Video Article

Isolation and Culture of Dissociated Sensory Neurons From Chick Embryos

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Abstract

Neurons are multifaceted cells that carry information essential for a variety of functions including sensation, motor movement, learning, and memory. Studying neurons *in vivo* can be challenging due to their complexity, their varied and dynamic environments, and technical limitations. For these reasons, studying neurons *in vitro* can prove beneficial to unravel the complex mysteries of neurons. The well-defined nature of cell culture models provides detailed control over environmental conditions and variables. Here we describe how to isolate, dissociate, and culture primary neurons from chick embryos. This technique is rapid, inexpensive, and generates robustly growing sensory neurons. The procedure consistently produces cultures that are highly enriched for neurons and has very few non-neuronal cells (less than 5%). Primary neurons do not adhere well to untreated glass or tissue culture plastic, therefore detailed procedures to create two distinct, well-defined laminin-containing substrata for neuronal plating are described. Cultured neurons are highly amenable to multiple cellular and molecular techniques, including co-immunoprecipitation, live cell imagining, RNAi, and immunocytochemistry. Procedures for double immunocytochemistry on these cultured neurons have been optimized and described here.

Video Link

The video component of this article can be found at http://www.jove.com/video/51991/

Introduction

Neurons are complex cells that carry information essential for a variety of functions including sensation, vision, motor movement, learning, and memory. Unique from other cell types, neurons extend arm-like processes, called axons, to form essential neural highways for communication. During development specialized compartments located at the tips of growing axons, called growth cones, navigate through a concert of extracellular cues to lead the axon to its appropriate destination. The intricate molecular mechanisms that underlie growth cone navigation are not fully understood. To better understand these mechanisms, investigators have used cell culture models to study neurons in a defined and simplified *in vitro* environment. Studying neurons in culture[1] has led to significant advances in our understanding of neuronal cell biology including: neuronal differentiation[2], cytoskeletal dynamics, endocytosis and trafficking, dendrite regulation[3,4], axonal regeneration[5], and clinical conditions such as neuropathies[6]. In addition, cultured neurons are highly amenable to a wide range of research techniques including immunocytochemistry, cell surface co-immunoprecipitation, Western blot, transfection, RNAi, and live imaging such as timelapse analysis of growth cone motility. Thus, culturing primary neurons is a powerful approach to elucidate numerous aspects of the cell biology of neurons.

The cell culture model provides investigators with detailed control over environmental conditions and variables. For example, the substrata on which neurons are plated (and grow upon) can be easily manipulated. Here, we provide detailed instructions for generating two distinct substrata, one with a low laminin-1 concentration and the other with saturating concentrations of laminin-1. Surprisingly, different concentrations of the same molecule can have dramatic effects on the internal state of neurons as well as their cell surface composition. For example, intracellular levels of cAMP and surface levels of integrins are significantly different in neurons plated on these two substrata[7,8]. Additional studies have shown that other molecules, including fibronectin and chondroitin sulfate proteoglycans, impact the expression of cell surface molecules and neuronal motility[7-11]. In addition, soluble molecules such as neurotrophins and neurotropins also impact cell membrane composition and neuronal motility[12-16] and can be easily and accurately manipulated in a cell culture model.

Here, we describe methods to isolate and culture dissociated sensory neurons from chick embryos. This procedure has been used to make significant breakthroughs in neurobiology, including axon outgrowth[5,7,8,10,11,16-21] and was modified from a procedure designed to isolate ganglion cells[22]. There are several advantages to this approach. First, many features of chick dorsal root ganglion (DRG) development are well characterized including the time frame for birth, axon extension, and protein expression profiles[2,23-28], thus providing an instructive basis upon which to build informative *in vitro* experiments. Second, dissociated neuronal cultures allow the investigator to more directly study neurons compared to alternative approaches using intact DRG explants (which contain neurons and non-neuronal cells) and/or mixed cultures containing both dissociated neurons and non-neuronal cells. Third, the procedure described here is straightforward, inexpensive and amenable to undergraduates. Therefore, this technique can be used for research as well as for teaching purposes. Furthermore, minor variations of this protocol should allow fast, high yield purification of neurons from sources other than DRGs. For example, this procedure could be modified to provide neuronally enriched cultures from other tissues such as embryonic forebrain or spinal cord.

