Technical Note

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SECTION I

Introduction:

Millicell Culture Plate Filter Inserts are a valuable cell biology tool designed to improve cell attachment, growth and differentiation over traditional plastic cell culture plates. Millipore has developed practical techniques for viewing, counting and analyzing cells grown on Millicell inserts. Millicell Culture Plate Inserts promote a more natural cell growth by allowing media access from both the apical and basolateral sides of the membrane. This allows for cell growth, structure and function to more closely mimic what occurs *in vivo*.

The following Technical Guide outlines the types of microporous membranes available in the Millicell Inserts and their recommended applications. Additional information is provided regarding various aspects of Millicell Insert use. See Index on page 2.

Most of the Millicell membrane types are available in a MultiScreen 96 well plate format for high throughput cell based applications. Procedures optimized in the single Millicell unit may be effectively transferred to a 96 well plate format.

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SECTION I

Membrane Selection Guide - by Application

Inset Type	Organotypic	HA	CM	PCF	PC
Embryo Slices	***		**		
Hippocampal Slices	***		**		
Tumor Cell Metastasis				***	
Transport Studies			*	***	**
Co-Culture		**	*	***	**
In-Vitro Toxicology	*	*	***	***	**
Chemotaxis				***	***
Permeability				***	*
Suspension Cells		*	**	**	***
Cell Surface Receptors		**	***	***	**
Polarized Uptake		**	***	***	
Electrophysiology	**	**	**	**	
Polarized Protein Secretions		*	***	**	
Fluorescent			***	***	***

*** Optimal
** Recommended
* Acceptable

<u>Definitions/Abbreviations</u>:
DMEM - Dulbecco's Modified Eagle Media
ECM - Extracellular Coating Matrix
MDCK Cells – Madin Darby Canine Kidney Cells ATCC #34

Membrane Selection by Physical/Chemical Properties

Membrane Type	Advantages	Limits
HA **[Mixed Cellulose Esters (MCE)]**	No ECM coating necessary – ability to direct seed High Pore Density Well referenced in literature	High Protein binding Slow transport across membrane Requires staining for viewing
Biopore CM and Organotypic **(Hydrophilic PTFE)**	Transparent for clear visualization of cells with phase contrast microscopy Extremely low protein binding Inert Low fluorescing	Requires ECM for attachment dependent cells (Organotypic applications **do not** require ECM)
PC **(Track-etched Polycarbonate w/PVP)**	Very thin, allowing for fast equilibrium across membrane Does not need coating Low protein binding For suspension cells	Lower pore density than HA or CM Not microscopically transparent Must be stained for viewing Contains PVP wetting agent
PCF **(Track-etched Polycarbonate, tissue culture treated)**	Very thin, allowing for fast equilibrium across membrane Does not need coating Contains no wetting agents (can be used with suspension and adherent cell lines) Low protein binding Tissue culture treated for attachment cells Low fluorescing	Lower pore density than HA or CM Not microscopically transparent Must be stained for viewing

*Note: An Extracellular Matrix (ECM) is only required for CM, but can be applied to all other Millicell inserts if desired. The coating procedures found in Section II describe four coatings that can be applied to Millicell inserts.

Millicell Specifications Chart

Membrane Type	HA [Mixed Cellulose Ester (MCE)]	CM Biopore [PTFE (Teflon)]	Organotypic (Low Height) [PTFE (Teflon)]	PCF PVP free (Polycarbonate, Tissue Culture Treated)	PC Polycarbonate
Pore sizes (µm)	0.45	0.4	0.4	0.4 3.0 8.0 12.0	0.4 3.0
Sterilization Mode	Ethylene Oxide	Ethylene Oxide	Ethylene Oxide	Gamma Irradiated	Gamma Irradiated
Available Diameters	12mm 30mm	12mm 30mm	30 mm	12mm 30mm (0.4 µm only)	12 mm 30mm (3.0 µm only)
Surface Area	0.6cm^2 4.2 cm^2	0.6 cm^2 4.2 cm^2	4.2 cm^2	0.6cm^2	0.6cm^2
Typical Protein Binding Capacity µg/IgG/12mm device	320	40	40	0.4 µm =50 3.0 µm =30	3.0 µm = 50 3.0 µm =30
Fluorescent Properties	High Background Due to Protein Binding	Low Fluorescing	Low Fluorescing	Low Fluorescing	Low Fluorescing
Porosity	~80%	~75%	~75%	~10 %	~10%

SECTION II

COATING PROCEDURES

Recommended ECM Coating Volumes for 12mm and 30 mm Inserts:

