ANATOMIC

Chrono™ Senso-DM
Technical Manual

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1.0 Introduction

Human pluripotent stem cells (hPSCs) have developmental capacity identical to that demonstrated by human embryonic inner cell mass cells. This means an entire adult organism can theoretically be produced using these cells, and any cell type by extension. Using hPSCs to produce cell types of the nervous system (such as neurons and glia) is especially attractive, as these cell types are difficult to acquire without damaging the organism.

The nervous system is exquisitely complex. An estimated 10,000 different neuronal subtypes originate within highly specific anatomical locations during development, and may or may not migrate to other anatomical locations later in development. Manufacture of specific neuron subtypes from hPSCs has proven to be a substantial technical challenge given this eventual complexity, but even the primary fate decision required to enter the ectodermal germ layer is a substantial barrier.

From the beginnings of hPSC culture and ectodermal differentiation, formation of ectoderm has required at least one week in vitro. This is only the first step in development with many more to occur, resulting in protracted 30 to 60-day protocols for producing neurons and glia. To put the difficulties working with ectoderm into context, terminally differentiated cardiomyocytes can be produced in seven days. An entire cardiomyocyte differentiation protocol can be executed in the same amount of time that is required for the first step in producing any particular neuron. This places neuroscience at a substantial disadvantage when attempting to utilize hPSC platforms, which are essential for understanding human neuroscience.

The Chrono™ Platform levels the germ layer playing field. For the first time, ectoderm can be produced from hPSCs efficiently within 24 hours, which grants immediate access to understanding the complexity of the nervous system. Chrono™ Senso-DM builds upon these findings to produce a highly pure population of immature sensory neurons within only 7 days. Chrono™ Senso-DM is useful for pain modeling or regenerative peripheral nerve grafting.

2.0 Related Products

The Chrono™ Platform by Anatomic provides an integrated ecosystem of products meant to streamline your research and production experiences. We provide a portfolio of sensory neuron products that encompass a broad range of use-cases. Whether you want to study early or intermediate developmental events, or are interested in the terminal cell type produced, the Chrono™ Platform provides a product to fit your specific needs.

Product	Cat #	Use
Chrono™ Senso-MM	1030	For the rapid maturation of sensory neurons
Chrono™ Sensory Neurons	1020	Cryopreserved neurons for downstream assays

Check our website frequently for an updated list of new and exciting products: www.anatomic.tech

3.0 Materials Required for Rapid Production of Sensory Neurons from hPSCs

Chrono™ Senso-DM is very simple to use and requires few materials and standard equipment to ensure success.

3.1 Product Contents and Storage

Component	Temperature	Packaging	Total Volume	Stability
Chrono™ Matrix 1	-20°C	1 mL Tube	25 µL	Until labeled expiry
Basecamp	4°C	60 mL PET Bottle	45 mL	Until labeled expiry
Senso DM1	-20°C	1 mL Tube	500 µL	Until labeled expiry
Senso DM2	-20°C	1 mL Tube	500 µL	Until labeled expiry
Senso DM3	-20°C	1 mL Tube	500 µL	Until labeled expiry
Senso DM4	-20°C	1 mL Tube	500 µL	Until labeled expiry
Senso DM5	-20°C	1 mL Tube	500 µL	Until labeled expiry
Senso DM6	-20°C	1 mL Tube	500 µL	Until labeled expiry
Senso DM7	-20°C	1 mL Tube	500 µL	Until labeled expiry

3.2 Materials Required but not Included

Product Name	Supplier	Cat #	Use
dPBS with Calcium and Magnesium	Corning™	21-030-CM	Coating Buffer
Y-27632	StemCell Technologies	72304	Day -1 Plating
Versene Solution	ThermoFisher	15040066	Day -1 Dissociation
DMEM F-12	ThermoFisher	11330107	Day -1 Dissociation
ACCUMAX™	Innovative Cell Technologies	AT105	Day 7 Dissociation
dPBS without Calcium and Magnesium	Corning™	21031CV	Day 7 Dissociation
40 µm cell strainer	Your preferred supplier	-	Day 7 Dissociation
15 & 50 mL conicals	Your preferred supplier	-	Day 7 Dissociation
CryoStor© CS10	BioLife Solutions	210102	Cryopreservation
Cryovials	Your preferred supplier	-	Cryopreservation
Hemocytometer	Your preferred supplier	-	Cryopreservation
Trypan Blue	Your preferred supplier	-	Cryopreservation

