

FM1-43 Photoconversion and Electron Microscopy Analysis at the *Drosophila* Neuromuscular Junction

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[Abstract] We developed a protocol for photoconversion of endocytic marker FM1-43 followed by electron microscopy analysis of synaptic boutons at the *Drosophila* neuromuscular junction. This protocol allows detection of stained synaptic vesicle even when release rates are very low, such as during the spontaneous release mode. The preparations are loaded with the FM1-43 dye, pre-fixed, treated and illuminated to photoconvert the dye, and then processed for conventional electron microscopy. This procedure enables clear identification of stained synaptic vesicles at electron micrographs.

Keywords: Electron microscopy, Photoconversion, FM1-43, *Drosophila*, Synaptic vesicle, Recycling pool, Neuromuscular junction

[Background] Neuronal transmitters are released via the fusion of synaptic vesicles with the neuronal plasma membrane. Vesicles can fuse spontaneously or in response to an action potential. Subsequently, vesicles become retrieved via endocytosis and recycled. Molecular mechanisms of synaptic vesicle recycling were investigated extensively with the tools of molecular biology, electrophysiology and microscopy (Slepnev and De Camilli, 2000; Sudhof, 2004; Rizzoli and Betz, 2005; Kavalali, 2006). Loading the endocytic marker FM1-43 coupled with the dye photoconversion followed by electron microscopy analysis is a powerful technique that allows the investigation and measurement of the recycling vesicle pools (Harata *et al.*, 2001; Schikorski and Stevens, 2001; Rizzoli and Betz, 2004). *Drosophila* neuromuscular junction (NMJ) is an advantageous preparation with clearly defined synaptic boutons, which enables rapid generation of lines with mutated synaptic proteins and rigorous evaluation of vesicle recycling pools (Akbergenova and Bykhovskaia, 2009; Denker *et al.*, 2009). A fundamental question in the field of synaptic transmission is whether the evoked and spontaneous transmission utilizes the same recycling pool. To address this question, the recycling pool utilized in the absence of stimulation needs to be measured. This is a challenging problem due to low rates of spontaneous release and recycling. We have developed a protocol for FM1-43 loading followed by the dye photoconversion and EM analysis, which enables rigorous evaluation of recycling pools utilized during spontaneous and evoked transmission at the *Drosophila* NMJ (Sabeva *et al.*, 2017).

Materials and Reagents

A. Sample preparation

1. Gloves
2. Long sleeve lab coat
3. Sample bottle with snap cap, size 4 ml (Electron Microscopy Sciences, catalog number: 64250)
4. Falcon 35 mm culture plates (Corning, NY)
5. *Drosophila melanogaster* fly stocks: Canton S (Bloomington *Drosophila* Stock Center, catalog ID: FBst1000081) and *cpx^{SH1}* (*cpx^{-/-}*) (Huntwork and Littleton, 2007)
6. Ca²⁺-free HL3 saline containing 75 µM advasep-7 (Biotium, catalog number: 70029) (see Note 1)
7. FM1-43 (Thermo Fisher Scientific, Invitrogen™, catalog number: T35356), 10 µM
8. 100 mM NH₄Cl (Sigma-Aldrich, catalog number: A9434) in HEPES
9. 1.5 mg/ml DAB (Agilent Technologies, DAKO, OEM) in HEPES
Note: The compound is available liquid or tablets in kits.
10. 1% osmium tetroxide (OsO₄) prepared in 90 mM cacodylate buffer using the 4% OsO₄ stock solution (Electron Microscopy Sciences, catalog number: 19150)
11. Ascending acetone series 50, 70, and 90% prepared from 100%
12. 2% uranyl acetate (Electron Microscopy Sciences, catalog number: 22400) prepared in water
13. Acetone, 100% (Sigma-Aldrich, catalog number: 270725) dehydrated with molecular sieve dehydrate Fluka (Fluka Analysis, Sigma-Aldrich, catalog number: 270725)
14. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9888)
15. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P3911)
16. Magnesium chloride hexahydrate (MgCl₂·6H₂O) (Sigma-Aldrich, catalog number: M9272)
17. Calcium chloride dihydrate (CaCl₂·2H₂O) (Sigma-Aldrich, catalog number: C5080)
18. Sodium bicarbonate (NaHCO₃) (Sigma-Aldrich, catalog number: S6014)
19. Trehalose (Sigma-Aldrich, catalog number: T9531)
20. Sucrose (Sigma-Aldrich, catalog number: S1888)
21. HEPES-Na salt (Sigma-Aldrich, catalog number: H7006)
22. HEPES (Sigma-Aldrich, catalog number: H3375)
23. Paraformaldehyde (Electron Microscopy Sciences, catalog number: 15710)
24. Glutaraldehyde (Electron Microscopy Sciences, catalog number: 16220)
25. Sodium cacodylate buffer (Electron Microscopy Sciences, catalog number: 11653)
26. HL3 *Drosophila* saline solution (see Recipes)
27. Pre-fixative solution (see Recipes and Note 2)
28. HEPES-buffered saline (see Recipes)
29. Fixative solution (see Recipes and Note 2)

