specific variations of the stimulation protocols, it is also an approach to investigate the recycling of different vesicle pools and/or endocytic mechanisms. In the face of the rapid development of large-volume automated electron microscopy approaches, a nanoscale readout of synaptic activity at the level of functional vesicle pools is likely to become an important tool.

Experimental design

This protocol describes an experimental procedure for the dye-labeling of functional synapses in living brain slice, followed by steps to fix the sample and photoconvert the fluorescence signal into an electron-dense form that is suitable for ultrastructural characterization. In this procedure, FM dye is applied by pressure injection into the target region of the slice and, at the same time, upstream axons are electrically stimulated to evoke vesicle turnover. A key issue for this type of experiment is to ensure saturating dye penetration to all areas of the region of interest. To achieve this, the dye is injected at a high concentration (20 µM) at a depth ~50 µm from the slice surface for an extended period before and after stimulation. The movement of the dye through the tissue can be directly visualized by imaging during dye injection, and in our experiments a high concentration of dye readily diffuses in the slice to occupy an approximately spherical volume extending up to the surface. To confirm that this leads to consistent synaptic labeling across the region of interest, we recommend performing pilot experiments after the completion of dye-loading and washing steps to test for widespread punctate fluorescence. This can be carried out by making comparisons of intensity measurements of fluorescent puncta near the injection site versus those of other regions displaced laterally or in the *z* plane. For additional steps to validate the presence of functional terminals, see **Box 1**.

Box 1 | Establishing the presence of functional FM dye–labeled terminals

● TIMING 10 min

This validation protocol is helpful in establishing that the punctate staining observed in Step 10 corresponds to functional dye-labeled presynaptic terminals. As the successful completion of this procedure results in the loss of synaptic dye labeling, it is not compatible with subsequent main protocol steps (Steps 11–46) that lead to the ultrastructural visualization of functional vesicles. The rationale for this validation step is the idea that functional synaptic labeling can be confirmed by observing a robust activity-evoked fluorescence decrease at terminals ('destaining') as FM dye–labeled vesicles undergo fusion and dye loss (**Fig. 4a**,**b**).

1. Carry out imaging in the CA1 region and identify the punctate staining as in Step 10.
2. Set up a time-lapse imaging experiment with an acquisition rate of 0.5–1 Hz. Establish a baseline of ten frames and then begin electrical stimulation (2–20 Hz for 1,200 pulses with parameter settings used for loading) while continuing to image at the same frequency.
3. In offline analysis, measure fluorescence intensities over time in regions of interest containing fluorescence puncta. Plots should appear as a stable baseline period followed by a decline in fluorescence that can be described by a single exponential decay profile. The time constant of dye loss should be inversely proportional to the stimulation frequency (**Fig. 4c**).

Another issue regarding penetration relates to the photoconversion reaction; establishing that photoconversion product is maximally and homogeneously distributed across the region of interest is a key requirement. A confounding factor is that photoconversion is necessarily achieved by illumination arising from a unidirectional light source above the sample. Thus, as the reaction proceeds, buildup of DAB photoconversion product at the surface (nearest to the light source) gradually occludes light penetration at greater depths, potentially limiting further photoconversion in less superficial tissue. As outlined below (see Equipment Setup), the measurement of transmitted light to monitor the accumulation of photoconverted product in the sample offers a useful indicator of the progression of the reaction. However, it is also important, particularly if the experimenter is interested in studying synapses over different depths in the tissue, to establish that the reaction product is uniformly distributed through the tissue depth at an ultrastructural level. For our system, we observe comparable levels of photoconverted vesicles across a depth range of 2–50 µm from the top surface of the slice. As a precaution, however, we typically target only a subset of this range (5–30 µm) to ensure that we are using the same conditions for comparison from slice to slice.

