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Imaging Thick Tissues with Confocal Microscopy

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1. Introduction

Confocal laser scanning microscopy (CLSM) can be used to obtain optical sections of thick tissues that are relatively free of interfering autofluorescence, and that do not strongly scatter or absorb either the excitation or emission light. This chapter provides protocols used to examine three such tissues: the cornea of the eye, the buccal mucosa (which lines the inner cheek), and the nasal respiratory epithelium. Although in each case our overall motivation was to study the transport of drugs or model compounds across the particular epithelium, the approaches taken were quite different.

1.1. Viability Assay for an Ex Vivo Tissue (Cornea)

Determining the viability of an ex vivo tissue sample is important in any experiment where the physiological state of the tissue may influence the results obtained. In particular, excised cornea viability is of interest for in vitro diffusion study design and ocular toxicity risk assessment ***(1)***. Simultaneous vital staining by calcein AM (CAM) and ethidium homodimer-1 (EH-1), as "live" and "dead" probes, respectively, has been used for viability determination in monolayer cultures, but has not found wide application with thick tissue sections ***(2)***. Using the CAM/EH-1 probe pair, we developed a confocal laser scanning microscopy (CLSM) assay to determine corneal epithelial and endothelial viability as well as cornea thickness. The assay described for cornea here can readily be adapted for other thick tissues.

1.2. Dynamic Visualization of Diffusion (Buccal Mucosa)

Diffusion cells are often used to measure permeation rates of compounds through a tissue sample. The tissue is mounted between two chambers, each of

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which typically contains a buffered salt solution. The compound of interest is present in the "donor" chamber, and its flux through the tissue is determined by its rate of appearance in the "acceptor" chamber (or its rate of disappearance from the donor). Although this provides important information about bulk flux, it does not show how and where the compound traversed the tissue, that is, how the distribution of the marker in the tissue changed as a function of time. To accomplish this, we developed an in situ model (essentially a miniature diffusion cell on a microscope stage) that made it possible to use CLSM to continuously monitor and measure changes in the distribution and transport pathways taken by a fluorescent marker as it permeated across a tissue, and thus to localize any regions of lower flux that indicated the presence of barriers to transport. Although our tissue of interest was the buccal mucosa (the lining of the cheek), this in situ cell can be used for other tissues. The flow probe used was fluorescein insothiocyatnate (FITC), but other probes may be used as appropriate.

An important feature of the in situ cell was that the tissue was imaged by making optical sections parallel to the plane of a mechanical cross-section and below the region where cells had been damaged by the razor blade. There is sufficient scattering and absorption of light in most tissues, as well as index of refraction mismatch between the oil immersion objective lenses and the aqueous sample, so that there is significant loss of fluorescence intensity and spatial resolution when imaging deep sections parallel to the tissue surface. By viewing the tissue "edge-on" we could visualize the penetration of the fluoroprobe across all cell layers simultaneously, and compare relative intensity between regions.

1.3. Imaging Transport Across Fragile Tissues (Nasal Epithelium)

In contrast to corneal and buccal tissue, the nasal respiratory epithelium consists of only two cell layers, with a thickness of approximately 20 μm, and is very fragile. Its viability and integrity are difficult to maintain in vitro, and it is also easy to damage the tissue during removal. The approach that we developed was to administer the fluorescent probe intranasally in vivo, and to fix the tissue in situ. Transport of the fluorescent probe thus occurs under exactly the same circumstances as in vivo drug absorption studies ***(3)***. The animal was then sacrificed, and the tissue removed from the nasal cavity, post-fixed, and mounted for examination with CLSM ***(4)***. The essential features of this approach are the use of a fixable probe and a rapidly acting in vivo fixation procedure. The fixative must be able to immobilize the compound rapidly during the initial fixation, and remain stable during storage and imaging.

2. Materials

In all cases, fluorescent probes are kept protected from light throughout the experiment until the tissue is imaged. It is also assumed that the tissue being used does not have unacceptably high autofluorescence.

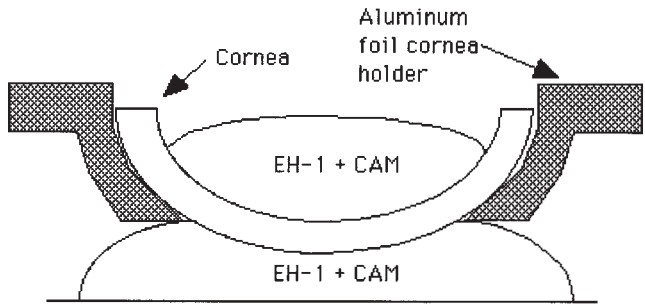

Fig. 1. Exposure of excised cornea to EH-1/CAM solution. About 100 mL of the EH-1/CAM solution is pipetted onto a glass slide and the cornea in the aluminum holder is gently floated onto the liquid, epithelium down. Another 100 mL of probe solution is then pipetted onto the concave endothelial side.