B. Sample embedding

1. ACLAR® 33C Embedding film (Electron Microscopy Sciences, catalog number: 50425-10)
2. Tri-Corn beakers: plastic, disposable (Electron Microscopy Sciences, catalog number: 60972)
3. Flat Bottom Embedding Capsules (Electron Microscopy Sciences, catalog number: 70021)
4. Epon (Embed-812) (Electron Microscopy Sciences, catalog number: 14900)
5. Nadic Methyl Anhydride (NMA) (Electron Microscopy Sciences, catalog number: 19000)
6. Dodecenyl Succinic Anhydride Specially Distilled (DDSA) (Electron Microscopy Sciences, catalog number: 13710)
7. 2,4,6-Tri(dimethylaminomethyl) phenol (DMP-30) (Electron Microscopy Sciences, catalog number: 13600)
8. ERL-4221 (Electron Microscopy Sciences, catalog number: 15004)
9. D.E.R. 736 Epoxy Resin (Used to simplify infiltration in combination with Embed 812) (Electron Microscopy Sciences, catalog number: 13000)
10. Nonenyl Succinic Anhydride Modified (NSA) (Electron Microscopy Sciences, catalog number: 19050)
11. Dimethylaminoethanol (DMAE) (Electron Microscopy Sciences, catalog number: 13300)
12. Embedding Mix A (see Recipes)
13. Embedding Mix B (see Recipes)

Equipment

A. Sample preparation

1. Sylgard 184 (World Precision Instruments, Sarasota, FL)
2. Fine tweezers (World Precision Instruments, models: #2 and #5)
3. Fine scissors (World Precision Instruments, model: 501233)
4. Insect Minutien Pins (0.1 mm) (Fine Science Tools, catalog number: 26002-10)
5. Dissecting stereoscopic zoom microscope (Nikon Stereozoom Microscope, Nikon Corporation)
6. A.M.P.I. Master 8 Stimulator (A.M.P.I., model: Master-8)
7. Suction electrode filled with HL3
8. Epifluorescence compound microscope with a filter cube with a long-pass emission filter customized for FM1-43 dye
9. 60x water-immersion objective (ZEISS, Thornwood, NY)
10. Mercury lamp
11. Biowave (Ted Pella, Redding, CA)
12. 480 ± 10 bandpass excision filter
13. PELCO® R1 Single Speed Rotator in the 35° positions (Ted Pella, model: PELCO® R1)
14. Fume hood

B. Sample embedding

1. Oven Binder (Tuttlingen, Germany)

C. Ultrathin Sections preparation and Image Capture

1. Ultramicrotome for ultrathin sectioning (Leica, model: Leica EM UC6)
2. Formvar/carbon-coated 2 x 1 mm slot copper grids (Electron Microscopy Sciences, catalog number: FCF-2010-Cu)
3. Ultra Diatome diamond sectioning knife, wet (Electron Microscopy Sciences, catalog number: 27-US)
4. UltraTrim Dry Room Temperature Diatome knife, 4.0 mm (Electron Microscopy Sciences, catalog number: UTT-40-R)
5. Eyelash with handle (Ted Pella, catalog number: 119)
6. Double edge stainless steel razor blades (Electron Microscopy Sciences, Hatfield, PA)
7. Forceps for grids (Electron Microscopy Sciences, Hatfield, PA)
8. JEOL 100 CX Electron Microscope equipped with Hamamatsu digital camera and AMT software

