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TITLE:

Isolation and Culture of Chick Ciliary Ganglion Neurons

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SUMMARY:

Chick ciliary ganglia (CG) are part of the parasympathetic nervous system. Neuronal cultures of chick CG neurons were shown to be effective cell models in the study of nerve muscle interactions. We describe a detailed protocol for the dissection, dissociation and in vitro culture of CG neurons from chick embryos.

ABSTRACT:

Chick ciliary ganglia (CG) are part of the parasympathetic nervous system and are responsible for the innervation of the muscle tissues present in the eye. This ganglion is constituted by a homogenous population of ciliary and choroidal neurons that innervate striated and smooth muscle fibers, respectively. Each of these neuronal types regulate specific eye structures and functions. Over the years, neuronal cultures of the chick ciliary ganglia were shown to be effective cell models in the study of muscle-nervous system interactions, which communicate through cholinergic synapses. Ciliary ganglion neurons are, in its majority, cholinergic. This cell model has been shown to be useful comparatively to previously used heterogeneous cell models that comprise several neuronal types, besides cholinergic. Anatomically, the ciliary ganglion is localized between the optic nerve (ON) and the choroid fissure (CF). Here, we describe a detailed procedure for the dissection, dissociation and in vitro culture of ciliary ganglia neurons from chick embryos. We provide a step-by-step protocol in order to obtain highly pure and stable cellular

cultures of CG neurons, highlighting key steps of the process. These cultures can be maintained in vitro for 15 days and, hereby, we show the normal development of CG cultures. The results also show that these neurons can interact with muscle fibers through neuro-muscular cholinergic synapses.

INTRODUCTION:

Ciliary ganglion (CG) neurons belong to the parasympathetic nervous system. These neurons are cholinergic, being able to establish muscarinic or nicotinic synapses[1–3]. Anatomically, the CG is located in the posterior part of the eye between the optic nerve (ON) and the choroid fissure (CF) and consists of around 6000 neurons in early embryonic stage[1,4]. For the first week in culture, ciliary ganglion neurons present a multipolar morphology. After one week, they start to transition to a unipolar state, with one neurite extending and forming the axon[5]. In addition, approximately half of CG neurons die between the 8th and 14th day of chick embryo development, through a programmed process of cell death. This decrease in the number of neurons results in a total population of the ciliary ganglion of around 3000 neurons[6–8]. In vitro, there is no reduction in the number of CG neurons when grown with muscle cells[9] and CG neurons can be cultured for several weeks[1,9].

The ciliary ganglion consists of a homogeneous population of ciliary neurons and choroidal neurons, each representing half of the neuronal population in the CG, innervating the muscle of the eye. These two types of neurons are structurally, anatomically and functionally distinct. Ciliary neurons innervate the striated muscle fibers on the iris and lens, being responsible for pupil contraction. Choroidal neurons innervate the smooth muscle of the choroid[1,10–12].

Cultures of chicken ciliary ganglion neurons have been shown to be useful tools for the study of neuromuscular synapses and synapse formation[1,5,9]. Considering that neuromuscular synapses are cholinergic[13], using a neuronal population that is cholinergic – CG neurons – emerged as a potential alternative to previous cell models[14]. These models consisted in an heterogenous neuronal population, in which only a small part is cholinergic. Alternatively, ciliary ganglion neurons develop relatively fast in vitro, and after approximately 15 hours already form synapses[1]. CG neurons have been used as a model system throughout the years for distinct research studies, due to its relatively ease of isolation and manipulation. These applications include optogenetic studies, synapse development, apoptosis and neuromuscular interactions[14,15].

We describe a detailed procedure for the dissection, dissociation and in vitro culture of ciliary ganglia neurons from embryonic day 7 (E7) chick embryos. We provide a step-by-step protocol in order to obtain highly pure and stable cellular cultures of cholinergic neurons. We also highlight key steps of the protocol that require special attention and that will improve the quality of the neuronal cultures. These cultures can be maintained in vitro for at least 15 days.

PROTOCOL:

1. Preparation of reagents

NOTE: The materials necessary for this procedure are the following: forceps (nº 5 and nº 55), surgical tweezers, dissection Petri dishes (black bottom), 24-well plates, plastic Pasteur pipette, fire-polished glass Pasteur pipette, 10 mL syringe, 0.22 µm syringe filter.

1.1. Prepare and sterilize all the material needed for the protocol including glass coverslips, forceps (nº 5 and nº 55), surgical tweezers, Petri dishes (black bottom), distilled H_2O, pipettes and material for surgery.

1.2. Prepare 0.1 mg/mL Poly-D-Lysine (PDL) solution.

1.2.1. Reconstitute PDL in 0.1 M borate buffer (pH 8.2) to a concentration of 1 mg/mL (10x solution).

1.2.2. Dilute 1:10 in 166.6 mM borate buffer (pH 8.2) to obtain a final concentration of 0.1 mg/mL.

1.3. Prepare 10 µg/mL laminin solution.

1.3.1. Dilute 1 mg/mL laminin in plain neurobasal medium to a final concentration of 10 µg/mL.

1.4. Prepare Hank's Balanced Salt Solution (HBSS): 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM NaHCO3, 0.34 mM $Na_2HPO_4 \cdot 2H_2O$, 5 mM glucose, 1 mM sodium pyruvate, 10 mM HEPES buffer, 0.001% phenol red. Adjust pH to 7.2.