2.1. Viability Assay for an Ex Vivo Tissue (Cornea)

1. Microscope used: upright CLSM.
2. Media optimized for tissue storage. For the cornea, we used Optisol™ (Chiron Vision, Irvine, CA) (*see* **Note 1**).
3. Tissue collection (cornea) requires an 11-mm trephine blade and holder (Solan Ophthalmic Products, Sacramento, CA), a pair of surgical scissors, and 2 pairs of microdissecting forceps (*see* **Note 2**).
4. The buffer solution used was bicarbonate Ringer (BR) solution pH 7.5 containing 115 m*M* NaCl, 5.5 m*M* KCl, 1.8 m*M* $CaCl_2$, 0.8 m*M* $MgCl_2$, 35.0 m*M* $NaHCO_3$, and 5.5 m*M* glucose was prepared fresh, and equilibrated with carbogen (a mixture of 95% O_2 and 5% CO_2) until pH stabilized (*see* **Note 3**).
5. Fluorescent probes used: Ethidium homodimer-1 (EH-1) and calcein AM (CAM) (Molecular Probes, Eugene, OR; *see* **Note 4**).
6. Stock solutions are prepared as follows: For the CAM stock solution, dissolve CAM powder in anhydrous dimethyl sulfoxide (DMSO) to form a 10 m*M* solution (*see* **Note 5**). For the EH-1 stock solution, use the 2 m*M* solution in DMSO/H_2O 1:4 (v/v) that is commercially available. Both stock solutions should be stored sealed, frozen (–20°C), protected from light, and desiccated (*see* **Note 6**).
7. For each cornea, 200 µL of EH-1/CAM working solution in BR is prepared immediately before use in a conical centrifuge tube by mixing 2.5 µL of the EH-1 stock solution and 1 µL of the CAM stock solution (the final concentrations are 25 µ*M* EH-1 and 50 µ*M* CAM). The working solution should be vortex-mixed and then degassed by vacuum for 1 min, keeping it protected from light at all times.
8. Cornea holder for probe exposure (**Fig. 1**). Make a simple holder out of aluminum foil to suspend a cornea in the probe solution (*see* **Note 7**).
9. Lucite sample holder for microscopy (**Fig. 2**). You will need a sample holder similar to one that we made for the rapid examination of thick tissue specimens in an

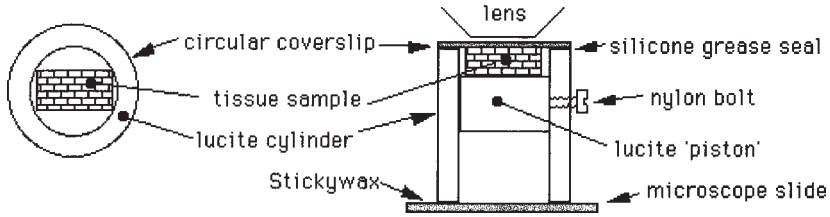

Fig. 2. Piston sample holder for CLSM viability assay.

upright microscope (*see* **Note 8**). Also have ready a clean 25-mm diameter circular glass no. 1 coverslip, silicone or vacuum grease, two pairs of tweezers, a wide glass microscope slide (75 × 38 mm), and either double-sided adhesive tape (Scotch, 3M, Minneapolis, MN) or a small piece of Stiki-wax (*see* **Note 9**).

2.2. Dynamic Visualization of Diffusion (Buccal Mucosa)

Because this protocol is focused on the construction of an in situ cell, only minimal information regarding the particular tissue and flow probe we used in our study is provided here. For more detail, *see* **ref. 5**.

1. Microscope used: inverted CLSM (*see* **Note 10**).
2. Fresh tissue should be stored chilled in an appropriate storage buffer (*see* **Note 11**). For the buccal mucosa, we used isotonic pH 7.5 Krebs buffer (*see* **Note 12**).
3. A flat, clean plastic cutting surface (e.g., a smooth nylon plate), and new de-oiled single-edged razor blades (*see* **Note 13**) to prepare the sample for the cell.
4. The fluorescent probe (the donor solution) should be prepared fresh in an appropriate buffer, and kept from light until use (*see* **Note 14**). We used 0.5 m*M* FITC in pH 7.5 Krebs buffer (*see* **Note 15**).
5. The acceptor solution, which is usually the same buffer used to prepare the probe (pH 7.5 Krebs buffer in this case).
6. Several round wooden toothpicks; two small (20-cc) hypodermic syringes, each with a no. 19 needle.
7. Sample holder for in-situ cell: A 1.5 inch × 3 inch × 0.25 inch piece of aluminum plate (approximately the length and width of a wide glass slide) is made with a (right circular) conical well bored through it at center (**Fig. 3**). This well provides clearance for the microscope objective lenses as the turret swings them into position. A 25.5 mm diameter cylindrical well is then bored at center from the other side, so that it intersects the conical well. The hole at the intersection of the well and the cone should be approx 15 mm in diameter. The sharp edge of the hole is removed, leaving a circular lip about 5 mm wide at the bottom of the cylindrical well. This lip supports the in situ cell described below. The completed cell holder should be anodized or painted black.
8. Preparation of coverslips for in situ cell: One such coverslip is needed to construct each in situ cell.

