1046
doi:10.1017/S1431927619005968
Microsc. Microanal. 25 (Suppl 2), 2019

A versatile en bloc staining procedure for large volume sample imaging

Graff Meyer Alexandra[1*], Titze Benjamin[1], Genoud Christel[1*]

[1.] Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland
* Corresponding author: alexandra.graff@fmi.ch.

Robust serial block-face scanning electron microscopy (SBEM) systems [1] as well as reliable automated acquisition software [2] enable us to image large volumes of biological samples at ultrastructural resolution. Other imaging techniques like FIB-SEM and multiSEM are also evolving towards faster and more reliable image acquisition of large tissue volumes [3] [4]. On the image analysis side, there have been various improvements in automatic/semi-automatic segmentation [5]. However, suitable EM protocols for sample preparation are the crucial first step to obtain high-quality datasets. Fixation must be fast enough to prevent tissue artefacts, staining must be homogeneous over large distances, conductivity must be sufficient to prevent charging artefacts, and the resin must permeate the entire sample volume. For decades, en bloc staining methods, like rOTO (reduced osmium-thiocarbohydrazide-osmium) protocols [6] [7] [8] [9] [10] have been successfully used for the staining of small samples (below 1 cubic mm) but uniform contrast was difficult to achieve when the sample diameter exceeded ~500 microns. More recently, a new protocol (BROPA, brain-wide reduced-osmium staining with pyrogallol-mediated amplification) for efficient staining of very large samples (entire mouse brains) has been introduced [11].

We explored modifications of this protocol and developed a faster procedure (fBROPA) for staining whole brains from adult zebrafish [12]. After an overnight fixation in 2.5% glutaraldehyde and 4% sucrose, two consecutive osmium steps (1. reduced osmium with formamide, and 2. osmium) are performed for 90min each. After washes in 0.1M cacodylate buffer, pH 7.4, the sample is placed in a freshly made pyrogallol solution for 30 min, followed by an additional 90min osmium step as described by Mikula and Denk [11]. After washes in ddH_2O and an overnight storage in ddH_2O at 4°C, the sample is immersed in Walton's lead aspartate solution at 60°C for 1hr. This additional step was necessary to increase sample conductivity to enable SBEM imaging in high vacuum. Finally, the sample is dehydrated in a graded alcohol series, immersed in 100% Epon resin (Serva) overnight, and the resin is cured at 60°C for at least 48h. If the sample is to be embedded in silver-containing resin to improve conductivity, proceed as described in [13]. Our images (Figure 1) were acquired on a Zeiss Merlin SEM (Zeiss, Oberkochen, Germany) equipped with an automated ultramicrotome inside the vacuum chamber for SBEM (3View; Gatan, Pleasanton, CA, USA; recently acquired by ThermoFisher Scientific).

With reduced incubation times and a lead aspartate incubation step to increase sample conductivity, the full procedure for staining whole brains from adult zebrafish was performed within 4 days. As shown in Figure 1A, we achieved homogeneous high-contrast staining throughout the brain. High-quality image stacks with voxel sizes of 10 x 10 x 25 nm^3 were obtained by serial block-face imaging using an electron dose of 15e^-/nm^2 (Figure 1B and 1C). Furthermore, we obtained high quality images with minimal charging for non-neural tissues like intestinal organoids. Recently, we have been investigating an fBROPA protocol with microwave use to achieve faster and stronger staining for heavy metal impregnation of many different samples. It seems that fBROPA with microwave heating is promising

for small samples like organoids or cells, but it is still a challenge to achieve homogeneous staining through larger samples like the entire zebrafish adult brain using the microwave approach.

