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Corresponding Author:	Elizabeth Engle Boston Children's Hospital Boston, MA UNITED STATES
Corresponding Author's Institution:	Boston Children's Hospital
Corresponding Author E-Mail:	Elizabeth.Engle@childrens.harvard.edu
Order of Authors:	Ryosuke Fujiki
	Joun Y. Lee
	Julie A. Jurgens
	Mary C. Whitman
	Elizabeth Engle
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TITLE:

Isolation and Culture of Oculomotor, Trochlear, and Spinal Motor Neurons from Prenatal *Isl^{mn}:GFP* Transgenic Mice

AUTHORS AND AFFILIATIONS:

Ryosuke Fujiki[1,2,3,4,9], Joun Y. Lee[1,2,10], Julie A. Jurgens[1,2,3,7], Mary C. Whitman[2,5,6], Elizabeth C. Engle[1,2,3,4,5,6,7,8]

[1]Department of Neurology, Boston Children's Hospital, Massachusetts, USA
[2]FM Kirby Neurobiology Center, Boston Children's Hospital, Massachusetts, USA
[3]Department of Neurology, Harvard Medical School, Boston, Massachusetts, USA
[4]Medical Genetics Training Program, Harvard Medical School, Boston, Massachusetts, USA
[5]Department of Ophthalmology, Boston Children's Hospital, Boston, Massachusetts, USA
[6]Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, USA
[7]Broad Institute of M.I.T. and Harvard, Cambridge, Massachusetts, USA
[8]Howard Hughes Medical Institute, Chevy Chase, Maryland, USA
[9] Present Address: Department of Neurology, Kokura Memorial Hospital, Kitakyushu, Fukuoka, Japan
[10]Present address: Department of Genetics, Albert Einstein College of Medicine, Bronx, New York, USA

Corresponding Author:

Elizabeth C. Engle (elizabeth.engle@childrens.harvard.edu)

Email Addresses of Co-authors:

Ryosuke Fujiki (yamoomatrix0609@gmail.com)
Joun Y. Lee (joun.lee92@gmail.com)
Julie A. Jurgens (Julie.Jurgens@childrens.harvard.edu)
Mary C. Whitman (Mary.Whitman@childrens.harvard.edu)

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motor neuron, oculomotor neuron, trochlear neuron, primary culture, mouse embryonic motor neuron culture, FACS, *Isl^{MN}:GFP* transgenic mouse, cell purification, cell isolation

SUMMARY:

This work presents a protocol to yield homogeneous cell cultures of primary oculomotor, trochlear, and spinal motor neurons. These cultures can be used for comparative analyses of the morphological, cellular, molecular, and electrophysiological characteristics of ocular and spinal motor neurons.

ABSTRACT:

Oculomotor neurons (CN3s) and trochlear neurons (CN4s) exhibit remarkable resistance to degenerative motor neuron diseases such as amyotrophic lateral sclerosis (ALS) when compared to spinal motor neurons (SMNs). The ability to isolate and culture primary mouse CN3s, CN4s,

and SMNs would provide an approach to study mechanisms underlying this selective vulnerability. To date, most protocols use heterogeneous cell cultures, which can confound the interpretation of experimental outcomes. To minimize the problems associated with mixed-cell populations, pure cultures are indispensable. Here, the first protocol describes in detail how to efficiently purify and cultivate CN3s/CN4s alongside SMNs counterparts from the same embryos using embryonic day 11.5 (E11.5) *Isl^{MN}:GFP* transgenic mouse embryos. The protocol provides details on the tissue dissection and dissociation, FACS-based cell isolation, and in vitro cultivation of cells from CN3/CN4 and SMN nuclei. This protocol adds a novel in vitro CN3/CN4 culture system to existing protocols and simultaneously provides a pure species- and age-matched SMN culture for comparison. Analyses focusing on the morphological, cellular, molecular, and electrophysiological characteristics of motor neurons are feasible in this culture system. This protocol will enable research into the mechanisms that define motor neuron development, selective vulnerability, and disease.

INTRODUCTION:

The culture of primary motor neurons is a powerful tool which enables the study of neuronal development, function, and susceptibility to exogenous stressors. Motor neuron cultures are particularly useful for the study of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS)[1,2], whose disease mechanisms are incompletely understood. Interestingly, despite the significant cell death of spinal motor neurons (SMNs) in both ALS patients and ALS model mice, cell death in oculomotor neurons (CN3s) and trochlear neurons (CN4s) are relatively scarce[1,3-9]. Therefore, comparative analyses of pure cultures of CN3s/CN4s and SMNs could provide important clues about mechanisms underlying relative vulnerability. Unfortunately, a major barrier to such analyses has been the inability to grow purified cultures of these motor neurons.

Many protocols have been described for the purification of SMNs from animal models. Most of these protocols use density gradient centrifugation[10-12] and/or p75^{NTR}-antibody-based cell-sorting panning techniques[13-16]. Density gradient centrifugation exploits the larger size of SMNs relative to other spinal cells, whereas p75^{NTR} is an extracellular protein expressed exclusively by SMNs in the spinal cord. Nearly 100% pure SMN cultures have been generated by one or both of these protocols[11,12,14]. However, these protocols have not been successful in generating CN3/CN4 cultures because CN3s/CN4s do not express p75^{NTR}, and other specific CN3/CN4 markers have not been identified. They are also smaller than SMNs and, therefore, more difficult to isolate based on size. Instead, in vitro studies of CN3s or CN4s have relied on dissociated[17-21], explant[17,22-26], and slice[27,28] cultures, which are composed of heterogeneous cell types, and no protocols have existed for the isolation and culture of primary CN3s or CN4s.

Here, a protocol is described for the visualization, isolation, purification, and cultivation of CN3s, CN4s, and SMNs from the same embryonic day 11.5 (E11.5) *Isl^{MN}:GFP* transgenic mice[29] (**Figure 1**,**Figure 2A**). *Isl^{MN}:GFP* specifically labels motor neurons with a farnesylated GFP that localizes to the cell membrane. This protocol enables species- and age-matched comparison of multiple types of motor neurons in order to elucidate pathological mechanisms in motor neuron disease.

PROTOCOL:

All experiments utilizing laboratory animals were performed in accordance with NIH guidelines for the care and use of laboratory animals and with the approval of the Animal Care and Use Committee of Boston Children's Hospital.

1. Setting up timed matings prior to the dissection

1.1. To generate prenatal embryonic mice for motor neuron harvest, weigh each female mouse and set up timed mating between adult *Isl^{MN}:GFP* transgenic mice 11.5 days prior to the day of neuron isolation. For the purpose of developing this protocol, 129S1/C57BL/6J *Isl^{MN}:GFP* mice, aged 2−9 months, were used and timed mating was set up in the evening.

1.2. Examine female mice for vaginal plugs the following morning. Consider the date on which the plug is identified as embryonic day (E) 0.5.

1.3. Weigh female mice and examine for pups using ultrasound (see **Table of Materials**) between E8.5−11. Check for the signs of successful mating.

1.3.1. Confirm the successful mating by detecting weight gain in female mice (usually >1.5 g on E9.5 if there are more than 5−6 embryos).

1.3.2. Visually confirm embryos under ultrasound. Embryos are easily detectable by ultrasound after E9.5. Ultrasounds are conducted only on females that have gained weight because they are more often pregnant than those that do not.

NOTE: Female mice can gain weight for reasons other than pregnancy, so weight gain alone is not a reliable indicator of pregnancy. Ultrasound confirmation prevents unnecessary sacrifice of females that are not pregnant but is not crucial if unavailable.

2. Dissection conditions and preparation of instruments

2.1. Perform all coating (except acid-cleaning of coverslips), media preparation, tissue dissociation (except centrifugation and incubation), and culture work in a laminar flow hood to ensure the sterility of the media and embryonic motor neurons.

2.2. With careful attention to sterile technique, conduct tissue dissection outside of a laminar flow hood with minimal risk of contamination.

2.3. Sterilize one dissection plate, one pair of microdissecting scissors, one pair of thumb dressing forceps, two pairs of Dumont #5 tweezers, one microdissecting knife, and one Moria mini perforated spoon by immersing in 70% ethanol prior to use.

3. PDL/laminin coating of dishes/coverslips

NOTE: Culture dissociated primary motor neurons in 96 well or 24 well plates, depending on the number of cells required for the application. Cells can be imaged directly in the tissue culture plate without the use of coverslips if the wells are optically transparent and the thicknesses are compatible with imaging.

3.1. For applications requiring coverslips, prepare acid-cleaned, sterilized, and air-dried coverslips at least 2 days prior to the neuron isolation as previously described[30]. Batches of coverslips can be prepared in this manner well in advance of experiments and can be stored up to 6 months without impact on experimental quality.

3.2. Prepare a working solution of 20 µg/mL poly D-lysine (PDL) in phosphate buffered saline (PBS) 2 days prior to the neuron isolation.

3.2.1. Aliquot PDL (1 mg/mL) in advance and store at -20 °C as a stock solution.

3.3. Cover the surface of each coverslip or the well of the tissue culture plate with enough PDL solution working solution (e.g., 100 µL per well on 96 well plates or 500 µL per well on 24 well plates with or without coverslips). Incubate overnight at 37 °C.

3.4. The following day wash 3x with sterilized water.

3.4.1. Seal the plates with paraffin film and store the washed PDL-coated plates at 4 °C for up to 1 month if they are dried completely after the final wash.

3.5. Prepare a working solution with 10 µL of laminin (1.1−1.2 mg/mL) in 1.2 mL of PBS.

3.5.1. Aliquot laminin stocks (1.1−1.2 mg/mL) in advance and store at -80 °C.

3.6. Cover the surface of each coverslip or well with enough laminin solution to evenly coat the surface. Incubate for at least 2 h at 37 °C prior to use.

NOTE: PDL/laminin-coated plates and coverslips can be stored up to 1 week at 37 °C, but freshly coated laminin is preferred. Remove laminin directly before plating[30]. Laminin should not be allowed to dry out. If the plates are to be stored for more than several hours, they should be wrapped in paraffin film.

4. Preparation of dissection, motor neuron culture media, and dissociation solutions

NOTE: Concentrations in parentheses indicate the final concentrations of each reagent.

4.1. Prepare the dissection medium.

4.1.1. Thaw the heat-inactivated horse serum overnight at 4 °C, prepare 1 mL aliquots, and store at -20 °C. Thaw aliquots at RT directly before use.

4.1.2. Store B27-supplement (50x) in 1 mL aliquots at -20 °C. Thaw aliquots at RT immediately before use. Avoid freeze/thaw cycles.

4.1.3. Store glutamine supplement (100x) at 4 °C or -20 °C. Divide into 0.5 mL aliquots if storing at -20 °C.

4.1.4. Store penicillin-streptomycin (10,000 U/mL) in 1 mL aliquots at -20 °C. Thaw aliquots at RT immediately before use.

4.1.5. To make the dissection medium, mix 9.4 mL of Hibernate E with 200 µL of horse serum (2%), 200 µL of 50x B27 supplement (1x), 100 µL of 100x glutamine supplement (1x) (e.g., GlutaMAX), and 100 µL of 10,000 U/mL penicillin-streptomycin (100 U/mL). Use this medium for collecting dissected tissues and making the final suspension of dissociated cells.

4.1.6. Add 500 µL of the dissection medium to individual 1.7 mL microcentrifuge tubes for the tissue collection the day before the neuron isolation. Prepare one tube for each tissue type that will be collected (e.g., positive control, negative control, CN3/CN4, SMN) and store at 4 °C prior to the use.

4.1.7. Combine 49 mL of Hibernate E low fluorescence media with 1 mL of B27 supplement (1x) and fill a 24 well plate with this medium (2 mL/well) the day before the neuron isolation. Use this dish for collecting mouse embryos. Store at 4 °C prior to use.

4.2. Prepare the motor neuron culture medium.

4.2.1. Prepare 250 µL aliquots of 25 mM 2-mercaptoethanol in Leibovitz's L15 medium. Store at -20 °C.

4.2.2. Generate 5 µL aliquots of 100 µg/mL solutions of BDNF, CNTF, and GDNF diluted in sterilized water. Store at -80 °C and thaw aliquots at RT immediately before use.