MATERIALS

REAGENTS

- Diaminobenzidine (DAB; Kem-En-Tec, cat. no. 4170) **! CAUTION** DAB may cause genetic defects and cancer. Wear gloves and, as far as possible, work in the fume hood.
- DDSA (TAAB Laboratories Equipment, cat. no. DO27) **! CAUTION** DDSA is irritant to skin. Wear gloves when you are handling the compound. All waste should be disposed of according to relevant hazardous chemical regulations.
- DMP-30 (TAAB Laboratories Equipment, cat. no. DO32) **! CAUTION** DMP-30 is harmful if swallowed; it is an irritant to eyes and skin. Wear gloves when you are handling the compound. All waste should be disposed of according to relevant hazardous chemical regulations.
- D-Glucose (Sigma-Aldrich, cat. no. G8270)
- DMSO (Sigma-Aldrich, cat. no. 472301)
- FM1-43FX (Invitrogen, cat. no. F-35355)
- Glutaraldehyde, 25% (vol/vol) solution (Agar Scientific, cat. no. AGR1312) **! CAUTION** This compound is toxic on inhalation; it is corrosive, is harmful if swallowed and causes irritation on inhalation and skin contact; it is dangerous to the environment. Wear gloves and work in the fume hood. All waste should be disposed of according to relevant hazardous chemical regulations.
- Glycine (Sigma-Aldrich, cat. no. G8898)
- Magnesium chloride ($MgCl_2$; Sigma-Aldrich, cat. no. M8266)
- MNA (TAAB Laboratories Equipment, cat. no. MO11) **! CAUTION** MNA is corrosive; it is an irritant to eyes and skin; it may cause allergic skin reactions and may cause allergy or asthma symptoms or breathing difficulties if inhaled. Wear gloves when handling it. All waste should be disposed of according to relevant hazardous chemical regulations.
- Osmium tetroxide (TAAB Laboratories Equipment, cat. no. O021) **! CAUTION** Osmium tetroxide is toxic on inhalation, on contact with skin and if swallowed. Keep it stored in a double container in a dangerous chemicals fridge. Wear gloves and work in the fume hood. All sample incubations need to also be placed in a sealable plastic container dedicated for use with osmium tetroxide. All waste should be disposed of according to relevant hazardous chemical regulations.
- Formaldehyde, 16% (wt/vol) solution (Agar Scientific, cat. no. AGR1026) **! CAUTION** This compound is harmful on inhalation, on contact with skin and if swallowed; it is an irritant to eyes, respiratory system and skin. Wear gloves and work in the fume hood. All waste should be disposed of according to relevant hazardous chemical regulations.
- Potassium chloride (KCl; Sigma-Aldrich, cat. no. P9333)
- Potassium ferrocyanide (Sigma-Aldrich, cat. no. 455989) **! CAUTION** This compound is harmful on inhalation, on contact with skin and if swallowed; it is an irritant to eyes. Wear gloves and work in the fume hood. All waste should be disposed of according to relevant hazardous chemical regulations.
- Propylene oxide (Agar Scientific, cat. no. AGR1080) **! CAUTION** This compound is toxic and it may cause cancer and heritable genetic damage; it is harmful on inhalation, on contact with skin and if swallowed; it is an irritant to eyes, respiratory system and skin; this compound is extremely flammable. It corrodes some plastics, so use glass Pasteur pipettes and glass dishes or vials for incubations. Wear latex gloves and work in the fume hood. All waste should be disposed of according to relevant hazardous chemical regulations.
- Sodium cacodylate (Agar Scientific, cat. no. AGR1104) **! CAUTION** This compound is toxic, and it may cause harm to the unborn child; it is harmful on inhalation, on contact with skin and if swallowed; it is an irritant to eyes. Wear gloves and work in the fume hood. All waste should be disposed of according to relevant hazardous chemical regulations.
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. 746398)
- Sodium hydrogencarbonate ($NaHCO_3$; Sigma-Aldrich, cat. no. 401676)
- Sodium phosphate, monobasic (Sigma-Aldrich, cat. no. S9638)
- TAAB 812 (TAAB Laboratories Equipment, cat. no. TO23) **! CAUTION** This compound is irritant to eyes and skin; it is dangerous to the environment. Wear gloves and work in the fume hood. All waste should be disposed of according to relevant hazardous chemical regulations.
- Uranyl acetate (Agar Scientific, cat. no. AGR1260A) **! CAUTION** This compound is very toxic on inhalation and if swallowed; it is dangerous to the environment. Wear gloves and work in the fume hood. All waste should be disposed of according to relevant hazardous chemical regulations.
- Hippocampal slices of interest. In this protocol, we describe the use of acute transverse hippocampal slices (300 µm thickness) from 21- to 28-d rats. For further information about preparing hippocampal slices, see refs. 46,47 **! CAUTION** All animal experiments must comply with relevant regulations. **▲ CRITICAL** Place slices into artificial cerebrospinal fluid (aCSF) bubbled with 95% O_2 and 5% CO_2 (Carbogen) immediately.

EQUIPMENT

- Antivibration table (e.g., 780 series, TMC)
- Amplifier (e.g., Multiclamp 700B, Molecular Devices)
- A-D converter (e.g., Digidata 1550, Molecular Devices)
- Charge-coupled device (CCD) camera (e.g., QIClick, Q-Imaging)
- Confocal microscope equipped with Argon laser; upright microscope (e.g., Olympus model: BX51WI or comparable) with confocal head (Olympus, model: Fluoview FV300 or comparable) and ×4 (PLN4X), ×40 (LUMPLFLN40XW) and ×60 (LUMPLFLN60XW) objectives
- Embedding capsules (Agar Scientific, cat. no. G360-1,00 BEEM capsules)
- Formvar-coated slot grids (TAAB Laboratories Equipment, cat. no. F218/050)
- Grid Box (e.g., Gilder SB50, TAAB Laboratories Equipment)
- Hg epifluorescence lamp for photoconversion (Omega Optical XF100-2, Dichroic mirror 500 nm to direct light for photoconversion)
- Illumination for stereomicroscope (e.g., KL 1500 LCD, Schott UK)
- Micromanipulator (LBM series or equivalent, Scientifica)
- Microwave oven (e.g., Panasonic NN-E289M) **▲ CRITICAL** Only required if using microwave fixation (**Box 2**).
- Peristaltic pump (e.g., Minipuls 3, Gilson)
- Pipette puller (e.g., PC-10, Narishige)
- Platinum wire, 0.5-mm thick (Alfa Aesar, cat. no. 10286)
- Pressure-injection system (e.g., Picospritzer, Parker)
- Stimulator (e.g., Grass SD-9)
- Stereomicroscope (e.g., Stemi 2000, Carl Zeiss)
- Thermometer (e.g., RS Components, 615-8212)
- Theta glass CG200T (Harvard Apparatus, cat. no. 300117)
- Tungsten wire, 0.075 mm thick (Alfa Aesar, cat. no. 00457)
- Ultramicrotome (e.g., EM UC7, Leica)
- Vibrating microtome (e.g., VT1200S, Leica)

REAGENT SETUP

Artificial cerebrospinal fluid This solution contains 125 mM NaCl, 2.5 mM KCl, 25 mM glucose, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 50 µM AP5 saturated with a gaseous mixture of carbogen (95% O_2 and 5% CO_2; pH 7.3). Prepare fresh solution on the first day of the experimental protocol.

