axol

Human iPSC-Derived Neural Stem Cells

System A, B, C and D

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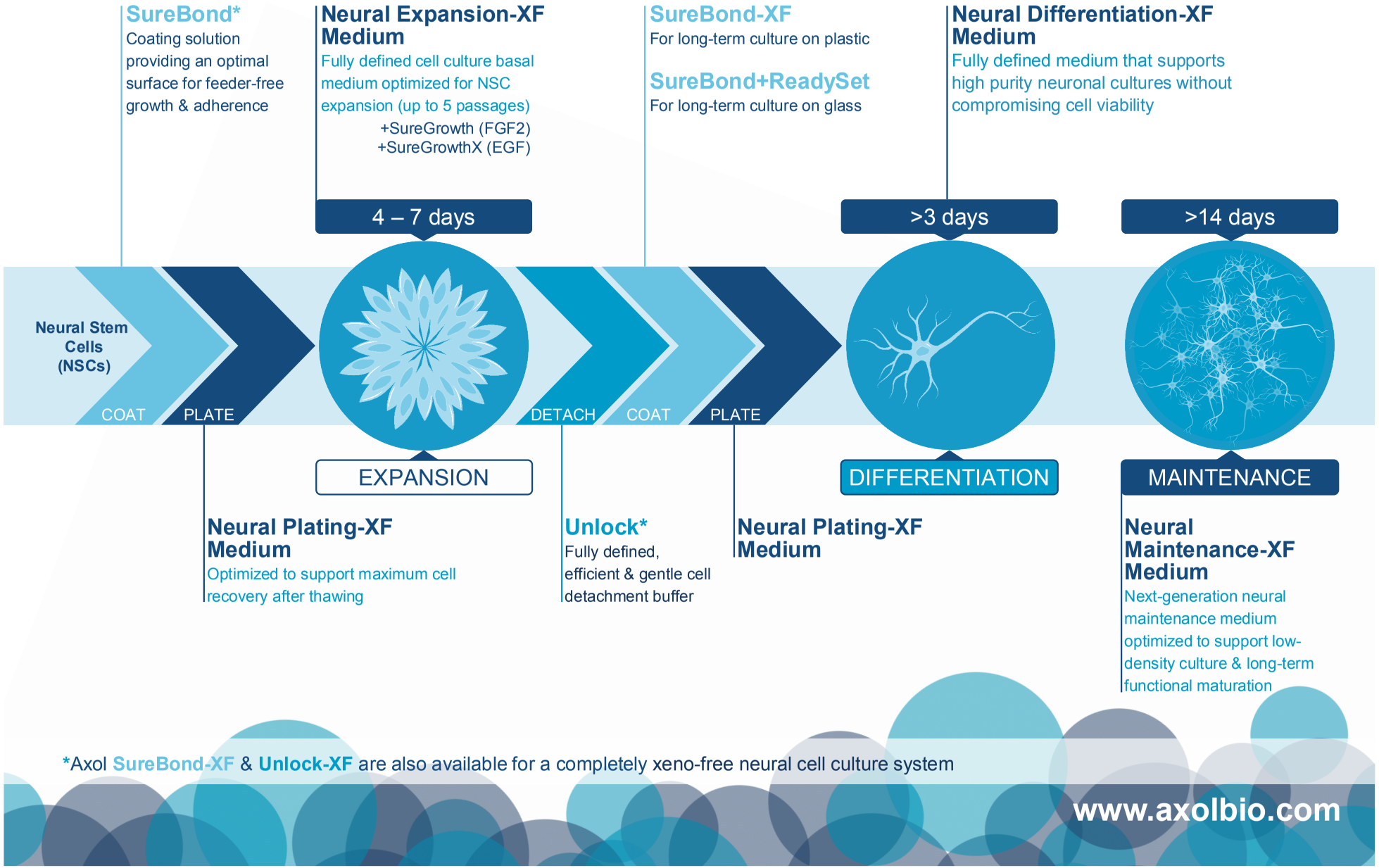
Human iPSC-Derived Neural Cell Culture System



*Axol **SureBond-XF** & **Unlock-XF** are also available for a completely xeno-free neural cell culture system

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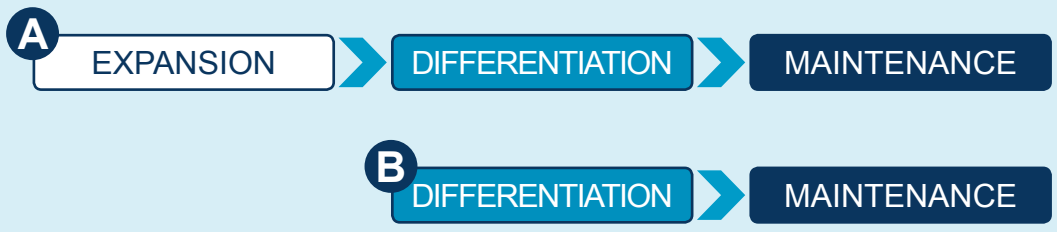
What's the Best System for Your Discovery?

Systems A and B – Synchronous Differentiation

Synchronous Differentiation

Pure population of cerebral cortical neurons



Following the protocol for **systems A and B** will result in the generation of a pure population of cerebral cortical neurons.

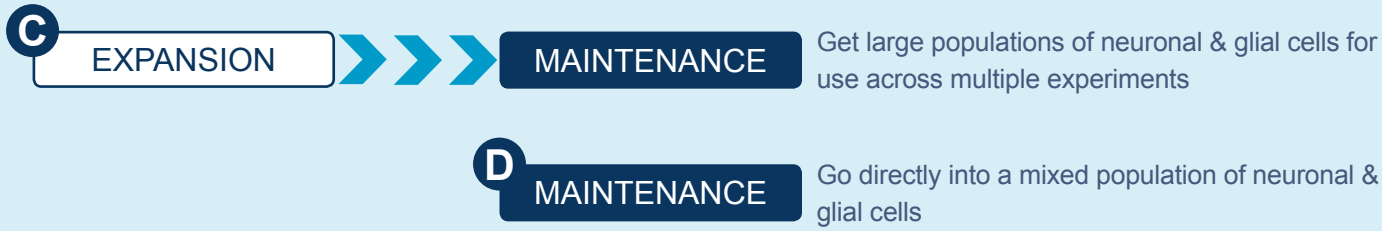
Using **system A**, **iPSC-Derived Neural Stem Cells** are expanded and then the synchronously differentiated into neurons, using **Neural Differentiation-XF Medium**.

