Purification and culture of nerve growth factor receptor (p75)-expressing basal forebrain cholinergic neurons

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The activity of the basal forebrain cholinergic neurons (BFCNs) that innervate the cerebral cortex and hippocampus is essential for normal learning and memory. Here, we present a method to isolate and culture BFCNs from the embryonic murine septum that takes advantage of their restricted expression of the nerve growth factor receptor (p75) in conjunction with fluorescence-activated cell sorting. The septal region dissection, cell dissociation and staining process, and cell sorting parameters are described in detail. Sufficient cell yield and optimized cell culture conditions make this protocol suitable for multiple assays including immunocytochemistry, reverse transcriptase PCR, microarray profiling, acetylcholine measurements and electrophysiological assessment. The study of these neurons as a purified population will greatly advance our understanding of factors that influence their development and maintenance.

INTRODUCTION

Cholinergic neurons have critical roles in the central (CNS) and peripheral nervous systems. In the brain, cholinergic projection neurons target the hippocampus, amygdala, cortex and thalamus, where they regulate multiple processes including attention, learning, memory and sleep. Cholinergic neurons in the basal forebrain provide afferents from the medial septum and the diagonal band of Broca to the hippocampus, and from the nucleus basalis magnocellularis to the neocortex[1–3]. Study of the development of BFCNs is fundamental to both the understanding of learning and memory and to the development of new treatments for diseases affecting these processes. However, the development of a purification technique has been hampered by a paucity of adequate markers of BFCNs and their low abundance in the septum.

Previously, *in vitro* studies examining BFCNs have been accomplished by culturing the whole septal cell population[4]. However, cholinergic neurons in this region represent only a minority population[5]. Thus, the ability to isolate and study these neurons alone could answer important questions concerning their development and unique characteristics. Cholinergic neurons first arise in the mouse basal forebrain *circa* embryonic day (E)11 and express cholinergic markers by E16 (see ref. 6). In the basal forebrain, specifically in the septum and the nuclei of the diagonal band, 90% of cholinergic neurons express the low-affinity nerve growth factor receptor p75 (p75+ cells) and 90% of the p75+ cells are cholinergic neurons[7–9]. Moreover, p75 is one of the earliest known cell surface markers expressed by these neurons[10].

Fluorescence-activated cell sorting (FACS) is a powerful tool used extensively in immunology to identify cell subsets in mixed populations, measure changes in the expression of surface determinants and physically sort cells based on such characteristics. Although increasingly used for the analysis of CNS cells[11,12], FACS remains an underutilized tool for the purification of CNS cell subsets. We have previously shown that freshly FACS-isolated p75+ septal cells are highly enriched in cholinergic markers, whereas p75− cells are greatly depleted of these markers[13]. Microarray analysis of these fractionated cells revealed the distinct transcriptome of differentiated BFCNs. Here, we detail an optimized method utilizing FACS for the fast isolation of p75-expressing basal forebrain cells that can be used for the generation of healthy BFCN cultures. This method has important advantages over alternative cell sorting methods, such as magnetic bead separation and immunopanning, including the elimination of dead and dying cells and the exclusion of cell aggregates containing antigen-negative cells. In conjunction with fluorescent protein-expressing transgenic animals and microarray analysis, among other technologies, this approach will greatly expand our understanding of BFCNs and the factors that are required for their development and maintenance. Moreover, these cells will prove useful in the studies of cholinergic synapse formation and synaptic activity[14]. The success of this method demonstrates the utility of FACS in the fractionation of neuronal subsets from highly heterogeneous CNS populations for their *in vitro* study.

MATERIALS

REAGENTS

- CD-1 mice (Charles River Labs) **! CAUTION** Experiments involving live animals must conform to national and institutional regulations.
- Dulbecco's modified Eagle's medium (DMEM; Invitrogen, 12800; filter-sterilized)
- Neurobasal medium (NB; Invitrogen, 21103)
- 50× B-27 supplement (Invitrogen, 17504)
- Fetal bovine serum, heat-inactivated (FBS; Invitrogen, 16140)
- L-Glutamine (Invitrogen, 25030)
- Bovine serum albumin (BSA; Sigma, A9430)
- Basic fibroblast growth factor (FGF; PeproTech, 100-18B)
- Poly-D-lysine (Sigma, P7280)
- Laminin (Invitrogen, 23017)
- Penicillin/streptomycin (Invitrogen, 15070)