Box 2 | Using microwave-enhanced fixation ● TIMING ~1 h

Microwave-enhanced fixation provides a means to achieve rapid fixation of thick tissue with excellent ultrastructural preservation[45]. The overall objective is to heat the brain slice for 8–15 s to 45–50 °C while it is immersed in fixative. This can be achieved by using a specialized microwave-based processing system or an inexpensive domestic microwave oven placed in a fume hood. Regardless of the system chosen, the intensity and duration of microwave irradiation needs to be initially calibrated. A suggested protocol for a domestic microwave is outlined below.

PROCEDURE

Calibration

1. Remove the microwave plate and cover the central rotating mechanism with an inverted Petri dish to create a stationary central platform.
2. Place a beaker containing 200 ml of ddH_2O at room temperature in one of the two rear corners of the microwave oven. Set the power of the microwave oven to 700 W and irradiate until the temperature of the water in the beaker reaches 40–45 °C. This can be determined by immediately removing the beaker after irradiation and measuring the temperature by using a fast-read temperature probe (e.g., RS 55II thermometer). The water in the beaker will absorb reflected irradiation, improving the uniformity of irradiation of the sample.
3. With the preheated beaker in the rear corner, place 4 ml of fixative in a Petri dish warmed to 37 °C on the central platform. Irradiate for 10 s and measure the temperature of the fixative immediately after irradiation (the use of a dedicated temperature probe is highly recommended). The final temperature of the fixative should be 45–50 °C. If this temperature is not achieved, repeat the process using a new fixative sample and adjusting the irradiation time accordingly. Once parameters are established to meet the target temperature, repeat with multiple samples to ensure consistency.

Sample fixation

4. Prepare the microwave as in calibration Step 1 above. As in Step 2, place a beaker containing 200 ml of reverse-osmosis water at room temperature in the rear corner of the microwave oven floor. Irradiate at 700 W for the time determined in Step 2.
5. Rapidly place a tissue sample in a Petri dish containing fixative (6% glutaraldehyde and 2% formaldehyde in PBS at 37 °C), and anchor it by using a glass O-ring. Place the Petri dish in the center of the microwave oven, and irradiate it at 700 W for the duration determined in Step 3.

Fixative On the first day of the experimental protocol, prepare a solution containing 1 ml of 10× PBS, 1.25 ml of 16% formaldehyde and 2.4 ml of glutaraldehyde, and bring it to a 10-ml volume by using ddH_2O. This produces a solution with final concentrations of 6% glutaraldehyde and 2% formaldehyde with pH 7.3. **! CAUTION** This fixative is harmful on inhalation, on contact with skin and if swallowed; it is an irritant to eyes, respiratory system and skin. Wear gloves and work in the fume hood. All waste should be disposed of according to relevant hazardous chemical regulations.

DAB Add one tablet of DAB-PBS to 10 ml of ddH_2O, and sonicate it for 10 min. Prepare DAB freshly immediately before use, and minimize exposure to light. **! CAUTION** DAB may cause genetic defects and cancer. Avoid contact with skin. After use, place all contaminated plastic and glassware in bleach for 48 h. Handle objective lens with gloves, and rinse it extensively with ddH_2O after use.

EPON solution In a disposable beaker on a measuring scale, add 24 g of TAAB 812, 9.5 g of DDSA, 16.5 g of MNA and finally 1 g of DMP-30; vortex the solution for 1 min and wait for ~5 min for air bubbles to surface. Prepare the solution freshly on the day of use, and allow 30 min for it to mix thoroughly. **! CAUTION** These reagents can be irritant to eyes and skin and dangerous to the environment. Wear gloves and work in the fume hood. All waste should be disposed of according to relevant hazardous chemical regulations.

FM1-43FX solution 10 mM FM1-43FX stocks should be kept at −20 °C in DMSO; they can be stored for up to 3 months. On the day of the experiment, make 500 µl of 20 µM FM dye solution by diluting the stock in bubbled aCSF.

Sodium cacodylate buffer Sodium cacodylate buffer is 0.1 M sodium cacodylate in ddH_2O adjusted to pH 7.4 with HCl. The solution can be stored at 4 °C for 3 months. **! CAUTION** Sodium cacodylate is toxic and harmful on inhalation, on contact with skin and if swallowed; it is irritant to eyes. Wear gloves and work in the fume hood. All waste should be disposed of according to relevant hazardous chemical regulations.

Uranyl acetate solution Prepare 4% (wt/vol) uranyl acetate in 70% (vol/vol) ethanol freshly on the second day of the experimental protocol. Vortex the solution for at least 5 min and filter it immediately before use. **! CAUTION** Uranyl acetate is very toxic on inhalation and if swallowed; it is dangerous to the environment. Wear gloves and work in the fume hood. All waste should be disposed of according to relevant hazardous chemical regulations.

EQUIPMENT SETUP

Bipolar tungsten-stimulating electrode A number of bipolar stimulating electrodes are commercially available (Metal microelectrode, WPI). Alternatively, stimulating electrodes can be fabricated by inserting one tungsten wire of 0.075 mm thickness in each of the two compartments of a theta glass capillary . Pull the capillary over the blue flame of a Bunsen burner to produce an electrode with a fine tip (~0.2 mm). Each tungsten wire is soldered at one end to a suitable electric wire for connection to the stimulator (e.g., SD-9, Grass stimulator; **Fig. 2a**).

