**Protocol for SBF-SEM**

1. Immersion fixed of brain tissue in 2.5 % glutaraldehyde/ 2 % formaldehyde (freshly made) in 150mM sodium cacodylate (pH 7.4) with 2 mM $CaCl_2$ at 4°C overnight.

2. Using sharp razor blade (vibratome to be tried ), small pieces of tissue were cut (approx. less than 0.5 mm) in ice-cold 150mM sodium cacodylate with 2 mM $CaCl_2$.

3. Samples were rinsed 5 times (3 min each) in 150 mM sodium cacodylate with 2 mM $CaCl_2$.

4. Right before use, an equal volumes of 4% $OsO_4$ (water) and 3% potassium ferrocyanide in 0.3 M sodium cacodylate with 4mM $CaCl_2$ were mixed, added to samples and left for 1 hour on ice.

5. Samples were rinsed in $ddH_2O$ for 5x3min at RT.

6. Fresh 1 % aqueous solution of thiocarbohydrazide (TCH) was prepared as follow: 0.1 g TCH in 10 ml of $ddH_2O$ was placed at 60°C for 1 h (gently agitated every 10 min) and filtered using 0.22 µm Millipore syringe filter. Samples were incubated in the solution for 20 min at RT.

7. Samples were rinsed in $ddH_2O$ for 5 x 3 min at RT.

8. Samples were incubated in 2 % $OsO_4$ in $ddH_2O$ for 30 min at RT.

9. Samples were rinsed in $ddH_2O$ for 5 x 3 min at RT.

10. Samples were immersed in 1% solution of uranyl acetate in $ddH_2O$ at left at 4°C overnight.

11. Samples were rinsed in $ddH_2O$ for 5 x 3min at RT.

12. Walton´s lead aspartate solution (0.998 g L-aspartic acid was diluted in 250 ml of $ddH_2O$ to prepare 0.03 M stock solution; 0.066 g lead nitrate was dissolved in 10 ml of 0.03 M L-aspartic acid, pH 5.5 adjusted with 1 N KOH).

Samples were incubated in that solution for 30 min at 60°C.

13. Samples were rinsed in $ddH_2O$ for 5 x 3min at RT.

14. Dehydration was performed in 20 %, 50 %, 70 %, 90 %, 100 %, 100 % ethanol (ice cold) for 2 x 7min each step.

15. 100 % acetone (ice cold) 10 min at RT.

16. 100 % acetone (RT) 10 min at RT.

17. Infiltration with 25 % Epon in 100% acetone for 2 h at RT. Will clarify volumes and masses after the next batch

18. Infiltration with 50 % Epon in 100% acetone for 2 h at RT.

19. Infiltration with 75 % Epon in 100% acetone for 3 h at RT.

20. Infiltration with 100 % Epon overnight on shaker at RT.

21. Infiltration with fresh 100 % Epon for 6 h at RT.

22. Embedding using flat molds and polymerization for 48 h at 60°C.

Protocol  without Microwave to start

Microvave protocol to be tested

Epon:

EMBED 812 (EMS) = 37 ml

DDSA =25 ml

NMA = 20 ml

DMP-30 = 1.3 ml

**Specimen Trimming and Mounting with Microtome**

1. Using a glass knife, the block with dimensions of 400 µm × 400 µm × 400 µm was trimmed (500 µm, 100 mm/s).

2. The block of 400 µm × 400 µm × 400 µm was cut off with a razor blade. The tissue have to be exposed in all 6 sides.

3. Using a cyanoacrylate glue, the block was mount onto aluminum specimen pins.

4. The connection between the block and the pin was covered by silver pain and the layer was let to dry.

5. New pyramid was trimmed using a glass knife with dimensions of 200 µm × 200 µm × 200 µm.

6. The entire surface was sputter coated with gold (with rotation of the sample) for 3 min. 2.1Kv and

7. The first 10-20 µm from the top of block was cut off with a glass knife.

8. Samples were checked by SEM using the BSE mode at 3 kV. **(see separate sheet)**