# 14

# Culture of Neuroendocrine and Neuronal Cells for Tissue Engineering

Peter I. Lelkes[1], Brian R. Unsworth[2], Samuel Saporta[3], Don F. Cameron[3], and Gianluca Gallo[4]

*[1] School of Biomedical Engineering, Science and Health Systems, Drexel University, Philadelphia, Pennsylvania; [2] Department of Biology, Marquette University, Milwaukee, Wisconsin; [3] Department of Anatomy and Center for Excellence for Aging and Brain Repair, University of South Florida Health Sciences Center, College of Medicine, Tampa, Florida; [4] Department of Neurobiology and Anatomy, Drexel College of Medicine, Philadelphia, Pennsylvania*

*Corresponding authors: pilelkes@drexel.edu, brian.unsworth@marquette.edu, ssaporta@hsc.usf.edu, gg48@drexel.edu*

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This chapter represents the joint effort of three distinct laboratories to describe their different approaches and methodologies for engineering neuroendocrine/neuronal tissues for the possible treatment of neurodegenerative diseases, such as Parkinson disease, which are related to the loss of catecholaminergic neurons. In the first part, Lelkes and Unsworth discuss the generation of three-dimensional catecholaminergic, adrenal medullary organoids with PC12 pheochromocytoma cells. In the second part, Saporta and Cameron focus on 3-D tissue constructs comprised of Sertoli cells and a neuronal cell line, NT2N. Finally, in the third part, Gallo discusses methods for isolating primary neuronal cells, using the chick dorsal root ganglion as a model system. Although each part was written by the authors mentioned above the overall responsibility for this enterprise rests with Peter I. Lelkes. The reader is advised to refer to each of the individual authors for specific questions regarding details of their particular methods.

## 1. THREE-DIMENSIONAL NEUROENDOCRINE/NEURONAL DIFFERENTIATION OF PC12 PHEOCHROMOCYTOMA CELLS

This section was contributed by Peter I. Lelkes and Brian R, Unsworth, to whom all related correspondence should be addressed.

### 1.1. Introduction

PC12 pheochromocytoma cells are cloned catecholaminergic cells derived from a spontaneous tumor (pheochromocytoma) of adrenal medullary chromaffin cells isolated from New England Deaconess rats [Greene and Tischler, 1976; Tischler and Greene, 1978]. Embryologically, chromaffin cells are derived from the sympathoadrenal (SA) lineage in the neural crest. In the rat, during migration out

of the neural crest, some SA cells arrive in the adrenal anlagen around embryonic day 10.5 and are arrested there both developmentally and functionally (a process termed neoteny), while other SA cells continue to migrate and differentiate eventually into sympathetic neurons [Anderson and Axel, 1986; Anderson, 1989, 1993]. In the adrenal anlagen, these SA cells differentiate into two distinct catecholaminergic chromaffin cell types, which are characterized as either noradrenergic or adrenergic, that is, each of these related, yet distinct, cell types synthesizes and secretes either noradrenaline (norepinephrine, NE) or adrenaline (epinephrine, E) [Moro et al., 1990; Weiss et al., 1996]. Recent studies indicate that the expression of noradrenaline in the NE cells during differentiation is independent of the presence of glucocorticoids (synthesized in the neighboring adrenal cortex). By contrast, the conversion of NE to E, which requires the functional expression of phenylethanolamine-$N$-methyltransferase (PNMT), the final enzyme in the catecholamine synthesizing cascade, might require induction by glucocorticoids, such as dexamethasone [Doupe et al., 1985; Anderson and Michelson, 1989; Finotto et al., 1999; Wong, 2003]. The details of the chromaffinergic differentiation process, as fascinating as they may be, are beyond the scope of this article and can be found in a recent review [Huber et al., 2002]. Suffice it to say that, regardless of the effect of glucocorticoids, homotypic and heterotypic 3-D cell-cell interactions during organogenesis appear to be pivotal for the organotypic differentiation of the parenchymal cells in the adrenal medulla [Lelkes and Unsworth, 1992]. Chromaffin cells have been used for many decades as an easily available model system for studying mechanisms of neurotransmitter synthesis and release, specifically of cholinergic neuronal or neuroendocrine stimulus-secretion-synthesis coupling. Indeed, a recent PubMed search for "chromaffin cells" (August 2005) revealed some 3900 relevant hits.

In contrast to bona fide chromaffin cells, PC12 pheochromocytoma cells are predominantly dopaminergic, that is, they express tyrosine hydroxylase (TH) and dopamine-$\beta$-hydroxylase (DBH), and synthesize and secrete small amounts of NE [Greene and Tischler, 1976]. In the hands of most (but not all) investigators, PC12 cells do not spontaneously express PNMT or epinephrine, thus suggesting that these cells originated from the NE phenotype of chromaffin cells. In the past 25 years PC12 cells have become an enormously popular model, mainly for studying fundamental mechanisms of neuronal differentiation and mechanisms of neurotransmitter synthesis and release. Currently (August 2005) there are more than 8500 PubMed citations describing a plethora of physiologic and molecular aspects of these cells, including the inducibility of the expression of functional PNMT [Byrd et al., 1986; Kim et al., 1993; Ebert et al., 1997; Lelkes et al., 1998, Unsworth and Lelkes, 1998b].

PC12 cells are in a unique position in that they maintain some of the features of the bipotentiality of fetal/embryonic SA cells, with the added advantage that PC12 cells are readily available and rather easy to culture. Thus, depending on the appropriate environmental cues, PC12 cells can differentiate along either the sympathetic neuronal or the neuroendocrine, chromaffinergic pathway. For

example, in the presence of neurotrophic agents, such as nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF), PC12 cells can differentiate into sympathetic neurons, whereas in the presence of glucocorticoids (and in organotypic culture) these cells acquire a neuroendocrine, chromaffin cell-like phenotype [Fujita et al., 1989; Mahata et al., 2002; Tischler, 2002; Vaudry et al., 2002]. This bipotentiality led to early attempts using PC12 cells as replacement cells or tissue for treating neurodegenerative disorders related to the destruction of catecholaminergic neurons, for example, in Parkinson disease [Jaeger et al., 1990; Aebischer et al., 1994; Lindner and Emerich, 1998]. The main caveat in using PC12 cells is the fact that these cells are spontaneously transformed.

About a decade ago we described that PC12 cells in coculture with adrenal medullary endothelial cells (but not endothelial cells from unrelated tissues) acquired structural and functional features reminiscent of acini of adrenal medullary chromaffin cells [Mizrachi et al., 1989, 1990]. This observation led to the extensive study of 3-D cultures of PC12 cells as a model system for tissue engineering neuroendocrine glands, specifically the adrenal medulla. [Lelkes et al., 1998; Lelkes and Unsworth, 1998, 2002]. In the wake of these studies we have been employing rotating wall vessel bioreactors (RWV, See below) as a unique cell culture environment for engineering functional tissue constructs [Unsworth and Lelkes, 1998a, 1998b, 2000; Lelkes and Unsworth, 2002]. The principles of RWV culture and the advantages of this system for generating differentiated tissue-like constructs have been described in detail in several recent reviews [Klaus, 2001; Hammond and Hammond, 2001; Lelkes and Unsworth, 2002].

In the following part of this chapter, we shall discuss the culture and 3-D assembly of PC12 cells as a source for engineering neuroendocrine tissue-like adrenal medullary organoids. Although primary chromaffin cells, especially from bovine adrenals, can be isolated in reasonable numbers and sufficient purity, and although isolated chromaffin cells can nowadays be fractionated into fairly homogeneous populations of epinephrine (E)- and norepinephrine (NE)-producing cells, no attempts have been reported, as yet, to engineer a functional adrenal medulla with primary isolates of chromaffin cells.

## 1.2. Preparation of Media and Reagents

### 1.2.1. Medium, Serum, and Plastics

In terms of the materials to be used for cell culture (Table 14.1), PC12 cells are quite tolerant with two exceptions:

i. All media formulations we have tried so far must contain at least 5% horse serum. Our current supplier is HyClone, but we have in the past equally successfully used horse serum from other vendors.

ii. We have found that PC12 cells do not grow well in tissue culture-treated flasks produced by Corning/Costar, but for optimal growth they require flasks produced by Nunc (blue cap).

**Table 14.1. Reagents required for PC12 culture.**

| Reagent | Supplier | Cat. # | Quantity | Estimate cost |
|---|---|---|---|---|
| DMEM high glucose | Fisher | 10-013-CV | 6 × 0.5 L | $71.55 |
| Fetal bovine serum | Hyclone | SH30071.03 | 0.5 L | $234.00 |
| Horse serum | Hyclone | SH30074.03 | 0.5 L | $39.00 |
| PBS without $Ca^{2+}$ and $Mg^{2+}$ (PBSA) | Fisher | 20031CV | 6 × 0.5 L | $66.00 |
| Trypsin-EDTA | Fisher | MT25053CI | 6 × 150 ml | $27.00 |
| Flasks (T175) | Fisher | 13-680-65 | 1 case | $70.00 |
| L-Glutamine | Fisher | MT25005CI | 6 × 150 ml | $27.00 |
| Antibiotic/antimycotic | Fisher | MT30004CI | 6 × 150 ml | $107.00 |
| NGF | Sigma | N2393 | 10 μg | $180.00 |

### 1.2.2. Source of PC12 Cells

The original strain of PC12 cells is available from American Tissue Type Culture Collection (ATTC; CRL-1721). As there are conflicting results on phenotypic expression of PC12 cells, including the presence and inducibility of PNMT [Lelkes, 1991], stock cultures should be obtained from a properly validated source, such as ATCC, and an authenticated seed stock should be frozen to provide working stocks for future use.

Among the major differences between the various strains of PC12 cells are the culture conditions, specifically the choice of culture medium. Originally, Tischler and Greene grew their PC12 cells in RPMI 1640 medium with a defined set of additives (See Table 14.2). Another group of investigators, mainly using PC12 obtained from the late Gordon Guroff's laboratory at NIH, use DMEM (high glucose) supplemented with 7.5% fetal bovine serum and 7.5% horse serum. Yet other groups have changed either the medium formulation (e.g., reducing glucose levels), the amount of serum, or both, or developed novel formulations [Lelkes et al., 1997]. Important, however, seems to be the inclusion of horse serum in virtually every formulation. To date, no comparative study has been performed that would evaluate the genotypic and phenotypic differences between PC12 cells maintained in the various culture media.