- DNase I (Sigma, D4263)
- Trypsin (0.25% (wt/vol); Invitrogen, 25200) ▲ CRITICAL The choice of proteolytic enzyme was found essential for the detection of cell surface p75. Although digestion with papain results in higher cell yield, we were unable to detect surface p75 on cells digested with this protease.
- Trypan blue solution (0.4% (wt/vol); Sigma, T8154)
- Phosphate-buffered saline (PBS; Sigma, P3813; filter-sterilized)
- Hibernate E (BrainBits)
- Anti-p75 antibody (Advanced Targeting Systems, cat. no. AB-N01AP); see **Supplementary Figure 1** online for evidence of specificity ▲ CRITICAL This antibody is affinity-purified using the extracellular domain of p75, and does not show any binding to septal cells prepared from p75-knockout mice, indicating its specificity for the receptor.
- Anti-rabbit IgG-Alexa488 antibody (Molecular Probes, A21441)
- Propidium iodide (Molecular Probes, P3566)
- Cytosine arabinoside hydrochloride (Sigma, C6645)
- GlutaMax (Invitrogen, 35050)
- Sigmacote (Sigma, SL2)
- 70% (vol/vol) ethanol

EQUIPMENT

- Humidified tissue culture incubator (37 °C, 5% CO_2)
- Laminar flow hood (safety cabinet)
- Inverted microscope
- Dissecting microscope (Zeiss, model 47 50 52)
- Microdissecting instruments (sterilized): two Dumont no. 5 standard tip forceps (FST, 11251-20); two angled to side spring scissors (FST, 15006-09 and 15008-08); one Noyes straight spring scissor (FST, 15012-12); one Adson 1 × 2 forceps (FST, 11027-12); one eye dressing forceps, serrated (FST, 11052-10)
- Fiber optic halogen illuminator (Nikon MKII)
- Tabletop centrifuge (IEC)
- Hemocytometer
- 15 ml conical tubes (Fisherbrand 05-539-5)
- 50 ml conical tubes (Fisherbrand 05-539-6)
- Serological pipettes (Fisherbrand 13-676-10H/J/K)
- 9-inch glass pipettes (Fisherbrand 13-678-20D)
- Flame (alcohol wick or natural gas burner)
- Pipette pump (2 ml; Scienceware 37897000)
- 48-well tissue culture plates (BD Falcon, 353078)
- 0.2 µm sterile filter units (Nalgene, 156-4020)
- 100 mm dishes (BD Falcon, 353003)
- 60 mm dishes (BD Falcon, 353002)
- 40 µm nylon mesh filters (BD Falcon, 352340)
- Polypropylene tubes (12 mm × 75 mm; BD Falcon, 352063)
- BD BioCoat poly-D-lysine/laminin-coated coverslips (BD Falcon, 354087)
- FACS (e.g., DakoCytomation, MoFlo)

REAGENT SETUP

Plating medium Sterile filtered DMEM, 10% (vol/vol) FBS, 50 U ml^{-1} each of penicillin and streptomycin; shelf life, 2 weeks at 4 °C. Add 20 ng ml^{-1} basic FGF immediately before use.
NB/B-27 NB, 1× B-27, 250 µM L-glutamine, 250 µM GlutaMax, 50 U ml^{-1} each of penicillin and streptomycin; shelf life, 2 weeks at 4 °C. Add 20 ng ml^{-1} basic FGF and 2 µM cytosine arabinoside hydrochloride immediately before use. See **Table 1**.
4% BSA 4% (wt/vol) BSA in PBS, pH 7.4. ▲ CRITICAL The pH must be readjusted with 1 N NaOH after addition of BSA to the PBS. Filter-sterilize.
DNase solution Resuspend one vial of DNase I containing 2,000 Kunitz units in 20 ml of Hibernate E; shelf life, 1 week at 4 °C.
Poly-D-lysine 5 mg ml^{-1} in filter-sterilized dH_2O and store in 3 ml aliquots at −20 °C.
Laminin 20 µg ml^{-1} in filter-sterilized dH_2O and store in 2.5 ml aliquots at −80 °C. ▲ CRITICAL Laminin must be thawed slowly to avoid gelatinization.