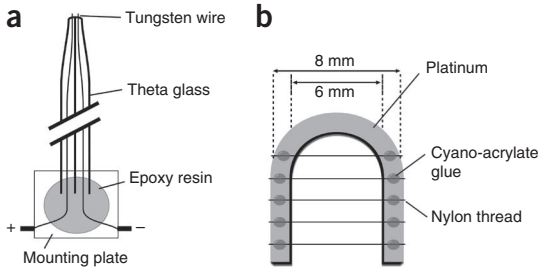

Figure 2 | Schematics of key equipment items. (**a**) Bipolar stimulating electrode. Heat-drawn theta glass contains tungsten wire in each barrel. The tip end is fire-polished to seal around each tungsten wire. The other end of each wire is glued securely onto a small mounting plate and soldered to plugs. (**b**) Harp-slice grid made from flattened platinum wire that is bent to a U shape. Nylon threads are stretched across this frame and secured with cyanoacrylate glue.

U-shaped harp-slice grid Harps can be purchased (e.g., Harvard Apparatus, cat. no. 64-0254) or manufactured by bending a segment of platinum wire in a U shape (0.5 mm in diameter and ~25 mm in length); the two arms of the U should be at least 6 mm apart. The wire is then flattened by using a bench-top jaw drill press, and 4–6 nylon threads are attached with cyanoacrylate glue between the two arms of the U-shaped wire with spacing of 1.5–2 mm (**Fig. 2b**).

Glass O-ring By using cyanoacrylate glue, attach 4–6 nylon threads onto one side of a glass ring (internal diameter, 15 mm; 5 mm thick).

Pressure-injection system Connect the pressure outlet of the pressure-injection system (e.g., Picospritzer and Parker) to the 'pressure' port of a patch-clamp electrode holder (e.g., Series Q holders, Harvard Apparatus), which is also connected to your amplifier.

Electrophysiological recording setup Set up according to the manufacturer's instructions. Further details and useful advice on how to set up an electrophysiology rig can be found in the Axon Guide (http://mdc.custhelp.com/euf/assets/content/Axon%20Guide%203rd%20edition.pdf).

Imaging setup Follow the manufacturer's instruction to set up the imaging system. Select a suitable excitation wavelength to visualize FM1-43FX (i.e., 488 nm with confocal, 840 nm with multiphoton laser). The emission range for FM1-43FX is broad with a peak at 580 nm; select suitable emission filter for visualization (e.g., BP530/30 or BP590/34).

Microwave fixation calibration If the preferred method of fixation relies on microwave fixation, this procedure will require calibration steps before the start of an experiment (**Box 2**).

PROCEDURE

FM dye-labeling and fluorescence imaging ● TIMING 3–4 h

1| Place the acute transverse hippocampal slices (300 µm thickness)[46,47] in aCSF bubbled with 95% O_2 and 5% CO_2 (Carbogen). Allow the slices to recover for 30 min at 37 °C, followed by 30 min at room temperature (20 °C).

2| Use a transfer pipette to move a slice from the recovery chamber to the imaging chamber containing bubbled aCSF, which is continuously perfused at a rate of 2–5 ml/min.

3| Place the harp-slice grid on top of the slice to anchor the tissue, and allow the slice to equilibrate for 15 min.

4| Place the tungsten-stimulating electrode on stratum radiatum to stimulate Schaffer collaterals (**Figs. 1a** and **3a**).

5| Place a recording electrode containing aCSF and 20 µM FM1-43FX in the stratum radiatum of CA1 region (**Fig. 3a**). The tip of the electrode should be ~50 µm below the surface of the slice.

6| Stimulate at 0.2 Hz (0.8–1 ms) by using a voltage between 0.1 V and 2 V; each tungsten electrode will need to be calibrated separately. The objective of this step is to obtain a repeatedly evoked field excitatory postsynaptic potential with an amplitude of at least 0.2 mV (**Fig. 3b**).

7| Once a robust postsynaptic response is achieved, change the perfused solution to aCSF with 20 µM CNQX to reduce recurrent excitation of the network.

8| Use the pressure-injection system to provide 15–20 p.s.i. positive pressure to the recording electrode to locally apply FM dye for 7 min. 3 min after the start of this dye-application step, begin 10-Hz stimulation through the stimulating electrode for 2 min by using the voltage settings determined in Step 6. The period of dye application after the end of the stimulation is necessary to allow complete uptake of the FM dye during endocytosis.

9| Carefully remove recording electrode from the slice chamber, and leave the slice for 10–20 min with continuous perfusion of aCSF with CNQX to wash residual FM dye from the extracellular membranes. This washing step may benefit from the use of chemical agents (e.g., Sulforhodamine[39] or Advasep-7 (ref. 46)) that can help improve the visualization of vesicular FM dye.

10| Perform confocal fluorescence imaging of the CA1 region, and establish the presence of FM dye–labeled synaptic terminals. These should appear as discrete fluorescent puncta at depths starting from 2 µm to 5 µm from the top of the slice (**Fig. 3c**). For steps to confirm the functionality of these synapses, see **Figure 4** and **Box 1**.