## 1.3. Maintenance of PC12 Cells

Undifferentiated PC12 cells in either medium are round, phase-bright cells approximately 12–15 μm in diameter (Fig. 14.1). These cells, although

**Table 14.2. Different culture media for PC12 cells.**

| Medium | ATCC (Tischler/Greene) | Guroff/Lelkes |
|---|---|---|
| Basal medium | RPMI 1640 | DMEM |
| L-Glutamine | 2 mM | 2 mM |
| Glucose | 4.5 g/L | 4.5 g/L |
| Sodium pyruvate | 1.0 mM | — |
| Sodium bicarbonate | 18 mM (1.5 g/L) | 26 mM (3.7 g/L) |
| HEPES | 10 mM | — |
| Serum | Heat-inactivated horse serum, 10%; fetal bovine serum, 5% | Horse serum 7.5%; fetal bovine serum 7.5% |

anchorage-dependent, are only loosely attached and do not spread even when cultured on tissue culture-treated surfaces. When maintained in suspension the cells readily form large, loose aggregates comprising up to hundreds of cells. In the presence of dexamethasone (0.1–10 μM), the cells remain similarly rounded and loosely attached and grow clonally in clusters. Of utmost importance, any cultures of PC12 cells should never become overcrowded and should be subcultured at 40–50% confluence. PC12 cells secrete neurotrophic factors (e.g., NGF and bFGF) and also other, as yet not fully characterized, differentiating factors, which when left to accumulate in the culture medium will a) induce PC12 cell differentiation toward the neuronal phenotype and b)give rise to a morphologically and functionally distinct phenotype of small, well-spread, anchorage-dependent cells. If these cells appear in our cultures, we abstain from any further use of the cells.

Addition of neurotrophic factors, specifically of nerve growth factor (NGF, 10–100 ng/ml), to undifferentiated PC12 cells causes a dramatic shift in their morphology: The cells rapidly flatten (<24 h) and begin (48–72 h) to extend neurites and to form (after ~120 h) neuronal networks (Fig. 14.1). Exposure of PC12 cells to NGF for <7 days results in reversible neuritogenesis, whereas after 10 days the cells have irreversibly differentiated into sympathetic neurons and thus have become dependent on NGF as a survival factor. In order to fully induce neuronal differentiation the initial seeding density of the cells must be rather low (<1000 cells/cm$^2$).

The key to expanding homogeneously undifferentiated PC12 cell populations is *never* to let the culture flasks become overconfluent and have differentiating growth factors accumulate in spent culture media. For this the cell cultures must be a) fed frequently (once every 48 h) and b) split weekly at a ratio of 1:8 (once the cells have reached some 50% confluence). In our laboratory, the routine protocol

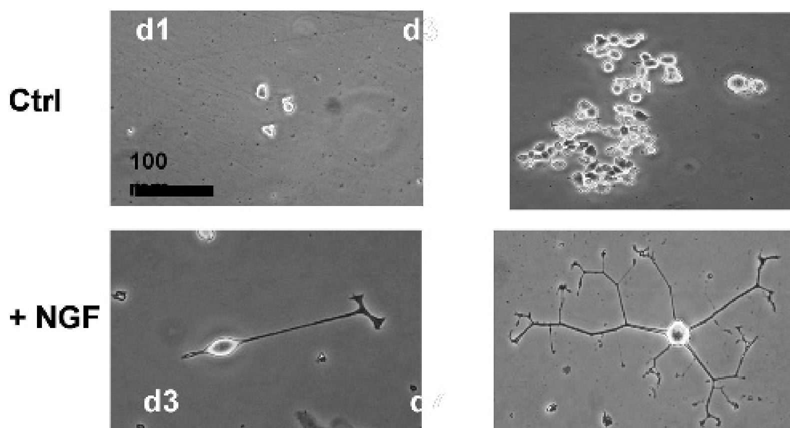


**Figure 14.1.** PC12 cells in 2-D culture. Top left: undifferentiated individual PC12 cells, 24 h after seeding; top right: cluster of undifferentiated PC12 cells after 7 days in culture; bottom left: single PC12 cells exposed to 25 ng/ml NGF, day 3; bottom right: neuronal network emanating from one single PC12 cell treated for 7 days with 25 ng/ml NGF. Original magnification 100×.

for passaging PC12 cells growing in T175 cell culture flasks is as described in Protocol 14.1 (all procedures are carried out under aseptic conditions).

### Protocol 14.1. Culturing and Passaging Undifferentiated PC12 Cells

***Reagents and Materials***

*Sterile*

- ❑ PC12 cells, 1 T175 (175 $cm^2$) flask, 50% confluent
- ❑ Culture flasks, T175, 8
- ❑ Growth medium (See Table 14.1)

***Protocol***

(a) For each T175 flask you are about to passage, prepare 8 new T175 flasks, containing 20 ml growth medium. The medium must be prewarmed and equilibrated with the air-$CO_2$ mix in your incubator. Cap the flasks and place them in the back of the hood in an upright position.

(b) Remove the culture to be passaged from the incubator and observe under the inverted microscope ($<10\times$ phase-contrast objective), to ascertain the status of your culture, especially the cell density, which should not exceed $>$50% confluence.

(c) Place the flask containing the cells upright in the hood, carefully aspirate as much of the growth medium as possible, and close the cap.

(d) Mechanically dislodge the cells by firmly tapping the side of the culture flask 5–10 times with the flat palm of your hand (***caveat***: too little force will fail to dislodge all the cells, too much force will crack the flask).

(e) Verify under the inverted microscope that all the cells have been dislodged, if not repeat Step (d).

(f) Open the flask, quickly wash the growth surface with $2 \times 10$ ml growth medium, and cap the flask.

(g) Place the flask upright and let it stand for 2 min so that all the cells can accumulate in the growth medium at the bottom of the flask.

(h) Gently swirl the suspension, open the cap, and, keeping the flask in an upright position, use a sterile disposable 1-ml pipette to remove 1 ml cell suspension for subsequent counting of the cells in a hemocytometer or an electronic cell counter. Place the cell suspension into a sterile Eppendorf microcentrifuge tube. For determining cell numbers follow the conventional procedures.

(i) Using a 10-ml disposable pipette, carefully mix the remaining cell suspension (by gently pipetting the solution $3\times$ up and down while avoiding air bubbles) to ensure even distribution of the cells.

(j) Aspirate $2 \times 8$-ml cell suspension. Between the first and the second aspiration repeat Step (i).

(k) Uncap 4 of the flasks prepared in step (a) and quickly dispense 2 ml cell suspension into each of them.

(l) Cap each flask and place it horizontally to allow even distribution of the cells.

(m) After adding cells to all of the prepared flasks (steps i–l), return all the flasks into the $CO_2$ incubator. Make sure to loosen the caps (unless you work with gas-permeable caps).

(n) For feeding, fully replace the medium every 48 h.

---

In following these simple steps, we have been able to maintain and passage undifferentiated PC12 cells for more than 40 passages (>300 population doublings). The expected cell yield from cultures maintained in this way is approximately $1.0–1.5 \times 10^7$ cells/T175 flask. In the past, we froze a large number of early-passage cells (seed stock). Every time we have to thaw one of our seed stocks, we refreeze 30–40 vials with $1 \times 10^6$ cells/vial of early-passage cells (working stock). Using the steps outlined above has allowed us to maintain (and supply others with) undifferentiated PC12 cells for the past 20 years.

## 1.4. Generation of 3-D PC12 Organoids

The ultimate goal of tissue engineering is to (re)create macroscopic, 3-D tissue-like constructs, aka "organoids." In the case of PC12 cells, this can be achieved by growing the cells on biological or synthetic scaffolds, in conventional static aggregation culture or in rotating wall vessel (RWV) bioreactors (also sold as Rotatory Cell Culture Systems (RCCS) by Synthecon Inc., Houston, TX) (Fig. 14.2) in the presence or absence of microcarriers (e.g., Cytodex 3, Sigma). In the following we describe several of the standard techniques used in our laboratory for generating 3-D PC12 organoids.

### 1.4.1. Aggregate Cultures

In our laboratory we use aggregate cultures mainly as static controls for the organoids dynamically generated in RWV bioreactors.

---

## Protocol 14.2. Aggregate Cultures of PC12 Cells

***Reagents and Materials***

*Sterile*

❑ Growth medium (See Table 14.1)

STLV HARV

**Figure 14.2.** Rotating wall vessel bioreactors (RWV). Left: Slow Turning Lateral Vessel (STLV); right: High Aspect Ratio Vessel (HARV). Photos courtesy of Synthecon Inc.

- ❑ Centrifuge tubes, 50 ml
- ❑ Petri dishes, non-tissue culture treated, 6.0 cm
- ❑ Petri dishes, glass or plastic, 25 cm
- ❑ Gas-permeable tissue culture bags

*Nonsterile*

- ❑ Gyratory shaker

***Protocol***

(a) Resuspend PC12 cells at an initial density of $1 \times 10^6$ cells/ml growth medium in up to 50 ml in a 50-ml centrifuge tube. Cap the centrifuge tube.

(b) Prepare 5 sterile, non-tissue culture-treated 6.0-cm Petri dishes.

(c) Carefully mix the cell suspension by inverting the capped tube 3 times

(d) Uncap and rapidly remove 10 ml cell suspension and place in a 6-cm non-TC Petri dish, replace the lid of the Petri dish, and recap the centrifuge tube.

(e) Repeat Steps (c) and (d). It is important to perform the mixing and dispensing of the cell suspension stepwise, to assure even distribution of the cells.

(f) Place 2 of the dishes inside the largest available (plastic or glass) Petri dishes (e.g., 25-cm diameter), replace the lids, and place the assembly into the incubator. The "dish in the dish" policy simplifies transport, prevents spillage and, most importantly, reduces the risk for contamination of both the cultures and of the incubators.

(g) Observe the cultures every 24 h to follow the formation of large multicellular aggregates. The formation of aggregates can be accelerated and enhanced (in terms of kinetics of aggregate formation, aggregate size, and number of cells/aggregate) if the cultures are gently shaken on a gyro-rotatory shaker placed inside the incubator for the first 24 h.

(h) As an alternative to the conventional aggregate cultures, PC12 cells at the same density as used for the dynamic cultures are placed in gas-permeable tissue culture bags (See Sources of Materials). Depending on the parameters to be analyzed, we use bags with a volume of either 7 ml or 35 ml with, respectively, one or two Luer-lock compatible inlet ports. These nonadhesive bags are placed horizontally into the incubators and serve as convenient, yet inexpensive, vessels for the aggregate cultures described above.

(i) Feed the aggregates (static controls) at the same frequency as the dynamic cultures.