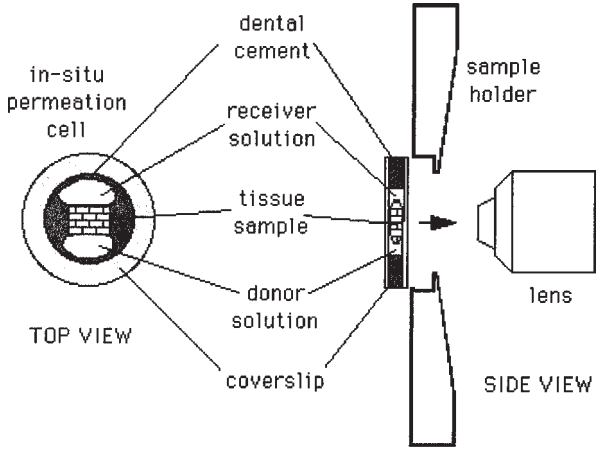

Fig. 3. In situ cell and sample holder. Cell sits in well in center of holder, and holder sits on stage of inverted microscope.

a. Thoroughly clean and dry a 25 mm diameter circular glass no. 1 coverslip
b. Dip the coverslip into a 0.5% (w/w) solution of Pioloform (polyvinylformaldehyde, Agar, The Netherlands) in chloroform.
c. Slowly lift the coverslip vertically out of the Pioloform solution
d. Dry it at ambient temperature.

The dried coverslip will be covered with a very thin hydrophobic film, which prevents "wicking" (capillarity) of the probe solution between the tissue surface and the coverslip. Other hydrophobic materials can also be used. One additional 25 mm circular coverslip (clean, but not prepared as above) is also required to construct the in situ cell.

9. Preparation of in-situ cell wall material: Two-component silicone dental clay is prepared by mixing Provil® L Base with Provil® L Catalyst (Bayer Dental, Leverkusen, Germany) in a ratio of 1:1. This adhesive elastomer ***(6)*** hardens in about 5 min, thus this step should be done just before it is to be used. Other substances may be used to form the in situ cell walls, provided that they are not fluorescent.

2.3. Imaging Transport Across Fragile Tissues (Nasal Epithelium)

1. Microscope used: either upright or inverted CLSM.
2. Preparation of fixative: For 100 mL of Bouin's fixative, add 75 mL of saturated picric acid (for microscopy, Fluka A.G. Buchs, Germany), 25 mL of formaldehyde solution (extra pure, Merck, Darmstadt, Germany), and 5 mL 98% of acetic acid (Baker Deventer, The Netherlands). Make up the fixative freshly before use (*see* **Note 16**).
3. Preparation of postfixative: After fixation in Bouin, tissue samples are postfixed and stored in a solution of 25 mL of formaldehyde solution (extra pure, Merck,

Darmstadt, Germany) and 75 mL of phosphate-buffered saline (PBS) (pH 8.0) (137 m*M* NaCl, 2.5 m*M* KCl, 8.8 m*M* $Na_2HPO_4 \cdot 2H_2O$, 0.45 m*M* KH_2PO_4). Make up the solution fresh before use.

4. PBS pH 8.0
5. Fluorescent probes:
   a. 0.5% (w/v) FITC-dextran solution is prepared by dissolving 1 mg of aldehyde fixable FITC-dextran (mol wt 3000 or 10,000, Molecular Probes, Eugene, OR) in 200 µL of physiological saline (0.9% NaCl in distilled water; w/v; *see* **Note 17**).
   b. 1% (w/v) of Evans blue solution is prepared by dissolving 10 mg Evans Blue (ICN Biomedicals Inc., Aurora, OH) in 1000 µL of physiological saline. Filter the solution through a Millipore filter.
   c. 0.5% (w/v) of DiIC18(5) solution is made by dissolving 5 mg of DiIC18(5) oil (Molecular Probes, Eugene, OR) in 500 µL ethanol. Add 375 mL of propylene glycol and 125 mL of PBS (pH 8.0).

   The fluorescent probe solutions should be stored at –20°C, protected from light.
6. Apparatus
   a. 5-cm silicone cannula.
   b. 20-cm silicone cannula.
   c. Small mirror.
   d. 100-µL syringe, with stub Luer fitting and 2 cm of polyvinyl chloride (PVC) tubing on it.
   e. Syringe for fixative.

3. Methods

For all the methods below, it is assumed that the CLSM has been turned on and allowed to warm up for at least 15 min prior to imaging, that the appropriate laser lines and filters have been selected, and that the optics of the system are aligned.

