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Mouse olfactory epithelium

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ISBN

9780199634859

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Publication Date

1995

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Mouse olfactory epithelium

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1. Introduction

The olfactory epithelium is unique in mammals in that proliferation of neuronal precursor cells and differentiation of their progeny into olfactory receptor neurons (ORNs) continue throughout the lifetime of the organism (1,2). Many of the studies in our laboratory are concerned with understanding how specific polypeptide growth factors and cell interactions regulate olfactory receptor neuron generation and survival (3–6). For these studies it is crucial to obtain and culture explants of olfactory epithelium that are free from stromal cells, since the stromal cells may provide signals that affect the development of olfactory receptor neurons. Thus, an entire section of this chapter is devoted to purification of the epithelium from its underlying stroma. For studies of growth factor effects on isolated ORNs, and for studies on olfactory receptor neuron adhesion, migration, and axon outgrowth, we prepare a neuronal cell fraction from embryonic mouse olfactory epithelium, as well as using explant cultures. This neuronal cell fraction consists solely of olfactory receptor neurons and their precursors, which we have called the immediate neuronal precursors (INPs) of olfactory receptor neurons, and does not contain basal cells or supporting (sustentacular) cells of the epithelium (6–8). Using this cell fraction allows us to work solely with neuronal cells in dissociated cell culture, which is a great advantage for quantitative studies.

Other authors have published methods for culturing olfactory epithelium from both embryonic and neonatal rats, and the reader is referred to these papers for culture methods for rat tissue (e.g. 9–17). However, because of the availability of many strains of transgenic mice that are of interest for studies on olfactory neurogenesis and development (6,18–22), as well as the greater ease of maintaining mouse as opposed to rat breeding colonies, the mouse is the preferred species for our studies.

2. Dissection and purification of olfactory epithelium from mouse embryos

We perform all the procedures described below on the bench-top in a room used exclusively for dissection. In order to maintain sterility, we wipe down all surfaces with 70% ethanol before beginning a preparation, and routinely dip our instruments in 95% ethanol and flame them. All procedures are performed using sterile, disposable polystyrene dishes and either sterile polystyrene tissue culture pipettes or autoclaved 9 inch Pasteur pipettes.

In order to work with precisely timed embryos, it is usually necessary to maintain your own breeding colony. For most of our experiments, we use random-bred CD-1 (Swiss) mice from Charles River Laboratories. The mice are naturally mated, and females are checked every morning for the presence of a vaginal plug, which indicates successful mating. The date of the vaginal plug is designated day 0.5 of pregnancy. For explant cultures, embryos are taken at day 14.5 or 15.5 of pregnancy. For dissociated cell culture, we purify epithelium from embryos taken any time from day 14.5 to day 17.5 of pregnancy, depending upon the goal of the particular experiment.

2.1 Dissection

Preparation of the olfactory epithelium (OE) for culture involves first, dissection of turbinates from the nasal cavities, and secondly, purification of the OE itself from its underlying stroma, which consists primarily of fibroblasts and chondrocytes. The dissection procedure has three parts:

- removal of the embryos from the pregnant mouse (see Barker and Johnson, Chapter 3 for procedure)
- dissection of heads into hemi-snouts
- removal of olfactory turbinates from the hemi-snouts

Once the embryos have been removed from the pregnant mouse, all subsequent procedures are performed using a dissection microscope equipped with bright-field and dark-field transillumination. Fibre-optic illumination is useful in the initial removal of turbinates from hemi-snouts.

Protocol 1. Dissection of olfactory turbinates

Equipment and reagents

You will need the following dissection instruments.

For initial removal of the uterus and dissection of embryos:

- 4 inch operating scissors
- 5 inch operating scissors
- 4.5 inch blunt forceps with serrated jaws
- 4.5 inch tissue forceps with teeth
- 4 inch microdissecting scissors, straight
- 4 inch microdissecting forceps with serrated jaws

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For removal of the olfactory turbinates from embryo heads:

- Tissue culture PBS with serum (for dissection of heads and removal of turbinates): to 500 ml of cold 1 x PBS (prepared as described in Appendix), add 25 ml bovine calf serum, defined, iron supplemented (Hyclone, A2151L, or equivalent high quality, virus and endotoxin-free bovine calf serum), and 2.5 ml penicillin-streptomycin, 10 000 U/ml (Gibco-BRL, 15140-015), store at 4°C
- Dumont No. 5 microdissecting forceps
- 3.5 inch Castroviejo ultra microscissors, angular, blades 11 mm, sharp ends (Roboz, RS-5658)^a