4.2.3. Prepare 10 mM forskolin solution by adding 64 µL of dimethyl sulfoxide (DMSO) to 5 mg (1.0670 µM) of forskolin and vortex well to dissolve completely. Then add sterilized water (1.003 mL) to the DMSO solution and vortex well. Store 12 µL aliquots of 10 mM forskolin at -20 °C and thaw aliquots at RT immediately before use.

4.2.4. Prepare 12 µL aliquots of 100 mM isobutylmethylxanthine (IBMX) diluted in DMSO. Store at -20 °C and thaw aliquots at RT immediately before use.

4.2.5. To make the motor neuron culture medium, mix 9.4 mL of neurobasal medium with 200 µL of horse serum (2%), 200 µL of 50x B27 supplement (1x), 100 µL of 100x glutamine supplement (1x), 100 µL of 10,000 U/m: penicillin-streptomycin (100 U/mL), and 20 µL of 25 mM 2-mercaptoethanol (50 µM), preferably directly before use. This step can be performed up

to 1 day before neuron isolation.

4.2.6. Just before the use, add 1 µL each of 100 µg/mL BDNF (10 ng/mL), CNTF (10 ng/mL), and GDNF (10 ng/mL) and 10 µL of 10 mM forskolin (10 µM), and 10 µL of 100 mM IBMX (100 µM) to the motor neuron culture medium. Prewarm the medium to 37 °C.

4.3. Prepare the dissociation solutions.

4.3.1. Prepare the papain solution (20 units/mL papain and 0.005% DNase) and an albumin-ovomucoid inhibitor solution (1 mg/mL ovomucoid inhibitor, 1mg/mL albumin, and 0.005% DNase) following the manufacturer's instructions.

4.3.2. Prepare 500 µL aliquots of each of ovomucoid inhibitor and papain solutions and store at -80 °C. Thaw aliquots at 37 °C immediately before use.

5. Ventral midbrain and spinal cord dissection

NOTE: Perform all of the following steps except for steps 5.1.1−5.1.3 and 5.1.5−5.1.6 under a fluorescence dissection stereomicroscope. Total dissection time per experiment is typically 3−5 h, depending on the proficiency at the dissection technique and the number of motor neurons required for each experiment.

5.1. Ventral midbrain dissection

5.1.1. Euthanize a pregnant mouse approximately 11.5 days postfertilization by carbon dioxide gas and cervical dislocation.

5.1.2. Spray the abdomen thoroughly with ethanol and remove the uterus using sterile microdissecting scissors and thumb dressing forceps. Wash the uterus briefly in sterile PBS, then transfer to the dissection plate filled with prechilled sterile PBS.

5.1.3. Remove the *Isl*^{MN}*:GFP*-positive embryos carefully from the uterus using sterile microdissecting scissors, thumb dressing forceps, and Dumont #5 tweezers in ice-cold sterile PBS under the bright light of the microscope. Using a sterile Moria mini-perforated spoon, transfer each embryo to a separate well of a 24 well plate filled with prechilled Hibernate-E low fluorescence medium supplemented with 1x B27. Keep the 24 well plate on ice.

5.1.4. Transfer one embryo to a sterile dissection plate and cover it completely with ice-cold sterile Hank's balanced salt solution (HBSS).

5.1.5. Ensure that the dissection steps are performed under fluorescein isothiocyanate (FITC) illumination of the microscope. Using tweezers, remove the tail and the face of the embryo without damaging the midbrain (**Figure 2Ba**). Place the embryo prone with limbs straddled underneath and tail pointing toward the front of the microscope, toward the dissector

(indicated by an asterisk, **Figure 2Bb**).

5.1.6. Using tweezers, slit open the roof of the fourth ventricle in order to generate a small opening. Use this opening to hook tweezers into the space created between the fourth ventricle and its roof. Dissect along the dorsal surface of the embryo rostral to the cortex and lateral to the floor plate and motor column (**Figure 2Ca**,**b**). Open the dissected tissue in an open-book manner to reveal the GFP-positive CN3 and CN4 nuclei.

NOTE: A small piece of tissue from the ventral midbrain containing mesenchyme, CN3, and CN4 will now be exposed.

5.1.7. Carefully separate the ventral midbrain from the embryo and remove meningeal tissue using tweezers and a microdissecting knife. Dissect the bilateral GFP-positive CN3 and CN4 nuclei away from the floor plate and other GFP-negative surrounding tissue using tweezers and a microdissecting knife (**Figure 2D**). Maximize the number of GFP-positive motor neurons in the excised tissue but avoid touching or damaging them.

5.1.8. If a collection of separate CN3 and CN4 nuclei is desired, cut along the midline of these two nuclei (yellow dotted line in **Figure 2D**). Using a P1000 pipette, collect the dissected ventral midbrain tissue with minimal HBSS and place it in a labeled 1.7 mL microcentrifuge tube filled with dissection medium (see step 4.1.5). Store on ice until dissociation.

5.1.9. Continue pooling ventral midbrains from additional embryos in the same tube until the total number meets the experimental requirement (refer to step 8 for ideal cell numbers).

NOTE: A pooled collection of at least 10 ventral midbrains yielding approximately 1 x 10^4 CN3/CN4 motor neurons is recommended because tissues are subject to stress during the dissociation and sorting.

5.2. Ventral spinal cord dissection

5.2.1. Keep the embryo prone with the head facing the front of the microscope, toward the dissector. Hold the embryo with one pair of tweezers and insert the tip of the other pair of tweezers into the unopened caudal part of the fourth ventricle.

5.2.2. Open the rest of the hindbrain and spinal cord dorsally over the whole rostrocaudal extent of the embryo. Open by cutting dorsal tissue, starting from the fourth ventricle and working toward the central canal of the caudal spinal cord using the forceps as scissors (**Figure 2Ca**,**b**). Take care to avoid touching or damaging the ventral spinal cord during this procedure.

5.2.3. Hold the embryo with one pair of tweezers and pinch off the flap of the dorsal tissue on each side with the other pair of tweezers (**Figure 2Ea**,**b**).

NOTE: Excised dorsal tissues contain dorsal skin, mesenchyme, dorsal root ganglia (DRGs), dorsal

hindbrain, and spinal cord. Remove as much of these tissues as possible without damaging the SMN nuclei, because they are adhesive and can trap SMNs during filtering or cause clogging during FACS sorting.

5.2.4. Remove the ventral spinal cord using the microdissection knife to pierce directly below the GFP-positive SMN. Lift the ventral spinal cord with saw-like movements on both sides (**Figure 2Fa**,**b**). Cut the floating ventral spinal cord transversely directly above C1, where the first GFP-positive anterior horn projects (**Figure 2G**). Also, cut transversely at the upper boundary of the lower limb (**Figure 2G**). Remove the cervical (C1)-lumbar (L2-L3) portion of the ventral spinal cord after this procedure.

5.2.5. Place the ventral spinal cord dorsal side up and hold by pressing the GFP-negative tissue between the GFP-positive SMN columns with one pair of tweezers. Remove the remaining attached mesenchyme, DRGs, and dorsal spinal cord by trimming both sides of the GFP-positive SMN column with the microdissection knife (**Figure 2H**). Take care to maximize GFP-positive motor neurons without damaging them.

5.2.6. Using a P1000 pipette, collect the dissected ventral spinal cord tissue with minimal HBSS and place in the SMN-labeled 1.7 mL microcentrifuge tube filled with dissection medium. Store on ice until dissociation. Continue pooling ventral spinal cords from additional embryos in the same tube until the total number meets the experimental requirements.

NOTE Collecting at least three ventral spinal cords yielding approximately 2.1 x 10^4 SMN is recommended because tissues are subject to stress during dissociation and sorting.

5.2.7. Collect facial motor neurons and extremities of the *Isl^{MN}:GFP* mouse embryos as GFP-positive and GFP-negative controls for fluorescence-activated cell sorting (FACS), respectively. Extremities are GFP-negative because the GFP-positive axons of the SMNs have not yet extended into the extremities at this embryonic age.

6. Tissue dissociation

NOTE: Total dissociation time is typically 1.5 h per experiment.

6.1. Warm papain and albumin-ovomucoid inhibitor solution aliquots to 37 °C 30 min prior to dissociation.

6.2. Briefly spin down microdissected tissues at a low speed.

6.3. Using a P100 pipette, carefully remove as much Hibernate E as possible without aspirating tissues.

NOTE: Be sure to remove all residual Hibernate E after this step to avoid reducing the efficacy of papain dissociation in the next step.

6.4. Add the appropriate volume of papain solution (**Table 1**) to each of the 1.7 mL microcentrifuge tubes containing the microdissected tissue samples.

NOTE: The appropriate volume of papain for the dissociation was determined in order to maximize the effective dissociation while minimizing stress on the cells.

6.5. Gently triturate 8x with a P200 pipette. Perform all trituration steps gently to preserve motor neuron viability.

6.6. Incubate the tubes containing the tissues for 30 min at 37 °C, agitating by finger flicking 10x every 10 min. Gently triturate each suspension 8x with a P200 pipette after incubation. Spin down the cells at 300 x *g* for 5 min.

6.7. To ensure the efficacy of ovomucoid inhibition in the next step, use a P1000 pipette to remove and discard as much supernatant as possible without aspirating the tissues.

6.8. Resuspend pellets in the appropriate volume of albumin-ovomucoid inhibitor solution (**Table 1**) by gently triturating 8x with a P200 pipette.

6.9. Wait for 2 min to allow any remaining pieces of undissociated tissue to settle to the bottom of the tube.

6.10. Collect as much supernatant as possible without aspirating undissociated tissues using a P200 pipette. Transfer the supernatant to fresh 1.7 mL microcentrifuge tubes.

6.11. If some chunks of tissue remain undissociated after step 6.10, repeat steps 6.8–6.10 for the undissociated tissues that remain in the original 1.7 mL microcentrifuge tubes to maximize the final yield of dissociated cells while minimizing stress on the cells dissociated previously, contained in the supernatant of step 6.10.

6.12. Spin down the cells at 300 x *g* for 5 min. Carefully remove and discard the supernatant using a P1000 pipette.

6.13. Resuspend the pellet in the appropriate volume of dissection medium (**Table 1**) by pipetting 8x using a P1000 pipette. The appropriate volume of final suspension was determined so that cell density does not exceed 10^7 cells/mL, which can block the stream of the flow cytometry machine, but also so that cells are not excessively diluted, which results in a slowed sorting speed.

6.14. Filter the suspensions through 70 µm cell strainers to eliminate any large clumps or undigested tissue. Transfer the suspensions into 5 mL round bottom polystyrene test tubes and store on ice until required.

7. Fluorescence-activated Cell Sorting (FACS)

NOTE: This protocol was optimized using a FACS sorter equipped with a 15 mw 405 nm violet laser, a 100 mw 488 nm blue laser, a 75 mw 594 nm orange laser, and a 40 mw 640 nm red laser. Cells were sorted as sheath fluid in sterile PBS under aseptic conditions through a 100 µm nozzle. In order to minimize cell stress, the flow rate was set to a sample pressure of 1−3, such that a maximum of 1,000−4,000 events per second were acquired. Total FACS time is typically 1−2 h per experiment.

7.1. Set up voltages for forward and side scatter so that the cell population can be visualized properly. Setting up appropriate voltages for cell sorting is complex and requires an experienced FACS operator.

7.2. To distinguish different cell populations, plot cells based on size as determined by the Forward Scatter Area (FSC-A) versus internal complexity as determined by the Side Scatter Area (SSC-A). Draw a gate around the live cells as indicated in **Figure 3Aa** and **Figure Ba** to exclude debris and dead cells. Group the cells within the gated region as population 1 (P1).

7.3. To exclude cell clumps and doublets, plot P1 cells next based on the Side Scatter Width (SSC-W) versus the SSC-A. Gate the population of single cells as population 2 (P2) (**Figure 3Ab** and **Figure Bb**).

7.4. Plot P2 cells based on the Forward Scatter Width (FSC-W) versus the FSC-A and gate the population of single cells as population 3 (P3) (**Figure 3Ac** and **Figure Bc**).

NOTE: Use of two consecutive gates in 7.3 and 7.4 excludes cell clumps and doublets (high FSC-W and high FSC-A).

7.5. Gate P3 cells based on GFP versus allophycocyanin (APC). The APC channel detects autofluorescence. Gating on this channel avoids capturing autofluorescent cells. Use GFP-negative cells to adjust the voltage for FITC/GFP fluorescent channels. Ideally, position gates for these cell populations around 10^2. Select gate thresholds for GFP-positive population 4 (P4) individually for each type of motor neuron (**Figure 3Ad** and **Figure Bd**).

