A Correlative Light-Electron Microscopy (CLEM) Protocol for the Identification of Bacteria in Animal Tissue, Exemplified by Methanotrophic Symbionts of Deep-Sea Mussels

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Abstract

Bacterial symbionts associated with animal tissues play major roles in the functioning of various ecosystems. Identification of bacteria often relies on marker gene comparative sequence analysis and fluorescence in situ hybridization (FISH). However, analysis of bacteria and host ultrastructure using transmission electron microscopy (TEM) can be equally important to understand the localization of bacteria and the degree of host-symbiont integration. We here provide a protocol which allows both FISH and TEM to be performed sequentially on a single section of tissue. Observations can then be superimposed, allowing ultrastructural investigation to be coupled with proper FISH-based identification of bacteria.

Keywords: Correlative microscopy, Fluorescence in situ hybridization, Symbiosis, Transmission electron microscopy

1 Introduction

Fluorescence in situ hybridization (FISH) is very often used in the assessment of microbial symbioses to identify bacteria associated with animals [1]. It most often uses 16S rRNA phylotype-specific oligonucleotide probes labeled with fluorochromes (FISH, DOPE-FISH) or enzymes that allow signal amplification (CARD-FISH) [2, 3]. However, FISH is constrained by poor resolution due to an upper threshold determined by the emission wavelength of the target signal observed and by the limits placed on separation power by fluorescence microscopy, preventing the visualization of fine structural details. Such information may be of importance when assessing the degree to which microbial symbionts are integrated into host tissues, for example, their intra- or extracellular localization. It is also important when investigating eventual ultrastructural differences between distinct symbionts in terms of size, internal structures, or the presence

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of inclusions [4]. It may therefore be desirable to examine the ultrastructure of tissues in more detail, using electron microscopy. Symbiosis studies often use low- and high-resolution approaches in tandem to examine discrete, complementary aspects of symbioses [5]. These are typically carried out on separate sections of tissue, as hybridization and counterstaining techniques for fluorescence and transmission electron microscopy (TEM), respectively, are assumed to be mutually exclusive. However, due to the relative size of microbial symbionts, neighboring sections cut in sequence will almost never feature the same bacterium. Consequently, any biological inferences made using FISH and TEM will not be based on the same set of bacteria. By making careful adjustments to each protocol (**Note 1**) and accepting certain technical compromises (**Note 2**), it is possible to employ correlative light-electron microscopy (CLEM) on a single section to overcome this problem, by first performing FISH and, following some washing and counterstaining steps, TEM [6]. If a sufficient number of micrographs are captured following each procedure, FISH and TEM image mosaics of identical regions in the same semi-thin section can then be superimposed directly upon one another for direct visual correlation, using the protocol presented below. This protocol is exemplified by methanotrophic symbionts present in gills of deep-sea cold seep mussels, though it can be adapted to other types of bacteria and animal tissues.

2 Materials

1. Ultramicrotome and accessories.
2. Epifluorescence microscope.
3. Hydrophobic PAP pen, available at Sigma Aldrich cat: Z377821.
4. Hybridization oven.
5. Liquid nitrogen.
6. Gelatine capsules (size 00, Electron Microscopy Sciences, UK) and holder.
7. LR white medium-grade resin (London Resin Company, UK).
8. Toluidine solution.
9. Carbon Film 200 Mesh, Nickel TEM grids and grid holders.
10. Precision forceps for handling EM grids.
11. Filter paper.
12. 8 mm diameter circular coverslips or coverslips of equivalent size.
13. Eppendorfs (PCR type).
14. Hybridization buffer containing 900 mM NaCl, 20 mM Tris HCl, 0.01% SDS, and 10–60 %vol. formamide depending on the probe(s) used (Table 1).

