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SHORT COMMUNICATION

## Easy flat embedding of oriented samples in hydrophilic resin (LR White) under controlled atmosphere: Application allowing both nucleic acid hybridizations (CARD-FISH) and ultrastructural observations

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### Summary

Hydrophilic resins present the advantage of making possible both hybridization experiments involving either antibodies or oligonucleotide probes and ultrastructural observations. Whereas various embedding protocols are available, only very few concern flat-embedded preparations. In this study we describe an easy protocol for flat embedding of small-oriented biological samples in hydrophilic resins (LR White). The most important constraints are (i) to polymerize the samples under argon-saturated atmosphere (avoiding oxygen which is an inhibitor of LR White polymerization) and (ii) to use transparent flat embedding molds. Two kinds of samples were analyzed: small pieces of large tissue that need to be accurately oriented for a valuable analysis and very small organisms such as free-living nematodes, which are very hard to investigate with conventional paraffin wax embedding techniques. Semi-thin sections strongly reinforce the quality of the observations from oligonucleotidic *in situ* hybridization experiments by reducing the background usually encountered in oligonucleotide probe hybridization experiments from sections. Such protocols could also permit a cheap alternative to the use of laser scanning confocal microscopes for oligonucleotidic *in situ* hybridization as in FISH and CARD-FISH experiments from histological sections. The interest of this

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embedding protocol is reinforced by the fact that molecular *in situ* hybridization experiments and ultrastructural observations from thin sections can be carried out from a single-small individual (<1 mm in length) sample.

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## Introduction

Some diagnostic areas in biology require greater cytological and nuclear detail than is provided by standard 4–5 µm paraffin-wax-embedded sections. This is especially the case with eukaryotic tissues containing bacterial symbionts. To achieve a high degree of detail, it is necessary to prepare sections less than 1 µm thick (semi-thin sections). Therefore, harder materials than paraffin wax are essential to provide adequate support to tissues during the cutting phase. Usually, epoxy resins are used for ultrastructural analyses but, as they are hydrophobic, sections cannot be used for other experiments. Acrylic resins are characterized by sufficiently low viscosity to allow short infiltration times and the use of various histological staining methods. Hydrophilic resins, such as LR White, present the advantage of permitting both ultrastructural observations and post-embedding hybridizations at various levels, including proteinic (immunohistochemistry) or nucleic (hybridization against DNA or rRNA) (Nussbaumer et al., 2006). The main problem involved is that resins such as LR White do not polymerize in the presence of oxygen. LR White may be polymerized either chemically (at room temperature or at elevated temperatures using a conventional oven) or at low temperature using ultraviolet light. Moreover, such cryotechniques need expensive equipment, high level technical skills and require long periods of infiltration at the temperature of liquid nitrogen.

Due to the fact that the embedding process of LR White always occurs in the absence of oxygen, tissues are classically embedded in gelatin capsules eliminating the possibility of controlling

the orientation of the samples. As a result, handling of the tissues is often delicate and analysis potentially difficult owing to bad section orientation. There is therefore a need for well-oriented tissues after LR White embedding to provide better and faster analysis. Moreover, because of the permeability properties of hydrophilic resins, combined analyses (ultrastructure, hybridization) may be done from the same block, i.e. on the same tissue, or even from a single organism, with the correct orientation.

The main objective of this paper is to describe an alternative to the usual protocols for hydrophilic resins with an easy protocol allowing flat embedding of small-oriented samples in LR White resin.