3.3 Required Equipment

- Biosafety cabinet certified for handling of biological materials
- 37°C, 5% CO2, 95% humidity incubator
- Pipette-aid with appropriate serological pipettes
- Inverted microscope
- -20°C freezer
- Refrigerator (2 - 8°C)
- Controlled rate freezer

4.0 Important Parameters for Successful Use of Chrono™ Senso-DM

Excellence, Defined. Chrono™ Senso-DM is currently the most modern system available for production of sensory neurons. Chrono™ Senso-DM utilizes serum-free, chemically defined media, and has been validated for use downstream from multiple defined systems for the undifferentiated culture of hPSCs. Chrono™ Senso-DM has not been validated for use on hPSCs grown in serum replacement-containing medium on human or murine feeder layers, or feeder-free using serum-replacement medium conditioned by feeders. The benefits of Chrono™ Senso-DM are best enjoyed looking toward the future, rather than living in the past.

Density is Critical. While use of Chrono™ Senso-DM is a robust process, every technology has its limits. Under-seeding of hPSCs before exposure to Chrono™ Senso-DM can result in substantial loss of viability, while over-seeding can reduce control of downstream differentiation processes. Best results are usually enjoyed using a seeding density of 15,000 cells per cm^2 (375,000 cells per T25 flask). Once 15,000 cells per cm^2 has been attempted, densities between 10,000 and 20,000 cells per cm^2 can be attempted to maximize yield and efficiency based on your hiPSC line's intrinsic growth rate. To ensure success, view Section 6.0: Production of Sensory Neurons Using Chrono™ Senso-DM for a guide to appropriate day-one density before addition of Chrono™ Senso-DM.

Volume Is Critical. While this will be clearly stated in the instructions, it's worth highlighting in its own section. Chrono™ Senso DM1 should be applied to hPSC cultures on day-one using 0.2 mL/cm^2. Exceeding this volume on day-one could result in a substantial loss in cell viability.

Apart from the previously stated caveats, Chrono™ Senso-DM is actually very easy to use. You should be ready to go!

5.0 Preparation of Reagents and Media

5.1 Coating with Chrono™ Matrix 1

5.1.1. Thaw Chrono™ Matrix 1 at room temperature
5.1.2. Dilute 25 uL of Chrono Matrix 1 into 2.5 mL dPBS with Calcium and Magnesium (1:100 dilution)
5.1.3. Add 2.5 mL of diluted Chrono™ Matrix to a T25 tissue culture-treated flask
5.1.4. Swirl the vessel to evenly spread the solution across the surface
5.1.5. Incubate the vessel overnight at 4°C or at least three hours at 37°C

!!!CRITICAL!!!: Do not let vessels dry out during storage and when aspirating matrix prior to cell seeding.

NOTE: Vessels can also be wrapped with parafilm and stored at 4°C overnight and up to two weeks before use

5.2 Preparation of Chrono™ Senso-DM

Compounding differentiation medium is a basal + supplement affair. For each day of differentiation, 0.5 mL of Senso DM(x) supplement will be added to 4.5 mL of Basecamp to create 5 mL of complete differentiation medium that can then be fed immediately to cultures or stored short-term in the refrigerator prior to feeding.

5.2.1. Thaw Chrono™ Senso-DM for the appropriate day at room temperature
5.2.2. Aliquot 4.5 mL Basecamp into a 15 mL conical
5.2.3. Combine the 500 uL Senso DM(x) supplement with the 4.5 mL Basecamp aliquot

Note: It is important that all volumes are combined to ensure the appropriate final concentrations are produced. The supplement can be briefly centrifuged to ensure no liquid remains in the cap, and the supplement tube can be rinsed with the combined supplement/basal to collect any residual-high concentration factors.

