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Research paper

An easy, fast and "low-tech"-equipment-requiring alternative method to optimize immunolabelling conditions for pre-embedding immunogold electron microscopy and to correlate light and electron microscopical immunogold labelling results

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ABSTRACT

Correlating light microscopic immunolabelling results with electron microscopic data is of great interest in many fields of biomedical research but typically requires very specialized, expensive equipment and complex procedures which are not available in most labs. In this technical study, we describe an easy and "low-tech"-equipment-requiring pre-embedding immunolabelling approach that allows correlation of light microscopical immunolabelling results with electron microscopic (EM) data as demonstrated by the example of immunolabelled synaptic ribbons from retinal rod photoreceptor synapses. This pre-embedding approach does not require specialized embedding devices but only commonly available equipment. The cryostat section-based procedure allows optimization of the pre-embedding immunolabelling conditions at the less laborious and time-consuming light microscopic (LM) level before the ultrastructural analyses of the immunolabelled structures can be performed on the same sample after ultrathin sectioning without further modification. The same photoreceptor synapse that has been first studied at the light microscopic level can be subsequently analyzed with this approach at the electron microscopic level at individual ultrathin sections or serial ultrathin sections from individual, identical synapses. Higher resolution EM analyses of the immunolabelled synapses can be performed with only minor modifications of the combined LM/EM procedure. The detergent-free procedure is applicable even for weakly fixed cryostat sections which is a relevant aspect for many antibodies that do not work with more strongly fixed biological samples.

1. Introduction

Correlating light microscopic immunolabelling results with electron microscopic data by CLEM (correlative light and electron microscopy) procedures is of great interest in many fields of biomedical research (for review, see Perkovic et al., 2014; Fonta and Humbel, 2015; De Boer et al., 2015; Begemann and Galic, 2016; Karreman et al., 2016). Different approaches have been developed that allow correlation of light and electron microscopic data at different levels of resolution (for review, see Watanabe and Jorgensen, 2012; Fonta and Humbel, 2015; De Boer et al., 2015; Begemann and Galic, 2016). DAB-dependent methods employ photo-oxidation (Grabenbauer et al., 2005; Meisslitzer-Ruppitsch et al., 2009, 2013; Grabenbauer, 2012; Horstmann et al., 2013) or enzymatic oxidation (Schikorski, 2010) of a light microscopic signal to generate an electron-dense reaction product that can be detected by electron microscopy. Other CLEM procedures involve ultracryotome sections that can be analyzed both at the light and electron microscopic level (Tokuyasu, 1973, 1980; Van Rijnsoever et al., 2008). With ultracryotomy, ultrathin frozen sections are obtained from frozen tissue samples by sectioning at ≈−100 °C with an ultracryotome. The frozen ultrathin sections are then processed for immunolabelling (Tokuyasu and Singer, 1976; Slot and Geuze, 2007). Ultracryotomy is technically challenging (Tokuyasu, 1980; Christensen and Komorowski, 1985) and requires specialized equipment. Recently, also sophisticated fixing and embedding procedures have been developed that preserve fluorescence in resin-embedded samples (Watanabe et al., 2011, 2014; for review, see Watanabe and Jorgensen, 2012). For the CLEM analyses, these resin-persisting fluorescent signals were first analyzed by super-resolution microscopy to obtain highly resolved light microscopic signals in the sample sections. Later on, these highly resolved light microscopic signals are overlayered onto the electron microscopic images. This approach provides a powerful, very high resolution approach to CLEM (nano-fluorescence electron microscopy; nano-fEM). But these procedures also require specialized and expensive equipment not readily available in most labs, e.g. high-pressure freeze substitution device/freeze substitution devices (Watanabe et al., 2011, 2014; Watanabe and Jorgensen, 2012); ultracryotomy (Al-Amoudi et

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al., 2004; Van Rijnsoever et al., 2008; Cortese et al., 2012) and challenging, complex analyses procedures or use of genetically enhanced probes (for review, see Watanabe and Jorgensen, 2012; De Boer et al., 2015; Kuipers et al., 2015; Souslova et al., 2016).

In the present study, we describe an alternative, technically very easy approach for pre-embedding immuno electron microscopy (immuno EM) that also allows correlation of light microscopic (LM) immunosignals with electron microscopic (EM), ultrastructural data in an uncomplicated manner using standard equipment, probes and procedures. It does not require specialized equipment and provides a simple, straightforward approach to obtain CLEM data also for non CLEM-specialized labs with a good level of resolution and ultrastructural preservation.

The usefulness and performance of the cryostat section-based procedure was exemplified by the analysis of the (bovine) retina. The retina is particularly suitable for morphological analyses because of its clear organization into well characterized and histologically clearly distinguishable layers. To establish the procedure, we analyzed photoreceptor synapses of the retina with a well characterized antibody against RIBEYE, a protein component of synaptic ribbons (Schmitz et al., 2000; for review, see Lagnado and Schmitz, 2015). Synaptic ribbons are relatively large, electron-dense structures associated with the active zone of ribbon synapses (for review, see Schmitz, 2009; Matthews and Fuchs, 2010). Ribbon synapses are tonically active synapses in the retina, inner ear and pineal gland that are able to maintain exocytosis for prolonged periods of time (Heidelberger et al., 2005; Matthews and Fuchs, 2010). RIBEYE is the main component of synaptic ribbons (Schmitz et al., 2000; Zenisek et al., 2004; Magupalli et al., 2008; Maxeiner et al., 2016). RIBEYE consists of a N-terminal A-domain and a carboxyterminal B-domain (Schmitz et al., 2000). The A-domain predominantly has a structural role in scaffolding the synaptic ribbon while the B-domain is most likely exposed on the surface of the synaptic ribbon (Magupalli et al., 2008). At that site, it can interact with proteins of the presynaptic terminal (Alpadi et al., 2008; Dembla et al., 2014; Wahl et al., 2016). The synaptic ribbon is considered to organize vesicle trafficking at the continuously active ribbon synapses and to provide the active zone with many release-ready vesicles (Matthews and Fuchs, 2010; Lagnado and Schmitz, 2015). It also contributes to the organization of active zone components (Maxeiner et al., 2016).

