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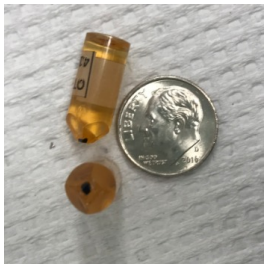
Post-Fixation Heavy Metal Staining and Resin Embedding for Electron Microscopy (EM)



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Protocol status: Working
We use this protocol and it's working

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Abstract

Tissues, cell monolayers, organoids and xenografts all image differently depending on the sample preparation method utilized. Moreover, the same tissue type from different species may also need different fixation solution and processing methods to have optimal contrast and charge mitigation when the same microscope is used (Borrett & Hughes, 2016; Kizilyaprak, Longo, Daraspe, & Humbel, 2015; Kopek et al., 2017). For example, brain tissue may be processed successfully using a protocol that yields poor images when applied to cancer tissues (unpublished data). Researchers are therefore encouraged to dive into the literature and test new sample preparation protocols for a specific sample-type. Also, and if available, having the ability to evaluate the use of FIB-SEM versus SBF-SEM will help the researcher to design a data collection strategy. The protocols evaluated during development of this workflow included the Dresden protocol (Paridaen, Wilsch-Bräuninger, & Huttner, 2013), Renovo (Mukherjee et al., 2016) and the Hua method (Hua, Laserstein, & Helmstaedter, 2015). We settled on the the Hua method with some modifications as described below, for human cancer biopsies. The final protocol described is sufficient for large format mapping and 3DEM FIB-SEM and SBF-SEM, eliminating the need for multiple samples processed with different protocols. In the case of biopsy tissue, where sample acquisition is limited and precious, flexibility is an important advantage of this workflow.

Day 1

1 Once the fixed samples are received, they are post fixed in 2% (v/v) OsO4 prepared in 0.1M Na Cacodylate (pH 7.4) for 1.5 hours at room temperature.
All these steps are performed using aluminum foil protected 2 ml centrifuge tubes. The three incubations done at room temperature are completed using a rotating platform.

2 Samples are incubated for 1.5 hours at room temperature in 2.5% (w/v) potassium ferricyanide ($K_3[Fe(CN)_6]$) dissolved in 0.1M Na Cacodylate (pH 7.4).

3 Samples are then extensively washed in dH2O for 5 minutes with 5 fresh exchanges.

4 After washing, the samples are incubated in a conventional oven for 45 minutes at 40°C in freshly prepared 1% (w/v) thiocarbohydrazide (TCH) solution.

5 This step is followed by another 5 exchanges of fresh dH2O over 25 minutes, and then incubated in 2% (v/v) OsO4 for 1.5 hours at room temperature. Water washes are then repeated as described above.

6 At the end of day 1, the samples are incubated overnight in the dark at 4°C in 1% (w/v) aqueous uranyl acetate.

Day 2

7 Samples are moved from 4°C incubation into a conventional oven set up at 50°C for two more hours.
Again, these steps are protected using aluminum foil covered 2 ml centrifuge tubes, as described above.

8 After this, the samples are washed in dH2O for 5 minutes with 5 fresh exchanges.

9 In the next staining step, the samples are transferred to a lead aspartate solution, previously warmed at 60°C, and incubated for 2 hours at 50°C using a conventional oven.

10 Another 5 exchanges of fresh dH2O over 25 minutes are completed. For this washing step the initial rinse is done in dH2O warmed in a 60°C oven.

11 The dehydration procedure is achieved in a series of acetone:water (50, 75, 85, 95%) mixtures with the final last step consisting of 100% acetone. It is worth mentioning that a new acetone bottle is utilized each time samples are processed using this protocol. Each dehydration step is completed twice for 5 minutes with one fresh exchange of the dehydrating solution.

12 The samples are then infiltrated in epoxy resin (EMS EMbed 812 cat# 14120). For this, samples are incubated in 1:1 acetone:resin for 40 minutes using a rotating platform followed by a 1:3 mixture and the same incubation period.

13 At the end of Day 2 the samples are transferred into vials with fresh 100% resin and incubated overnight on a rotating platform.

Day 3

14 Fresh epoxy resin is prepared and the samples are incubated with fresh resin for 30 minutes with 4 fresh exchanges.

15 After resin infiltration, the 1 mm2 sample blocks are wicked onto filter paper (Whatman #1) to remove the excess resin (Schieber et al., 2017) and then placed onto “Mini Pin” gunshot residue stubs (Ted Pella, Inc. Product #16180). If preferred, samples may also be preserved in a traditional BEEM capsule or mold and polymerized in the same manner.

16 The samples are polymerized at 60°C for 48 hours using a conventional oven.

Day 5

17 Samples are removed from the oven and allowed to cool to room temperature.

18 The advantage of mounting the samples directly to the stub in this way is that there is no need for extra trimming to expose the sample block. Furthermore, these mini pins can be fitted to a microtome sample holder for trimming and sectioning as needed without risk to the trim tool or histology diamond knives. If the researcher decided to polymerize the sample in a traditional BEEM capsule or mold, then the final sample needs to be trimmed and exposed as routinely done in an EM sample preparation laboratory.