Using **system B**, **iPSC-Derived Neural Stem Cells** are plated into the final assay format and then synchronously differentiated, using **Neural Differentiation-XF Medium**, from **iPSC-Derived Neural Stem Cells** to neurons.

Systems C and D – Spontaneous Differentiation

Spontaneous Differentiation

Mixed population of neuronal and glial cells



Following the protocol for **systems C and D** will result in the generation of a mixed population of cerebral cortical neurons and glial cells.

Using **system C**, **iPSC-Derived Neural Stem Cells** are expanded and then spontaneously differentiated, using **Neural Maintenance-XF Medium**, from **iPSC-Derived Neural Stem Cells** to neuronal and glial cells.

Using **system D**, **iPSC-Derived Neural Stem Cells** are plated into the final assay format and then spontaneously differentiated into neuronal and glial cells, using **Neural Maintenance-XF Medium**.

Caution!

Increasing the number of days the **iPSC-Derived NSCs** are in culture and the more expansions the NSCs undergo will increase the population of glial cells in the final culture. We recommend a maximum of 5 passages for healthy/control lines and a maximum of 3 passages for the disease/patient lines. Expansion must be conducted with caution and the cells should not be pushed to proliferate for too long before differentiation.

Product Information

Catalog. No.	Product Name	Format	Stock Conc.	Storage on Arrival	Thawing Instructions	Storage Once Thawed
ax0015 ax0016 ax0018 ax0019	Human iPSC-Derived Neural Stem Cells (Healthy Donors)	1.5 million cells/ vial	N/A	Liquid Nitrogen	Follow protocol	N/A
ax0111 ax0112 ax0113 ax0114 ax0115	Human iPSC-Derived Neural Stem Cells (Alzheimer's Disease Donors)					
ax0212	Human iPSC-Derived Neural Stem Cells (Huntington's Disease Donor)					
ax0411	Human iPSC-Derived Neural Stem Cells (Epilepsy Donor)					
ax0030-500	Neural Expansion-XF Medium	500 mL	1x	Aliquot & store at -80ºC for up to 6 months	Overnight at 4ºC	Once thawed, store aliquot at 4ºC for up to 1 week
ax0032-500	Neural Maintenance-XF Medium	500 mL	1x	Aliquot & store at -80ºC for up to 6 months. Keep in the dark.	Overnight at 4ºC	Once thawed, store aliquot at 4ºC for up to 1 week
ax0033	Neural Plating–XF Medium	30 mL	1x	-80ºC	Overnight at 4ºC	Must be used immediately once thawed
ax0034-125	Neural Differentiation-XF Medium	125 mL	1x	Aliquot & store at -80ºC for up to 6 months. Keep in the dark.	Overnight at 4ºC	Once thawed, store aliquot at 4ºC for up to 1 week
ax0041	SureBond	3 x 120 µL	50x	-80ºC	Overnight at 4ºC	Store at 4ºC for up to 2 weeks
ax0041XF	SureBond-XF	1 mL	200x	4ºC	N/A	Store at 4ºC for up to 1 month
ax0041+	SureBond+ ReadySet	SureBond 3 x 120 µL ReadySet 2 x 10 mL	SureBond 50x ReadySet 1x	SureBond -80ºC ReadySet 4ºC	SureBond Overnight at 4ºC ReadySet N/A	SureBond Store at 4ºC for up to 2 weeks ReadySet Store at 4ºC for up to 1 month
ax0044	Unlock	25 mL	1x	Aliquot & store at -80ºC for 6 months	Overnight at 4ºC	Once thawed, store aliquot at 4ºC for up to 1 week
ax0044XF	Unlock-XF	100 mL	1x	Aliquot & store at -80ºC for 5 months	Overnight at 4ºC	Once thawed store aliquot at 4ºC for 1 week
ax0047	SureGrowth Recombinant Human FGF2	100 µg Lyophilized Powder	N/A	-20ºC	N/A	Reconstituted protein should be used immediately or stored in working aliquots at -20ºC
ax0047X	SureGrowthX Recombinant Human EGF	100 µg Lyophilized Powder	N/A	-20ºC	N/A	

Lot-specific information such as specifications and quality control details are stated in the Certificate of Analysis.

Important!
Axol Neural Cell Culture Media

DOES NOT contain antibiotics or antifungal agents. Axol Bioscience does not recommend the use of antimicrobial agents such as penicillin, streptomycin and amphotericin. Antimicrobial agents should not be necessary if proper aseptic technique is adopted

Preparation of Reagents

Neural Expansion-XF Medium (required for systems A and C)

- Upon receipt, store **Neural Expansion-XF Medium** at or below **-80ºC** protected from light. Stored at **-80ºC**, medium is stable for 6 months from date of manufacture.
- When ready to use, thaw medium overnight at **4ºC** in the dark, aliquot and store at **-80ºC**.
- On the day of thawing **Human iPSC-Derived Neural Stem Cells (NSCs)**, prepare growth factors as follows:

SureGrowth Recombinant Human FGF2

Prepare **100 µg/mL** solution (5000x) of **SureGrowth Recombinant Human FGF2** by resuspending the 100 µg of lyophilized powder in 1 mL of PBS (1x) supplemented with 0.1 % human serum albumin.