Method

1. After removing the embryos from the pregnant mouse, decapitate the embryos and place the heads on ice in a 100 mm Petri dish containing cold PBS with serum. Distribute the heads into 60 mm Petri dishes, with three or four heads in each dish. Make the first, coronal incision from the top to the base of the head, and remove the entire back of the head (see *Figure 1A*). Do this for all the heads in the dish. Remove the waste tissue (backs of heads) from the dissection dish.
2. Place each head standing up on the surface that was just cut, and make the second, horizontal incision to remove the lower jaw and tongue (*Figure 1B*). Remove this waste tissue from the dissection dish. Make the third, sagittal incision from the front of the snout through the back of the head, splitting the front part of the head and the nasal cavity into two halves ('hemi-snouts').
3. Lay the two hemi-snouts on their sides, so that the inside of the nasal cavity is face up. The olfactory turbinates (O.T.) appear as oval buds covered by a shiny, translucent layer of cells (the olfactory epithelium) (*Figure 1C*). The invaginations in the surface of the turbinates appear as dark lines running crosswise across the ovals. In about half of the hemi-snouts, the turbinate will not be visible because it is covered by the nasal septum (N.S.). If the septum is peeled away with fine forceps, then the turbinate should be visible. Place the tips of the fine forceps underneath each turbinate, and pinch gently to remove it (*Figure 1D*).

^a While most of the instruments are readily available from a number of different sources, we have found that the Castroviejo ultra microdissecting scissors from Roboz (or Downs Surgical, see Appendix) work particularly well for the incisions of the embryo heads.

Protocol 2. Purification of OE

Reagents

- Holding medium for turbinates: 575 ml of cold 1 x PBS, 0.6 ml phenol red, 0.5% (Gibco-BRL, 630-5100AG), 3 ml penicillin-streptomycin (10000 U/ml), 6 ml 30% glucose, dissolved in H₂O, 25 ml fetal bovine serum (Hyclone, A-1111-L)—filter sterilize with 0.2 μ m filter, and store at 4°C
- Post-trypsin rinse: 500 ml cold 1 x PBS, 5 ml 30% glucose, 0.5 ml phenol red; 0.5%, 2.5 ml penicillin-streptomycin (10000 U/ml), 250 mg albumin, bovine, crystalline (BSA; ICN Biomedicals, 103700), 125 mg trypsin inhibitor, Type I-S from soybean (Sigma, T-9003)—sterilize through 0.2 μ m filter, and store at 4°C
- Trituration medium: 500 ml cold 1 x PBS, 5 ml 30% glucose, 2.5 ml penicillin-streptomycin (10000 U/ml), 0.5 ml phenol red, 0.5%, 250 mg crystalline BSA (ICN Biomedicals, 103700)—sterilize through 0.2 μ m filter, and store at 4°C
- Trypsin-pancreatin solution (for separation of olfactory epithelium from stroma): in a 250 ml polypropylene centrifuge bottle add 1 g pancreatin, from porcine pancreas, Grade II (Sigma, P-1500), 3 g trypsin 1: 250 (Difco Laboratories, Detroit, MI), 0.5 ml phenol red, 0.5%, 1 ml penicillin-streptomycin (10000 U/ml), 100 ml cold 1 x PBS. Mix well to dissolve, keeping at 4°C. Spin 20 min at ~ 16300 g to remove insoluble material. Filter supernatant twice through Whatman No. 1 filter paper, keeping solution on ice. Filter sterilize through 0.2 μ m filter unit with pre-filter. Make 8 ml aliquots and store in sterile polypropylene tubes at -20°C.

Method

1. As you dissect the turbinates out of the heads in each dish, collect the turbinates by mouth pipette, using a sterile, cotton plugged 9 inch Pasteur pipette attached to a plastic mouthpiece with latex tubing (Figure 1E). Transfer the turbinates to a separate 60 mm Petri dish, on ice, containing holding medium. Collect all the turbinates from a dissection into this dish, then remove the holding medium using a sterile Pasteur pipette (being careful not to lose any turbinates), rinse the turbinates once in ice-cold PBS, remove the PBS, and add 8 ml of trypsin-pancreatin solution.
2. Incubate the turbinates in the trypsin-pancreatin solution for 40-55 min on ice, depending on the age of the embryos (40 min for E14.5, 50-55 min for E16.5-17.5). Then remove the enzyme solution and add 8-10 ml of post-trypsin rinse to the dish. Keep dish on ice.
3. Transfer the turbinates into 35 mm Petri dishes each containing 2.5 ml of cold trituration medium (Figure 1F). Distribute two turbinates into each dish, again by mouth pipette.
4. To remove the OE from its underlying stroma, gently triturate the turbinates using a hand-held 9 inch Pasteur pipette whose opening has been flamed down to approximately the width of the turbinates (Figure 1F, 1). Under dark-field illumination, draw the turbinates up and down in the pipette, and the epithelium will separate from the stroma (Figure 1F, 2). The epithelium may still have some stroma

