BD Biosciences

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# Preparation of BD Falcon™ Cell Culture Inserts for Transmission Electron Microscopy

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## Introduction

BD Falcon™ Cell Culture Inserts (Cat. Nos. 353090 and 353095) feature smooth, transparent, microporous membranes that provide an excellent substrate for cell culture. For most histological procedures, the culture inserts can be processed intact, using standard techniques, by passing them through a series of fixation and staining solutions. The membrane, which offers excellent chemical resistance to organic solvents, can be easily cut with a razor blade or scalpel in order to remove samples for embedding, sectioning or staining. BD Falcon Cell Culture Inserts are highly recommended for both transmission and scanning electron microscopy (SEM) procedures. We recommend the following procedure for the preparation of BD Falcon Cell Culture membrane Inserts for transmission electron microscopy (TEM).

## Materials

- Monobasic potassium phosphate $KH_2PO_4$
- Dibasic sodium phosphate $NA_2HPO_4•2H_2O$
- Monobasic sodium phosphate $NaH_2PO_4•H_2O$
- Sodium Hydroxide NaOH
- Sodium citrate $Na_3C_6H_5O_7•2H_2O$
- Calcium Chloride $CaCl_2$
- Reagent grade glucose and sucrose
- Dulbecco's phosphate buffered saline (PBS, with calcium and magnesium salts)
- Toluidine blue stain
- Glutaraldehyde (e.g., Polysciences, Inc. #18428, 50%, E.M. Grade)
- Osmium tetroxide (e.g., Polysciences, Inc. #223C, 0.5 g ampules)
- Uranyl acetate (e.g., Polysciences, Inc. #0379)
- Propylene oxide (e.g., Polysciences, Inc. #0236, E.M. Grade)
- Lead nitrate (e.g., Polysciences, Inc. #0785)
- Spurr non-aqueous embedding medium (e.g., Polysciences, Inc. #1916, Spurr embedding kit, four components), ***or***
- Water-miscible embedding medium (e.g., Polysciences, Inc. #15923, Lowicryl K4M embedding kit, or #18272, Nanoplast embedding kit)

### Fixation and En Bloc Staining

**Steps 1-6 are done at 4°C.**

1. Remove culture medium and gently rinse insert with Dulbecco's phosphate buffered saline (PBS + $Ca^{++}$, $Mg^{++}$, pH 7.4). Add PBS to both insert and well and allow to stand for one to two minutes. Repeat wash with PBS.
2. Remove PBS and replace with primary glutaraldehyde fixative: 0.1 M phosphate buffer, 2% glutaraldehyde, pH 7.4, 470 mos (osmolality). Add fixative to insert and well and allow fixation to proceed overnight at 4°C. The glutaraldehyde fixative (500 ml) is prepared by mixing 195 ml of 0.2 M $Na_2HPO_4•2H_2O$, 11 ml of 1.0 M $KH_2PO_4$, 20 ml of 50% glutaraldehyde and 274 ml distilled water.
3. Remove primary fixative and wash insert with phosphate buffered sucrose: 0.1 M phosphate buffer, 0.22 M sucrose, pH 7.4, 450 mos (osmolality). Add buffered sucrose to insert and well and allow to stand for five minutes. Repeat wash with phosphate buffered sucrose (five minutes). Phosphate buffered sucrose (500 ml) is prepared by dissolving 37.5 g sucrose in a mixture of 195 ml of 0.2 M $Na_2HPO_4•2H_2O$, 11 ml of 1.0 M $KH_2PO_4$ and 294 ml distilled $H_2O$.

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*Note: Omit Step 4 (secondary osmium fixation) if specimen is to be used for immunocytochemistry or if specimen is to be embedded in Nanoplast melamine resin or any resin that requires UV light for polymerization.*

4. Remove phosphate buffered sucrose and replace it with Millonig's phosphate buffered osmium (1%) fixative: 0.22 M disodium phosphate buffer, 0.03 M glucose, 0.45 mM $CaCl_2$, 1% osmium tetroxide, pH 7.3.[3] Add Millonig's fixative to both insert and well and allow secondary fixation to proceed (under a fume hood) for two hours. To prepare Millonig's phosphate buffered osmium fixative (50 ml), first mix together 41.5 ml of 0.164 M (2.26%) monosodium phosphate and 8.5 ml of 0.63 M (2.52%) sodium hydroxide. Remove 5 ml of this solution and test the pH, which should be 7.3, if properly prepared. To the remaining 45 ml add 5.0 ml of 0.3 M (5.4%) glucose and 0.25 ml of 0.09 M (1.0%) calcium chloride, which will result in 50 ml of vehicle solution. Dissolve 0.5 g of osmium tetroxide in this vehicle solution to make the final 1% Millonig's fixative.[1,3]

5. Following secondary osmium fixation, wash the insert for 10 minutes in high-quality distilled water; repeat the 10-minute wash with distilled $H_2O$ twice (total 3 x 10-minute washes).

