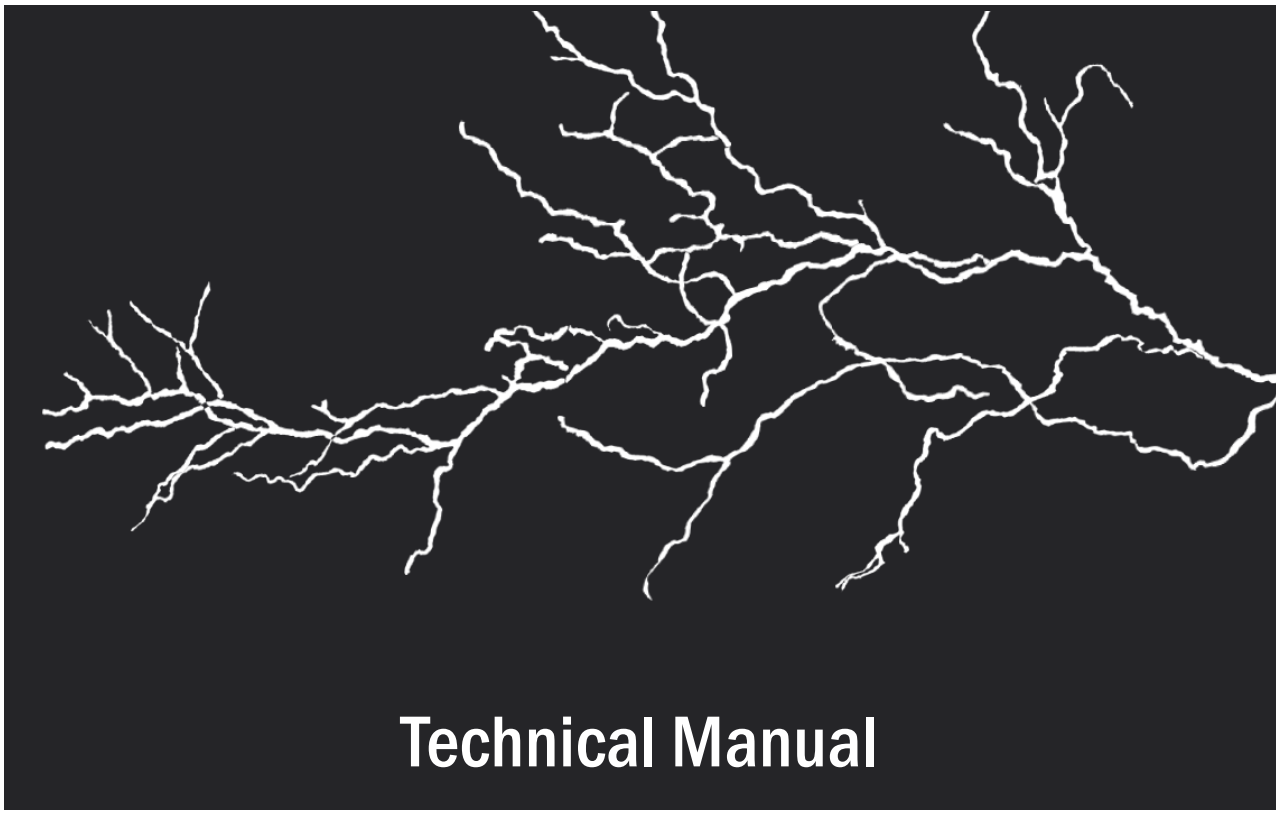

Technical Manual

Rapid Production of Sensory Neurons from Human Pluripotent Stem Cells Using Chrono™ Senso-DM

ANATOMI

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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 fully differentiated, primarily sensory neurons within 8 days. Chrono™ Senso-DM is useful for somato-sensory pain modeling or regenerative peripheral nerve grafting. Table 1 lists additional Chrono™ Platform products that are available to further your research.

2.0 Related Products Coming Soon

The Chrono™ Platform by ANATOMI™ Corp. provides an integrated ecosystem of products meant to streamline your research and production experiences. We are developing a portfolio of culture media formulations 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.