Table 1
Composition of hybridization buffer depending on the formamide concentration employed

Formamide used	10%	20%	30%	40%	50%	60%
NaCl (5 M)	1.08	1.08	1.08	1.08	1.08	1.08
Tris HCl (1 M)	0.12	0.12	0.12	0.12	0.12	0.12
Milli-Q	4.2	3.6	3	2.4	1.8	1.2
SDS (20%)	0.003	0.003	0.003	0.003	0.003	0.003
Formamide	0.6	1.2	1.8	2.4	3	3.6

Concentrations of stock solutions are displayed, and volumes are given in mL for a final volume of ~6 mL, to be used for wetting tissue in hybridization chambers and for mixing with probes

Table 2
Composition of washing buffer depending on the formamide concentration employed during hybridization

Formamide used	10%	20%	30%	40%	50%	60%
NaCl (5 M)	900	430	204	92	36	8
Tris HCl (1 M)	200	200	200	200	200	200
EDTA (0.5 M)	0	100	100	100	100	100
SDS (20%)	5	5	5	5	5	5
Milli-Q	8,900	9,300	9,500	9,600	9,650	9,700

Concentrations of stock solutions are displayed, and volumes are given in μL for a final volume of 10 mL

15. Washing buffer, composition depends on formamide concentration used for hybridization (Table 2).
16. Anti-fade mounting medium, such as SlowFade with DAPI available at Life Technologies cat: S36938, with or without DAPI.
17. Proper DNA probes labeled with various fluorochromes in their 5′ end can be ordered from various companies such as Eurogentec (examples in Duperron [8], this volume, Table 3).
18. Uranyl acetate solution containing 1.25 g uranyl acetate per 25 mL Milli-Q water. Prepare on the day of use and keep in the dark.
19. Lead (II) citrate solution containing 0.1 mL NaOH (10 M) and 0.02 g lead (II) citrate per 10 mL CO_2-free Milli-Q water.
20. Plastic petri dishes.
21. Pelletized potassium hydroxide.

22. Extraction hood.
23. Glass microscope slides.
24. Install ImageJ and plugin MosaicJ on a computer [7].

3 Methods

3.1 Tissue Fixation, Embedding, and Grid Preparation for CLEM

Tissue fixation using formaldehyde with serial transfer to 80% ethanol is recommended (detailed Duperron [8]; this volume, Sect. 4.2.3). The resin used for embedding animal tissue intended for CLEM-type analyses must meet specific FISH and TEM requirements in order to optimize both techniques. The protocol below uses thermal-cured LR white medium-grade resin, a low toxicity, ultralow viscosity polyhydroxy-aromatic acrylic. Once polymerized, the resin is both hard and hydrophilic, permitting the cutting of sections sufficiently thin for TEM, while ensuring probe permeability during FISH. Several means of polymerization exist for this resin; however, only anaerobic thermal curation is presented here.

1. Bring LR white resin to room temperature.
2. In a suitable capsule holder, prepare two uncapped gelatin capsules (size 00) for each tissue sample to be embedded.
3. Half-fill first capsule with unpolymerized LR white.
4. Remove target tissue from ethanol (**Note 3**), blot dry with filter paper, and immerse in resin in first capsule.
5. Leave for 30 min so resin can infiltrate.
6. Remove used resin completely with transfer micropipette. If tissue samples are small, the use of a dissecting microscope can help to prevent their accidental removal.
7. Place the waste LR white into a dedicated container to be disposed of later following polymerization into a solid.
8. Refill the capsule containing the tissue with fresh resin.
9. Repeat **steps 6–8** eight more times, excluding **step 9** on the eighth and last repeat.
10. Half-fill the second capsule with fresh unpolymerized LR white and carefully transfer the fully infiltrated tissue into it.
11. Once the tissue has sunk to the bottom, orientate using a stiff hair or nylon thread by rolling the tissue into position (under a dissecting microscope if necessary).
12. Fill capsule to brim with resin until a convex meniscus is formed, and carefully replace the cap down fully until it clicks, catching overflowing resin with tissue. This minimizes the volume of air trapped at the top. Ensure the exterior is cleaned of resin.