Immunocytochemistry protocols have been optimized for these dissociated neuronal cultures and are described in detail here. The procedure for double immunocytochemistry against neural cell adhesion molecule (NCAM) and β1 integrins is provided. Data generated from these immunocytochemical methods have been used to examine the spatial patterning and intensity of several molecules in cultured neurons[8,16].

Protocol

1. Coverslip Preparation: Acid Wash and Bake

1. At least 2 days prior to dissection (step 2), begin the following steps to prepare coverslips. Perform the acid wash step to remove oils and thoroughly clean coverslips.
2. Load coverslips in porcelain holder that vertically positions coverslips and prevents them from touching each other. Submerge holder and coverslips into glass histology container filled with 2 M hydrochloric acid (HCl). Alternatively, if porcelain holder is not available, spread ~20 coverslips horizontally to cover the bottom of 100 mm glass Petri dish and submerge coverslips in 2 M HCl.
3. Place glass vessel (with coverslips submerged in acid) on rocker table in fume hood and allow gentle rocking for at least 5 hr at room temperature. Alternatively, acid wash coverslips overnight under the same conditions.
4. Wash coverslips with distilled H_2O(dH_2O) by transferring porcelain holder loaded with coverslips from 2 M HCl to a clean glass histology container filled with dH_2O. Alternatively, decant 2 M HCl from glass Petri dish with coverslips and refill with dH_2O.
5. Wash by decanting dH_2O again and refilling glass vessel with fresh dH_2O. Repeat for a total of 3 quick washes.
6. Begin longer washing regimen by returning glass vessel (with coverslips and dH_2O) onto rocker. Allow for gentle agitation for 10-30 min at room temperature. Then, decant dH_2O and add fresh dH_2O. Repeat for a total of at least ten washes. Alternatively, continue washing overnight.
7. Place washed coverslips into furnace preheated to 350 °C. If using porcelain holder, remove holder from dH_2O-filled glass container and then place holder with coverslips directly into the furnace. If using a glass Petri dish, decant dH_2O and place the glass dish with coverslips into the furnace.
8. Bake coverslips for at least five hours or overnight at 350 °C.
9. After baking is complete, remove coverslips from porcelain holder with fine forceps and place into sterile 100 mm glass Petri dish. Leave coverslips that were not originally placed in porcelain holder in the glass Petri dish.