1.5. Prepare 0.1% trypsin solution.

1.5.1. Dissolve 5 mg of trypsin 1:250 powder in 5 mL of HBSS for a final concentration of 0.1%.

1.5.2. Place in a roller mixer at 4 °C until completely dissolved.

1.5.3. Filter using a 10 mL syringe and a 0.22 µm syringe filter.

1.6. Prepare ciliary ganglia **incomplete medium**: neurobasal medium without glutamine, 1X B27 (photo-sensitive), 10% heat-inactivated horse serum, 2% heat-inactivated FBS, 12.5 U/mL penicillin/streptomycin (0.25x) and 2 mM glutamine. Use sterile reagents and prepare the medium under sterile conditions.

1.7. Prepare ciliary ganglia **complete medium** (supplemented with growth factors): to the incomplete medium, add 5 ng/mL GDNF and 5 ng/mL CNTF.

2. Preparation of glass coverslips for 24-well plates

2.1. Place the desired number of glass coverslips inside an acid resistant container and add 65% nitric acid until all coverslips are submerged. Place the container in an orbital shaker and incubate overnight at room temperature (RT) at a speed of 1000 rpm.

2.2. The next day, carefully transfer the nitric acid to a small reservoir and store for further use. Nitric acid can be re-used 2-3x.

2.3. Carefully, add distilled H_2O to the coverslips to remove the remaining nitric acid. Place in agitation for 30 minutes, discard the washing solution and repeat this 5x.

2.4. Rinse the coverslips with 75% ethanol twice.

2.5. Carefully separate and place individual coverslips in a metal rack covered with aluminum foil and incubate at 50 ºC for 10-15 minutes or until fully dry.

NOTE: Do not autoclave glass coverslips as they will stick to each other.

2.6. Sterilize the coverslips under UV light for 10-15 minutes. Maintain coverslips sterile for neuronal tissue culture.

3. Coating of glass coverslips for 24-well plates

3.1. Using a sterile tweezer, place one coverslip in each well of a 24-well plate.

3.2. Add 500 µL of 0.1 mg/mL PDL and incubate overnight at 37 °C.

3.3. The next day, rinse the coverslips twice with sterile distilled H_2O. Then, add 500 µL of distilled water to each coverslip and leave for 30 minutes at room temperature.

3.4. Discard the water and add 350 µL of 10 µg/mL laminin solution in each well.

3.5. Place in a 37 °C incubator for 2 h.

3.6. Before cell plating, remove the laminin solution and wash twice with plain neurobasal medium.

NOTE: It is important that the coverslips do not dry at any time.

3.7. Add 300 µL of complete medium and leave in an incubator at 37 °C and 5% CO_2 until plating time. Before plating cells, remove this medium.

4. Culture of ciliary ganglia from chicken embryo (**embryonic day 7**)

4.1. Dissection of ciliary ganglia (CG)

4.1.1. Remove eggs from incubator and spray them with 75% ethanol.

NOTE: Eggs are stored at ~16 °C before being incubated at 37.7 °C for 7 days (or the desired embryonic stage). Eggs used here are from Ross chicken species.

4.1.2. Cut the top of the egg with a scissor and take out the embryo using a spoon. Place the embryo in a Petri dish with ice-cold HBSS and separate the head from the body by cutting in the neck region.

NOTE: As soon as the embryo is removed from the egg, it can produce proteases that are responsible for cell death. It is important to rapidly separate the head from the body once the embryo is outside the shell to minimize cell death.

4.1.3. Keep the head of the embryo in ice-cold HBSS.

4.1.4. Hold the embryo head up and fix it in the beak of the chick with nº 5 forceps. Then with nº 55 forceps, start to remove the thin layer of skin around the eye.

4.1.5. Carefully remove the eye and rotate it to access the posterior part. While separating the eye from the head of the chick, notice the optic nerve being sectioned. This will help to localize the ciliary ganglion.

4.1.6. Once the eye is separated, keep it with the posterior side up and notice the ciliary ganglion adjacent to the sectioned optic nerve and the choroid fissure. The preganglionic nerve might still be attached to the ciliary ganglion, which facilitates its identification.

4.1.7. Dissect the ciliary ganglion from each eye and clean very well by removing the excess tissue around each ganglion.

NOTE: To have a yield of ~$1x10^6$ cells/mL, dissect ~70 CGs. Please note that the cell population obtained contains non-neuronal cells as well. To decrease the number of non-neuronal cells and, consequently, increase the purity of the neuronal population, it is very important to clean the ciliary ganglia as much as possible, removing all the excess tissue.

4.2. Dissociation and culture of ciliary ganglia

4.2.1. Collect all ciliary ganglia to a 15 mL tube using a sterile plastic Pasteur pipette.

NOTE: It is important to pre-wet the Pasteur pipette to minimize the attachment of the ganglia to the wall of the pipette.

4.2.2. Centrifuge the ciliary ganglia for 2 minutes at 200 x *g*.

4.2.3. Carefully, remove all the HBSS medium using a Pasteur pipette and then a P1000 micropipette. Add 1 mL of 0.1% trypsin solution and incubate for 20 minutes at 37 °C in a water bath, without agitation.

4.2.4. Centrifuge for 2 minutes at 200 x *g*.

4.2.5. Immediately remove the trypsin solution and add 1 mL of **incomplete medium**.

NOTE: Incomplete medium contains serum which will immediately stop the effect of trypsin.