## Materials and methods

### Fixation and embedding

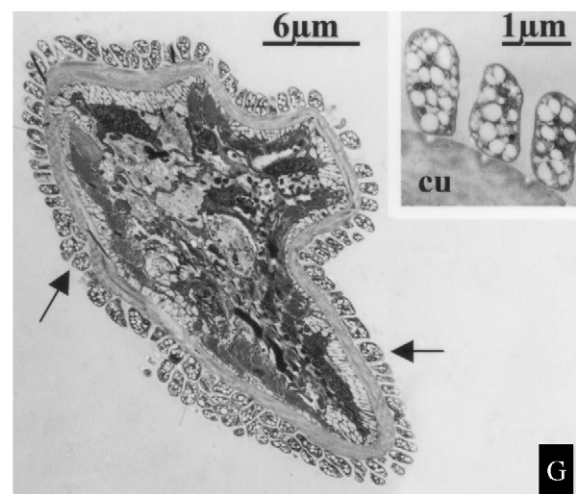
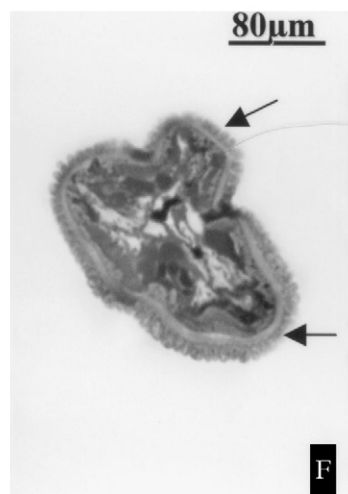
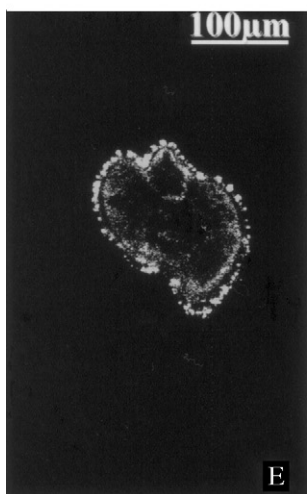
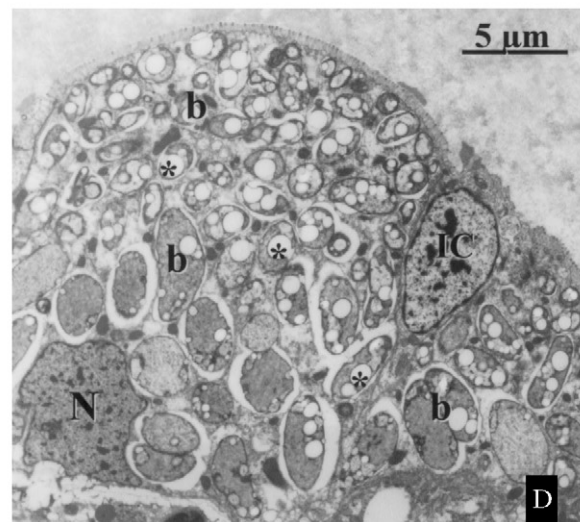
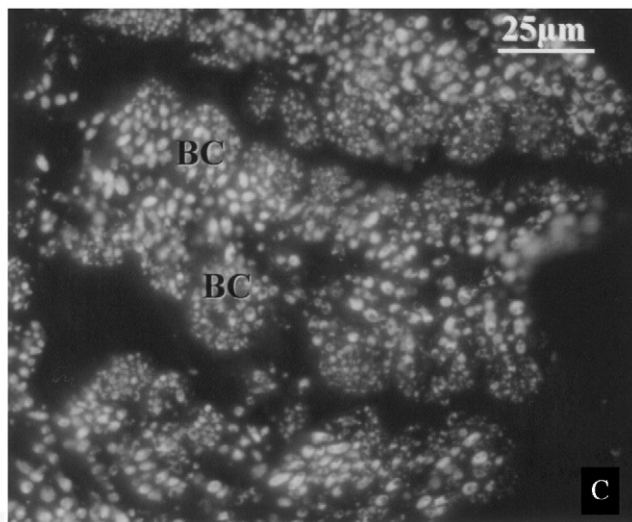
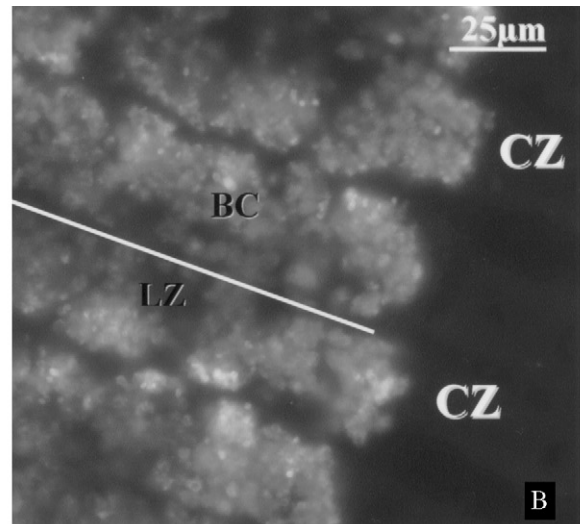
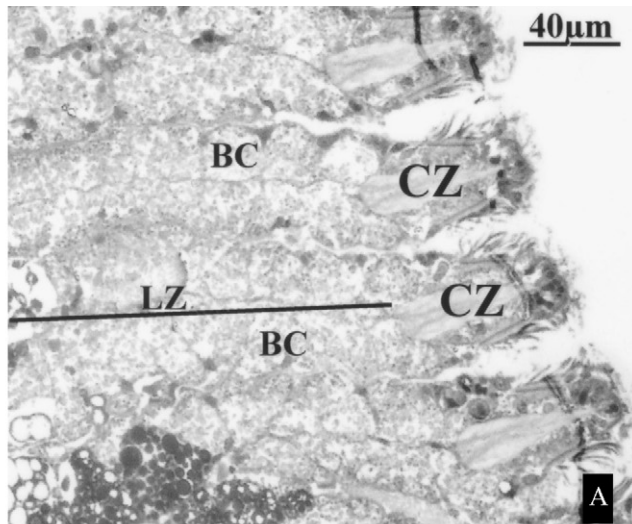
Pieces of gill tissue from the bivalve *Codakia orbicularis* and free-living marine nematodes, belonging to the Stilbonematidae family, as small organisms (length <1 mm; width <0.3 mm), were used in this study. Samples were fixed for 1–3 h at 4 °C in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2) or in seawater. The specimens were then washed three times, for 10 min each, at room temperature in seawater, dehydrated through an ascending series of ethanol, then stored in 100% ethanol at 4 °C until embedding.