NOTE: Set the GFP gate much higher for SMNs than for CN3s/CN4s in order to obtain a pure culture (**Figure 3Ad** and **Figure Bd**). A lower GFP gate for SMN cultures leads to contamination of the cultures by glia and non-motor neurons. This is likely because there is low-level GFP expression in some glia and non-motor neurons due to a leaky promoter. The percentage of GFP-positive cells as compared to total cells is typically 0.5−1.5% for CN3s/CN4s and 1.5−2.5% for SMNs. If the dissection was successful, these numbers can be used as a benchmark to determine the appropriate position for the GFP-positive gate (**Figure 3Ae** and **Figure Be**).

7.6. Perform FACS according to the manufacturer's protocol. Collect P4 cells into fresh 1.7 mL microcentrifuge tubes filled with 500 µL of motor neuron culture medium. Store on ice until

plating.

NOTE: Although the cells can be sorted directly into the wells, this results in an uneven number of cells per well. Sort the cells into 1.75 mL microcentrifuge tubes and then plate manually in order to achieve a more even plating distribution.

8. Culture of purified primary motor neurons

8.1. Dilute FACS-isolated CN3/CN4 and SMN suspensions with motor neuron culture medium prewarmed to 37 °C to densities of 5 x 10^3 and 1 x 10^4 cells/mL, respectively.

NOTE: One E11.5 embryo yields approximately 1 x 10^3 CN3/CN4 and 7 x 10^3 SMN. However, these yields rely heavily on the purity of dissected tissues, the thoroughness of cell dissociation, and the appropriate thresholding of GFP gates during FACS.

8.2. Transfer 96 well plates precoated with PDL and laminin from the 37 °C tissue culture incubator to the laminar flow hood and aspirate laminin from each well. Use plates and coverslips immediately without washing.

8.3. Add 200 µL of diluted CN3/CN4 and SMN suspensions into the each well of PDL/laminin-coated 96 well plates. Final cell densities should be 1 x 10^3 and 2 x 10^3 cells/well for CN3/CN4 and SMN, respectively.

NOTE: Initial plating density of SMNs in 96 well plates (2 x 10^3 cells/well) is double that of CN3s/CN4s (1 x 10^3 cells/well) in order to obtain similar final motor neuron numbers and densities at 2 and 9 days in vitro (DIV) (4–6 x 10^2 and 2–4 x 10^2 cells per well, respectively).

8.4. Culture neurons in a 37 °C, 5% CO_2 incubator.

8.5. Feed neurons every 5 days by removing half of the old media (100 µL) and replacing with the same volume of fresh motor neuron culture medium. Ensure that neuronal processes become visible on 1 DIV and become thicker and longer by 14 DIV (**Figure 4**). Neuronal cell bodies become enlarged and tend to aggregate in long-term cultures, particularly for SMNs (**Figure 4**).

NOTE: Perform all medium and solution changes by leaving half of the original medium volume in order to avoid detaching cultured cells. This includes fixation and immunocytochemistry (ICC) steps. If all media is removed, regardless of how gently, most of the cells will detach and be washed away.

REPRESENTATIVE RESULTS:

The aim of this protocol was to highly purify and culture both primary CN3s/CN4s and SMNs long-term to enable comparative analyses of the mechanisms underlying motor neuron disorders (see **Figure 1** and **Figure 2** for overview).

Once neurons were successfully isolated and grown in culture, nearly pure primary CN3/CN4 and SMN cultures were obtained (**Figure 5A**,**B**) and maintained for at least 14 DIV (**Figure 4** and **Figure 6**). The purities of CN3/CN4 and SMN cultures at 2 DIV were 93.5 ± 2.2% and 86.7 ± 4.7%, respectively, when assessed by ICC using the motor neuron marker Islet1 and neuronal marker TUJ1 (**Figure 5B**). However, these high purities relied heavily on the age of the embryos and on setting appropriate thresholds for GFP gates during FACS (**Figure 3**). Dissection of embryos at E10.5 is more difficult than dissection at E11.5 due to increased softness and adhesiveness of tissues, resulting in decreased motor neuron yields. However, the purities of E10.5 CN3s/CN4s and SMNs were comparable to those for E11.5 embryos (92.8% and 82.2% at 2 DIV, respectively; data obtained from a single experiment). The purities of CN3s/CN4s and SMNs dramatically decreased when E13.5 embryos were used, even if only the highest GFP-positive population was collected (20.7% and 7.4% at 2 DIV, respectively; data obtained from a single experiment), probably due to the expression of GFP in non-motor neurons (**Figure 7**). This same tendency also held true for E12.5 cultures, although it was much less dramatic. Therefore, embryos at E12.5 or older are inappropriate for use in the purification of motor neurons using this protocol.

Pure motor neuron cultures are valuable for understanding isolated growth patterns, behaviors, and vulnerabilities of motor neurons. This example demonstrates how these cultures can be used to test motor neuron responses to chemical treatment. To determine if primary CN3s/CN4s and SMNs show differential responses to endoplasmic reticulum (ER) stressors, primary monocultures of CN3s/CN4s and SMNs were obtained using this protocol and treated with varying concentrations of an ER stressor, cyclopiazonic acid (CPA). Neurons were treated with CPA (5, 10, 15, 20, 25, or 30 µM) or vehicle control (DMSO) at 2 DIV and fixed 3 days later for ICC to evaluate survival ratios (**Figure 8A**). The number of viable neurons in each sample was counted and survival ratios were calculated as the number of viable cells in drug-treated wells divided by the number of viable cells in the wells treated with DMSO. CN3/CN4 monocultures were significantly more resistant to CPA treatment (10−25 µM) as compared to SMN monocultures (**Figure 9** and **Figure 8B**)[31].

In conclusion, this protocol allows for the generation of highly purified primary mouse embryonic CN3/CN4 and SMN cultures that provide a powerful and reliable system for the investigation of neuronal behavior.

FIGURE LEGENDS:

Figure 1: Scheme for preparation of mouse embryonic motor neurons. The schematic illustrates the steps involved in the isolation and culture of mouse embryonic motor neurons and the approximate time in hours or days for each step. The order of the dissection procedure for CN3/CN4 and for SMN are each labelled sequentially 1 through 4. Abbreviations: CN3/CN4 = oculomotor neuron/trochlear neuron; SMN = spinal motor neuron; FACS = fluorescence-activated cell sorting; h = hour; d = day.

Figure 2: Dissection of the ventral midbrain and the cervical (C1)-lumbar (L2-L3) portion of the ventral spinal cord. (**A**) Lateral (**a**) and dorsal (**b**) views of GFP-positive motor neurons in an

E11.5 *Isl^{MN}:GFP* transgenic mouse embryo under fluorescein isothiocyanate (FITC) illumination. A whole mount E11.5 embryo was prepared as previously described[32] in order to make the embryo transparent. Subsequently, the embryo was analyzed by immunofluorescence labeling with anti-GFP staining (green). Images were captured under a confocal microscope. Scale bars: 200 µm (lateral view) and 400 µm (dorsal view). Abbreviations: S = superior; I = inferior; V = ventral; D = dorsal. (**B**-**H**) Dissection steps highlighted on images of E11.5 ventral midbrain and ventral spinal cord tissues taken with an equipped camera under bright light (Bb) or FITC illumination using a fluorescence dissection stereomicroscope. Scale bars = 200 µm in (D) and = 1 mm (A−C, E−H). (**B**)(**a**) Removal of the face and tail of the embryo by cutting along the red lines. (**b**) Embryo positioned for dissection. Positioning of the front of the microscope is indicated by an asterisk. (**C**) Cutting along the solid red line in order to slit open the roof of the fourth ventricle (**a**) lateral view and (**b**) dorsal view. Use of this opening to cut along the surface of the embryo dorsal to the brain (trajectory indicated by dashed red arrow). This exposes the tissue containing mesenchyme, CN3, and CN4, which can be lifted out of the cranium. For SMN dissection, insertion of forceps into the same opening between the fourth ventricle and its roof, then cutting toward the caudal side of the embryo (trajectory indicated by dashed yellow arrow). (**D**) Final view of the ventral midbrain containing bilateral GFP-positive CN3 and CN4 nuclei. The edges of the tissue are highlighted by a red rectangle. Cutting along yellow dotted line to collect CN3 and CN4 nuclei separately, if desired. (**E**) After opening the rest of the hindbrain and spinal cord, flapping dorsal tissues pinched off above the red lines on both sides with tweezers (**a**) before, and (**b**) after. (**F**) Bilaterally removal of excess tissue ventral to the spinal cord along the red line (**a**) before, and (**b**) after. (**G**) Cutting of the ventral spinal cord at the two locations indicated by the red lines. On the rostral side, cutting of the floating ventral spinal cord transversely above C1 where the first GFP-positive anterior horn projects. Cutting of the caudal end of the spinal cord transversely at the upper boundary of the lower limb. Once these cuts are made, the cervical (C1) through lumbar (L2-L3) portion of the ventral spinal cord can be dissected away. (**H**) Final view of the ventral spinal cord containing GFP-positive SMN columns.

Figure 3: Representative sort plots of ventral midbrains (A) and ventral spinal cords (B). (**Aa** and **Ba**) Forward Scatter Area (FSC-A) versus Side Scatter Area (SSC-A) sorted plot before exclusion of debris and dead cells. (**Ab, Bb, Ac, Bc**) Sorted plots for exclusion of cell clumps (**b**) and doublets (**c**) based on Width (SSC-W) versus SSC-A and Forward Scatter Width (FSC-W) versus FSC-A, respectively. (**Ad** and **Bd**) Sorted plots to isolate *Isl^{MN}:GFP* -positive motor neurons. In order to obtain a pure culture, the GFP gate must be set higher for SMNs (**Bd**) than for CN3s/CN4s (**Ad**). (**Ae** and **Be**) Percentages of cells gated for collection by FACS sorting. **%Parent** represents the percentage of cells in the current gated population relative to the number of cells in the previous gated cell population, whereas **%Total** represents the percentage of gated cells relative to total cells. Expected percentages of GFP-positive cells as compared to total cells (boxed in red) are 0.5−1.5% for CN3/CN4 and 1.5−2.5% for SMN. If the dissection was performed successfully, these percentages can be used as a benchmark to set up the GFP-positive gate in (**Ad** and **Bd**).

Figure 4: Phase-contrast images of primary CN3/CN4 and SMN monocultures at 2, 7, and 14 DIV. Representative differential interference contrast images of primary CN3/CN4 and SMN

cultures were captured at 2, 7, and 14 DIV with inverted fluorescence microscope using corresponding image acquisition and processing software and 40x objectives. Neuronal processes became thicker and longer by 14 DIV. Neuronal cell body sizes became enlarged and tended to aggregate in long-term cultures, especially for SMNs. Both cultures can be maintained at least 14 DIV. Scale bar = 50 µm.

Figure 5: Characterizations of isolated E11.5 mouse CN3/CN4 and SMN cultures. (**A**) Representative immunocytochemistry images of E11.5 mouse CN3s/CN4s (top) and SMNs (bottom) cultured for 2 DIV. Immunofluorescence labeling with the neuronal marker TUJ1 (green) and the motor neuron marker Islet1 (red) performed to analyze neurons and nuclei were counterstained with DAPI (blue). Almost all the cultured cells were motor neurons ($TUJ1^+$, $Islet1^+$). Images were captured with an inverted fluorescence microscope using corresponding image acquisition and processing software and 20x objectives. Samples were imaged and processed to achieve maximum signal intensity without saturated pixels. All of the microscopic work and image processing in the following figures were performed in these conditions unless otherwise specified. Scale bar = 100 µm. (**B**) The purities of E11.5 mouse CN3/CN4 and SMN cultures at 2 DIV. The purities of CN3/CN4 and SMN cultures were 93.5 ± 2.2% and 86.7 ± 4.7%, respectively. Dead neuronal cell bodies were assessed by screening for pyknotic nuclear morphology and membrane swelling. Neuronal processes were classified as degenerating processes when signs of beading and swelling were observed. Cells with neither cell body death nor degenerating processes were considered viable non-motor neurons ($TUJ1^+$, $Islet1^-$) or viable motor neurons ($TUJ1^+$, $Islet1^+$)[33]. The purities of motor neuron cultures were calculated as the number of viable motor neurons divided by the total number of viable non-motor neurons plus viable motor neurons. Values represent the mean ± SEM of three separate experiments. Not significant ($p > 0.05$) by Student's t test. Cell counting was performed manually under 20x magnification. Abbreviations: SEM = standard error of the mean.