EQUIPMENT SETUP

Fire-polished, siliconized pipettes Polish the tips of 9-inch glass pipettes over a small flame (alcohol wick or natural gas burner) such that the edges are smoothed and the aperture is slightly narrowed (**Fig. 1**). ▲ CRITICAL If the aperture is too narrow, the cell yield will be greatly diminished. Siliconize the inside of each pipette by brief aspiration of Sigmacote solution. Allow the pipettes to dry and sterilize by autoclaving. Generally, one or two pipettes are used for every 20 dissected septa. See **Table 1**.
Poly-D-lysine/laminin-coated plates Apply enough poly-D-lysine solution to each well such that the entire culture surface is coated. Because the surface only needs to be in brief contact with the coating solution, one can use aspirated solution to coat subsequent wells. Vacuum-aspirate any remaining solution and allow the culture surface to dry (generally for 30–60 min, depending on ambient conditions). Apply enough laminin to coat each culture well. Use aspirated laminin to coat subsequent wells. Allow plates to dry. We have stored dry, coated plates at 4 °C for up to 2 months with no reduction in performance. See **Table 1**.
Poly-D-lysine/laminin pre-coated coverslips Pre-incubate BD BioCoat 12 mm coverslips with 75 µl of plating medium for at least 1 h before the culture of the sorted neurons.

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PROCEDURE

Dissection of the embryonic forebrain septa ● TIMING 2–4 h

1| Euthanize the required number of E17 or E18 timed-pregnant dams (typically 4/5, yielding ∼50 embryos).
▲ CRITICAL STEP The entire procedure should be executed as quickly as possible. Prolonged storage of the tissue or cells at any point will reduce cell yield and viability. Ice should be used at all times during the temporary storage of specimens and during the procedure, unless otherwise noted. All centrifugation steps may be done at 4 °C or room temperature (20–25 °C).

2| Spray the abdomen with 70% ethanol. Make a midline abdominal incision starting at the level of the upper limits of the bladder to the sternal apophysis, followed by two small mid-lateral incisions on each side at the lower end of the previous one, to open two flaps of the abdominal wall. Using the Adson forceps, lift the uterine sacs at the level where they meet and cut the vaginal attachment to remove the uterus by gently pulling upward. Store uteri in 50 ml conical tubes in 1× PBS on ice until you are able to proceed with the dissection.

3| Place one uterus in a 100 mm Petri dish on ice.

4| Remove embryos from the uterus and amniotic sac and decapitate with the Noyes straight spring scissors and the eye dressing forceps. Place up to 12 heads in a 60 mm dish containing just enough Hibernate E to cover the heads.

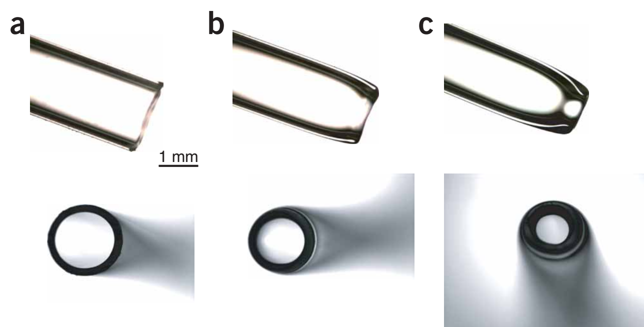

Figure 1 | Fire-polished glass pipettes. The end of a 9-inch glass pipette (**a**) is briefly exposed to flame to smooth its edges and slightly narrow the aperture (**b**). An aperture that is too narrow (**c**) results in increased cell loss.

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5| Repeat Steps 3 and 4 for the remaining uteri. Heads can be kept in ice-cold Hibernate E.

6| Place one 60 mm dish containing the heads under a dissecting microscope on a container with ice. For better visualization, slip a small sheet of dark plastic under the dish to enhance contrast.

7| Orient a head on its right side and hold it in place with the Dumont no. 5 forceps on the ventral side. Using the FST 15006-09 scissors, make small incisions just above the eye and ear from the forehead to the back of the head (6–8 incisions are generally required to cut all the way through the base of the skull) (**Fig. 2a,b**).