Coating	12mm (24 well)	30mm (6 well)
Collagen	50µL Collagen/Ethanol mix	400 µL Collagen/Ethanol mix
Fibronectin*	100 µL Fibronectin/DMEM mix	700 µL Fibronectin/DMEM mix
Laminin*	100 µL Laminin/DMEM mix	700 µL Laminin/DMEM mix
Matrigel™	100 µL Matrigel/H2O solution	700 µL Matrigel/H2O solution

*** Can be used on any Millicell membrane**

(A) Collagen Coating – Type I (Dried Film)

Materials:

- Millicell-CM (12 mm or 30mm) inserts
- 70% Ethanol [filter sterilized with a Millex™-GV filter unit (catalogue number SLGV033RS) or a Stericup™-GV (catalogue number SLGVU11RE)]
- Rat Tail Collagen, Type 1, ~3 mg/ml Sigma, St. Louis, MO*. Handle and store according to manufacturer's instructions.
- Sterile pipette syringe(s) and tip(s) for repetitive 50 µL (12mm inserts) or 400µl (30mm inserts) aliquots

> ***Note:*** *An alternate collagen source (Type I) of suitable concentration diluted in 0.01 N HCl or acetic acid may also be used.*

Method:

1. Dilute the collagen 1:4 in 70% ethanol (1 part collagen and 3 parts 70% ethanol) and vortex until the collagen is solubilized.
2. Place the applicable number of 12mm Millicell inserts into the well(s) of a 24- well cell culture plate. For 30mm inserts use 6-well cell culture plate.
3. Using a sterile pipette syringe add 50µL of the collagen/ethanol mixture to each of the 12 mm Millicell-CM insert(s). For 30mm inserts add 400µl.
4. Gently shake the cell culture plate until the collagen and 70% ethanol mixture evenly coats the inside of the Millicell-CM insert.

5. Air-dry inserts in a laminar flow hood. Leave cell culture plate cover ajar to allow airflow and prevent condensation. This typically takes anywhere from 3 hrs to overnight. Overnight drying is recommended.

6. Seed Millicell-CM insert with appropriate cell density (for example, MDCK cells: 5 x 10^4 to 5 x 10^5 cells/cm^2 in 10% fetal calf serum/DMEM

(B) Fibronectin Coating (Dried Film)

Materials:

- Millicell-CM (12 mm or 30mm) inserts
- DMEM-serum free culture media [filter sterilized with a Millex™-GP filter unit (catalogue number SLGP033RS) or a Stericup™-GP (catalogue number SLGPU11RE)]
- CR-Human Fibronectin, 1 mg/ml Sigma, St. Louis, MO). Reconstitute, handle and store according to manufacturer's instructions.
- Sterile pipette and tip(s) for repetitive 100 µL (12mm inserts) or 700µl (30mm inserts) aliquots

Method

1. Dilute the fibronectin 1:10 in the serum-free DMEM (1 part fibronectin in 9 parts DMEM) and vortex until fibronectin is solubilized.

2. Place the applicable number of 12mm Millicell inserts into the well(s) of a 24-well cell culture plate. For 30mm inserts use 6-well cell culture plate.

3. Using a sterile pipette, add 100µL fibronectin/DMEM coating mixture to each of the 12 mm Millicell-CM insert(s). For 30mm inserts add 700µl.

4. Gently shake the cell culture plate until the fibronectin/DMEM mixture evenly coats the Millicell-CM insert(s).

5. Air dry inserts overnight in a laminar flow hood. Leave cell culture plate cover ajar to allow airflow and prevent condensation.

6. Seed Millicell-CM insert with appropriate cell density (for example, MDCK cells: 5 x 10^4 to 5 x 10^5 cells/cm^2 in 10% fetal calf serum/DMEM

(C) Laminin Coating (Dried Film)

Materials:

- Millicell-CM (12 mm or 30mm) inserts
- DMEM-serum free culture media [filter sterilized with a Millex™-GP filter unit (catalogue number SLGP033RS) or a Stericup™-GP (catalogue number SLGPU11RE)]
- CR-Laminin, 1 mg/ml (Sigma, St. Louis, MO) Handle and store according to manufacturer's instructions.
- Sterile pipette and tip(s) for repetitive 100 µL (12mm inserts) or 700µl (30mm inserts) aliquots

Method:

1. Dilute the CR- Laminin 1:10 in DMEM (1 part laminin in 9 parts DMEM) and vortex until the laminin is solubilized.
2. Place the applicable number of 12mm Millicell inserts into the well(s) of a 24-well cell culture plate. For 30mm inserts use a 6 well cell culture plate.
3. Using a sterile pipette, add 100µL of laminin/DMEM mixture to each of the 12 mm Millicell-CM insert(s).For 30mm inserts add 700µL.
4. Gently shake the cell culture plate until the laminin/DMEM mixture evenly coats the Millicell-CM inserts.
5. Air-dry inserts overnight in a laminar flow hood. Leave the cell culture plate cover ajar to allow airflow and prevent condensation.
6. Seed Millicell-CM insert with appropriate cell density (for example, MDCK cells: 5×10^4 to 5×10^5 cells/cm^2 in 10% fetal calf serum/DMEM

