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A semi-correlative technique for the subcellular localization of proteins in *Drosophila* synapses

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ABSTRACT

The neuromuscular junction (NMJ) in *Drosophila* larvae serves as an important model system to study synaptic vesicle recycling, plasticity, development, and organelle trafficking in nerve terminals. Here we provide a protocol that reliably localizes proteins subcellularly in this system in combination with good preservation of the ultrastructure of the NMJ. In this protocol, the NMJs are cut open to increase access of reagents to the interior of the synapse, stained with primary antibodies followed by secondary antibodies conjugated to Fluoronanogold particles to allow selection of the best-stained areas for electron microscopic (EM) analysis using confocal microscopy. To improve visualization at the EM level, the nanogold particles are silver enhanced. Good penetration of the antibodies in this protocol allows for a three-dimensional reconstruction of the labeling pattern from serial ultrathin sections. To explore the utility and resolution of this approach, we determined the ultrasturcutral localization of the cell adhesion molecule Fascicline II (Fas II) at rest using an antibody raised against the cytoplasmic epitope. We observed that Fas II-ir is accumulated at the cytoplasmic surfaces of pre- and postsynaptic elements of the synapse at equal distances from the membranes, thus supporting the model that it has a similar orientation in these opposing synaptic structures. Fas II-ir delineates active zones and is compartmentalized from CSP, Dap160, and Bruchpilot immunolabeling. We conclude that Fas II immunolabeling in *Drosophila* can be reliably used to evaluate changes in the size of the active zone region at rest in genetic and functional experiments.

1. Introduction

The possibility of genetic manipulation makes *Drosophila* a powerful tool in the study of how specific molecules function in the operation of the synaptic vesicle cycle and in synaptic plasticity and development (Prokop, 1999; Prokop and Meinertzhagen, 2006). Larval *Drosophila* NMJs are most widely used in genetic experiments, since most synaptic mutants do not survive until adulthood. In a majority of the studies in *Drosophila* larvae, synaptic molecules are successfully localized at the light microscopic (LM) level using immunofluorescence or by expression of proteins containing fluorescent tags (Ashley et al., 2005; Koh et al., 2007; Wagh et al., 2006; Prokop and Meinertzhagen, 2006). Reliable subcellular localization of proteins at the ultrastructural level in combination with good ultrastructural preservation of the tissue is, however, difficult to achieve. There are several reasons for this. Most of the classical preembedding methods include the treatment of specimens with detergents that strongly damage the ultrastructure of the synapses. The use of postembedding electron microscopic (EM) methods is also limited. Exclusion of osmium postfixation, which is often required for preserving antigenisity, results in a poor ultrastructure in studied objects (Fouquet et al., 2009). Cryo-EM immunolabeling techniques are also very rarely used, since it is technically difficult to obtain good cryosections from muscles attached to the cuticle and to find synapses.

We recently tried to improve the standard pre-embedding technique for *Drosophila* synapses by cutting nerve terminals open on a vibratome instead of using detergents. This approach allowed for successful localization of the endocytic protein Eps15 in synaptic boutons (Koh et al., 2007). It was, however, still difficult to find the labeled boutons in this study. We have now developed a semi-correlative LM-EM protocol, which allows for the reliable selection of labeled nerve terminals and thus significantly accelerates the subsequent detection of antigens of interest at the subcellular level. In addition, we show that this approach permits a three-dimensional (3D) reconstruction of labeling patterns. We tested this protocol by detecting the subcellular localization of the cell adhesion molecule, Fasciclin II (Fas II) (Ashley et al., 2005; Mao and Freeman, 2009; Schuster et al., 1996; Kristiansen and Hortsch, 2008), and compared it with the localization of three other markers of *Drosophila* NMJs, which are widely used in immunofluorescence experiments: antibodies against cystein string protein, CSP (Arnold

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et al., 2004; Zinsmaier et al., 1994), dynamin associated protein, Dap160 (Koh et al., 2004; Marie et al., 2004), and NC82 antibody, which signals for Bruchpilot protein (Wagh et al., 2006).

2. Methods

2.1. Animals and dissection procedures

Flies, strain w^{1118}, were maintained at room temperature on normal food. Third instar larvae were mounted and dissected on pieces of Sylgard (Dow Corning) in HL3 buffer without Ca^{2+} (see, e.g., Stewart et al., 1994; Verstreken et al., 2002). The body wall, brain, and connecting nerve were kept as a fillet, while other inner tissues were removed. To reduce spontaneous activity and achieve the resting condition we incubated NMJs in HL3 buffer containing 0.5 mM EGTA for 10 min prior to fixation. In several experiments NMJs were exposed to HL3 containing 1.5 mM Ca^{2+} and incubated for 10 min in high potassium (60 mM) to induce synaptic vesicle recycling.

2.2. Semi-correlative labeling procedure

Sequential steps of the semi-correlative labeling procedure are shown in Fig. 1A and described in detail below.