4.2.6. Centrifuge for 2 minutes at 200 x *g* and remove all medium.

4.2.7. Add 350-500 µL of **complete medium**.

NOTE: The necessary volume to dissociate cells depends on the number of ciliary ganglia obtained and, thus, on the obtained pellet size. For ~70 CG it is recommended to use 500 µL of medium.

4.2.8. Dissociate CGs by pipetting up and down 10-15x first using a P1000 followed by 10-15x using a fire-polished glass Pasteur pipette. Avoid air bubble formation to minimize cell loss.

NOTE: Keep the cellular suspension on ice until plating.

4.2.9. Determine cellular density using a Trypan blue solution and a standard Neubauer chamber.

4.2.10. Plate 1 x 10^4 cells/mL in each well of the 24-well plate by diluting the appropriate volume of cell suspension in 500 µL of complete medium (supplemented with 10 µM 5'-FDU).

4.2.11. Incubate cells in a 37 °C, 5% CO_2 incubator.

5. Immunocytochemistry and image analysis of ciliary neurons

5.1. Perform the immunocytochemistry assay presented in this paper as previously described[16,17].

5.2. Use the following primary antibodies: mouse monoclonal β-III tubulin (1:1000, T8578), chicken monoclonal neurofilament M (1:1000, AB5735), mouse monoclonal SV2 (1:1000, AB2315387).

5.3. As secondary antibodies, use Alexa Fluor 568-conjugated goat anti-mouse antibody (1:1000, A11031), Alexa Fluor 568-conjugated goat anti-chicken antibody (1:1000, A11041), Alexa Fluor 647-conjugated goat anti-mouse antibody (1:1000, A21235).

5.4. Mount coverslips using mounting medium with DAPI, for nuclear staining (P36935).

REPRESENTATIVE RESULTS:

The estimated duration for this procedure tightly depends on the yield needed for each specific experiment and, thus, on the number of ciliary ganglia that need to be isolated. For an estimated yield of 1 x 10^6 cells/mL, isolate around 70 ciliary ganglia (35 eggs). For this number of ganglia, it will take 2-3 hours for the dissection procedure and a total of 4-5 hours for the total procedure. A step-by-step illustration of the isolation protocol is shown in **Figure 1A**. The identification of the ciliary ganglion can be difficult, especially when performing this protocol for the first time. The ciliary ganglion is localized near the optic nerve and the choroid fissure (**Figure 1B**). The key steps of the dissection procedure are shown in **Figure 2**. First, the embryo is removed from the egg and placed in ice-cold HBSS. The head is separated from the body and, once again, placed in ice-cold HBSS in a dissection Petri dish (**Figure 2A-2C**). Then, the eye is removed from the head of the chick and the ciliary ganglion is isolated (**Figure 2D-2H**).

The cultures obtained with this protocol are highly pure. However, cleaning the ganglia and removing the excess tissue strongly dictates the success and purity of the culture. The cells develop fast and can be used already in the first days in culture if the overall experiment requires so. Nevertheless, the cultures can be maintained for 15 days, or more. If using the cultures for longer than 7-8 days, make sure to replace a third of the culture medium with fresh medium every 2-3 days. After 1 day in vitro, CG neurons show a multipolar morphology. However, neurite extension occurs rapidly, and a primary neuronal network is already established after 24 hours. After 8 days in vitro, neurons already transitioned to a unipolar state, where one of the neurites extends and forms the axon. The neuronal network is very dense at this stage of development (**Figure 3** and **Figure 4**).

Ciliary ganglion neurons are cholinergic neurons that belong to the parasympathetic nervous system. In vivo, these neurons are responsible for muscle innervation in the eye. These neuronal cultures are very well suited for the study of neuromuscular synapses. For this, CG neurons can be plated on top of muscle cells. The chick pectoral muscle was dissected and allowed to develop and maturate in vitro until DIV 4. CG neurons were then plated on top of the muscle layer and the co-culture allowed to develop for 3 more days. At this time point, muscle fibers are formed and can be easily identified by the presence of multiple nuclei (blue). Synaptic vesicle glycoprotein 2A (SV2) immunostaining, a presynaptic marker shows the presence of synapses that are established between the CG neurons axons and the muscle fibers (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1: Scheme of the dissection protocol and the ciliary ganglion. (**A**) Diagram of the isolation and culture protocol. (**B**) Scheme of the chick ciliary ganglion localization in the posterior part of the eye. Optic nerve, ciliary ganglion and choroid fissure are indicated by arrows.

Figure 2: Dissection of E7 chick ciliary ganglion. (**A**) Cut the top of the egg using scissors. (**B**) Remove the embryo from the egg with a spoon and place it in a dissection Petri dish with ice-cold HBSS. (**C**) Separate the head from the body by cutting in the neck region. (**D**) Fix the head of the embryo in the beak, holding with forcep nº 5. (**E**) Remove the eye by gentle rotation using forcep nº 55. (**F**) Posterior view of the eye. Arrows indicate the localization of the optic nerve, choroid fissure and ciliary ganglion. (**G**) Dissect the ciliary ganglion. (**H**) Dissected ciliary ganglion. Excess tissue should be removed.

Figure 3: Ciliary ganglion neurons development in vitro. Phase contrast images of CG neurons at DIV 1, 3, 8 and 15. As CG neurons are plated, they immediately initiate neurite outgrowth. At DIV 15, the axonal network is very dense and at this stage neurites are completely differentiated into dendrites and axons. Phase contrast-images were acquired using a confocal microscope with a plan-Apochromat 20x ph2 objective. Scale bar: 50 μm.