(D) Matrigel ™ Coating (Dried Film)

Materials:

- Millicell-CM (12 mm or 30mm) inserts
- Milli-Q® water [filter sterilized with a Millex-GV unit (catalogue number SLGV033RS) or a Stericup-GV (catalogue number SCGVU11RE)] or serum free culture media
- Basement Membrane Matrigel (Becton Dickinson Life Science, Bedford, MA). Handle on ice, according to manufacturer's instructions

- Sterile pipette and tip(s) for repetitive 100 µL (12mm inserts) or 700µl (30mm inserts) aliquots

Method:

1. Dilute Matrigel according to manufacturers instructions.

2. Place the applicable number of 12mm Millicell inserts into the well(s) of a 24- well cell culture plate. For 30mm inserts use 6 well cell culture plate.

3. Using a sterile pipette syringe, add 100 µl of Matrigel solution to each of the 12mm Millicell inserts. For 30mm inserts, use 700µl. Gently shake the cell culture plate until the Matrigel solution evenly coats the Millicell-CM insert.

4. Air-dry inserts overnight in a laminar flow hood. Leave cell culture plate cover ajar to allow airflow and prevent condensation.

5. Seed Millicell-CM insert with appropriate cell density (for example, MDCK Cells: 5 x 10^4 to 5 x 10^5 cells/cm^2 in 10% fetal calf serum/DMEM

SECTION III

FIXATION AND STAINING PROCEDURES

(A) Toluidine Blue Staining*

Materials:

- Millicell Culture Plate Inserts
- Milli-Q water
- Millex-GV unit, catalogue number SLGV033RS
- Toluidine blue (Sigma, St. Louis, MO)
- 3% glutaraldehyde in Phosphate Buffered Saline (PBS)
- Triton X-100 (Sigma, St. Louis, MO), 0.5%

Method:

1. Prepare a 0.3 % solution (gram percent) of toluidine blue in Milli-Q water, stir, and filter through a Millex filter unit.

2. Remove the Millicell insert from the plate and wash gently with PBS to remove growth media.

3. Fix the cells for 15 minutes with 3% glutaraldehyde in PBS.

4. Rinse gently with Milli-Q water. Repeat twice.

5. Permeabilize the cells with 0.5% Triton X™-100 for 5 mins.

6. Rinse gently with Milli Q water. Repeat twice.

7. Apply stain to the apical cell side of membrane for 30-60 secs.

8. Observe as a wet mount.

******Recommended as a quick and easy stain*

(B) WRIGHT'S STAINING PROCEDURE

Materials:

- Millicell-CM Culture Plate Inserts
- 3% glutaraldehyde in Phosphate Buffered Saline (PBS)
- Milli-Q water
- 100% Methanol
- Wright's stain

Method:

1. Remove the Millicell-CM insert from the plate and wash gently with PBS to remove growth media.

2. Fix the cells for 15 minutes with 3% glutaraldehyde in PBS.

3. Rinse gently with Milli-Q water. Repeat twice.

4. Rinse once with methanol and incubate in fresh methanol for 5 minutes.

5. Aspirate the methanol. Add Wright's stain to cover the inside membrane.
6. Incubate for 30 seconds.
7. Rinse gently with Milli Q water. Repeat three times.
8. Observe as a wet mount.

(C) FIXATION AND HEMATOXYLIN PROTOCOL FOR MILICELL HA

Materials:

- Millicell HA Culture Inserts
- Millex GV Filter Unit (catalogue number SLGV033RS)
- 3% glutaraldehyde in PBS, store at 4°C
- Phosphate Buffered Saline (PBS)
- 0.5% Triton® X-100 (Sigma, St. Louis, MO) in Milli-Q water
- Hematoxylin solution (HHS-1, 7.5 g/L, Sigma, St. Louis, MO)
- Dilute ammonium hydroxide (8-10 drops concentrated ammonium hydroxide in 100 ml of water)
- 0.5% hydrochloric acid in 70% ethanol
- Milli-Q water

Method:

1. Filter hematoxylin solution through a Millex-GV unit and cover the cell layer.
2. Incubate for 15 minutes at room temperature.
3. Rinse the Millicell-HA insert with Milli-Q water to remove stain. Repeat twice.
4. Destain by adding 0.5% hydrochloric acid in 70% ethanol for 2-3 minutes.
5. Rinse with Milli-Q water. Repeat twice.
6. Add dilute ammonium hydroxide into the Millicell-HA insert to cover the membrane.