? TROUBLESHOOTING

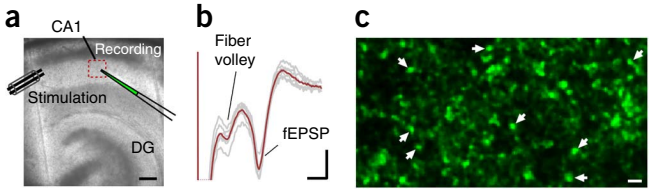

Figure 3 | Labeling functional synapses in acute slice. (**a**) Example brightfield image of a slice with approximate placement of stimulation and recording electrodes. Scale bar, 200 µm. DG, dentate gyrus. (**b**) Typical example of field excitatory postsynaptic potential recordings (fEPSPs; gray lines) from the CA1 region triggered by Schaffer collateral stimulation (stimulus: 1 ms). Average trace is shown in red. Vertical line shows the end of stimulus artifact. Scale bars, 0.5 mV (vertical) and 2 ms (horizontal). (**c**) Typical sample image of FM dye–positive fluorescent puncta in CA1 (white arrows). Scale bar, 1 µm. Parts of this figure have been adapted with permission from Marra *et al.*[40] (Elsevier) and Ratnayaka *et al.*[35] (Nature Publishing Group). Experiments were performed in accordance with the UK Animal (Scientific Procedures) Act 1986 and complied with local institutional regulations.

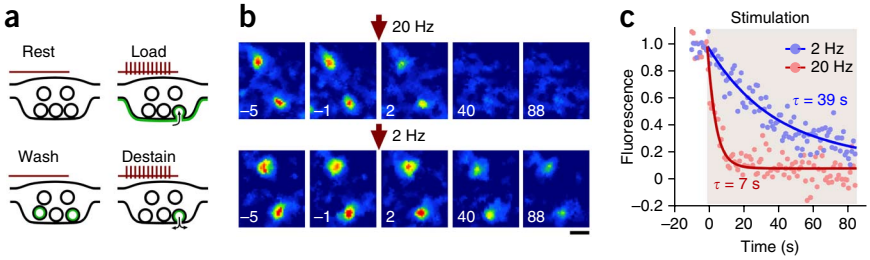

Figure 4 | Testing activity dependence of synaptic labeling. (**a**) Cartoon illustrating the rationale for experiment to determine functional integrity in dye-filled puncta. Fluorescence signal in synapses related to recycling vesicles (Load, Wash) should be lost when terminals are subjected to further stimulation (Destain). (**b**) Example frames from time-lapse sequences illustrating stimulation-frequency dependence of FM dye loss. Scale bar, 1 µm. Numbers in the lower corners indicate time to stimulation in seconds. (**c**) Dye-loss profiles for sample destaining protocols (from **b**). *x* axis shows time with respect to the onset of stimulation. *y* axis shows fluorescence normalized to the mean of the three values immediately preceding stimulation onset. Time constant of dye loss, described by single exponential fits, is inversely proportional to the stimulation frequency. Experiments were performed in accordance with the UK Animal (Scientific Procedures) Act 1986 and complied with local institutional regulations.

11| By using a CCD camera, take ×60 and ×4 magnification brightfield images of the target region to assist with its relocation in Step 17.

Fixation and photoconversion of the sample ● TIMING 100–120 min

12| Remove the stimulating electrode and harp-slice grid, and use a transfer pipette to move the slice to a plastic Petri dish containing freshly bubbled aCSF plus CNQX. Ensure that the slice is correctly oriented in the dish (same side up), and place the glass O-ring on the slice to anchor the tissue.

13| Transfer the Petri dish to the fume hood for fixation.

14| Fix the tissue. For a discussion of optimal approaches to fix thick brain tissue, we refer the reader to Jensen *et al.*[45]. The established method we use relies on microwave fixation, by using a calibrated microwave, and it is described in **Box 2**.
? TROUBLESHOOTING

15| Replace the fixative with 100 mM glycine in PBS and leave tissue in it for 1 h.

16| Rinse tissue in 100 mM NH_4Cl in PBS (1 min), and then rinse it well with fresh PBS.

17| Transfer the chamber back to the imaging rig, and relocate the target region by using the brightfield image(s) collected in Step 11.

18| Raise the objective and replace the extracellular solution with carbogen-bubbled DAB solution (1 mg/ml). Leave it to incubate for 10 min.
! CAUTION DAB may cause genetic defects and cancer. Avoid contact with skin. After use, place all contaminated plastic and glassware in bleach for 48 h. Handle the objective lens with gloves and rinse it extensively with ddH_2O after use.

19| Replace with fresh DAB solution and continue to bubble with carbogen. Lower the objective and focus on the top of the slice in the target region. Note that a dedicated objective is required for DAB photoconversion steps (e.g., ×40, numerical aperture (NA) 0.8 water-immersion objective, objective power density of ~1,500 mW/cm^2).
! CAUTION See Step 18.

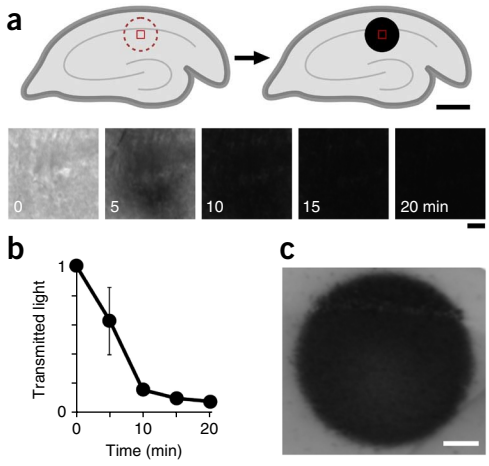

Figure 5 | Monitoring the progress of photoconversion. (**a**) Development of the photoconversion product can be followed by measuring the level of transmitted light in the target region (top, red square). Scale bar, 500 µm. Sample images (bottom) show the photoconversion reaction in the region of interest with time. Scale bar, 50 µm. (**b**) Mean ± s.e.m. line plot showing reduced brightfield light transmission as photoconversion reaction progresses ($n = 3$). (**c**) Image showing the appearance of the target region after 20 min of photoconversion. Scale bar, 100 µm. Parts of this figure have been adapted with permission from Marra *et al.*[40]. Experiments were performed in accordance with the UK Animal (Scientific Procedures) Act 1986 and complied with local institutional regulations.

