Technical Bulletin #459

Preparation of BD Falcon™ Cell Culture Inserts for Confocal Indirect Immunofluorescence: Fixation and Staining of Caco-2/bbe (C2) Cells with Various Dyes

[1]*Mark W. Musch, Ph.D., Margaret Mary Walsh-Reitz, Ph.D.*, [2]*Marshall Kosovsky, Ph.D.*,
[1]*The Martin Boyer Laboratories, Inflammatory Bowel Disease Research Center, Department of Medicine, The University of Chicago, Chicago, IL;* [2]***BD Biosciences** – Discovery Labware, Billerica, MA*

Introduction

BD Falcon™ Cell Culture Inserts (Cat. Nos. 353090 and 353095) feature smooth, transparent, microporous membranes that provide an ideal substrate for culturing a wide variety of cell types. For most histological procedures, the cell 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 transmission and scanning electron microscopy procedures. BD Biosciences recommends the following procedure for the preparation of BD Falcon Cell Culture Inserts for confocal indirect immunofluorescence.

Materials and Methods

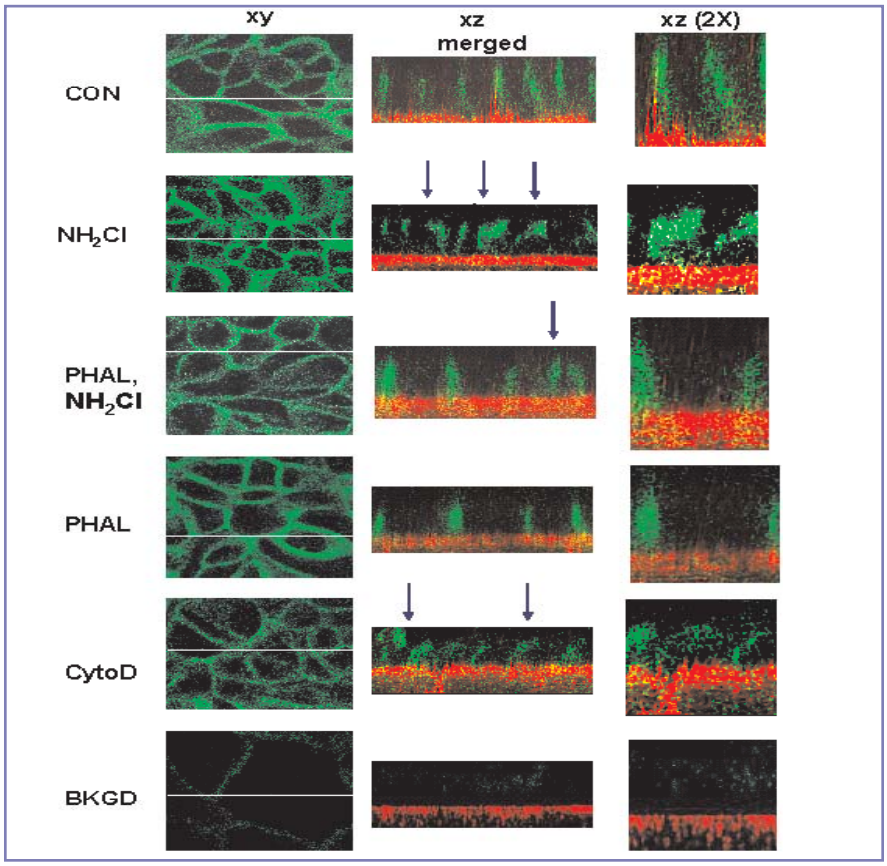
Solutions

- Prepare the following formulations of PBS:
  1. PBS mixture (1 mM $CaCl_2$ and 1 mM $MgCl_2$). *Include these cations in all PBS buffers. These cations ensure that cells do not lift off when tight junctions loosen, which occurs in the absence of divalent cations.*
  2. PBS/TRITON mixture (1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.2% (v/v) TRITON™ X-100)
  3. PBS/BSA mixture (1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1% BSA)
- K-PIPES fixation buffer (80 mM K-PIPES, 5 mM EDTA, 2 mM $MgCl_2$, pH = 6.5)
- $NaBO_4$ fixation buffer (100 mM $NaBO_4$, pH = 11.0)
- N-propyl gallate(npg): dissolve 500 mg of npg in 7 ml gylcenol (***do not use heat***). Increase volume to 10 ml with PBS. Add 163 mg of Tris base to ensure that ph is >8.5 (additional Tris base can be added if necessary). Store at -20°C in dark.