Incubate for 3 minutes or until a uniform blue color is observed on the membrane.

7. Rinse with Milli-Q water. Repeat twice.

8. Using a cork borer (available through Fisher Scientific, Pittsburgh, PA) remove membrane. Mount membrane on a slide in a commercial mounting medium. Make sure the cell layer is facing the microscope objective.

(D) HEMATOXYLIN PROTOCOL FOR MILLICELL CM

Note: *Standard histological hematoxylin and eosin staining techniques can be performed on thin sections.*

Materials:

- Millicell-CM Culture Plate Inserts
- Millex Filter Unit
- Milli-Q water
- Sterile Phosphate Buffered Saline (PBS)
- 3% glutaraldehyde in PBS, store at 4°C
- Methanol
- 0.5% Triton X™-100 in Milli-Q water
- Hematoxylin solution (Gill No. 1),filter sterilized with a Millex-GV unit (catalogue number SLGV033RS)
- 0.5% hydrochloric acid in 70% ethanol
- Dilute ammonium hydroxide (8-10 drops concentrated ammonium hydroxide in 100 ml of water)

Method:

1. Remove the Millicell-CM insert from the plate and wash gently with PBS to remove growth media.

2. Add 3% glutaraldehyde (at 4°C) in PBS (pH 7.3) to the inside and outside (cell culture plate well) of the Millicell-CM device. Leave for 15 minutes.

3. Carefully remove glutaraldehyde. Add methanol to the inside and outside (cell culture plate well) of the Millicell-CM unit. Leave for 10 minutes.

4. Carefully remove methanol. Add 0.5% Triton X™-100 to the inside and outside (cell culture

plate well) of the Millicell-CM unit. Leave for 5 minutes.

5. Carefully remove Triton X™-100. Add hematoxylin solution (Gill No. 1, Sigma Chemical, filtered with a Millex unit). Leave for 15 minutes.

6. Wash the Millicell-CM insert gently with Milli-Q water. Repeat twice.

7. Add 0.5% hydrochloric acid in 70% ethanol for 45 seconds to remove excess stain.

8. Rinse with Milli-Q water. Repeat twice.

9. Add diluted ammonium hydroxide and leave for approximately 45 seconds.

10. Rinse with Milli-Q water. Repeat twice.

11. Store wet or mount for microscopy.

(E) Hema-3 Quick Stain for Millicell-PCF

The Hema-3 stain kit is a quick staining procedure that can be used with the Millicell PCF cell culture insert. The kit is available through Fisher scientific (catalogue number 23-123869). Please contact the manufacturer for more information.

SECTION IV

GENERAL IMMUNOFLUORESCENT PROTOCOL FOR MILLICELL CM/PC/PCF

Principle: Immunofluoresence is a technique employing fluorescently labeled antibody, by which cells can be localized, labeled and examined via fluorescent microscopy.

Materials*:

Millicell-CM Culture Plate Inserts
Sterile Phosphate Buffer Saline (PBS)
Methanol, 100%
Glycerol
FITC-conjugated antibody (Sigma Chemical Co.)
1% BSA in PBS
Glass slides
Fluorescent microscope

***Storage and Product Stability:** Store all assay solutions according to manufacturer's recommendations.

Method:

1. Remove Millicell-CM insert from plate and wash gently with PBS to remove media.

2. Fix in methanol at 4°C for 10 minutes.

3. Wash with PBS.

4. Incubate the Millicell-CM insert(s) in 1% BSA/PBS at room temperature for 5 minutes.

5. Add appropriate dilution(s) of primary antibody and controls.

6. Incubate at 37°C in a moist chamber for 45 minutes to 1 hour.

7. Wash with PBS. Repeat two to three times.

8. Incubate the Millicell-CM insert(s) in 1% BSA/PBS at room temperature for 5 minutes

9. Add appropriate dilution of FITC-conjugated secondary antibody in 1% BSA/PBS (a 1:250 dilution if Sigma Chemical Co. conjugate is used).

10. Incubate at 37°C in a moist chamber for 45 minutes to 1 hour.

11. Wash with PBS. Repeat three times.

12. Remove excess moisture from Millicell-CM unit.

13. Using a cork borer (available from Fisher Scientific, Philidelphia, PA) cut out the membrane.

14. Mount the membrane on a glass slide with a 90% glycerol/10% PBS solution.

15. Read under fluorescent microscope in a dark room.

SECTION V

SODIUM FLUORESCEIN PERMEABILITY ASSAY

Sodium Fluorescein Permeability Assay- Adapted from Tchao, Ruy, Progress in In-Vitro Toxicology 6 (2).