---

### 1.4.2. Dynamic Formation of PC12 Organoids in RWV Bioreactors

RWV bioreactors create a suitable environment for generating macroscopic aggregates, which, because of the unique properties of this system, can differentiate into functional, tissuelike constructs, or, as we call them, "organoids" [Lelkes and Unsworth, 2002]. The principles of RWV bioreactors are described elsewhere in detail [Begley and Kleis, 2000; Hammond and Hammond, 2001] In this part of our chapter we will describe methods to generate PC12 organoids with and without

cell culture beads, using, respectively, HARV and STLV type RWVs. Both systems are commercially available from Synthecon, whose instructions describe in great detail the procedures for preparing and using the RWV systems. We routinely use disposable HARVs (10 and 50 ml) and reusable STLVs (55 ml). We generate beadless aggregate cultures in HARVs only for short-term experiments (<48 h) in which we study the effects of the RWV environment on the initial stages of cell assembly and differentiation. For long-term experiments (7–30 days) the use of beadless PC12 cell cultures in HARVs (or in STLVs) is inappropriate because it will result in large organoids with necrotic cores. By contrast, when using Cytodex 3 and/or Cultisphere microcarriers as anchorage surfaces for long-term studies, we can generate and maintain large macroscopic (>5 mm) aggregates without necrotic cores (See Fig. 14.3, Color Plate 9A) for up to 30 days.

## Protocol 14.3. Generation of PC12 Organoids in HARVs Without Beads

### *Reagents and Materials*

*Sterile*

- ❑ HARVs, 10 or 50 ml
- ❑ Growth medium (See Table 14.1)
- ❑ Luer-lock syringes, tuberculin, 10 ml and 60 ml
- ❑ Centrifuge tubes, 50 ml

### *Protocol*

(a) To generate PC12 organoids in the absence of beads, follow the procedures described in Protocol 14.2. Aggregation-based organoids have been generated for short-term experiments, of between 10 min and 24 h, in which we

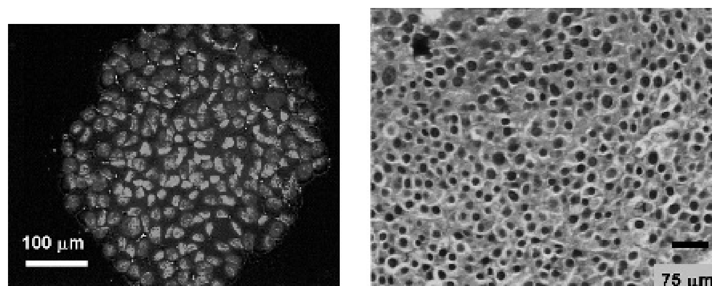


**Figure 14.3.** 3-D assemblies of PC12. Left: Aggregate of PC12 cells generated and maintained for 14 days under static conditions in LiveVue™ tissue culture bags. Cells/nuclei were stained with Hoechst 22358/Bisbenzimide. Right: 3-D organoid generated in the presence of Cytodex 3 beads and maintained for 20 days in STLV. Note the dense tissue like organization of the cells and the absence of a necrotic core. (See Color Plate 9A.)

have evaluated gene expression, intracellular signaling, and protein-protein interactions.

(b) Depending on the subsequent analysis and its demands for material (RNA, DNA, proteins, etc.), use either the 10-ml or the 50-ml HARVs.

(c) Use $1 \times 10^6$ cells/ml for routine experiments. We have used inoculation concentrations as low as $2 \times 10^5$ cells/ml and as high as $5 \times 10^6$ cells/ml; any concentration $<2 \times 10^5$ cells/ml will yield large number of cells that remain single and undergo apoptosis, whereas very high seeding concentrations will yield very large aggregates (5–10 mm in diameter) in 2–3 h, accompanied by rapid depletion of the nutrients in the medium and the formation of necrotic cores.

(d) Prepare an appropriate volume of cell suspension, that is, the net volume + 20% excess, i.e., 12 and 60 ml, respectively (See also Step (k)).

(e) Fill all RWVs, HARVs aseptically inside the hood, making sure that all air bubbles are removed from the vessels. Add the cell suspension via the lower syringe/Luer lock port, with the upper Luer lock having an empty syringe barrel attached. This syringe will serve as the receptacle for the excess medium and cell suspension, as the vessel is being filled through the lower port. Of utmost importance, greatest care must be exercised to remove every last air bubble from the assembly.

(f) After filling, sterilize the front (top plastic face) of the vessels with the 70% ethanol and cap the Luer-lock inlet ports of the HARVs with 1-ml tuberculin syringes, to prevent leakage and contamination.

(g) Screw the HARVs firmly onto the rotatory bases (either single or quadruple), and initiate the dynamic cell culture at a rotation speed of 10 rpm.

(h) Normally, under the above-mentioned routine conditions, feeding of the culture for up to 24 h is not necessary. The HARVs being well aerated, the levels of dissolved gases ($pO_2$, $pCO_2$) will remain identical to those in the incubator, and the glucose levels will remain normal provided the high-glucose culture medium is used. By contrast, for very high initial inoculation densities ($>2.5 \times 10^6$ cells/ml) and for experiments lasting $>24$ h, feed the vessels after 24 h, by replacing 75% of the cell culture medium.

(i) To feed the cultures, dismount the HARVs and aseptically place vertically in the hood, allowing the aggregates/organoids to settle to the bottom within 15 min. In the meantime prepare 2 Luer-lock syringes (either 10 ml or 60 ml), one containing medium (8 ml or 40 ml, respectively), the other empty syringe to serve as the receptacle for the spent medium. Before replacing the medium, remove a small aliquot of the spent medium, using one of the smaller syringes attached to the ports (See Step (f)) and analyze for blood gases and glucose content.

(j) To replace the medium in the HARV, remove the old syringes and attach new ones. Open the stopcocks in the front of the HARV and carefully empty the syringe containing the fresh medium (slowly, gently, so as not to disturb the

aggregates/organoids sitting at the bottom), while simultaneously aspirating the spent medium into the empty syringe.

(k) To avoid creating air bubbles during the refeeding, first gently push a small volume (1 ml) of the new medium into the HARV and then open the stopcock and start manually aspirating the spent medium. Remove any air bubbles that, despite all precautions, may have found their way into the HARV, by alternatively filling and emptying in an oscillatory movement small volumes of excess medium through the two syringes. This is the main reason to prepare some 20% excess of fresh medium when filling the vessels (See Step (f)).

(l) Once all bubbles are removed, close both stopcocks and turn the HARVs upside down (the front plate facing downwards). Gently pull the pistons in both syringes (equivalent to 1–2 ml); remove both of them and replace them with small empty 1-ml tuberculin syringes. This prevents spillages that can occur when changing the syringes.

(m) To remove the organoids, open the main, central filling port and simply pour the contents into a 50-ml centrifuge tube. For further sample treatment proceed according to your specific protocol.

---

## Protocol 14.4. Generation of PC12 Organoids with Beads in STLVs

### *Reagents and Materials*

*Sterile*

- ❑ T175 flask culture of PC12 cells, semiconfluent
- ❑ Cytodex 3, collagen-coated beads
- ❑ Petri dishes, non-TC treated, 6 cm
- ❑ STLV
- ❑ Syringes, 10 ml and 60 ml
- ❑ Centrifuge tubes, 50 ml

### *Protocol*

(a) Prepare the Cytodex 3 beads according to the manufacturer's instructions and store the suspension aseptically in the refrigerator.

(b) Attach cells to beads:

  i) To a semiconfluent cell culture (just ready for passaging, See Section 1.3) add beads aseptically at an approximate ratio of 1 bead per 10 cells. We routinely add approximately $1.75 \times 10^6$ beads to a T175 flask.

  ii) Return the flasks to the incubator and continue to feed the cultures gently for 5 days. This will allow the cells to migrate onto the beads (PC12 cells preferentially attach to collagen-coated surfaces) and to form a epitheloid monolayer on the beads.

  iii) After 5 days dislodge the cell-covered beads by firmly tapping on the side of the flask (See Protocol 14.1, Step (d)).

(c) Alternatively, to attach cells to beads:
  i) Incubate cells and beads at the ratio of 10:1 for 24 h in a non-tissue culture-treated Petri dish.
  ii) Incubate under gentle rocking on the gyratory shaker inside the incubator (See Protocol 14.2, Step (g)).

Firm attachment of the PC12 cells on the bead surface before inoculation into the STLV is critical. In contrast to other cell types, coincubation of PC12 cells with cell culture beads in the STLV will not lead to the attachment of the cells to the beads and formation of a sufficiently cell coverage to allow subsequent cell-cell contact-mediated aggregation of the beads.

(d) Resuspend the bead suspension in 5 ml medium to yield approx. $2.5 \times 10^6$ beads obtained as above (either Step (b) or (c)) covered with $\sim 2.5 \times 10^7$ cells to yield a final cell concentration of $\sim 5 \times 10^5$ cells/ml.
(e) Sterilize (autoclave) and prepare the reusable STLV as per the manufacturer's instructions. Aseptically assemble the STLV in the hood, and fill the vessel through the central filling port with 40 ml medium.
(f) Inoculate STLV with the cell-covered beads:
  i) Aspirate the bead suspension (See Step b (iii)) with a 10-ml disposable pipette.
  ii) Add the suspension through the central filling port to the vessel lumen.
  iii) Close the central filling port tightly.
(g) Open the two luer-lock compatible venting ports and firmly attach 2 empty 10-ml syringes with their plungers removed.
(h) Tilt the STLV so that the remaining air bubble rests at the top part underneath one of the venting ports. Using a 25-ml pipette gently, add up to 20 ml cell culture medium to the lower venting port, until all air has been removed inside the STLV and the medium rises up in the upper syringe (while some medium still remains in the lower syringe).
(i) At this time push the pistons into first the lower syringe and then the upper syringe and filling ports, making sure not to add any additional air bubbles into the STLV.
(j) Close the stopcocks of the venting ports, and by gently tapping the assembled STLV ascertain that there are no air bubbles inside. Frequently air is retained in some of the crevices of the STLV and dislodged by the tapping. In this case, open the stop cock and repeat Steps (i) and (j) (See also Protocol 14.3, step (k)).
(k) After all the air bubbles have been meticulously removed (which can be quite tedious and time consuming, at least in the beginning when you learn how to operate the system), mount the STLV on the aerated rotatory base and begin your experiments with a rotational speed that will prevent sedimentation of the beads. In our experience an initial rotatory speed of 14 rpm is optimal. However, be aware that as the aggregate/organoid size increases, you will have to empirically and gradually increase the rotatory speed to compensate for the enhanced sedimentation rate of the growing aggregates/organoids.

(l) Feed long-term cultures regularly, to maintain appropriate levels of $pO_2$, $pCO_2$, and nutrients, specifically glucose:
   i) Remove, while the vessels continue to rotate, 1 ml spent culture medium and determine the "vital signs" as described above (Protocol 14.3, Steps (i–k)).
   ii) If feeding is indicated, dismount the vessels from the bases, aseptically transfer the vessels to the hood, and place them upright. This allows the organoids to settle (in about 10 min) on the bottom of the STLV.
   iii) Exchange up to 75% of the spent medium (depending on the extent of glucose utilization of the growing aggregates) with new medium, essentially following the steps described above (Protocol 14.3, Steps (i–k)), using two 60-ml syringes.