2. Chick Dissection and Isolation of DRGs

1. Remove fertile, staged White Leghorn chick egg from incubator (held at 37-38.5 °C with gentle rocking).
   NOTE: Previous studies have used embryonic day 7-10 for isolation of DRGs[2,8,11-13,16].
2. Place selected egg in laminar flow hood. Conduct all procedures listed below under aseptic conditions in the laminar flow hood with sterile solutions and instruments. Pre-warm all solutions in a 37 °C water bath.
3. Crack egg and place contents into a plastic 100 mm Petri dish. Use standard forceps to transfer embryo to another 100 mm Petri dish.
4. Using a Pasteur pipette, add warmed 1x PBS to Petri dish to keep tissue wet. Decapitate embryo by pinching neck with fine forceps. Transfer body to another clean 100 mm Petri dish.
5. Add warm F12HS10 (Ham's F12, 10 mM HEPES, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 10% fetal bovine serum) to dish with Pasteur pipette to keep tissue wet. Use forceps to position embryo on its dorsal side (**Figure 1A**).
6. Place 100 mm plastic Petri dish containing embryonic tissue onto a binocular stereovision dissecting microscope in laminar flow hood. Use fine forceps to make a vertical midline incision from tail to neck by pinching the developing dermis to expose the heart, lung and other organs. Alternatively, make a series of small tears to expose internal organs.
7. Gently use 2 sets of sterile fine forceps to: a) grasp the neck and b) grasp the aorta or other tissue immediately superior to the heart and pull towards the tail, eviscerating the animal.
8. Apply warm F12HS10 with Pasteur pipette to tissue. Use forceps to remove any remaining cardiovascular, respiratory or digestive organs to expose the developing ribs, vertebral column and DRGs (**Figures 1B-1D**).
9. Under the dissecting microscope, use fine forceps to further remove organs and collect DRGs along both sides of lumbar vertebral column, inferior to the ribs. Collect DRGs by plucking intact DRGs from the embryo. Pinch and sever the roots that reach from the DRG towards the spinal cord and grasping the developing nerve that reaches from the DRG towards the periphery or vice versa.
10. Place intact DRGs into a 35 mm culture dish filled with warm F12HS10. Remove any excess tissue, such as the dorsal or ventral roots that may have adhered to DRGs, from the intact DRGs in 35 mm dish by pinching with fine forceps.
11. To obtain DRGs superior to the lumbar region, remove the ventral half of the thoracic and cervical vertebral column. This is done by making a cut in the developing vertebral column perpendicular to the spinal cord axis in the upper cervical region and another cut in the upper lumbar region. Then, make cuts parallel to the spinal cord axis along the right and left sides of the vertebral column. Lift the ventral half of the vertebral column from the embryo, which exposes the exposes the spinal cord (**Figures 1E-1F**).
12. To allow easy access to DRGs, remove the thoracic and cervical spinal cord by grasping the spinal cord with fine forceps. Use the forceps to lift the spinal cord out of the vertebral column. As an approximation, the thoracic spinal cord is at the level of ribs and the cervical area is superior to the ribs.
13. Collect intact thoracic and cervical DRGs with fine forceps and place them in warm F12HS10 with other DRGs. Remove excess tissue that may adhere to DRGs.
14. Rinse interior of a new glass Pasteur pipette with F12HS10 to help prevent DRGs from sticking to walls of pipette. Use rinsed pipette to transfer all DRGs and F12HS10 from small Petri dish to a sterile 15 ml conical tube and immediately proceed to step 3.
    NOTE: Additional embryos can be dissected to increase number of DRGs. DRGs should be kept in F12HS10 at 37 °C until ready for step 3.

3. Dissociation, Enriching, and Culturing DRG Neurons – Part 1

1. Place conical tube with intact DRGs into centrifuge and gently spin (200 x g) for 2-3 min. Confirm that DRGs have sunk to bottom of tube. If not, spin again.
2. Using a pipette, gently remove F12HS10, leaving DRG pellet undisturbed. Add 2 ml of 1x calcium and magnesium free (CMF) PBS, dislodging the pellet of DRGs. Spin again and remove CMF PBS while leaving the DRG pellet intact. Repeat this wash step for a total of three washes to remove fetal bovine serum in F12HS10, which would interfere with trypsin digestion in next step.
3. Add 2 ml of warmed trypsin to 15 ml tube containing 2 ml CMF PBS and DRGs. Place tube in 37 °C water bath for 10-15 min to digest DRGs into dissociated cells.
4. During incubation, make F12H + supplement containing media (Ham's F12, 10 mM HEPES 100 units/ml penicillin, 0.1 mg/ml streptomycin, 10 ng/ml NT3, 10 ng/ml NGF, 200 mM l-glutamine and N2) and warm in 37 °C water bath. Typically, 10 ml of total media is sufficient for 4-5 35 mm dishes of primary sensory neurons. Also begin thawing laminin-1 for coating coverslips (step 4).
5. Gently triturate DRGs in trypsin solution with p1000 pipette and visually inspect for absence of intact DRGs. Continue trituration until intact DRGs are no longer seen by eye and/or allow for longer trypsin incubation in 37 °C water bath until intact DRGs are dissociated. To protect DRG neurons from damage, avoid air bubbles during trituration and limit trypsin digestion to <30 min.
6. Centrifuge tube containing dissociated DRGs and trypsin at 200 x g for 3-5 min to form pellet. Spin longer if needed until pellet is formed.
7. Carefully aspirate trypsin without disrupting pellet. Add 2 ml of F12HS10 to pellet. Gently triturate DRGs with a p1000 pipette tip.
8. Transfer all contents from 15 ml conical tube into 100 mm culture dish. Rinse the tube with 8 ml F12HS10 and transfer it to the culture dish, 2 ml at a time for a total of 10 ml. This rinsing step helps collect cells that may have remained adhered to the walls of the tube.
9. Place 100 mm culture dish (with 10 ml F12HS10 and dissociated DRG cells) into a humidified 37 °C incubator for 3 hr.
   NOTE: CO_2 is not required in the incubator because HEPES buffers media pH in the absence of CO_2. During incubation, glial cells bind to surface of tissue culture plastic and neurons tend to loosely adhere to glial bed.