3.1. Viability Assay for an Ex Vivo Tissue (Cornea)

3.1.1. Tissue Collection and Preparation

1. As soon as possible after animal sacrifice, both corneas are excised (*see* **Note 2**) and immediately placed into either storage medium or test medium, depending upon the experimental purpose.
2. Immediately prepare the CAM/EH-1 working solution as described previously.
3. Rinse cornea with BR, position it in the cornea holder (**Fig. 1**), and expose it to the fluorescent probe solution for 120 min, away from light (*see* **Note 7**).
4. During this time, set up the microscope for 488 and 568 nm excitation if this has not already been done (*see* **Note 18**).
5. Rinse the corneas with BR and use the forceps and surgical scissors to isolate the circular area that was exposed to the fluorescent probes from the rest of the tissue to create a small, roughly circular "button" about 7 mm in diameter.

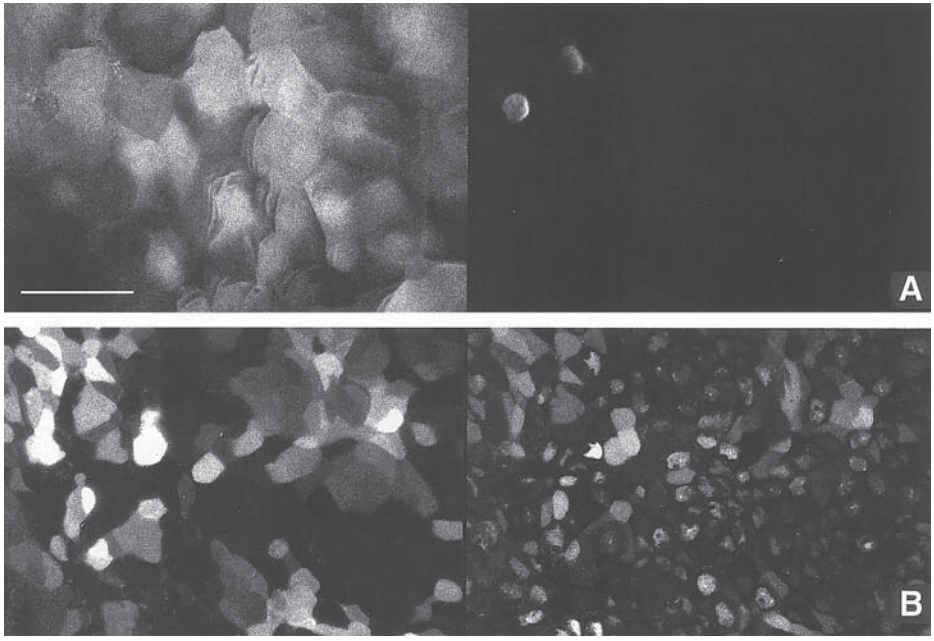

Fig. 4. Epithelium viability of freshly excised corneas stored at room temperature in PBS. Calcein fluorescence is shown on the **left**, EH-1 fluorescence is shown on the **right**. (**A**) Healthy control cornea; (**B**) After 4 h in PBS. All images are at the same magnification. Scale bar = 50 μm.

6. Mount this corneal button with the epithelium side up in the lucite sample holder (**Fig. 2**) without further treatment (*see* **Note 19**).

3.1.2. Imaging

1. Scan the sample: Use a 40× or 60× lens to survey the sample with a fast scan (typically F3 on MRC 600), identifying any heterogeneous regions and/or representative areas. Be careful to stay away from the edges of the sample.
2. Imaging the epithelium: Collect full screen averaged images of areas of interest at normal speed. Begin with the calcein image (calcein is more susceptible to photobleaching than the EH-1; *see* **Notes 20** and **21**). Typical calcein and EH-1 signals observed on control cornea samples are shown in **Fig. 4A.** After 4 hours at room temperature in PBS (**Fig.** 4**B**), severe damage to the epithelium can be observed.
3. Imaging the endothelium: Remove the coverslip from the sample holder, rinse the corneal button in BR, and place it back in the sample holder with the endothelium side up (*see* **Note 22**). Endothelium is imaged as described previously for the epithelium. Healthy New Zealand white rabbit endothelium is shown on **Fig.** 5**A**. After 6 h in PBS, massive endothelial cellular death is seen (**Fig. 5B**).