Software

1. ImageJ (National Institutes of Health) for image analysis
2. Adobe Photoshop (Adobe Systems) software for image analysis

Procedure

A. Dissection of *Drosophila melanogaster* larvae in HL3 solution

1. Select third-instar larvae (L3) and place it in a Sylgard dish.
2. Pin the L3 on the anterior (head/mouth apparatus) and the posterior part, and the dorsal side-up.
3. Immerse the pinched larvae in 4-5 ml HL3 solution (Recipe 1) to the Sylgard dish.
4. Section the dorsal side longitudinally and remove the internal organs (intestines, fat bodies and trachea).
5. Stretch and pin the rest of the L3.
6. Several muscles could be used. In this study experiments were performed at 1b boutons in muscles 6 and 7. (Figure 1A)

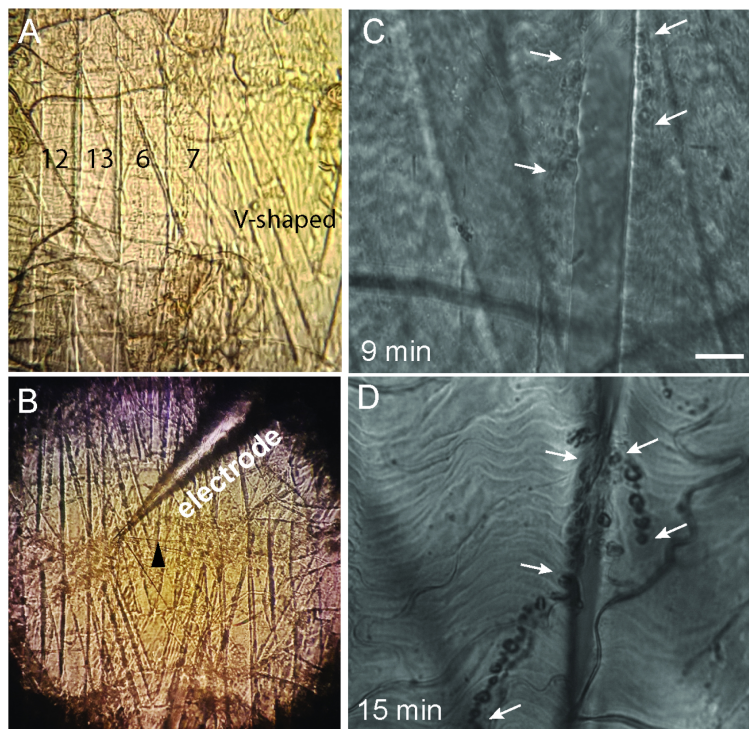


Figure 1. Third-instar larvae dissection. A. High magnification image of muscle fibers 7, 6, 13, 12 and V-shaped; B. Image of the ventral muscles in L3 where a nerve (black arrowhead) is sucked into the suction electrode connected to a stimulator; C and D. Adjusting the illumination times of the pre-fixed sample loaded with FM1-43 (WT larvae loaded for 30 sec). C. The DIC image shows that the illumination time of 9 min produces a successful photoconversion. Synaptic boutons can be identified as dark spots barely visible in the surface of the muscle (white arrows). D. The illumination time of 15 min is excessive, as evident from strong DAB precipitation, observed as large dark spots over the length of the NMJ (white arrows). Scale bar = 10 μ m.

B. Activity dependent labeling of Synaptic Vesicles with FM1-43 dye

Note: Perform the following steps in low light conditions to protect the fluorophore component of FM1-43 dye. Cover the FM1-43 dye with foil or work in the dark at room temperature.