(m) At the termination of the experiments:
   i) Demount the vessels, place in the incubator, and allow the aggregates/organoids to settle, as above.
   ii) Open the venting ports and the inlet ports and, using 10-ml disposable pipette, aseptically remove 20 ml of the medium (among others for one final determination of oxygen consumption and glucose utilization).

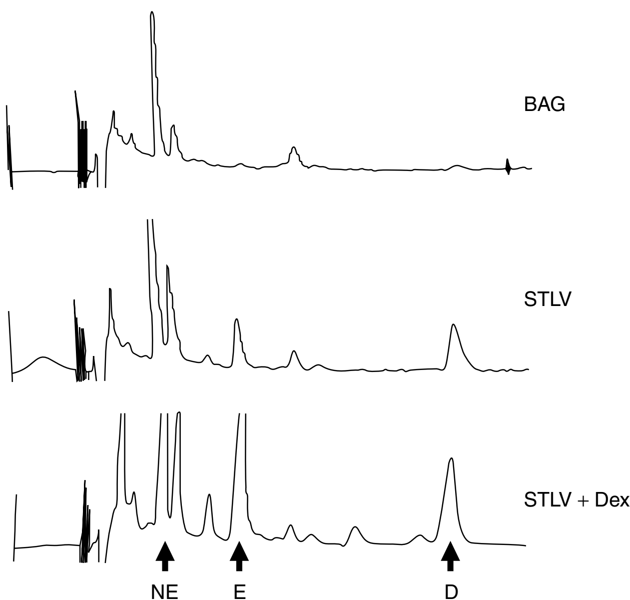


**Figure 14.4.** Generation of functional PC12 adrenal medullary organoids. Functionality of the paternal/maternal cells (chromaffin and PC12 cells) and of PC12 organoids grown in the absence or presence of 10 μg/ml dexamethasone was assessed by HPLC with electrochemical detection (for technical details on the HPLC methodology See Lelkes et al., 1994). In the bags (aggregates, See Fig. 14.3.), PC12 cells synthesize norepinephrine (NE) and dopamine (D) but no discernable quantities of epinephrine (E). By contrast, in the STLV, the cells synthesize epinephrine, the hallmark of adrenergic chromaffinergic differentiation; epinephrine synthesis in STLV is significantly potentiated by dexamethasone.

iii) Remove the aggregates/organoids by pouring the remaining vessel contents ($\sim$35 ml) through the central filling port into a 50-ml centrifuge tube.

Subsequent processing of the samples will follow the specific protocols for the particular analysis of protein, RNA, morphology, etc.

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### 1.5. Conclusions

Using the techniques described above, specifically the ones detailed in Protocol 14.4, we have been able to generate functional PC12-based adrenal organoids (See Fig. 14.4). Remarkably, these organoids express significant levels of PNMT and, moreover, synthesize both norepinephrine and epinephrine, the hallmark of adrenergic differentiation, thus indicating the functional differentiation of the tissuelike constructs toward the parental, chromaffin-like phenotype. Ongoing and future studies are focused on evaluating the genotype and phenotype of the ensuing organoids and will address the role of heterotypic cocultures (e.g., addition of organ-specific mesenchymal, cortical, and endothelial cell)s as well the implantation/vascularization of the tissuelike organoids into host animals.

## 2. FORMATION OF SERTOLI-NT2N TISSUE CONSTRUCTS TO TREAT NEURODEGENERATIVE DISEASE

This section was contributed by Samuel Saporta and Don F. Cameron, to whom all correspondence should be addressed.

### 2.1. Background

Parkinson disease (PD), a neurodegenerative loss of dopaminergic neurons in the substantia nigra pars compacta, results in the loss of dopamine in the corpus striatum, among other places. This loss of dopaminergic input to the striatum results in progressively increasing tremor, bradykinesia, and rigidity, as well as alterations of cognition and affect, that, as the disease progresses, make the activities of daily living nearly impossible. Current pharmacological treatments to replace dopamine are initially effective, although palliative, and eventually lose their efficacy. Therefore, the use of dopaminergic cell replacement therapy as a long-term treatment of neurodegenerative disease has been of great interest.

Transplantation of fetal dopaminergic neurons ameliorates behavioral deficits in animal models of PD [Baker et al., 2000; Bjorklund et al., 1981; Bjorklund and Lindvall, 2000; Johnston and Becker, 1999; Palmer et al., 2001; Winkler et al., 2000]. Human fetal neurons have been transplanted into human patients with PD [Freed et al., 1990; Freeman et al., 1995; Lindvall et al., 1990], and initial controlled human clinical trials indicate the potential of this type of therapy for long-term treatment of PD, although a surgical protocol that yields consistent benefit is not yet established [Bjorklund et al., 2003; Freed et al., 2001; Olanow et al., 2003]. Alternative cell sources for cell replacement therapy have also been

considered. The unique ability of embryonic stem cells to give rise to all somatic cell lineages makes them an especially promising source for cell therapy [Bjorklund et al., 2002; Freed, 2002; Kim et al., 2002]. However, it has proven difficult to reliably control their expansion and differentiation [McKay, 2002; Panchision and McKay, 2002]. Additionally, the therapeutic use of human embryonic stem cells and fetal cells is fraught with ethical issues. Some success has been reported for the expansion and differentiation of multipotent adult stem cells toward desired neuronal phenotypes in vitro [Englund et al., 2002; Espinosa-Jeffrey et al., 2002; Ourednik et al., 2001; Sanchez-Ramos et al., 2001; Uchida et al., 2000; Yang et al., 2003; Zigova et al., 2002], although, again, the extracellular instructive signals controlling their engraftment and differentiation in vivo are inadequately understood.

#### 2.1.1. Engineered Tissue Constructs for Transplantation

The ideal cellular source for transplantation therapy in PD would be an easily expanded cell that reliably differentiates to a dopaminergic phenotype, releases dopamine, and remains dopaminergic for the life of the graft. Additionally, the ideal cellular source should elicit a minimal host-graft response in the transplant recipient. No single cell, to date, has been proven capable of demonstrating all of these desirable features. Therefore, it may be reasonable to consider combining cells for transplantation that would provide a tissue graft with these desirable properties. We have reasoned that it may be advantageous not only to combine cells with desirable properties for cell replacement therapy, but to engineer a tissue construct of these cells, ensuring aggregation and cell-to-cell contact. As will be seen below, this strategy has proven to be useful in creating a transplantable tissue construct composed of NT2N dopaminergic neurons and Sertoli cells that may, indeed, fulfill the enumerated qualities of an ideal source of dopaminergic neurons for cell replacement therapy.

#### 2.1.2. The NT2 Neuronal Precursor Cell

The NTera-2/clone D1 (NT2) cell, a cell line derived from a human teratocarcinoma has been used extensively to study neuronal differentiation [Bani-Yaghoub et al., 2001; Guillemain et al., 2000, 2003; Iacovitti and Stull, 1997; Leypoldt et al., 2001; Misiuta et al., 2003]. This cell line is easily expanded and differentiates into immature neurons after 4–5 weeks of treatment with retinoic acid [Andrews, 1984, 1998; Pleasure and Lee, 1993]. These differentiated postmitotic neurons, designated NT2N, have phenotypic and morphological characteristics of immature neurons [Andrews, 1984; Daadi et al., 2001; Guillemain et al., 2000; Pleasure and Lee, 1993; Saporta et al., 2001]. Approximately 10% of NT2N neurons express tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis [Iacovitti and Stull, 1997; Zigova et al., 2000]. Dopaminergic NT2N neurons have been shown to engraft within the central nervous system and have proven useful in ameliorating the behavioral deficits associated with stroke [Nelson et al., 2002; Saporta et al., 1999; Watson et al., 2003; Willing et al., 2002], neurodegenerative disease [Willing et al., 1999], and spinal cord injury [Saporta et al., 2002].

### 2.1.3. Sertoli Cells

Sertoli cells (SC), the "nurse" cell of the testis, secrete a number of growth factors that are believed to help maturation of spermatids and protect developing sperm from immunosurveillance [Skinner, 1993]. Surprisingly, many of these growth factors, such as transforming growth factor-$\beta$1 and -$\beta$2, insulin-like growth factor I, glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor, basic fibroblast growth factor 1 and 2, platelet derived growth factor, and neurturin, are neurotrophic. We have previously shown that coculturing rat fetal midbrain neurons with SC enhances the number of TH-positive neurons in vitro [Othberg et al., 1998a, b] and in vivo [Willing et al., 1999]. Additionally, a number of lines of evidence suggest that SC may provide immunoprotection to transplanted cells [Baker et al., 2000; Pollanen et al., 1988; Pollanen and Uksila, 1990]. They do not express major histocompatibility factor (MHC) II and express little MHC I [Pollanen and Maddocks, 1988]. Both allografts of rat and xenografts of pig SC survive in the rat brain for 2 months without immunosuppression of the recipient [Saporta et al., 1997]. Moreover, SC produce an unknown soluble factor that inhibits interleukin-2 (IL-2) production as well as IL-2-induced lymphocyte proliferation [Pollanen et al., 1990; Selawry et al., 1985], and when cotransplanted with dopaminergic neurons they enhance the survival of the transplanted neurons in recipient rats that are not systemically immunosuppressed [Willing et al., 1999].

## 2.2. Sertoli-NT2N-Aggregated-Cell (SNAC) Tissue Constructs

Cells cultured in the rotating wall vessel (RWV) bioreactors, originally developed by NASA to simulate conditions of microgravity, organize into tissue constructs that express tissue-specific markers [Becker et al., 1993; Lelkes et al., 1994, 1998]. This culture system has also been shown to effect the differentiation of cells grown in the RWV, as compared to those grown in conventional static cultures. For example, PC12 cells have been shown to aggregate and adopt a neuroendocrine phenotype when grown in the RWV, but not in conventional culture [Lelkes et al., 1998] (See Section 1.3). Similarly, neural stem cells [Wang and Good, 2001] and progenitor cells [Low et al., 2001] can be induced to differentiate when cultured in the RWV. We have previously reported aggregation of NT2 cells with Sertoli cells, using the High Aspect Ratio Vessel (HARV) RWV bioreactor to create a transplantable tissue construct that may provide a readily available source of dopaminergic neurons [Saporta et al., 2002].

### 2.2.1. NT2 Cells Rapidly Differentiate into NT2N Neurons

Immunohistochemical analysis of SNAC tissue constructs grown in the HARV RWV for 3 days reveals the presence of SC, TH-positive cells, and type III $\beta$-tubulin-positive NT2 cells (Fig. 14.5, See Color Plate 9B). These data suggest that TH-positive cells within the SNAC tissue constructs are NT2 cells that have differentiated to become NT2N dopaminergic neurons. Approximately 9% of NT2 cells differentiate to NT2N neurons after 3 days of culture in the HARV.