attached (Figure 1F, 3), however, so it is necessary to continue triturating until there is no stroma attached to the epithelium (Figure 1F, 4). The epithelium will appear as shiny, translucent sheets in dark-field transillumination, whereas the stroma will appear as irregularly shaped, opaque masses of cells and collagen fibrils.

5. Transfer purified pieces of epithelium by mouth pipette through two successive rinses of low calcium culture medium (see Protocol 4), each in a new 60 mm Petri dish held on ice. Inspect each piece of epithelium during the first rinse, and carefully remove any remaining stroma before transferring the epithelium to the second, medium-containing dish.

3. Culturing olfactory epithelium explants

In our explant culture system, small pieces of OE are plated on to substrata that maximize neuronal migration. Three cell types are readily identifiable within one day of initiating olfactory epithelium explant cultures:

- (a) Basal cells remain within the body of the explant, and grow as sheets of epithelial cells that express keratins (3,4).
- (b) Immature olfactory receptor neurons migrate readily from explants that have been cultured on merosin- or laminin-treated substrata (3,7,8). The immature olfactory receptor neurons are postmitotic cells that can be easily recognized in culture by their neurites; in addition, they are the only cells in the OE that express the neural cell adhesion molecule NCAM, which can be recognized by several different antibodies (3-5,7).
- (c) The immediate neuronal precursors (INPs) of olfactory receptor neurons are also present in these cultures within the first 24-48 h (3-6). INPs do not express keratins or NCAM, and they can be recognized as migratory, round cells that rapidly sort out from the body of the explant, synthesizing DNA and dividing as they migrate. [³H]Thymidine incorporation analysis has shown that the INPs are the direct precursors of olfactory receptor neurons (3-5). Thus, the disappearance of NCAM-negative INPs from olfactory epithelium cultures is a result of these cells giving rise to postmitotic olfactory receptor neurons, which express NCAM.

In addition to the three major cell types described above, our recent studies—using an antibody to a mucin expressed by sustentacular cells of the olfactory epithelium (23)—suggest that sustentacular cells and basal cells may be intermixed within some of the epithelial sheets that develop in embryonic olfactory epithelium cultures (24) (A. Calof and S. Whitehead, unpublished results). We have also shown that olfactory Schwann cells (ensheathing cells) may