4. Coating Acid Washed and Baked Coverslips with Laminin-1

1. Thaw sterile aliquots of frozen laminin-1 on ice.
2. Under sterile conditions, add 1 ml of sterile 1x PBS to 50 µl aliquot of laminin-1.
3. Use a spectrophotometer to obtain an absorbance value of laminin-1 at 280 nm. Use this value in the following equation to determine the concentration of laminin-1.

ABS_{280} = (concentration mg/ml) * (extinction coefficient of protein)
The extinction coefficient for laminin-1 is 0.86.

4. Under sterile conditions, dilute laminin-1 with 1X PBS to obtain the concentrations 1 mg/ml and 20 mg/ml. Use these two applied concentrations of laminin-1 to achieve a ten-fold difference of laminin-1 *bound* to the coverslip (30 ng/cm^2 and 300 ng/cm^2, respectively) after incubation[7,8,10].
5. In a laminar flow hood, use fine forceps to place one acid-washed and baked coverslip into the bottom of one 35 mm culture dish. Repeat as necessary to obtain the number of dishes needed.
6. Subject the coverslips to UV light for 20-30 min to further ensure sterilization. Keep dish lid off during this step.
7. Add 400 µl of prepared laminin-1 to each glass coverslip. Use pipette tip to spread laminin-1 to ensure full coverage of coverslip. Surface tension will allow laminin-1 to remain on coverslip and not spread onto bottom of culture dish, which is required.
8. Place lid onto dish and allow laminin-1 to incubate for 2-3 hr at room temperature (3 hr is optimal).
9. After laminin-1 incubation, rinse coverslips using aseptic technique with sterile PBS in laminar flow hood. Remove solution on coverslip and add 400 µl of PBS. Wait 5-10 min. Repeat for a total of 3 rinses.
10. Leave PBS on coverslip after last rinse. Do not break surface tension during rinsing and do not allow coverslips to dry out. Leave lid on until ready to plate dissociated neurons onto the coverlip.

5. Dissociation, Enriching, and Culturing DRG Neurons – Part 2

1. After incubation, use a 10 ml sterile pipette to gently rinse the bottom of the 100 mm dish containing dissociated DRG cells. Hold dish at ~45° angle, pull ~7 ml of F12HS10 with pipette aid, then gently expel 7 ml onto approximately 1/3 of dish. Repeat 5x, gently dislodging neurons.
2. Rotate dish and repeat rinsing procedure, for another six rinses, on another 1/3 of the dish. Repeat procedure again for remaining 1/3 of dish. In this way, gently rinse the entire bottom of the dish with F12HS10 to remove neurons.
3. Using same pipette, transfer F12HS10 containing neurons from 100 mm dish to 15 conical tube. Centrifuge for 5-10 min at 200 g to pellet cells.
4. Remove F12HS10, leaving pellet undisturbed. Resuspend pellet with 2 ml of warmed F12H + supplements.
5. Use a hemocytometer to determine cell density.
6. Dilute cells as necessary with F12H + supplements to obtain desired plating concentration (120,000 cells/ml for coverslips coated with 1 µg/ml laminin-1 and 40,000 cells/ml for coverslips coated with 20 µg/ml laminin-1[8,16]).
7. Remove PBS from laminin-coated coverslips and immediately place 400 µl of cells onto coverslips. Carefully transfer dish to a humidified, 37 °C cell culture incubator. Allow to incubate for at least 1 hr and up to 3 hr to allow neurons to adhere to laminin-1.
8. Under sterile conditions, gently flood 35 mm dishes containing neurons with 1.6 ml of F12H + supplements.
   NOTE: At this time, surface tension on coverslip can be broken. Neurons have adequately adhered to laminin-1 on coverslip.
9. Return cells to incubator and incubate overnight. Perform experiments such as immunocytochemistry the following day.