Figure 4: Immunocytochemistry of CG neurons at DIV 8. CG neurons show a well-established neuronal network after 8 days in vitro. Nuclei were stained with DAPI (blue) and axons were stained with β-III tubulin (red). Fluorescence imagens were acquired using a confocal microscope with a plan-Apochromat 20x objective. Scale bar: 50 μm.

Figure 5: Cultured CG neurons establish synapses with muscle fibers. Immunocytochemistry images of CG neurons-pectoral muscle co-cultures. Muscle fibers identified by dashed lines present multiple nuclei, which were stained with DAPI (blue). Axons were labeled against neurofilament (red) and synaptic vesicles were labeled against SV2 (cyan). Images were acquired using a confocal microscope with a plan-Apochromat 63x oil objective. Scale bar: 20 μm.

DISCUSSION:

In this protocol, we demonstrated how to prepare and culture CG neurons. The identification and dissection of the ciliary ganglion can be difficult for unexperienced users. Therefore, we present a detailed and step-by-step procedure to efficiently dissect E7 chick ciliary ganglia, dissociate the tissue and prepare neuronal cultures that can be maintained for at least 15 days. The ciliary ganglion neurons obtained with this protocol are also suitable for co-culture with muscle cells.

Ciliary ganglia at different developmental stages of chick embryonic development can be used as a cell model, depending on the purpose of the study. However, for cultures of CG neurons it is suggested that they be isolated from chick embryo between embryonic days 7 and 8[18]. In the embryonic stage E8, CG neurons have not yet undergone neuronal death processes and the number of non-neuronal cells is reduced comparatively with neuronal cells[18]. This, in combination with a rigorous dissection procedure and very well cleaned ganglia, will contribute for a highly pure culture of ciliary ganglion neurons, with little contamination by non-neuronal cells, such as fibroblasts or glial cells.

During the isolation of CG neurons, one of the critical points is the identification and the cleaning of the CG. The dissection of such a small structure, as the ciliary ganglion, can be difficult considering the localization, the ability to identify the ganglion as well as the size of the ganglion

itself. It is normal that the ganglia might attach to the forceps during dissection. High quality dissection instruments are very important for a successful dissection and will minimize the attachment of the ganglia to the forceps. Cleaning the GC is important to prevent contamination with non-neuronal cells. It is necessary to isolate approximately 70 ganglia to obtain a cellular density of ~1x10^6 cells/mL, in contrast with other neuronal tissues of the peripheral nervous system that have a 5-15x greater number of ganglia[3].

In culture, the addition of 5'-FDU to the complete medium decreases the contamination of the GC culture with non-neuronal cells. 5'-FDU is an anti-mitotic compound that inhibits cell proliferation, namely the proliferation of glial cells and fibroblasts. The concentration of 5'-FDU added to the medium is enough to stop the cell cycle in the S phase but is not detrimental to the normal development of CG neurons[3,19,20]. The time of treatment with 5'-FDU can be adjusted. However, since CG neurons establish a dense axonal network in a short time, 5'-FDU should be added to the culture as early as the time of plating.

One of the main limitations of this model is that it is not representative of the normal development of CG neurons under physiological conditions. In ovo, about half of CG neurons die between the 8th and 14th day of chick embryo development. In culture, there is no decrease in the number of CG neurons when the medium is supplemented with neurotrophic factors that allow its survival[1,6,14].

The neuronal population obtained from the dissection of the chick ciliary ganglion is a homogenous population of cholinergic neurons, belonging to the autonomic nervous system. It should be noted that the expression of neurotransmitters in the choroid population of the CG is target-driven, which might be hampered depending on the type of muscle used in the co-culture[24]. If the aim of the study is related to the genetic identity or sub-type of the motor neuron itself, then CG neurons might not be the best suitable neuronal model. Also, the specificity of motor neurons in the innervation of muscle fibers may not be accomplished when using CG neuron co-cultures since, in this case, the muscle fibers can be multiply innervated[25]. However, this neuronal culture has several advantages, it only requires basic equipment to maintain and incubate the eggs, it is a reasonably inexpensive procedure and, more importantly, provides an excellent model for the study of neuromuscular synapses[1], since CG neurons neurotransmission mechanisms are very similar to the ones occurring in spinal motor neurons. The cell models previously used for these type of studies were sensory neurons from the spinal cord[12,21–23]. However, these co-cultures were composed of an heterogeneous population of neurons, not all cholinergic and, thus, only a small part of the neurons were able to establish functional contacts with the muscle cells[1]. Besides the developmental analysis (immunocytochemistry) demonstrated in this work other assays can be performed in CG cultures like electrophysiology and neuronal survival.

Based on this protocol additional scientific questions can be addressed, for example how subcellular localization of specific mRNAs and proteins regulate synapse formation and function. Moreover, nerve-muscle co-cultures can be easily established and be further used to study neuromuscular diseases when the site of injury is the neuromuscular junction. Neuromuscular

diseases are heterogeneous in nature in the sense that the dysfunction might be associated with the muscle itself, the peripheral nerves or the neuromuscular junctions[26]. Thus, through these co-cultures it would be possible to study the neuromuscular junction alterations that ultimately underlie the development and progression of neuromuscular diseases. Another interesting possibility would be to adapt this protocol to the mouse trigeminal system. These neurons are easily accessible, and their developmental pattern is well-known[27]. Because mice are amenable to genetic manipulation and the trigeminal system is well characterized in terms of topographic map formation new possibilities arise by using a trigeminal-based protocol to study neuronal development.