1. Replace HL3 solution with HL3 containing 10 μ M FM1-43.
2. For loading in the absence of stimulation (spontaneous recycling pool) leave the preparation for the time of loading (10, 30, 120, and 600 sec were used in our studies).
3. For active loading, using the head stage controllers pull the nerve into a suction electrode connected to a stimulator. Suck the end of the nerve. Use the stimulation protocol for low-frequency stimulation, 5 min, 5 Hz. Quickly remove the FM1-43 dye once the stimulation is complete (Figure 1B). The technique as adopted from Verstreken *et al.*, 2008.
4. Upon dye loading, rapidly wash the preparations three times for 30 sec in Ca^{2+} -free HL3 saline containing 75 μ M Advasep-7.

C. Fixation

1. Prefix the preparation (Recipe 2) for 15 min at room temperature.
2. Wash three times for 5 min in HEPES buffer saline (Recipe 3).
3. Leave the preparation covered in 100 mM NH₄Cl for 10 min to quench autofluorescence of the fixative.
4. Wash out the NH₄Cl solution with HEPES buffer 2 times for 5 min.

D. FM1-43 photoconversion

1. Preincubate the preparation for 10 min in HEPES buffer containing 1.5 mg/ml DAB.
2. Place the sample under an epifluorescent microscope. Identify the area of interest under 60x water immersion objective, at room temperature.
3. Illuminate the sample for 8-10 min using a mercury lamp with a 480 ± 10 bandpass excision filter using the maximum light intensity. The dye will bleach with time. Observe the process of photoconversion by switching periodically to the bright field. As photoconversion takes place, dark brown DAB precipitate localizes at the illuminated area. The illumination time needs to be adjusted, as illustrated in Figures 1C and 1D.

E. Post-fixation

1. Wash the sample in HEPES three times 5 min.
2. Immerse the preparation in the fixative solution (Recipe 4) and fix the sample in BioWave for 2 min at 100 W. Place the temperature insert close to the preparation to monitor temperature fluctuations. Do not allow the temperature to exceed 25-26 °C.
3. Unpin the preparation carefully from the Sylgard dish. Transfer the preparation into a sample bottle with a snap cap.
4. Wash the sample in 90 mM sodium cacodylate buffer, pH 7.4 twice for 5 min.

F. EM sample processing

Note: The following steps (unless specified) are performed at room temperature.

1. Osmium tetroxide post-fixation and uranyl acetate staining
 - a. Incubate the preparation in 1% OsO₄ solution for 1 h at room temperature. The unused solution can be stored refrigerated for up to 1 month. Perform all steps under a fume hood, using gloves and long sleeve lab coat. Use 500 µl of this solution to immerse the sample. (Note 4)
 - b. Wash with 90 mM cacodylate buffer for 5 min.
 - c. Wash with deionized water for 5 min.
 - d. Incubate the preparation in 2% uranyl acetate for 30-60 min. The unused solution can be stored refrigerated for up to 2 weeks.
 - e. Wash with deionized water for 5 min twice.

2. Dehydration

- a. Prepare separate solutions containing 50, 70, 90, and 100% of acetone.
- b. Add 1 ml of 50% acetone to the sample for 10 min.
- c. Add 1 ml of 70% acetone to the sample for 10 min.
- d. Add 1 ml of 90% acetone to the sample for 10 min.
- e. Add 1 ml of 100% acetone to the sample twice for 10 min.

3. Embedding

- a. Mix 2 volumes of embedding resin (Note 3) with one volume of 100% acetone. Add it to the preparation under continuous rotation for 1 h or overnight.
- b. Keep the sample in embedding resin for at least 18 h (overnight).
- c. Under a stereomicroscope, place the preparation over ACLAR film, and then identify and trim the area of interest.
- d. Place the preparation over a new ACLAR film with the muscles facing down and the cuticle facing up. Position an embedding capsule filled with polymerized resin upside-down on the top of the specimen. Polymerize at 60 °C for 24-36 h.
- e. Remove the ACLAR film. This will leave sample attached to the embedding capsule.