8| Gently open the incision in two halves and tease away the brain from the skull (**Fig. 2c–e**; **Supplementary Video 1** online).

9| Remove the olfactory tubercles (**Fig. 2f**; **Supplementary Video 1**).

10| Repeat Steps 7–9 for the remaining heads in the dish.

11| Transfer all the brains to a new 60 mm dish containing Hibernate E.

12| Proceed with Steps 13–17 for each brain using the FST 15008-08 scissors and Dumont no. 5 forceps.

13| Orient the brain on its ventral side and gently spread apart the cortices. It may be necessary to cut through the corpus callosum to expose the septum (**Fig. 2g,h**; **Supplementary Video 1**).

14| Make one cut on each side of the septum to liberate it from the telencephalic hemispheres[15] (**Fig. 2i**; **Supplementary Video 1**).

15| Make a transversal cut to separate the posterior side of the septum from the anterior pole of the diencephalon (i.e., the thalamus)[15] (**Fig. 2j**; **Supplementary Video 1**).

16| Use the scissors to gently tease the septum away from the meninges (**Fig. 2k**; **Supplementary Video 1**).

17| Once all the septa have been dissected, mince each into 6–8 pieces and transfer the minced septa with a P1000 pipette to a 15 ml conical tube containing 1.5 ml Hibernate E on ice. Each conical tube should contain minced pieces from not more than six septa.
? TROUBLESHOOTING

18| Repeat Steps 6–17 for the remaining 60 mm dishes containing heads.

Dissociation of embryonic forebrain septal cells ● TIMING 1 h

19| In a laminar flow hood, aspirate the supernatant Hibernate E from each 15 ml conical tube containing the sedimented, minced septal pieces.

20| Add 1 ml trypsin and 0.5 ml DNase solution to each tube and gently mix the pieces.