6.0 Production of Sensory Neurons from hPSCs using Chrono™ Senso-DM

Chrono™ Senso-DM will promote the conversion of hPSCs into sensory neurons within 7 days. Resulting neurons can be characterized by immunolabeling using a standard antibody panel, which includes primary antibodies that are immunoreactive toward sensory neuron specific markers PERIPHERIN, BRN3A, and ISL1, and general neuron marker TUJ1. Follow standard immunocytochemical protocols for verification of appropriate immunolabeling.

The following scheme provides a visual representation of the steps required to promote formation of sensory neurons from hPSCs using Chrono™ Senso-DM. As can be seen, conversion using Chrono™ Senso-DM is relatively simple to execute when compared to legacy methods.

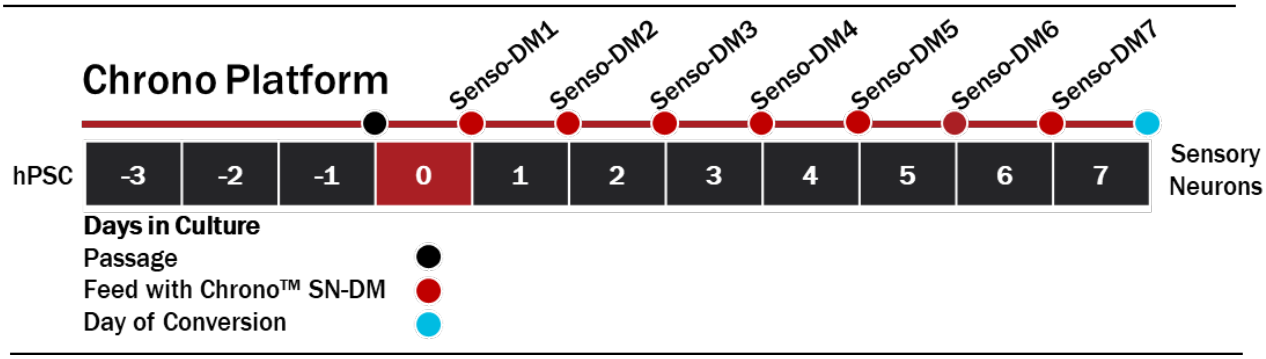


Figure: Schematic for production of sensory neurons using Chrono™ Senso-DM.

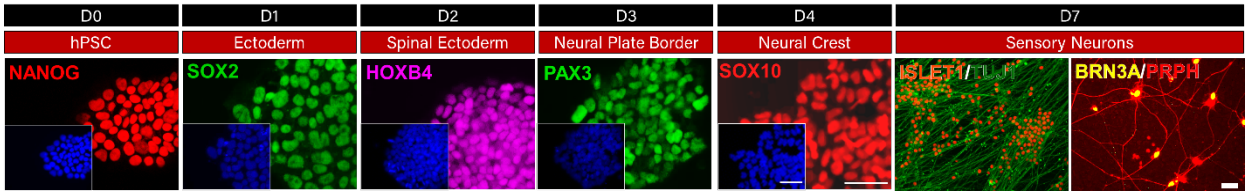
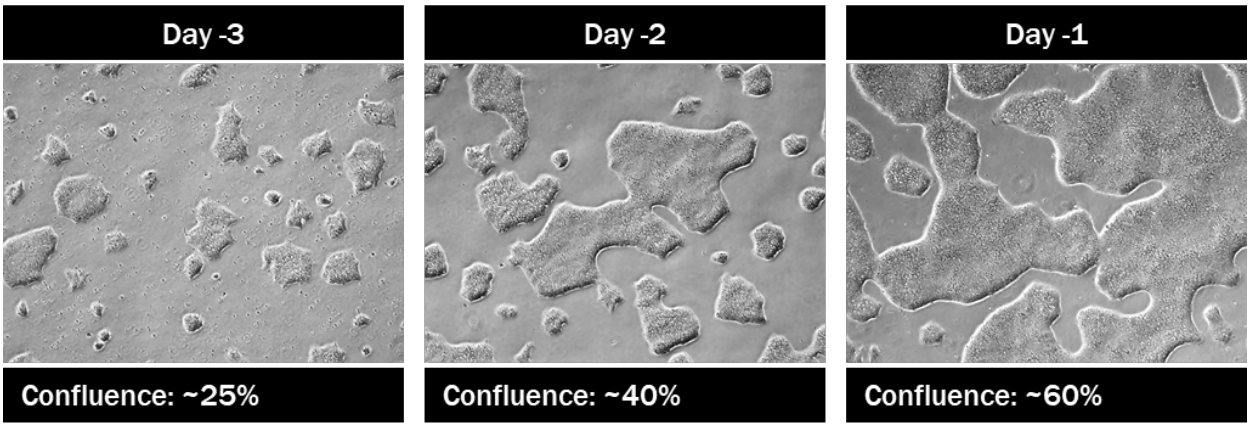


Figure: Immunocytochemistry panel characterizing sensory neurons days 0-7.