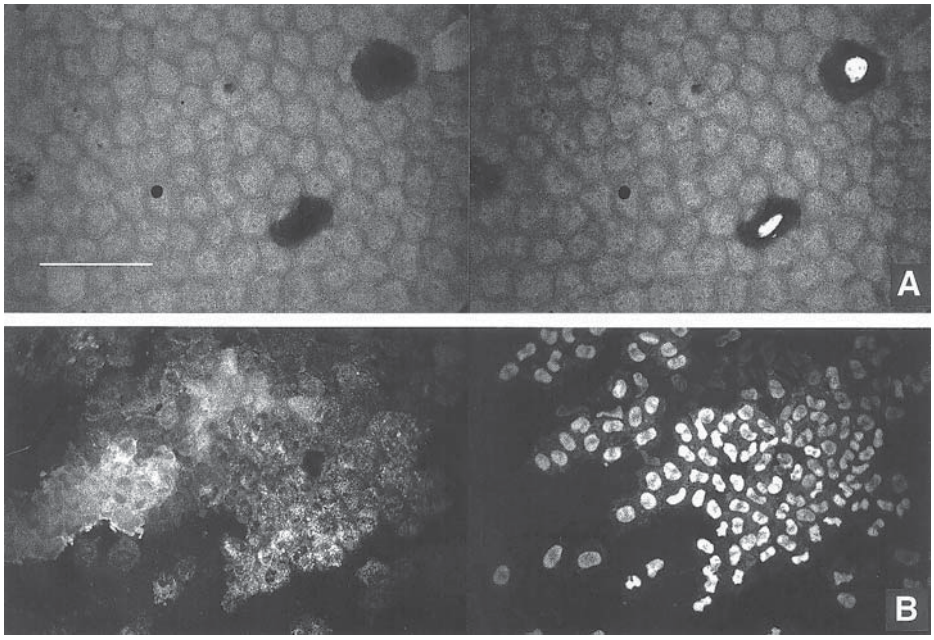

Fig. 5. Endothelium viability of freshly-excised corneas stored at room temperature in PBS, as determined by CLSM. Calcein fluorescence is shown on the **left**, EH-1 fluorescence is shown on the **right**. **(A)** Healthy control cornea; **(B)** After 6 h in PBS. All images are at the same magnification. Scale bar = 50 µm.

Confocal Z-sections through the entire sample of cornea can provide a measure of relative thickness, and therefore of corneal swelling *(7)*.

3.1.3. Display of Dual-Label Images

Simultaneous display of both calcein and EH-1 signals on a single color-coded image can be accomplished with the confocal software or with NIH Image (*see* **Note 23**), or any other image processing software.

3.2. Dynamic Visualization of Diffusion (Buccal Mucosa)

3.2.1. Preparation of Tissue for Cell

1. If the tissue sample is not already thin (0.5–2.0 mm) and flat, then it should be sectioned so that it is (*see* **Note 24**). This tissue slice will also establish which surfaces will face the donor and receptor chambers in the cell (*see* **Note 12**).
2. Use a de-oiled razor blade to make the slice, balancing the pressure evenly across the razor's edge; i.e., push straight down rather than rocking or sliding the blade (which may tear the tissue) (*see* **Note 25**). Be careful to maintain the orientation of the sample (*see* **Note 26**). The thickness of the tissue slice is "Th."

3. Now orient this tissue slice so that one side is flat against the cutting surface (*see* **Note 25**). It's helpful if you can now cut out a square of tissue with sides slightly shorter than the width of the razor.
4. Carefully make a clean cross-section cut (pushing straight down as in **step 1** above) parallel to one edge of the tissue.
5. Move the blade over 1–2 mm from the new edge and cut again to make a strip with parallel sides about 1–2 mm wide and as long as the blade.
6. Make several such strips, and select the best cut. A 6 mm long portion of the best strip is your sample. Again, be careful to maintain the orientation of the sample throughout.

3.2.2. Construction of In Situ Cell

1. Position the sample along the diameter of one hydrophobic coverslip so that one "Th" edge is against the surface of the coverslip (**Fig. 3**) (*see* **Note 27**).
2. Surround the tissue slice with the freshly mixed dental clay so that a crude Greek "theta" (θ) is formed. The tissue is the bar across the center of the letter, and the open areas above and below the tissue are the donor and receptor chambers of the cell (**Fig. 3**). Use a toothpick to move the clay around (*see* **Note 28**). The top surface of the clay should be about even or just below the height of the tissue strip (1–2 mm).
3. Cover the top side of the θ loosely with the second coverslip to prevent the tissue sample from drying out, but do not press it down (*see* **Note 29**).
4. Insert this coverslip/tissue/coverslip sandwich (the in situ cell) into the sample holder so that the center bar of the θ is along the line of either X or Y stage motion.

3.2.3. Imaging

1. Mount the sample holder with the cell assembly on the stage of the inverted microscope, and check the sample for autofluorescence using the laser and confocal settings that will be employed during the experiment (*see* **Note 30**).
2. Position the sample holder so that the field of view includes the area through which transport is expected to occur, and determine the Z-location of the tissue/coverslip interface (*see* **Note 31**). Make a note of this position as Ztc.
3. Move the focal place about 30 μm away from the interface, deeper into the tissue and away from the damaged cells at the cut edge. Note the Z-location position here as well, will be Zim.
4. Fill one 20-cc hypodermic with the probe (donor) solution, and the other with the buffer (acceptor) solution.
5. At $t = 0$ (the start of the experiment), use the appropriate syringe to fill the acceptor chamber with a drop (about 10 μL) of Krebs buffer, and the donor chamber with a drop of probe solution.
6. After filling the chambers, quickly move the focal plane to Ztc, and check to see is there is any leakage of the probe (seen as a bright fluorescent film) along this interface.
7. If leakage is observed, discard the in situ cell. If not, continue with the experiment, returning to Zim for image acquisition (*see* **Note 32**).