Principle: MDCK cell growth on a microporous membrane more closely mimics *in vivo* growth and differentiation when compared to growth on plastic. The morphological and functional similarities are due to media access to both the apical as well as the basolateral side of the cells. This results in distinct apical and basolateral domains as well as tight junction formation. This epithelial monolayer provides a barrier to movement of indicator dye, such as sodium fluorescein,

Once cells are grown to a monolayer on the Millicell-HA Cell Culture Inserts fluorescein dye can be added to the apical compartment. This monolayer should prevent passage of the dye to the basolateral compartment. The penetration of the dye to the basolateral compartment would indicate disruption of tight junctures. An insufficient monolayer would be reflected in the dose/response curve of the permeability test.

Suitability: The Sodium Fluoroscein Permeability Assay is suited to score the irritancy of compounds and mixtures that are miscible in water, most commonly soaps and detergents. These results have been correlated to Draize eye irritancy scores or Average Maximum Draize (AMD) scores*. The assay may be used to establish rank orders of the irritancy of soaps and detergents and may potentially be used to assess and predict irritancy-related compounds.

Other diluents may be substituted for less miscible materials. Suggestions include coconut oil, mineral oil, and dimethyl sulfoxide (DMSO). Corn oil, cotton seed oil, soy bean oil, and mineral oil (heavy and light) have shown no adverse effects in this assay. Sodium dodecyl sulfate (SDS) in homogenized mineral oil produced an effect similar to SDS in Hank's Balanced Saline Solution (HBSS).

This test quantitatively determines the integrity of in vitro MDCK cell to cell junctions in an epithelial monolayer. It has been shown that increased permeability of compounds is related to increased irritancy. The irritancy of unknown samples is determined by comparison to an SDS positive control. This assay also allows evaluation of cell recovery after injury. For example, disruption of tight junctions (without cell detachment and loss) will require two to four hours for complete recovery. If cell detachment and loss occur, the recovery may require several days depending on the severity of the injury. A limitation of the assay is that it can not be used to test compounds of extreme pH (lower than pH 3 or higher than pH 11) due to the buffering capacity of the diluent used. In general, compounds at these extreme pH levels are by definition considered irritants and therefore would not be tested.

* All Draize test scores were obtained from the Soap and Detergent Association. Millipore was not involved in Draize eye irritancy testing.

Recommended Materials:

- 24-well tissue culture plates (3 plates for each compound)
- 12mm Millicell-HA culture plate inserts (catalogue number PIHA01250)
- MDCK epithelial cells (American Type Culture Collection: No. CCL 34)
- T-75 tissue culture flasks
- MEM media with 10% fetal bovine serum and gentamycin (1µg/ml)
- Hanks' Balanced Salt Solution (HBSS)
- Trypsin-Versene™ Gibco Lab (Life Technologies)
- Sodium Fluorescein 1mg/vial (Molecular Probes)

Storage and Product Stability:
Store all assay solutions according to the manufacturer's recommendations.

Method [Adapted from Tchao, Ruy, Progress in In-Vitro Toxicology 6 (2)]**:**

1. Grow MDCK cell line in T-75 culture flask with MEM (10% FBS, 1µg/ml gentamycin) and pass at weekly intervals using trypsin-versene.

2. Place inserts into a 24 well culture plate containing 0.5ml media per well. One 24 well plate can be used to test a compound at 6 concentrations in quadruplicate.

3. Seed 1.5 x 10^5 cells per insert; confluency should occur in 3 days. Test for monolayer before proceeding. During this incubation period the media should be changed daily.

4. When cultures are confluent, remove media and rinse inserts with HBSS.

5. Place inserts into a new 24 well plate containing 0.5ml HBSS and a specific amount of the test compound. Incubate at 24°C for 15 minutes.

6. Decant solution and wash inserts 3 times, each with 1ml HBSS.

7. Place inserts into a new 24 well plate containing 0.5ml HBSS. Add 0.5ml HBSS containing 0.02% sodium fluorescein to the apical side of the inserts. Incubate at 24°C for 30 minutes.

8. Remove inserts and fix for examination via light and electron microscopy.

9. Dilute sodium fluorescein solution in each well to 3ml and measure in a spectrophotometer at 490nm.

SECTION VI

CELL REMOVAL

Method:

1. Remove Millicell insert from plate and wash gently with PBS to remove growth media.

2. Gently wash both sides of the Millicell insert membrane with Versene (Gibco Laboratories, part number 670-6040AG.

3. Add Versene to the inside of the Millicell insert and to the outer chamber (cell culture plate

well), to expose both sides of the membrane. Incubate for 5 minutes at room temperature, then remove the Versene solution.