Days -4 to -1: Maintaining undifferentiated cultures in defined media.

Chrono™ Senso-DM is best used for hPSC cultures maintained under defined conditions. Day -3 represents the first day following passage when cultures are at their lowest confluence. Cultures should be fed as recommended, usually every day to maintain cultures in good condition. Confluence on Day -3 should be roughly 20%, and confluence should increase to roughly 60% by day -1 when cultures will be passaged. The following figure represents the usual confluence and growth rate from days -3 to -1.



Day -1: Seeding undifferentiated hPSC colonies for conversion with Chrono™ Senso-DM

By day -1, your hPSC cultures should have achieved roughly 60% confluence and are ready to passage for the purposes of differentiation. You will have already Chrono™ Matrix 1 coated T-25 flask as described in Section 5.1: Coating with Chrono™ Matrix 1. Now you will single-cell dissociate the culture using the non-enzymatic passaging solution Versene, a solution containing 0.55 mM EDTA.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from hPSC culture vessel
3. Dispense 2.5 mL Versene Solution into the T25 flask
4. Aspirate Versene Solution from culture vessel
5. Dispense 2.5 mL Versene Solution into the T25 flask
6. Aspirate Versene Solution from culture vessel
7. Dispense 2.5 mL Versene Solution into the T25 flask
8. Return culture vessel to incubator and treat for 20 minutes

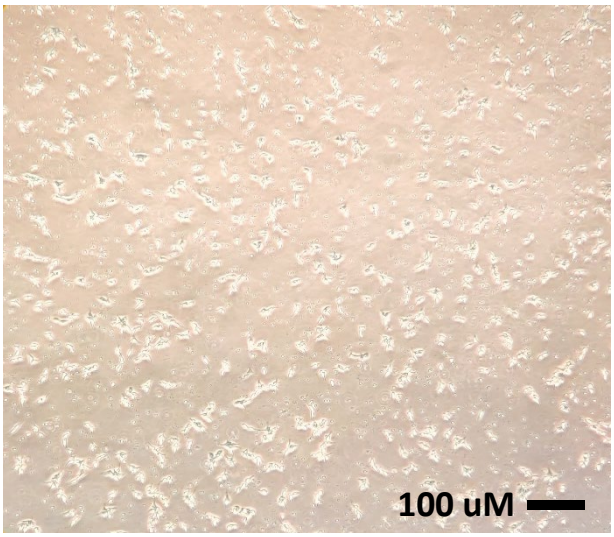
NOTE: Following 20 minutes incubation, cells will have completely dislodged from culture surface, but have not yet singularized.

9. Transfer treated culture to biosafety cabinet
10. Tilt flask vertically to collect dislodged culture into the bottom of the flask
11. With a 5 mL pipet, triturate culture 5 to 10 times to dissociate the cells
12. Collect hPSC culture into sterile 15 mL conical tube

13. Rinse previous culture vessel with 2.5 mL DMEM F-12 medium to collect remaining hPSCs
14. Add this rinse to the hPSC culture in the 15 mL conical tube.
15. Evenly mix the culture to reduce sampling error
16. Sample roughly 25 uL of culture for hemacytometer counting (or appropriate amount necessary given your counting method.)
17. Centrifuge the culture 5 minutes at 300 x G.
18. Observe pellet has formed and carefully aspirate supernatant
19. Resuspend culture to 1 million cells per mL in your hPSC medium of choice
20. Inoculate 5 mL hPSC medium containing 10 uM Y-27632 with 375 uL of cell suspension.
21. Aspirate the Chrono™ Matrix 1 coating from its culture vessel
22. Dispense the roughly 5.4 mL of hPSC culture into the coated flask
23. Return flask to incubator and distribute cells evenly by motioning flask front-to-back and side-to-side

Day 0: Addition of Chrono™ Senso-DM1 for production of ectoderm from hPSCs.