6. Immunocytochemistry

1. Prior to immunocytochemistry, warm fixative solution (4% paraformaldehyde, 30% sucrose 2X PBS) in 37 °C water bath.
   NOTE: Before fixing cells, treat with various reagents, such as netrin-1, Mn^{2+}, sonic hedgehog, semaphorin, and ephrin[16,29-31].
2. Slowly remove 1 ml of the 2 ml of F12H + supplements from culture dish. Gently add 1 ml of warmed fixative solution. Allow to incubate at room temperature for 10-15 min.
3. Carefully remove fixative solution and add 2 ml PBS. Apply solution towards edge of dish, avoiding possible dislodging of fixed cells due to application of PBS. Incubate at room temperature for 5 min.
4. Repeat PBS wash for a total of 5 washes. Do not allow cells to dry during any step of immunocytochemistry.
5. Remove PBS. Apply 1 ml of blocking solution (0.1% Triton-X, 1X PBS, 5% normal goat serum). Incubate for 1 hr at room temperature. Alternatively, to stain surface molecules, do not use Triton-X in blocking solution[8].
6. Dilute primary antibodies into blocking solution per manufacture's recommendation. Use 1:500 for antibodies against NCAM and activated β1 integrins[16]. Remove blocking solution, add 1 ml of primary antibody solution and incubate for 1 hr at room temperature or overnight at 4 °C.
7. Remove primary antibody solution and add 2 ml of 1X PBS. Incubate for 5-10 min at room temperature. Repeat for a total of 4 PBS washes. Washes can be extended, if needed.
8. Dilute secondary antibody 1:1,000 in blocking solution. Remove PBS, add 1 ml secondary antibody solution and incubate for 1 hr at room temperature.
   NOTE: Secondary antibodies are light sensitive. Keep solutions and cells in the dark as much as possible from this point forward.
9. Remove secondary antibody solution and rinse 5x with 1x PBS, as done previously.
10. Apply ~60-80 µl of Fluoromount G onto a microscope slide. Using fine forceps, gently lift coverslip out of dish and lower coverslip into Fluoromount G onto microscope slide. Avoid air bubbles.
11. Store slides horizontally at 4 °C in the dark. After 12-24 hr, Fluormount G will polymerize and coverslip will be firmly attached to microscope slide. Image cells after this polymerization step is complete.

Representative Results

The protocol described here enables investigators to culture an enriched population of dissociated embryonic sensory neurons with very few (*e.g.*, <5%) non-neuronal cells[7,8,10,16]. Numerous DRGs can be obtained from the lumbosacral, thoracic and cervical regions. Depending upon the needs of the investigator, DRGs from these distinct anatomical regions can be easily isolated. For example, **Figure 1** shows images of the chick embryo through various stages of dissection with the lumbar DRGs highlighted in **Figures 1C** and **1D** and thoracic DRGs in **Figures 1E** and **1F**.

Cultured cells are easily labeled by immunocytochemistry. Here, cultured cells are immunostained with antibodies against β1 integrin and NCAM (**Figure 2**). Surprisingly, we've been able to visualize fluorescent staining for up to one year after these ICC procedures, if the slides were kept horizontally at 4 °C and in the dark. However, the longevity of the ICC stains needs to be determined by user and per antibody.