8. Images are then acquired at set time intervals from the same plane in the sample, without changing any settings, and the time/date stamped images stored without image enhancement (*see* **Note 33**).
9. Successive images will show the gradual spread of fluorescence through the tissue as the probe molecule moves through it ***(5)***.

3.3. Imaging Transport Across Fragile Tissues (Nasal Epithelium)

3.3.1. In Vivo Intranasal Administration of Fluorescent Dextran Solution

1. Anesthetize the animal and place it on its back.
2. When the rat is sedated, insert a 5-cm silicone cannula into the trachea via tracheotomy to enable the animal to breathe.
3. Insert a second 20-mm silicone cannula via an incision in the esophagus into the posterior end of the nasal cavity (*see* **Note 34**). This cannula will be used to flush the nasal cavity with fixative.
4. Administer the fluorescent probe solution (FITC-dextran in our study) to both nostrils with a 100 µL syringe with a PVC tube attached to it. Insert the PVC tube at least 0.5 cm into each nostril, and administer at least 50 µL of the probe solution.
5. Keep the animals supine during exposure to the probe solution (15 min in our experiments).

3.3.2. Fixation and Postfixation

1. Give an intravenous overdose of anaesthetic 30 s before flushing the nasal cavity with fixative to ensure the complete sedation of the animal.
2. Perfuse 5 mL of Bouin's fixative through the nasal cavity via the esophageal cannula. The fixative will flush from the nasal cavity through the nostrils.
3. Remove the nasal septum, divide it into three equal parts (*see* **Note 35**).
4. Post-fix the tissue by immersing the parts in Bouin's fixative for another 2 h.
5. Store the fixed tissue in a solution of 10% formaldehyde at pH 8.0 (*see* **Note 36**).

3.3. Counterstaining the Tissue

Identification of cell types in the tissue is aided by counterstaining with Evans blue, which binds to proteins, or dioctadecyl indodicarbocyanine (DiIC18(5)), which stains lipids (*see* **Note 37**) 24 h after the completion of the in vivo portion of the procedure.

1. Counterstain the tissue by immersing the septum parts in 1 mL of Evans Blue solution for 15 min, or in 1 mL of DiIC18(5) solution for 60 min.
2. Rinse the tissue after staining in PBS pH 8.0.
3. Carefully remove the fixed epithelium from both sides of the septum with a sharp scalpel.
4. Arrange the epithelium with the apical side facing the objective lens (*see* **Note 38**), using either of the sample holders described previously.
5. Examine the fixed tissue.

4. Notes

1. Optisol (Chiron Vision, Irvine, CA) is a medium-term storage medium for human cornea. It is no longer commercially available, but can be replaced by Optisol-GS, also from Chiron Vision. These transplant media, however, although very elaborate, are also rather expensive. In most instances, simpler media can be prepared in the laboratory or purchased from cell culture facilities. The most commonly used of such cost-effective media include glutathione bicarbonate Ringer solutions [e.g., ***(7,8)***] and medium 199 ***(8)***. PBS should be avoided whenever possible as it has been shown to alter excised cornea integrity within a few hours ***(1,9)***.
2. Cornea collection and storage: A clear and illustrated protocol for the excision of cornea from the enucleated ocular globe can be found in **ref. *11***. A similar procedure can be followed without prior enucleation of the globe. In our study, New Zealand white rabbit corneas were excised immediately after sacrifice, sans scleral rim, without prior enucleation, using the trephine blade and surgical scissors.
3. BR can also be stored at 4°C overnight. If so, after equilibration at room temperature, pH should be checked and readjusted if necessary with carbogen.
4. CAM is a nonfluorescent cell-permeant dye (mol wt 994.8) that is cleaved by intracellular esterases to fluorescent calcein (mol wt 666.5, λ_{max} absorption = 490-510 nm, λ_{max} emission = 515-535 nm). EH-1 (mol wt 856.7, λ_{max} absorption = 528 nm, λ_{max} emission = 617 nm) passes through damaged cell membranes to bind DNA and undergo a 40-fold enhancement in fluorescence.
5. DMSO is a permeation enhancer. In an effort to minimize the amount of DMSO used in the assay, we made our own CAM stock solution rather than using the commercial version.
6. Solutions of EH-1 in H_2O/DMSO can be stored frozen for at least 1 yr. CAM is subject to hydrolysis when exposed to moisture, and the CAM stock solution will take up some moisture from the air when it is made. It thus should be used within 30 d.
7. The cornea holder (which we made out of aluminum foil) was used because we found that immersion of the whole cornea in the EH-1/CAM solution resulted in extracellular CAM hydrolysis, probably due to leakage of enzyme from the edge of the tissue into the probe solution.
8. The sample holder is essentially a short piston in a cylinder, with a setscrew to hold the piston in place. The cylinder is made by using a lathe to bore a cylinder of diameter 0.5015 inch about 1.2 inches deep through the center of a short section of 1-inch lucite rod. The projecting end of the rod is then faced (made smooth) while it is still in the lathe, and then cut off about 1.1 inches from the faced end. The other end of the resulting cylinder is faced, and a hole for a nylon bolt (size 8 or larger) is drilled and tapped along a line that would intersect the center of the bore, about 0.5 inches from one face. This bolt acts as a setscrew for the piston, which is made by first turning lucite rod stock to an exact diameter of 0.5 inch in a lathe (rod stock is typically not truly round) and then cutting and facing an 0.75 inch section of the turned rod. When lubricated by tissue fluids or buffer solution, this piston moves easily with the 1.5 thousands clearance pro-