Phenotype	Product
Schwann Cells	Chrono™ Schwann-DM
A9 Midbrain Dopaminergic Neurons	Chrono™ Dope-DM
Striatal GABAergic Neurons	Chrono™ GABA-DM
Cortical Glutamatergic Neurons	Chrono™ Gluta-DM
Serotonergic Neurons	Chrono™ Sero-DM
V0-V3 Spinal Neurons	Chrono™ Spinal-DM
Spinal Motor Neurons	Chrono™ Moto-DM

Check our website frequently for an updated list of new and exciting products: www.anatomicorp.com/products

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	75 µL	Until labeled expiry
Chrono™ Matrix 2	4°C	1 mL Tube	75 µL	Until labeled expiry
Senso-DM1	-20°C	30 mL PET Bottle	15 mL	Until labeled expiry
Senso-DM2	-20°C	30 mL PET Bottle	15 mL	Until labeled expiry
Senso-DM3	-20°C	30 mL PET Bottle	15 mL	Until labeled expiry
Senso-DM4	-20°C	30 mL PET Bottle	15 mL	Until labeled expiry
Senso-DM5	-20°C	30 mL PET Bottle	15 mL	Until labeled expiry
Senso-DM6	-20°C	30 mL PET Bottle	15 mL	Until labeled expiry
Senso-DM7	-20°C	30 mL PET Bottle	15 mL	Until labeled expiry
Senso-DM8	-20°C	30 mL PET Bottle	15 mL	Until labeled expiry

3.2 Materials Required but not Included

Product Name	Supplier	Cat #	Use
dPBS without Calcium and Magnesium	Your preferred supplier	-	Coating Buffer
Versene Solution	ThermoFisher	15040066	Day -1 Dissociation
ACCUTASE™	Innovative Cell Technologies	AT104	Day 5 Dissociation
40 µm cell strainer	Your preferred supplier		Day 5 Dissociation
DMEM	Your preferred supplier		Day 5 Dissociation
B-27™ Plus Neuronal Culture System	ThermoFisher	A3653401	Long-term culture
DMSO	Your preferred supplier	-	Cryopreservation

3.3 Required Equipment

- Biosafety cabinet certified for Level II 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)

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 ectoderm. 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 enjoyed using a split ratio of 1.5x to 2x of hPSC maintenance ratio for a three-day passage cycle. Our hPSCs are passaged every third day at ~60% confluence, and are maintained with a 1-to-8 to 1-to-10 split ratio. This means for the purposes of using Chrono™ Senso-DM, cultures maintained 1-to-8 should be seeded between 1-to-12 and 1-to-16; and cultures maintained 1-to-10 should be seeded between 1-to-15 and 1-to-20. 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.

Colony Size Is Critical. Chrono™ Senso-DM was designed to transform hPSC *colonies* into sensory neurons. This is very convenient, as hPSC lines are generally maintained as colonies to maintain euploid cultures, which means separate cultures don't need to be partitioned for single-cell passaging to initiate differentiation. Generally, the ideal colony size useful for maintaining hPSC lines is also useful for differentiation using Chrono™ Senso-DM. Substantial loss of cell viability will be seen after exposure of single-cell hPSC cultures or hPSC cultures comprised of primarily very small (10 to 20-cell colonies) to Chrono™ Senso-DM. Very large hPSC colonies (200 cells or greater) will survive, however control of downstream differentiation processes may be reduced. An ideal hPSC colony size range for differentiation using Chrono™ Senso-DM is between 50 and 100 cells. This isn't commonly quantified in practice, so please take note of our colony sizing guide to visually identify ideal hPSC colony size parameters. To ensure success, view Section 6.0: Production of Sensory Neurons Using Chrono™ Senso-DM for a guide to appropriate day-one colony size before addition of Chrono™ Senso-DM.