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DISCLOSURES:

The authors declare that they have no competing interests.

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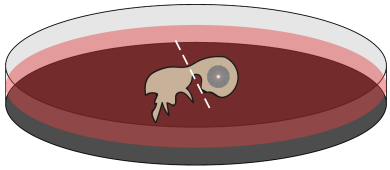
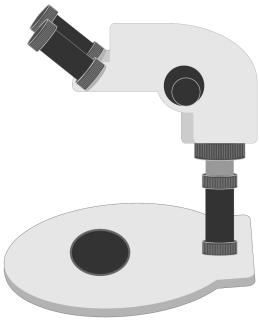
A

E7 chick embryo



Remove the embryo, place in ice-cold HBSS and separate the head from the body



Dissect ciliary ganglion

Trypsinization and tissue dissociation

Dilute cellular suspension at desired density

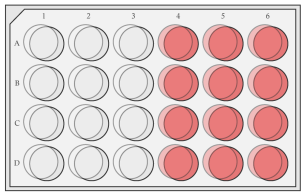
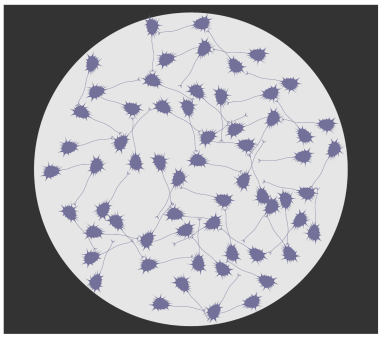