Your culture should be scattered with small spiky hPSC colonies typical of single-cell dissociation with ROCK inhibitors. The figure below depicts a potential culture you may witness.

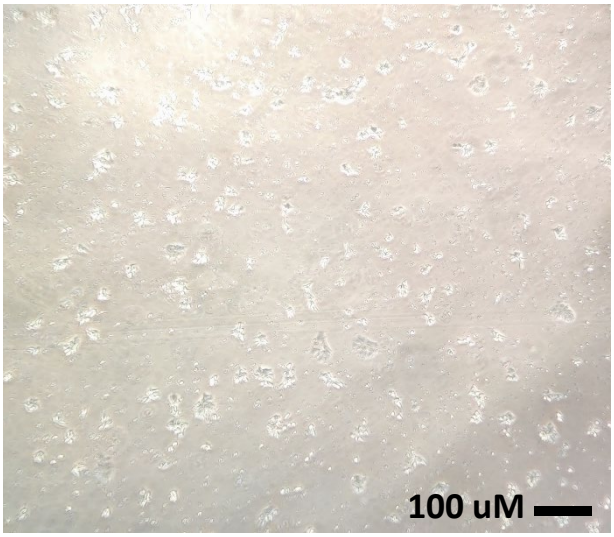


Prepare Chrono™ Senso-DM1 as indicated in Section 5.2: Preparation of Chrono™ Senso-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from hPSC culture vessel
3. Dispense 5 mL Chrono™ Senso-DM1 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 1: Observing ectoderm following conversion

The culture of hPSCs exposed to Chrono™ Senso-DM1 the previous day should have converted to homogenous ectoderm. The culture may appear less dense due some minor cell death, as well as due to the merging of smaller colonies into larger colonies that occupy less surface area in aggregate.

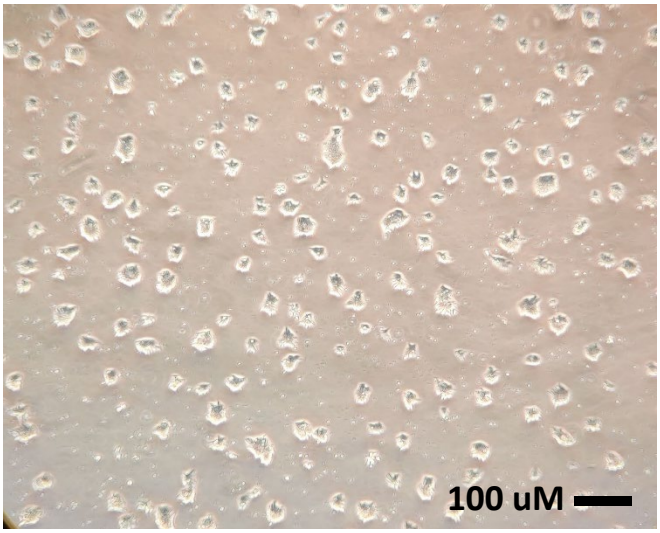


Prepare Chrono™ Senso-DM2 as indicated in Section 5.2: Preparation of Chrono™ Senso-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 5 mL Chrono™ Senso-DM2 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 2: Observe spinal neural culture

Cultures exposed to Senso-DM2 produce spinal neural populations indicated by immuncytochemical staining with antibodies specific to HOXB4 and SOX2. Colonies become more bright and edges are smoother. The center of colonies become increasingly more dense over the next couple of days.