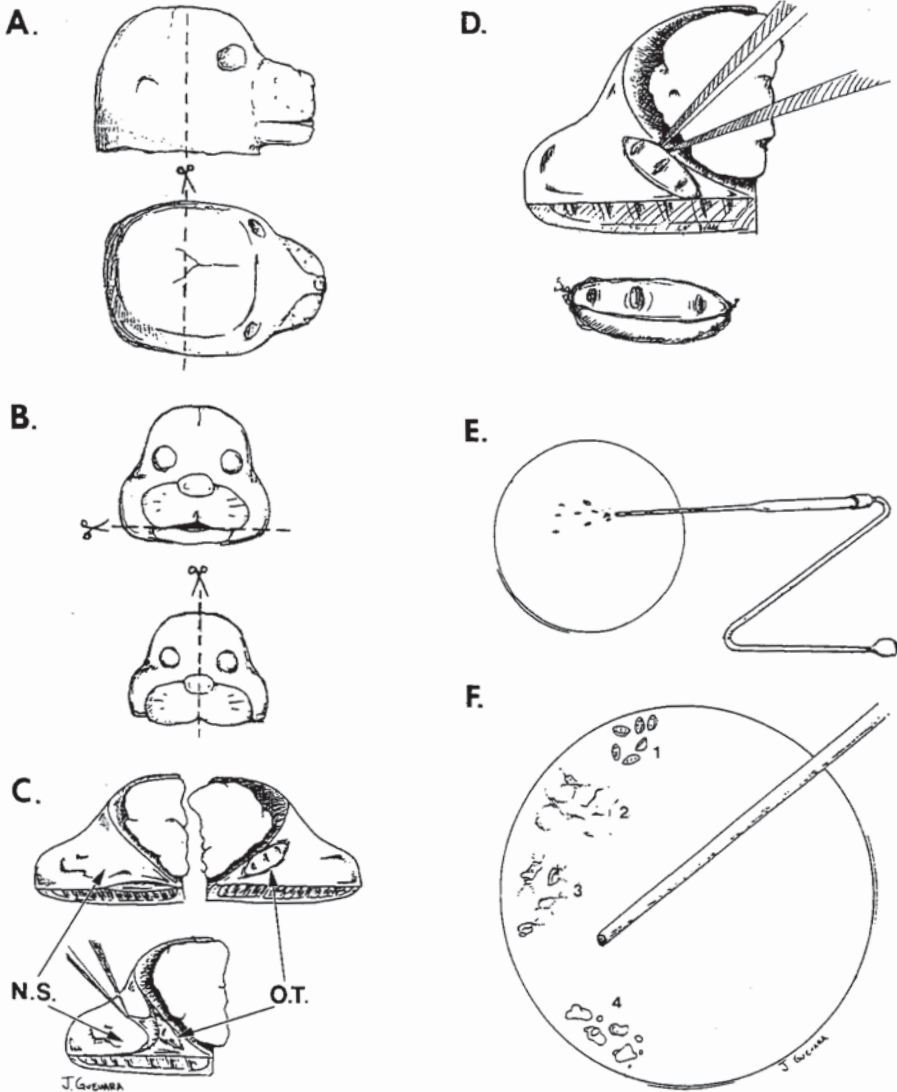


Figure 1. Dissection of olfactory turbinates and purification of olfactory epithelium. See *Protocols 1 and 2.*

appear in long-term OE cultures (24). For immunocytochemical procedures see section 5.

3.1 Preparation of substrata for explant cultures

For explant culture of OE, we use acid-cleaned glass coverslips that have been coated with one of several extracellular matrix molecules. For olfactory

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receptor neuron axon outgrowth and cell migration, laminin or its homologue merosin are the best substrata; however, fibronectin is an excellent substratum for epithelial attachment (3,7). We have found the best 'compromise' is to coat coverslips with poly-D-lysine (Sigma, P-0899, 1 mg/ml in ddH₂O, 4 h to overnight), then wash them four times with ddH₂O, and sterilize by ultraviolet irradiation. The coverslips are then coated with merosin (Gibco-BRL, 12162-012) at 10 µg/ml in Ca²⁺/Mg²⁺-free Hank's balanced salt solution (CMF-HBSS; Cellgro Mediatech, 21-021-LM) for 3 h at 37 °C. After rinsing four times in sterile CMF-HBSS, the coverslips are placed into the wells of a 24-well tissue culture tray (we use Falcon, 3047), covered in low calcium culture medium, and explants are plated on to them.

Protocol 3. Acid cleaning glass coverslips for tissue culture

1. Place one ounce of round glass coverslips (12 mm, No. 1 thinness; Propper Manufacturing, Long Island City, NY) into 500 ml of 1% HCl in ddH₂O in a 2 litre Pyrex beaker.
2. In a fume hood, boil the coverslips in the HCl solution for 1-3 h, stirring frequently with a glass rod.
3. Pour off the HCl solution, and add 500 ml ddH₂O to the beaker. Bring the water to the boil, and then pour off. Do this three times.
4. After the third rinse in boiling ddH₂O, rinse the coverslips once in 95% ethanol, swirling gently.
5. Discard the ethanol, cover the beaker loosely with aluminium foil, and dry the coverslips in a vacuum oven set at 60 °C overnight.

3.2 Culture media

All of our tissue culture media are made with a lower level of calcium than is present in most tissue culture media (0.1 mM as opposed to 2 mM), since we have found this to be optimal for cell sorting and cell migration in cultures of mouse OE (3,7). The basal medium used for culturing OE consists of two-thirds calcium-free DME (see *Protocol 4*), and one-third Ham's F-12; this medium is supplemented with additives to promote cell growth.

3.3 Culturing explants

In many of our studies, we analyse the effects of pharmacological agents and polypeptide growth factors on purified olfactory epithelium grown on the substrata described above. Chopping the OE into small pieces increases the number of explants which can be analysed.