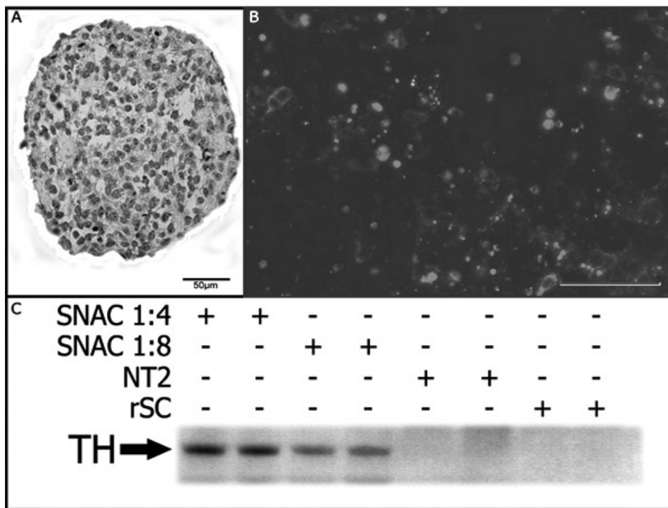


**Figure 14.5.** Morphology and TH content of SNAC tissue constructs. A) Photomicrograph of a SNAC tissue construct immunostained for human nuclei to show the presence of NT2 cells within the SNAC. The distribution of NT2 cells does not appear to be organized. B) Double immunostained fluorescent photomicrograph showing the presence of Sertoli cells (green) and TH-positive NT2N neurons (red) within a SNAC tissue construct. Scale bar = 100 μm C) Immunoblot of SNAC tissue constructs, NT2 cells, and Sertoli cells grown in the HARV rotating wall vessel for 3 days. The SNAC tissue construct with a starting ratio of Sertoli cells to NT2 cells of 1:4 contains the most TH. NT2 cells and Sertoli cells grown under the same conditions do not contain detectable TH. (See Color Plate 9B.)

Although TH is a good indicator of catecholaminergic neurons within the nervous system, it does not provide direct evidence that TH-positive cells contain dopamine (DA), or whether DA-containing neurons are capable of releasing DA. Therefore, we stimulated SNAC tissue constructs, NT2 cell aggregates, and SC aggregates grown in the HARV for 3 days with 60 mM KCl to stimulate release of DA into the medium, and analyzed medium from stimulated SNAC tissue constructs and cell aggregates, as well as normal medium, by high-performance liquid chromatography (HPLC) with electrochemical detection, for the presence of DA. Unstimulated SNAC tissue constructs constitutively release a small, but detectable, amount of DA into the culture medium. When stimulated with 30 mM KCl for 20 min, SNAC tissue constructs released a significant amount of DA (Table 14.3). No DA was detected in the stimulated or unstimulated NT2 aggregates or SC aggregates, or in medium.

### 2.2.2. Tissue Constructs Survive and Retain Their Dopaminergic Phenotype When Transplanted into the Brain

Our goal in creating the SNAC tissue construct was to develop a transplantable tissue source that would allow dopaminergic neurons to engraft within the brain, maintain the dopaminergic phenotype of the transplanted neurons, and allow survival of the graft without immunosuppression of the recipient. We have transplanted SNAC tissue constructs into nonimmunosuppressed male and female

**Table 14.3. Effect of Sertoli cells on dopamine production by NT2 cells.**

| Sample | 30 mM KCl | Dopamine concentration |
|---|---|---|
| SNAC | + | 0.39 ± 0.07 nM |
| SNAC | − | 0.039 nM |
| NT2 | + | Not detectable |
| NT2 | − | Not detectable |
| SC | + | Not detectable |
| SC | − | Not detectable |
| Medium | + | Not detectable |
| Medium | − | Not detectable |

HPLC analysis of dopamine release from three independent experiments. Unstimulated SNAC released a small amount of dopamine into the medium. However, SNAC stimulated with 30 mM KCl released an order of magnitude more dopamine.

rats that have had the nigrostriatal pathway, the source of dopaminergic input to the striatum, disrupted. We have recovered viable grafts from these animals 4 weeks after transplantation and have identified dopaminergic neurons within these grafts (See Color Plate 9C). At this time point, there is little evidence of neurite extension, which may take longer than 30 days to fully develop [Saporta et al., 2002].

## 2.3. Conclusions

The present studies show that SC and NT2 cells aggregate into a tissue construct containing TH-positive neurons when grown in the HARV RWV under conditions of simulated microgravity in the absence of retinoic acid. These SNAC tissue constructs are a readily obtainable source of dopaminergic neurons that, when transplanted, may provide a long-lasting source of dopamine to the dopamine-deprived striatum in PD without the usual immunosuppression of the graft recipient. Longer-term transplant studies will assess the long-term survivability of these grafts and their degree of engraftment.

## 2.4. Preparation of Media and Reagents

### 2.4.1. Maintenance Medium (MM)

Dulbecco's modified minimal essential medium (DMEM)-Ham's F-12 nutrient mixture supplemented with retinoic acid, ITS+ Premix, and gentamycin sulfate. To 500 ml DMEM-F-12 add:

5.0 ml gentamycin sulfate
5.0 ml ITS+ Premix
13 μl working solution (50 mg/25 ml DMSO) of retinoic acid

Make sterile, store at 4 °C for up to 2 weeks.

#### 2.4.2. Incubation Medium (IM)

Maintenance Medium (MM) supplemented with 10% fetal calf serum (GIBCO) and 1.0% Growth Factor Reduced Matrigel™ ($MG_{GFR}$). Make sterile, store at 4 °C for up to 2 weeks.

#### 2.4.3. NT2 Medium (NT2M)

Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 nutrient mixture supplemented with 10% fetal calf serum and 5.0 ml of gentamycin sulfate.

#### 2.4.4. Freezing Medium (FM) for NT2 Cells

DMEM supplemented with 10% fetal calf serum and 5% dimethyl sulfoxide (DMSO).

#### 2.4.5. Trypsin Solution (for Tissue Digestion)

Dissolve 75 mg trypsin (type 1; T-8003) in 30.0 ml of MM in sterile 50.0-ml conical tube, to give 0.25%, immediately before use.

#### 2.4.6. Trypsin-EDTA Solution (for Lifting Plated Sertoli Cells and NT2 Cells)

Trypsin, 0.25%, EDTA, 2 mM (Sigma; T-4049) is stored at −18 °C indefinitely.

#### 2.4.7. Collagenase Solution

Dissolve 60 mg of collagenase (type IV, GIBCO) in 30.0 ml of MM in a sterile 50.0-ml conical tube, to give 0.2%, immediately before use.

#### 2.4.8. Tris Buffer

Prepare 20 mM Tris buffer by dissolving 2.422 g of Trizma Base in 1.0 L dd$H_2O$ and adjust pH to 7.4 with 12 N HCl. Sterilize by filtration and store at 4 °C for up to 2 months.

#### 2.4.9. DNase Stock Solution

Dissolve 100 mg DNase in 50 ml of NaCl (0.14 M) to give 2 mg/ml. Sterilize by filtration and store at −20 °C. Dilute 1 ml with 70 ml MM as needed.

### 2.5. Isolation of Rat Sertoli Cells

Sertoli cells are isolated from prepubertal male rat pups (16–19 days old). Briefly, rat pups are sacrificed and testes are removed through a scrotal incision. Excised testes are placed in sterile MM and transferred to a sterile tissue culture hood. The rest of the protocol is performed observing sterile technique. The capsules (tunica albugenea) are removed from all testes, after which the parenchyma is minced into small fragments with sterile scissors and subjected to sequential enzymatic treatment at 37 °C with 0.25% trypsin and 0.2% collagenase. The resulting Sertoli cell aggregates are distributed in a volume of 50.0 ml MM into T150 cell culture flasks (Corning/Costar) and incubated at 39 °C in 5% $CO_2$-95% air for 48 hours. Sertoli cell-enriched monocultures are then subjected to hypotonic treatment

with sterile 20 mM Tris-HCl buffer (2 min at room temperature) to expedite the removal of contaminating germ cells (15). After two washes with MM, cell culture flasks are replenished with 50.0 ml of MM and returned to the incubator at 37 °C in 5% $CO_2$-95% air. The resulting pretreated Sertoli cell-enriched monocultures contain greater than 95% Sertoli cells. Before coculture with NT2 cells, pretreated SC are lifted with 0.25% trypsin-EDTA (2 min at room temperature), washed (3×) with MM, counted by hemocytometric analysis, and assayed for viability by trypan blue exclusion.

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## Protocol 14.5. Preparation of Sertoli Cells

### *Reagents and Materials*

*Sterile*

- ❑ Sodium pentobarbital (Nembutal)
- ❑ MM (See Section 2.4.1)
- ❑ IM (See Section 2.4.2)
- ❑ Trypsin, 0.25% (See Section 2.4.5)
- ❑ Collagenase, 0.2% (See Section 2.4.7)
- ❑ DNase (See Section 2.4.9)
- ❑ Tris buffer, 20 mM (See Section 2.4.8)
- ❑ Erlenmeyer flask, 125 ml
- ❑ Sterile beaker, 250 ml
- ❑ Scissors

*Nonsterile*

- ❑ Rats, 16–19 day old, 10
- ❑ Ethanol, 70%

### *Protocol*

*Isolation*

(a) Sacrifice ten 16–19 day old rats with overdose of sodium pentobarbital (Nembutal).
(b) For each rat, wet scrotum with 70% ethanol.
(c) Remove testes and place in sterile MM in sterile tissue culture dish. Transport to sterile hood.
(d) Rest of protocol performed observing sterile technique.
(e) Remove tunica albugenea from each testis with scissors and place testicular parenchyma in clean MM.
(f) Mince parenchyma with scissors.
(g) Place minced tissue in 125-ml Erlenmeyer flask in 30 ml MM. Wash 3× with MM by resuspending and settling.
(h) Replace MM with 30 ml 0.25% trypsin solution and incubate at 37 °C in shaking water bath for 15 min.
(i) Add DNase (0.5 ml stock solution) 5.0 min before end of trypsin digestion incubation.

(j) Let tissue settle to the bottom of the flask, remove trypsin solution, and wash 3× with MM by resuspension and settling. Optional—add 1% trypsin inhibitor (Sigma) in first wash.
(k) Replace MM with 30 ml 0.2% collagenase and incubate at 37 °C in shaking water bath for at least 20 min or until most aggregates do not display peritubular cells (this is determined by microscopic evaluation of sample using 10× objective lens on inverted microscope).
(l) Let tissue settle to bottom of flask, remove collagenase solution, and wash 3× with MM by resuspension and settling.
(m) Transfer Sertoli cell aggregates to 250-ml sterile beaker and resuspend in 100 ml MM.
(n) Place beaker on stir plate, gently suspend aggregates in MM with sterile magnetic stir bar, and plate at recommended volume for culture vessels.