Figure 6: Representative immunocytochemistry of primary CN3/CN4 and SMN monocultures at 2, 7, and 14 DIV. Primary CN3/CN4 and SMN cultures were analyzed at 2, 7, 14 DIV by immunofluorescence labeling with TUJ1 (green), and nuclei were counterstained with DAPI (blue). Neuronal processes become thicker and longer by 14 DIV. Neuronal cell body sizes became enlarged and tended to aggregate in long-term cultures, particularly for SMNs. Both CN3/CN4 and SMN cultures can be maintained at least 14 DIV. Images were captured under 10x magnification. Scale bar = 200 µm.

Figure 7: Characterization of E13.5 mouse CN3/CN4 and SMN isolated cultures. E13.5 CN3/CN4 and SMN were isolated and cultured using this protocol and analyzed at 2 DIV by immunofluorescence labeling with TUJ1 (green) and Islet1 (red), and the nuclei were counterstained with DAPI (blue). Many non-motor neuronal cells ($TUJ1^+$, $Islet1^-$) were present (arrows) resulting in a drastic decrease in both CN3/CN4 and SMN purity, with the decrease more pronounced in SMN cultures. Images were captured under 20x magnification. Scale bar = 100 µm.

Figure 8: Representative application of primary motor neuron culture demonstrating that

CN3s/CN4s are selectively resistant to ER stress induced by CPA. (**A**) Experimental outline: primary CN3/CN4 and SMN monocultures were treated with CPA or vehicle control (DMSO) at 2 DIV and cell viabilities were evaluated through immunocytochemistry analysis after 3 days of treatment. This outline has been modified from published work[31]. (**B**) Quantification of survival ratios of CN3s/CN4s and SMNs treated with 5−30 µM CPA for 3 days from 2 DIV. Neurons were analyzed by immunofluorescent labeling of cells with TUJ1, and nuclei were counterstained with DAPI. Survival ratios were calculated as the number of viable cells (see **Figure 5B** legend) in drug-treated wells divided by the number of viable cells in wells containing vehicle alone (DMSO). Cell counting was performed manually under 20x magnification. Values represent the mean ± SEM of four separate experiments. *$p < 0.05$; ***$p < 0.005$ by Student's *t* test. This figure has been modified from previously published work[31].

Figure 9. Representative immunocytochemistry of primary CN3/CN4 and SMN monocultures after a 3-day exposure to increasing concentrations of CPA beginning at 2 DIV. Neurons were analyzed by immunofluorescent labeling of cells with TUJ1 (green) and nuclei were counterstained with DAPI (blue). Primary CN3s/CN4s were more resistant to CPA treatment than primary SMNs. Images were captured under 10x magnification. Scale bar = 200 µm.

Table 1: Appropriate volumes of papain, albumin-ovomucoid, and final suspension used in dissociation steps. The appropriate volumes of papain and albumin-ovomucoid to be used with various numbers of ventral midbrain and ventral spinal cord tissues were modified from the manufacturer's instructions after several rounds of optimization. Because tissues are subject to stress during dissociation and sorting, a pooled collection of more than 10 ventral midbrains and more than three ventral spinal cords is recommended. The volume of papain was determined by considering the balance between effective dissociation and the stress of this procedure. The volume of albumin-ovomucoid inhibitor solution is half of that of papain. The appropriate volume of Hibernate E final suspension was determined such that cell density does not exceed 10^7 cells/mL, but the cells do not become excessively diluted.

DISCUSSION:

Historically, in vitro studies of CN3 and/or CN4 motor neurons have relied on heterogeneous cultures such as dissociated[17-21], explant[17,22-26], and slice[27,28] cultures, because these cells cannot be distinguished from surrounding cells based on size, and specific markers for these cells have not been reported. The present protocol is a comprehensive method for the isolation and culture of primary E11.5 murine CN3s/CN4s, and SMNs from the same embryos and confirm the high purity of the cultures. By generating pure SMN and CN3/CN4 cultures from the same mouse embryos, the protocol enables controlled comparisons of the in vitro behaviors of CN3s/CN4s versus SMNs isolated from wild type as well as mutant embryos.

The pure cultures of CN3s/CN4s and SMNs generated by this protocol allow comparative studies of morphological, cellular, molecular, and electrophysiological characteristics of these motor neurons. In theory, because other cranial motor neuron populations can be visualized and dissected from this *Isl^{MN}:GFP* transgenic mouse line (including abducens, motor trigeminal, facial, and hypoglossal), this protocol could be expanded for their isolation and culture as well,

provided that FACS GFP gates are adjusted appropriately. Finally, the FACS-sorted motor neurons derived from this protocol can be subjected to genomic (e.g., Assay for Transposase-Accessible Chromatin using sequencing, or ATAC-seq) and/or transcriptomic (e.g., RNA sequence[31]) analyses to study normal development and the selective vulnerability of specific motor neuron subtypes in neurodegenerative disorders[31].

There are multiple steps in this protocol that are critical to maximize the number of pure, healthy motor neurons in the isolated culture system. During the dissection, the GFP-negative tissues (e.g., mesenchyme and DRGs) should be maximally removed without damaging the motor neurons, because these tissues are adhesive and can trap SMNs during filtering or cause clogging during FACS sorting. During tissue dissociation, the minimal essential volume of papain should be used, and the cells must be treated gently with minimal but sufficient trituration. Papain was used for the tissue dissociation step in this protocol, because preliminary data indicated that it is less destructive than trypsin to both CN3/CN4 and SMN. Survival ratios based on plated numbers of CN3s/CN4s and SMNs at 2 DIV increased from 39.5% to 52.7% and from 52.3% to 58.4%, respectively, when papain was used instead of trypsin (0.25%, 4 min incubation). Although these numbers are derived from a single experiment performed before full optimization, additional reports also suggest that trypsin is suboptimal for cell extraction from nervous system tissues[12,34-36]. During FACS, fluorescent vital dyes (propidium iodide and calcein blue) and small sorting nozzles (e.g., 70 µm) should not be used, because they are deleterious to motor neuron survival. Use of large sorting nozzles (100 µm or larger) is highly recommended, because SMN cell death increases significantly when a 70 µm nozzle is used. Setting the appropriate gating thresholds for GFP-positive cells in FACS is a critical step in order to obtain pure cultures. Motor neuron cultures are supplemented with forskolin, IBMX, and growth factors (BDNF, CNTF, and GDNF). Forskolin and IBMX have been reported to additively promote SMN survival[37,38]. Preliminary data from the present studies suggest that forskolin and IBMX also additively increase CN3/CN4 survival. Survival ratio based on plated numbers of CN3s/CN4s at 2 DIV increased from 17.5% to 26.9%, 31.9%, and 37.0% when IBMX, forskolin, and IBMX+forskolin were added, respectively (numbers are based on a single experiment performed prior to full optimization of cell culture conditions). It is best to perform all medium changes and washes of cultured cells by leaving half of the original volume to avoid detaching cultured cells. Finally, it is also ideal to reduce the time spent between dissection and plating of motor neurons (e.g., by shortening dissection time using multiple dissectors) to improve the viability of the cultures.

There are four major potential problems that may arise when following this protocol. The first is low yield of motor neurons after FACS. Potential causes for low yields include using young embryos (e.g., E10.5), which have fewer motor neurons, insufficient removal of adhesive GFP-negative tissues during dissection (e.g., mesenchyme and DRGs), which can trap motor neurons and lead to their removal during filtration, insufficient papainization/trituration during dissociation, and/or setting the GFP-positive gate too high during FACS. The second potential problem is low purity of the motor neuron cultures, which most likely arises from the use of older embryos (e.g., E12.5) and/or from setting the GFP-positive gate too low during FACS. Third, a low number of attached motor neurons in culture may be observed due to inappropriate FACS

sorting and/or inadequate PDL/laminin coating of plates/coverslips. Fourth, motor neurons can show low viability in culture. Potential causes of low viability include rough and/or prolonged dissection, excessive papainization/trituration during dissociation, inappropriate handling of cells throughout the protocol (e.g., rough pipetting of cells, failure to place cells on ice, failure to pre-chill PBS and HBSS), and/or excessive time between euthanization of pregnant mice and final plating of the cells. Use of reagents that are not fresh and/or inappropriate concentrations can also impair experimental outcomes.

There are three major limitations of this protocol. *Isl^{MN}:GFP* transgenic mice and FACS sorting are both fairly expensive. They are, however, crucial for this protocol as there is currently no alternative method capable of generating highly purified CN3s/CN4s in a more economical fashion. There is a small E10.5-E12.5 age window for the embryonic mice, and it is difficult to confirm that appropriately aged embryos are present, especially if an ultrasound machine is not available. If only pure SMNs are required, they can be derived from E12.5-15.0 mouse embryos using methods such as gradient centrifugation[10-12] and/or p75^{NTR}-antibody-based cell-sorting panning techniques[13-16]. Finally, protein-based assays that require a large amount of starting material (e.g., Western blot analysis) are not feasible from these cultures due to the small yield of motor neurons (especially CN3s/CN4s). Stem cell-derived motor neurons[31,39], which can be generated limitlessly, could in theory be substituted for this purpose.

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DISCLOSURES:

The authors declare no conflict of interest.