Protocol 4. Low calcium OE culture medium

Reagents

- Calcium-free DME: to a 500 ml tissue culture bottle (preferably a new polystyrene bottle) add 50 ml 10 x Earle's balanced salt solution with phenol red ($\text{Ca}^{2+}/\text{Mg}^{2+}$ -free; Gibco-BRL, 14160-022), 5.833 ml 30% glucose, 5 ml 100 x $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (20 mg/ml in ddH_2O), 10 ml MEM amino acids solution without L-glutamine, 50 x (Gibco-BRL, 11130-010), 5 ml MEM non-essential amino acids, 10 mM, 100 x (Gibco-BRL, 320-1140AG), 5 ml MEM vitamins solution, 100 x (Gibco-BRL, 11120-11), 0.5 ml phenol red 0.5%, 0.5 ml 1000 x $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ (10 mg/litre in ddH_2O), 1.1 g NaHCO_3 , take up to final volume of 500 ml with ddH_2O —filter through 0.2 μm filter to sterilize, and store at 4 °C
- Serum-free additives (100 x stock): to 60 ml $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's balanced salt solution (CMF-HBSS; Cellgro Mediatech, 21-021-LM) add 10 ml insulin (Sigma, I 5500), 10 mg/ml in 10 mM HCl, 100 mg transferrin (Sigma, T 1283), 100 μl progesterone (Sigma, P 8783), 2 mM in 100% ethanol, 10 ml putrescine (Sigma, P 5780), 100 mM (16.11 mg/ml) in CMF-HBSS, 100 μl sodium selenite (Sigma, S 5261), 3 mM (519 $\mu\text{g}/\text{ml}$) in CMF-HBSS, 200 mg crystalline BSA, take to a final volume of 100 ml with CMF-HBSS—filter sterilize (0.2 μm), make 1-2 ml aliquots in sterile polypropylene tubes, and store at -80 °C

Serum-free additives are a modification of the additives devised by Bottenstein and Sato for the growth of neuroblastoma cells (25). The final concentration of the additives in the culture medium is: insulin, 10 $\mu\text{g}/\text{ml}$; transferrin, 10 $\mu\text{g}/\text{ml}$; progesterone, 20 nM; putrescine, 100 μM ; and selenium, 30 nM.

Method

1. To prepare the medium, first add 500 mg crystalline BSA to 70 ml calcium-free DME, and mix until the BSA has dissolved. The resulting solution will be acidic (the medium will be yellow), and should be neutralized by adding 1 M NaOH dropwise (50-100 μl is usually sufficient to restore the pH, turning the medium pink again).
2. Add the following:
 - 35 ml Ham's F-12 with L-glutamine (Cellgro Mediatech, 10-080-LV)
 - 0.5 ml penicillin-streptomycin (10 000 U/ml)
 - 1 ml L-glutamine, 200 mM, 100 x (Gibco-BRL, 320-5030AG)
 - 1.15 ml serum-free additives, 100 x
3. Sterilize by filtering through a 0.2 μm filter.
4. Store at 4 °C.

Protocol 5. Explant culture

1. After transferring the isolated epithelium to the second dish of low calcium medium (Protocol 2), chop the OE into fine pieces using a sharp blade or knife (we use tungsten wire etched in 10% KOH).