SureGrowthX Recombinant Human EGF

Prepare **100 µg/mL** solution (5000x) of **SureGrowth Recombinant Human EGF** by resuspending the 100 µg of lyophilized powder in 1 mL of PBS (1x) supplemented with 0.1 % human serum albumin.

- Supplement **Neural Expansion-XF Medium** with **100 µg/mL SureGrowth Recombinant Human FGF2** and **SureGrowth Recombinant Human EGF** to a final concentration of **20 ng/mL** of FGF2 and EGF.
- Growth factors should be **added fresh each time** an aliquot of **Neural Expansion-XF Medium** is thawed.
- A thawed and supplemented aliquot of **Neural Expansion-XF Medium** can be stored at **4ºC** for **1 week**.

Neural Maintenance-XF Medium (required for all systems)

- Upon receipt aliquot and store **Neural Maintenance-XF Medium** at or below **-80ºC** protected from light. Stored at **-80ºC**, medium is stable for 6 months from date of manufacture.
- When ready to use, thaw an aliquot of **Neural Maintenance-XF Medium** overnight at **4ºC** in the dark.
- A thawed aliquot of **Neural Maintenance-XF Medium** can be stored at **4ºC** for **1 week**, protected from light.
- **Neural Maintenance-XF Medium** is fully supplemented and ready to use.

Neural Plating–XF Medium (required for all systems)

- Upon receipt store **Neural Plating–XF Medium** at or below **-80ºC** protected from light.
- When ready to use, thaw **Neural Plating–XF Medium** overnight at **4ºC** in the dark.
- Once thawed, **Neural Plating–XF Medium** must be used and cannot be refrozen.

Neural Differentiation-XF Medium (required for systems A and B)

- Upon receipt, aliquot and store **Neural Differentiation-XF Medium** at or below **-80ºC** protected from light. Stored at **-80ºC**, the medium is stable for 6 months from date of manufacture.
- When ready to use, thaw an aliquot of **Neural Differentiation-XF Medium** overnight at **4ºC** in the dark.
- A thawed aliquot of **Neural Differentiation-XF Medium** can be stored at **4ºC** for **1 week**, protected from light.
- **Neural Differentiation-XF Medium** is fully supplemented and ready-to-use.

Unlock (required for systems A and C)

- Upon receipt aliquot and store **Unlock** at or below **-80ºC** protected from light. Stored at **-80ºC**, the reagent is stable for 6 months from date of manufacture.

Preparation of Coating Reagents and Coating the Culture Vessel

SureBond Coating Solution (required for systems A and C)

- Upon receipt store **SureBond** at or below **-80ºC**.
- Thaw the **SureBond** coating solution overnight at **4ºC**.
- Calculate the total surface area that requires coating.
- Dilute the **SureBond** stock solution (50x) in Dulbecco's-PBS (1x) (D-PBS, without calcium or magnesium) to make 1x working solution e.g. **120 µL** in **6 mL**.
- Coat the surface of your culture vessel with the **SureBond** 1x working solution. We recommend coating at a volume of **200 µL per cm²** however, please optimize for your experiments.
- Incubate your culture vessel **overnight** at **37ºC**.

Important!

Make sure that the coating does not evaporate overnight.
Do not let the **SureBond** coating dry out before seeding the cells.
DO NOT wash the vessel after coating with **SureBond**.

SureBond-XF Coating Solution (required for all systems for final plating on plastic or a fully xeno-free system in systems A and C)

- Upon receipt store **SureBond-XF** at **4ºC**.
- Calculate the total surface area that requires coating.
- Dilute the **SureBond-XF** stock solution (200x) in D-PBS (1x) (without calcium or magnesium) to make 1x working solution e.g. **30 µL** in **6 mL**.
- Coat the surface of your culture vessel with the **SureBond-XF** 1x working solution. We recommend coating at a volume of **200 µL per cm²** however, please optimize for your experiments.
- For final culture in a 96-well plate format see **Protocol Supplement (version 1.0): Culture of Human iPSC-Derived Neural Stem Cells in a 96-well Plate Format**.

Important!

Make sure that the coating does not evaporate.
Do not let the **SureBond-XF** coating dry out before seeding the cells.
DO NOT wash the vessel after coating with **SureBond-XF**.

SureBond+ReadySet Coating Solution (required for all systems for final plating on glass)

- Upon receipt store **SureBond** at or below **-80ºC** and store **ReadySet** at **4ºC**.
- Thaw the **SureBond** coating solution overnight at **4ºC**.
- Calculate the total surface area that requires coating.
- Pre-coat your culture vessel with **ReadySet** at a volume of **250 µL per cm²**.
- Incubate at **37ºC** for **45 minutes**.
- Wash the plate thoroughly **four times** using an equal volume of sterile distilled H_2O (e.g. if 250 µL of **ReadySet**, use 250 µL sterile distilled H_2O). During each wash rock the dish to ensure thorough washing.
- Do not let the **ReadySet** dry out following washing, proceed straight to coating with **SureBond**.
- Dilute the **SureBond** stock solution (50x) in D-PBS (1x) (without calcium or magnesium) to make 1x working solution e.g. **120 µL in 6 mL**.
- Coat the surface of your culture vessel with the **SureBond** 1x working solution. We recommend coating at a volume of **200 µL per cm²**.
- Incubate for **1 hour** at **37ºC**.
- For final culture in a 96-well plate format see **Protocol Supplement (version 1.0): Culture of Human iPSC-Derived Neural Stem Cells in a 96-well Plate Format**.

Important!

Do not let the **SureBond** coating dry out before seeding the cells.
DO NOT wash the vessel after coating with **SureBond**.

System A
Expansion and Synchronous Differentiation

The expansion of Human iPSC-Derived Neural Stem Cells followed by the generation of a pure population of cortical neurons

Following the protocol for **system A** will result in the generation of a pure population of cerebral cortical neurons. **System A** includes the expansion of **iPSC-Derived Neural Stem Cells** and then the synchronized differentiation of **iPSC-Derived Neural Stem Cells** to neurons.

