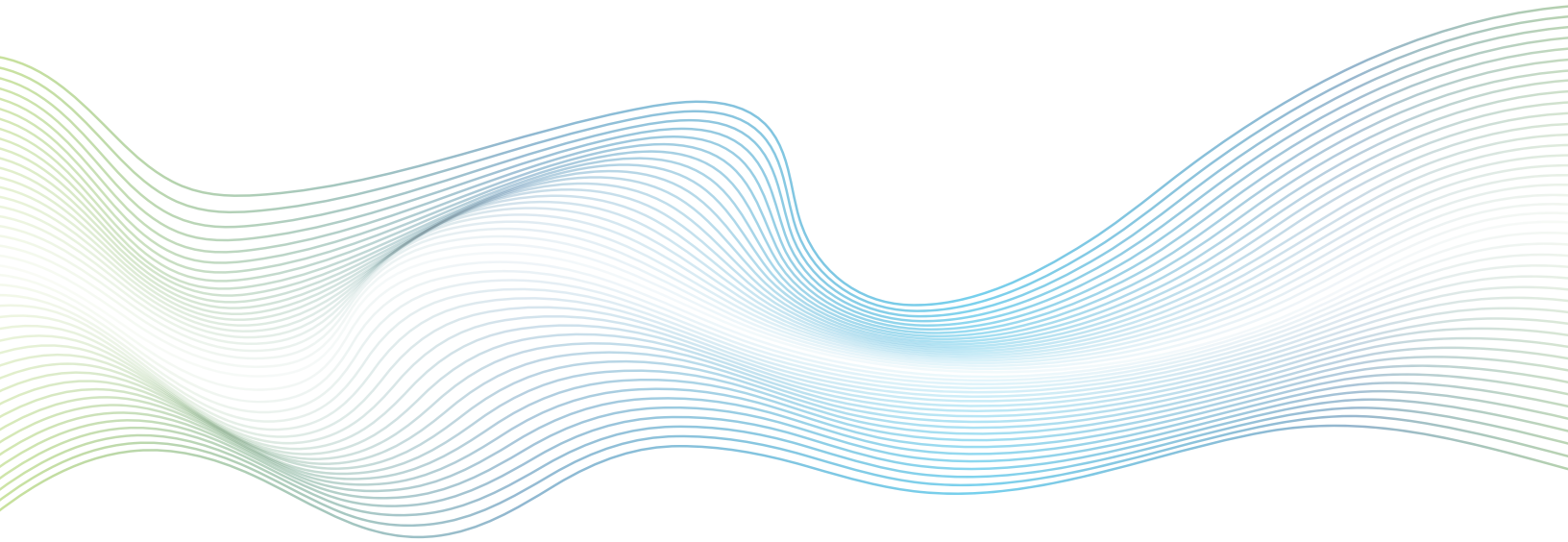
NeuCyte
Translatable Neuroscience

Human iPSC-Derived Induced Neurons and Co-Culture System



www.neucytelabs.com

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NeuCyte does not in any way guarantee or represent that you will obtain satisfactory results from using the SynFire® Technology as described herein. The only warranties provided to you are included in the Limited Warranty enclosed with this guide. You assume all risk in connection with your use of SynFire® Human iPSC-Derived Induced Neurons.

Ethical Statement

NeuCyte is a leading provider of human iPSC derived neural cells. For our business practices, we strictly comply with the following policy:
See www.neucytelabs.com.

Product Use Statement

See user restrictions at www.neucytelabs.com/neucyte-product-user-notice.

Origin

The components in our SynFire® Technology are manufactured in the United States of America.

Restricted Use

This product is for research use only and not intended for human or animal diagnostic or therapeutic uses.

Revision History

April 2023

Table of Contents

1

Introduction to the SynFire® Technology

NeuCyte manufactures pure ready-to-use human induced pluripotent stem cells (iPSCs)-induced Glutamatergic or GABAergic neurons, the SynFire iNs (induced neurons). SynFire iNs are generated using a patented procedure for direct reprogramming and exhibit the main characteristics of human neurons, such as expression of typical pan-neuronal markers and complex electrophysiology, including spontaneous and evoked action potentials and synchronized network activity. Neuronal subtype identities have also been confirmed by immunostaining and patch clamping.