Protocol

1. Cells can be grown on square glass Coverslips or on BD Falcon Cell Culture Inserts containing polyethylene terephthalate (PET) membranes (Cat. No. 353090). Coverslips must be sterilized and cleaned before use, preferably by dipping in 100% ethanol and flaming in the hood, or by autoclaving. All the volumes, except as noted, are 2 ml per well in 6-well plates. ***NOTE:** If using 6-well BD Falcon Cell Culture Inserts (Cat. No. 353090), use 1.5 ml in the apical chamber (within the insert) and 2 ml in the basolateral chamber for all incubations. When using cell culture inserts, add the buffer to the apical and basolateral sides for all incubations.* Perform required experimental treatment(s) of the cells.
2. Aspirate the media and then add K-PIPES buffer containing 3.75% formaldehyde at RT (use 1:4 dilution of 16% paraformaldehyde from EM Sciences). Incubate for 5 minutes at RT.
3. Aspirate K-PIPES containing formaldehyde. Add $NaBO_4$ buffer containing 3.75% formaldehyde. Rotate gently and fix for 10 minutes at RT.
4. Aspirate and wash with PBS. Replace with fresh PBS and incubate for 5 minutes at RT.
5. Aspirate and then permeabilize by adding PBS/TRITON. Incubate 15 minutes with gentle rotation at RT.
6. Aspirate PBS/TRITON and replace with blocking solution comprised of PBS/BSA. Perform three incubations/washes (5 minutes each). Add the appropriate dilution of primary antibody to the top (apical) and underside (basolateral) of the cell culture insert membrane in the following manner:
   - Cut a circle of parafilm to fit the well (two pieces will be needed for each well).
   - Place 50 µl of primary antibody diluted in PBS/BSA onto the top surface of the membrane and cover with a parafilm circle.
   - Turn the insert over and repeat for the underside of the membrane.
   - Add 50 µl of diluted primary antibody to the underside of the membrane, and cover with a parafilm circle. In the vast majority of cases, the parafilm covers will remain in place.
   - Keep in a humidified box at 4°C overnight.
7. Remove the parafilm circles and wash both sides of the membrane 3x with PBS at RT. For each wash, wash BOTH sides of the membrane, and incubate for at least 5 minutes at RT.
8. Block the membrane with PBS containing a species appropriate serum corresponding to the species used to generate the secondary antibody. Dilute the secondary antibody approximately 1:1000 in PBS and place on the apical and basolateral sides. Incubate for 60 minutes at RT.

9. Wash 3x for 5 minutes with PBS. Add appropriate dilution of secondary antibody (can start with 1:1000), usually conjugated to either a Cy3 or Alexa dyes, and place 50 µl on apical and basolateral sides. Cover with the parafilm circles (which can be reused) and incubate for 60 minutes at RT. ***NOTE:*** *If needed, a 1:1000 dilution of FITC or Rhodamine-conjugated phalloidin can be added at this time to stain microfilaments.*
10. Wash the membranes thoroughly with PBS. Perform at least three washes for 5 minutes each (additional washes may be required for some antibodies).
11. Place four small drops of nail polish on a glass slide to mark the location corresponding to the corners of a square No. 1 Coverslip. Remove the PET membrane from the insert housing with a razor blade or scalpel. Cut the edges off to prepare a square cut piece of membrane that is smaller than the square No. 1 Coverslip. Place the square cut membrane in the middle of the four spots of nail polish, cell side up, and place 38 µl of a solution of 50 mg/ml n-propyl gallate directly onto the membrane. Place a square No. 1 Coverslip over the membrane to seal the area. Remove excess propyl gallate/glycerol from the sides, and then use nail polish to seal all of the edges. Allow the nail polish to dry at RT, keeping the slides in a dark box at 4°C. Perform imaging analysis as soon as possible.



Results

Caco-2/bbc (C2) cells were analyzed using confocal imaging to assess distribution of the α-subunit of Na^+-K^+-ATPase after exposure to NH_2Cl and the actin destabilizer cytochalasin D.[1] The images presented in ***Figure 1***, are en face (*xy*) images with the α-subunit of Na^+-K^+-ATPase given the pseudocolor green. The red color present in some panels is transmitted reflected light used to monitor monolayer integrity. As can be observed, a large majority of the ATPase subunit appears at the outer plasma membrane (***Figure 1***). To observe the α-subunit on the lateral, basal, and apical aspects of the cells, *xz* axes were chosen so that the data was selected as shaving the sides of a cylindrical-shaped cell and is denoted by a line through the *xy* images. This ATPase is not localized to the apical pole in untreated monolayers; however, after NH_2Cl treatment, green staining appeared at the apical pole (***Figure 1*** middle and right).

Figure 1*. Confocal micrographs of Cy2-tagged α-subunit of Na^+-K^+-ATPase in C2 cells. The cells were treated for 60 minutes with 0.6 mM NH_2Cl alone or after 1-hour treatment with phalloidin (100 µM) or with cytochalasin D (2 µM). The red color is transmitted reflected light used to observe monolayer integrity. Apical slices are shown in the* ***left****. Vertical stack slices (xz) from regions marked with lines are shown in the* ***middle*** *and* ***right****, and a portion of this is enlarged (2x) to the* ***right****. White dashed lines have been included as representative xz reconstructions (n>4 from each panel) obtained from the series of xy slices (0.2 µm apart). Arrows are indicated on apical membranes of monolayers to indicate the apical movement of the α-subunit.*

BD Biosciences
Two Oak Park
Bedford, MA 01730 USA
tel: 877.232.8995
fax: 800.325.9637

BD
Akasaka Garden City,
Akasaka 4-15-1, Minato-ku,
Tokyo, 107-0052 Japan
tel: (81) 24 593 5405
fax: (81) 24 593 5761

BD Biosciences
Erembodegem-Dorp 86
9320 Erembodegem, Belgium
tel: (32) 53 720 211
fax: (32) 53 720 450
contact_bdb@europe.bd.com

BD
2280 Argentia Road
Mississauga, Ontario
Canada L5N 6H8
tel: 866.979.9408
fax: 800.565.0897

BD Biosciences
Singapore Branch
30 Tuas Avenue 2
Singapore 639461
tel: (65) 6861 0633
fax: (65) 6860 1590

BD Biosciences
4 Research Park Drive
Macquarie University Research Park
North Ryde NSW 2113 Australia
tel: (800) 656 100
fax: (612) 8875 7200
aus_customerservice@bd.com

References

1. Musch, M.W., Walsh-Reitz, M.M., and Chang, E.B. Roles of ZO-1, occludin, and actin in oxidant-induced barrier disruption. *Am J Physiol - Gastrointest. Liver Physiol.* **290**:222 (2006).