Video Article

Dissection and Culture of Chick Statoacoustic Ganglion and Spinal Cord Explants in Collagen Gels for Neurite Outgrowth Assays

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Abstract

The sensory organs of the chicken inner ear are innervated by the peripheral processes of statoacoustic ganglion (SAG) neurons. Sensory organ innervation depends on a combination of axon guidance cues[1] and survival factors[2] located along the trajectory of growing axons and/or within their sensory organ targets. For example, functional interference with a classic axon guidance signaling pathway, semaphorin-neuropilin, generated misrouting of otic axons[3]. Also, several growth factors expressed in the sensory targets of the inner ear, including Neurotrophin-3 (NT-3) and Brain Derived Neurotrophic Factor (BDNF), have been manipulated in transgenic animals, again leading to misrouting of SAG axons[4]. These same molecules promote both survival and neurite outgrowth of chick SAG neurons *in vitro*[5,6].

Here, we describe and demonstrate the *in vitro* method we are currently using to test the responsiveness of chick SAG neurites to soluble proteins, including known morphogens such as the Wnts, as well as growth factors that are important for promoting SAG neurite outgrowth and neuron survival. Using this model system, we hope to draw conclusions about the effects that secreted ligands can exert on SAG neuron survival and neurite outgrowth.

SAG explants are dissected on embryonic day 4 (E4) and cultured in three-dimensional collagen gels under serum-free conditions for 24 hours. First, neurite responsiveness is tested by culturing explants with protein-supplemented medium. Then, to ask whether point sources of secreted ligands can have directional effects on neurite outgrowth, explants are co-cultured with protein-coated beads and assayed for the ability of the bead to locally promote or inhibit outgrowth. We also include a demonstration of the dissection (modified protocol[7]) and culture of E6 spinal cord explants. We routinely use spinal cord explants to confirm bioactivity of the proteins and protein-soaked beads, and to verify species cross-reactivity with chick tissue, under the same culture conditions as SAG explants. These *in vitro* assays are convenient for quickly screening for molecules that exert trophic (survival) or tropic (directional) effects on SAG neurons, especially before performing studies *in vivo*. Moreover, this method permits the testing of individual molecules under serum-free conditions, with high neuron survival[8].

Video Link

The video component of this article can be found at http://www.jove.com/video/3600/

Protocol

Recipes

Chick Ringers

7.2 g	NaCl
0.23 g	$CaCl_2 + 2H_2O$
0.37 g	KCl
0.115 g	Na_2HPO_4
900 ml	Water (Tissue culture grade)

Note: Adjust pH to 7.4. Bring final volume to 1000 ml with water.

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10X PBS

40 g	NaCl
1 g	KCl
7 g	Na_2HPO_4
1.2 g	KH_2PO_4
450 ml	DEPC water

Note: Adjust pH to 7.4. Bring final volume to 500 ml with water. Working concentration (1X) is made by diluting 1 part 10X stock with 9 parts of tissue-culture grade water, such as that obtained with a Millipore UV irradiation system to generate water with 18 MΩ-cm conductivity.

SAG explant holding medium

- DMEM/F12 with L-glutamine, 10 mM Hepes
- 10% Insulin, Transferring, Selenium (ITS+1)
- 1% pen-strep

Explant culture medium

- SAG explant holding medium
- 10 ng/ml Neurotrophin-3 (NT-3)
- 10 ng/ml ciliary neurotrophic factor (CNTF)

Spinal cord dissection and holding medium

- L-15
- 10% fetal calf serum
- 1% pen-strep

Incubate eggs at 37-38°C. Sterilize dissecting tools and Sylgard dissecting dish in 70% ethanol. Dissecting dishes and tools can also be UV sterilized overnight.

1. Bead preparation

1. Place beads in a microtube with 1 ml sterile PBS, mix, and wait for the beads to settle to the bottom of the tube.
2. Wash the beads several times by removing the supernatant after the beads have settled and resuspend in PBS.
3. Incubate the beads in purified protein (diluted in PBS) or PBS alone (Control) for 1 hour at room temperature.
4. Rinse the beads in PBS and store in a 24-well plate with 1ml PBS until placed in collagen.