Thawing and Plating of Human iPSC-Derived Neural Stem Cells

The day before thawing **Human iPSC-Derived NSCs**:

- Thaw **Neural Plating–XF Medium** overnight at **4ºC**.
- Prepare culture vessels with **SureBond** and incubate **overnight** at **37ºC** (if using a completely xeno-free system pre-coat the culture vessels with **SureBond-XF** **4 hours** prior to thawing cells). **Axol recommends**: coating and seeding into 2 wells of a 6-well plate for initial plating post-thaw.

On the day of thawing **Human iPSC-Derived NSCs**:

- Pre-warm all media and culture vessels to **37ºC** before use.
- To thaw cells – transfer the cells from storage by transporting cells buried in dry ice. Remove the cells from dry ice and transfer the cells to a **37ºC** water bath.
- Quickly thaw the vial of cells in a **37ºC** water bath. Do not completely submerge the vial (only up to 2/3rd of the vial). Remove the vial before the last bit of ice has melted, after ~1-2 minutes.
- **Do not shake the vial during thawing**.
- Take the vial of cells to a biological safety cabinet, spraying the vial and hood thoroughly with 70% ethanol and wiping with an autoclaved paper towel before placing the vial in the hood.

Important!

Do not mix the cells vigorously. Avoid generating bubbles.

- Once thawed, use a P1000 pipette to transfer the cells into a 15 mL sterile conical tube drop-wise add **10 mL** of pre-warmed, **37ºC**, **Neural Expansion-XF Medium**. Gently wash the cryogenic vial with **1 mL** of **Neural Expansion-XF Medium**. Transfer this to the 15 mL sterile conical tube containing the cells.
- Centrifuge cells at **200 x *g*** for **5 minutes** at room temperature.
- During the centrifugation step, remove the coating solution.
- **Axol Recommends**: Once the coating solution has been removed add **1.95 mL** of **Neural Plating–XF Medium** into 2 wells of the pre-coated 6-well plate, ensuring that the culture vessel does not dry. Return the plate containing the **Neural Plating–XF Medium** to the incubator until ready for cell seeding.
- Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet in **1 mL** of **Neural Plating–XF Medium** until they are in a single cell suspension.
- **Perform a cell count to ensure optimal seeding density**.
- Plate the resuspended cells drop-wise and evenly **at a density ranging from 70,000 - 200,000 cells/cm^2** on **SureBond** coated culture vessel.
- To ensure an even plating of **Human iPSC-Derived NSCs**, gently rock the culture vessel back and forth and side to side several times.
- Incubate the cells at **37ºC**, **5% CO_2**.
- The day after plating, replace the medium with fresh pre-warmed, **37ºC**, **Neural Expansion-XF Medium** (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)), to remove any dead cells/debris and to promote proliferation.

Caution!

Increasing the number of days the **iPSC-Derived NSCs** are in culture and the more expansions the NSCs undergo will increase the population of glial cells in the final culture. We recommend a maximum of 5 passages for healthy/control lines and a maximum of 3 passages for the disease/patient lines. Expansion must be conducted with caution and the cells should not be pushed to proliferate for too long before differentiation.

EXPANSION

Expansion of Human iPSC-Derived Neural Stem Cells

- **Every 2 days**, replace all of the medium with fresh, pre-warmed, **37ºC**, **Neural Expansion-XF Medium** (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)).
- When the culture is 70-80% confluent, it is ready to undergo passaging for expansion.
- **Pre-coat culture vessels**: Make sure to pre-coat the surface of your culture vessel that the cells will be passaged into. If continuing to expand **Human iPSC-Derived NSCs**, pre-coat culture vessels with **SureBond**.
- Thaw an aliquot of **Unlock**, **Neural Expansion-XF Medium** and **Neural Plating–XF Medium** overnight at **4ºC** before use and store at **4ºC**.
- Pre-warm all media and culture vessels to **37ºC** before use.
- Remove all spent medium from cell culture vessels.
- Gently rinse the surface of the cell layer once with the Dulbecco's-PBS (1x) (D-PBS, without calcium or magnesium, **2 mL D-PBS (1x) per 10 cm^2 culture surface area**).
- Discard the D-PBS.
- To detach the cells from a coating of **SureBond** use **Unlock**.
- Add **1 mL per 10cm^2** of culture surface area of cold/room temperature **Unlock**. Evenly distribute it over the entire cell layer. Incubate the cells for **5 minutes** at **37ºC**.
- Use a P1000 pipette to transfer the cells drop-wise into a 15 mL sterile conical tube add gently **four volumes** of pre-warmed, **37ºC**, **Neural Expansion-XF Medium**. (For example if 1 mL of **Unlock** is used, then add 4 mL of the medium to stop the reaction). Gently pipette up and down a few times to disperse the medium.
- Centrifuge cells at **200 x *g*** for **5 minutes** at room temperature.
- During the centrifugation step, remove the coating solution.
- Once coating solution has been removed add **Neural Plating–XF Medium** to the empty well. Add ~65% of the total well volume so that the culture vessel does not dry out (e.g. 6-well plate maximal volume is < 3 mL, add 1.95 mL of **Neural Plating–XF Medium**). Return the plate containing the **Neural Plating–XF Medium** to the incubator until ready for cell seeding.
- Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet in **1 mL** of **Neural Plating–XF Medium** until they are in a single cell suspension.
- **Perform a cell count to ensure optimal seeding density**.
- Plate the resuspended cells drop-wise and evenly **at a density ranging from 70,000 - 200,000 cells/cm^2** on **SureBond** coated culture vessel.
- To ensure an even plating of **Human iPSC-Derived NSCs**, gently rock the culture vessel back and forth and side to side several times.
- Incubate the cells at **37ºC**, **5% CO_2**.
- The day after plating, replace the medium with fresh pre-warmed, **37ºC**, **Neural Expansion-XF Medium** (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)), to remove any dead cells/debris and to promote proliferation.
- **Every 2 days**, replace all of the medium with fresh, pre-warmed, **37ºC**, **Neural Expansion-XF Medium** (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)).
- When the culture is 70-80% confluent, it is ready to undergo another passage for further expansion or seeded for differentiation.