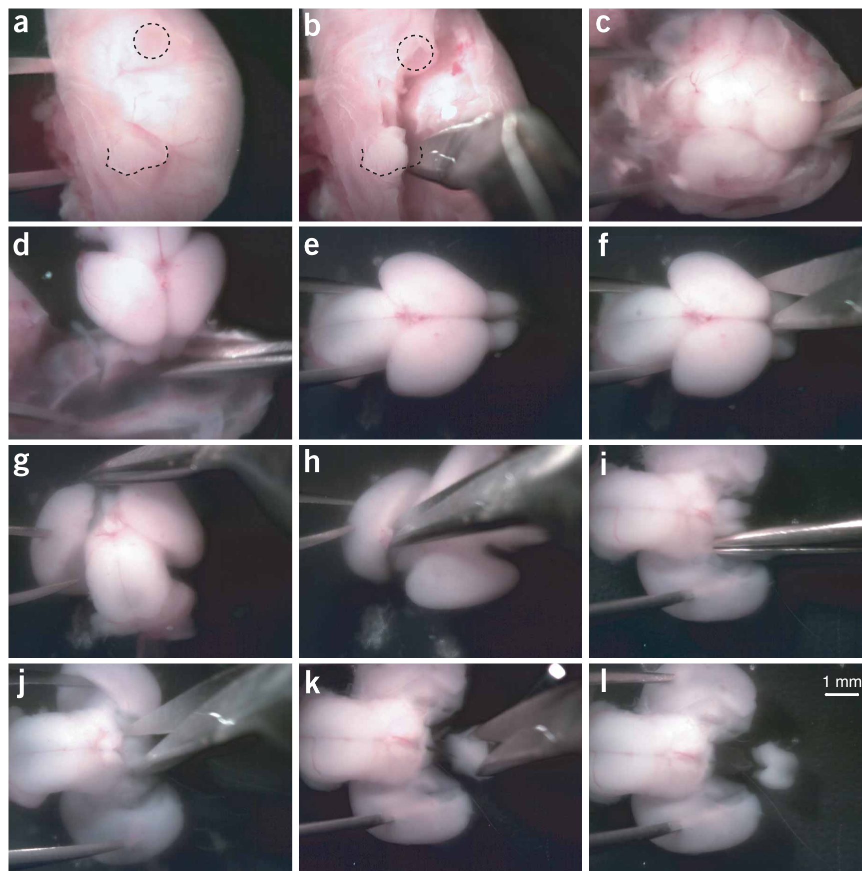

Figure 2 | Dissection of the late embryonic septum. (**a**,**b**) While steadying the head on its right side, cut just above the eye and ear (indicated with dotted lines), all the way through the base of the skull. (**c**,**d**) Remove the brain by gently lifting it away from the skull and severing the connections at the olfactory bulbs. (**e**,**f**) Remove the olfactory bulbs. (**g**,**h**) Cut the corpus callosum on each hemisphere to expose the septum. (**i**,**j**) Cut on either side of the septum and at its posterior side. (**k**,**l**) Gently tease the septum away from the meninges. See **Supplementary Video 1** showing **e**–**l**.

21| Incubate the tubes at 37 °C for 10 min, agitating gently after the first 5 min.

22| Aspirate the trypsin/DNase solution and add 0.5 ml of 4% BSA, 1 ml plating medium and 2 ml DNase solution to each tube. All solutions are pre-chilled. Keep the septa-containing tubes on ice.

23| To achieve single-cell suspensions, pipette the septal pieces repetitively through a fire-polished, siliconized glass pipette using a 2 ml pipette pump. The first approximately 10 passes should be slow and gentle, followed by increased speed and force as the tissue dissociates. 2 to 4 minutes are generally required to dissociate all the septal pieces in each tube.
? TROUBLESHOOTING

24| Once single-cell suspension has been achieved for each tube, underlay the suspensions with 2.5 ml of cold 4% BSA by dispensing the solution at the bottom of the conical tube from a 5 ml serological pipette.

25| Centrifuge the tubes at 250*g* for 5 min in a swinging bucket rotor.

26| Aspirate the supernatants and resuspend pellets into 5 ml of 0.2% BSA in 1× PBS, pH 7.4. Use an additional 5 ml of 0.2% BSA to rinse the tubes and combine with the first 5 ml.

27| Strain the cell suspension through a 40 μm nylon mesh filter into a 50 ml conical tube. Rinse the mesh with an additional 5 ml of 0.2% BSA. Keep the cells on ice.

Isolation of p75+ septal cells ● TIMING 4–5 h

28| Increase the volume of suspended cells with cold 0.2% BSA in 1× PBS, pH 7.4, such that there is 7.5 ml for every ten dissected septa, rounding to the nearest 10 (e.g., if 43 septa were dissected, increase the volume to 30 ml).

29| Aliquot 7.5 ml of the cell suspension to 100-mm tissue-culture-treated Petri dishes without additional extracellular matrix coating.

30| Incubate the Petri dishes undisturbed for 20 min at room temperature.
? TROUBLESHOOTING

31| Observe the cells with an inverted microscope. A low frequency (∼1% of total cell number) of round, flat cells that have tightly adhered to the culture surface should be apparent. If not, incubate the cells for an additional 10 min.

32| Transfer the non-adherent cells to 15 or 50 ml conical tubes and remove a small aliquot to count the cells as follows: mix the cells with Trypan blue solution (see the manufacturer's instructions) and count with a hemocytometer.

33| Centrifuge the tubes at 250*g* for 5 min.

34| Pool all the cells into one tube in 0.2% BSA in Hibernate E such that the concentration is 20×10^6 cells per ml.

35| Place 0.5×10^6 cells in a polypropylene tube. This will serve as the secondary antibody control.

36| Prepare a second polypropylene tube with 100 μl of 0.2% BSA in Hibernate E and anti-p75. When combined with the cell suspension, the final concentration of anti-p75 should be 5 μg ml^{-1}.

37| Add the cell suspension to the primary antibody-containing tube and mix gently.

38| Incubate the cells with the primary antibody for 35 min at 4 °C, gently mixing halfway through the incubation.

39| Add enough 0.2% BSA in Hibernate E to both the primary antibody tube and the control tube to fill them. Cap and centrifuge the tubes for 5 min at 250*g*.

40| Prepare enough Alexa488-conjugated anti-rabbit IgG secondary antibody (1:500 dilution in 0.2% BSA in Hibernate E) for the control cells and anti-p75 antibody-stained cells such that the final concentration will be 20×10^6 cells per ml.