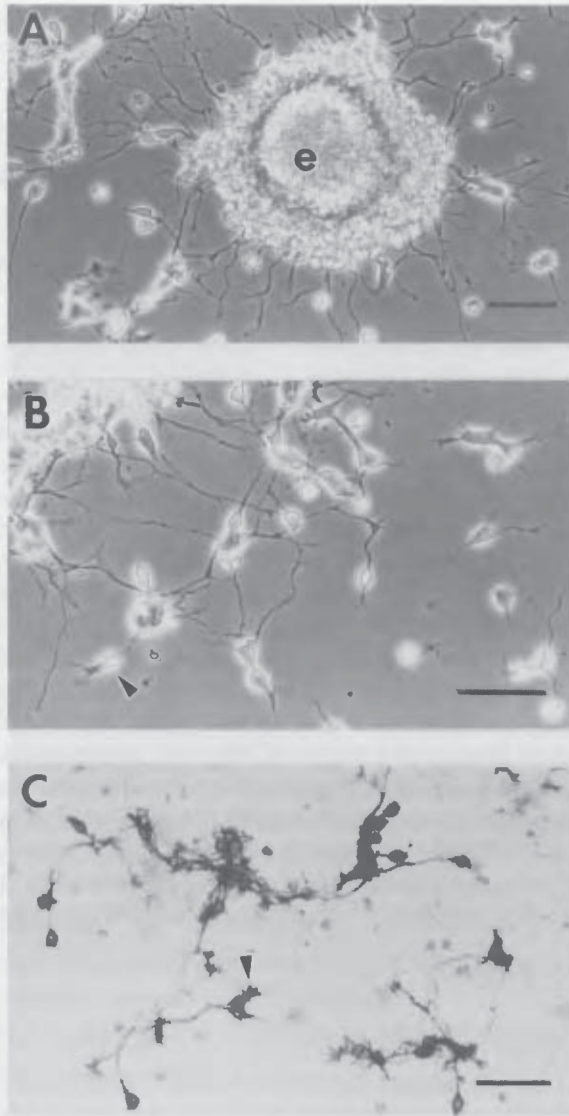
2. After chopping, gently swirl the dish until all of the tissue is in the centre of the dish. Using a sterile Pasteur pipette, gently take up the pieces of epithelium, and distribute by mouth pipette on to acid-cleaned, treated coverslips which have been placed into the wells of a 24-well tissue culture tray and covered with low calcium medium (1.5 ml/well). Optimal density is obtained by using one 12 mm round coverslip for every three or four embryos dissected.
3. Incubate at 37 °C in a 5% CO₂ atmosphere.

3.4 Growth factor requirements

Explants prepared and cultured as described above will survive for several days. However, the INPs cease to divide unless they receive appropriate growth factor stimulation (5), and olfactory receptor neurons begin to die within this period unless they are grown in agents that inhibit programmed cell death (6,18,21). For example, aurintricarboxylic acid (100 µM; Sigma, A 0885) promotes the survival of dissociated olfactory receptor neurons (see section 4) at late times in culture (see *Figure 2C*). Basal cells of the epithelium require epidermal growth factor for their continued proliferation and survival (3,4). Ongoing studies in our laboratory are directed toward identifying specific polypeptide growth factors that will stimulate proliferation of INPs and promote long-term survival of olfactory receptor neurons. To date, we have found that members of the fibroblast growth factor family stimulate proliferation by INPs in explant cultures (5,18), and that individual members of the neurotrophin family will promote survival of small numbers of dissociated olfactory receptor neurons (see section 4), although no single neurotrophin will allow survival of all olfactory receptor neurons in a culture (6,18,21).

4. Culture of isolated neurons and neuronal precursors

In many of our experiments, we isolate the olfactory receptor neurons and their precursors (the immediate neuronal precursors, or INPs) from the basal cells of the epithelium. Immunocytochemical analysis has demonstrated that the cells in this fraction, referred to as the neuronal cell fraction, are either olfactory receptor neurons (~ 74%) or INPs (~ 26%) (5-7). Culturing these cells in the absence of the other OE cell types (i.e. basal cells and sustentacular cells) allows us to study directly the effects of growth factors and pharmacological agents on the neuronal population.



4.1 Preparation of substrata for dissociated neuronal cell fraction

Cultures of the dissociated neuronal cell fraction grow well at high density on tissue culture plastic coated with poly-D-lysine. For these studies, we coat the wells of 96-well tissue culture trays (Costar, 3596) overnight at 4°C with 50 μ l/well of poly-D-lysine (1 mg/ml in ddH₂O). The plates are then washed five times in ddH₂O and sterilized by ultraviolet irradiation.

Figure 2. Olfactory epithelium and olfactory receptor neurons in explant and dissociated cell culture. (A) Explant of olfactory epithelium purified from E14 CD-1 mouse embryos and grown for 18 h in low calcium culture medium. The substratum is an acid-washed glass coverslip, coated with merosin. Note the many axonal processes and olfactory receptor neuron cell bodies that have migrated out of the epithelial explant ('e'), even at this early time in culture. Bar = 50 μ m. (B) Higher power view of olfactory receptor neurons that have migrated out of an olfactory epithelium explant grown in the same conditions as in (A). The *arrowhead* points to an olfactory receptor neuron with a prominent dendrite. Bar = 50 μ m. (C) Olfactory receptor neurons grown in dissociated cell culture in a polylysine coated 96-well tissue culture tray. After being cultured for 72 h in low calcium medium containing 100 μ M aurintricarboxylic acid (6,18), the cells were fixed in Omnifix II and processed *in situ* for NCAM expression as described in section 5.1(b), using the Vector ABC-AP kit followed by a blue chromagen (Vector SK-5300 blue). Note the thick apical dendrites (*arrowhead*) and the thin axonal processes of the neurons. Bar = 50 μ m.