I. Fix the fillets pinned down to Sylgard pieces in freshly prepared 4% paraformaldehyde in 0.1 M PBS buffer (pH 7.2) for 15 min and wash in PBS for 30 min.
II. Apply the razor blade against the cuticle and slide it gently along the cuticle to detach the muscles. Place them in an aliquot containing PBS. Depending on the type of experiment, a selected muscle group from several fillets can be collected if needed.
III. Transfer the muscles into 5% agarose (Sigma) in PBS heated to melting temperature and cool the preparation down to room temperature.
IV. After agarose solidification, cut the block containing muscles into 100 μm sections on a vibratome (e.g., Leica VT 1000S was used in our experiments). Many NMJs become randomly cut open. Cut sections at a medium vibration rate to reduce damage to nerve terminals.
V. Incubate vibratome sections in 1% human serum albumin for 30 min in TPBS (Tris-phosphate buffered saline; pH 7.4). Add primary antibody and incubate on a shaker overnight at 4 °C. Wash sections in TPBS for 3 h and then block for 30 min in 5% milk (Nestle) and 1% goat serum (Invitrogen) in TPBS. Incubated with Fluoronanogold (Nanoprobe) conjugated secondary antibodies (diluted 1:10) overnight at 4 °C. Wash sections for 2 h in TPBS, and then mount them on glass slides in a drop of TPBS. Use cover slips and work quickly to avoid drying.
VI. Observe the sections in an upright confocal microscope (e.g., Bio-Rad Radiance 2100 was used in our experiments) to identify the 3D position of presynaptic boutons. Collect serial digital slices from the objects of interest to evaluate the depth of the Fluoronanogold penetration and visualize details of the labeling pattern (Fig. 1B and Fig. 2D inset).
VII. Transfer the section into a glass container and wash in PBS for 30 min. After this, fix them again in 3% glutaraldehyde in 0.1 M PB overnight at 4 °C.
VIII. Following fixation, wash sections again in ddH_2O for an hour, and then perform silver enhancement to make gold particles more visible for EM studies. In our experiments the time of the reaction ranged from 3 to 3.5 min. Smaller size of gold particles can be obtained if the time of the reaction is reduced. Add ddH_2O to quench the reaction.
IX. Post-fix the floating sections in 0.2% osmium tetraoxide in 0.1 M PB for 30 min and then dehydrate in a graded series of ethanol/water (30%, 50%, 70%, 85%, 95%, 100%; 20 min for each step), and then embed in a thin layer of Durcupan ACM (Fluka) mixed following manufacturer's instructions. After polymerization of the resin at 50 °C for 48 h, re-locate target regions in the sections using images collected in Step V with a conventional microscope (Fig. 1C). Use graphics software (e.g., Photoshop or Xara Xtreme) to align images if necessary. Cut the region of interest out with a sharp scalpel and glue it with a Super Attak glue (Loctite) to a blank surface of an epoxy block prepared in advance.
X. Trim the block around the area of interest under a dissection microscope. Cut serial ultrathin sections in an ultratome (e.g., Ultratome UCT (Leica), was used in our experiments) with a diamond knife (e.g., Diatome was used in our experiments). Mount the sections on formvar-coated copper slot grids. Counterstain sections with uranyl acetate (7 min) and lead citrate (3 min), and examine and photograph them in an electron microscope. In the present study, images were taken in a Tecnai 12 electron microscope (FEI) equipped with a bottom mounted 2k × 2k TemCam F224 CCD Camera (TVIPS). For 3D reconstruction of serial sections, trace membrane contours and gold particles in electron micrographs with a digitizer and use 3D-reconstruction software to obtain surfaces of the objects. Maya 8.0® 3D-reconstruction program (Autodesk) was used in the present study for 3D reconstruction and surface rendering.

2.3. Antibodies

Four different antibodies were used in our experiments: anti-Fas II monoclonal antibody (1D4) from DSHB, which recognizes the cytoplasmic domain of the molecule, was used at a dilution of 1:100; anti-CSP monoclonal antibody was used at a dilution of 1:50 (Zinsmaier et al., 1994); NC82 monoclonal antibody was used at a dilution of 1:100 (Wagh et al., 2006); and anti-Dap160 rabbit polyclonal antibodies were used at a dilution of 1:500 (Roos and Kelly, 1998). 1.4 nm Fluoronanogold conjugated secondary antibodies (used at a dilution 1:10) and a silver enhancement kit were purchased from Nanoprobes, USA and GE Healthcare. The NC82, anti-CSP and anti-Dap160 antibodies were the kind gifts of Dr. Erick Bucher, Dr. Hugo J. Bellen and Dr. Regis Kelly.

3. Results

The *Drosophila* NMJ, like conventional vertebrate synapses, is composed of the active zone, at which neurotransmitter-filled vesicles fuse with the plasma membrane, and the periactive zone, where vesicles reform by endocytosis. Cell adhesion molecules, signaling molecules, and molecular scaffolds are believed to be present at the active and periactive zones to coordinate the proteins involved in vesicle fusion and endocytosis, and also to control the shape and size of the neuromuscular junction. Many functions of these proteins in *Drosophila* are evolutionary conserved (Broadie, 2004; Chang and Balice-Gordon, 2000; Prokop and Meinertzhagen, 2006).

Fasciclin II represents the structural and functional ortholog of the vertebrate NCAM (Kristiansen and Hortsch, 2008; Mao and Freeman, 2009). It is believed that in adult NMJs it is accumulated in the synaptic periactive zone where it functions as a homophilic cell adhesion molecule (Ashley et al., 2005; Marie et al., 2004; Schuster et al., 1996; Kristiansen and Hortsch, 2008; Mao and Freeman, 2009). Immunofluorescence revealed that the Fas II immunoreactivity is not precisely co-localized with another component of the