In the retina, photoreceptors and bipolar cells form ribbon synapses to communicate with their postsynaptic targets. Photoreceptor synapses are located in the outer plexiform layer (OPL) and have particularly large synaptic ribbons. Photoreceptor synaptic ribbons are plate-like, horseshoe-shaped structures in side views (with a contour length of about 1.5–2 μm) and EM reconstructions (for review, see Schmitz, 2009; Matthews and Fuchs, 2010). In EM cross-sections, they typically appear as bar-shaped structures. The OPL is dominated by ribbon synapses whereas the inner synaptic layer of the retina, the inner plexiform layer (IPL), contains a mixed population of ribbon synapses and "conventional", non-ribbon-containing synapses. Synaptic ribbons in the IPL are smaller than in the OPL (Heidelberger et al., 2005; Schmitz, 2009; Sterling, 2013).

2. Materials and methods

2.1. Materials

2.1.1. Bovine retina

Immunolabelling experiments were performed with bovine retinas freshly isolated from bovine eyes (see below). Bovine eyes were obtained from a local slaughterhouse.

2.1.2. Primary antibodies

2.1.2.1. Anti-RIBEYE. Polyclonal rabbit antiserum (U2656) raised against RIBEYE(B)-domain. This antibody has been extensively characterized previously (Schmitz et al., 2000; Magupalli et al., 2008; Alpadi et al., 2008; Maxeiner et al., 2016). Omitting primary antibody (i.e. using only secondary antibody) or irrelevant primary antibodies served as negative control incubations.

2.1.3. Secondary antibodies

2.1.3.1. Secondary antibodies conjugated to nanogold particles for light microscopy and electron microscopy. Goat *anti*-rabbit IgG conjugated to ultrasmall gold particles (1.4 nm gold particle size) (NanoProbes order #2003; via Biotrend, Cologne, Germany); used for pre-embedding immunolabelling at a 1:40 dilution in PBS to which 0.5% BSA has been added. Silver enhancement kit:HQ SILVER™ Enhancement kit (NanoProbes order #2012; via Biotrend, Cologne, Germany).

2.1.4. Epon embedding medium

Epon embedding medium was prepared by mixing 6.5 g of epoxy embedding medium (Epon 812, Fluka-Sigma-Aldrich; Munich, Germany) with 2.75 g 2-Dodecenyl Succinic Anhydride (DDSA; Electron Microscopy Sciences; Science Services; Munich, Germany) and 4 g Methyl-5-Norbornene-2,3-Dicarboxylic Anhydride (MNA; Electron Microscopy Sciences). 2,4,6-Tri(dimethylaminomethyl)phenol (DMP-30; Electron Microscopy Sciences) served as accelerator. After thoroughly mixing of Epon 812, DDSA and MNA, 0.1 g of DMP-30 was added to the mixture. Epon resin was degased using an exsiccator and directly used for embedding.

2.2. Methods

2.2.1. Gelatin coating of glass slides

Standard glass slides were coated with a relatively thick layer of gelatin. This was done 1.) for allowing a better attachment of the cryostat sections and semithin sections to the glass surface and 2.) to promote the detachment of the cryostat/semithin sections-containing Epon capsules from the surface of the glass slides at the very end of the pre-embedding procedure (see below). For gelatin coating, a 1% gelatin solution (w/v) in deionized H_2O was freshly prepared just before use with gentle heating (60 °C). To 100 ml of this solution, 0.05 g of potassium chromium-III-sulfate was added. Dust-free glass slides were inserted into cuvette-holders. For coating, the cuvette-holders with the loaded glass slides were submerged into cuvettes containing the freshly prepared gelatin solution and were incubated at RT for 5–15 min with gentle agitation. Excess gelatin solution was drained from the slides. The slides were incubated at 60 °C for ≈1 h to completely dry the gelatin-coated glass slides. This coating procedure was repeated five times. After the last drying step slides were placed in dust-free slide boxes and kept at RT until needed. This method allowed a very flat mounting of the immunolabelled cryostat sections/semithin sections. A flat embedding of the immunolabelled semithin sections is particularly important for making serial ultrathin sections from these samples (Fig. 3).

2.2.2. Ultra-small immuno gold staining of cryostat sections for pre-embedding light microscopy and electron microscopy

Freshly isolated bovine retinas were flash-frozen in liquid nitrogen-cooled isopentane, as previously described (Schmitz et al., 1996). In a liquid nitrogen-filled styrofoam box, an aluminum tray filled with isopentane was fixed in the way that the aluminum tray was surrounded by the liquid nitrogen. For freezing, small pieces of retina were rapidly inserted into the solidifying isopentane using metal tweezers. Frozen retinas were stored at −80 °C until the samples were mounted in the cryostat for sectioning. The samples were transported from the −80 °C freezer to the cryostat in a liquid nitrogen-containing dewar. Frozen retinas were mounted in freezing medium (Thermo Scientific Richard-Allan Scientific Freezing medium "NEG-50") at −20 °C and 20 μm-thick cryosections were prepared with a LEICA CM 1950 cryostat (LEICA Biosystems, Wetzlar, Germany). For sectioning, an anti-role plate (Leica, #14047742497) was used to obtain flat, well-