SynFire iNs are suitable for imaging, electrophysiological, genomic[1] and biochemical assays. Different neuronal subtypes can be cultured with human glia for making a defined human mixed neural subtype culture for experimental purposes. Once thawed and cultured following our protocols, the cells rapidly mature and exhibit complex neuronal morphologies. Mixed neuronal cultures display synchronous network bursting activity on MEA within 3 to 4 weeks.

These human iPSC-derived neurons are valuable because of lack of a natural source of human neurons for use in research. These neurons are an essential tool for in vitro disease modeling, compound efficacy assessment and drug discovery. SynFire iNs can also be used for pre-clinical safety assessment and chemical neurotoxicity evaluation.

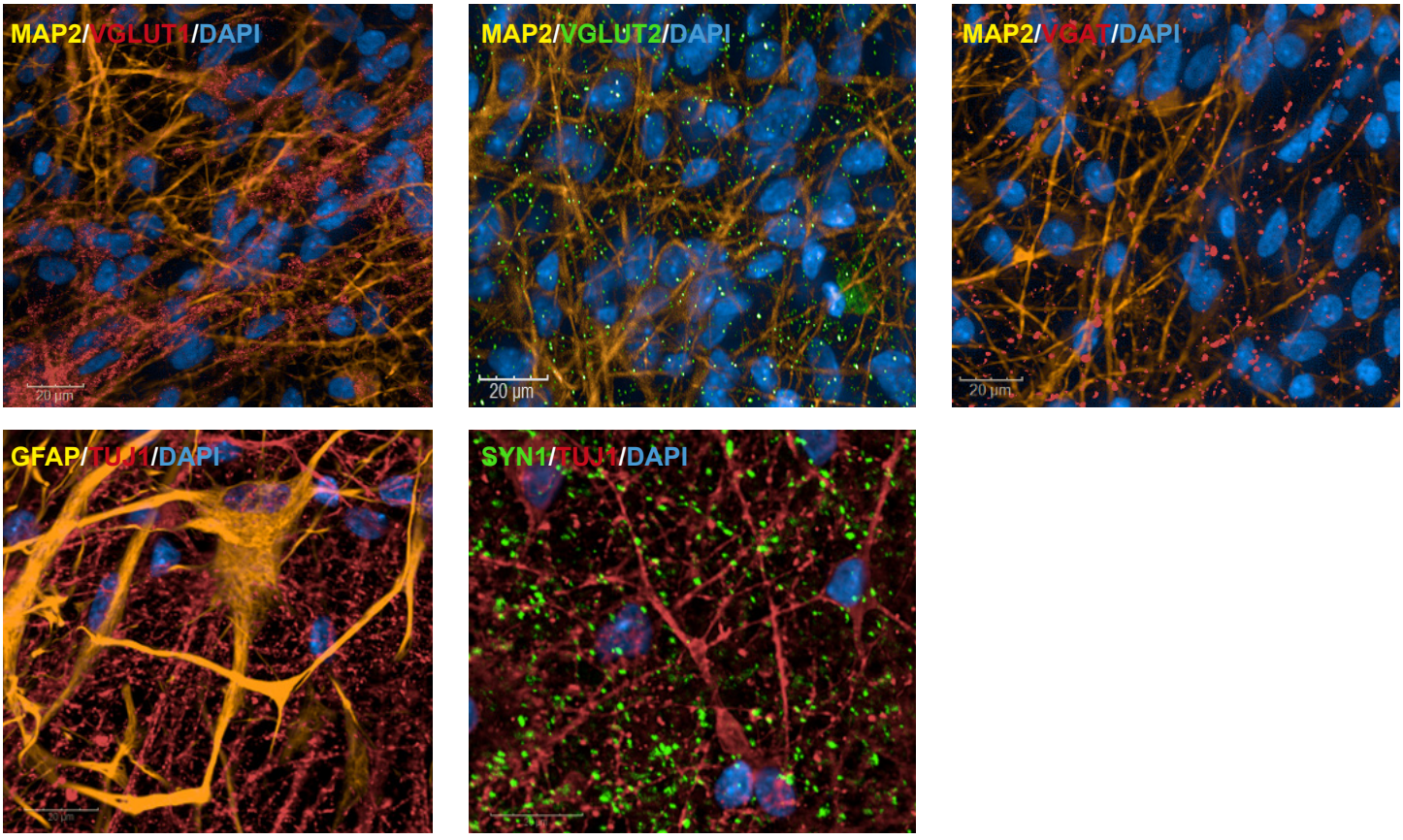


Figure 1. SynFire iNs exhibit representative neuronal characteristics by immunohistochemical analysis

SynFire iNs express pan-neuronal and subtype specific markers and rapidly mature to form complex networks and cellular morphologies. The modular aspect of SynFire neural cells allow for defined co-culture conditions and specific ratios of mixed neuronal subtypes, including excitatory glutamatergic and inhibitory GABAergic neurons. The markers shown here include pan-neuronal marker β3-Tubulin (TUJ1), microtubule-associated protein 2 (MAP2), NEUN, Synaptic marker SYNAPSIN1, PSD95, glutamatergic neuron-specific marker VGLUT2, GABAergic neuron-specific marker VGAT, nuclei stained with DAPI.