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Figure 1

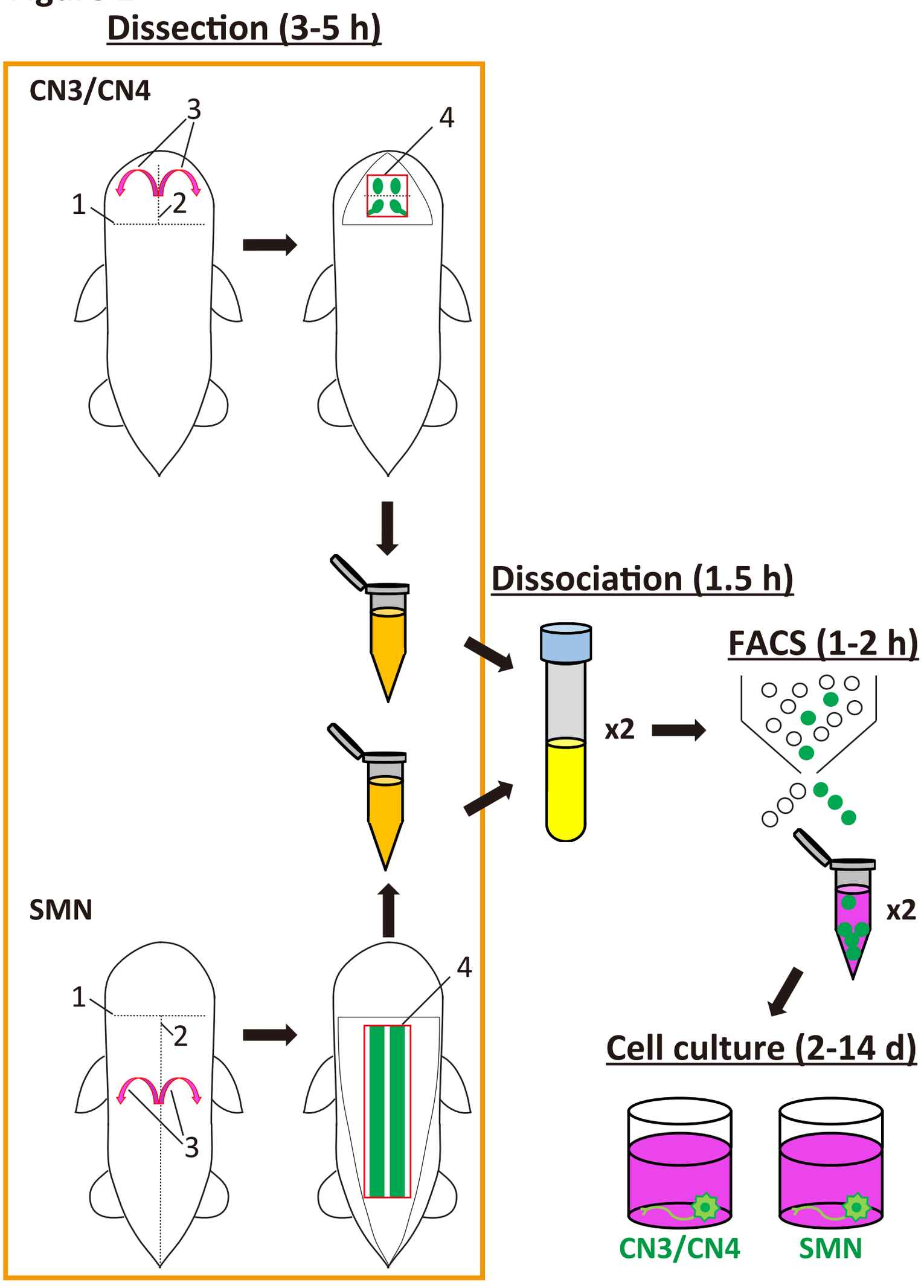

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Figure 2

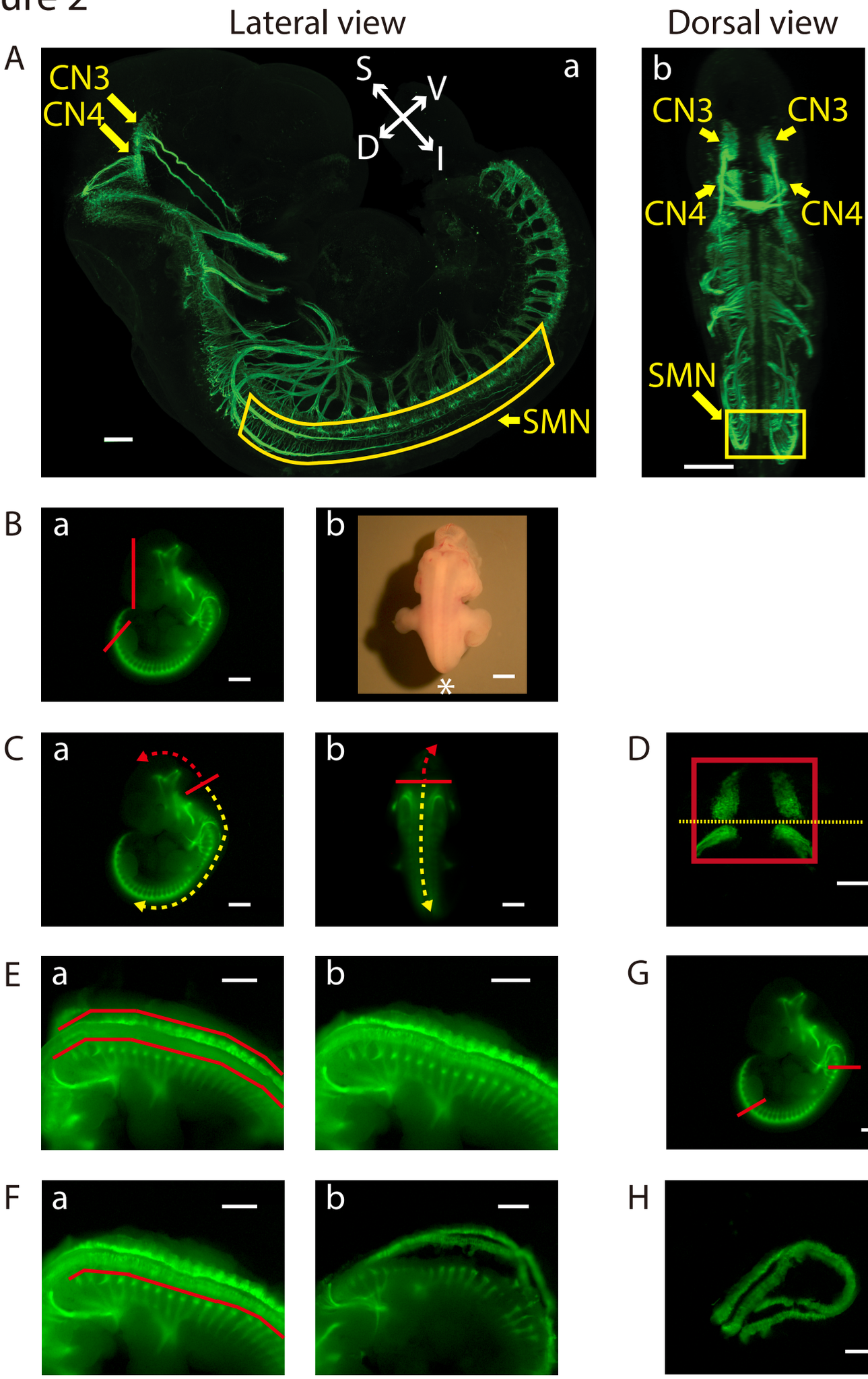

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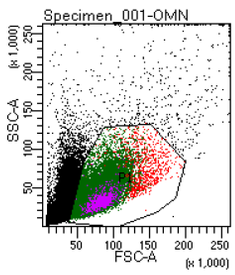
Figure 3

A Ventral midbrain (CN3/CN4)

a



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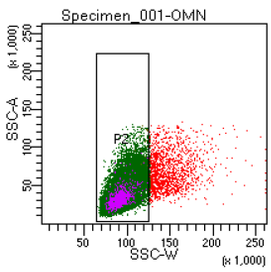

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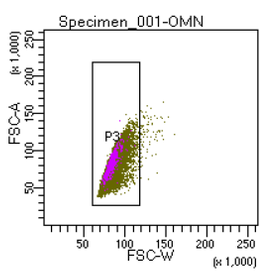
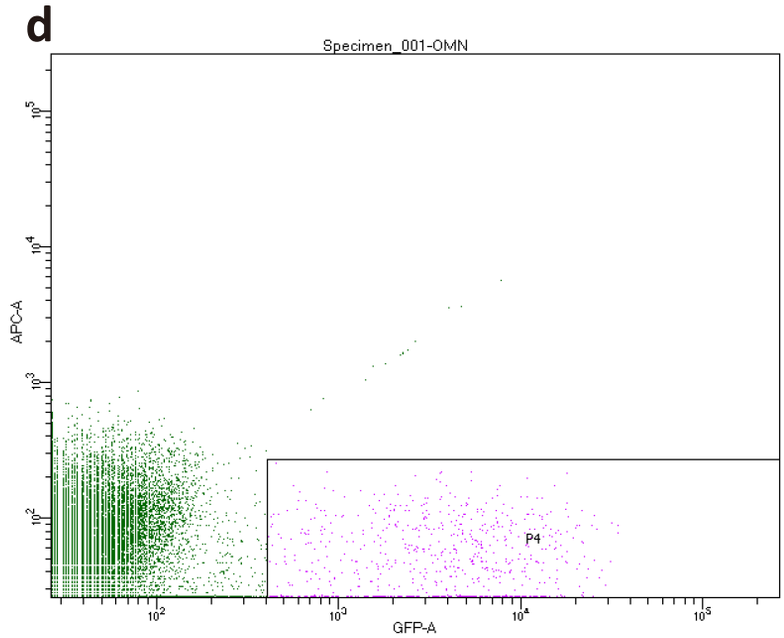


e

Tube: OMN

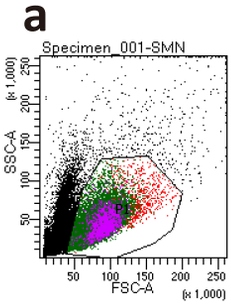
Population	#Events	%Parent	%Total
All Events	100,062	####	100.0
P1	62,843	62.8	62.8
P2	61,341	97.6	61.3
P3	61,260	99.9	61.2
P4	769	1.3	0.8

d

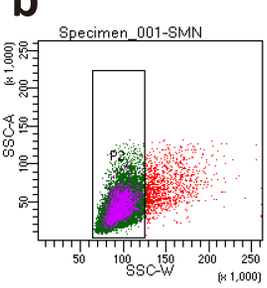


B Ventral spinal cord (SMN)

a



b



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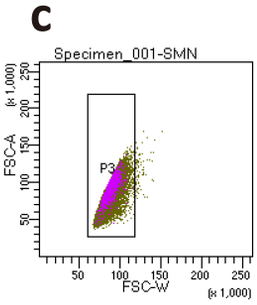

e

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P2	54,306	98.1	54.3
P3	54,220	99.8	54.2
P4	1,466	2.7	1.5