4. Thin sectioning and EM imaging

- a. Remove the block from the embedding capsule and mount the block into a specimen holder of an ultramicrotome.
- b. Under a stereomicroscope, mark the area of interest with a razor blade. The marked area should be a trapezoid that would fit onto the slot grid.
- c. Trim the block over the outlined area.
- d. Insert specimen holder with the block in the ultramicrotome and align the long edge of the trapezoid to the trimming knife edge. Trim 15-20 μ m and shift the knife to trim the second long edge of the trapezoid. Trim another 15-20 μ m. Repeat the same for the short sides of the trapezoid (Figure 2 and Videos 1-5).
- e. Align the block face exactly parallel to the edge of the diamond sectioning knife.
- f. Cut thin sections (50-60 nm).
- g. Collect individual sections (or collect sections in series) and place them on the 2 x 1 mm slot grids.
- h. Allow the sections to dry before taking images.
- i. Image the sections using conventional TEM.

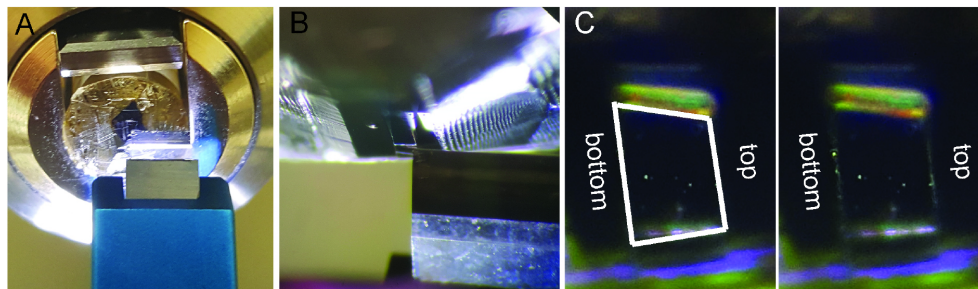


Figure 2. Sample block fine trimming. In the fixed and flat embedded *Drosophila* larvae the region of interest, muscles 6 and 7 are outlined with razor blade. Upon rough block trimming with razor blade and elimination of resin the face of the block is shaped with trimming knife. A. Fine trimming. The edge of the knife is aligned to the trapezoid top. B. Vertically positioned trapezoid with the left edge of the trimming knife aligned to the top of the trapezoid. C. Fine trimmed trapezoid with parallel top and bottom, and slightly tilted sides.

Note: The paralleled top and bottom are important for forming a ribbon during serial sectioning.

Video 1. Rough trapezoid trimming



Video 2. Fine trapezoid trimming, part one. Trimming trapezoid top.



Video 3. Fine trapezoid trimming, part 2. Once sectioned the trapezoid top the trimming knife is retracted and positioned to trapezoid bottom without additional alignment.



Video 4. Fine trapezoid trimming, part 3. Trimming trapezoid bottom.



Video 5. Serial sectioning



Data analysis

- Employing the photoconversion procedure, we investigated the recycling vesicle pool in the *Drosophila* larvae lacking the presynaptic protein complexin (*cpx*^{-/-}) (Huntwork and Figure 3A), which enables the elevated rate of spontaneous release. In contrast, the recycling pool observed in WT boutons during spontaneous loading was negligibly small (data not shown, see Sabeva *et al.*, 2017). In *cpx*^{-/-} preparations loaded during the nerve stimulation, the recycling pool was also increased compared to WT preparations (Figures 3A and 3B), suggesting that separate recycling pools are utilized for the spontaneous and evoked release modes (Sabeva *et al.*, 2017).