13. Carefully place the sealed capsule securely in the holder in a vertical position, and relocate to an oven preheated to 55°C to polymerize, for a minimum 20 h.
14. Following polymerization, gelatin can be removed with hand-hot water.
15. Having trimmed the resin pellet, wet-cut sections on a suitable ultramicrotome (glass knives with boats are sufficient), employing periodic toluidine staining to identify the cutting axis and suitability of tissue for CLEM analyses, according to standard TEM protocols.
16. When target tissue region is located (i.e., well-preserved suitable host tissue and the presence of putative symbionts), ramp down to 300 nm and begin cutting semi-thin sections until cut consistency and iridescent hue becomes uniform (300 nm sections should be slightly transparent and somewhere between purple and blue green).
17. Let sections rest in the boat for a period of time, to minimize compression effects during cutting.
18. Transfer sections onto the darker, coated side of Carbon Film 200 Mesh, nickel grids, avoiding pleats (**Note 5**).
19. Leave the grid to air-dry and carefully transfer to and store in a dust-free grid holder.

3.2 CLEM Part 1: Adapted FISH Protocol and Fluorescence Imaging

As with standard FISH, symbiont- or group-specific oligonucleotide probes targeting ribosomal RNA (usually 16S or 23S) are applied during CLEM-type FISH. Probe specificity and suitable formamide concentrations are best assessed on a tissue by tissue basis according to the same criteria. This can be done in advance of CLEM using the standard FISH protocol, but carried out on thinner 300-nm LR white sections on Superfrost Plus slides (rather than grids, for simplicity) with an extended hybridization step of 20 h. Once the formamide concentration has been established (**Note 5**), FISH can be performed on the grid-mounted samples. Note that in the following protocol, the composition of the hybridization and washing buffers is prepared as indicated in Tables 1 and 2 (i.e., Tris HCl is retained, contrary to Halary [6]).

1. If not already, bring grids to room temperature in their holder.
2. Preheat hybridization chambers containing a piece of tissue wetted with hybridization buffer to 46°Cs.
3. Using a PAP pen on microscope slides (they need not be Superfrost Plus), trace an empty encircled area (min diameter 5 mm) for each of the grid-loaded sections to be hybridized, with a maximum of 4 per slide.
4. Note what each circle is intended to hold with regard to buffer, grid, and associated probes. At least one of the encircled areas should be for a control on each slide (refer again to **Note 3**).

5. First distribute the hybridization buffer and probes chosen for each encircled area, ensuring adjacent aliquots do not mix (1:15 dilution of the 50 ng/μL probe stock solution in buffer). Several probes with distinct fluorochromes can be mixed: each probe contributes to the overall aliquot volume, which is best kept below 30 μL. Probes are light sensitive, so the following steps should be performed under low, indirect light.
6. Using precision forceps suitable for handling TEM grids, place grids section-side down floating on buffered, probe-loaded aliquots.
7. Once all grids are loaded, place the glass slides into preheated hybridization chamber carefully, close and gently seal chamber, and leave to hybridize for 20 h at 46°C in darkness.
8. Prior to the end of the hybridization, prepare two Eppendorfs filled with washing buffer for each grid being processed (volume depends on incubation approach, **Note 6**) and preheat them to 48°C.
9. Carefully retrieve the slides from the hybridization chambers. Using fine forceps, carefully extract each grid, touch grid edge to filter paper to remove retained hybridization buffer, and quick dip several times in the first washing buffer Eppendorf tube. Then transfer the grid to the second washing buffer Eppendorf tube, seal it, and incubate for 15 min in the dark. Wipe the forceps clean between grids.
10. Remove grids carefully from Eppendorfs using forceps, touch grid edge to filter paper to remove retained washing buffer, and quick dip several times in Milli-Q water (RT). Leave each grid to dry on filter paper, keeping note of which grid is which throughout.
11. Place a small amount of anti-fade (1–2 μL) on the underside of each grid while inverted (opposite side to the tissue section), to minimize trapped microbubbles.
12. Place up to four grids (section-side up) on each slide, spaced apart, resting on the small droplet of anti-fade.
13. Add a similar volume of anti-fade to the section side of each grid near their edges and gently cover with coverslip; the smaller the coverslip, the easier it is to remove later (individual 8 mm diameter circular coverslips work very well, **Note 4**).
14. Press each coverslip down gently until it begins to resist lateral movement and remove excess anti-fade using a filter paper edge, if necessary.
15. Store at −20°C until observation. Overnight storage often improves the signal-to-noise ratio by decreasing tissue autofluorescence.