41| Aspirate the supernatants and add 50 μl of secondary antibody solution to the control tube, and an appropriate amount to the anti-p75-stained tube.

42| Incubate the cells with secondary antibody for 25 min at 4 °C, gently mixing halfway through the incubation.

43| Add enough 0.2% BSA in Hibernate E to both the primary antibody tube and the control tube to fill them. Cap and centrifuge the tubes for 5 min at 250*g*.

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44| Resuspend anti-p75-stained cells at 15×10^6 cells per ml in 0.2% BSA in Hibernate E, and cells stained with only secondary antibody in 250 µl of 0.2% BSA in Hibernate E.

45| Strain cells through a 40 µm nylon mesh filter into an appropriate tube for cell sorting. For example, 12 mm × 75 mm polypropylene tubes are compatible with the MoFlo cell sorter (DakoCytomation).

46| Immediately before sorting, add propidium iodide to a final concentration of 1 µg ml^{-1}.

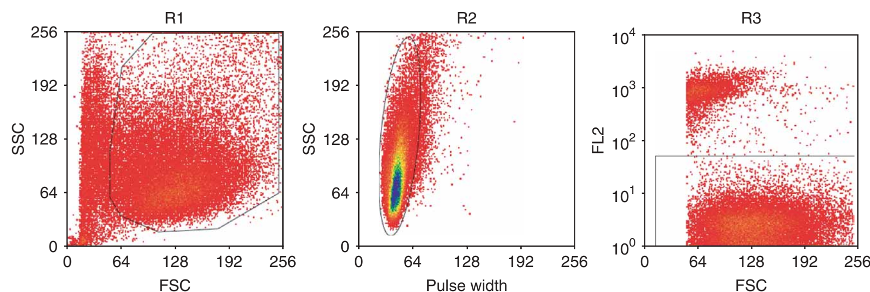


Figure 3 | Live, single-cell sorting gates for septal cells. Non-adherent E17 septal cells were isolated, stained with anti-p75 antibody and analyzed by FACS as described in Steps 1–47. Events depicted in regions R2 and R3 were gated through region R1. Percentage of events within the regions: R1, 72%; R2, 96%; R3, 90%.

47| Set the sorting gates using cells stained only with the secondary antibody. For example, we gate all cells to be collected through the forward-/side-scatter whole-cell gate (**Fig. 3,** R1), the pulse-width single-cell gate (**Fig. 3,** R2) and the propidium iodide-negative gate (**Fig. 3,** R3). The p75+ gate is set to the right of the cell population stained only with the secondary antibody, on the FL1 scale, such that less than 0.5% of these cells fall within the p75+ gate (**Fig. 4a,** R7).

48| Sort the anti-p75-stained cells into p75+ and p75− (if desired) populations using the appropriate instrument parameters and collect into 20% FBS in Hibernate E. Optimal parameters for MoFlo are as follows: nozzle width, 100 µm; trigger rate, 5,000 events per s; sheath pressure, 30 p.s.i.
▲ CRITICAL STEP Cell viability is greatly decreased with sorting durations of longer than 1.5 h.
? TROUBLESHOOTING

Culture of p75+ septal neurons (optional) ● TIMING ≥18 h

49| Centrifuge sorted cells for 5 min at 250*g*.

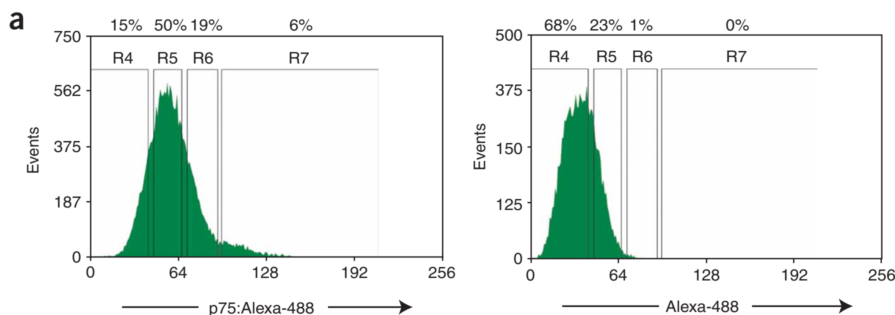
50| Resuspend cells in plating medium containing 20 ng ml^{-1} basic FGF added just before use. Recommended plating densities are as follows: 5×10^4 cells per 12 mm coverslip; 1.5×10^5 cells per well of the 48-well plate; 2.5×10^5 cells per well of the 24-well plate.