DIFFERENTIATION

Synchronous Differentiation of Human iPSC-Derived Neural Stem Cells

When you have expanded the cells to your desired passage/ cell quantity and are ready to start the differentiation carry out the following procedure:

Note:

Differentiation will take a minimum of 3 days and can take up to 10 days depending on the confluency of the **Human iPSC-Derived NSCs**. The optimal confluency for synchronous differentiation of **Human iPSC-Derived NSCs** to neurons is 60%.

- **Pre-coat culture vessels**: Make sure to pre-coat the surface of your culture vessel that the cells will be passaged into. If seeding cells onto **plastic** culture vessels for the final assay format, then pre-coat the vessels with **SureBond-XF**. If seeding cells onto **glass** culture vessels for the final assay format, then pre-coat the vessels with **SureBond+ReadySet**.
- Thaw an aliquot of **Unlock**, **Neural Expansion-XF Medium** and **Neural Plating–XF Medium** overnight at **4ºC** before use and store at **4ºC**.
- Pre-warm all media and culture vessels to **37ºC** before use.
- Remove all spent medium from cell culture vessel.
- Gently rinse the surface of the cell layer once with the Dulbecco's-PBS (1x) (D-PBS, without calcium or magnesium, **2 mL D-PBS (1x) per 10 cm² culture surface area**).
- Discard the D-PBS.
- To detach the cells from a coating of **SureBond** use **Unlock**.
- Add **1 mL per 10cm²** of culture surface area of cold/room temperature **Unlock**. Evenly distribute it over the entire cell layer. Incubate the cells for **5 minutes** at **37ºC**.
- Use a P1000 pipette to transfer the cells drop-wise into a 15 mL sterile conical tube add gently **four volumes** of pre-warmed, **37ºC**, **Neural Expansion-XF Medium**. (For example if 1 mL of **Unlock** is used, then add 4 mL of the medium to stop the reaction). Gently pipette up and down a few times to disperse the medium.
- Centrifuge cells at **200 x *g*** for **5 minutes** at room temperature.
- During the centrifugation step, remove the coating solution.
- Once coating solution has been removed add **Neural Plating–XF Medium** to the empty well. Add ~65% of the total well volume so that the culture vessel does not dry out (e.g. 6-well plate maximal volume is < 3 mL, add 1.95 mL of **Neural Plating–XF Medium**). Return the plate containing the **Neural Plating–XF Medium** to the incubator until ready for cell seeding.
- Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet with **1 mL** of **Neural Plating–XF Medium** until they are in a homogeneous single cell suspension.
- **Perform a cell count to ensure optimal seeding density.**

- Plate the resuspended cells drop-wise and evenly **at a density ranging from 70,000 - 200,000 cells/cm²** on pre-coated culture vessels.
- To ensure an even plating of **Human iPSC-Derived NSCs** gently rock the culture vessel back and forth and side to side several times.
- Incubate the cells at **37ºC**, **5% CO_2**.
- The day after plating, conduct a full medium change with fresh, pre-warmed, **37ºC**, **Neural Expansion-XF Medium WITHOUT EGF** or **FGF2**.
- After a further **24 hours**, conduct a full medium change with fresh, pre-warmed, **37ºC**, **Neural Differentiation-XF Medium**.
- Replace half the volume of medium with fresh pre-warmed, **37ºC**, **Neural Differentiation-XF Medium** every **3 days**.
- Detection of a pure population of neurons is visual. Neurite formation will begin to occur and you will see an increase in neuronal cell types. Check the culture each day to observe neuronal development. When the cells are 60% confluent before starting differentiation, a pure neuronal population will take at least 3 days to develop. If the culture is >60% confluent it may take longer (up to 10 days). If the culture is fully confluent, a pure population of neurons will be difficult to obtain.

MAINTENANCE

Maintenance of Human iPSC-Derived Cortical Neurons

- Once the culture has reached a pure population of neurons, replace half the volume of medium with fresh, pre-warmed, **37ºC**, **Neural Maintenance-XF Medium**.
- **24 hours** after the half medium change to **Neural Maintenance-XF Medium**, replace half the volume of medium with fresh, pre-warmed, **37ºC**, **Neural Maintenance-XF Medium**.
- To maintain a healthy neuronal culture, replace half the volume of medium with fresh, pre-warmed, **37ºC**, **Neural Maintenance-XF Medium** every **3 days**.
- It is important that the medium is pre-warmed and that the medium changes are conducted gently by pipetting to the side of the culture dish.
- Terminally differentiated neurons can now be maintained in culture until ready for experimental assays.

System B
Synchronous Differentiation

The generation of a pure population of cortical neurons without the expansion of Human iPSC-Derived Neural Stem Cells

Using **system B**, **iPSC-Derived Neural Stem Cells** are plated into the final assay format and then synchronously differentiated from **iPSC-Derived Neural Stem Cells** to neurons, using **Neural Differentiation-XF Medium**.

Thawing and Plating of Human iPSC-Derived Neural Stem Cells

The day before thawing **Human iPSC-Derived NSCs:**

- Thaw **Neural Plating–XF Medium** overnight at **4ºC**.
- **Prepare culture vessels:** If seeding cells onto **plastic** culture vessels for the final assay format, then pre-coat the vessels with **SureBond-XF**. If seeding cells onto **glass** culture vessels for the final assay format, then pre-coat the vessels with **SureBond+ReadySet**.