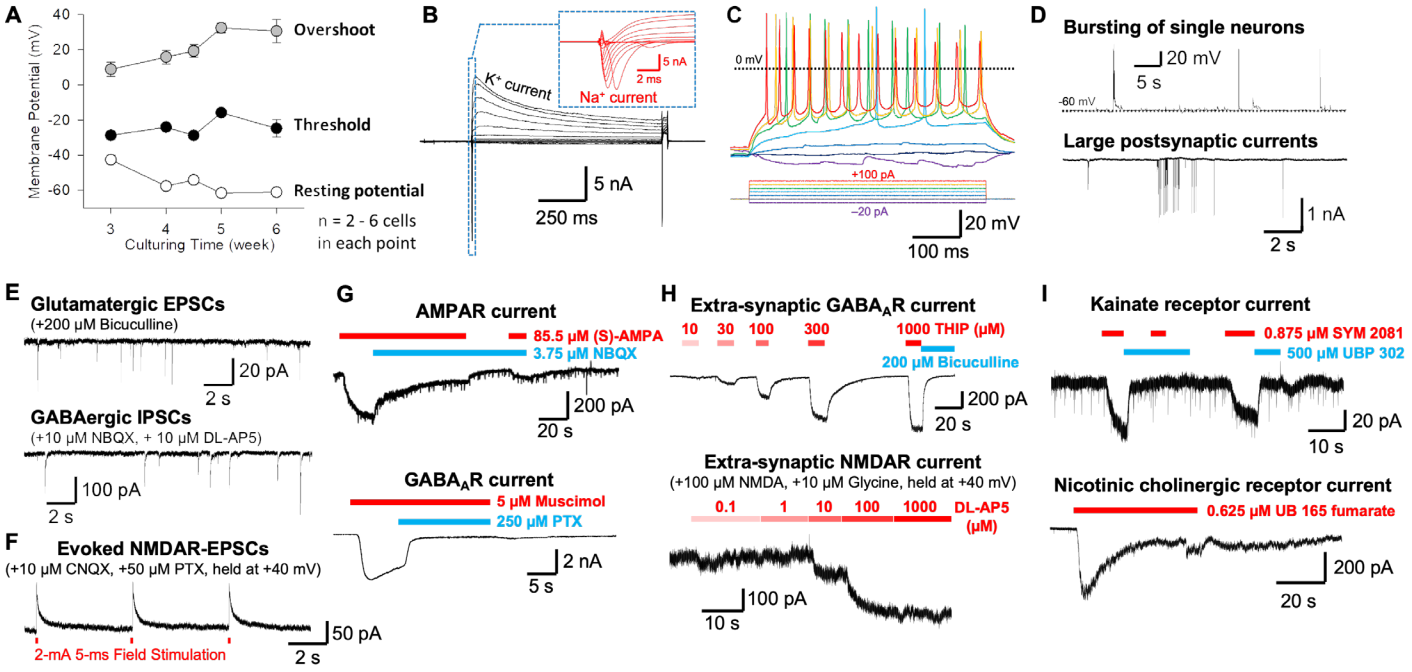


Figure 2. SynFire® iNs demonstrate principal neurophysiological properties

(A) SynFire neural cultures rapidly mature with five weeks, reaching a resting membrane potential ≤ −60 mV and showing stable excitability in both action potential threshold and overshoot value. Patch-clamp studies show intrinsic and extrinsic properties in mature SynFire neural cultures, including **(B)** voltage-dependent K^+- and Na^+-currents, **(C)** evoked action potential firings, **(D, top)** bursting of single neurons, and **(D, bottom)** large postsynaptic currents indicating advance synaptic competence. **(E)** Pure SynFire subtype cultures of either **(top)** only excitatory iNs or **(bottom)** only inhibitory iNs exclusively show glutamate mediated excitatory postsynaptic currents (EPSCs) or GABA-mediated inhibitory postsynaptic currents (IPSCs), respectively. **(F)** Showing robust