*Pretreatment*

(a) Place culture vessels with aggregates in 5% $CO_2$ incubator at 39 °C and incubate for 48 h.
(b) Transfer vessels to sterile hood.
(c) Remove MM and replace with 20 mM Tris-HCl buffer (just enough to cover the cells) and incubate for 2.5 min at room temperature.
(d) Remove Tris-HCl buffer, wash once with IM. and refill tissue/cell culture vessel with MM.
(e) Return tissue/cell culture vessels to 5% $CO_2$ incubator and incubate at 33 °C.
(f) Replace IM every 48–72 h.

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### 2.6. Preparation of NT2 Cells

NTera-2/D1 cells are obtained from ATTC and kept frozen in liquid nitrogen until needed. Before the experiment, the frozen vial is thawed (as prescribed by the supplier), reconstituted in NT2M (See Section 2.4.3) in 15-cm cell culture dishes (Corning) and incubated at 37 °C in 5% $CO_2$. When the cells reach 70–80% confluence (about 2–3 days), cells are subcultured with trypsin-EDTA (See Section 2.4.6) and split 1:4 into four T150 cell culture flasks. These cells are incubated for 48 h at 37 °C, subcultured with trypsin-EDTA, pooled, washed (3×) with NT2M, counted by hemocytometer, and assayed for viability by Trypan Blue exclusion.

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## Protocol 14.6. Propagating NT2 Cells

***Reagents and Materials***

*Sterile*

- ❑ NT2M (See Section 2.4.3)
- ❑ Trypsin-EDTA (See Section 2.4.6)
- ❑ FM: freezing medium (See Section 2.4.4)
- ❑ Vial of NT2 cells (ATTC)

- ☐ Conical centrifuge tube, 15 ml
- ☐ Petri dish, 15 cm

*Nonsterile*
- ☐ Ethanol, 70%
- ☐ Water bath at 37 °C

***Protocol***

(a) Place frozen vial of NT2 cells in 37 °C water bath and agitate until just a "sliver" of ice is left in the vial, i.e., almost completely thawed (about 30 s).
(b) Swab vial with 70% EtOH, open, and pipette entire contents of vial (about 1.0 ml) into 10 ml NT2M in a 15-ml sterile conical centrifuge tube.
(c) Wash (3×) with NT2M by centrifugation (800 rpm [135-mm radius] for 3 min, 120 *g*) and plate cells into 15-cm cell culture dish.
(d) Incubate cells at 37 °C in 5% $CO_2$ until 70–80% confluence (3–4 days).
(e) Subculture with 0.25% trypsin-EDTA and seed total yield into four T150 cell culture flasks. Return to 37 °C in 5% $CO_2$-95% air.
(f) Repeat Steps (d) and (e)—this will give you 16 flasks.
(g) Subculture cells from all flasks with trypsin-EDTA, wash (3×) with NT2M and pool.
(h) Prepare 1.0-ml aliquots of $5 \times 10^6$ cells/ml in freezing medium (FM) and freeze.
(i) Store in liquid nitrogen. Each vial should be labeled "Working Stock of NT2 cells."

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### Protocol 14.7. Preparation of NT2 Cells for Coculturing

***Reagents and Materials***

*Sterile*
- ☐ NT2M (See Section 2.4.3)
- ☐ Trypsin-EDTA (See Section 2.4.6)
- ☐ One vial of NT2 cells (Working Stock)

***Protocol***

(a) Thaw and plate one vial of NT2 cells in 15-cm cell culture dish and incubate in NT2M at 37 °C in 5% $CO_2$.
(b) Grow to near confluence (80%) and subculture (1:4) in NT2M until near confluence.
(c) Repeat subcultures (Step (b)) if more cells are required.
(d) Harvest cells with trypsin-EDTA.
(e) Wash (3×) with NT2M. Cells are now ready to use.

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### 2.7. Preparation of Sertoli-NT2-Aggregated-Cell (SNAC) Tissue Constructs

Sertoli cells and NT2 cells are combined at a 1:4 ratio (SC:NT2) to a total of $2.0 \times 10^7$ cells per construct. The combined cells are suspended in 10 ml of IM

(See Section 2.4.2) and placed in the HARV bioreactor. The biochamber is secured on its motor mount and the entire apparatus is placed in the incubator at 37 °C in 5% $CO_2$. The rotational speed is set a 62 rpm. The coculture period is for a total of 72 h, after which the aggregate is retrieved from the biochamber and washed (3×) with NT2M by resuspension and settling. The SNAC tissue construct is now ready for transplantation or assay.

---

### Protocol 14.8. Tissue Constructs of Sertoli and NT2 Aggregated Cells (SNAC)

***Reagents and Materials***

*Sterile or Aseptically Prepared*

- ❑ SC (See Protocol 14.5)
- ❑ NT2 cells (See Protocol 14.7)
- ❑ IM
- ❑ Syringe, 5 ml or 10 ml
- ❑ HARV bioreactor

***Protocol***

(a) Suspend $4 \times 10^6$ SC and $1.6 \times 10^7$ NT2 cells in 5 ml IM at room temperature.
(b) Inject the suspended cells by syringe into the HARV bioreactor.
(c) Completely fill the 10-ml biochamber with additional IM, making sure that no bubbles exist within the biochamber (See Protocol 14.4 Steps (i), (j)).
(d) Secure biochamber on motor mount and place apparatus (HARV bioreactor on motor mount) in incubator at 37 °C in 5% $CO_2$.
(e) Connect motor mount to rheostat outside of the incubator.
(f) Turn motor on and rotate biochamber at 26 rpm. (***Note***: rotational speed at this point in time is adjusted so that the aggregate inside the biochamber remains in one spot in the biochamber. This speed will vary, depending on the size of the aggregate. We attempt to maintain multiple small aggregates, rather than one large aggregate, and the rotational speed is adjusted to maintain the position of the majority of the aggregates.)
(g) Incubate coculture for a total of 72 h.
(h) Retrieve biochamber from motor mount and place in sterile hood.
(i) Gently remove the SNAC tissue construct from the biochamber and place in 15-ml sterile conical centrifuge tube and let sediment by gravity. Wash SNAC (3×) with IM by resuspension and settling.
(j) SNAC is now ready for transplantation or assay.

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## 3. THE DORSAL ROOT GANGLION AS A MODEL SYSTEM

This section was contributed by Gianluca Gallo, to whom all correspondence should be addressed.

## 3.1. Introduction

Dorsal root ganglia (DRG) are ball-shaped clusters of neurons, Schwann cells, and fibroblasts found outside of the dorsal portion of the spinal cord. The neurons in the DRG project axons to the periphery and into the spinal cord, thereby forming a relay system for sensory information received from skin and muscle. DRG have proven of great use in modern neurobiology and cell biology. For example, the initial discovery and elucidation of nerve growth factor used the DRG as a standard bioassay [Hamburger, 1993]. DRG are also useful in studying the effects of inhibitory molecules associated with spinal cord injury [Fournier et al., 2002]. The DRG is a reliable and robust model system for studying sensory neurons in a variety of conditions. The DRG system also provides the advantage over neuronal cell lines that it is reflective of the biology of primary neurons. Furthermore, because DRG axons project from the periphery into the spinal cord, the DRG system is of relevance to investigations of spinal cord injury and recovery.

The purpose of this section is to describe in detail the method used routinely in the Gallo laboratory to prepare both explant and dissociated cultures of DRG cells obtained from embryonic chickens and their culture on two-dimensional substrata (Fig. 14.6). The chicken DRG can also be cultured in three-dimensional gels [Pond et al., 2002]. Embryonic chickens provide an affordable and high-throughput system for experimentation. In addition, the chicken embryo is not under animal usage regulation until embryonic day 14 in the United States and day 11 in most

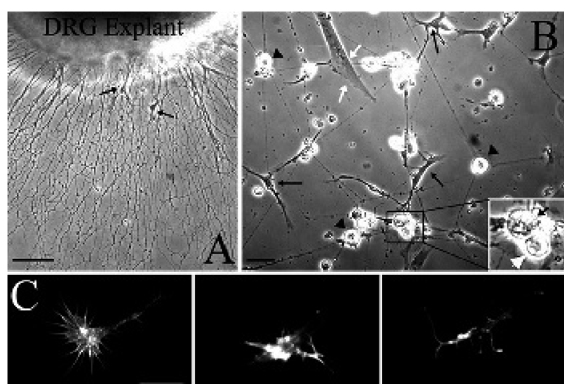


**Figure 14.6.** Phase-contrast microscopy examples of DRG axons and growth cones invitro. Pictures of an E10 DRG explant (A) and (B) dissociated E10 DRG cells cultured on laminin in 20 ng/ml NGF for 24 and 72 h, respectively. Black arrows in A and B denote Schwann cells. White arrows in B denote a fibroblast; note the classic fibroblast morphology relative to the multipolar Schwann cells. Black arrowheads in B denote DRG neuronal cell bodies. Inset in B shows two neuronal cell bodies. The cell body labeled with the white arrowhead is phase bright and healthy. The adjacent cell body, labeled by the black arrow, appears rough with phase imaging and represents a dying neuron. C) F-actin in growth cones of E10 DRG axons growing for 24 h from an explant (rhodamine-phalloidin stained). Note that growth cones can exhibit varied morphologies ranging from elaborate (left) to minor (right), exhibiting lamellipodia and/or filopodia. Bars = 100, 20, and 10 μm in A, B, and C, respectively.

of Europe, allowing experiments with this model system to commence and continue without the need to have approved animal use protocols.

Depending on the experiment, it is worthwhile considering whether explants or dissociated cells would be of greatest use. When explanted, chicken embryo DRG are spheres approximately 300–1000 μm in diameter depending on the embryonic age and position along the vertebral axis. Axons extend radially from explanted DRG, forming a "halo." This provides numerous growth cones, the tips of axons of which are not in contact with other cells. For this reason, explants have been of great use in studying the mechanism of growth cone collapse [Luo et al., 1993]. However, in explants, the neuronal cell bodies and initial segments of axons are not readily accessible. On the other hand, dissociated DRG cells allow investigation of cellular mechanisms acting throughout the neurons. Dissociated cells at low density also allow for the axons of specific neurons to be followed from cell body to their termini, the growth cones.

## 3.2. Preparation of Materials: Embryos, Reagents, and Dissection Tools

### 3.2.1. Eggs and Embryos

Fertilized chicken eggs can be obtained from Charles River Laboratories (www.criver.com). The maintenance of chicken eggs in the laboratory requires minimal equipment: (1) a refrigerator set at 10 °C and (2) a humid incubator set at 39 °C for the eggs. Chicken eggs can remain viable in a state of developmental stasis for up to 1 week when stored at 10 °C. Our laboratory receives 40 eggs a week, and on each day of the week seven eggs are moved from the refrigerator to the incubator. The eggs are disposed of after 14 days in the incubator because at this point they are considered animals and continued maintenance requires approved animal care protocols. A chicken egg incubator is necessary for two reasons, the maintenance of physiological temperature and the mechanized rotation of the chicken eggs (rotation of the eggs is required for normal development to occur). Various models of incubators are available depending on the needs of the laboratory. Our laboratory uses a model 1205 incubator (G.Q.F. Manufacturing Co.) that can accommodate up to 180 eggs. Additional models are available for laboratories working with smaller numbers of eggs.