On the day of thawing **Human iPSC-Derived NSCs**:

- Pre-warm all media and culture vessels to **37ºC** before use.
- To thaw cells – transfer the cells from storage by transporting the cells buried in dry ice. Remove the cells from dry ice and transfer the cells to a **37ºC** water bath.
- Quickly thaw the vial of cells in a **37ºC** water bath. Do not completely submerge the vial (only up to 2/3rd of the vial). Remove the vial before the last bit of ice has melted, after ~1-2 minutes.
- **Do not shake the vial during thawing**.
- Take the vial of cells to a biological safety cabinet, spraying the vial and hood thoroughly with 70% ethanol and wiping with an autoclaved paper towel before placing the vial in the hood.
- Once thawed, use a P1000 pipette to transfer the cells into a 15 mL sterile conical tube drop-wise add **10 mL** of pre-warmed, **37ºC**, **Neural Expansion-XF Medium**. Gently wash the cryogenic vial with **1 mL** of **Neural Expansion-XF Medium**. Transfer this to the 15 mL sterile conical tube containing the cells.

Important!

Do not mix the cells vigorously. Avoid generating bubbles.

- Centrifuge cells at **200 x *g*** for **5 minutes** at room temperature.
- During the centrifugation step, remove the coating solution.
- Once coating solution has been removed add **Neural Plating–XF Medium** to the empty well. Add ~65% of the total well volume so that the culture vessel does not dry out (e.g. 6-well plate maximal volume is < 3 mL, add 1.95 mL of **Neural Plating–XF Medium**). Return the plate containing the **Neural Plating–XF Medium** to the incubator until ready for cell seeding.
- Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet with **1 mL** of **Neural Plating–XF Medium** until they are in a single cell suspension.
- **Perform a cell count to ensure optimal seeding density**.
- Plate the resuspended cells drop-wise and evenly at **a density ranging from 70,000 - 200,000 cells/cm²** on pre-coated culture vessels.
- To ensure an even plating of **Human iPSC-Derived NSCs** gently rock the culture vessel back and forth and side to side several times.
- Incubate the cells for **24 hours** at **37ºC**, **5% CO_2**.

DIFFERENTIATION

Synchronous Differentiation of Human iPSC-Derived Neural Stem Cells

Note:

Differentiation will take a minimum of 3 days and can take up to 10 days depending on the confluency of the **Human iPSC-Derived NSCs**. The optimal density for synchronous differentiation of **Human iPSC-Derived NSCs** to neurons is 60%.

- **24 hours** after plating, replace half the volume of medium with fresh, pre-warmed, **37ºC**, **Neural Maintenance-XF Medium**. This will allow the cells to recover after thawing.
- After a further **24 hours**, replace the medium fully with fresh pre-warmed, **37ºC**, **Neural Differentiation-XF Medium**.
- Replace half the volume of medium with fresh, pre-warmed, **37ºC**, **Neural Differentiation-XF Medium** every **3 days**.
- Detection of a pure population of neurons is visual. Neurite formation will begin to occur and you will see an increase in neuronal cell types. Check the culture each day to observe neuronal development. When the cells are 60% confluent before starting differentiation, a pure neuronal population will take at least 3 days to develop. If the culture is >60% confluent it may take longer (up to 10 days). If the culture is fully confluent, a pure population of neurons will be difficult to obtain.

MAINTENANCE

Maintenance of Human iPSC-Derived Cortical Neurons

- Once the culture has reached a pure population of neurons, replace half the volume of medium with fresh, pre-warmed, **37ºC**, **Neural Maintenance-XF Medium**.
- **24 hours** after the half medium change to **Neural Maintenance-XF Medium**, conduct another half medium change with fresh, pre-warmed, **37ºC**, **Neural Maintenance-XF Medium**.
- To maintain a healthy neuronal culture, replace half the volume of medium with fresh, pre-warmed, **37ºC**, **Neural Maintenance-XF Medium** every **3 days**.
- It is important that the medium is pre-warmed and that the medium changes are conducted gently by pipetting to the side of the culture dish.
- Terminally differentiated neurons can now be maintained in culture until ready for experimental assays.

System C
Expansion and Spontaneous Differentiation

The expansion of Human iPSC-Derived Neural Stem Cells followed by the generation of a mixed population of cortical neurons and glia

System C includes the expansion of **iPSC-Derived Neural Stem Cells** and then spontaneous differentiation of **iPSC-Derived Neural Stem Cells** to neuronal and glial cells, using **Neural Maintenance-XF Medium**.

Thawing and Plating of Human iPSC-Derived Neural Stem Cells

The day before thawing **Human iPSC-Derived NSCs**:

- Thaw **Neural Plating–XF Medium** overnight at **4ºC**.
- Prepare culture vessels with **SureBond** and incubate **overnight** at **37ºC** (if using a completely XF system pre-coat the culture vessels with **SureBond-XF** **4 hours** prior to thawing cells). **Axol recommends**: coating and seeding into 2 wells of a 6-well plate for initial seeding post-thaw.

On the day of thawing **Human iPSC-Derived NSCs**:

- Pre-warm all media and culture vessels to **37ºC** before use.
- To thaw cells – transfer the cells from storage by transporting the cells buried in dry ice. Remove the cells from dry ice and transfer the cells to a **37ºC** water bath.
- Quickly thaw the vial of cells in a **37ºC** water bath. Do not completely submerge the vial (only up to 2/3rd of the vial). Remove the vial before the last bit of ice has melted, after ~1-2 minutes.
- **Do not shake the vial during thawing**.
- Take the vial of cells to a biological safety cabinet, spraying the vial and hood thoroughly with 70% ethanol and wiping with an autoclaved paper towel before placing the vial in the hood.

Important!

Do not mix the cells vigorously. Avoid generating bubbles.