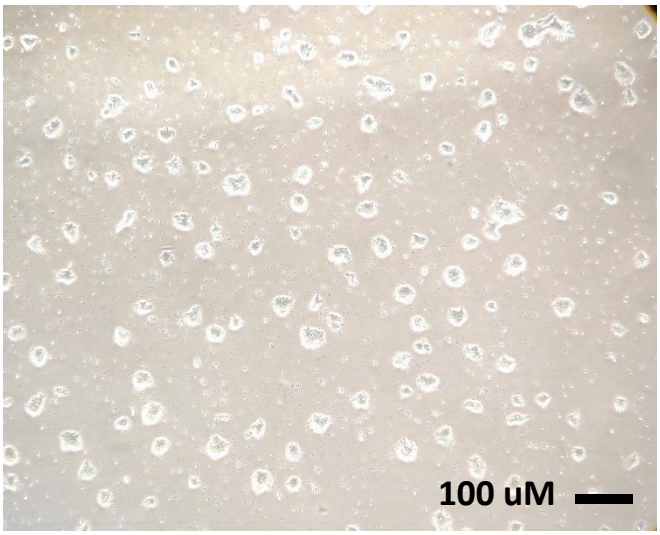


Prepare Chrono™ Senso-DM3 as indicated in Section 5.2: Preparation of Chrono™ Senso-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 5 mL Chrono™ Senso-DM3 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 3: Observe neural plate border culture

Cultures exposed to Senso-DM3 produce a neural plate border population evident with immuncytochemical staining with antibodies specific to PAX3. The edges of colonies should become spikier. This culture is now competent to produce neural crest by applying Senso-DM4.

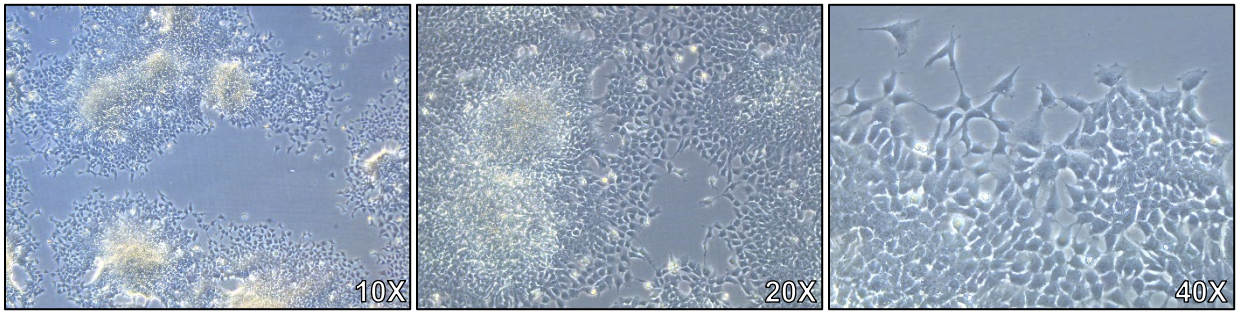


Prepare Chrono™ Senso-DM4 as indicated in Section 5.2: Preparation of Chrono™ Senso-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 5 mL Chrono™ Senso-DM4 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 4: Observe neural crest culture

Cultures exposed to Senso-DM4 initiate commitment into neural crest. This is evident with immuncytochemical staining with antibodies specific to PAX3 and SOX10. Neural crest progenitors begin to extensively migrate in a sheet-like mass from the edges of colonies. Centers of colonies continue to become more three-dimensional and yellow in coloration. This culture now requires users to expand the neural crest by applying Senso-DM5.

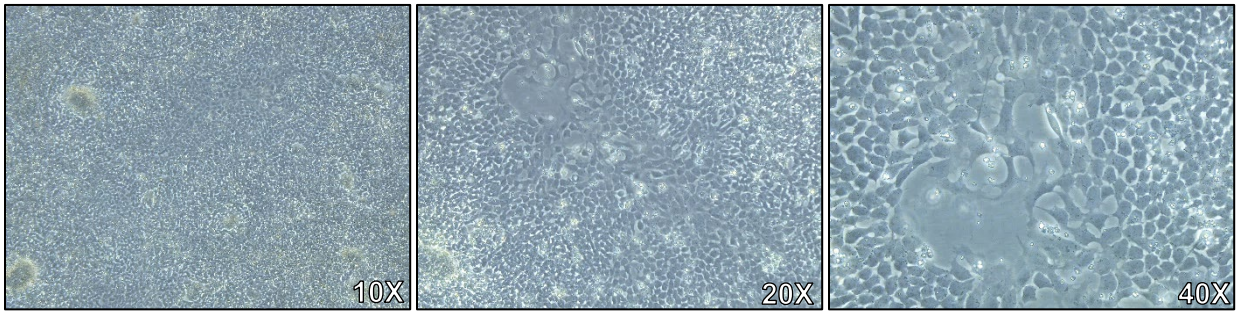


Prepare Chrono™ Senso-DM5 as indicated in Section 5.2: Preparation of Chrono™ Senso-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 5 mL Chrono™ Senso-DM5 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 5: Observe expanded neural crest culture