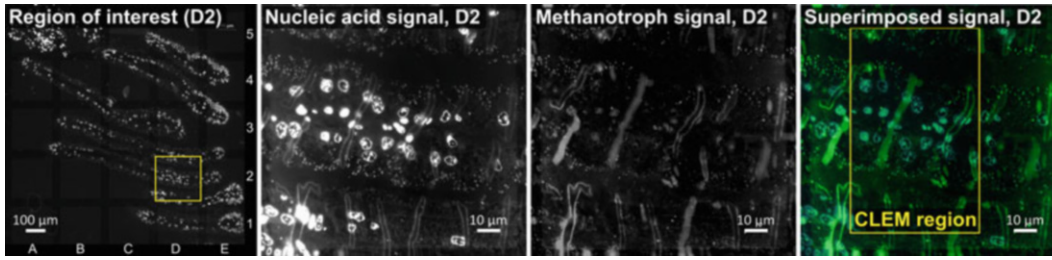

Fig. 1 FISH imaging of bacteria upon bivalve gill filaments using micrograph mosaics. The images from left to right are of mosaics made up of individual aligned micrographs either at 20× (first) or 100× objectives (remaining images). Pictured are views of gill tissue in sagittal section from a chemosymbiotic bivalve, *Idas modiolaeformis*. The latter three micrographs are magnified views of region D2 in the first reference micrograph, labeled accordingly. The overlaid methanotroph-specific signal upon DAPI in the last image reveals corresponding signal between the two labeling techniques (and also a general eubacterial probe, which is not pictured). The "CLEM region" refers to the region imaged with TEM, allowing correlative imaging to be performed (*see* Figs. 2 and 3). The methanotroph signal was obtained using probe MedM-138 [9]

Sections can then be observed under an epifluorescence microscope (suitability of confocal microscopy may depend on the plane of laser excitation, as completely flat grids are rare), and images are acquired using excitation wavelengths corresponding to the different fluorochromes (Fig. 1). In order to construct mosaic images for CLEM, sequences of overlapping images are captured manually under the 100× objective to permit the alignment of tiled images of target tissues, identified during fluorescence imaging (*see* Fig. 1). This procedure can be automated on microscopes equipped with a motorized stage. At this magnification, four overlapping images will cover one grid square. Mosaics can be created using Adobe Photoshop (*File* > *Automate* > *Photomerge* > *Reposition only* or *Interactive layout*) or by using the MosaicJ plugin of ImageJ, discussed in more detail later using the TEM images [7]. Ideally several target squares within which target tissue and symbionts appear should be imaged in this way for each grid and each emission wavelength.

3.3 CLEM Part 2: Grid Removal and Temporary Storage

1. Plunge the coverslip to be removed into liquid nitrogen, and once the nitrogen ceases to boil, remove the slide, and using a scalpel or feather blade, lever off the coverslip. The grid should remain in place.
2. Remove grid using fine forceps (heating the underside of the glass with a finger tip if necessary) and rinse with ultrapure water and dry on filter paper.
3. Place in a dust-free grid holder and store in the fridge.