51| Culture cells overnight at 37 °C in a humidified, 5% CO_2 incubator.

52| Exchange media with NB/B-27 containing 20 ng ml^{-1} basic FGF and 2 µM cytosine arabinoside hydrochloride added just before use.
▲ CRITICAL STEP The sorted neurons are highly susceptible to lifting and drying upon aspiration of medium. Gently exchange medium in one well at a time. If culturing for longer than 3 d, exchange half of the medium with fresh NB/B-27 every 48 h.

● TIMING

Steps 1–18, dissection of embryonic forebrain septa: 2–4 h
Steps 19–27, dissociation of embryonic forebrain septal cells: 1 h
Steps 28–48, isolation of p75+ septal cells: 4–5 h
Steps 49–52, culture of p75+ septal neurons: ≥18 h



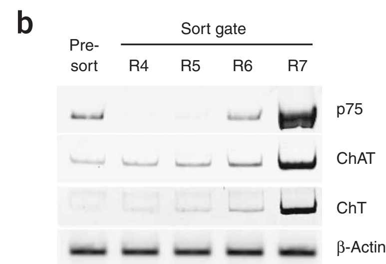


Figure 4 | Isolation of p75+ BFCNs via FACS. Non-adherent E18 septal cells were isolated, stained with anti-p75 antibody and sorted by FACS as described in Steps 1–48. Cells gated through R1, R2 and R3 (**Fig. 3**) were sorted based on p75 expression level into fractions R4–R7 (**a**) and analyzed by RT-PCR (**b**) for expression levels of the indicated mRNAs.

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? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1.**

TABLE 1 | Troubleshooting table.

Step(s)	Problem	Possible causes and solutions
17	Incomplete cell dissociation	Mince septa into finer pieces prior to tryptic digestion
Equipment setup		Fire-polished pipette aperture may be too wide
23		Use more force during repetitive pipetting
1–18	Low cell yield pre-sort OR	Dissection duration is too long Unless otherwise indicated, tissue and cells must be kept on ice at all times
Equipment setup	High % of cells outside of R1, or PI+ cells	Fire-polished pipette aperture may be too narrow
23		Repetitive pipetting force is too high Formation of air bubbles during repetitive pipetting Incomplete cell dissociation (see above)
47–48	Low cell yield post-sort	Sorting duration should be less than 1.5 hours Adjust sheath pressure and/or trigger rate Sort sample is too dense
28–30	Non-neuronal cells in the culture	Cell density during non-neuronal cell adhesion is too high
Reagent setup		Non-neuronal cells initially present will expire after approximately 3 days in culture. An expansion of any non-neuronal cells present at the time of media exchange to NB/B-27 may indicate that the concentration of AraC is not high enough
Equipment setup	Low survival in cell cultures	Culture surface is not adequate. Use fresh aliquots of poly-d-lysine and laminin
Reagent setup		AraC concentration is too high. The optimal concentration may need to be determined by titration Media, serum, and supplements must be fresh

ANTICIPATED RESULTS

The percentage of p75+ septal cells varies with age, but is 5–7% at E17 or E18. We typically sort p75+ cells from 50 to 60 septa in 1 d, yielding an average of 9,273 (± 806 s.e.m.) p75+ cells per E17 or E18 septum. This yield is in agreement with previous evaluations of the number of p75-expressing cells in the postnatal mouse septum[16]. We have found the late embryonic stages to be the most robust for the isolation of cholinergic neurons from the forebrain. For example, at E17 or E18, we typically observe greater than two-thirds (70.2% ± 0.7%) of the cells within the viable cell gate (**Fig. 3,** R1). However, at P0, this population is diminished to approximately 30% (data not shown). As we also exclude cell aggregates that fall outside the R2 gate (R2 = 94.6% ± 1.3%) and PI-positive cells that fall outside the R3 gate (R3 = 91.2% ± 1.6%), the yield of p75+ cells collected from the total events measured by the cell sorter is typically 3–4%.