Box 3 | Monitoring the photoconversion reaction

Accurate determination of the time necessary to photopolymerize DAB in the presence of FM1-43FX is a crucial requirement for this protocol. A number of parameters including the objective used for the photoconversion step, the specific wavelength and power of the excitation light, and the depth of the target region in the tissue are factors that influence the length of this process. It is strongly recommended that, initially, pilot experiments be carried out to establish this time precisely for a given imaging system. For the actual photoconversion of samples, we suggest proceeding with photoillumination periods of 3–5 min. After each period, take an image with a CCD camera of the transmitted brightfield light (**Fig. 5a**). As the photoconversion reaction develops, the target region should become darker as transmission light is reduced, and this can be quantified and plotted (**Fig. 5**). An approximate rule of thumb is to photoconvert for an additional 5 min after the decline in transmitted signal has reached a steady state.

20| Illuminate the region of interest with intense blue light (<500 nm from a 100 W Mercury lamp) for 15–25 min (e.g., Olympus UMNB-2 with excitation filter removed). The objective here is to irradiate the slice with a wavelength that maximally excites across the peak absorption wavelengths of the FM dye (see **Fig. 5** and **Box 3** for calibration of the photoconversion reaction). With the appropriate steps, the photoconverted region is clearly identifiable as a dark area in the slice (**Fig. 5c**).
! CAUTION See Step 18.
? TROUBLESHOOTING

21| Replace the DAB solution with PBS and wash the sample three more times with fresh PBS solution to ensure the removal of DAB. Collect brightfield images of the photoconverted region in the tissue. This is necessary to ensure precise relocation of the target region with respect to the remaining slice structure, after embedding.

22| Transfer the sample to the fume hood. Replace PBS with 0.1 M sodium cacodylate buffer, and wash the sample three more times. This is to ensure that phosphate groups have been removed.
! CAUTION Perform Steps 22–36 in a fume hood. Sodium cacodylate is toxic and harmful on inhalation, on contact with skin and if swallowed; it is an irritant to eyes. Wear gloves and work in the fume hood. All waste should be disposed of according to relevant hazardous chemical regulations.
■ PAUSE POINT Samples can be stored at 4 °C overnight.

Sample embedding ● TIMING ~90 h
23| Replace 0.1 M sodium cacodylate buffer with 1.5% potassium ferrocyanide/1% osmium tetroxide (wt/vol) in 0.1 M sodium cacodylate buffer for 1 h.
! CAUTION See Step 22.

24| Wash the samples thoroughly in cacodylate buffer (five washes of 10 min each).
! CAUTION See Step 22.

25| Replace with 1% (wt/vol) osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h.
! CAUTION Osmium tetroxide is toxic on inhalation, on contact with skin and if swallowed. Store it in a double container in a dangerous chemicals fridge. Wear gloves and work in the fume hood. All sample incubations need to also be placed in a sealable plastic container dedicated for use with osmium tetroxide. All waste should be disposed of according to relevant hazardous chemical regulations. Also see Step 22.
▲ CRITICAL STEP Osmium tetroxide is used in multiple steps to maximize the contrast in the block, and therefore to avoid the need for additional staining on the sections.

26| Wash the samples thoroughly in cacodylate buffer until all traces of the osmium fixative have been removed.
! CAUTION See Step 22.
■ PAUSE POINT Samples can be stored at 4 °C overnight.

27| Replace cacodylate buffer with 50% (vol/vol) ethanol and incubate for 10 min.
! CAUTION See Step 22.

28| Stain the samples *en bloc* with 4% uranyl acetate in 70% ethanol for 1 h.
! CAUTION Uranyl acetate is very toxic on inhalation and if swallowed; it is dangerous to the environment. Wear gloves and work in the fume hood. All waste should be disposed of according to relevant hazardous chemical regulations.

29| Prepare EPON solution as described in Reagent Setup. Prepare a 1:1 EPON:propylene oxide mixture in a glass vial, and vortex it thoroughly.
! CAUTION Store all solutions in the fume hood.

30| Dehydrate the samples in ethanol stepwise (2 × 75%, 2 × 90% and 2 × 100%, 5 min each).

31| Use blunt forceps to transfer the samples to a glass Petri dish containing 1:1 propylene oxide:EPON. Take care, as the sample will be brittle. Ensure that the samples are completely immersed in the mixture. Cover the sample, and leave it overnight in the fume hood.
■ **PAUSE POINT** Samples are stored overnight in the fume hood.

32| Prepare fresh EPON solution as described in Reagent Setup, and carefully replace propylene oxide:EPON mix with 100% EPON. Replace it with fresh EPON after 12 h and leave it for a further 12 h.

33| Detach the lid of a BEEM capsule, and use a razor blade to remove the conical end (**Fig. 6a**).

34| Use blunt forceps to transfer the samples into BEEM capsule lids, ensuring that the photoilluminated face of the slice is facing down (**Fig. 6b**). Each sample needs to be in the center of a separate lid.