4.2 Suspension culture and isolation of the neuronal cell fraction

The initial preparation of the neuronal cell fraction is identical to that of the olfactory epithelium explants. After isolating the purified epithelium, however, three new steps are added:

- (a) The isolated epithelium is cultured in suspension for 6–12 h (during this time, olfactory receptor neurons and their precursors sort out from, but remain loosely attached to, the basal epithelial cells, which form a ball of tightly packed cells).
- (b) The neuronal cell fraction is isolated from the basal cells by sequential enzymatic digestion and mechanical dissociation.
- (c) Cells in the neuronal cell fraction are plated into the wells of tissue culture trays.

Protocol 6. Dissociation and isolation of olfactory neurons and neuronal precursors from suspension cultures

Equipment and reagents

The solutions should be sterilized by filtering with a 0.2 μ m filter and stored at -20°C in 1–2 ml aliquots.

- Nylon mesh, 10 μ m (CMN-10-D; Small Parts, Inc., Miami, FL)
- Trypsin solution (5 mg/ml): dissolve 100 mg trypsin (Sigma, T-8253) in 20 ml Leibovitz's L-15 medium (Gibco, 320-1415AJ)
- Trypsin inhibitor solution (5 mg/ml): dissolve 250 mg trypsin inhibitor (Type I-S from soybean; Sigma, T-9003) in 50 ml L-15
- Deoxyribonuclease (DNase) solution (1 mg/ml): dissolve 100 mg DNase I (Sigma, D-5025) in 100 ml Dulbecco's modification of Eagles medium (DME; Mediatech, 15-013-LV)
- 4% crystalline BSA: dissolve 4 g crystalline BSA in 100 ml CMF-HBSS

Protocol 6. Continued

Method

1. After collecting purified epithelium in the second dish of low calcium medium (see *Protocol 2*), transfer the epithelium to a third 60 mm Petri dish containing 8–10 ml low calcium medium. Incubate the cells in this dish for 6–12 h at 37 °C in a 5% CO₂ atmosphere.
2. Transfer the tissue from the suspension culture into a sterile 15 ml conical polystyrene centrifuge tube.
3. Rinse the suspension culture dish with 4–5 ml CMF-HBSS, and add the rinse to the centrifuge tube.
4. Spin for 5 min at 100 *g*.
5. After carefully aspirating the supernatant, add 4 ml CMF-HBSS to the tissue pellet.
6. Add 0.4 ml trypsin (5 mg/ml), and incubate in a 37 °C water-bath for 4 min.*
7. Add 1 ml of 1 mg/ml DNase to the cell suspension, swirl to mix, and return to the water-bath for 6 min.
8. Add 1 ml of 5 mg/ml trypsin inhibitor.
9. Mechanically dissociate the cells by triturating ten times using a flame-polished Pasteur pipette.
10. Let sit for 3 min (this allows the larger clumps, composed primarily of basal cells, to settle to the bottom of the tube).
11. Remove 4.5–5 ml from the top of the cell suspension, and filter this through sterile (autoclaved) 10 µm nylon mesh into another 15 ml conical centrifuge tube.
12. To the remaining cell suspension (approx. 1.5–2 ml) add 4 ml CMF-HBSS and 1 ml of 5 mg/ml trypsin inhibitor.
13. Repeat the trituration, settling, and filtration steps (9–11), collecting both filtrates in the same 15 ml conical centrifuge tube.
14. Underlay the filtered cell suspension with 2 ml 4% crystalline BSA (in CMF-HBSS), and spin for 10 min at 100 *g*.
15. Remove the supernatant by aspirating with a flame-polished Pasteur pipette, and resuspend the pellet in a small volume (~ 1.5 ml) of low calcium medium.
16. Use a haemocytometer to count the cells, and plate cells at the desired density into polylysine coated 96-well tissue culture trays (see section 4.1). For high density cultures, we typically plate ~ 3 x 10⁴ cells/well of a 96-well tray.

* Alternatively, the trypsin may be replaced with 200 µl of 1 mg/ml dispase (Grade I, > 6 U/mg, made in L-15; Boehringer Mannheim, 210455), and incubated for 10 min at 37 °C; or the dissociation may be done without the use of proteolytic enzymes, by eliminating step 6 and proceeding directly to step 7 (eliminate all 37 °C incubations if no enzymes are used).

