

Visualization of Cell Complexity in the Filamentous Cyanobacterium *Mastigocladus laminosus* by Transmission Electron Microscopy (TEM)

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[Abstract] The cyanobacterium *Mastigocladus laminosus* (*M. laminosus*) is one of the most morphologically complex prokaryotes. It forms long chains of cells that are connected via septal junction complexes; such complexes allow diffusion of metabolites and regulators between neighboring cells. Cellular division occurs in multiple planes, resulting in the formation of true branches, and cell differentiation leads to the formation of specialized cell types for nitrogen fixation (heterocysts) and culture dispersal (hormogonia and necridia). Here, we describe a detailed protocol for the preparation of *M. laminosus* for TEM in order to visualize the ultrastructural properties of the organism. The presented preparation method is based on adding potassium permanganate as fixative which has been shown to increase the contrast of membranes (Luft, 1956), making it suitable for studies in cyanobacteria where the visualization of the photosynthetic membranes is important.

Materials and Reagents

1. Liquid culture of *Mastigocladus laminosus*
2. Potassium phosphate monobasic (KH₂PO₄) (Thermo Fisher Scientific, catalog number: BP362)
3. Sodium phosphate dibasic (Na₂HPO₄) (Thermo Fisher Scientific, Acros Organics, catalog number: 204855000)
4. Glutaraldehyde (25%, EM grade) (Agar Scientific, catalog number: R1020)
5. Low gelling temperature agarose (Sigma-Aldrich, catalog number: A9414)
6. Potassium permanganate (KMnO₄) (VWR International, BDH, catalog number: 296444N)
7. 100% ethanol
8. Propylene oxide (Agar Scientific, catalog number: R1080)
9. Araldite CY212 (Agar Scientific, catalog number: R1042)
10. Methyl nadic anhydride (MNA) (Agar Scientific, catalog number: R1083)
11. Dodeceny succinic anhydride (DDSA) (Agar Scientific, catalog number: R1052)
12. Benzyl dimethylamine (BDMA) (Agar Scientific, catalog number: R1061)

13. Sodium tetraborate (borax) (Sigma-Aldrich, catalog number: 221732)
14. Toluidine blue (Sigma-Aldrich, catalog number: 89640)
15. Uranyl acetate [$\text{UO}_2(\text{CH}_3\text{COO})_2 \cdot 2 \text{H}_2\text{O}$] (SPI Supplies, catalog number: 02624-AB)
16. Lead (II) nitrate [$\text{Pb}(\text{NO}_3)_2$] (Sigma-Aldrich, catalog number: 228621)
17. Tri-sodium citrate (TAAB, catalog number: S011)
18. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalog number: 221465)
19. 0.125 M Sørensen's phosphate buffer (PB) (see Recipes)
20. 4% (v/v) glutaraldehyde in PB (see Recipes)
21. 2% (w/v) low gelling temperature agarose (see Recipes)
22. 2% (w/v) potassium permanganate (see Recipes)
23. Araldite (see Recipes)
24. 1% (w/v) toluidine blue (see Recipes)
25. Uranyl acetate (see Recipes)
26. Reynold's lead citrate stain (see Recipes)

Equipment

1. Centrifuge
2. Copper grids (300 mesh) (Agar Scientific, catalog number: G2740C)
3. Dental wax
4. Disposable plastic Pasteur pipettes
5. Disposable polyethylene beaker
6. Eppendorf tubes (1.5 ml)
7. Glass or diamond knife
8. Hotplate
9. Light microscope
10. Oven (60 °C)
11. Razor blades
12. Rotator
13. Rubber embedding mould
14. Sealable glass vials (7 ml)
15. Shaker
16. Spatula
17. Sterilization filters (pore size: 0.22 μm)
18. Syringe
19. TEM (JOEL, model: JEM-1230)
20. Tweezers

21. Ultramicrotome (Reichert Ultracut E)
22. Vortexer
23. SPI Slide-A-Grid™ storage box (SPI Slide-A-Grid™, catalog number: 02450-AB)

Procedure

Note: Some chemicals used in this protocol are highly toxic and should be used only under the fume hood with adequate precautions. Please check the MSDS before using them.

Preparation of samples for ultra-thin section transmission electron microscopy requires multiple days. The timeline for the different phases of preparation is shown in Table 1.