### 3.2.2. F12H Medium

(i) Prepare 1 L of F12H medium as per manufacturer's directions, using a HEPES buffer.

(ii) To 1 L of F12H add 10 ml of 1 M HEPES and adjust pH to 7.4.

(iii) Filter sterilize.

(iv) Before use, add 5 mL of 200 mM L-glutamine to 500 ml of F12H medium and supplement with additives (See Section 3.2.3) and 5 ml of 100× Pen/Strep/Fungizone (PSF).

(v) Medium containing additives (See Section 3.2.3), PSF, and glutamine can be stored at 4 °C for up to 1 month.

### 3.2.3. F12HS10 Medium

F12HS10 consists of F12H medium, without the additives solution, but supplemented with 10% fetal bovine serum and filter sterilized. Storage is at 4 °C.

### 3.2.4. Medium Additives

Prepare the medium additives cocktail by adding to 20 ml of water:

(i) 5.1 g of phosphocreatine (vortex after adding to get into solution)

(ii) 400 mg of apo-transferrin

(iii) 20 μl of 20 mg/ml sodium selenate in water

(iv) 20 mg of insulin

(v) 20 μl of 4 μM progesterone in absolute ethanol

(vi) Filter sterilize.

The cocktail is added at 5 μl/ml of F12H medium.

(vii) Add 40 μl of 10 mg/ml sodium pyruvate in PBSA, pH 7.4, filter sterilized, per ml of F12H medium.

The additive solutions are stable for up to 3 months when stored at 4 °C.

### 3.2.5. Nerve Growth Factor (NGF)

Prepare a stock solution at 100 μg/ml in PBSA containing 0.1% BSA. Filter sterilize and store 100-μl aliquots at −20 °C. Working stocks of NGF are prepared by diluting to 10 μg/ml by addition of 900 μl of PBSA+0.1% BSA. Store at 4 °C for up to 3 months. The final concentration of NGF that provides optimal growth in F12H medium is 20 ng/ml. The minimum concentration suggested is 0.1 ng/ml.

### 3.2.6. Calcium-, Magnesium-Free Phosphate-Buffered Saline (PBSA)

PBSA (CMF-PBS, Invitrogen) is prepared according to manufacturer's directions (pH 7.4) and filter sterilized.

### 3.2.7. Trypsin Solution

Trypsin, 0.25%, 0.1% EDTA in Hanks' BSS PBSA is divided into 5-ml aliquots and stored at −20 °C.

### 3.2.8. Dissection Tools and Microscope Setup

The dissection will require two #55 forceps and a pinning forceps. The dissection requires a dissection microscope with 0.7–1.0× magnification and is vastly

facilitated by the use of fiber optic illumination. For best imaging, we suggest placing the fiber optic light source above the embryo at approximately 45° to the dissection surface.

#### 3.2.9. Laminin and Polylysine

Laminin (LN) and polylysine (PL) are commonly used culture substrata for primary neurons. PL provides a strong adhesive substratum, whereas LN is a component of the extracellular matrix and provides a more biologically relevant substratum. Prepare and store laminin stocks as suggested by the supplier. We prepare a 1 mg/ml solution of PL and store it in 100-μl aliquots at −20 °C. PL solution is prepared and diluted to the final concentration with borate buffer (add 190 mg of borax and 124 mg of boric acid to 40 ml of water). Sterilize the PL stock and borate buffer by filtration.

#### 3.2.10. Coverslips and Substratum Coating

Explants and dissociated cells are routinely cultured on 18 × 18-mm German glass coverslips coated with either laminin or PL. Coverslips are first flamed for approximately 1 second on each side with a Bunsen burner; this removes residues from the manufacturing procedure and renders the glass hydrophilic. Prolonged exposure to the flame will crack the coverslips. Insufficient flaming will result in a hydrophobic surface, and solutions will not spread well. Each coverslip is then placed in a sterile bacteriological grade culture dish and coated overnight at 39 °C with a minimum of 100 μl of either 25 μg/ml laminin or 100 μg/ml PL. Coverslips can also be double-coated with both substrata by first coating with PL for 4 h, followed by overnight coating with laminin. When using PL it is important to suction off the solution and wash three times with PBSA to remove soluble PL that can be toxic to cultured cells. We never let the PL or laminin dry on the coverslip, and the solutions are removed just before adding culturing medium (500 μl of medium/18 × 18-mm coverslip).

### 3.3. Dissection Procedure

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### Protocol 14.9. Dissection and Culture of Dorsal Root Ganglion from Chick Embryo

***Reagents and Materials***

*Sterile or aseptically prepared*

- ❑ Embryonated eggs, 10 days
- ❑ F12HS10 (See Section 3.2.3)
- ❑ F12H medium containing 20 ng/ml NGF (See Section 3.2.5)
- ❑ CMF-PBS (PBSA)
- ❑ Trypsin (See Section 3.2.7)
- ❑ Laminin (See Section 3.2.9)
- ❑ Sterile dishes (plastic or glass; one 15 × 100 mm and one 15 × 60 mm)

- ❑ Bacteriological grade culture dish
- ❑ Conical centrifuge tube, 15 ml
- ❑ Dissection instruments (See Section 3.2.8); sterilize with 70% ethanol

*Nonsterile*

- ❑ Ethanol, 70%
- ❑ Pipettor with 100-μl tips
- ❑ Pipettor, 1 ml
- ❑ Microscope and light source placed in a laminar flow hood
- ❑ Coffee can with a small plastic bag inserted to collect waste during the dissection
- ❑ Water bath at 37 °C

*Preparation for the dissection*

UV illuminate the whole dissection setup for 15 min.

*A. Removing the embryo from the egg:*

(a) Add F12HS10 to the small dish.

(b) Obtain an egg that has been in the incubator for 10 days (embryonic day 10; E10).

(c) Spray the surface of the egg with 70% ethanol.

(d) With the pointed end of the pinning forceps make quick stabbing motions to crack the egg shell, proceed circumferentially and remove the "cap."

(e) You will now see a white extraembryonic membrane within the egg. Rip this membrane with your forceps and move it aside. This will expose the yolk, albumin, and embryo.

(f) The embryo will be beneath additional clear, but vascularized, extraembryonic membranes. Using the forceps, rip these membranes and move them to the side.

(g) At this point you should be able to see the embryo clearly. If the embryo is not evident; insert the forceps about 3–5 cm into the contents of the egg and gently sweep the forceps from one side of the egg to the other; this should "stir" the contents and allow you to see the embryo. Throughout this procedure make sure not to break the yolk sac, as this will obscure the remainder of the egg contents.

(h) Once you have identified the embryo, use the tips of the forceps to grab hold of the neck, just below the head, and gently lift the embryo from the remainder of the egg contents. While lifting the embryo make sure not to squeeze the tips of the forceps, as this will result in severing of the head and the body will fall back into the egg.

(i) After lifting the embryo by the head you may have to break remaining membranes that connect the embryo to the egg.

(j) Once the embryo has been removed from the egg, place it in the large dissection dish and decapitate it by squeezing the neck, just below the head, with the tips of your forceps.

*B. Dissecting the embryo*

(k) Wash the embryo with 1–2 ml F12HS10 to remove attached albumin and/or yolk.

(l) Place the embryo on its back and, with the dissection forceps, cut the skin over the viscera.

(m) Spread the hindlimbs apart and rotate the embryo so that the neck faces toward you.

(n) Using the tips of the dissection forceps, break the skin above the sternum and rib cage along the midline.

(o) Using the forceps, break the sternum and pull the two sides of the rib cage apart, exposing the heart, lungs, and viscera.

(p) Removing the internal organs without creating a lot of tissue debris is usually one of the biggest challenges for a novice. We suggest the following approach based on the concept that the organs are interconnected by connective tissue and can thus be thought of as a unit.

   i) Starting at the top of the rib cage, use the tips of the dissection forceps to sever connective tissue between the heart and lungs with the surrounding tissues.

   ii) Next, insert the tips of the forceps (held approximately 3–4 mm apart), between the heart/lungs and the rib cage/vertebral column. As you do this, make slight upward motions. This should allow you to lift the organs within the rib cage away from the ribs.

   iii) Continue with this procedure while moving caudally, toward the tail of the embryo.

(q) With practice you will be able to remove the majority of the organs in one continuous motion. Mastering the removal of the internal organs will greatly simplify the rest of the dissection by providing a "clean" working environment free from tissue debris. Blood from the embryo can obscure the dissection, but this is easily overcome by washing the embryo with F12HS10 by pipette.

(r) In most cases, even after removal of all the organs there will still be one obstacle to overcome before the DRG will be exposed. In the chicken embryo the late embryonic kidneys (metanephros) are in close physical apposition to the lumbosacral DRGs, and are often not removed along with all the other organs (as described above). The metanephros appears as a white, "fluffy" organ that runs from the caudal 2–3 ribs all the way down to the end of the vertebral column. The DRG will be just beneath the metanephros. In addition, in E10 embryos the metanephros interdigitates between the peripheral nerves of the DRGs. Thus it is imperative to carefully remove the metanephros without damaging the DRG. This is accomplished by lifting the metanephros away from the ribs and vertebral column, using the tips of the dissection forceps in an antero-caudal manner. You may have to remove pieces of the metanephros that remain between the DRG nerves and in the caudal-most region of the embryo.

(s) After the removal of the metanephros, the DRG and peripheral nerves should be partially visible. However, the vertebral column is covered by a connective

tissue layer and it is advisable to remove this layer by picking at it with the forceps.

(t) In addition, it is important to remove the sympathetic chain ganglia before attempting the removal of individual DRG. The sympathetic chain is a strip of white nervous tissue that contains NGF-responsive neurons, and could thus contaminate the culture with non-DRG neurons. To remove the sympathetic chain, we suggest closing the tips of the forceps and gently running them over the sides of the vertebral column while moving from the caudal-most rib toward the tail of the embryo.

(u) After these obstacles have been removed, the DRG will appear as white balls of tissue lined up in a row next to the vertebral column. If the dissection was performed expertly the peripheral nerves will remain connected to the respective DRG, further facilitating identification of the DRG.

