

Freeze substitution in Potassium Permanganate

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Robby Weimer (Bessereau Lab)

weimer@biologie.ens.fr

Freezing:

Several animals (~10) were loaded into a 100um deep chamber surrounded by *E. coli* and frozen in a BalTech HPM 010.

Freeze substitution and embedding:

The freeze substitution was carried out in a Reichart AFS.

-90C for 3 days 0.1% KMnO₄ in anhydrous acetone

-90C to -45C at 4C/hr, total of 11.25 hours

-45C for 2 hours in anhydrous acetone, changing the acetone several times

-45C for 5 days during embedding in Lowicryl HM20

-45C for 3 days to UV polymerize resin

-45C to 20C at 6C/hr, total of 10.8

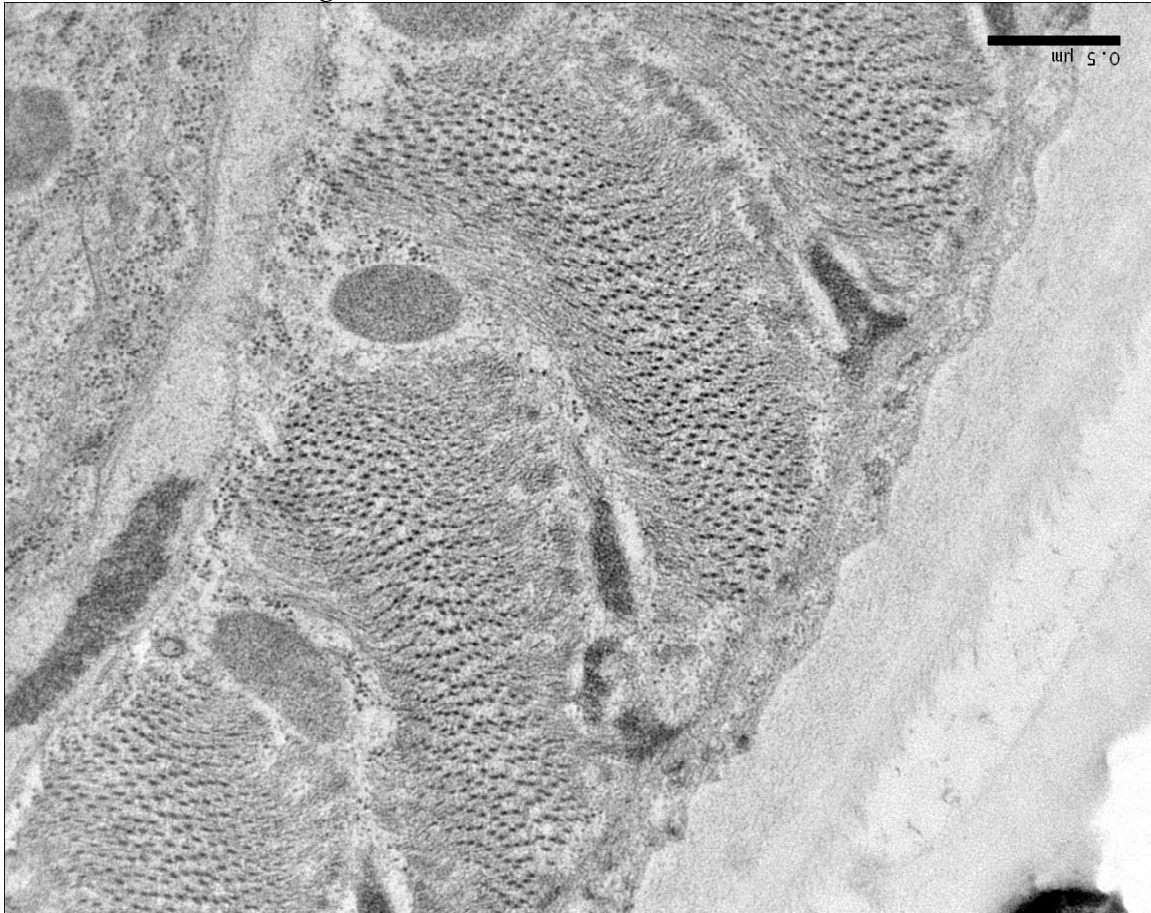
Sectioning:

40nm sections were collected on formvar covered grids and counterstained (4 minutes in 2.5% uranyl acetate in 70% methanol, 2 minutes in Reynolds lead). Micrographs were collected using a Tecnai 12 electron microscope at 60kV or 80kV. Prior to counterstaining, select sections were immunolabeled in the following manner: 15 min in 0.5% glycine in PBS, 30 min in goat sera, 3X5 min in PBS, 1hr in primary antibody diluted in PBS with 0.001% tween20, 6X5 min PBS, 30 min secondary gold-conjugated antibody diluted in PBS with 0.001% tween20, 9X5 min PBS, 5 min in 2% gluteraldehyde, 5 min in PBS and 2X5 min in water.

Observation:

Most tissues were well preserved and most membranes (including, synaptic vesicles and mitochondrial membranes) are well contrasted before immunostaining (see following micrographs). In comparison to methanol fixation, immunoreactivity appears to be the same if not improved. However, during immunostaining much of the membrane contrast is lost (see following micrographs), but examples of each type of membrane structure can be observed (in other words, it does not look like every vesicle can be seen but you can see vesicles).

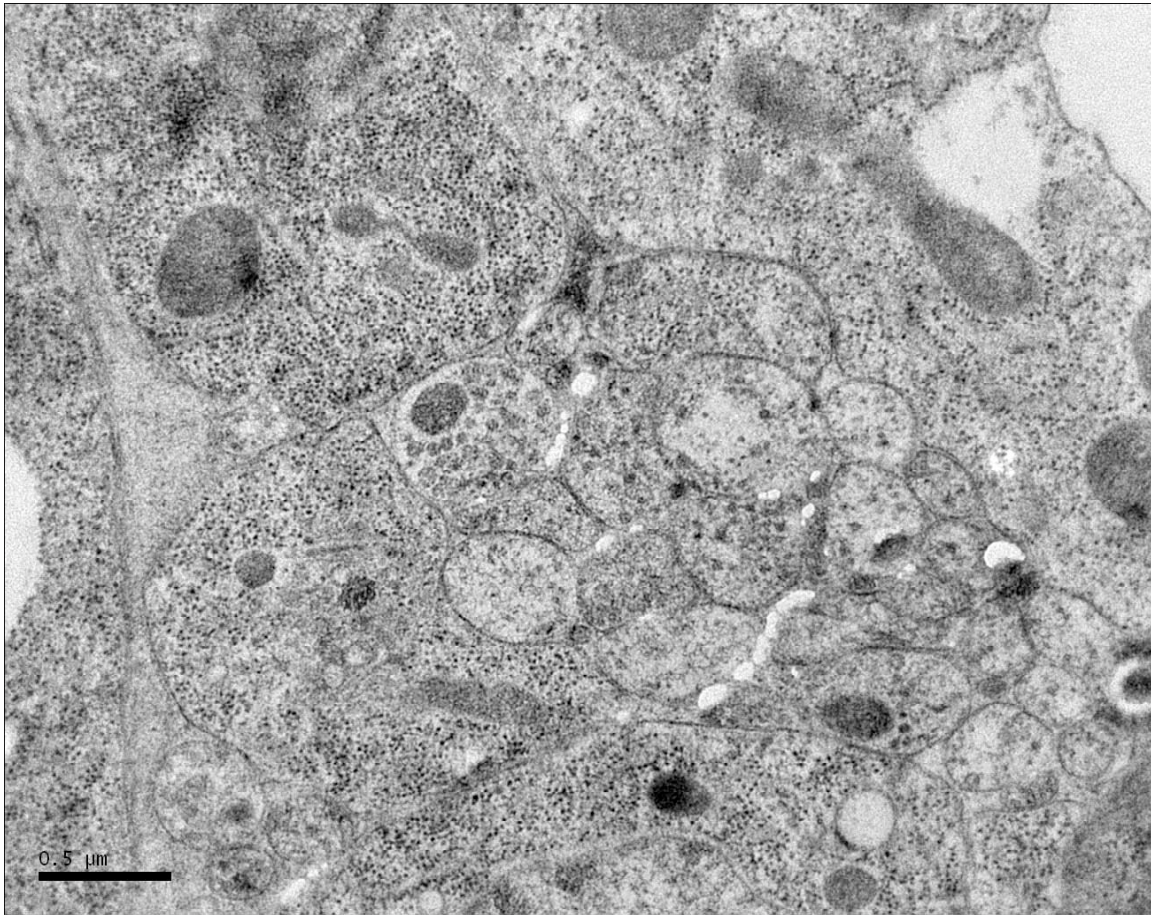
before immunostaining



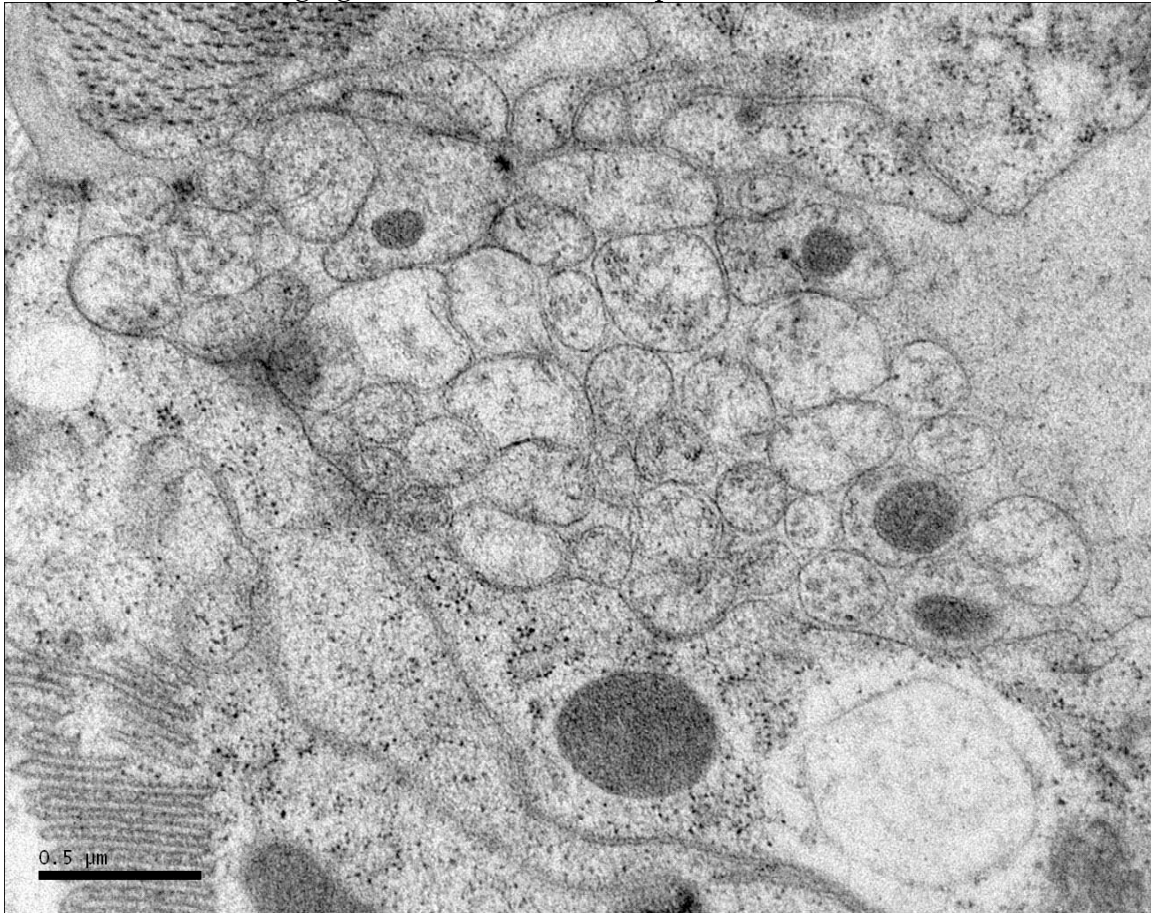
after immunostaining with antiVAB-10A (different section)



before immunostaining



after immunostaining against a non-neuronal protein (different section)



after immunostaining against a neuronal protein