2. Statoacoustic ganglion dissection

1. On E4 remove the embryo from the egg and place it in a dish with chick Ringer's solution to remove embryonic membranes.
2. Place the embryo in a Sylgard© lined petri dish filled with cold HBSS and position the embryo on its side with the otic vesicle facing up. Pin the embryo to the dish using dissecting pins.
3. Using two dissecting pins, make a horizontal cut through the skin immediately ventral to the canal pouches, approximately where it meets the cochlear duct. Make a vertical cut anterior and posterior to the SAG. Use forceps or a dissecting pin to lift and remove the flap of skin bordered by the three cuts.

   Note: The SAG is located at the anteroventral edge of the otocyst, about midway between the dorsal pouch which is readily visible with brightfield base illumination, and the ventral cochlear duct which projects ventromedially and is obscured by the pharyngeal arches. The cochlear duct elongates so its dorsoventral dimension will progressively increase on E4 between Hamburger-Hamilton stages 23-25 9.
4. Pull the otocyst in a posterior direction to separate it from the SAG. Displace tissue surrounding the SAG with dissection pins and carefully remove the SAG from the embryo using #55 forceps. Remove large chunks of mesenchymal tissue and protruding nerve bundles from the SAG.
5. Using a wide-mouth pipet tip, transfer the explant to a 24-well plate with 0.5 ml SAG explant holding medium. Keep explants on ice or at room temperature for up to 4 hours.

3. Spinal cord dissection

1. Remove the E6 embryo from the egg and place it in a dish containing chick Ringer's solution. Remove the head and embryonic membranes.
2. Place the body in a Sylgard© dissecting dish containing cold spinal cord dissection medium. Position and pin the embryo "ventral down" and caudal facing the experimenter. Place dissecting pins through each limb and through the anterior end.

3. Using forceps and/or dissecting pins, carefully remove skin and tissue, moving in a ventral direction, until the dorsal surface of the spinal cord is visible.
4. Cut the dorsal midline of the spinal cord with a dissecting pin, in a posterior to anterior direction, along the entire length of the spinal cord. This creates an "open book" as the left and right sides of the spinal cord separate.
5. Remove surrounding tissue, laterally, to isolate the spinal cord and expose the dorsal root ganglia (DRG). Remove the DRG and meninges by rubbing a dissecting pin or forceps between the spinal cord and DRG.
6. Remove the spinal cord from the body by holding the caudal-most end of the spinal cord with forceps and lifting up and away from the experimenter. Remove remaining DRG and meninges.
7. Hold one end of the the spinal cord with forceps (or pin it to the dish) and use Vannas scissors to cut along the ventral midline, to bisect the spinal cord.
8. Hold one end of the explant with forceps to immobilize the tissue and use Vannas scissors to cut small explants (100-500 um in length) along the length of the tissue. The floor plate can be recognized as a clear thickening of tissue along one explant edge.
9. Use a wide-mouth pipet tip to transfer explants to a 24 well plate with 0.5 ml cold dissection medium. Keep explants on ice to maintain tissue viability, for up to 4 hours.

4. Explant culture with purified proteins to test neurite responsiveness

1. Prepare a 1.5 mg/ml collagen solution in a 15 ml conical tube, on ice, according to the manufacturer's instructions.
2. Verify the collagen solution has a pH of 7-7.4 using pH indicator paper. Adjust the pH by adding NaOH in 1 µl volumes.
3. Transfer explants to a 24-well plate using a wide mouth pipet tip and aspirate excess liquid. Multiple explants can be cultured in one well, but should be placed at least 500 µm apart from each other.
4. Add 0.5 ml of collagen to the well containing the explant. Position the explant on the bottom surface of the well. Be sure to set each culture completely before proceeding with the next well because the collagen will begin to polymerize at room temperature.
5. Place the culture plate on a 37°C slide warmer for 30-45 min to polymerize the collagen. When the collagen has polymerized it will resemble a gel.
6. Add 0.5 ml warm explant culture medium supplemented with either purified proteins (diluted in PBS) or PBS (Control) and incubate at 37°C, 5% CO_2. Effects on neurite outgrowth should be visible by 24 hours.

NOTE: We have used the same protocol to culture SAG explants up to 42 hours.