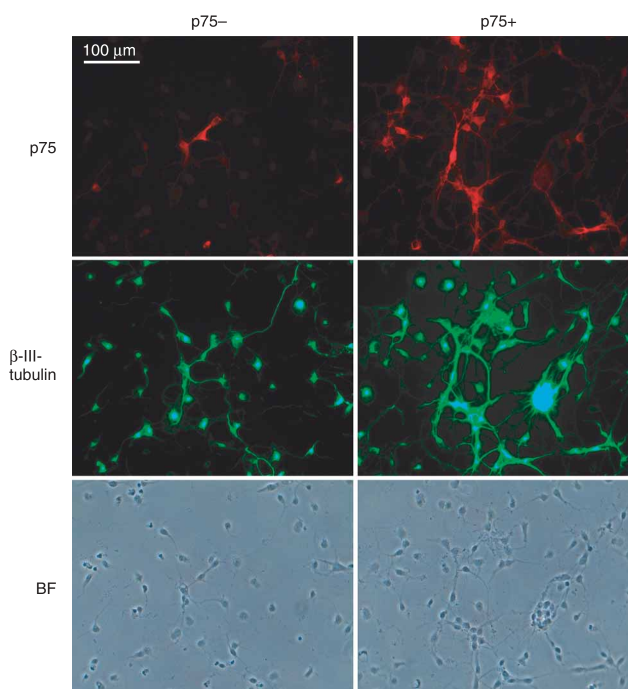

Figure 5 | Sorted p75− and p75+ septal cells persist in culture. p75− and p75+ E17 septal cells were dissociated, stained, sorted and cultured as described in Steps 1–52. After culturing for 3 d in NB/B-27, cells were fixed with 3% PFA, permeabilized with methyl alcohol and stained with anti-p75 and anti-rabbit IgG-Alexa594, or anti-β-III-tubulin and anti-mouse IgG-Alexa-488 antibodies. BF, bright field.

Freshly isolated p75+ septal cells collected from region R7 (**Fig. 4a**) are highly enriched in the expression of p75 and the cholinergic markers choline acetyltransferase (ChAT) and choline transporter 1 (ChT1, SLC5A7) (**Fig. 4b**). The cellular fractions collected through R4–R6 are depleted in the expression of these markers, in conjunction with p75 expression. We estimate that 50–80% of the p75-expressing cells collected from the cell sorter are viable 24 h after plating and throughout the subsequent culture period. After 3 d of culture in NB/B-27, the p75+ cells express high levels of p75 protein and are highly neuronal as determined by morphology and β-III-tubulin staining (**Fig. 5**). Conversely, the p75− cells have low and sparse p75 protein expression, but are also highly neuronal.

A cell-isolation approach utilizing FACS has been developed that will allow fundamental questions to be addressed concerning the development and nature of p75+ cholinergic neurons and the factors that regulate their phenotype. The use of surface marker-specific antibodies to label the cells of interest likely renders this method easily applicable to a range of neuronal cell subtypes. This method highlights the utility of FACS for the isolation of neurons within the different brain regions that have yet to be defined or characterized. With common equipment and reagents and access to a flow cytometry facility, researchers will be able to generate robust cell cultures from FACS-sorted primary neurons.

Alternative approaches

We have developed this method using separate primary and secondary antibodies to maximize the flexibility of the protocol. However, the use of a fluorescently conjugated primary antibody would allow the user to eliminate Steps 35 and 40–43, saving 30 min. Moreover, an increasing number of transgenic animals that express fluorescent proteins under specific promoters are becoming available, presenting an efficient alternative to the use of antibodies for labeling cells of interest. We have isolated BFCNs from ChAT-eGFP mice[17] with minor modifications to our protocol. In place of trypsin, we used papain (Worthington; follow manufacturer's instructions) to dissociate septal cells, thereby eliminating Steps 35–44, saving approximately 75 min. Similar to p75-stained septal cells, approximately 4% of septal cells from ChAT-eGFP E18 embryos are eGFP-positive, and these cells are enriched in cholinergic marker expression (data not shown).

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Note: Supplementary information is available via the HTML version of this article.

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