d

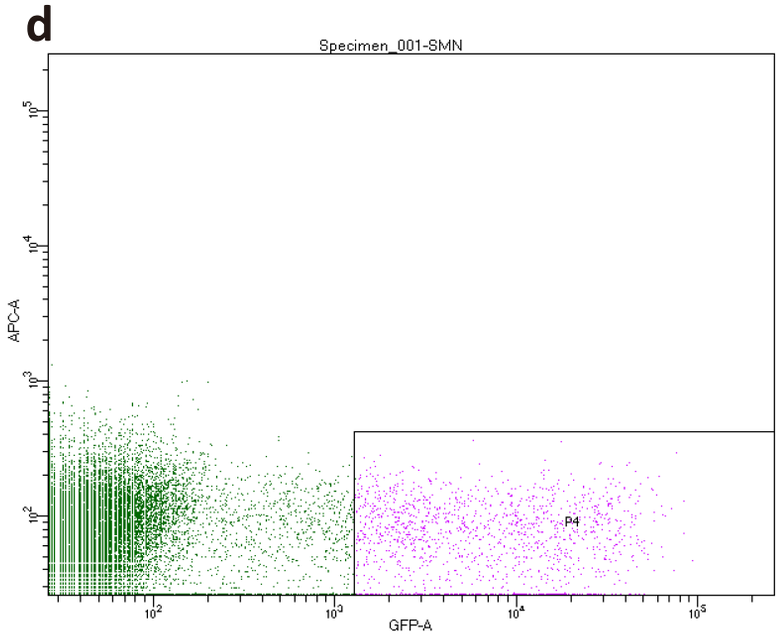

Figure 4

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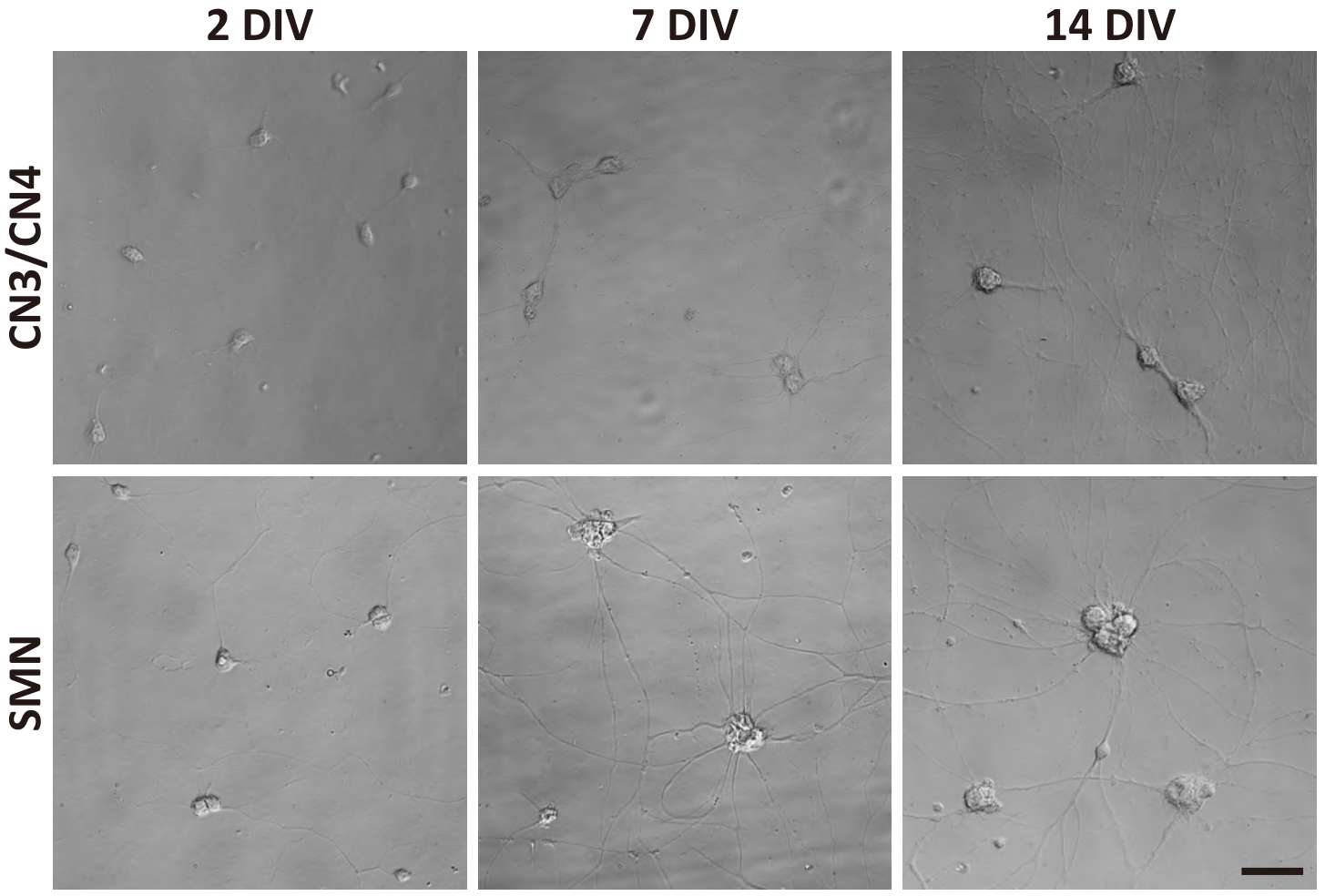
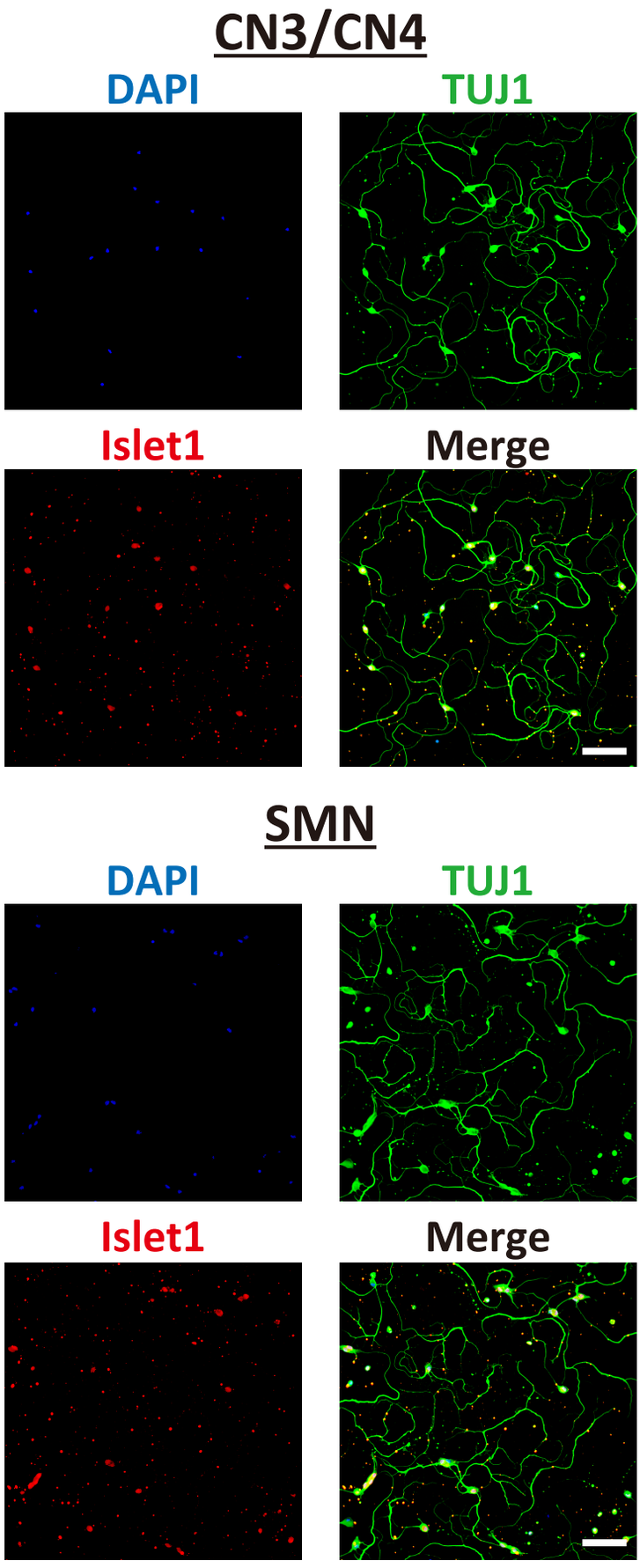