NMDA currents, mature SynFire neural cultures are suited for studying short- and long-term plasticity. **(G–I)** The function of ionic receptors expressed in SynFire iNs were determined by micro perfusion of their agonists (red) or antagonists (blue), including **(G, top)** the AMPA-, **(G, bottom)** the GABAA-, **(H, top)** the extra-synaptic $GABA_A$-, **(H, bottom)** the extra-synaptic NMDA-, **(I, top)** the kainate-, and **(I, bottom)** the nicotinic cholinergic receptor.

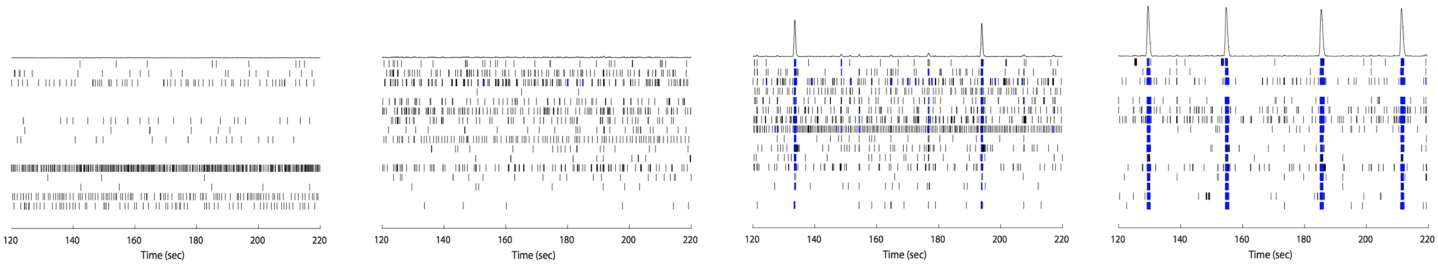


Figure 3. MEA ontogeny demonstrates neural network activity maturation of the SynFire co-culture platform.

SynFire iNs in co-culture (52% Glutamatergic, 22% GABAergic neurons and 26% human astrocytes) mature and exhibit synchronous bursting after 4 weeks when assessed by MEA (Axion 48-well plates). Representative raster plots from the MEA recordings of SynFire co-cultures are shown (weeks 1-4).