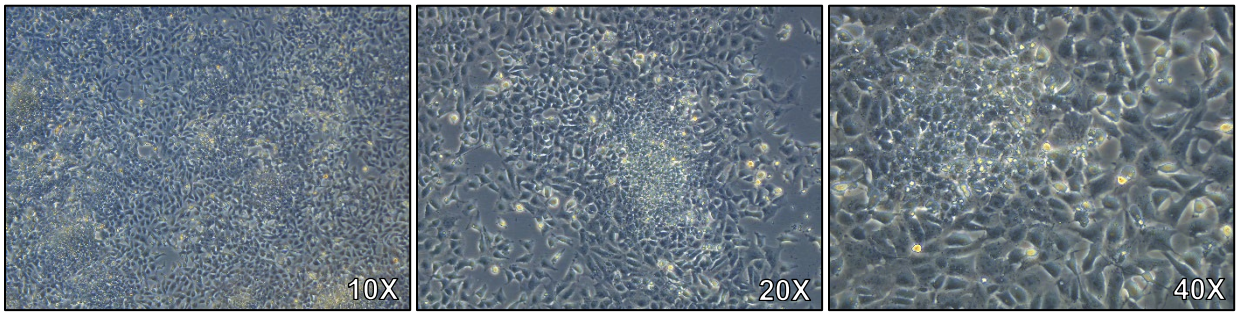
Cultures exposed to Senso-DM5 increase the number of PAX3 and SOX10 expressing neural crest. Day 5 cultures is now competent to produce a population of sensory neuroblasts by applying Senso-DM6.



Prepare Chrono™ Senso-DM6 as indicated in Section 5.2: Preparation of Chrono™ Senso-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 5 mL Chrono™ Senso-DM5 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 6: Observe initial axon extension

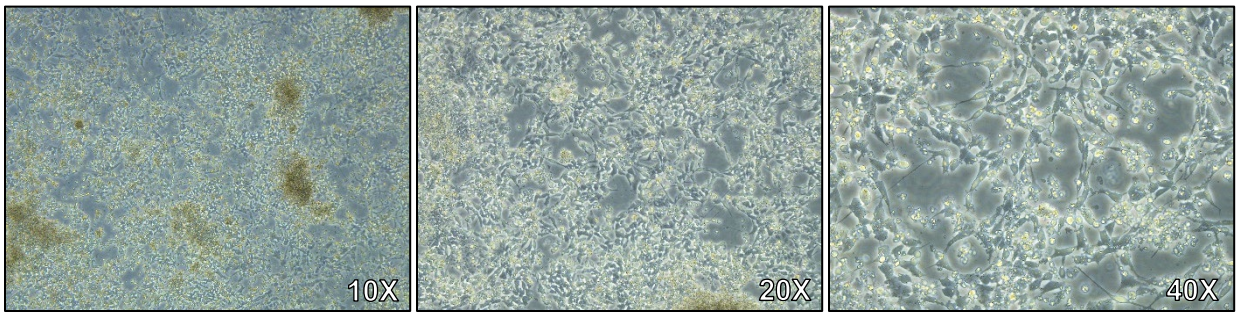


Day 5 cultures exposed to Senso-DM6 initiated commitment into a mix of sensory neuroblasts evident with immuncytochemical staining with antibodies specific to BRN3A, ISL1, PERIPHERIN and TUJ1 on the periphery of colonies. The culture should begin extending axons with phase bright cell bodies. There may also be cell debris associated with enrichment for sensory neuroblasts. The BRN3A/ISL1/PERIPHERIN/TUJ1-positive sensory neurons will further enrich with application of Senso-DM7.