3.4 CLEM Part 3: TEM Protocol and Imaging

Pre-embedding contrast staining using osmium tetroxide must be omitted by necessity when performing CLEM. Although it is not explored here, in theory it may be feasible to include a post-FISH

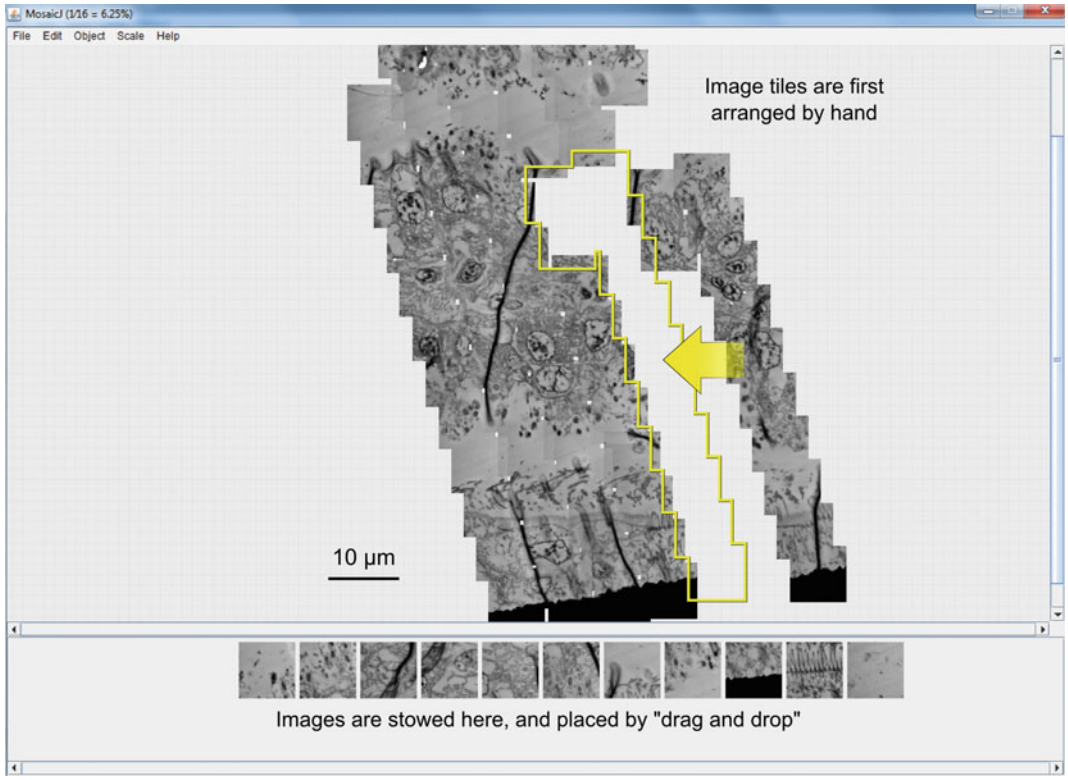

Fig. 2 Using MosaicJ in ImageJ. The screenshot shows the TEM images midway through the process of creating the mosaic, made up of individual hand-aligned micrographs at a relatively high magnification (2,000×), in the main window pane. These images correspond to a region in square D2 indicated in Fig. 1. Images can be dragged as groups seen here in *yellow*. The images stowed in the scrolling pane beneath are yet to be added. Final precision alignments and moderate blending are performed automatically

osmium tetroxide treatment on individual grids at this stage. However, the contrast staining described here involves uranyl acetate and lead (II) citrate only.

1. Prepare two plastic petri dishes under an extraction hood: the first is for uranyl acetate staining and must be in complete darkness, and the second is for lead citrate staining, which is performed in the absence of CO_2 (**Note** 7). Turn the hood's fan on.
2. Prepare the uranyl acetate and lead (II) citrate solutions fresh.
3. Prepare a washing solution of 50% ethanol (aqueous) and a separate one of Milli-Q.
4. Immediately prior to use, filter the uranyl acetate solution (e.g., using a 20-µm syringe filter) and dilute in absolute ethanol (1:1).