- Once thawed, use a P1000 pipette to transfer the cells into a 15 mL sterile conical tube drop-wise add **10 mL** of pre-warmed, **37ºC**, **Neural Expansion-XF Medium**. Gently wash the cryogenic vial with **1 mL** of **Neural Expansion-XF Medium**. Transfer this to the 15 mL sterile conical tube containing the cells.
- Centrifuge cells at **200 x *g*** for **5 minutes** at room temperature.
- During the centrifugation step, remove the coating solution.
- Once coating solution has been removed add **Neural Plating–XF Medium** to the empty well. Add ~65% of the total well volume so that the culture vessel does not dry out (e.g. 6-well plate maximal volume is < 3 mL, add 1.95 mL of **Neural Plating–XF Medium**). Return the plate containing the **Neural Plating–XF Medium** to the incubator until ready for cell seeding.
- Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet with **1 mL** of **Neural Plating–XF Medium** until they are in a single cell suspension.
- **Perform a cell count to ensure optimal seeding density**.
- Plate the resuspended cells drop-wise and evenly **at a density ranging from 70,000 - 200,000 cells/cm²** on **SureBond** coated culture vessels.
- To ensure an even plating of **Human iPSC-Derived NSCs** gently rock the culture vessel back and forth and side to side several times.
- Incubate the cells at **37ºC**, **5% CO_2**.
- The day after plating, replace the medium with fresh pre-warmed, **37ºC**, **Neural Expansion-XF Medium** (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)), to remove any dead cells/debris and to promote proliferation.

Caution!

Increasing the number of days the **iPSC-Derived NSCs** are in culture and the more expansions the NSCs undergo will increase the population of glial cells in the final culture. We recommend a maximum of 5 passages for healthy/control lines and a maximum of 3 passages for the disease/patient lines. Expansion must be conducted with caution and the cells should not be pushed to proliferate for too long before differentiation.

EXPANSION

Expansion of Human iPSC-Derived Neural Stem Cells

- **Every 2 days**, replace all of the medium with fresh, pre-warmed, **37ºC**, **Neural Expansion-XF Medium** (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)).
- When the culture is 70-80% confluent, it is ready to undergo passaging for expansion.
- **Pre-coat culture vessels**: Make sure to pre-coat the surface of your culture vessel that the cells will be passaged into. If continuing to expand **Human iPSC-Derived NSCs** pre-coat culture vessels with **SureBond**.
- Thaw an aliquot of **Unlock**, **Neural Expansion-XF Medium** and **Neural Plating–XF Medium** overnight at **4ºC** before use and store at **4ºC**.
- Pre-warm all media and culture vessels to **37ºC** before use.
- Remove all medium from cell culture vessels.
- Gently rinse the surface of the cell layer once with the Dulbecco's-PBS (1x) (D-PBS, without calcium or magnesium, **2 mL D-PBS (1x) per 10 cm^2 culture surface area**).
- Discard the D-PBS.
- To detach the cells from a coating of **SureBond** use **Unlock**.
- Add **1 mL per 10cm^2** culture surface area. of cold/room temperature **Unlock**. Evenly distribute it over the entire cell layer. Incubate the cells for **5 minutes** at **37ºC**.
- Use a P1000 pipette to transfer the cells drop-wise into a 15 mL sterile conical tube add gently **four volumes** of pre-warmed, **37ºC**, **Neural Expansion-XF Medium**. (For example if 1 mL of **Unlock** is used, then add 4 mL of the medium to stop the reaction). Gently pipette up and down a few times to disperse the medium.
- Centrifuge cells at **200 x *g*** for **5 minutes** at room temperature.
- During the centrifugation step, remove the coating solution.
- Once coating solution has been removed add **Neural Plating–XF Medium** to the empty well. Add ~65% of the total well volume so that the culture vessel does not dry out (e.g. 6-well plate maximal volume is < 3 mL, add 1.95 mL of **Neural Plating–XF Medium**). Return the plate containing the **Neural Plating–XF Medium** to the incubator until ready for cell seeding.
- Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet with **1 mL** of **Neural Plating–XF Medium** until they are in a single cell suspension.
- **Perform a cell count to ensure optimal seeding density**.
- Plate the resuspended cells drop-wise and evenly **at a density ranging from 70,000 - 200,000 cells/cm^2** on **SureBond** coated culture vessels.
- To ensure an even plating of **Human iPSC-Derived NSCs** gently rock the culture vessel back and forth and side to side several times.
- Incubate the cells at **37ºC**, **5% CO_2**.
- The day after plating, replace the medium with fresh pre-warmed, **37ºC**, **Neural Expansion-XF Medium** (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)), to remove any dead cells/debris and to promote proliferation.
- **Every 2 days**, replace all of the medium with fresh, pre-warmed, **37ºC**, **Neural Expansion-XF Medium** (freshly supplemented with EGF (20 ng/mL) and FGF2 (20 ng/mL)).
- When the culture is 70-80% confluent, it is ready to undergo another passage for further expansion or seeding for differentiation.

MAINTENANCE

Spontaneous Differentiation and Maintenance of Human iPSC-Derived Neural Stem Cells

When you have expanded your cells to the desired passage or cell quantity and are ready to start the spontaneous differentiation carry out the following procedure:

- **Pre-coat culture vessels**: Make sure to pre-coat the surface of your culture vessel that the cells will be passaged into. If seeding cells onto **plastic** culture vessels for the final assay format, then pre-coat the vessels with **SureBond-XF**. If seeding cells onto **glass** culture vessels for the final assay format, then pre-coat the vessels with **SureBond+ReadySet**.
- Thaw an aliquot of **Unlock**, **Neural Expansion-XF Medium** and **Neural Plating–XF Medium** overnight at **4ºC** before use and store at **4ºC**.
- Pre-warm all media and culture vessels to **37ºC** before use.
- Remove all medium from cell culture vessel.
- Gently rinse the surface of the cell layer once with the Dulbecco's-PBS (1x) (D-PBS without calcium or magnesium, **2 mL D-PBS (1x) per 10 cm² culture surface area**).
- Discard the D-PBS.
- To detach the cells from a coating of **SureBond** use **Unlock**.
- Add **1 mL per 10cm²** culture surface area of cold/room temperature **Unlock**. Evenly distribute it over the entire cell layer. Incubate the cells for **5 minutes** at **37ºC**.
- Use a P1000 pipette to transfer the cells drop-wise into a 15 mL sterile conical tube add gently **four volumes** of pre-warmed, **37ºC**, **Neural Expansion-XF Medium**. (For example if 1 mL of **Unlock** is used, then add 4 mL of the medium to stop the reaction). Gently pipette up and down a few times to disperse the medium.
- Centrifuge cells at **200 x *g*** for **5 minutes** at room temperature.
- During the centrifugation step, remove the coating solution.
- Once coating solution has been removed add **Neural Plating–XF Medium** to the empty well. Add ~65% of the total well volume so that the culture vessel does not dry out (e.g. 6-well plate maximal volume is < 3 mL, add 1.95 mL of **Neural Plating–XF Medium**). Return the plate containing the **Neural Plating–XF Medium** to the incubator until ready for cell seeding.
- Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet with **1 mL** of **Neural Plating–XF Medium** until they are in a single cell suspension.
- **Perform a cell count to ensure optimal seeding density**.
- Plate the resuspended cells drop-wise and evenly **at a density ranging from 70,000 - 200,000 cells/cm²** on pre-coated culture vessels.
- To ensure an even plating of **Human iPSC-Derived NSCs** gently rock the culture vessel back and forth and side to side several times.
- Incubate the cells at **37ºC**, **5% CO_2**.
- **24 hours** after plating, replace half the volume of medium with fresh, pre-warmed, **37ºC**, **Neural Maintenance-XF Medium**.
- After a further 24 hours, again replace the medium fully with fresh, pre-warmed, **37ºC**, **Neural Maintenance-XF Medium**.
- To maintain a healthy neuronal and glial culture, replace half the volume of medium with fresh, pre-warmed, **37ºC**, **Neural Maintenance-XF Medium** every **3 days**.
- It is important that the medium is pre-warmed and that the medium changes are conducted gently by pipetting to the side of the culture dish.
- Terminally differentiated neurons and glia can be maintained in culture until ready for experimentation.

System D
Spontaneous Differentiation

The generation of a mixed population of cortical neurons and glia without expansion of Human iPSC-Derived Neural Stem Cells

D MAINTENANCE

Using **system D**, **iPSC-Derived Neural Stem Cells** are plated into the final assay format and then spontaneously differentiated, using **Neural Maintenance-XF Medium**, from **iPSC-Derived Neural Stem Cells** to neuronal and glial cells.

Thawing, Plating, Spontaneous Differentiation and Maintenance of Human iPSC-Derived Neural Stem Cells

The day before thawing **Human iPSC-Derived NSCs**:

- Thaw **Neural Plating–XF Medium** overnight at **4ºC**.
- **Prepare culture vessels**: If seeding cells onto **plastic** culture vessels for the final assay format, then pre-coat the vessels with **SureBond-XF**. If seeding cells onto **glass** culture vessels for the final assay format, then pre-coat the vessels with **SureBond+ReadySet**.

On the day of thawing **Human iPSC-Derived NSCs**:

- Pre-warm all media and culture vessels to **37ºC** before use.
- To thaw cells – transfer the cells from storage by transporting the cells buried in dry ice. Remove the cells from dry ice and transfer the cells to a **37ºC** water bath.
- Quickly thaw the vial of cells in a **37ºC** water bath. Do not completely submerge the vial (only up to 2/3rd of the vial). Remove the vial before the last bit of ice has melted, after ~1-2 minutes.
- **Do not shake the vial during thawing**.
- Take the vial of cells to a biological safety cabinet, spraying the vial and hood thoroughly with 70% ethanol and wiping with an autoclaved paper towel before placing the vial in the hood.
- Once thawed, use a P1000 pipette to transfer the cells into a 15 mL sterile conical tube drop-wise add **10 mL** of pre-warmed, **37ºC**, **Neural Expansion-XF Medium**. Gently wash the cryogenic vial with **1 mL** of **Neural Expansion-XF Medium**. Transfer this to the 15 mL sterile conical tube containing the cells.
- Centrifuge cells at **200 x *g*** for **5 minutes** at room temperature.

Important!

Do not mix the cells vigorously. Avoid generating bubbles.

- During the centrifugation step, remove the coating solution.
- Once coating solution has been removed add **Neural Plating–XF Medium** to the empty well. Add ~65% of the total well volume so that the culture vessel does not dry out (e.g. 6-well plate maximal volume is < 3 mL, add 1.95 mL of **Neural Plating–XF Medium**). Return the plate containing the **Neural Plating–XF Medium** to the incubator until ready for cell seeding.
- Aspirate and discard the supernatant carefully. Gently resuspend the cell pellet with **1 mL** of **Neural Plating–XF Medium** until they are in a single cell suspension.
- **Perform a cell count to ensure optimal seeding density**.
- Plate the resuspended cells drop-wise and evenly **at a density ranging from 70,000 - 200,000 cells/cm^2** on pre-coated culture vessels.
- To ensure an even plating of **Human iPSC-Derived NSCs** gently rock the culture vessel back and forth and side to side several times.
- Incubate the cells for **24 hours** at **37ºC**, **5% CO_2**.
- **24 hours** after plating, replace half the volume of medium with fresh, pre-warmed, **37ºC**, **Neural Maintenance-XF Medium**. This will allow the cells to recover after thawing.
- After a further **24 hours**, again replace the medium fully with fresh, pre-warmed, **37ºC**, **Neural Maintenance-XF Medium**.
- To maintain a healthy neuronal and glial culture, replace half the volume of medium with fresh, pre-warmed, **37ºC**, **Neural Maintenance-XF Medium** every **3 days**.
- It is important that the medium is pre-warmed and that the medium changes are conducted gently by pipetting to the side of the culture dish.
- Terminally differentiated neurons and glia can now be maintained in culture until ready for experimental assays.

Got any questions? Need help with the protocol?
Contact Axol Technical Support at **support@axolbio.com**
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