Prepare Chrono™ Senso-DM7 as indicated in Section 5.2: Preparation of Reagents and Media.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 5 mL Chrono™ Senso-DM7 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 7: Differentiation is complete



Day 6 cultures exposed to Senso-DM7 should have fully differentiated into a monolayer of intertwining axons. The percentage of sensory neurons is variable depending on optimized seeding density, but should be no worse than 70%, and can be used for downstream uses.

This is the optimal timing for cryopreservation, as these neurons are immature and dissociate and survive cryopreservation well.

7.0 Dissociation of neurons produced with Chrono™ Senso-DM.

Neurons produced with Chrono Senso-DM are best cryopreserved on Day 7 when neurons are immature. The following procedure can be followed to dissociate these neurons.

1. Pre-warm 5 mL dPBS -/- in a 37°C water bath
2. Carefully aspirate culture medium (some neurons might detach)
3. Add 2.5 mL cold ACCUMAX™ to culture
4. Incubate Room Temp for 3 minutes
5. Tap flask to dislodge neurons
6. Neurons will peel off in sheets leaving behind more adherent contaminants
7. Tip flask so that neural sheets flow into corner
8. Rinse remaining sheets into corner with another 2.5 mL ACCUMAX™
9. Collect this 5 mL Accumax + neural sheets into a 15 mL Conical
10. Cap conical loosely and incubate room temperature for ~1 hour

Note: This incubation time may seem excessive. However, this extended incubation ensures that the neurons will be singularized by enzyme, rather than shear force from vigorous trituration (which drastically reduces neuronal viability). Viability post-treatment and trituration will be greater than 90%.

11. After incubation, pipet culture vigorously to singularize neural sheets. This may require 10 to 20 pipettings to completely dissociate culture.
12. Confirm dissociation progress under microscope

NOTE: Single-cells can be observed in solution when viewing conical over objective lens.

13. Use 5 mL of warmed DMEM F-12 to rinse out 15 mL conical
14. Sample well-mixed culture for a cell count
15. Centrifuge cells 5 minutes at 400 x g
16. Perform a cell count during centrifugation
17. Observe compacted cell pellet
18. Carefully aspirate supernatant by tipping supernatant toward aspirator
19. Smear pellet by flicking conical vigorously, which aids in resuspension
20. Re-suspend pellet into an appropriate volume of cryopreservative according to application

8.0 Cryopreservation of neurons produced using Chrono™ Senso-DM

Neurons produced using Chrono™ Senso-DM can be cryopreserved when differentiation has been completed, 24 hours following application of Senso-DM7. Following step 7.23, after the centrifuged cell pellet has been smeared, ice cold CryoStor® CS10 can be used for resuspension following manufacturer instructions. This suspension can be vialed (>1 million cells per vial) and placed into an isopropanol bath at -80°C for controlled rate freezing before long-term storage in liquid nitrogen. The expected recovery rate (total viable cells post-thaw divided by number of viable cells vialed) ranges from 30 to 60% when using isopropanol containers.

9.0 Maturation of sensory neurons using Chrono™ Senso-MM

Neurons produced using Chrono™ Senso-DM can be rapidly matured using Chrono™ Senso-MM to become electrophysiologically active within one week and express important key ion channels important for function.

Please feel free to reach out to us for more information on Chrono™ Senso-MM and to discuss your research goals with the Chrono™ Senso Products.

10.0 Troubleshooting

Things go wrong. It's understandable. The following are potential issues that may occur when using Chrono™ Senso-DM. This is not an exhaustive list, so please contact us at by email: info@anatomic.tech to report any issues.

PROBLEM	SOLUTION
Everything immediately died 24 hours after using Chrono™ Senso-DM1	• Ensure no more than 0.2 mL/cm^2 of Chrono™ Senso-DM1 was used on Day 0
I don't notice a morphology change on day 1	• Look more closely (20x to 40X magnification)
Cells died mid-differentiation	• Manually extract culture media with serological pipet rather than vacuum aspirate to prevent cultures from drying out
My cultures didn't characterize to be highly positive for sensory neuronal markers	• Ensure use of Senso-DM in proper sequence Senso-DM1→Senso-DM2→Senso-DM3→Senso-DM4→Senso-DM5→Senso-DM6→Senso-DM7 • Ensure cultures display neuronal morphology as undifferentiated neural crest will stain negative for sensory neuron markers.