Volume Is Critical. This will be clearly stated in the instructions you will surely ignore, so we broke this critical parameter out from the requisite list-of-too-many-steps so that you will notice it. 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 Chrono Matrix 1 1:100 into dPBS without Calcium and Magnesium
5.1.3. Add 0.1 mL/cm^2 of Chrono™ Matrix to tissue culture-treated vessels
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 Coating with Chrono™ Matrix 2

5.1.1. Dilute Chrono Matrix 2 1:100 into dPBS without Calcium and Magnesium
5.1.2. Add 0.1 mL/cm^2 of Chrono™ Matrix to tissue culture-treated vessels
5.1.3. Swirl the vessel to evenly spread the solution across the surface
5.1.4. 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

5.3 Preparation of Chrono™ Senso-DM

5.2.1. Thaw Chrono™ Senso-DM for the appropriate day at room temperature or overnight in the refrigerator

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

While Chrono™ Senso-DM can be used several different ways, we recommend the following monolayer protocol for best results. Chrono™ Senso-DM will promote the conversion of hPSCs into sensory neurons within 8 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 ectoderm from hPSCs using Chrono™ Senso-DM. As you can see, conversion using Chrono™ Senso-DM is relatively simple to execute when compared to legacy methods.

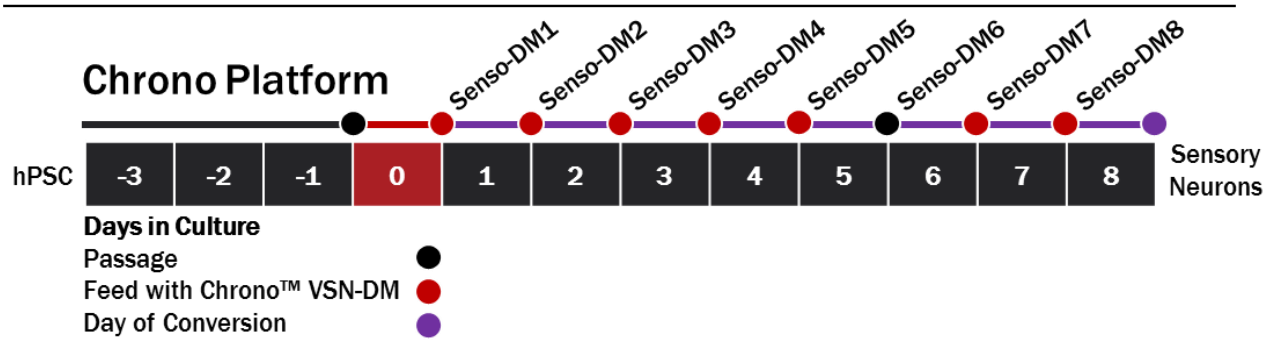


Figure: Schematic for production of sensory neurons using Chrono™ Senso-DM.