Samples were embedded in the hydrophilic LR White resin (Biovalley Inc.) and the infiltration steps were similar to those previously described by Nussbaumer et al. (2006). Briefly, dehydrated

**Figure 1.** (A) Semi-thin section of gill filament of *Codakia orbicularis* (bivalve) showing the ciliated zone (CZ) devoid of bacteria and the lateral zone (LZ) which contains bacteriocytes (BC) harboring intracellular bacteria; (B) CARD-FISH from a 4-µm-thick paraffin wax section of *C. orbicularis* gill with the universal probe Eub 338 showing a strong hybridization inside the bacteriocytes (BC) through the lateral zone (LZ); the ciliated zone (CZ) presents no signal; (C) CARD-FISH from a semi-thin section (1 µm thick) with the universal probe Eub 338. Bacteria inside the bacteriocytes (BC) are easily distinguishable and present a strong positive signal; (D) Ultrastructure (TEM) of a bacteriocyte whose cytoplasm is filled with envacuolated bacteria (b) characterized by numerous sulfur granules that appear as empty vesicles (asterisks). N: nucleus; IC: intercalary cell; (E) CARD-FISH with the universal probe Eub 338 from a semi-thin section (1 µm thick) of a free-living marine nematode. Bacteria, located outside the organism, are positively hybridized; (F) Semi-thin section (0.5 µm thick) stained with toluidine blue from the same marine free-living nematode as shown in (E). Bacteria are located outside the nematode (arrows); (G) Ultrastructure (TEM) of bacteria (arrows), which are located only on the surface of the cuticle (cu) of the nematode, are characterized by numerous intracellular sulfur granules (inset).

samples were transferred into 100% LR White resin, passed through three changes of resin (2 h each), then left overnight in fresh 100% resin. Samples were then transferred to flat transparent polyethylene molds filled with pure resin, where they

were carefully oriented under stereomicroscope control by moving the specimens inside the cavity using a dissecting needle. No special lid or others equipment was added to protect against oxygen during polymerization. Oriented samples were



polymerized at 37 °C for 48 h in a small embedding oven placed inside a glove box filled with a saturated argon atmosphere ( $O_2 = 0.4$  ppm;  $H_2O = 5.3$  ppm).

As a control for classical *in situ* hybridization experiments, some pieces of bivalve gills were also processed and embedded in paraffin wax in order to provide 4- $\mu$ m-thick standard histological sections.

Semi-thin (1  $\mu$ m thick) or thin (60 nm thick) sections were obtained from the resin blocks using an ultramicrotome Ultracut E (Leica). Semi-thin sections were used either for hybridization experiments or histological staining using toluidine blue in 1% borax buffer. Thin sections were contrasted for 30 min in 2% aqueous uranyl acetate and 10 min in 0.1% lead citrate before examination using a Philips 201 transmission electron microscope at 75 kV.