2

List of Catalog Products

Catalog #	Product name	Part #	Component	Details
1010-1.5	SynFire Co-Culture Kit (Basic)	1001-1.5	Glutamatergic Induced Neurons	>1.5 x 10^6 cells/ vial
		1002-1.5	GABAergic Induced Neurons	>1.5 x 10^6 cells/ vial
		1003-1.5	Astrocytes	>1.5 x 10^6 cells/ vial
		2001-10	Seeding Basal Media	Enough for 10 ml
		2001S-10	Seeding Supplement	Enough for 10 ml
		2002-20	Short-Term Basal Media	Enough for 20 ml
		2002S-20	Short-Term Supplement	Enough for 20 ml
		2003-60	Long-Term Basal Media	Enough for 60 ml
		2003S-60	Long-Term Supplements	Enough for 60 ml
1010-7.5	SynFire Co-Culture Kit (MEA)	1001-7.5	Glutamatergic Induced Neurons	>7.5 x 10^6 cells/ vial
		1002-3.5	GABAergic Induced Neurons	>3.5 x 10^6 cells/ vial
		1003-3.5	Astrocytes	>3.5 x 10^6 cells/ vial
		2001-20	Seeding Basal Media	Enough for 20 ml
		2001S-20	Seeding Supplement	Enough for 20 ml
		2002-40	Short-Term Basal Media	Enough for 40 ml
		2002S-40	Short-Term Supplement	Enough for 40 ml
		2003-120	Long-Term Basal Media	Enough for 120 ml
		2003S-120	Long-Term Supplement	Enough for 120 ml
1001-10	SynFire Glutamatergic Induced Neuron Kit (Small)	1001-1.5	Glutamatergic Induced Neurons	>1.5 x 10^6 cells/ vial
		1003-1.5	Astrocytes	>1.5 x 10^6 cells/ vial
		2001-10	Seeding Basal Media	Enough for 10 ml
		2001S-10	Seeding Supplement	Enough for 10 ml
		2002-20	Short-Term Basal Media	Enough for 20 ml
		2002S-20	Short-Term Supplement	Enough for 20 ml
		2003-60	Long-Term Basal Media	Enough for 60 ml
		2003S-60	Long-Term Supplements	Enough for 60 ml
1001-20	SynFire Glutamatergic Induced Neurons (Small)	1001-1.5	Glutamatergic Induced Neurons	>1.5 x 10^6 cells/ vial

Catalog #	Product name	Part #	Component	Details
1001-50	SynFire Glutamatergic Induced Neuron Kit (Large)	1001-7.5	Glutamatergic Induced Neurons	>7.5 x 10^6 cells/ vial
		1003-3.5	Astrocytes	>3.5 x 10^6 cells/ vial
		2001-20	Seeding Basal Media	Enough for 20 ml
		2001S-20	Seeding Supplement	Enough for 20 ml
		2002-40	Short-Term Basal Media	Enough for 40 ml
		2002S-40	Short-Term Supplement	Enough for 40 ml
		2003-120	Long-Term Basal Media	Enough for 120 ml
		2003S-120	Long-Term Supplement	Enough for 120 ml
1001-60	SynFire Glutamatergic Induced Neurons (Large)	1001-7.5	Glutamatergic Induced Neurons	>7.5 x 10^6 cells/ vial
1002-10	SynFire GABAergic Induced Neuron Kit (Small)	1002-1.5	GABAergic Induced Neurons	>1.5 x 10^6 cells/ vial
		1003-1.5	Astroglia	>1.5 x 10^6 cells/ vial
		2001-10	Seeding Basal Media	Enough for 10 ml
		2001S-10	Seeding Supplement	Enough for 10 ml
		2002-20	Short-Term Basal Media	Enough for 20 ml
		2002S-20	Short-Term Supplement	Enough for 20 ml
		2003-60	Long-Term Basal Media	Enough for 60 ml
		2003S-60	Long-Term Supplements	Enough for 60 ml
1002-20	SynFire GABAergic Induced Neurons (Small)	1002-1.5	GABAergic Induced Neurons	>1.5 x 10^6 cells/ vial
1002-50	SynFire GABAergic Induced Neuron Kit (Large)	1002-3.5	GABAergic Induced Neurons	>3.5 x 10^6 cells/ vial
		1003-3.5	Astroglia	>3.5 x 10^6 cells/ vial
		2001-20	Seeding Basal Media	Enough for 20 ml
		2001S-20	Seeding Supplement	Enough for 20 ml
		2002-40	Short-Term Basal Media	Enough for 40 ml
		2002S-40	Short-Term Supplement	Enough for 40 ml
		2003-120	Long-Term Basal Media	Enough for 120 ml
		2003S-120	Long-Term Supplement	Enough for 120 ml
1002-60	SynFire GABAergic Induced Neurons (Large)	1002-3.5	GABAergic Induced Neurons	>3.5 x 10^6 cells/ vial
2010-10	SynFire Complete Media Kit (Small)	2001-10	Seeding Basal Media	Enough for 10 ml
		2001S-10	Seeding Supplement	Enough for 10 ml
		2002-20	Short-Term Basal Media	Enough for 20 ml
		2002S-20	Short-Term Supplement	Enough for 20 ml
		2003-60	Long-Term Basal Media	Enough for 60 ml
		2003S-60	Long-Term Supplement	Enough for 60 ml