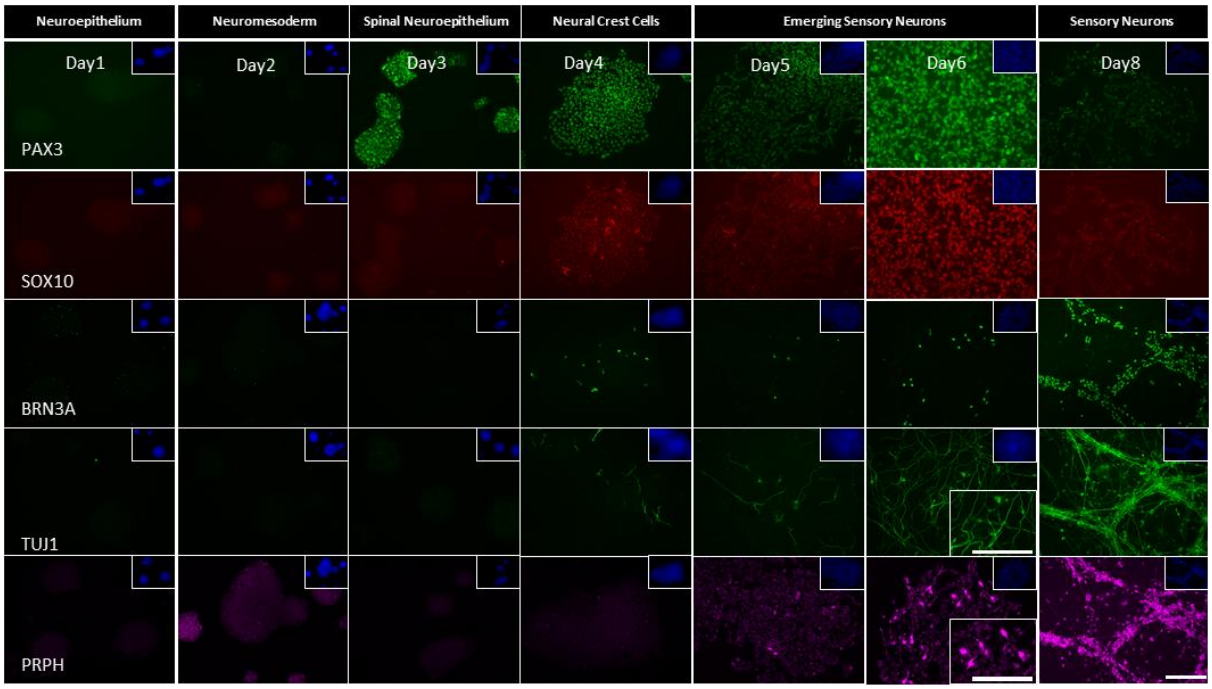
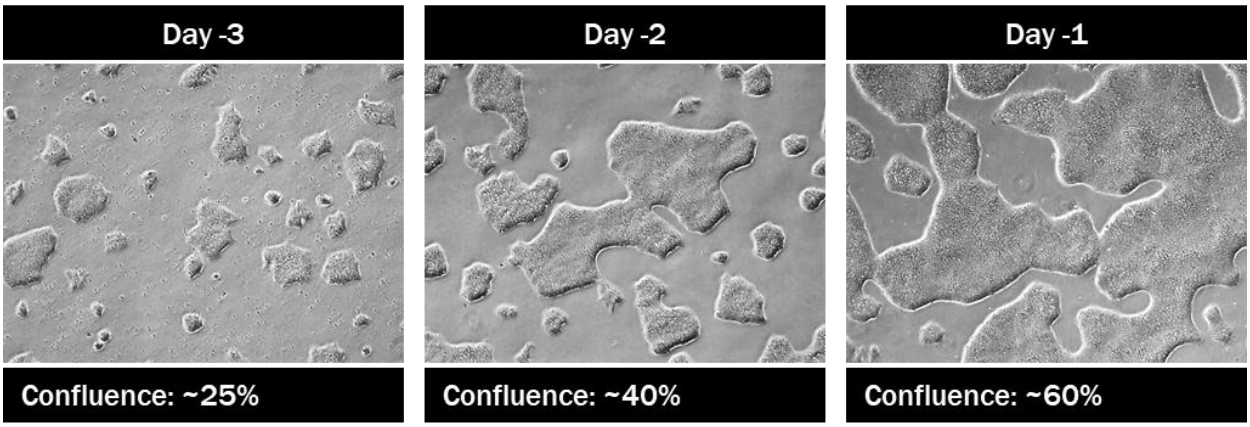


Figure: Immunocytochemistry panel characterizing sensory neurons days 0-8.

Days -3 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 tissue culture-treated vessels as described in Section 5.1: Coating with Chrono™ Matrix 1. Now you will dissociate the culture using a non-enzymatic passaging solution such as 0.55mM EDTA.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from hPSC culture vessel
3. Dispense 0.1 mL/cm^2 (i.e 2.5 mL/T25) Versene Solution into the culture vessel
4. Aspirate Versene Solution from culture vessel
5. Dispense 0.1mL/cm^2 Versene Solution into the vessel
6. Return culture vessel to incubator and treat approximately 6 minutes
7. Transfer treated culture to biosafety cabinet
8. Aspirate Versene Solution from culture vessel
9. Dislodge hPSC colonies from culture surface using 0.1mL/cm^2 hPSC medium
10. Collect hPSC culture into sterile 15 mL conical tube
11. Rinse previous culture vessel to collect remaining hPSC culture
12. Combine hPSC culture with all rinses
13. Process culture into colonies roughly 50 to 100 cells in size with vigorous pipetting
14. Dilute culture as necessary to seed at a 1-12 ratio or as described in Section 4.0 Important Parameters for Successful Use of Chrono™ Senso-DM
15. Dispense the required volume for hPSC culture maintenance

16. Aspirate the Chrono™ Matrix 1 coating from its culture vessel
    !!!CRITICAL!!! Do not allow the culture surface to dry, as the matrix will become inactivated.
17. Dispense the required volume of hPSC culture for differentiation
18. Return culture to incubator and distribute colonies evenly by rocking culture vessel

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

On day 0, your culture may be approximately 20% confluent, composed of a number of hPSC colonies of various shapes and sizes. The figure below depicts a potential culture you may witness, along with colony sizing guide.