5. Explant co-culture with protein-coated beads to test directional neurite outgrowth

1. Begin with explant culture steps 4.1- 4.4.
2. Using a pipet, transfer 1-5 beads from PBS to the well containing the collagen solution and explant.
3. Use forceps to position beads 50-500 µm from the edge of the explant.
4. Place the plate on a 37°C slide warmer for 30-45 min to polymerize the collagen. The tissue or bead may become displaced as the collagen polymerizes. Check the cultures and re-position the tissue and beads during the first 5 min.
5. Add 0.5 ml SAG media to each well and incubate for 24 hours.

6. Visualization of neurite outgrowth by immunohistochemistry

1. Rinse cultures in PBS and fix for 1 hour at room temperature in 4% paraformaldehyde in PBS.
2. Release the gels from the walls of the wells by tracing around the edge of the gels with the rounded end of a teflon micro spatula. Perform the immunostain with the gels in the wells. Rinse several times in PBS.
3. Incubate in 0.5 ml blocking solution (10% calf serum, 0.1% Triton X-100, 0.1% sodium azide in PBS) overnight at 4°C.
4. Incubate in 0.5 ml primary antibody (anti-β-Tubulin antibody diluted 1:500 in blocking solution) overnight at 4°C.
5. Rinse several times in PBS. Incubate in 0.5 ml secondary antibody (Alexa fluor 488 goat anti-mouse IgG_{2b} antibody diluted 1:500) overnight at 4°C. From this step forward, keep plates in dark or wrap in foil, due to light sensitivity of secondary antibodies.
6. Rinse several times in PBS and store at 4°C in PBS for up to one week.

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7. Representative Results

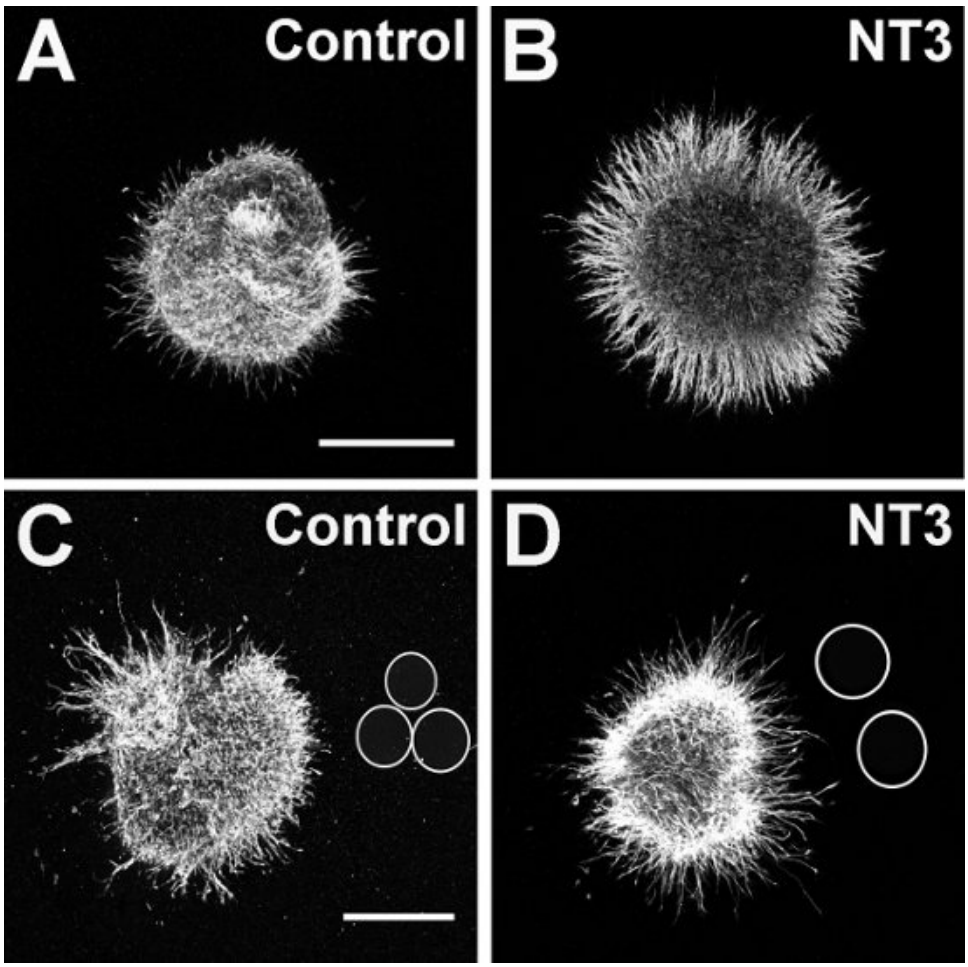

Figure 1. Representative results of the neurite-promoting effects of purified NT-3 and NT-3-coated beads on E4 chick SAG explants.