Plate 500 µl/well of cell suspension in a 24-well plate



Immunocytochemistry

B

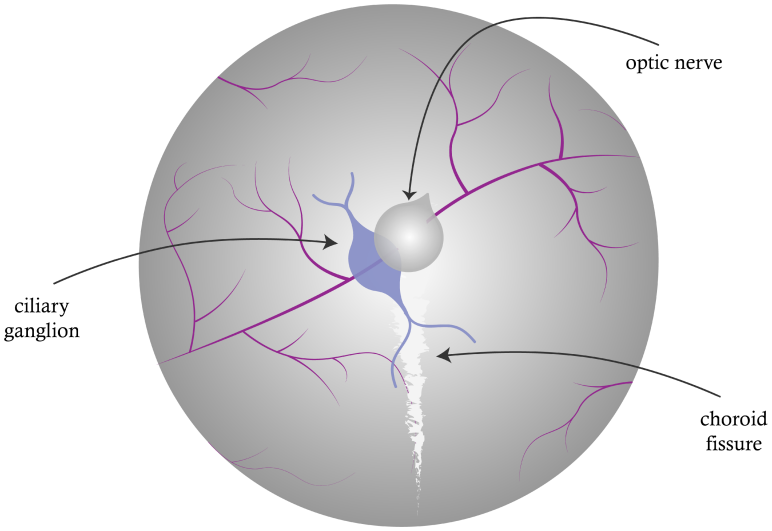


Figure 2

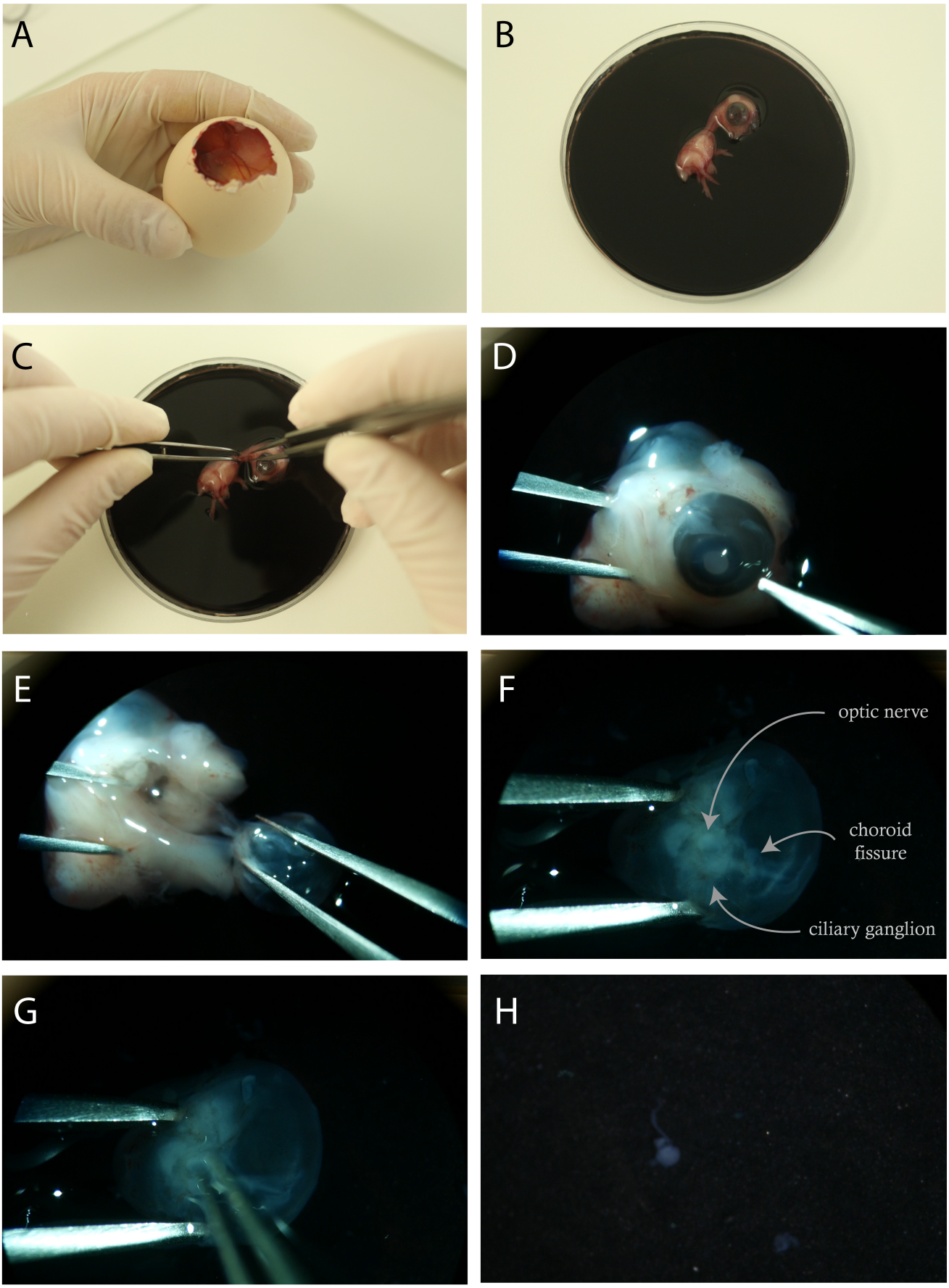