5. Analysis of cell types within olfactory epithelium cultures

Cell type specific markers have proved invaluable for analysing the generation, differentiation, and maturation of mouse olfactory receptor neurons, both *in vivo* and *in vitro* (3-6,18,21,22). Immunocytochemical analysis of the cell types present in mouse olfactory epithelium cultures can be performed using a number of different antibodies. We list below those antibodies that have been used successfully in our laboratory, along with the fixation conditions, and immunocytochemical procedures that we currently use.

5.1 Olfactory receptor neuron markers

- (a) H28 anti-NCAM (neural cell adhesion molecule) (rat hybridoma): H28 rat IgG (kind gift of Christo Goridis) recognizes an extracellular domain common to all three forms of the NCAM polypeptide, and is used as full strength tissue culture supernatant (26) (also available from AMAC, Inc., Westbrook, ME; Cat. No. 0270). Fixation: 3.7% formaldehyde in PBS, 10 min at room temperature. Visualize with Texas red goat anti-rat IgG (Jackson), 1 : 50 dilution.
- (b) AG1D5 anti-NCAM: mouse anti-NCAM ascites fluid diluted 1 : 500, or full strength tissue culture supernatant (3); recognizes a cytoplasmic domain common to the 140 and 180 kDa forms of NCAM. Fixation: acetone, room temperature, 5 min; or Omnifix II (An-Con Genetics, Inc., Melville, NY), 10 min. Visualize with rhodamine goat anti-mouse IgG (Tago), 1 : 100 dilution. This antibody can also be visualized with goat anti-mouse IgG biotin (Vector, 2.5 µg/ml), followed by alkaline phosphatase conjugated to avidin (Vector, AK-5000; alkaline phosphatase ABC kit).
- (c) Anti-GAP43: sheep antiserum to GAP-43 (generous gift of L. Benowitz) used at a 1 : 1000 dilution (3) (a monoclonal anti-GAP-43 also available from Boehringer Mannheim, 1379 011). Fixation: 3.7% formaldehyde in PBS, 10 min at room temperature; permeabilize with 0.1-0.2% Triton X-100 in PBS. Visualize with rabbit anti-sheep biotin (Vector; 2.5 µg/ml) followed by avidin-alkaline phosphatase (as specified in Vectastain ABC-AP kit).

5.2 Olfactory Schwann cell (ensheathing cell) markers

- (a) Anti-S100: rabbit anti-S100 (Dako, Z311) use at a 1 : 200 dilution (24,27). Fixation: PPG (4% paraformaldehyde/0.2% picric acid/0.05 % glutaraldehyde/0.1 M sodium phosphate, pH 7.0), 15 min. Permeabilize with 0.1-0.2% Triton X-100 in PBS. Visualize with FITC-conjugated goat anti-rabbit IgG (Capell), 1 : 100 dilution.

- (b) Anti-GFAP (glial fibrillary acidic protein): rabbit anti-GFAP IgG (Dako, Z334) is used at 1 : 100 (24,27,28). Fixation: acetone. Visualize with FITC-conjugated goat anti-rabbit IgG (Capell), 1 : 100 dilution.

5.3 Sustentacular cell marker

3C2 anti-mucin: mouse hybridoma producing anti-mucin IgM (kind gift of Pat Levitt and Steve Prouty). Full strength hybridoma supernatant (23). Fixation: PPG, acetone, or Omnifix II. Visualize with Texas red goat anti-mouse IgM (Jackson), 1 : 100 dilution.

5.4 Basal cell marker

Anti-cytokeratin: Dako rabbit polyclonal anti-keratin (wide spectrum screening; Dakopatts, Z622) used at 1 : 400 dilution (3). Fixation: Omnifix II or 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7, either fixative for 10 min at room temperature. Permeabilize with 0.1–0.2% Triton X-100 in PBS. Visualize with FITC goat anti-rabbit IgG (Jackson or Kirkegaard-Perry), 1 : 50 dilution.

Acknowledgements

The authors thank Jeff Mumm, Sara Whitehead, and Arthur Lander for their contributions to developing many of the procedures that are referred to in this manuscript. We are grateful to Shelley Plattner and John Busse for photographic work. This work was supported by grants DC02180 and NS32174 from the National Institutes of Health to A. L. Calof.

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