5. Add as many separate aliquots of each solution as there are grids to be stained, to the base of their respective petri dish staining chambers. Replace the lid of the second CO_2-free dish immediately.
6. Float the grids section-side down on the aliquots of the uranyl acetate, replace the lid, and leave in complete darkness for 7 min.
7. Remove the lid (first dish), lift each grid and dip several times in the 50% ethanol solution, and touch-dry on filter paper.
8. Open the second petri dish and float each grid section-side down on the aliquots of lead (II) citrate and return the Parafilm-sealed lid immediately. Leave for 7 min.
9. Remove the lid (second dish), lift each grid and dip several times in the Milli-Q, and touch-dry on filter paper.
10. Place in a dust-free grid holder and store in the fridge.

Sections can then be observed under a transmission electron microscope so long as the power of the microscope is sufficient to penetrate 300-nm sections. As this is not always the case, be sure to confirm this with the operating technician. Using the lowest magnification that entirely fills the field of view (i.e., no circular border from the microscopes narrowest aperture), take sequences of overlapping images to cover the grids selected during FISH imaging. This may involve a large number of images and is far easier to perform if it can be automated sequentially between saved grid references. Specific microbes and target host tissues visible in reference FISH images can then be selected and viewed at full magnification. If a mosaic-type feature is not available (or fails to work) in the TEM software, mosaics can be created using Adobe Photoshop (*File* > *Automate* > *Photomerge* > *Reposition only* or *Interactive layout*), though with large numbers of images, mosaics are best reconstructed using the MosaicJ plugin of ImageJ (Abramoff et al. [7], Fig. 3). The images can be dragged and dropped into the MosaicJ window once the plugin is running. Initially, images will appear in the scrolling window below. These can then be selected and dragged into the upper window for rough alignment by hand. The sequence of images is best named and ordered incrementally. When complete, the plugin aligns overlapping tiles more precisely based on to the registration engine TurboReg, which must be installed independently of MosaicJ. For details see http://bigwww.epfl.ch/thevenaz/mosaicj/. It remains to identify the overlapping regions of the FISH and CLEM mosaics and superimpose one upon the other. The superimposed images in Fig. 4 were performed manually in a vector graphics package (Inkscape v. 0.48).