Depending on the density of neurons, growth cones at the tips of extending neurites can also be visualized (**Figures 2D-2F**). Neurons plated at 40,000 and 120,000 cells/ml on coverslips coated with 20 µg/ml and 1 µg/ml of laminin-1 respectively, have numerous free neurites up to 26 hr post plating. These cultures can be used for specifically analyzing growth cones in addition to analyzing the entire neuron. Previously, this procedure has been used to evaluate growth cone velocity and behaviors, such as growth cone collapse[8,9,11,13,16]. Lower plating densities on these coverslips results in dead neurons that are not adhered to the substrata. The exact appropriate density needed for each experiment needs to be optimized per investigator.

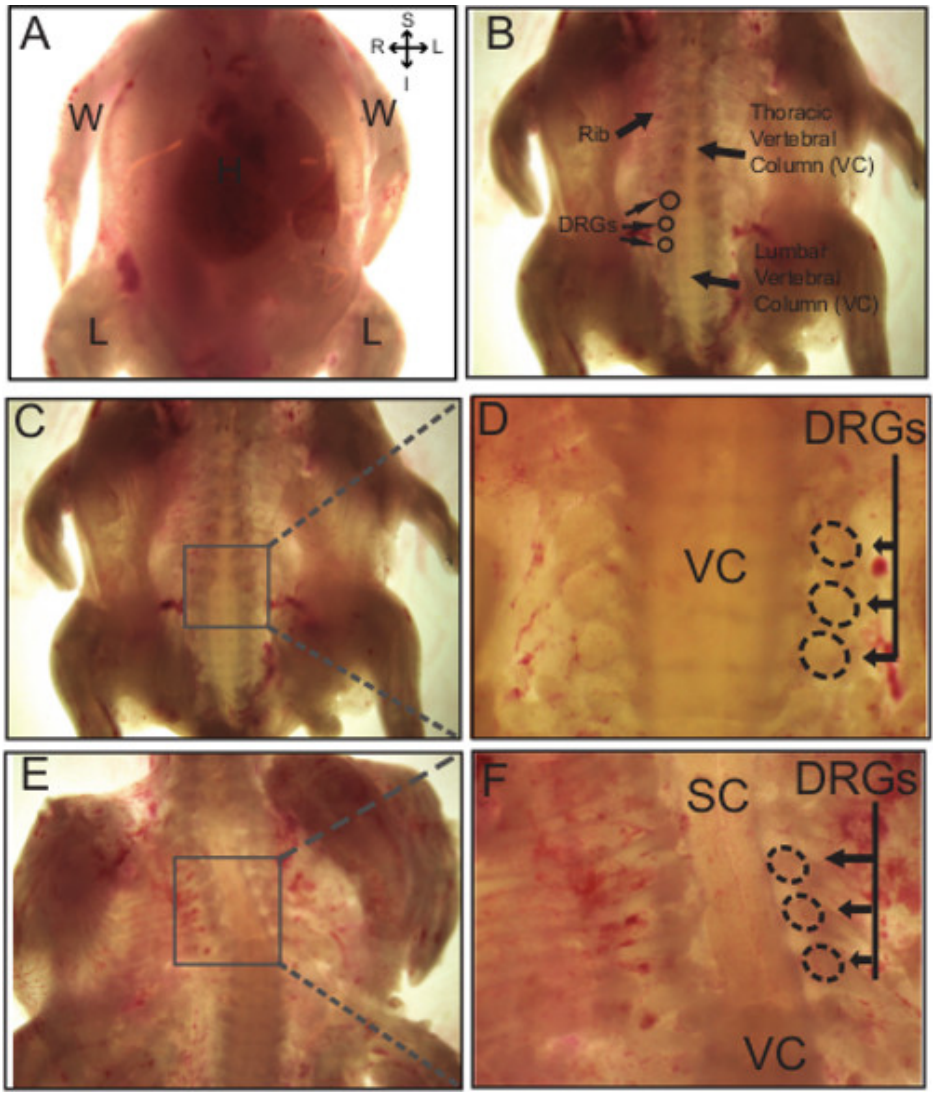

Figure 1. Stages of embryonic chick dissection to obtain DRGs. A) Chick embryo lying on its dorsal side. The heart (H), wings (W) and legs (L) are labeled. The top of the image is towards the head, or superior (S). The bottom of the image is towards the tail, or inferior (I). R= right side of animal, L= left side of animal. All subsequent images are in the same orientation. **B)** The internal organs have been removed from the embryo in A. The vertebral column, ribs and DRGs are seen. For simplicity, one rib is labeled, three representative DRGs are identified by black circles and the thoracic and lumbar vertebral column is labeled. **C)** Same chick embryo image as B with a grey box identifying the region shown at higher magnification in D. **D)** Higher magnification of lumbar vertebral column (VC) region. Three of the eleven DRGs in this view are identified by dashed circles. DRGs are round and encapsulated. These features make it easy to obtain intact DRGs with forceps. **E)** A chick embryo after removal of internal organs and ventral half of thoracic vertebral column. Grey box indicates region shown at higher magnification in F. **F)** Spinal cord (SC) is seen in region where vertebral column has been removed. Towards the bottom of the image, the lumbar vertebral column (VC) is still intact. 3 of 10 DRGs are identified by dashed circles. DRGs are found between each set of ribs.