35| With the lid resting on a flat surface, insert the BEEM capsule cylinder (**Fig. 6c**).

36| Carefully fill the capsule with EPON from the open end by ensuring that the EPON is applied above the sample to minimize lateral movement of the tissue (**Fig. 6c**).

37| Place the capsules in an oven at 60 °C for 48 h to polymerize EPON.

38| Remove the capsules and store them carefully at room temperature.
■ **PAUSE POINT** The samples can be stored for an extended period at room temperature (months to years).

Sectioning, electron microscopy and reconstruction ● **TIMING 10–14 h**
39| Carefully remove the BEEM capsule plastic surrounding the polymerized EPON by using a razor blade. The tissue should appear very dark, approximately central in the cylinder and flat to the surface of the capsule (**Fig. 6d**,**e**).

40| Use a stereomicroscope to identify the target photoconverted region. This can be readily achieved using local structural landmarks by aligning the pre-embedding brightfield images of the tissue (see Step 21) with images of the embedded tissue (**Fig. 6f**,**g**). Alternatively, by altering the incidence angle of brightfield illumination, the photoconverted region can be visualized by its different light-scattering properties (**Fig. 6g**, inset). Mark the region by lightly scoring it with a scalpel blade.

41| Asymmetrically trim the block up to the scalpel marks. Use an ultramicrotome to cut silver/gold (60–70 nm thickness) serial sections. Assuming that the sample is flat and precisely parallel to the sectioning face, we recommend collecting ribbons of serial sections over a range of 2–30 µm from the top of the slice. 2 µm represents the minimal tissue depth below

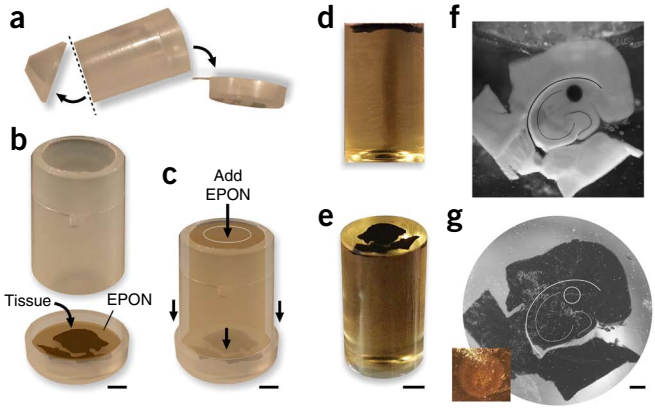

Figure 6 | Approach for embedding tissue in EPON. (**a**) Prepare BEEM capsule by detaching the lid and removing the conical end with a razor blade. (**b**) Transfer the inverted slice into the lid, with the photoconverted side facing down. Scale bar, 2 mm. (**c**) Insert the BEEM capsule cylinder into the lid, and fill it with EPON from the open end, by ensuring that it is applied directly above the sample (white circle). Scale bar, 2 mm. (**d**,**e**) Side (**d**) and half-face (**e**) appearance of polymerized EPON block with embedded tissue at the surface. Scale bar, 2 mm. (**f**,**g**) Appearance of the tissue immediately after fixation with photoconverted region (**f**) and the same tissue after embedding in EPON (**g**). Inset illustrates how the target photoconverted region is visible in EPON if light settings are adjusted. Scale bar, 500 µm. Experiments were performed in accordance with the UK Animal (Scientific Procedures) Act 1986 and complied with local institutional regulations.

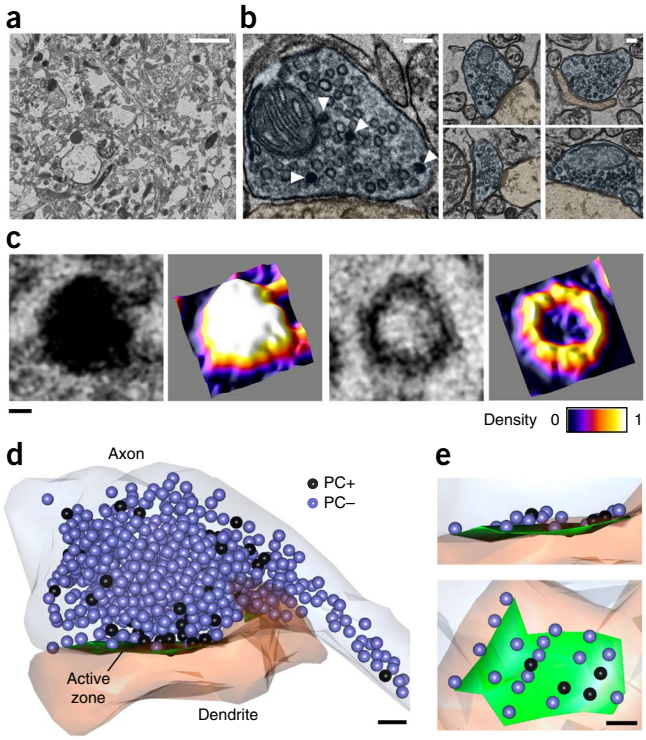

Figure 7 | Ultrastructural readout of functional vesicles. (**a**) Low-magnification electron micrograph showing the appearance of the slice in ultrastructure. Scale bar, 2 µm. (**b**) Sample images of photoconverted vesicles (arrowheads) in presynaptic terminals. Scale bar, 100 nm. (**c**) Density profiles of photoconverted vesicles (left) and nonphotoconverted vesicles (right). Scale bar, 10 nm. (**d**) Example reconstruction based on 11 consecutive sections showing photoconverted and nonphotoconverted vesicles. Scale bar, 100 nm. (**e**) Detail on vesicle composition at active zone (green). Scale bar, 100 nm. Experiments were performed in accordance with the UK Animal (Scientific Procedures) Act 1986 and complied with local institutional regulations.

the superficial cut surface of the slice, where there is a likelihood for intact axons and photoconverted vesicles.