Table 1. Timeline for preparation of samples for transmission electron microscopy

Days 1-7	Growth of <i>M. laminosus</i> (A)
Day 8	Fixation and pre-embedding (B); Storage for up to 3 months possible
Days 9-11	Dehydration and embedding in Araldite (C); Indefinite storage possible
Day 12	Sectioning and post-staining (D); Storage for up to 1 year possible
Day 13	Visualization at the transmission electron microscope

A. Growth of *M. laminosus*

M. laminosus was grown in gas washing bottles filled with Castenholz D or ND medium (lacking combined nitrogen) (Castenholz, 1988) by bubbling with sterile air. Cultures were kept at 40 °C under constant white light at approximately 20 $\mu\text{E}/\text{m}^2/\text{s}$ until a dense cell mat was visible at the surface (Figure 1).



Figure 1. Dense cell mat of *M. laminosus* grown in Castenholz D medium

B. Fixation and pre-embedding

1. Transfer a piece of the cell mat (corresponding to a volume of approximately 100 μ l) into an Eppendorf tube using a 1 ml pipette.
2. Add 1 ml of PB, centrifuge for 2 min at 3,000 \times g and remove supernatant.
3. Resuspend the cells in 500 μ l of 4% (v/v) glutaraldehyde (*toxic!*) and leave the tube standing for 2 h at room temperature.
4. Centrifuge cells for 2 min at 3,000 \times g and remove supernatant. Wash cells 3x in 1 ml of PB with 10 min of incubation time between each step to remove the fixative. Resuspend cells by inverting the tube.
5. Remove supernatant after the final centrifugation step.
6. Resuspend cells in an approximately equal volume of 2% (w/v) liquid agarose (place vial at 60 $^{\circ}$ C ca. 30 min prior usage to melt the agarose and keep it fluid until usage). Leave sample at room temperature until it is fully polymerized. It is possible to stop the protocol at this step. Samples can be stored in the fridge for up to 3 months.
7. Cut the cell pellet with a razor blade into small cubes of about 1-2 mm length.

8. Transfer cubes to a glass vial and add 2% (w/v) potassium permanganate solution to fully cover the cubes.
9. Incubate for 12 h under gentle shaking at 4 °C.
10. Remove potassium permanganate solution and wash the sample several times for 10 min with distilled water until the supernatant remains clear.

C. Dehydration and embedding in Araldite

Note: All incubation steps were performed using a rotator with a rotation speed of 3 rpm.

1. Dehydrate cells by adding 30%, 50%, 70% and 90% (v/v) ethanol for 15 min and 3x 100% (v/v) ethanol for 20 min. Remove ethanol after each incubation step.
2. Incubate the sample for 5 min in propylene oxide (*toxic!*), remove supernatant and repeat washing step with propylene oxide for 5 min to remove remaining ethanol. Remove supernatant.
3. Add freshly prepared Araldite until the cubes are fully covered and incubate for 1 h.
4. Remove Araldite and replace by a fresh solution of Araldite. Incubate over night.
5. Transfer samples to an embedding mould which is filled with fresh Araldite solution and remove all air bubbles with tweezers.
6. Polymerize samples by placing the Araldite filled embedding moulds for 48 h at 60 °C. After polymerization samples can be stored indefinitely.

D. Sectioning and post-staining

Note: Sectioning requires a lot of experience. Contacting an expert is recommended for beginners.

1. Trim blocks with a razor blade and cut sections of 1 µm thickness with an ultramicrotome.
2. Transfer a section to a drop of distilled water on a glass slide by using an eyelash attached to a matchstick or a metal loop. Dry section on a hotplate (circa 60 °C).
3. Stain sections with 1% (w/v) toluidine blue for 30 sec on hotplate.
4. Drain excess stain and rinse section briefly with distilled water.
5. Dry on a hotplate and localize cells in a light microscope.
6. Reduce size of block further to get the smallest possible block area including the sample of interest and prepare ultrathin sections of approximately 70 nm thickness.
7. Transfer the sections to a copper grid.
8. Place droplets of saturated aqueous uranyl acetate (*toxic!*) onto a piece of dental wax in a Petri dish and float the grids (sections downwards) on the droplets for 4 min (Video 1).

Video 1.



9. Wash the grids by dipping approximately 10 times into distilled water. Repeat three times. (Video 1)
10. Blot off excess water with the edge of a piece of filter paper, and transfer the grids to droplets of Reynold's lead citrate placed on dental wax. Stain for 4 min (Video 1).
11. Wash with distilled water as described in step 9.
12. Blot off excess water and leave the sections to dry before viewing at the TEM. Grids can be used for TEM at any time. For long-term storage (up to 1 year) grids are kept in grid storage boxes (e.g. SPI Slide-A-Grid™ storage box). A sample micrograph is shown in Figure 2.