Figure 3

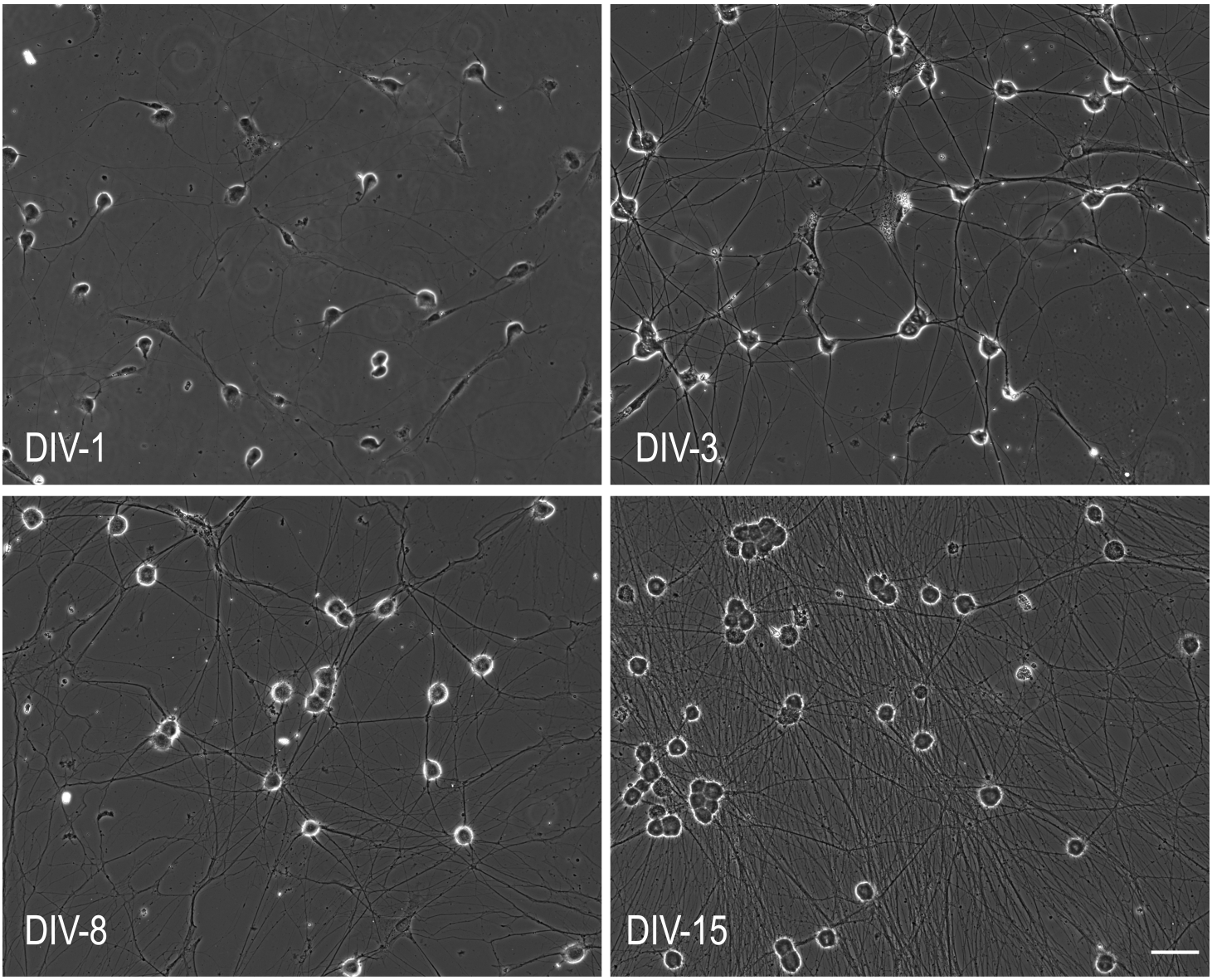


Figure 4

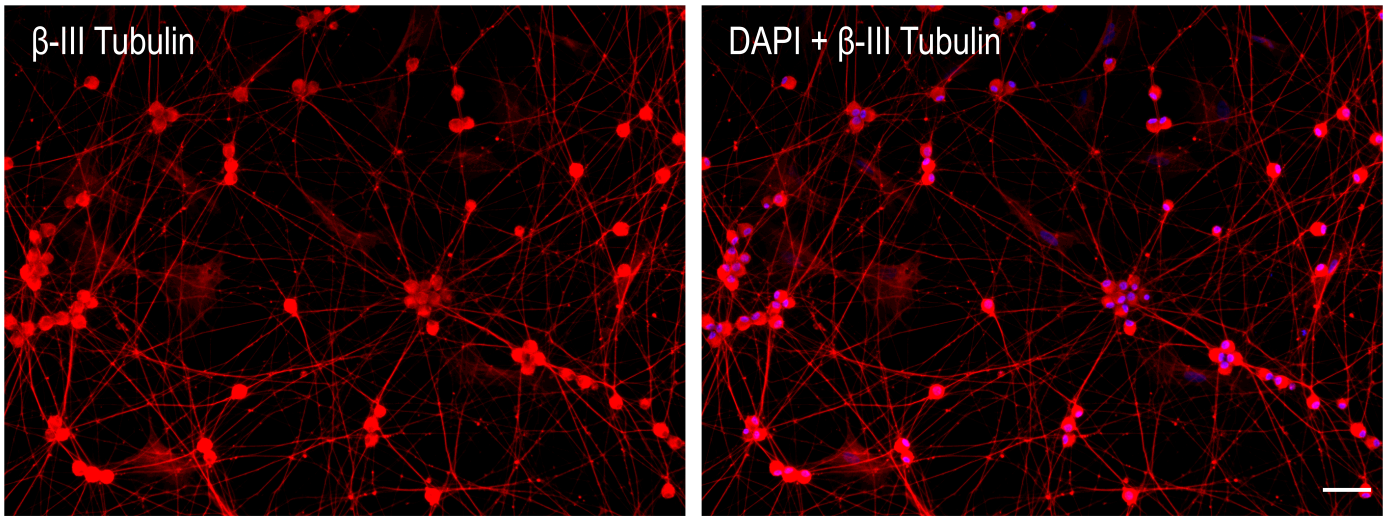
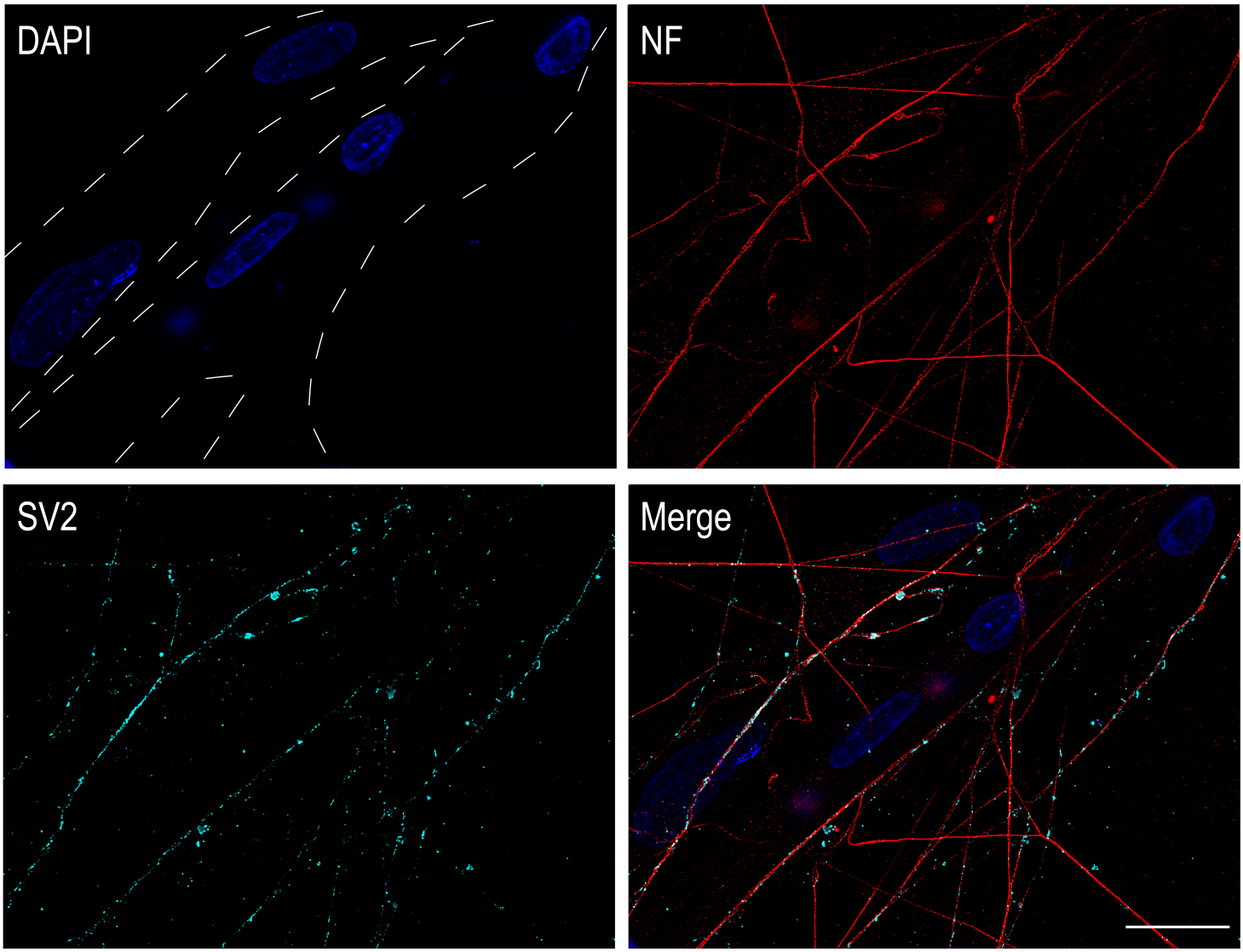