Click here to access/download;Figure;Figure 5.ai

Figure 5

A



B

Purity of Cultures (%)	
CN3/CN4	SMN
93.5 ± 2.2	86.7 ± 4.7

Click here to access/download;Figure;Figure 6.ai

Figure 6

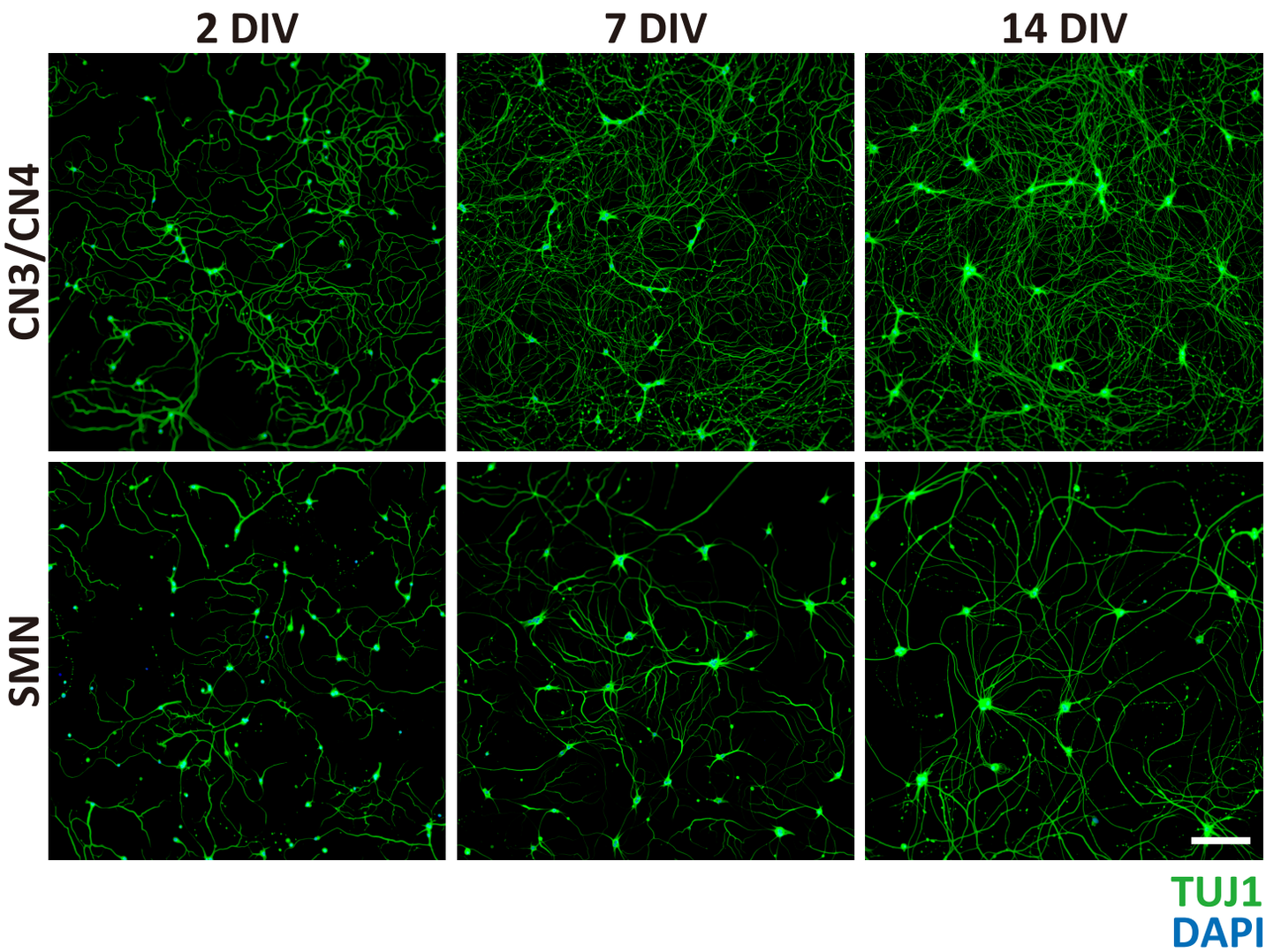


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Figure 7

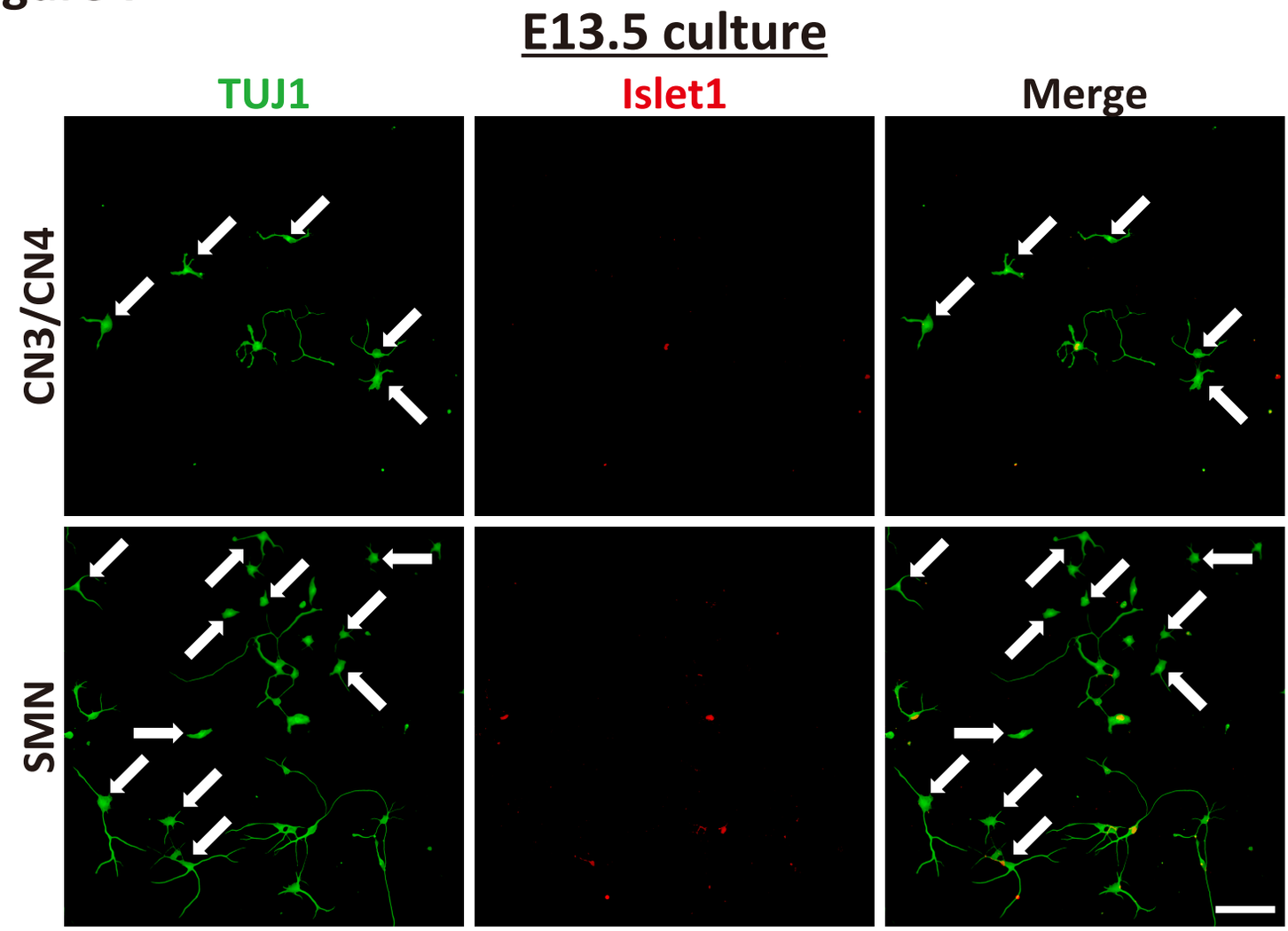

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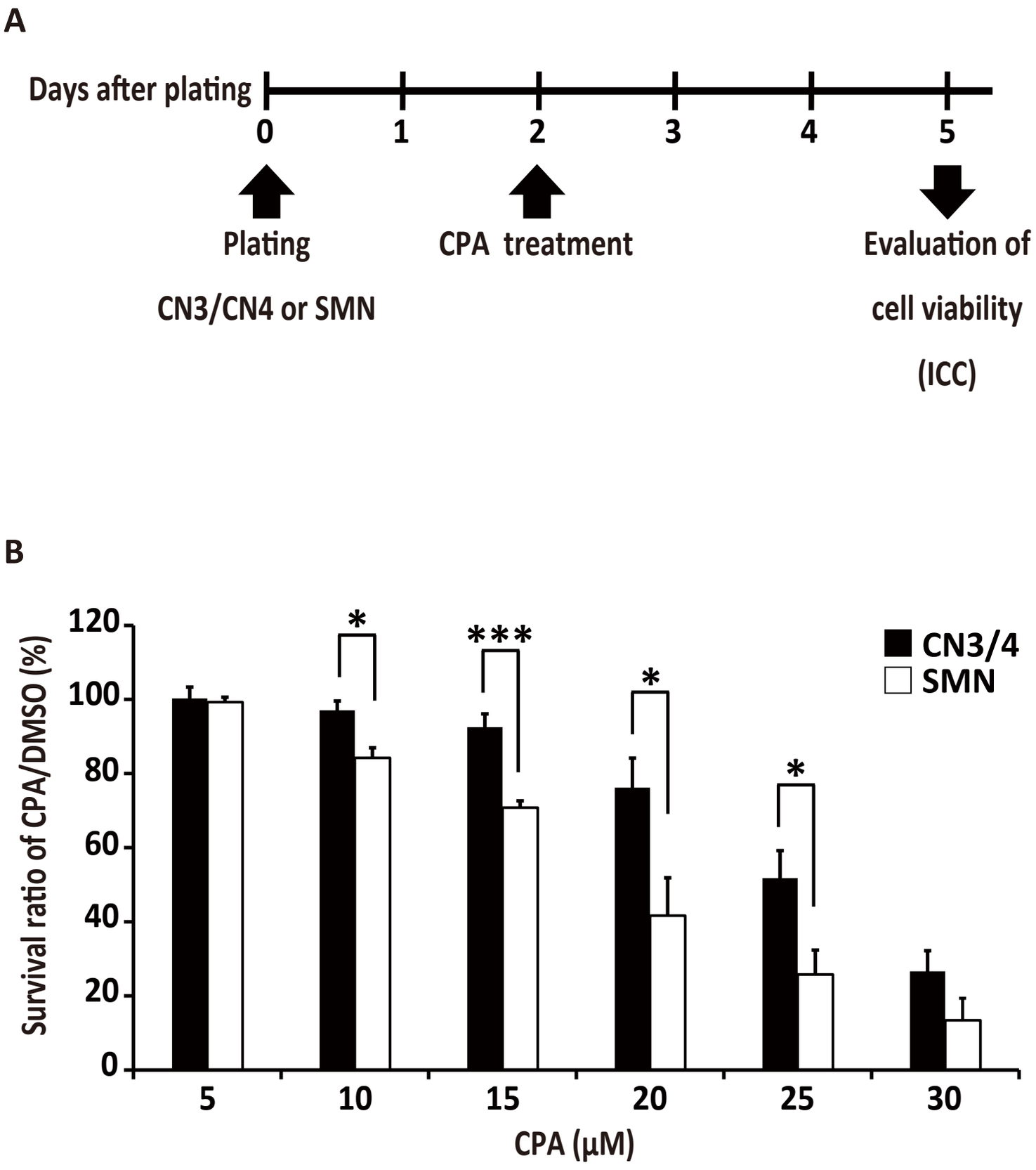
Figure 8



Click here to access/download;Figure;Figure 9.ai

Figure 9

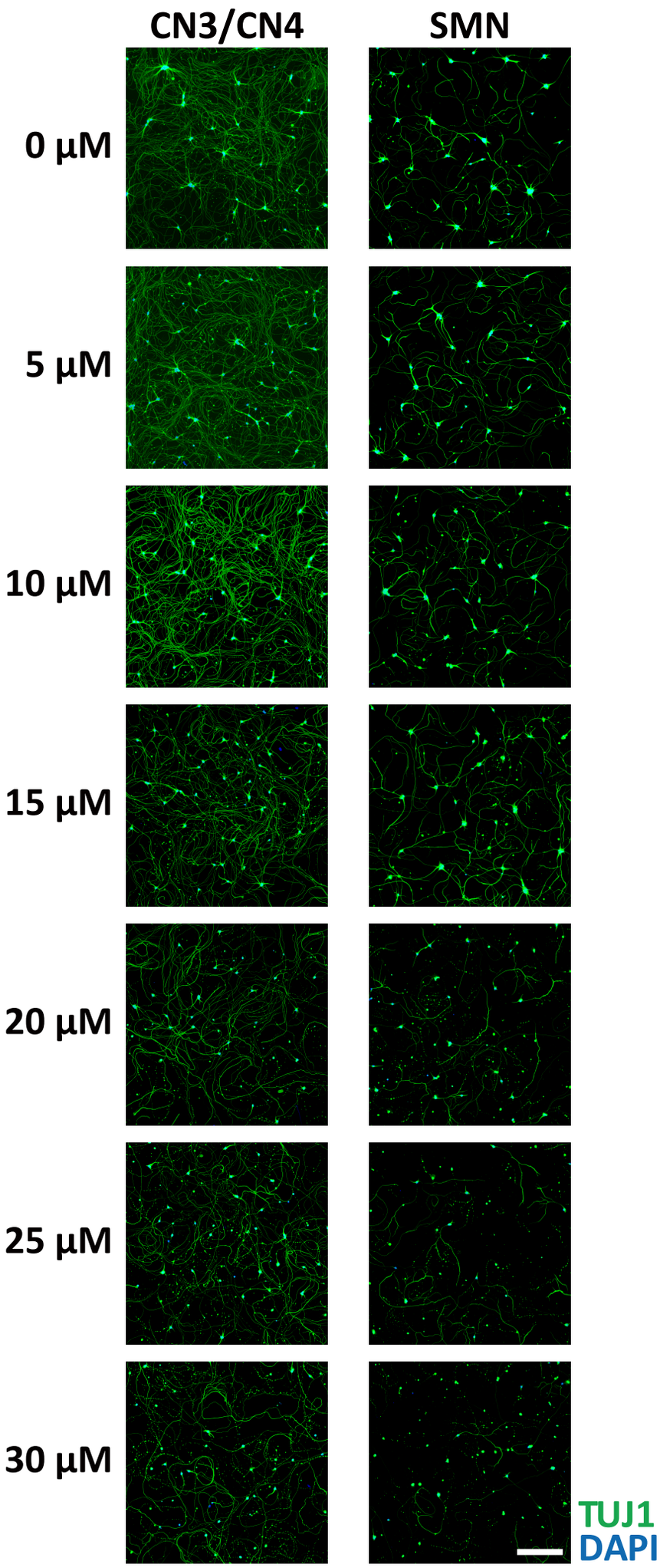


Table 1

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Number of midbrains (X)	Papain	Albumin-ovomucoid	Hibernate E
$10 \leqq X \leqq 20$	200 µl	100 µl	600 µl
$20 < X \leqq 30$	300 µl	150 µl	700 µl
$30 < X \leqq 40$	400 µl	200 µl	800 µl
Number of spinal cords (Y)	**Papain**	**Albumin-ovomucoid**	**Hibernate E**
$3 \leqq Y \leqq 5$	200 µl	100 µl	500 µl
$5 < Y \leqq 10$	400 µl	200 µl	800 µl
$10 < Y \leqq 15$	600 µl	300 µl	1200 µl

Click here to access/download;Table of Materials;JoVE_Materials_RF edit (alphabetical order).xls

Name of Material/ Equipment	Company	Catalog Number
Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L)	Thermo Fisher Scientific	A-11001
Alexa Fluor 594-conjugated F(ab')2 goat anti-rabbit IgG (H+L)	Thermo Fisher Scientific	A-11072
B27 Supplement (50X), serum free	Thermo Fisher Scientific	17504-044
BD FACSAria llu SORP Flow Cytometer	BD Bioscience	-
BD Falcon 70µm Nylon Cell Strainers	CORNING	352350
BD Falcon Round Bottom Test Tubes With Snap Cap	CORNING	352054
BDNF Human	ProSpec-Tany TechnoGene, Ltd.	CYT-207
Cell Culture microplate, 96 well, PS, F-bottom (Chimney Well)	Greiner Bio-One International	655090
Circular Cover Glasses for microscopy	Karl Hecht & Assistent	1001/14
CNTF Human	ProSpec-Tany TechnoGene, Ltd.	CYT-272
Cyclopiazonic acid from *Penicillium cyclopium*	Sigma-Aldrich	C1530
4',6-diamidino-2-phenylinodole (DAPI)	Thermo Fisher Scientific	D1306
Dimethyl sulfoxide	Sigma-Aldrich	D2650
Dumont #5 Forceps Inox Tip Size .05 x .01 mm Biologie Tips	Roboz Surgical Instrument	RS-5015
Forskolin	Thermo Fisher Scientific	BP25205
GDNF Human	ProSpec-Tany TechnoGene, Ltd.	CYT-305
GlutaMAX supplement	Thermo Fisher Scientific	35050-061
Hanks' Balanced Salt Solution (HBSS)	Thermo Fisher Scientific	14175-095
Hibernate E	BrainBits	HE
Hibernate E low fluorescence	BrainBits	HELF
Horse serum, heat inactivated, New Zealand origin	Thermo Fisher Scientific	26050-070
IBMX	Tocris Cookson	2845
Laminin	Thermo Fisher Scientific	23017-015
Leibovitz's L15 medium	Thermo Fisher Scientific	11415064
2-Mercaptoethanol	Sigma-Aldrich	M6250
Micro Dissecting Scissors	Roboz Surgical Instrument	RS-5913

Micro Knife 4.75" 1.7 x 27 mm blade	Roboz Surgical Instrument	RS-6272
Moria Mini Perforated Spoon	Fine Science Tools	10370-19
mouse monoclonal antibody to neuronal class III β-tubulin (TUBB3)	BioLegend	801202
Nikon Perfect Focus Eclipse Ti live cell fluorescence microscope and Elements software	Nikon	-
Nitric Acid 90%, Fuming (Certified ACS)	Fisher Scientific	A202-212
Olympus 1.7ml Microtubes, Clear	Genesee Scientific	22-281
Papain Dissociation System	Worthington Biochemical Corp	LK003150
Penicillin-streptomycin (10,000 U/ml)	Thermo Fisher Scientific	15140-122
Phosphate buffered saline (PBS)	Thermo Fisher Scientific	10010-023
Poly D-lysin (PDL)	MilliporeSigma	A-003-E
rabbit monoclonal antibody to Islet1	Abcam	ab109517
SMZ18 and SMZ1500 zoom stereomicroscopes with DS-Ri1 camera	Nikon	-
Sylgard 170 Black Silicone Encapsulant - A+B 0.9 Kg kit	Dow Corning	1696157
TC treated Dishes, 100 x 20 mm	Genesee Scientific	25-202
Thum Dressing Forceps 4.5" Serrated 2.2 mm Tip Width	Roboz Surgical Instrument	RS-8100
Transducer for LOGOQ e VET	GE Healthcare	L8-18i-RS
Veterinary ultrasound machine	GE Healthcare	LOGOQ e VET
Zeiss LSM 700 series laser scanning confocal microscope and Zen Software	Carl Zeiss	-

Comments/Description
1:400
1:400
This has 4 laser system equipped with 405, 488, 594, and 640 nm lasers. For filtering the dissociating cells before FACS.
We tried multiple 96-well dishes and this was the best one for culture and analyses after ICC We used thie coverslip since the area was large (diamater: 14 mm).
CPA. One of ER stressors.
DMSO
Fluorescence which hinders observation of embryo's GFP expressions should be low.
Isobutylmethylxanthine

1:500, TUJ1

Differential interference contrast images and immunocytochemistry images of the cell cultures were captured with these equipmer
For rinsing coverslips
These are the tubes that we discribed "1.7 mL microcentrifuge tubes" in the context.

