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FIB SEM protocol for anaerobic ciliates to visualize their prokaryotic endosymbionts and association with MROs

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Fixation of the cells

1 Individually pick several hundreds of cells with a micropipette from 2 ml of a well-grown culture of the targeted ciliate strain. Subsequently, follow fixation protocol by Rotterová et al. (2018) - see steps briefly described below or see full protocol "Transmission electron microscopy protocol for anaerobic ciliates" listed at protocols.io (dx.doi.org/10.17504/protocols.io.85uhy6w).

Rotterová J, Bourland W, Čepička I (2018). Tropidoatractidae fam. nov., a Deep Branching Lineage of Metopida (Armophorea, Ciliophora) Found in Diverse Habitats and Possessing Prokaryotic Symbionts.. Protist.
https://doi.org/10.1016/j.protis.2018.04.003

2 Fix picked cells with 2.5% (v/v) glutaraldehyde (Polysciences) and subsequently centrifuge at 800×g for 5 min at 4 °C.

3 Carefully replace supernatant with 1 ml of 2.5% (v/v) glutaraldehyde in 0.2M SCB (Sodium Cacodylate buffer, pH 7.2) and incubate 1 hour on ice.

4 Then, rinse the pellet carefully with SCB three times per 15 minutes.

5 Postfix the cells with 1% OsO4 in distilled water for 1 hour on ice.

6 Rinse the cells with distilled water, dehydrate them in a graded ethanol series from 30% to absolute ethanol, and infiltrate them with ethanol-acetone mixture, 100% acetone, and acetone-resin mixture.

7 Embed cells in absolute EPON resin (Poly/Bed 812, Polysciences) and let the cells get polymerized at 70 ◦C for 48 hours.

8 Cut serial ultrathin sections on an Ultracut E ultramicrotome (Reichert) using a diamond knife and stain with lead citrate and uranyl acetate (2–3%).

FIB SEM sample preparation

9 Separate the resin layer with fixed cells and wash thoroughly.

10 Select a region containing enough vertically oriented cells within the slice of resin using dissecting microscope with transmitted illumination.

11 Subsequently, process the sample following a protocol adapted from Carpenter et al. (2013), Loyola Machado et al. (2017), and Karnkowska et al. (2019) and optimized for larger cells with archaeal endosymbionts (7, 8, 9).

Carpenter KJ, Weber PK, Davisson ML, Pett-Ridge J, Haverty MI, Keeling PJ (2013). Correlated SEM, FIB-SEM, TEM, and NanoSIMS imaging of microbes from the hindgut of a lower termite: methods for in situ functional and ecological studies of uncultivable microbes.. Microscopy and microanalysis : the official journal of Microscopy Society of America, Microbeam Analysis Society, Microscopical Society of Canada.
https://doi.org/10.1017/S1431927613013482

Loyola-Machado AC, Azevedo-Martins AC, Catta-Preta CMC, de Souza W, Galina A, Motta MCM (2017). The Symbiotic Bacterium Fuels the Energy Metabolism of the Host Trypanosomatid Strigomonas culicis.. Protist.
https://doi.org/10.1016/j.protis.2017.02.001

Karnkowska A, Treitli SC, Brzoň O, Novák L, Vacek V, Soukal P, Barlow LD, Herman EK, Pipaliya SV, Pánek T, Žihala D, Petrželková R, Butenko A, Eme L, Stairs CW, Roger AJ, Eliáš M, Dacks JB, Hampl V (2019). The Oxymonad Genome Displays Canonical Eukaryotic Complexity in the Absence of a Mitochondrion.. Molecular biology and evolution.
https://doi.org/10.1093/molbev/msz147

12 Cut out square around the region of target cells, glue to the top of blank resin block. Trim and cut out the stub, leaving the height of the block smaller than 5 mm.

13 To ensure imaging the correct side, glue the block to an aluminium stub and coat it with a thin layer of platinum with high vacuum sputter coater (e.g., Leica EM ACE600).

3D FIB SEM imaging and acquisition

14 Processed the sample using scanning electron microscope with focused ion beam milling (e.g., FEI Helios NanoLab 660 G3 UC).

Orient the block using low magnification and secondary electron imaging (30 kV, 0.8 nA).

15 Apply a protective layer of carbon onto the surface, above the region of interest.

16 Remove a narrow band of resin from the front of the target region using an ion beam current of 13 - 27 nA at 30 kV.

17 Mill the target region of the block using a current of 700 pA until no artefacts are present.

18 Select optimal parameters and take serial images of the targeted cell by automatic slicing (30 kV, 0.79 nA) and imaging (2 kV, 0.2 nA) of backscattered electrons.

19 Select a region (10 x 10 µm) with sufficient amount of mitochondrion related organelles (MROs) and symbionts for detailed serial imaging with the highest resolution.