Figure 5

Click here to access/download;Figure;Figure 5.pdf



Name of Material/Equipment	Company
5-fluoro-2’-deoxiuridina (5'-FDU)	Merck (Sigma Aldrich)
Alexa Fluor 568-conjugated goat anti-chicken antibody	Thermo Fisher Scientific
Alexa Fluor 568-conjugated goat anti-mouse antibody	Thermo Fisher Scientific
Alexa Fluor 647-conjugated goat anti-mouse antibody	Thermo Fisher Scientific
B27 supplement (50x), serum free	Invitrogen (Gibco)
Chicken monoclonal neurofilament M	Merck (Sigma Aldrich)
D-(+)-Glucose monohydrate	VWR
Fetal Bovine Serum (FBS), qualified, Brazil	Invitrogen (Gibco)
HEPES, fine white crystals, for molecular biology	Fisher Scientific
Horse Serum, heat inactivated, New Zealand origin	Invitrogen (Gibco)
L-Glutamine (200 mM)	Invitrogen (Gibco)
Mouse laminin I	Cultrex (R&D systems)
Mouse monoclonal β-III tubulin	Merck (Sigma Aldrich)
Mouse monoclonal SV2	DSHB
Multidishes, cell culture treated, BioLite, MW24 (50x)	Thermo Fisher Scientific
Neurobasal medium without glutamine	Invitrogen (Gibco)
Penicillin/streptomycin (5,000 U/mL)	Invitrogen (Gibco)
Phenol red, bioreagent, suitable for cell culture	Merck (Sigma Aldrich)
Poly-D-Lysine	Merck (Sigma Aldrich)
Potassium chloride	Fluka (Honeywell Reaarch Chemicals)
Potassium di-hydrogen phosphate (KH_2PO_4) for analysis, ACS	Panreac Applichem
Prolong Gold Antifade mounting medium with DAPI	Invitrogen (Gibco)
Puradisc FP 30mm Syringe Filter, Cellulose Acetate, 0.2µm, sterile 50/pk	Fisher Scientific
Recombinant human ciliary neurotrophic factor (CNTF)	Peprotech
Recombinant human glial cell-derived neurotrophic factor (GDNF)	Peprotech
Sodium chloride for analysis, ACS, ISO	Panreac Applichem
Sodium dihydrogen phosphate 2-hydrate ($Na_2HPO_4 \cdot 2H_2O$), pure, pharma grade	Panreac Applichem
Sodium Pyruvate 100 mM (100x)	Thermo Fisher
Syringe without needle, 10 mL	Thermo Fisher
Trypsin 1:250 powder	Invitrogen (Gibco)

Catalog Number	Comments/Description
F0503	
A11041	
A11031	
A21235	
17504-044	
AB5735	
24371.297	
10270-106	
10397023	
26050-070	
25030-081	
3400-010-02	
T8578	
AB2315387	
11874235	
21103-049	
15070-063	
P3532	
P7886	
31248-1KG	
131509-1000	
P36935	
10462200	
450-13	
450-10	
131659-1000	
141677-1000	
11360039	
11587292	
27250-018	

Response to reviewers

Revision of manuscript JoVE61431 – "Isolation and Culture of Chick Ciliary Ganglion Neurons," by Costa *et al*.

We would like to thank the editor for the suggestions to improve our video protocol. We submitted a new version of the video and we believe we have addressed all the suggested alterations.

We would also appreciate if you could consider the new version of the manuscript (uploaded in the present revision step) as we detected a mistake the funding sources. This is the only alteration that needs to be made in the manuscript and it is highlighted in grey (page 9, lines 368 and 370).

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. No changes are required.

Changes to be made by the Author(s) regarding the video:

1. Video Glitches:

"• 06:05 There seems to be a video glitch here. Is this glitch in the original footage or is it only in the exported file?

We corrected the glitch.

• 07:18 Video and audio drops out here briefly as well."

We corrected the glitch.

4. Narration:

• 08:25 The line here about "collecting all ganglia to 15 mL tube" is slightly jumbled. It needs some careful editing to eliminate the repeat word "15 mL tube".

We corrected the voice-over in this section. We believe that the narration is now completely understandable.

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Author(s):	Filipa J. Costa*, Marta S. Dias*, Rui O. Costa, Joana R. Pedro, Ramiro D. Almeida

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