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Analysis of acute brain slices by electron microscopy: A correlative light–electron microscopy workflow based on Tokuyasu cryo-sectioning

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ABSTRACT

Acute brain slices are slices of brain tissue that are kept vital *in vitro* for further recordings and analyses. This tool is of major importance in neurobiology and allows the study of brain cells such as microglia, astrocytes, neurons and their inter/intracellular communications via ion channels or transporters. In combination with light/fluorescence microscopies, acute brain slices enable the *ex vivo* analysis of specific cells or groups of cells inside the slice, e.g. astrocytes. To bridge *ex vivo* knowledge of a cell with its ultrastructure, we developed a correlative microscopy approach for acute brain slices. The workflow begins with sampling of the tissue and precise trimming of a region of interest, which contains GFP-tagged astrocytes that can be visualised by fluorescence microscopy of ultrathin sections. The astrocytes and their surroundings are then analysed by high resolution scanning transmission electron microscopy (STEM). An important aspect of this workflow is the modification of a commercial cryo-ultramicrotome to observe the fluorescent GFP signal during the trimming process. It ensured that sections contained at least one GFP astrocyte. After cryo-sectioning, a map of the GFP-expressing astrocytes is established and transferred to correlation software installed on a focused ion beam scanning electron microscope equipped with a STEM detector. Next, the areas displaying fluorescence are selected for high resolution STEM imaging. An overview area (e.g. a whole mesh of the grid) is imaged with an automated tiling and stitching process. In the final stitched image, the local organisation of the brain tissue can be surveyed or areas of interest can be magnified to observe fine details, e.g. vesicles or gold labels on specific proteins. The robustness of this workflow is contingent on the quality of sample preparation, based on Tokuyasu's protocol. This method results in a reasonable compromise between preservation of morphology and maintenance of antigenicity. Finally, an important feature of this approach is that the fluorescence of the GFP signal is preserved throughout the entire preparation process until the last step before electron microscopy.

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1. Introduction

Despite the increased use of *in vivo* recordings on conscious animals to provide new insights into cellular neurophysiology, acute brain slices remain the tool of choice for patch clamp recording to analyse biological features such as ion channels and calcium flux (Khurana and Li, 2013). Since their introduction 50 years ago by Henry McIlwain (Collingridge, 1995; McIlwain, 1958), acute brain slices have been studied in detail by using advanced fluorescence microscopy techniques such as Stimulated Emission-Depletion (STED) microscopy, Stochastic Optical Reconstruction Microscopy (STORM) and Photoactivated Localization Microscopy (PALM), bringing the resolution under the micrometre level (Huang et al., 2010). Even more recently, a combination of two photon excitation and pulsed STED microscopy (Bethge et al., 2013) improved the imaging resolution on acute brain slices to a point-spread function

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of 62 nm for 40 nm fluorescent beads. However, one disadvantage of these imaging techniques is that only fluorescent structures (labelled or auto-fluorescent) can be observed and the reference structure needs to be fluorescently counterstained (Griffiths, 2001; Schwarz and Humbel, 2007). For this reason, electron microscopy is still the preferred method to observe subcellular organisation of biological samples. This is also due to its higher resolving power and when used in conjunction with gold immunolabelling, specific proteins can be localised and studied within their context, as the subcellular structure is directly visible (Schwarz and Humbel, 2007).

Electron microscopy analysis of acute slices (Kirov et al., 1999; Takano et al., 2014) combined with the low magnification overview and localisation capabilities of light/fluorescence microscopy, i.e. correlative light–electron microscopy, appears the most appropriate way to access the complexity of biological samples (Loussert et al., 2012; Mironov and Beznoussenko, 2009; Modla and Czymmek, 2011; Robinson and Takizawa, 2009). The light micrograph can supply *in vitro* analysis or maps of the regions of interest displaying for instance a fluorescent signal, which can then be imaged at higher resolution in an electron microscope. Nevertheless, correlating light and electron micrographs has proven to be challenging due to the different constraints imposed by the type of microscopy performed. To ensure easy transfer of a precisely located biological region from the light microscope to the electron microscope, we developed a complete process based on the cryo-sectioning method for sample preparation established by Tokuyasu (Griffiths et al., 1983; Slot and Geuze, 2007; Tokuyasu, 1973). This method was chosen for two main reasons:

(1) The type of fixation is suitable for immunolocalisation;
(2) The absence of dehydration and resin embedding preserves GFP fluorescence throughout the entire process.

The aim of the protocol was to identify and recover the cells of interest during all processing steps from the original location in the organ to nanometre-scale observation in the electron microscope. As a model system, we chose mouse brain from transgenic animals, which expressed eGFP in astrocytes (Nolte et al., 2001). The first step of our protocol involved slicing the brain into thick vibratome sections. From that point onwards, the GFP-expressing astrocytes could be observed at all steps during sample preparation, ultrathin cryo-sectioning until high-resolution imaging by electron microscopy.