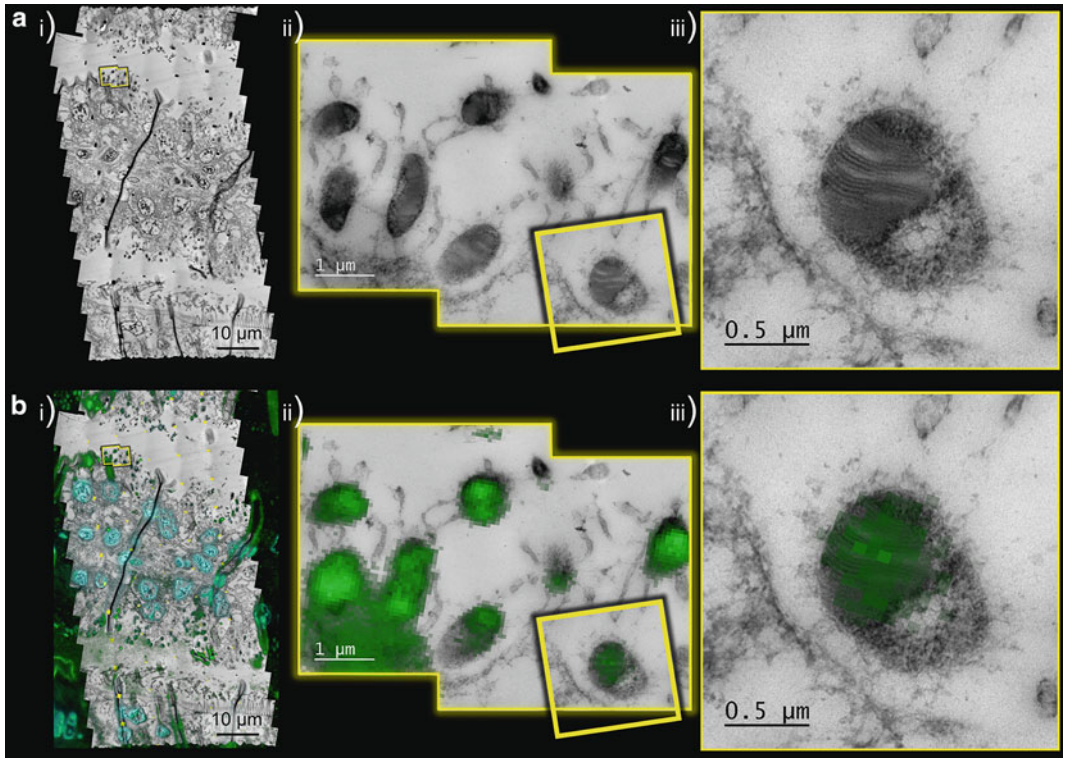

Fig. 3 Manual CLEM superimposition of FISH and TEM mosaics. Images are (**a**) TEM mosaics or single images at increasing magnifications from (i)–(iii). Regions of interest are indicated in *yellow* in the preceding image from left to right. In (**b**) the same TEM mosaics are overlaid with FISH image mosaics (see full images in Fig. 1) which correspond to the same region. In (i) both a blue signal (DAPI) and a green signal (methanotroph probe) have been overlaid on top. In (ii) and (iii) only methanotroph signal is shown for clarity. Ultrastructural details from high-resolution TEM images thus augment the data available from the specific fluorescent labeling through FISH. In this case, intracellular stacked membranes seen in both figures (iii) are typical of type I methanotrophic Gammaproteobacteria, supporting the phylogenetic relatedness already identified using specific FISH probes. Note that FISH signal is highest in intact bacteria. Images were superimposed in the vector graphics software Inkscape, having removed the black background in Photoshop CS6

4 Notes

1. Main adjustments include the following: (a) the use of a hard methacrylate embedding resin and an ultramicrotome for semi-thin sectioning; (b) FISH hybridizations being performed upon sections mounted on custom TEM grids, resistant to corrosion; (c) the extension of FISH hybridization times to accommodate reduced section thicknesses; (d) the washing of grids immersed in Eppendorfs rather than as sections on slides in a rack/falcon tube; (e) transitory sample treatment to allow post-FISH counterstaining for TEM; and (f) an electron microscope powerful enough to penetrate semi-thin sections.

2. Principal compromises include (a) semi-thin sections (300 nm) at thicknesses falling between those employed in FISH and TEM as standard; (b) the retention of embedding resin and use of TEM grids during FISH, which can result in refracted-light aberrations and variability in focal planes, respectively; and (c) the complete omission of osmium tetroxide counterstaining.
3. When using LR white, tissue stored in ethanol at >70% does not need to be dehydrated any further prior to infiltration within resin. However if preferred, serial transfer to higher-% ethanol grades will not affect the quality of embedding adversely.
4. This can be achieved either by drawing the grid upward, from below the water's surface in the knife boat, or by using a suitable EM loop and transferring the section to a grid on a dry slide, drawing the excess water laterally (because of impermeable carbon film) by touching filter paper to the edge.
5. A default concentration of 30% formamide is recommended as a starting point.
6. The best is to use PCR-type Eppendorfs placed in a thermocycler set at 48°C (the temperature will be stringently controlled in this way). Alternatively, larger Eppendorfs may be used in a rack, placed in a water bath maintained at 48°C. In either case use caution: due to the viscosity of the washing buffer, the grids will likely sink. Retrieval is best achieved using forceps, having gently rotated the grid to access its edge if necessary. Avoid bending the grid.
7. Cover the first petri dish with an opaque lid, since the uranyl acetate solution is photolabile. Fifteen mins ahead of use, place KOH pellets within the second dish at the perimeter of the base, balance a square of Parafilm so as to cover the open rim, and replace the lid on top to seal the petri dish. The KOH absorbs CO_2 which otherwise readily forms precipitous, electron dense lead carbonate.

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