SAG neuron cell bodies and neurites were immunostained with β-Tubulin and imaged with a 10x objective on a confocal microscope. After 24 hours *in vitro*, SAG explants displayed greater neurite outgrowth in the presence of 100 ng/ml purified human NT-3 added to the cell culture medium (1B), compared to controls (1A). SAG neurons were co-cultured with beads soaked in100 ng/ml NT-3 to demonstrate that a point source of NT-3 can locally promote neurite outgrowth. Explants displayed longer and denser neurite outgrowth on the side of the explant facing the bead compared to the opposite side (1D) and compared to control cultures with beads soaked in PBS (1C). Scale bar = 200 µm.

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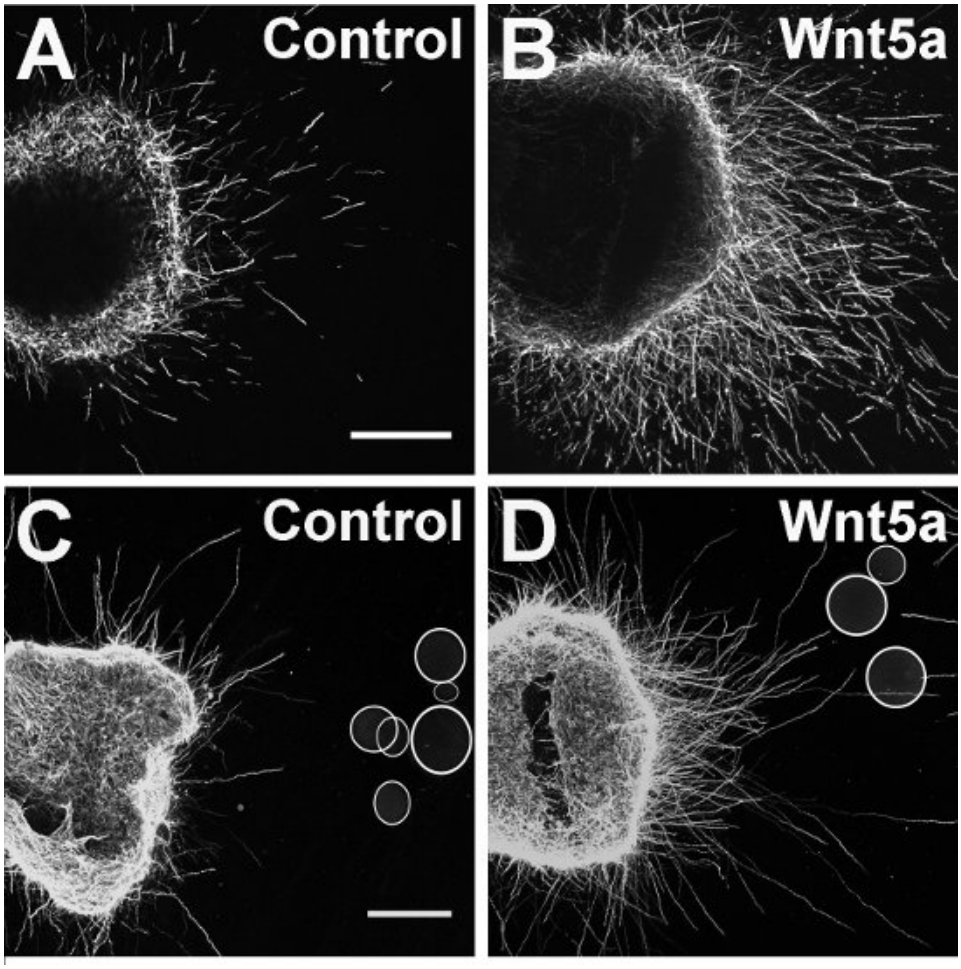

Figure 2. Representative results of the neurite-promoting effects of purified Wnt5a and Wnt5a-coated beads on E6 chick spinal cord.