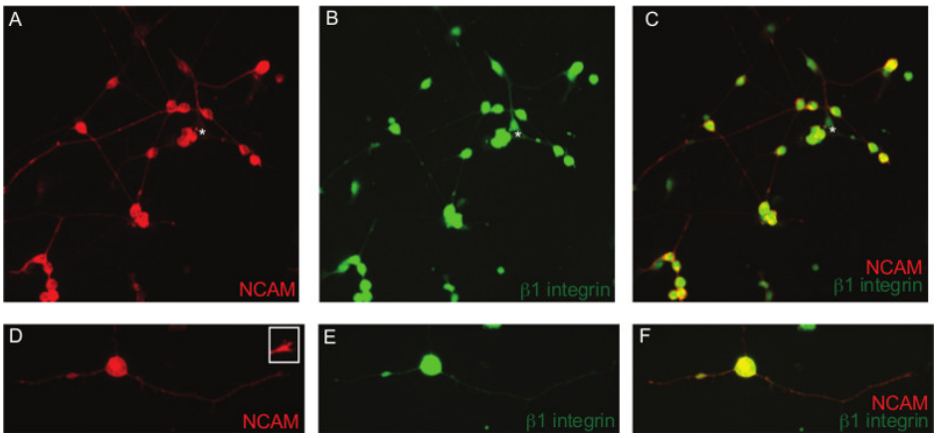

Figure 2. Cultured primary sensory neurons immunolabeled for NCAM and β1 integrins. Chick DRG neurons cultured on high concentrations of laminin-1 are immunopositive for NCAM (**A**) and β1 integrin (**B**). **C)** Merged image of two stains reveals most cells are positive for both NCAM and β1 integrin. These avian sensory neurons are immunopositive for both of these markers. However, there is a cell, marked by an asterisk that is NCAM negative and β1 integrin positive, consistent with a non-neuronal cell. **D-F)** Higher magnification of a cultured sensory neuron shows neurites extending from the cell body. **D)** Inset in white box shows higher magnification of growth cone at the tip of a neurite.

Discussion

Here we present detailed protocols for isolating and culturing dissociated sensory neurons from a chick embryo. This procedure generates an enriched population of robustly growing neurons *in vitro*[7,8,10,16]. Numerous cellular and molecular techniques can be applied to these cultured neurons, including immunocytochemistry, which is described here. This protocol was recently used to quantitatively assess the intensity of immunolabeled activated integrins in sensory growth cones[8,16]. In these previous studies, a software program was used to accurately create an outline of the distal 20 micron end of the growth cone. The outline was made based on NCAM staining. The ubiquitous expression of NCAM in neurons enabled the software program to identify the entire growth cone rather than a limited portion of the growth cone. This growth cone outline was then identified as a region of interest and the integrin staining intensity within that region of interest was assessed by the software. In this manner, the intensity of a protein of interest can be easily measured in a specific region within cultured sensory neurons.