4. Add enough trypsin/EDTA to the inside and to the outer chamber (cell culture plate well) of the Millicell insert to cover the membrane. Incubate at 37°C for 10 minutes.

5. Carefully remove the trypsin/EDTA without dislodging the cell layer.

6. Add growth medium and pipette up and down extensively along the rim and membrane surface to dislodge and disperse cells.

7. Pipette suspension into a test tube.

8. The suspension of cells can now be stained with trypan blue for counting and viability.

9. If desired, stain the Millicell insert membrane with hematoxylin (see appropriate procedures for Millicell-HA and Millicell-CM units) to check for residual cells on the membrane.

Note: *The key to successful removal of cells from the Millicell insert is to expose both sides of the membrane to trypsin. Method does not always yield quantitative removal of cells.*

SECTION VII

TEM TECHNIQUES

Note: *It is assumed that user(s) of this procedure will be knowledgeable in TEM procedures.*

(A) Processing/ Cell Preparation:

Note: Steps 1 - 5 should be done on an *intact* Millicell unit.

1. Wash cells briefly (2 times for 5 minutes each) at room temperature with phosphate buffered solution without fixative.

2. Fix cells in 2% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.5 at room temperature for 15 minutes to 2 hours.

3. Wash cells (2 times, for 5 minutes each) in 100 mM sodium cacodylate buffer at room temperature.

   Note: At this point, cells can be stored in the above buffer with 7 g sucrose/100 ml buffer at 4 degrees C.

4. Fix cells 1% osmium tetroxide in either 100 mM sodium cacodylate or suitable phosphate buffer.

5. Dehydrate cells in the following concentrations of Ethanol:

Ethanol Concentration	Time (minutes)
30%	15
50%	15
70%	15
95%	15
100%	3 X 15

Notes: Dehydration of Millicell-HA units should be performed in a metal pan which will be used as the embedding tray due to the tendency of the cellulosic membranes to be less rigid during the dehydration process. ***Attempts to transfer the membranes during these steps could lead to mechanical damage to the cells.***

6. For infiltration, EPON812, an EDPON substitution, or LX112 is suitable for both devices (do not use Spurr's).

The following is a general infiltration scheme:

Ethanol concentration/tray	Time (minutes)
75% ethanol/25% plastic	30 on a shaker
50% ethanol/50% plastic	30 on a shaker
100% plastic	30 each / 3X on a shaker
100% plastic	Overnight

Notes: With the plastic, it is not necessary to use any other agent such as propylene oxide. The latter will dissolve the cellulosic filters. In addition, the standard inversion/rotation of specimens used in these steps is not advised since either (1) damage to the cell layer or (2) stretching of the cellulosic filter may occur. Mild shaking on a gel shaker apparatus is sufficient for successful infiltration.

Note: Before the next step the membrane must be detached from the surrounding plastic ring. Sometimes this will occur without manipulation since the EPON may loosen the membrane-to-ring bond. If this does not occur, use a sharp scalpel and cut the membrane. Under no circumstances should the membrane be left attached to the ring during polymerization.

9. Transfer to fresh plastic and polymerize at 68º C overnight.

(B) Sectioning Notes:

1. Nitrocellulose (HA) Membrane: The nitrocellulose membranes can be sectioned in any plane without difficulty.

2. CM (Biopore) Membrane: The Biopore membrane must be processed in on of two alternative ways dictated by the final thickness of the section.

   (a) Sections of 90 nanometers or thicker -for standard transmission electron

   In this case, sections of cells will adhere to the Biopore membrane. Membranes should be oriented with the axis of the membrane/cells perpendicular to the knife-edge. This orientation will allow sections of cells to be made without passing them over the knife that has been previously used to section the underlying Teflon. Wrinkling of the section occurs to some degree since the plastic expands somewhat after it is sectioned, but the Biopore membrane does not. However, this problem is minimal and minor wrinkles are spread out under the heat of the electron beam.

   Note: Best sectioning of the CM membrane is achieved with a diamond knife.

   (b) Sections of less than 90 nanometers-for exceedingly extra-thin sections.

If sections of this thickness are desired, the cells will not adhere to the Biopore membrane and will be separated from the membrane after sectioning. Sections in this case must be mounted on a formvar-coated grid so that edges will not curl when they are mounted. The separation of the cell layer from the Teflon does not rest in subsequent damage to the membrane surface of the cell. Thus, even in this situation, studies examining the basal surface of cells can be accomplished with this technique.