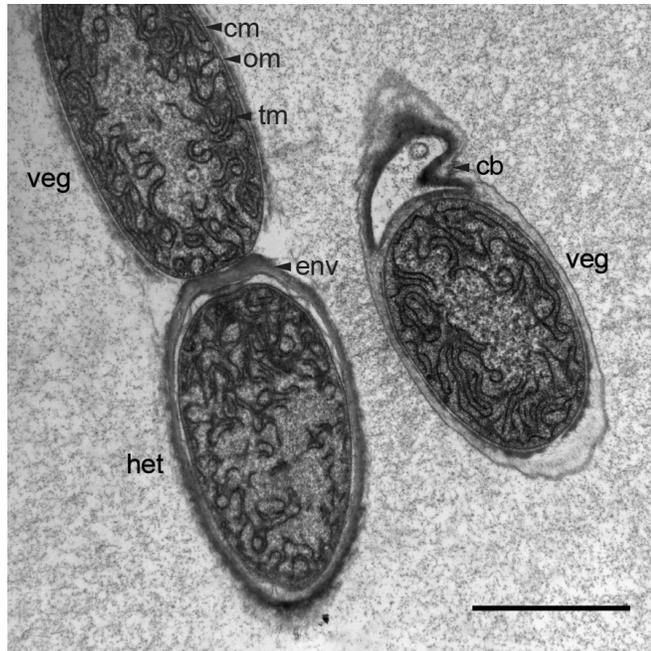


Figure 2. Electron micrograph of an ultra-thin section through a trichome of *M. laminosus* grown in Castenholz ND medium. Under nitrogen deprivation *M. laminosus* differentiates photosynthetically active vegetative cells (veg) into nitrogen-fixing heterocysts (het) which possess an additional multilayered envelope (env) as gas diffusion barrier to reduce the entrance of O₂ into heterocysts. When filaments break by the formation of necridia ("releasing cells") a small fraction of the former cell remains attached to the trichome (cb). Different membranes are highlighted by black arrows: thylakoid membrane (tm), cytoplasmic membrane (cm) and outer membrane (om). Scale bar, 2 μm

Recipes

1. 0.125 M Sørensen's phosphate buffer (PB) (pH 7.2-7.4)
 - 450 ml 0.2 M sodium phosphate dibasic (28.4 g in 1,000 ml distilled water)
 - 150 ml 0.2 M potassium phosphate monobasic (27.2 g in 1,000 ml distilled water)
 - 381 ml distilled water
 - Stored at 4 °C
2. 4% (v/v) glutaraldehyde in PB
 - 4 ml 25% glutaraldehyde
 - 21 ml PB
 - Stored at 4 °C
 - Use within 1 month
3. 2% (w/v) low gelling temperature agarose

- Add 0.2 g agarose to 10 ml PB
Place in the oven at 80 °C and stir occasionally until the agarose is fully dissolved
Stored at 4 °C
4. 2% (w/v) potassium permanganate
 - Add 2 g potassium permanganate to 10 ml distilled water
 - Dissolve by vortexing and filter
 - Prepare freshly before usage
 5. Araldite
 - 40 ml Araldite CY212
 - 60 ml dodecenylsuccinic anhydride
 - 2 ml methyl nadic anhydride
 - 1 ml benzyl dimethylamine
 - Stir with a spatula for at least 5 min in a disposable polyethylene beaker; introduce as little air as possible
 - Leave for 1 h at room temperature
 - Prepare freshly before usage
 6. 1% (w/v) toluidine blue
 - Add 1 g toluidine blue to 100 ml 1% (w/v) sodium tetraborate in distilled water
 - Dissolve by boiling and stir occasionally
 - Filter and stored at room temperature
 7. Uranyl acetate
 - Add 1 g uranyl acetate to 100 ml distilled water
 - Shake vigorously and leave for 48 h to dissolve at room temperature
 - If all the uranyl acetate has dissolved, add a further 1 g and shake vigorously
 - Leave for a further 48 h at room temperature
 - Repeat until no more uranyl acetate dissolves and some undissolved reagent remains
 8. Reynold's lead citrate stain
 - Dissolve 2.66 g lead nitrate in 30 ml distilled water
 - Dissolve 3.52 g tri-sodium citrate in 30 ml distilled water
 - Combine solutions and shake vigorously for 30 sec every 5 min over a period of 30 min
 - Add 16 ml 1 M sodium hydroxide solution
 - Mix until solution is clear
 - Add distilled water to a final volume of 100 ml
 - Stored at 4 °C

Acknowledgments

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References

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