Papain solution and alubumin-ovomucoid inhibitor solution are prepared from this kit.

1:200

Dissection was performed and images of dissected embryos and tissues are captured under these fluorescence microscopes.
We make dissection dishes using this kit.
We make dissection dishes using this dish.

For ultrasound on female mice

For ultrasound on female mice

Confocal image of the embryo was captured with these equipments

ıts

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Title of Article: Isolation and culture of oculomotor, trochlear, and spinal motor neurons from prenatal IslMN:GFP transgenic mice

Author(s): Ryosuke Fujiki, Joun Y. Lee, Julie A. Jurgens, Mary C. Whitman, and Elizabeth C. Engle

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Name:	Elizabeth Engle
Department:	Neurology
Institution:	Boston Children's Hospital
Article Title:	Isolation and culture of oculomotor, trochlear, and spinal motor neurons from prenatal IslMN:GFP transgenic mice
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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have proofread the manuscript.

2. Please provide an email address for each author.

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Joun Y. Lee: joun.lee92@gmail.com
Julie A. Jurgens: Julie.Jurgens@childrens.harvard.edu
Mary C. Whitman: Mary.Whitman@childrens.harvard.edu
Elizabeth C. Engle: Elizabeth.Engle@childrens.harvard.edu

These have been added to the title page.

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

We have rephrased the short abstract, which is now 40 words in total. It now reads:

Here, we present a protocol to yield homogeneous cell cultures of primary oculomotor, trochlear, and spinal motor neurons. These cultures can be used for comparative analyses of the morphological, cellular, molecular, and electrophysiological characteristics of ocular and spinal motor neurons.

4. Please ensure that the Introduction contains all of the following with citations:

a) A clear statement of the overall goal of this method

b) The rationale behind the development and/or use of this technique

c) The advantages over alternative techniques with applicable references to previous studies

d) A description of the context of the technique in the wider body of literature

e) Information to help readers to determine whether the method is appropriate for their application

We have confirmed.

5. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Greiner Bio-One #655090 96-well microplates, Hibernate E, GlutaMAX, Eppendorf tube, GFP-positive 317 SMN column, BD FACSAria IIu 4 Laser system, Zeiss LSM 700 series laser 546 scanning confocal microscope, Zen Software (Carl Zeiss), Nikon Perfect Focus Eclipse Ti live cell fluorescence 616 microscope using Elements software (Nikon), etc.

We have changed the names of most commercial items in the manuscript, including Eppendorf tube, BD FACSAria IIu 4 Laser system, Zeiss LSM 700 series laser scanning confocal microscope, Zen Software (Carl Zeiss), Nikon Perfect Focus Eclipse Ti live cell fluorescence microscope, and Elements software (Nikon). We could not identify any items called "GFP-positive 317 SMN column" in the manuscript. Although substituting non-commercial aliases was reasonable for most items, we believe a few should remain as originally named in order to avoid confusion. For instance, Hibernate E is the specific type of medium used in the protocol, so identifying it as "medium" would not be suitably informative. Similarly, GlutaMAX is similar but not identical to L-glutamine, so we would not want readers to generate results inconsistent with ours by using regular L-glutamine.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have addressed this comment in the manuscript. In certain instances, sentences were maintained as non-imperative statements, since they represent optional but not mandatory outcomes (e.g. plates can be prepared in advance, but advance preparation is not necessary).

7. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Two notes cannot follow one action step.

We have removed unnecessary notes as requested.

8. Please revise the protocol section to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

We have modified the manuscript to remove personal pronouns.

9. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

To respond to this comment, we have moved 6-Note2, 7-CAUTION1and2, 8-Note into the discussion.

10. Please ensure you answer the “how” question, i.e., how is the step performed?

We have ensured the “how” question is addressed to the best of our abilities.

11. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

We have modified the protocol to reduce the number of actions per step.

12. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable

content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have confirmed that whole protocol is less than 10 pages and the highlighted part in yellow is less than 2.75 pages.

13. Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.

We believe we have discussed all of the result figures in the representative results.

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The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We have updated the legend of Figure 8 to incorporate this suggestion.

15. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the

Figure Legends, but rather the Protocol. In this case, please shorten the figure 2 legend.

We did our best to shorten the Figure 2 legend, but kept a few key portions of the methodology, since this figure is crucial for visualizing the dissection method.

16. Figure3: This is a table instead. Please convert to .xlsx file. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xls file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

We have changed Figure 3 to Table 1 and followed the instruction above.

17. Please do not abbreviate the journal titles in the references section.

We have unabbreviated the journal titles of all of our references.

18. Please sort the materials table in alphabetical order.

We have sorted the material table in alphabetical order.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This is a really important technical achievement, which I believe will be used by many in the field. The manuscript is well written, and it is written so that the method is extremely clear. I have not reviewed a manuscript this excellent in quite a while. I find this innovation very exciting, and I look forward to all the scientific questions we can now answer in a much more definitive manner.

I have no concerns. Great work!

We appreciate reviewer 1's comments.

Reviewer #2:

Manuscript Summary:

This manuscript outlines a protocol to extract, purify and culture oculomotor/trochlear neurons in parallel with spinal motoneurons extracted from Isl1::GFP transgenic mice.

As a novice to cell culture, I found the protocol nicely detailed and I felt that I could follow most of the steps required. Of course, the dissection part it the most critical and I had some troubles following the exact procedure just based on the text, but that is precisely the added value of the video, that I'm looking forward to watching.

Major Concerns:

None

Minor Concerns:

I only have some minor suggestions that could improve the readability of the text, especially for a novice like me.

p4. How critical is the use of an ultrasound machine? A discussion of this point could be useful to help other investigators decide whether the purchase of this equipment is necessary to test this protocol.

We thank reviewer 2 for these suggestions. In response to the question about ultrasound usage, sometimes female mice gain weight without getting pregnant, especially when they are old or very young and still growing. To reduce unnecessary sacrifice of female mice that are not pregnant, ultrasound is preferred but not crucial.

We revised this section of the manuscript to clarify as follows:

1.3. Weigh female mice and examine for pups using ultrasound (see Table of Materials) between E8.5-11. In addition to recognition of vaginal plugs, successful mating can be confirmed by detection of weight gain in female mice (usually >1.5g on E9.5, if there are more than 5-6 embryos) and visual confirmation of embryos under ultrasound. Embryos are easily detectable by ultrasound after E9.5. Females that gain weight as described above are more often pregnant than those that do not gain weight, so ultrasounds are conducted only on females that have gained weight.

However, female mice can gain weight for reasons other than pregnancy, so weight gain alone is not a reliable indicator of pregnancy. Ultrasound confirmation prevents unnecessary sacrifice of females that are not pregnant, but is not crucial if unavailable.

Step 4.2.3 what is the volume of DMSO vs water needed to prepare the aliquots of forskolin? I checked thermofisher's website but could not find the "commercial recommendations".

Your concern was right. We were making the forskolin solution by referring to the instruction sheet from Thermo Fisher Scientific, which was accessible only upon specific request from the company. Thank you so much for catching this error. We have now added details for preparation of forskolin solution in Step 4.2.3. It now reads:

Add DMSO (64 µl) to 5 mg (1.0670 µmol) of forskolin and vortex well to dissolve it completely. Then add sterilized water (1.003 ml) to the DMSO solution and vortex well. This will give a 10 mM forskolin solution. Store 12 µl aliquots of 10 mM forskolin at -20 °C and thaw aliquots at RT immediately before use.

p7 step 5 Note 1. I don't understand this sentence. Are extremities MNs the same as spinal MNs? And are they GFP-negative?

We have rearranged this sentence to improve clarity. It now reads:

5.2.9. Collect facial motor neurons and extremities of *Isl^{MN}:GFP* mouse embryos as GFP-positive and -negative controls for fluorescence-activated cell sorting (FACS), respectively. Extremities are GFP-negative because the GFP-positive axons of the spinal motor neurons have not yet extended into the extremities at this embryonic age.

p8 step 5.2.1. From my understanding, the 4th ventricle has been opened rostrally to dissect the midbrain. At this step, shouldn't it be the "unopened *caudal* part of the 4th ventricle" that need to be opened?

This is correct, thank you for catching this error. We have changed the wording from "rostral" to "caudal."

p9 Step 7 Note 3. I found the last sentence of this note very confusing. I initially understood that half the volume of papain was to be used for the dissociation and the second half to be added to the inhibitor solution. I finally understood that the authors meant that the volume of inhibitor is always half the volume of papain used, but that's apparent from the table and its legend, therefore I believe that last sentence of Note 3 could be omitted entirely.

We have completely omitted Note 3.

p10 Step 7.5 Note. The wording of this note is confusing. I initially thought that the authors suggested that thus step could be reduced (in duration?) if papain is left in the tube.

To make the whole sentence easier to understand, we have changed the sentence as follows:

To ensure the efficacy of ovomucoid inhibition in the next step, use a P1000 pipette to remove and discard as much supernatant as possible without aspirating the tissues.

p11 Step 8.4. where does the APC signal comes from?

The APC channel is used to detect autofluorescence. Autofluorescent cells excite both the 633 and GFP channels, and are excluded. We have changed the text to read:

7.4. Gate P3 cells based on GFP versus allophycocyanin (APC). The APC channel detects auto-fluorescence and gating on this channel avoids capturing auto-fluorescent cells. Use GFP-negative cells to adjust the voltage for FITC/GFP fluorescent channels. Ideally, position gates for these cell populations around 10^2. Select gate thresholds for GFP-positive population 4 (P4) individually for each type of motor neuron (Figure 3Ad and Bd).

p11 step 8.4 Note. Why is the GFP gate for SMNs need to be much higher than for CN3s/CN4s? Although the authors discuss the effect of setting that gate and the resulting purity of the culture, it could be discussed why the gate must be different for both populations of MNs, and what kind of GFP-positive cells are contaminating the culture if the gate is set incorrectly.

We have added the following sentence:

A lower GFP gate for SMN cultures leads to contamination of the cultures by glia and non-motor neurons. This is likely because there is low-level GFP expression in some glia and non-motor neurons due to a leaky promoter.

Reviewer #3:

Manuscript Summary:

This is a nicely written description of the isolation and culture of oculomotor, trochlear and spinal motor neuron isolation and culture. The authors acknowledge the limitations of this approach. Overall, I think this report is a contribution to the field and offer "minor" comments for improvement of the report.

Minor Concerns:

1. In the introduction, a sentence or two to explain "why" isolation and culture of CN3 and CN4 motor neurons has been a challenge would provide a bit more background.

We have added the following sentence:

These protocols have not been successful for generating CN3/CN4 cultures because CN3s/CN4s do not express $p75^{NTR}$, are smaller than SMNs and therefore more difficult to isolate based on size, and other specific CN3/CN4 markers have not been identified.

2. Also in the introduction, describe the Islmn:GFP mouse for the non-motor neuron reader. A sentence or two is all that is needed here.

We have added the following sentence:

***Isl^{MN}:GFP* specifically labels motor neurons with a farnesylated GFP that localizes to the cell membrane.**

3. in Step 3.6- note that laminin should not dry out. In my experience, if the laminin dries out, the cultures are not successful. Add a comment that if the plates are to be stored for more than a few hours, they should be wrapped in parafilm.

We have added the following sentence:

Laminin should not be allowed to dry out. If the plates are to be stored for more than several hours, they should be wrapped in parafilm.

4. In the last paragraph of the Discussion, the authors address the limitations of the protocol. The second limitation is noted as the small age window. A few sentences to expand here that the real limitation is that it is difficult to confirm that appropriately aged embryos are present this early. The use of the ultrasound to confirm this is wonderful, but not everyone has access to this equipment.

We have added this to the discussion:

(2) The small E10.5-12.5 age window for the embryonic mice, and the difficulty of confirming that appropriately aged embryos are present, especially if an ultrasound machine is not available.

5. In Figure 5, the spinal MNs are clumped. This sometimes occurs when the laminin concentration is too low- or perhaps the plates sat too long? A note to this effect would be helpful to those who attempt the protocol. Also, if the authors have a better imaging without the clumping, would be good.

Thank you very much for pointing out this phenomenon. We have found that no matter how sparsely the cells are plated, motor neurons tend to aggregate in long-term cultures, particularly for SMNs. These images are representative.

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