SECTION VIII

ORGANOTYPIC PROTOCOL

Principle: Organotypic cell culture is a novel method of maintaining tissue explants in a system that duplicates, as closely as possible, the integrity of structure, proximity and function of a specific group of cells in-vitro as in-vivo. Organotypic cell culture method was created as an experimental environment that would more closely resemble that found within a living organism. It assesses the effect that factors such as contact between different cell types, chemotactic signals and the like may have on the development and eventual maturation of cells into an organized tissue. The Millicell method for organotypic culture involves placing a tissue slice on the Millicell-CM cell culture insert, placing the insert in a petri dish (35-50 ml) containing a small amount of culture medium, and wetting the exposed surfaces of the explant but not submerging them. With this method and careful aseptic technique, cultures have been grown for more than 40 days.

Suitability: Millipore Millicell-CM organotypic inserts are sterile, single-use devices consisting of a filter sealed to a cylindrical polystyrene holder. The devices are specially designed for customers who need to do long-term organotypic culture. Membrane-based organotypic techniques pioneered by Stoppini *et al*. - previously needed to cut inserts to fit petri dishes. The use of petri dishes, not plates, is preferred to avoid cross-contamination concerns. Millicell

organotypic inserts have a low wall height (5 mm) which fits inside covered petri dishes. The inserts enable the use of optical microscopes and electro-physiology equipment above the inset, without having to remove and handle the slices. The membrane in the Millicell-CM organotypic inserts is Biopore™ CM, a 0.4 µm hydrophilic PTFE, the same membrane as in all Millicell-CM units.

Materials (Culturing and Microscopy)

Millicell-CM (PICM0RG50)
Thinly dissected tissue explant
Culture medium

- 50% MEM (Gibco 079-01.012)
- 25% Horse Serum, 25% HANKS solution (buffered by addition of 5mM TRIS and 4mM $NaHCO_3$, pH 7.2)
- penicillin and streptomycin may be added
- glucose may be added to render concentration of 6.5mg/ml in each solution made

Rinse solution: 0.1M phosphate buffer, pH 7.3
Fixative: 1.5% glutaraldehyde and 1.0% paraformaldehyde in 0.1M phosphate buffer, pH 7.3
Primary Dehydration Solution: 25%, 50%, 75%, 95% and 100% acetone solutions
Secondary Dehydration Solution: PO (propylene oxide)
Infiltration Solution: 1:1 and 1:3 solutions of PO:EPON
Embedding Solution: 100% EPON
Light Microscopy Stain: methylene blue and azur II in borax
Electron Microscopy Stain: aqueous uranyl acetate and lead citrate
Polyester Foils
Sterivex GP (catalogue number SLGPB1010) or Stericup GP (catalogue number SCGPU11RE)

CULTURING

1. Excise the area of interest from the tissue sample and store in medium until all tissue is harvested.

   Note: All media should be pre-filtered using the Serivex™– or a Stericup ™-GP

2. Pretreat the Millicell CM with 10ul of a 1mg/ml solution of polyornithine.

3. Place the explants (using a cut Pasteur pipette) on the membrane, being certain to maintain a drop of original culture medium in the pipette with the explant. This small droplet will help form the minute film of media that must be maintained on the surface of the ex-plant to keep it humid. In general, only enough media should be added to the ex-plant to cover the tissue slice with a thin film of liquid. Tissues must be well exposed to the air (CO^2 enriched) to promote healthy development and maintenance of tissue slices for long term culture. Additionally, several slices may be placed on a single membrane. The Millicell-CM then may be placed in a petri dish containing medium. In general, for a 35 mm petri dish, 1.1 ml of media is used.

MICROSCOPY

Dehydration and Fixation

1. Rinse slices in 0.1M phosphate buffer, pH 7.3
2. Fix slices in 1.5% glutaraldehyde and 1.0% paraformaldehyde in 0.1M phosphate buffer (pH 7.3) for 2 hours at 4°C
3. Slice away membrane around culture
4. Rinse explant in phosphate buffer (pH 7.3, cold solution) for 1 hour at 20°C in the dark
5. Rinse three more times for 15 minutes each in phosphate buffer
6. Dehydrate samples through an ascending series of acetone concentrations (25%, 50%, 75%, 95%) for 10 minutes each followed by 3 changes of 100% acetone for 20 minutes each
7. Replace acetone with propylene-oxide (PO), 2 changes of 5 minutes each
8. Infiltrate samples through graded PO:EPON mixtures (1:1, 1:3), 2 hours each
9. Store overnight in 100% EPON

Embedding

1. Embed slices flat in EPON between transplant foils for 48 hours at 60°C
2. Remove polyester foils and the thin blocks of tissue re-embedded in EPON
3. Polymerize at 60°C for 2 days

Staining

- 1-2µm thin sections can be stained in a solution of methylene blue and azur II in borax for light microscopy
- Ultra thin sections of 60-80 nm can be mounted on uncoated copper grids and stained with aqueous uranyl acetate and lead citrate
- Sections can be examined with Philips EM300 electron microscope at 80kV (or equivalent)

SECTION IX

CO-CULTURE

This protocol involves growing two cell types on the Millicell membrane, one inside the Millicell and the other on the underside of the membrane. Two basic methods are described below, the first one being simpler to set up. The individual needs to decide which method is best suited to his/her specific area of research.