In order to obtain reliable results, the following recommendations are provided. First, ensure that coverslips are well washed with water after HCl incubation in steps 1.4-1.6. Otherwise, molecules such as laminin-1 will not disperse well on the coverslip. Second, confirm that DRGs are adequately dissociated (step 3.5) after trypsin digestion, otherwise clumps of cells will form and this will decrease neuronal purity. Third, it is optimal to incubate DRG cells in step 3.9 for a full 3 hr to obtain a purer population of neurons. If this incubation step is shortened, then decrease rinsing steps 5.1 and 5.2 to limit the number of non-neuronal cells that are dislodged after a shorter incubation time. Fourth, ensure that surface tension is maintained while applying laminin-1 and dissociated neurons to the coverslips. If surface tension is lost, the solution will extend beyond the coverslip and dry. This will kill the neurons. Fifth, optimal cell density needs to be determined by each investigator. Previous studies have plated 120,00 neurons/ml and 40,000 neurons/ml on coverslips coated with 1 µg/ml laminin-1 and 20 µg/ml laminin-1, respectively[8,16]. This concentration was low enough to allow the investigators to study free axon endings (endings that had not yet connected with another neuron). Sixth, during immunocytochemistry, be certain to gently rinse cells. Removing or applying solutions too quickly can dislodge neurons from the coverslip.

Limitations of this procedure include the somewhat-limited time window of chick DRG dissection. DRGs can easily be obtained from embryonic day (E) 7-10 chick embryos. While it is possible to obtain DRGs earlier than E7, it is somewhat challenging. After E11, more cartilage transitions to bone and this makes the dissection more difficult. Thus, studies that aim to compare embryonic versus adult neurons would not be ideal for this chick model, however, previous studies have used the rat model for age comparison in DRGs[32]. It should be noted that rat DRG dissection is more expensive, less robust and technically more challenging than chick. Another point to consider is the ability of cultured embryonic DRG neurons to change over time in culture. For example, these sensory neurons express different levels of receptors within 48 hr depending upon the presence of select neurotrophins[2,30].

Two subclasses of DRG neurons can be enriched by use of selective neurotrophins in the media[2,12,30]. For example, nerve growth factor (NGF) and neurotrophin-3 (NT3) primarily support the survival of cutaneous and proprioceptive neurons, respectively[33]. The media used in this experiment has both NGF and NT3, however, this can be easily modified to select for either cutaneous or proprioceptive sensory neurons.

Dissociated neurons are amenable to numerous live imaging techniques including timelapse imagining of growth cone motility and calcium imaging. The media used here is advantageous for live cell imaging because it does not require CO_2 for pH buffering of the media. Thus, data acquisition from live cells can be performed on a microscope with a warmed stage but does not require maintenance of adequate CO_2 levels. Growth cone velocity, collapse[8,16,30], and calcium levels[17] have been studied in live primary chick sensory neurons isolated by the technique described here. In addition, RNAi can successfully decrease protein levels in cultured chick DRG neurons[34].

Insights gained from well-defined *in vitro* models can be used as an essential foundation to design experiments in a more complex *in vivo* model. For example, various approaches were used in the Silver lab[35] to stimulate axon outgrowth in an *in vitro* model of the glial scar. Of the variety of reagents tested *in vitro,* only two (inflammation induction and digestion of chondroitin sulfate proteoglycans) could foster axon regeneration. Interestingly, a combination of these two treatments *in vivo* stimulated dramatic and functional axon regeneration into the spinal cord[35]. In

this case, the *in vitro* experiments provided an important tool to rapidly screen for reagents that increased axon outgrowth. The reagents that successfully promoted axon growth *in vitro* also proved successful *in vivo*, which further supports the value of cell culture experiments.

Disclosures

The authors have nothing to disclose.

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