Prepare Chrono™ Senso-DM1 as indicated in Section 5.3: Preparation of Chrono™ Senso-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from hPSC culture vessel
3. Dispense 0.2 mL/cm^2 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 ectoderm. This should be obvious morphologically, as the culture will acquire a specific appearance under phase-contrast microscopy (see following figure).

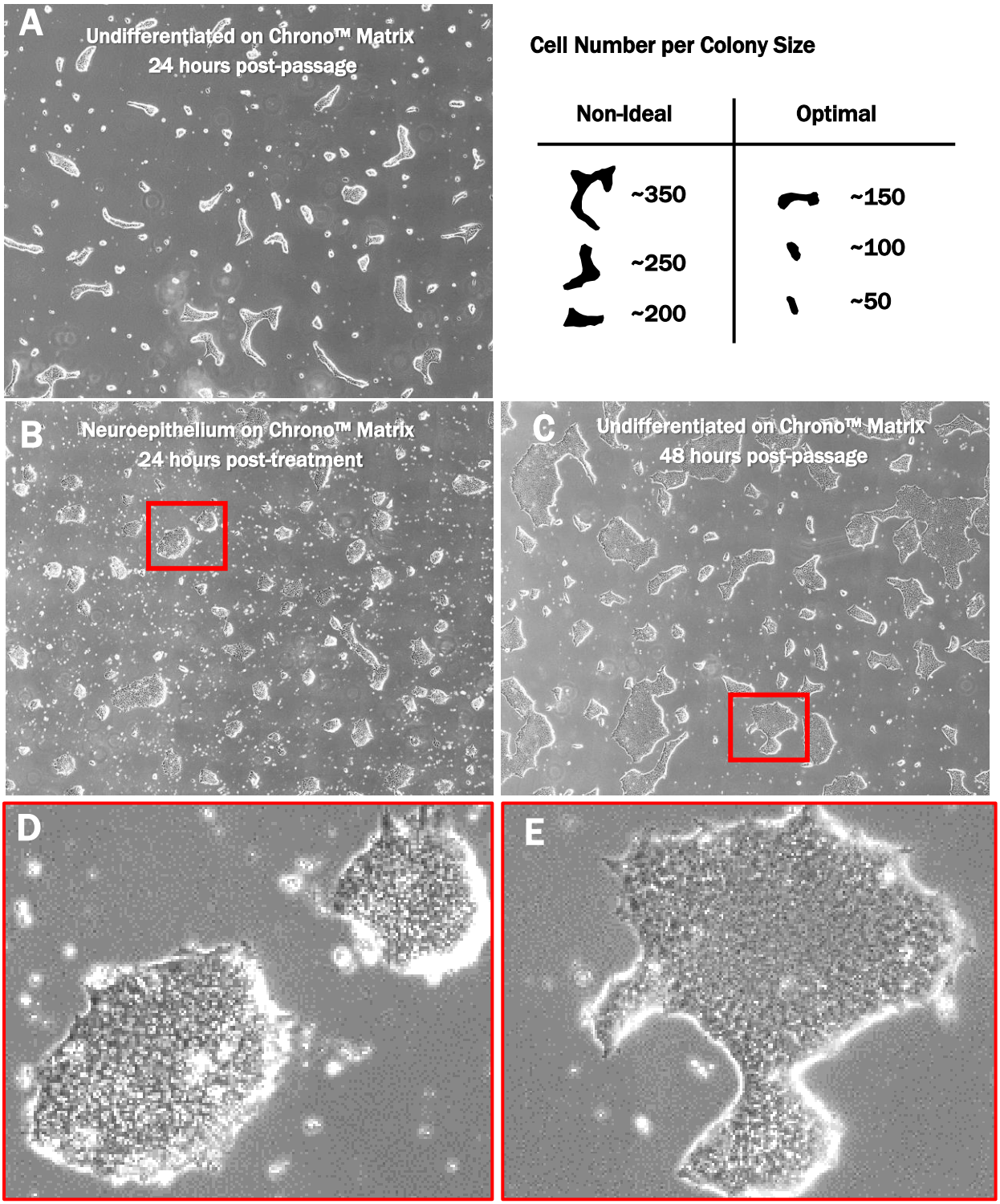


Figure: A) Colony size ruler following plating of hPSCs onto Chrono™ Matrix 24 hours post-passage. B) Low magnification phase-contrast morphology of neuroepithelium 24 hours post-treatment. C) Low magnification phase-contrast morphology of hiPSCs 48 hours following undifferentiated culture. D) High-magnification phase-contrast morphology of red-boxed area in B demonstrating morphology of neuroepithelium 24-hours post-treatment. E) High-magnification phase-contrast morphology of red-boxed area in C demonstrating morphology of hiPSCs following 48 hours of undifferentiated culture post-passage.

Prepare Chrono™ Senso-DM2 as indicated in Section 5.3: Preparation of Chrono™ Senso-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 0.2 mL/cm² Chrono™ Senso-DM2 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 2: Observe neuromesoderm culture

Day 1 cultures exposed to Senso-DM2 produce neuromesoderm. This is evident with immuncytochemical staining with antibodies specific to SOX2, T, and HOXB4. This culture is now competent to produce spinal neuroepithelium by applying Senso-DM3.

Prepare Chrono™ Senso-DM3 as indicated in Section 5.3: Preparation of Chrono™ Senso-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 0.2 mL/cm² Chrono™ Senso-DM3 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 3: Observe sensory neuroepithelial culture

Day 2 cultures exposed to Senso-DM3 produce sensory neuroepithelium. This is evident with immuncytochemical staining with antibodies specific to SOX1, OLIG2, and HOXB4. This culture is now competent to produce neural crest by applying Senso-DM4.

Prepare Chrono™ Senso-DM4 as indicated in Section 5.3: Preparation of Chrono™ Senso-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 0.2 mL/cm² Chrono™ Senso-DM4 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 4: Observe neural crest culture