For optimal cell-cell interaction, the 12mm Millicell-PCF is recommended for these protocols. The Millicell may be coated with a basement membrane if desired.

Note: *Both methods should be performed in a laminar flow hood using sterile technique.*

Method #1:

1. Wet out the Millicell membrane with media in a 24 well plate.

2. Turn the Millicell upside down and add approximately 200µl of one cell type to the bottom of the Millicell.

3. Incubate the Millicell in a 37°C CO_2 incubator for 1 to 4 hours to allow the cells to attach to the membrane.

4. Gently turn the Millicell over in the 24 well plate and seed the second cell type inside the Millicell. Put media in the plastic well outside the Millicell.

5. Place the Millicell in the incubator to allow the second cell type to attach.

Method #2

1. Seed the first cell type inside of the Millicell.

2. Allow these cells to grow in a 37°C CO_2 incubator.

3. Gently turn the Millicell over in a large, sterile Petri dish.

4. Seed the second cell type on the underside of the Millicell. Seed these cells at confluence in a small amount of media (100 to 200µl).

5. Place the Millicell in the incubator for 1 to 4 hours to allow the second cell type to attach.

6. Gently turn the Millicell upright into a 24 well plate.

SECTION X

TROUBLESHOOTING

Problem	Solution
Membrane not properly wetted	Follow the procedure in the package insert for wetting the membrane. Allow several minutes for the membrane to become wetted with medium before seeding cells.
Cells do not attach or grow poorly	Cells should be seeded at 5 X 10^5 cells/cm^2 minimum densities. If cells do not grow well or form monolayers, seed at higher densities, and ensure that the ECM coating is appropriate for the particular cell line in use..
Cells do not form confluent, columnar monolayers	Some cells (i.e. Sertoli cells) do not form well-developed polarized monolayers even on membranes and need extracellar matrix (ECM) to attach and grow properly. Most researchers will have already been using some type of ECM on plastic and should also try it on Millicell. Due to the individuality of cells lines, growth characteristics must be individually determined.
Cells do not form confluent monolayers on coated Millicell	Millicell-CM must be coated for attachment. Some cells have a preference for specific ECM matrixes. It may be necessary to experiment with many ECMs before determining the most appropriate one. Another alternative for Millicell-Cm is to double the coating volume. Some cells are very sensitive to the inert Biopore membrane and double coating will eliminate any possible bare spots.
Cells detach from membrane	Medium on the outside should be slightly higher than the medium on the inside of the insert. For the 12 mm inserts, the inside volume should be 400µL and the outside volume 600µL. For the 30mm inserts, the inside and outside volumes should be between 1.5 to 2.0 mL, with a slightly higher volume on the outside.

SECTION XI

ORDERING INFORMATION

Membrane Description	Diameter	Qty/pk	Cat.#
Biopore Organotypic CM 0.4 µm (PTFE)	30mm	50	PICM0RG50
Biopore CM 0.4µm (PTFE)	12mm	50	PICM01250
Biopore CM 0.4µm (PTFE)	30mm	50	PICM03050
MF-Millipore, HA 0.45 µm (MCE)	12mm	50	PIHA01250
MF-Millipore, HA 0.45 µm (MCE)	30mm	50	PIHA03050
PC 0.4 µm (polycarbonate)	12mm	50	PIHT01250
PC 3 µm (polycarbonate)	12mm	50	PITT01250
PC 3 µm (polycarbonate)	30mm	50	PITT03050
PCF 0.4 µm (polycarbonate)	12mm	50	PIHP01250
PCF 0.4 µm (polycarbonate)	30mm	50	PIHP03050
PCF 3 µm (polycarbonate)	12mm	50	PITP01250
PCF 8 µm (polycarbonate)	12mm	50	PI8P01250
PCF 12µm (polycarbonate)	12mm	50	PIXP01250

Accessories:

Millicell ERS voltammeter, electrode pair	1	MERS00001
Replacement electrodes, 1 pair	1	MERSSTX01

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Versene™ is a trademark of Gibco Lab (Life Technologies)

SECTION XII

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