   *Note: This step (en bloc staining with uranyl acetate) is optional, and should be omitted if there is a possibility that it may interfere with other planned procedures (e.g., it is incompatible with preservation and localization of glycogen particles).*

6. Block stain the intact insert in 1% uranyl acetate for one hour to enhance contrast. Uranyl acetate (1%) should be prepared fresh daily in distilled water; add stain to both well and insert as usual.

## Dehydration Procedure

Steps 7-9 are done at room temperature.

7. Following block staining with uranyl acetate allow the insert to come to room temperature and wash the insert twice for 10 minutes in distilled $H_2O$ (2 x 10-minute washes).

8. Perform the initial dehydration of the intact insert by sequentially immersing it in the following ethanol solutions made up with distilled water (fill well and insert, as usual):
   - 50% ethanol for 10 minutes
   - 95% ethanol for 10 minutes
   - 95% ethanol for 10 minutes
   - 100% ethanol for 15 minutes
   - 100% ethanol for 15 minutes

9. Using a scalpel, remove the flexible membrane from the base of the intact insert. Cut it with fine scissors or a scalpel into the individual specimens that will be embedded. Work rapidly and keep the membrane and individual specimens saturated with ethanol during this manipulation. Do not allow them to air dry during this dissection. Transfer the small membrane specimens to glass vials containing propylene oxide (100%) and continue the dehydration and clearing process that was interrupted to remove the membrane:
   - 100% propylene oxide for 15 minutes
   - 100% propylene oxide for 15 minutes

   It is also possible to complete the dehydration, clearing, and embedding of the membrane with the insert intact, since the insert housing (molded from PET, poly-ethylene terephthalate) is also quite chemically resistant. In this case carry out the propylene oxide clearing and embedding with the insert in a glass dish or other chemically resistant vessel, since propylene oxide will dissolve polystyrene multiwell plates.

## Embedding in Non-Aqueous Resin

10. Follow the instructions that come with the specific embedding kit (e.g., Spurr, Polysciences, Inc.) to prepare the liquid embedding medium. We routinely infiltrate with Spurr by transferring the specimen from 100% propylene oxide to a mixture of ½ propylene oxide, ½ Spurr:
    - 50% propylene oxide, 50% Spurr for 30 minutes

11. Place the individual membrane specimens in 100% Spurr embedding medium in BEEM capsules, and transfer the capsules to a vacuum oven.

12. Evacuate the oven to 25 mm Hg for 30 minutes. Cure the embedded samples in the oven overnight at 65ºC. The cured resin blocks can then be removed from the capsules and sectioned. The blocks should be hard and able to be trimmed and sectioned easily without crumbling.

## Sectioning and Counterstaining

13. Rough cut the block to form a pyramid having the side of the membrane of interest at the apex, or having the membrane oriented perpendicular to the plane of the apex (for sectioning through the membrane, if this is desired).

14. If necessary, cut one micron thick sections on ultratome or pyramatome for selection of regions of interest. Place the thick sections on a drop of water on a glass microscope slide and allow to dry at 25-100ºC. Stain with 1% toluidine blue in distilled water on hot plate for three minutes, and examine in the light microscope.

15. Cut thin sections on the ultratome at 80 nm or less at high speed and mount them on copper or nickel grids.

16. Counterstain the sections with 1% uranyl acetate (prepared fresh), and then with Reynolds' lead citrate formulation using standard methods.[4]

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## Safety Precautions

Many, if not most, of the chemicals used in electron microscopy are potentially hazardous.[2] Glutaraldehyde and osmium tetroxide are toxic and must be handled with standard precautions in a fume hood. Glutaraldehyde and osmium fixative solutions have volatile toxic components and should be dispensed under a fume hood. Propylene oxide is flammable and potentially carcinogenic and must be used in a fume hood. The lead and uranium salts used for staining are hazardous; extreme caution must be taken to avoid contact with uranyl acetate in its powdered crystalline form, since it is highly toxic, both chemically and radiologically.

Many of the components of the standard embedding resins are hazardous. For example, the VCD (ERL 2406) component of the Spurr mixture is carcinogenic, and Nanoplast may release free formaldehyde during curing. All unpolymerized resins should be manipulated in fume hoods. The resins also emit considerable vapor during polymerization. Curing ovens should be vented to the outside. In summary, fume hoods and gloves should be used extensively when preparing and using materials for EM.

## Discussion

Good results may also be obtained by embedding the transparent membrane from the BD Falcon™ Cell Culture Insert in a water-miscible embedding medium (e.g., Nanoplast melamine resin, Lowicryl K4M, etc.). Some resins (e.g., Nanoplast melamine) require no dehydration following fixation. Many water-miscible embedding media (e.g., Lowicryl K4M) can be used to infiltrate intact inserts directly from 100% ethanol. Infiltration with water miscible media may be accomplished with the insert and membrane intact, or the membrane may be removed from the insert after dehydration and cut into small samples for embedding.

Some resins (e.g., Lowicryl K4M) are designed for UV polymerization, which is the method of choice if osmium staining has been omitted. It is usually also possible to polymerize these resins with a more classical thermal technique (e.g., 60ºC) if the osmium stain has been used. Follow the instructions that come with the specific embedding kit.

### References


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