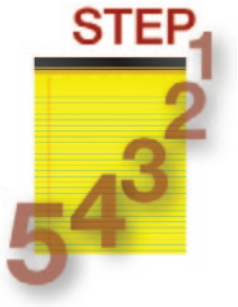
p-Phenylenediamine: An Adjunct to and a Substitute for Osmium Tetroxide



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p-Phenylenediamine (PPD) has been used to retard photobleaching in fluorescence microscopy, to increase the intensity of osmium staining [1, 2], and to improve lipid preservation [3, 4]. Phend et al. [5] used PPD to improve post-embedding immunolocalization in epoxy-embedded specimens; more recently, Brorson et al. [6] recommended including 1% (wt/vol) PPD in all dehydration steps for post-embedding immunolocalization. Experience with a number of animal tissues and microorganisms has resulted in the following protocol, which has been used to improve post-embedding immunolocalization in specimens embedded in either epoxy resins or acrylic resins such as LR White and LR Gold.

1. Fixation: In a properly functioning fume hood, fix specimens for 1 hour in freshly prepared 4% (wt/vol) paraformaldehyde-0.5% (vol/vol) glutaraldehyde in 0.1 M PIPES or HEPES buffer, pH 7.2–7.4 at room temperature. For microorganisms, 2.5–5% (vol/vol) acrolein in the same buffers or growth medium can be used. (Safety alert: If acrolein (tear gas) is used, potassium disulfite should be on hand to neutralize the used fixative and for use if there are accidental spills.) All subsequent steps until 100% ethanol (ETOH) should be done in an ice bath on a shaker with slow agitation.

2. Buffer Washes: Wash the specimens 4 × 15 minutes with cold (on an ice bath) 0.1 M buffer (the same buffer as used above) containing 0.5–1.0% (vol/vol) dimethylsulfoxide (DMSO). The specimen can then be kept in cold buffer without DMSO in the refrigerator overnight if necessary. Wash specimens 2 × 15 minutes in cold buffer plus 0.1 M glycine to remove any unbound aldehydes. Then rinse with cold buffer without glycine for 15 minutes.

3. Tannic Acid Treatment: Incubate the specimens for one hour in 1% (wt/vol) tannic acid in 0.1 M sodium maleate buffer, pH 6.0, followed by two quick rinses with 0.1 M sodium maleate buffer. Some protocols use 1% (wt/vol) tannic acid in the primary fixative, and thus the incubation in tannic acid in sodium maleate buffer is not necessary.

4. Dehydration: Dehydrate in 40% and 60% (vol/vol) ETOH that contains 1% PPD (wt/vol) for 15 minutes each and then incubate overnight in freshly prepared 1% (wt/vol) PPD in 70% ethanol. Continue the next day with further dehydration in 80% and 95% ethanol that contains 1% (wt/vol) PPD. Dehydration can be stopped here or continued to 100% ethanol containing 1% PPD.

5. Resin Infiltration: Specimens can then be infiltrated with the appropriate embedding medium. If epoxy resins are used and propylene oxide is used as the transitional solvent, 1% (wt/vol) PPD should be included in all propylene oxide containing steps. Low-viscosity epoxy resins based on the corrected Spurr formulation [7] or any straight-chain epoxy-resin formulation have been used successfully. Epoxy resins containing Araldites 502, 506, or 6005 are not recommended because the labeling efficiency is still very poor as a result of the benzene ring structure of these epoxies. If LR White acrylic resin is used, infiltration should be continued on an ice bath on a shaker for 30 minutes for each infiltration step (2 parts 95% ETOH + PPD: 1 part LR White; 1 part 95% ETOH + PPD: 1 part LR White; 1 part 95% ETOH + PPD: 2 parts LR White; three changes of pure LR White for 30 minutes each followed by a fourth change overnight). The usual precautions to exclude oxygen from the acrylic embedding medium by bubbling dry nitrogen or argon through an aliquot of the embedding medium should be done to ensure uniform polymerization of the specimen block.

PPD (Sigma catalog No. P-6001), DMSO, and sodium maleate were all purchased from Sigma, St. Louis, MO. (Safety alert: PPD is potentially allergenic and care should be taken to avoid skin or other contact with this compound.)