2. Materials and methods

2.1. Animal experiments

All animal work was carried out according to the recommendations of the Swiss Federal Laws on animal experimentation, approved by the Cantonal Veterinary Office (Vaud, Switzerland). All efforts were made to minimise suffering.

2.2. Tissue preparation

Adult wild type mice and adult eGFP-GFAP mice (Nolte et al., 2001) were anaesthetised with isoflurane in a closed chamber and euthanased by decapitation. The brain was removed and placed in ice-cold, gassed (95% O_2, 5% CO_2) Artificial CerebroSpinal Fluid (ACSF) medium containing 118 mM NaCl, 2 mM KCl, 1.3 mM $MgCl_2$, 2.5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4 and 10 mM glucose, pH 7.4. Thick coronal sections of 300 μm containing hippocampus were then cut with a vibratome (Leica VT1200, Leica Microsystems, Vienna, Austria) and immediately transferred into phosphate buffer (0.1 M pH 7.4) containing 4% formaldehyde and 0.5% glutaraldehyde. Fixation was done for 2 h at room temperature, then the samples were stored in fixative for 12 h at 4 °C. The slices were washed in 0.1 M sodium phosphate buffer (PB: pH 7.4) and cut into small pieces. Each piece of sample was isolated and cryo-protected by infiltration with 2.3 M sucrose in 0.1 M sodium phosphate buffer (PB: pH 7.4) overnight at 4 °C.

2.3. Imaging

Brain slices in fixative solution were imaged with a Zeiss Imager.Z2 fluorescence microscope (Carl Zeiss Microimaging GmbH, Germany) equipped with an AxioCam MRc5 digital CCD camera (Carl Zeiss). Bright-field images and fluorescence images using filter set 38, excitation BP 470/40, beam splitter FT 495, emission BP 520/50 (Carl Zeiss) were acquired sequentially at the same focal plane through the entire volume. The brain slice was imaged with a 100 μm step in Z (through the entire depth of the tissue) with 2.5× objective (Carl Zeiss Microimaging Inc.; Plan-Neofluar, NA = 0.075) using the MosaiX module of the Zeiss Axiovision rel.4.8 software (Carl Zeiss).

Sucrose infiltrated brain pieces, mounted on aluminium pins, were imaged with the same Zeiss Imager.Z2 fluorescence microscope with 10× objective (Carl Zeiss; Plan-Neofluar, NA = 0.3) with 5 μm steps in Z and 20× objective (Carl Zeiss; Plan-Neofluar, NA = 0.5) with 1 μm steps in Z. During acquisition, the samples were kept hydrated and ice cooled to avoid dehydration. This was achieved by mounting the pins in a hole drilled into a Petri dish filled with ice. After imaging, the samples were immediately plunged into liquid nitrogen and stored until further processing.

2.4. Cryo-sectioning and fluorescence imaging of the thawed sections

For cryo-sectioning, pins with frozen brain tissue were mounted in a modified cryo-ultramicrotome (Ultracut UC6/FC6, Leica Microsystems, Vienna, Austria). The original binocular was replaced by a M205 FA stereo-fluorescence microscope equipped with a DFC 345 FX camera (Leica, Heerbrugg, Switzerland), suspended on a large swing arm (Leica, Heerbrugg, Switzerland). Tissue blocks were trimmed at −90 °C with a cryotrim diamond knife (Diatome, Biel, Switzerland) and ultrathin cryo-sections, 70 and 100 nm thick, were cut at −110 °C with an immuno diamond knife (Diatome). Sections were picked up with a drop containing an equal mixture of 2% methylcellulose and 2.3 M sucrose (Liou et al., 1996), warmed up to room temperature, and transferred onto a Formvar film-coated, carbon-stabilised 100 mesh copper finder grid (Electron Microscopy Sciences, Hatfield, PA, USA).

100 nm thin cryo-sections collected on grids were incubated four times for 2 min on a PBS solution to remove the methylcellulose/sucrose mix and then mounted on a glass microscope slide in PBS. This slide was overlaid with a glass coverslip and the cryo-sections were examined immediately with the Zeiss Imager.Z2 fluorescence microscope in bright-field mode and with GFP filters using 20× air (Carl Zeiss, Plan-Neofluar, NA = 0.5) and 40× oil objectives (Carl Zeiss, Plan-Neofluar, NA = 1.3).

Alternatively, sections were imaged with an epifluorescence microscope (CorrSight, FEI Company Eindhoven, The Netherlands) equipped with a ORCA-03G camera (Hamamatsu, Hamamatsu City, Japan) in bright-field mode and with GFP filter set using 5× air (Carl Zeiss, EC Plan-Neofluar, NA = 0.15), 20× air (Carl Zeiss, EC Plan-Neofluar, NA = 0.5) and 40× air (Carl Zeiss, EC Plan-Neofluar, NA = 0.9) objectives. Imaging was driven by a correlative and automated acquisition software ('Maps', FEI Company Eindhoven, The Netherlands).

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