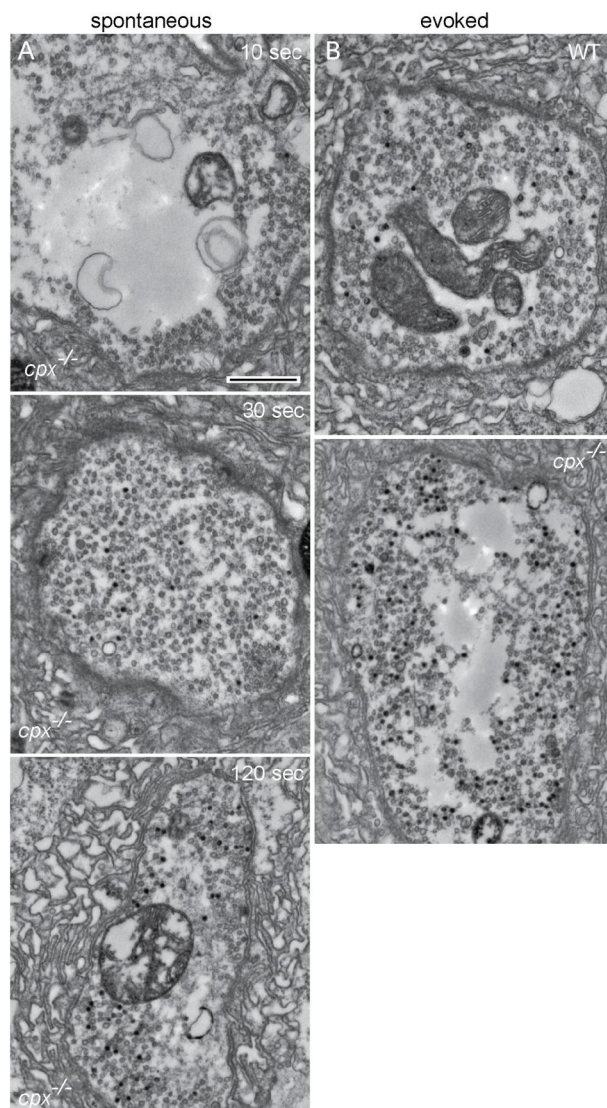


Figure 3. Representative micrographs showing the recycling vesicle pools. A. *cpx*^{-/-} boutons loaded in the absence of stimulation for 10, 30 or 120 sec. B. WT and *cpx*^{-/-} boutons loaded during the nerve stimulation at 5 Hz for 5 min. Photoconverted labelled vesicles (black), non-labelled vesicles (grey). Scale bar = 500 nm.

- Detailed data processing analysis and replicates including applied statistical tests could be found in the original manuscript, see Sabeva *et al.*, 2017.

Notes

- Advasep-7 is a β -cyclodextran derivative. It works as a dye scavenger and reduces background fluorescence.
- 16% paraformaldehyde solution and 25% glutaraldehyde solution can be purchased in 10 ml ampoules.
- Embedding resin: Prepare in a new disposable 250 ml beaker by mixing equal volumes of Embedding mix A (Recipe 5) and Embedding mix B (Recipe 6). Use a wooden tongue depressor to mix thoroughly. Degas (eliminate the air bubbles) for 5 min in a vacuum desiccator or leave 30 min at room temperature. Store tightly sealed at room temperature and use at the same day or aliquot and store at -20 °C.
- All steps during EM sample processing are performed in shaker or sample rotator.

Recipes

- HL3 *Drosophila* saline solution

Ingredients	Con. mM
NaCl	70
KCl	5
MgCl ₂	20
CaCl ₂	1
NaHCO ₃	10
Trehalose	5
Sucrose	115
HEPES-HCl	2.5
HEPES-NaOH	2.5
pH 7.2-7.4 at 25 °C	

- Pre-fixative solution

Ingredients	Con.
Paraformaldehyde	4%
Glutaraldehyde	0.1%
CaCl ₂	0.2 mM
Sodium cacodylate buffer	90 mM
pH 7.4	

3. HEPES-buffered saline

Ingredients	mM
HEPES	20
NaCl	145
CaCl ₂	0.2
pH 7.2	

4. Fixative solution

Ingredients	Con.
Paraformaldehyde	4%
Glutaraldehyde	2.5%
CaCl ₂	0.2 mM
Sodium cacodylate buffer	90 mM
pH 7.4	

5. Embedding mix A

Prepare by mixing:

20 g EMBed-812

11 g DDSA

9 g NMA

0.8 ml DMP-30

In 250 ml Tri-Corn disposable beaker

Use a wooden tongue depressor to mix thoroughly

6. Embedding mix B

Prepare by mixing:

10 g ERL

6 g DER

26 g NSA

0.4 ml DMAE

In 250 ml Tri-Corn disposable beaker

Use a wooden tongue depressor to mix thoroughly

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