vided. It is important that the faces of the piston be smooth and unscratched. O-rings can be mounted on the sides of the piston if a water-tight seal is desirable.

9. The type of silicone or vacuum grease is not important, as long as it is somewhat sticky and not too liquid. Stiki-wax, which was used to apply adhesive to small notes before the advent of Post-Its, is easier to use than double-sided adhesive tape, and may still be available in some stationery stores.
10. This technique requires an inverted microscope to prevent loss of fluid from the cell. We used a Bio-Rad MRC 600 confocal mounted on a Zeiss IM-35, and a Zeiss Plan-Neo-Neofluar 25×/0.8 oil/water/glycerin objective set to oil. The FITC label was detected with the blue high sensitivity (BHS) filterblock. Ten scans were obtained and Kalman-averaged to obtain an image.
11. Sample viability is a concern, and should be verified in parallel experiments using an appropriate method.
12. We used porcine buccal mucosa dermatomed to 500 μm (which includes the epithelium and part of the connective tissue) and washed with cold Krebs pH 7.5 buffer before storage in the same buffer. The mucosal surface faced the donor chamber of the cell, and the serosal surface (the cut edge) faced the receptor side. The Krebs buffer was prepared fresh using twice-distilled water and equilibrated with carbogen for 15 min before use.
13. New razor blades come coated with a protective film, usually oil or silicone. This film should be removed before use, or it may form small globules on the sample which will interfere with imaging (and the protective material may also be fluorescent).
14. Be sure to use an isotonic buffer solution. Also, note that fluorescently labeled compounds used as flow probes may have unreacted label present when purchased, and can also lose their labels during the course of a long experiment. The presence of fluorescence in the tissue can then be an artifact. It is therefore advisable to test the purity and stability of the label, e.g., by HPLC.
15. The chosen concentrations depend very much on the fluorescent compounds used. FITC-labeled dextrans, e.g., are available from various sources with different labeling efficiency, and donor concentrations have to be adjusted accordingly.
16. We found Bouin's fixative to be superior to other formaldehyde-containing fixatives in immobilizing the intranasally administered dextrans and preserving the general morphology of the tissue well. However, Bouin's fixative is less appropriate for other uses, e.g., immunocytochemistry, as it contains high amounts of picric acid and methanol, both of which destroy cell organelles. When the loss of ultrastructural elements is unacceptable, Bouin's fixative can be replaced by other fixatives that contain smaller amounts of picric acid and replace formalin with paraformaldehyde. One such fixative is Zamboni's fixative, which consists of 2% formaldehyde and 0.2% picric acid ***(12,13)***.
17. FITC-labeled aldehyde fixable dextrans with molecular weights of 3000 and 10,000 ***(4)*** were used as model compounds in our studies. These dextrans have covalently bound lysine residues that permit them to be covalently linked to biomolecules by aldehyde fixation.