Day 3 cultures exposed to Senso-DM4 initiate commitment into neural crest. This is evident with immuncytochemical staining with antibodies specific to PAX3 and SOX10. This culture now requires additional maturation to produce pure populations of sensory neuroblasts by applying Senso-DM5.

Prepare Chrono™ Senso-DM5 as indicated in Section 5.3: Preparation of Chrono™ Senso-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 0.2 mL/cm² Chrono™ Senso-DM5 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 5: Observe peripheral neuroblastic culture & passage to purify culture

Day 4 cultures exposed to Senso-DM5 initiate 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. Contaminant CNS at the center of colonies should be removed by passaging and filtering with a 40 µm cell strainer. Sensory neuroblasts should be re-seeded at a 1:1 ratio.

Prepare Chrono™ Senso-DM6 as indicated in Section 5.3: Preparation of Chrono™ Senso-DM. You will have already Chrono™ Matrix 2 coated tissue culture-treated vessels as described in Section 5.2: Coating with Chrono™ Matrix 2. Now you will dissociate the culture using Accutase™ and filter with a 40 µm cell strainer.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from hPSC culture vessel
3. Dispense 0.1 mL/cm² (i.e 2.5 mL/T25) Accutase™ into the culture vessel
4. Return culture vessel to incubator and treat approximately 2 minutes
5. Transfer treated culture to biosafety cabinet
6. Tap the culture to dislodge sensory neuroblasts from the center of colonies
7. Collect sensory neuroblast culture and strain through the 40 µm cell strainer
   NOTE: The goal is to remove the center of colonies through the 40 µm cell strainer so be careful not to break up the centers
8. Gently rinse the previous culture vessel using 0.2mL/cm² DMEM to collect and combine remaining sensory neuroblasts
9. Combine sensory neuroblast culture with all rinses
10. Centrifuge the cells at 300 x g for 3 minutes
11. Gently re-suspend the culture into 0.2mL/cm² Chrono™ Senso-DM6 to seed at a 1-1 ratio
12. Aspirate the Chrono™ Matrix 2 coating from its culture vessel
    !!!CRITICAL!!! Do not allow the culture surface to dry, as the matrix will become inactivated.
13. Dispense the required volume of sensory neuroblast culture for differentiation
14. Return culture to incubator and distribute cells evenly by rocking culture vessel