Spinal cord explants were immunostained with β-Tubulin and imaged with a confocal microscope. After 24 hours of growth in the presence of purified mouse Wnt5a (400ng/ml), neurite outgrowth from chick spinal cord explants was increased (2B) compared to controls cultured without Wnt5a (2A). Beads soaked in 500 ng/ml Wnt5a, placed 300-500 µm from the edge of spinal cord explants, also promoted neurite outgrowth (2D) compared to control cultures (2C). Similar co-culture results were previously published with Wnt5a-expressing cells[8]. Scale bar = 200 µm.

Discussion

We present a method to dissect and culture E4 SAG and E6 spinal cord explants, from chick, under serum-free conditions. This procedure is currently used in our lab to study the effects of various secreted ligands on SAG neuron survival and neurite outgrowth. Novel aspects of this protocol include the use of a serum-free system for culturing explants and the use of beads soaked in growth factors to study effects on SAG neurite outgrowth. A bead method similar to ours has been described for the chick spinal cord[10]. Traditionally, beads have been used in studies with classic axon guidance factors and morphogens. Here, we have shown that the bead assay can also be used to investigate the effects of neurotrophins (or other growth factors) on neurite outgrowth (Fig. 1).

There are critical steps in the procedure that may need to be modified or optimized for different applications. These steps are also important variables for trouble-shooting experiments that appear to be unsuccessful. First, Neurotrophin-3 (NT-3), Ciliary Neurotrophic factor (CNTF) and ITS (Insulin, Transferrin, Selenium) were added to the culture medium to promote SAG neuron survival and enhance neurite outgrowth[6,8]. These growth factors are included in the spinal cord experiments in order to test the bioactivity of molecules under the same assay conditions that the SAG explants were tested. However, other growth factors may be more appropriate for these and other tissue types. Second, a 1.5 mg/ml concentration of collagen was chosen after testing a range of collagen concentrations (0.5, 1.0, 1.5 and 2 mg/ml) because it produced the most robust neurite outgrowth from the E4 chicken SAG after 24h. Other sources of neurons may display a different optimum of collagen. Finally, the protein concentrations, the number of beads, and the distance between the explants and beads should also be optimized for each application. Some growth factors, such as morphogens, are expressed in gradients and exert concentration-dependent effects on growing axons during development[11]. Therefore, a range of concentrations should always be tested with neurite outgrowth assays. Care should be taken to avoid toxicity: we have observed elevated levels of cell death with high concentrations of some proteins (eg., Sonic hedgehog).

This method can be adapted for additional applications, including other ages, tissue types, co-culture methods, and additional immunostaining (eg., cell survival assays). We have successfully cultured E3-E5 SAG, E6 retina, and E7 olfactory bulb explants, from chick, using the same

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culture conditions. When outgrowth is robust in control samples, this method can also reveal repulsive responses to secreted factors. Similar methods were used for testing effects of morphogens on mammalian neurons[12,13], and we are currently investigating the effects of BMPs, FGFs, and Shh on SAG neurite outgrowth. Also, since the collagen gels are polymerized in a 24-well plate, they are large enough for subsequent embedding and sectioning with a cryostat. We routinely perform TUNEL assays on cryosections of collagen gel cultures, which enables us to correlate the level of cell survival with the amount of neurite outgrowth from the same sample[8].

There are limitations to using the collagen gel assays in the way that we have presented them here. For example, responses observed in the deliberately simplified *in vitro* environment may not reflect responses to the same factor when present in the more complex *in vivo* situation. As well, we cannot distinguish peripheral from central processes and cannot distinguish auditory from vestibular neurites. Therefore, heterogeneity in responsiveness among these populations could yield irregular or non-uniform outgrowth or, if the responding population is a small fraction of the total, the ganglion may be scored as showing no effect. Finally, we have presented a method that can be used to culture E4 SAG explants under serum-free conditions with significant neuron survival, with a variety of applications from studying cell survival to axon guidance. Overall, this system is beneficial because it allows one to monitor effects due to the addition of purified factors alone or in combinations without the confounding variables of other secreted factors and axon guidance cues that may be present in the *in vivo* environment.

Disclosures

No conflicts of interest declared.

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