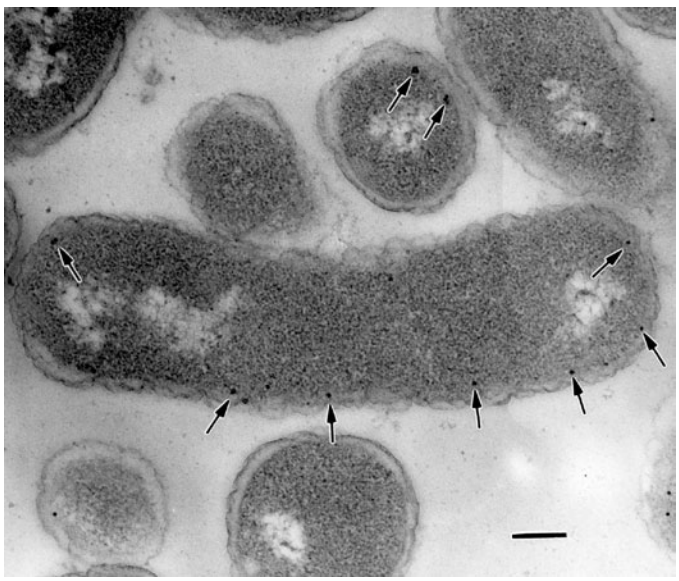

Figure 1: Immunolocalization of chemotaxis protein *tsr* in *Eschericia coli* with 12-nm colloidal gold (arrows) labeled secondary antibodies. Scale bar equals 200 nm.

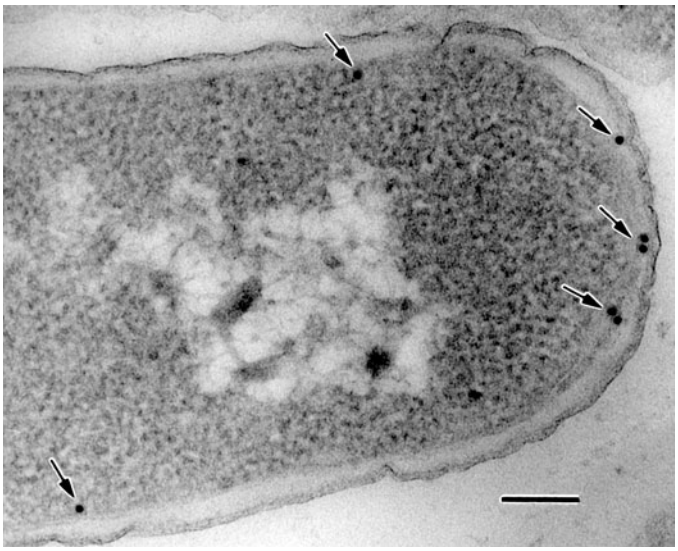

Figure 2: Higher magnification of localization of chemotaxis protein *tsr* in *E. coli* with 12-nm colloidal gold (arrows) labeled secondary antibodies. Scale bar equals 100 nm.

Results

Sections of specimens embedded in epoxy resins require oxidation with periodic acid in the immunolabeling protocol, whereas sections from specimens embedded in acrylic resins usually do not require oxidation. Overall, the antigenic preservation and immunolabeling efficiency is greatly improved by use of this protocol. In addition, the morphological preservation is improved to the extent that specimens appear similar in contrast to those post-fixed with osmium tetroxide. Use of 1% PPD during the dehydration of cell cultures permits visualization of the cell cultures after osmication without the use of a dissecting microscope to locate areas of high cell density. Figures 1 and 2 show specimens that were fixed in buffered acrolein with 1% (wt/vol) tannic acid, dehydrated and infiltrated with 1% (wt/vol) PPD in all the ETOH containing steps and embedded in LR White followed by polymerization in gelatin capsules at 50–55 C. Improved immunolocalization has been accomplished using conventional bench methods for specimen processing and labeling as well as with cold microwave assisted protocols.

References

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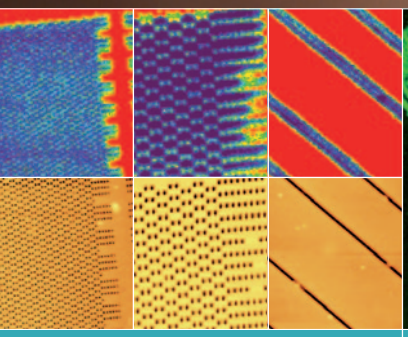
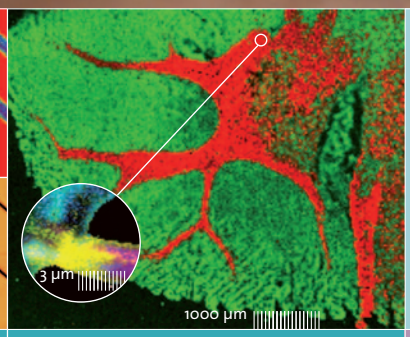
[7] EA Ellis, *Microscopy Today* 14(4) (2006) 32.
[8] Appreciation is expressed to Dr. Rosemary McAndrew for use of the micrographs in Figures 1 and 2.

MT

Confocal Raman Imaging Atomic Force Microscopy



Raman stress and AFM topography images recorded automated at three different areas of a Si-device.



Raman large area scan of a hamster brain cross-section and high-resolution zoom-in Raman image at the marked area.

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