Day 6: Observe initial axon extension

Day 5 cultures passaged and exposed to Senso-DM6 should be at 80-100% confluency and begin extending axons with phase bright cell bodies. The BRN3A/ISL1/PERIPHERIN/TUJ1-positive sensory neurons will further enrich with application of Senso-DM7 and Senso-DM8.

Prepare Chrono™ Senso-DM7 as indicated in Section 5.3: Preparation of Chrono™ Senso-DM.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 0.2 mL/cm^2 Chrono™ Senso-DM7 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 7: Observe extensive axon extension

Day 6 cultures exposed to Senso-DM7 begin extending substantial axons. There may be a large amount of cell debris due from contaminant cells dying off. The BRN3A/ISL1/PERIPHERIN/TUJ1-positive sensory neurons will further enrich with application of Senso-DM8.

Prepare Chrono™ Senso-DM8 as indicated in Section 5.0: Preparation of Reagents and Media.

1. Transfer hPSC culture vessel to biosafety cabinet
2. Aspirate culture medium from culture vessel
3. Dispense 0.2 mL/cm^2 Chrono™ Senso-DM8 into the culture vessel
4. Return culture to incubator
5. Incubate approximately 24 hours

Day 8: Differentiation is complete

Day 7 cultures exposed to Senso-DM8 should have fully differentiated into a monolayer of intertwining axons. The percentage of sensory neurons is nearing 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.

Aaaaand, that's it, actually. Wow, that was anticlimactic. Now that the sensory neurons have been produced, they're useful for study in isolation or for downstream maturation.

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

Neurons produced with Chrono Senso-DM are best cryopreserved on Day 8 when neurons are immature. The following procedure can be followed to dissociate these neurons.

1. Aspirate culture medium
2. Rinse culture with 0.1 mL/cm^2 Accutase
3. Aspirate Accutase
4. Add 0.1 mL/cm^2 Accutase
5. Incubate culture at 37™C for 5 to 10 minutes

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).

6. Agitate culture vessel to dislodge neurons
7. Using a 10 mL serological pipet, triturate cell suspension 10 to 20 times
8. Observe that neurons are a single-cell suspension
9. Quench Accutase with addition of 2 to 3 volumes of B-27™ Plus Neuronal Culture System or equivalent
10. Collect cell suspension into 50 mL conical and centrifuge for 4 minutes at 300 x g
11. Observe compacted cell pellet
12. Resuspend pellet into an appropriate volume according to application

8.0 Cryopreservation of neurons produced using Chrono™ Senso-DM

Neurons produced using Chrono™ Senso-DM can be cryopreserved on day 8, after Senso-DM8 has been applied. Freezing medium can be made by supplementing B-27™ Plus Neuronal Culture System with 10% v/v DMSO. This freezing medium can be used to disrupt and resuspend the cell pellet produced in 7.12. This suspension can be vialed and placed into an isopropanol bath at -80°C for controlled rate freezing before long-term storage at -275°C in liquid nitrogen.

9.0 Plating and maturation of neurons produced using Chrono™ Senso-DM

Neurons produced using Chrono™ Senso-DM can be plated onto laminin coated tissue culture vessels for downstream maturation and study using media systems such as the B-27™ Plus Neuronal Culture System supplemented with neurotrophins

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@anatomicorp.com or on our contact page at www.anatomicorp.com/contact to report any issues.

PROBLEM	SOLUTION
Everything immediately died 24 hours after using Chrono™ Senso-DM1	• Ensure hPSCs were seeded to ~20% confluence • Ensure colony sizes are greater than 50 cells • 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→Senso-DM8 • Ensure colonies distributed on Day -1 are between 50 and 100 cells at roughly 20% confluence