### Fluorescent *in situ* hybridization experiments

The oligonucleotide probe Eub338 (5'-GCTGC-CTCCCGTAGGAGT-3'), directed against rRNA, was used for this study. It targets most members of the eubacteria (Amann et al., 1990). This probe was used either as Cyanine3- or horseradish peroxidase (HRP)-labeled probe. Oligonucleotidic *in situ* hybridization experiments were similar to those previously described by Dubilier et al. (1995) while CARD-FISH hybridizations were done accordingly to Pernthaler et al. (2001, 2002) using FITC-labeled tyramide, both without special modification related to the hydrophilic resin used. As there are fewer ribosomes inside a semi-thin section compared to a 4- $\mu$ m-thick paraffin wax section, we recommend using HRP-labeled probes (CARD-FISH experiment) rather than fluorochrome-labeled probes (FISH experiment) to provide a stronger signal. *In situ* hybridization of microbial cells with HRP-labeled oligonucleotide probes and catalyzed fluorescent reporter deposition (known as CARD-FISH) is a useful technique for quantification of bacteria in aquatic environments (Pernthaler et al., 2001, 2002; Sekar et al., 2004). The fluorescence intensity of hybridized cells after CARD-FISH is up to 20-fold higher than after FISH with monolabeled fluorescent probes (Schönhuber et al., 1997). Thus, this technique allows visualization of bacterial cells with low ribosomal content that could not be detected with FISH. CARD-FISH has also been used for bacterial detection in slides of eukaryotic tissues embedded in paraffin wax in studies of symbiotic or parasitic relationships (Pernice et al., 2007).

### Results and discussion

Each embedding assay was successful using this easy protocol for flat embedding of oriented samples. Few disturbances were observed in the orientation of very small samples when the vacuum was broken too quickly during gaseous exchange with the intermediate lock chamber of the glove box. Successful embedding was also obtained with a vacuum oven connected to an argon source (results not shown). However in this case, the parameters of the atmosphere during embedding were not controlled.

Hybridization experiments were successful using 0.5–2- $\mu$ m-thick semi-thin sections. We typically used 1- $\mu$ m-thick sections during this investigation. For gill filaments (illustrated in Figures 1A–C), only the lateral zone is positively hybridized while the ciliated zone, which is devoid of bacteria, remains negative. These results are consistent with the ultrastructural data previously published on this tropical lucinid (Frenkiel and Mouëza, 1995). All the bacteriocytes through the lateral zone contain numerous intracellular bacteria and the low thickness of the semi-thin sections (Figure 1C) reduces the green background usually observed with paraffin wax sections (Figure 1B) improving the quality of the observation. Consequently, using a conventional epifluorescence microscope, bacteria can be individually distinguished inside each bacteriocyte (Figure 1C). The symbiont distribution inside the bacteriocyte is also available with a gradient of bacterial cell size from the apical pole (with a majority of small bacteria) to the basal pole (filled with larger bacteria) of the host cell. This coexistence of small and large bacterial cells inside the gill of *C. orbicularis* was recently confirmed by flow cytometry analysis (Caro et al., 2007), therefore, it is not due to a particular section orientation. Such information is not available when using hybridization with paraffin wax sections due to the high background (Figure 1B). Usually, only the use of a laser confocal microscope can sufficiently reduce such background.

With very small samples, such as free-living nematodes (Figures 1E–F) or protozoa (not shown), the interest of using this embedding protocol is increased by the fact that both analyses (molecular *in situ* hybridization experiments and ultrastructural observations) can be carried out from a single-oriented individual. In paraffin wax sections, small organisms (i.e. protozoans) are very difficult to observe as it is hard to locate them through the block and discern their structural organization. By using semi-thin sections for hybridization, the internal organization can be observed with toluidine blue



staining (Figure 1F) and bacterial ectosymbionts can be located on the surface of the stilbonematid nematode (Figures 1E–F) after *in situ* hybridization. TEM observations of thin sections from the same individual clearly show the bacteria located on the cuticle of the nematode (Figure 1G). Such small bacteria (smaller than those observed from the gill of the bivalve *C. orbicularis*) also possess numerous sulfur granules in their cytoplasm (Figure 1G inset) according to their cytological aspects. Thus, combined analyses (TEM and *in situ* hybridization) can be done from a single individual avoiding potential bias observations due to the use of several individuals. However, when using very small samples (for example, protozoa) pre-embedding positioning can be considered as secondary compared to the possibility to locate the samples during embedding and sectioning processes. In such cases, samples can be embedded using preshaped moulds of polyethylene with a hinged lid closure commonly used for biological preparation (i.e. BEEM capsules), but under argon-saturated atmosphere, without orientation.

Moreover, LR White sections can also be coupled with chemical analyses such as Raman spectroscopy (Pasteris et al., 2001), EDX analyses using STEM (Liberge et al., 2001), Nano-SIMS (Audinot et al., 2004), and/or MIMS (Kleinfeld et al., 2004; Peteranderl and Lechene, 2004) complementing the information obtained from a specific individual. Such analytic techniques can reveal important data on the chemical content of a single-studied organism with regard to heavy metals, isotope molecules (Kleinfeld et al., 2004), spatial identification of labeled-protein molecules, etc., and open a large field of analysis with an easy embedding method.

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