42| Collect continuous ribbons of serial sections on 1 mm × 2 mm Formvar-coated slot grids.

43| Air-dry the grids for 15 min and store them in a grid box. ■ **PAUSE POINT** The grids can be stored for several months at room temperature.

44| View sections by using an electron microscope fitted with a cooled CCD camera (**Fig. 7**). Acquire images of synapses, and use local landmarks to identify the same target synapse in consecutive sections to facilitate a serial reconstruction. **? TROUBLESHOOTING**

45| Classify vesicles as photoconverted (PC+) or nonphotoconverted (PC–). PC+ vesicles have a characteristically electron-dense lumen compared with the clear lumen of nonrecycling vesicles (**Fig. 7b**,**c**). Most vesicles can be readily categorized by visual assessment. However, there are also a number of established quantitative approaches to aid in vesicle classification on the basis of comparisons of membrane and lumenal optical densities[25,30,31,33].

46| Use reconstruction software such as 'Reconstruct' (http://synapses.clm.utexas.edu/tools/reconstruct/reconstruct.stm) or 'Fiji' (http://fiji.sc/Fiji) to align images, and build a cartoon representation of a target synapse of interest (**Fig. 7d**). These software packages provide clear and comprehensive guidance for reconstruction approaches in online manuals (e.g., http://synapses.clm.utexas.edu/tools/reconstruct/ReconstructUserManualv1.1.0.0.pdf) and video tutorials (e.g., http://fiji.sc/TrakEM2_tutorials).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
10	No punctate staining is visible	Poor health of slice	Visually assess the health of the slice. At low magnifications, unhealthy slices have a fuzzy, low-contrast appearance with poor definition between anatomically defined layers. By contrast, in stratum radiatum of a healthy slice, tissue has a more defined and striated appearance. At higher magnifications, visually assess the health of the pyramidal neuron cell bodies in the most superficial part of the slice. They should appear densely packed and intact with smooth membranes. Use a different slice if health of cells appears to be suboptimal

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
		Suboptimal stimulation	Repeat Step 6 until a better fEPSP is evoked
		Plane of focus is too deep or too superficial	Perform *z*-stack imaging of the *x*–*y* region of interest using large *z* steps (2–4 μm) over a large *z* range (50 μm) to identify depth with good staining
		Too much background signal relative to punctate staining, even with a strong stimulation loading protocol	Extend the washout period to reduce nonspecific staining; optimize imaging conditions (e.g., slow scan speed, increase laser power and zoom in)
14	Poor preservation of the tissue	Microwave irradiation is too short or too long	Recalibrate microwave irradiation system as described in **Box 2**
20	No photoconverted region is identifiable	Photoillumination is too brief	Size and chromaticity of the photoconverted region can vary; however, a darkening of the photoilluminated region should always be observed; repeat procedure to follow the photoconversion reaction described in **Box 3**
		Inefficient carbogen bubbling	Reduce the bore of the carbogen outlet; ensure that bubble size does not exceed 2–3 mm
	Photoconverted region has variable appearance between experiments	Photoillumination is not stable	Mercury burners are not necessarily stable in their power output across their burn history. For your system, calibrate the irradiation power when photoconversion is optimal and ensure that this power is maintained for all subsequent experiments. Change the bulb if power output begins to reduce. Alternatively, test whether an alternative light source (e.g., an LED-based system), which has more stable output over its lifetime, is effective for your needs
44	Electron-dense debris	Sections contain only the most superficial part of the sample	Use samples collected deeper in the embedded sample
	No photoconverted vesicles	Sections are taken from too deep below the surface of the slice	The penetration of photoconversion reaction is limited to relatively superficial depths in the slice (up to ~50 μm). Use samples collected at sites closer to the tissue surface

● TIMING

Day 1, Steps 1–11, FM dye labeling and fluorescence imaging: 3–4 h, depending on the samples
Day 1, Steps 12–22, fixation and photoconversion of the sample: 100–120 min
Day 2, Steps 23–31, sample embedding: 90 h
Day 3, Step 32: 24 h
Day 4, Steps 33–38: 49 h
Day 6, Steps 39–43, sectioning, electron microscopy and reconstruction: 10–14 h, depending on the samples
Day 7, Steps 44–46: 8 h, depending on the samples

ANTICIPATED RESULTS

Figure 7 provides an overview of typical results. Within the target region (**Fig. 7a**), presynaptic terminals with PC+ vesicles can be readily identified (**Fig. 7b**). The characteristic electron-dense profile of such vesicles contrasts with PC– vesicles characterized by a clear lumen (**Fig. 7c**). 3D reconstruction based on consecutive serial sections provides a representation of the organization of a recycled vesicle pool in the context of the nonrecycled pool, as well as other ultrastructural features such as the active zone (**Fig. 7d**). This offers substantial scope for exploring the details of ultrastructure-function relationships. Here we briefly demonstrate one possible application: characterization of the PC+ and PC– composition and position of vesicles comprising the anatomically defined docked pool at the active zone (**Fig. 7e**).

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