(v) The embryo is now ready for the removal of individual DRG. The DRG send nerves to the periphery and into the spinal cord. Therefore, to remove DRG from the embryo both of these nerves must be severed. Hold the tips of the forceps approximately 0.5–1 mm apart and insert them between the DRG and the vertebral column, followed by closing the tips. This should result in the severing of the DRG nerve root that projects into the spinal cord. If the peripheral nerve has been retained you should be able to the lift the DRG away from the vertebral column and observe a "ball on a chain" (i.e., DRG attached to a nerve).

(w) To free the DRG from the nerve, simply cut the nerve by crushing it with the tips of the forceps where it meets the DRG. If the nerve had been severed during earlier phases of the dissection, the DRG is ready to be removed from the embryo.

(x) Removing the isolated DRG from the embryo can be challenging at first. Resist the temptation to impale or grab the DRG with the forceps, as these procedures will damage the tissue. Instead, lift the DRG out from the embryo by "scooping" it up between the tips of the forceps held approximately 0.5 mm apart (less than the average diameter of the DRG). If the DRG tend to escape the scooping, hold them in place with the closed tips of the other forceps. With this procedure, the DRG will be caught in a drop of medium between the slightly splayed tips of the forceps. Place the DRG in the dish containing F12HS10.

(y) In our experience, after 6–10 dissections individuals develop sufficient expertise to efficiently remove a total of 12 DRG (6 from each side of the vertebral column). The lumbosacral DRG are the easiest to obtain, and are thus the focus of this description. However, with expertise, up to 40 DRG can be harvested from a single embryo along the entire axis of the vertebral column up to the cervical region. Briefly, to harvest the remaining DRG the ribs and overlying tissue/membranes must be removed along the entire axis, exposing the DRG. Removal of these DRG can be performed as described above.

(z) Clean the DRG. Before culturing, the DRG must be cleaned from attached tissue debris. During this procedure do not impale the DRG or try to grab hold of it with the forceps. It is important to clean the DRG as some of the

associated tissues (e.g., the metanephros) can have contact-mediated inhibitory effects on axon extension (G. Gallo and L. Silver, unpublished observations). The "clean" DRG will have the appearance of a white sphere.

*C. Culturing the DRG as an explant*

(a) After the DRG are cleaned they are now ready for culturing. Remove the laminin solution (See Section 3.2.10) from the coverslip and add 500 μl F12H medium containing 20 ng/ml NGF.
(b) Pick up individual DRG, using a pipette set at 10–15 μl (100-μl tips work well).
(c) Slowly expel the DRG from the pipette tip in the center of the coverslip. If the DRG does not fall in the center, you can move the DRG around by repeated cycles of expelling medium from the pipette you used to bring the DRG into the dish.
(d) Transfer the dish to the incubator and culture overnight.
(e) After a 24-h culture period explanted DRG should exhibit a dense halo of axons extending to a mean length of 700–1000 μm (Fig. 14.6). Fibroblasts and Schwann cells will also be found migrating outward from the explant.

*D. Dissociating the DRG*

After the DRG have been cleaned (See Step (p) in B above) they can be dissociated as an alternative to primary explantation (See C above).

(a) Place all the DRG in a cluster and pick them up by aspiration through a Pasteur pipette, minimizing the amount of F12HS10 medium transferred with the DRG.
(b) Deposit the DRG into 5 ml CMF-PBS, in a 15-ml conical tube, and incubate at 37 °C in a water bath for 10 min.
(c) Six minutes into the CMF-PBS treatment, put a 5-ml aliquot of frozen trypsin solution into a 37 °C water bath.
(d) At the end of the 10 min, centrifuge the DRG very briefly (15 s at 1000 *g*).
(e) Remove the CMF-PBS, taking care not to aspirate the DRG.
(f) The trypsin solution should be fully thawed by this point, and it is ready to add to the DRG. Incubate the DRG in trypsin for 12 min in the 37 °C water bath.
(g) Briefly centrifuge the DRG, as described above (Step (d)) and remove all the trypsin solution, taking care not to aspirate the DRG.
(h) Immediately add 2 ml of F12HS10 (the proteins in the serum inactivate the trypsin).
(i) Using a pipettor set at 1 ml, mechanically triturate the DRG.

Place the tip of the pipette half way into the 2 ml of medium and repeatedly aspirate and expel 50% of the medium 15 times at a rate of approximately 1 s per aspiration/expel cycle.

Do not generate excess bubbles during this procedure.

Determine, by visual inspection, whether the DRG have fully dissociated and are no longer visible.

If remnants of the DRG are visible, continue with additional round of trituration until the DRG are no longer visible.

(j) Bring the volume up to 5 ml with additional F12HS10.
(k) Centrifuge the cells into a pellet (5 min at 1000 *g*). If dissociating more than four DRG, a pellet should be visible after centrifugation.
(l) Remove the F12HS10 medium, resuspend the dissociated cells in F12H medium containing 20 ng/ml NGF, and plate the cells on laminin-coated coverslips (we suggest a 500-μl volume per 18 × 18-mm coverslip). The cell density will have to be determined empirically to fit the user's experimental needs. For standard bioassay procedures we suggest plating 2–3 dissociated DRG per 18 × 18-mm coverslip.

After a 24-h culturing period axons will be 700–1000 μm in length and the neurons will be intermixed with Schwann cells and fibroblasts (Fig. 14.6). On a laminin substratum axon extension will commence between 3–6 h after plating.

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## SOURCES OF MATERIALS

| *Item* | *Catalog #, type or size* | *Supplier* |
|---|---|---|
| Antibiotic/antimycotic | | GIBCO, Invitrogen, 1600 Faraday Ave, Carlsbad CA |
| Antibiotic/antimycotic (Lelkes and Unsworth) | MT30004CI | Fisher |
| Bacteriological grade culture dish | 08-757-100A | Fisher Scientific |
| Borate buffer | B-9876 and B-0252 | Sigma, 3050 Spruce St, St. Louis, MO 63103 |

| *Item* | *Catalog #, type or size* | *Supplier* |
|---|---|---|
| $CO_2$ incubator | Model MCO-17AIC | SANYO Scientific, distributed by Southeastern Scientific, PO Box 585147, Orlando, FL 32858 |
| Cell culture dish | 15 cm | Corning, Fisher Scientific, 3970 John's Creek Ct, Suawanee, GA 30024 |
| Cell culture flasks | T150 | Corning/Costar, Fisher Scientific |
| Centrifuge | CRU5000 | Damon IEC Division, 300 Second Av, Needham Heights, MA 02194 |
| Centrifuge tubes | 50 ml | Falcon, B-D Biosciences |
| CMF-PBS | 14200-075 | Invitrogen |
| Collagenase powder | Type IV | GIBCO, Invitrogen |
| Conical centrifuge tube | 15 ml | Nunc, Fisher Scientific |
| Cryogenic vial | 2.0 ml | Nalgene, Fisher Scientific |
| Culture bags | VueLife™ | American Fluoroseal Co |
| Dimethyl sulfoxide (DMSO) | | ATCC, PO Box 1549, Manassas, VA 20108 |
| Dissection microscope with 0.7–1.0× magnification | MX1583 | Daigger, Vernon Hills |
| DNase II | Type V from bovine spleen | Sigma |
| Dulbecco's modified Eagle's medium (DMEM) | | Cellgro, Fisher Scientific |
| DMEM high glucose (Lelkes and Unsworth) | 10-013-CV | Fisher |
| Dulbecco's modified minimal Eagle's medium- Ham's F-12 nutrient mixture | DMEM-F12 | Cellgro, Fisher Scientific |
| F12H medium | cat # 21700-075 | Invitrogen |
| Fetal bovine serum | | Gibco, Invitrogen |
| Fetal bovine serum | SH30071.03 | HyClone |
| Fetal bovine serum | cat # MTT-350-11cv | Fisher Scientific, Pittsburgh, PA |
| Fiber optic illumination | 12-455-20 | Fischer Scientific |
| Flasks (T175) | 13-680-65 | Fisher |
| Forceps #55 | 72707-01 | EMS, Hatfield, PA |
| Gas-permeable tissue culture bags | | American Fluoroseal |
| Gentamycin sulfate | | Sigma |
| German glass coverslips | ww-63-3093 | Carolina Biological Supplies, Burlington NC |
| L-Glutamine | MT25005CI | Fisher |
| L-Glutamine, 200 mM | Cat # G-7513 | Fisher Scientific |
| Growth Factor Reduced Matrigel™ | $MG_{GFR}$ | BD Biosciences, Fisher Scientific |
| Gyratory shaker | | Stovall Belly Dancer, Fisher Scientific |
| HARV bioreactor | | Synthecon Inc., 8054 El Rio, Houston TX 77054 |

| *Item* | *Catalog #, type or size* | *Supplier* |
|---|---|---|
| Hen's eggs | | Charles River Laboratories (www.criver.com) |
| HEPES buffer | Cat # H-3375 | Sigma |
| Horse serum | SH30074.03 | HyClone |
| Hydrochloric acid | | Fisher Scientific |
| Incubator | Model 1205 | G.Q.F. Manufacturing Co., Savannah GA |
| Insulin | I-5500 | Sigma |
| ITS+ Premix culture supplement | | BD Biosciences, Fisher Scientific |
| Laminin | 23017-015 | Invitrogen |
| NGF | 256-GF-100 | R&D Systems, Minneapolis, MN |
| NGF (Lelkes and Unsworth) | N2393 | Sigma |
| PBS without $Ca^{2+}$ and $Mg^{2+}$ (PBSA) | 20031CV | Fisher |
| Pen/Strep Fungizone mix | Cat # BW17-745E | Fisher Scientific, |
| Pentobarbital sodium (Sodium Nembutal) | | Abbott Laboratories, North Chicago, IL 60064 |
| Petri dishes | 15 cm | Corning |
| Petri dishes | 25 cm, glass or plastic | Corning, Falcon (B-D Biosciences), Pyrex |
| Petri dishes, | Non-TC grade, 6 cm | Nunc, Falcon (B-D Biosciences) |
| Phosphate-buffered saline,s 1× (PBS) | | GIBCO, Invitrogen |
| Phosphocreatine | P-7936 | Sigma |
| Pinning forceps | 10-270 | Fisher Scientific |
| Polylysine | P-9011 | Sigma |
| Progesterone in absolute ethanol | P-8783 | Sigma |
| Retinoic acid, sodium | | Sigma |
| Rotatory Cell Culture Systems | RCCS | Synthecon, Inc. |
| Sodium pyruvate | P-2256 | |
| Sodium selenate in water | S-8295 | Sigma |
| apo-Transferrin | T-2252 | Sigma |
| Trizma Base (Sigma) | | Sigma |
| Trypan Blue | | Gibco, Invitrogen |
| Trypsin | | Sigma |
| Trypsin inhibitor | | Sigma |
| Trypsin, 0.25%, 0.1% EDTA in PBSA | MT-25-053-c1 | Fisher Scientific |
| Trypsin-EDTA solution, 0.25% | | Sigma |

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