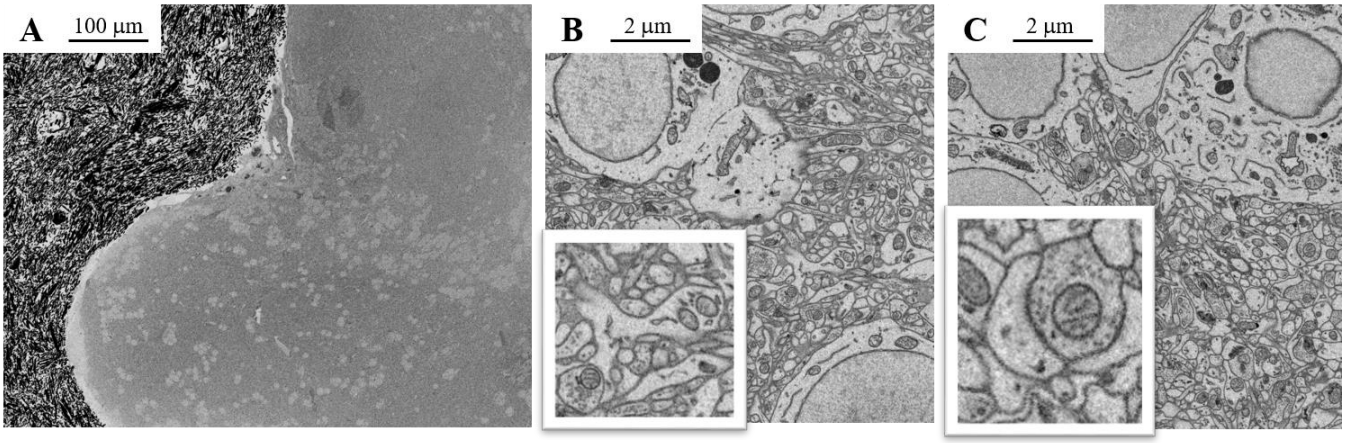

Figure 1. SBEM images of an adult zebrafish brain prepared with fBROPA protocol. **(A)** Coronal section through the telencephalon of adult zebrafish at the location of Dp (posterior zone of the dorsal telencephalon). Note the homogeneous staining. Black particles outside the tissue are silver particles in the surrounding resin to optimize conductivity. **(B)** Neuropil close to the surface. **(C)** Neuropil 300 µm below the surface. The images in the white frames of (**B**) and (**C**) show a zoomed-in region of (**B**) and (**C**).

[1] Denk W, Horstmann H, PLoS Biol. **11** (2004), e329.
[2] Titze B, Genoud C, Friedrich RW, Front Neural Circuits **12** (2018), 54.
[3] Xu CS, Hayworth KJ, Lu Z, Grob P, Hassan AM, García-Cerdán JG, Niyogi KK, Nogales E, Weinberg RJ, Hess HF, Elife **6** (2017), 25916.
[4] Eberle AL, Mikula S, Schalek R, Lichtman J, Tate MLK, Zeidler D., J Microsc. **259** (2015), 114.
[5] Januszewski M, Kornfeld J, Li PH, Pope A, Blakely T, Lindsey L, Maitin-Shepard J, Tyka M, Denk W, Jain V, Nat Methods **15** (2018), 605.
[6] Tapia JC, Kasthuri N, Hayworth KJ, Schalek R, Lichtman JW, Smith SJ, Buchanan J, Nat Protoc. **7(2)** (2012),193-206.
[7] Hua Y, Laserstein P, Helmstaedter, M.Nat Commun. **6** (2015), 7923.
[8] Seligman AM, Wasserkrug HL, Hanker JS, J Cell Biol. **30** (1966), 424.
[9] Malick LE, Wilson RB, Stain Technol. **50** (1975), 265.
[10] Deerinck TJ, Bushong EA, Thor A, Ellisman MH, Nat. Center Microsc. Imag. Res. methods for 3D EM Microscopy (Oxf) (2010), 6.
[11] Mikula S, Denk W, Nat. Methods **12** (2015), 541.
[12] Genoud C, Titze B, Graff-Meyer A, Friedrich RW, Front Neuroanat. **12** (2018), 76.
[13] Wanner AA, Genoud C, Masudi T, Siksou L, Friedrich RW, Nat. Methods **19** (2016), 816.

https://doi.org/10.1017/S1431927619005968 Published online by Cambridge University Press