18. The confocal microscope system used in our study was a Bio-Rad MRC600 equipped with a krypton/argon laser and mounted on a Nikon Optiphot microscope. Samples were simultaneously excited with the 488- and the 568-nm lines and imaged with Nikon Planapochromat air (10×/0.5) or oil immersion (40×/1.3 and 60×/1.4) objectives and a zoom of 1. Calcein and EH-1 fluorescence were detected using the Bio-Rad K1/K2 filterblock set.
19. The piston is lowered about 3/8" inches below the top edge of the cylinder, and kept in place either by the setscrew or by a finger inserted up the bore from the other side. The tissue sample is then placed on the head (top surface) of the piston. A thin layer of silicone grease is applied to the rim of the cylinder above the sample, and a small drop of liquid (e.g., buffer) is placed on top of the tissue. The coverslip is set carefully onto the grease and lightly pressed down on its edges to seal the top of the cylinder. The piston is then slowly advanced by pushing it up from the other side until complete contact between the top of the tissue surface and the bottom of the coverslip has been established (the drop of liquid ensures an air-free coupling between the tissue and the coverslip). Stiki-wax or double-sided tape is applied to the bottom rim of the cylinder, which can thus be firmly attached to a wide glass slide.
20. Calcein signal was found to bleach faster than EH-1 signal. On the MRC 600, full images can only be acquired consecutively; therefore the calcein image was collected first followed by the EH-1 image of the same field. For image acquisition, normal scanning speed was usually satisfactory (1 s/frame, 512 lines/frame; F2 on Bio-Rad COMOS) to obtain a good quality Kalman-averaged image, and slow speed scanning (3 s/frame, 512 lines/frame; F1) was not necessary.
21. Typical microscope settings for epithelium or endothelium imaging (Kalman averaging, n = 3–5) with the Bio-Rad MRC 600 using K1/K2 filterblocks, full horizontal box, zoom = 1, and scan speed F2, were: ND = 1–3%, aperture 3–5 units, gain 4–6, black level 4.5–5.0, enhancement off.
22. When flipping corneal button over, be careful to keep track of tissue orientation. The curvature of the button is minimal, and thus this is not a good indicator for which side is which.
23. NIH Image is a public domain program developed at the U.S. National Institutes of Health. NIH Image and the Bio-Rad confocal macros can both be downloaded from the NIH Image homepage on the Internet at http://rsb.info.nih.gov/nih-image/).
24. The object is to prepare a small rectangular block of tissue about 6 mm long, 1–2 mm deep, and anywhere from 0.5 to 2.0 mm thick for the surface against the coverslip. For epithelia, this latter dimension is usually the thickness of the particular epithelium.
25. If one side of the tissue is more difficult to cut than the other (e.g., contains connective tissue), place this side facing the cutting surface to avoid unnecessarily compressing the tissue.
26. One way to do this is to sprinkle a tiny amount of graphite powder on one side of the tissue. The powder grains are easy to see by eye and in the microscope, and are not chemically reactive. Scrapings from a pencil lead work well.

27. Be careful not to wrinkle the tissue slice, and avoid trapping a liquid film or air bubbles beneath it. Inspection with a magnifier is advisable.
28. Be sure to arrange the clay so that the left and right edges of the tissue strip are captured and a good seal is formed.
29. The coverslip needs to be loose to allow access to the donor and acceptor chambers in the clay.
30. Tissue autofluorescence varies between individual animals, and interior autofluorescence cannot be determined until a cross-section is made. If significant autofluorescence is present in the tissue at the gain, aperture, etc. settings to be used in the experiment, discard the preparation.
31. This can be accomplished in several ways, but the easiest is to epiilluminate the tissue with laser light, and increase detection sensitivity until its residual autofluorescence can be used to determine the position of the interface.
32. Repeat **step 11** at least once during the experiment, and at the end as well, to verify that no leakage along the interface has taken place.
33. Be sure to note all settings that are not automatically recorded by the imaging software, including the magnification of the lens used.
34. Check that the cannula is inserted into the nasal cavity and not the buccal cavity by holding a mirror in front of the animal's nostrils. Blow through the esophageal cannula, and water vapor should condense on the mirror.
35. After euthanasia and fixation the rat is decapitated. The skin and the mandibles are removed to expose the palate of the buccal cavity. Separate the front of the nose from the skull by cutting between the second palatal ridge and the first molars. Carefully excise the nasal septum by cutting the dorsal and ventral portions of the skull and removing these from both sides of the septum. Remove the ridge on the ventral part of the septum and divide the septum in three equal parts: anterior, medial, and posterior.
36. The tissue is post-fixed and stored in a 10% formaldehyde (pH 8.0) solution because formaldehyde fixation is reversible, and thus the tissue must be stored in formaldehyde to preserve the fixation. In addition, Bouin's fixative can quench fluorescein fluorescence to some extent, but when the tissue is stored in 10% formaldehyde (pH 8.0) overnight after Bouin fixation, the FITC fluorescence is bright enough for confocal microscopic imaging (fluorescein fluorescence intensity is maximal between pH 8.0–10.0).
37. Evans blue (FW 960.8, λ_{max} absorption = 611 nm) and DiIC18(5) (mol wt 960, λ_{max} absorption = 644 nm, λ_{max} emission = 665 nm) were used as counterstains because they fluoresce in the far red area, and thus avoid overlap with FITC fluorescence.
38. Confocal microscopic visualization. We used a Nikon Planapo 60×/1.40 oil immersion lens, 488 nm excitation for the FITC and 633 nm excitation for both of the red fluorescent probes, Evans blue and DiIC18(5). There is no crosstalk between FITC and Evans blue/DiIC18(5). The depth of imaging in the fixed tissue is generally not deeper than 15–20 μm. Depth resolution can be improved by the use of mounting media with a refractive index close to the refraction index of the immersion medium ***(12,14)***.

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