Catalog #	Product name	Part #	Component	Details
2010-20	SynFire Complete Media Kit (Large)	2001-20	Seeding Basal Media	Enough for 20 ml
		2001S-20	Seeding Supplement	Enough for 20 ml
		2002-40	Short-Term Basal Media	Enough for 40 ml
		2002S-40	Short-Term Supplement	Enough for 40 ml
		2003-120	Long-Term Basal Media	Enough for 120 ml
		2003S-120	Long-Term Supplement	Enough for 120 ml
2003-1	SynFire Media (Long Term Maintenance)	2003-120	Long-Term Basal Media	Enough for 120 ml
		2003S-120	Long-Term Supplement	Enough for 120 ml
1003-1.5	Astrocytes	1003-1.5	Astrocytes	>1.5 x 10^6 cells/ vial
1003-3.5	Astrocytes	1003-3.5	Astrocytes	>3.5 x 10^6 cells/ vial
20011-SDCM	Seeding Complete Media (small)	2001-10	Seeding Basal Media	Enough for 10 ml
		2001S-10	Seeding Supplement	Enough for 10 ml
20012-SDCM	Seeding Complete Media (large)	2001-20	Seeding Basal Media	Enough for 20 ml
		2001S-20	Seeding Supplement	Enough for 20 ml
20021-STCM	Short Term Complete Media (small)	2002-20	Short-Term Basal Media	Enough for 20 ml
		2002S-20	Short-Term Supplement	Enough for 20 ml
20022-STCM	Short Term Complete Media (large)	2002-40	Short-Term Basal Media	Enough for 40 ml
		2002S-40	Short-Term Supplement	Enough for 40 ml
2003-2	Long Term Complete Media (small)	2003-60	Long-Term Basal Media	Enough for 60 ml
		2003S-60	Long-Term Supplement	Enough for 60 ml
1010-CUS	Custom Packaging of Cells			
1020-CUS	Custom Packaging of Media			

3

General Guidelines

Handling, Storage and Use of Consumables

Shipping

	Dry Ice	Blue Ice	Room Temperature
Cells	X		
Culture Supplements	X		
Basal Media		X	X

Storage and Stability

Store everything properly and immediately upon receipt, according to the specifications in this section.
Astrocytes should always be stored in the vapor phase of liquid nitrogen. Store iNs at −80°C for short-term storage for no more than 3 months. For long-term storage for longer than 3 months, iNs should be stored in the vapor phase of liquid nitrogen. Basal media should be stored at 4°C. Media supplements should be stored at −80°C.

Note: This product is stable for at least 6 months from the date of receiving when stored as directed.

Quality Control

Structural validation of SynFire iNs and astrocytes was performed by immunostaining. Functional validation of maturation and neural network formation potential has been done on MEA. All cells have been tested for the absence of pathogens (i.e., mycoplasma, etc). Full characterization is included in the Certificate of Analysis.

Safety Precautions

Important: **BEFORE HANDLING FROZEN VIALS:** Always wear the appropriate Personal Protection Equipment (lab coat, thermal gloves, safety goggles and a face shield) when handling the cells. Handle the frozen vials with due caution.

Warning: Be aware that the following scenario can occur: Liquid nitrogen can leak into the vials when the vials are submerged in liquid nitrogen. Upon thawing, the liquid nitrogen returns to the gas phase, resulting in a dangerous build-up of pressure within the vial. This can result in the explosion of the vial and expelling not only the vial contents but also the vial cap and plastic fragments of the vial.

Media

Important: Before use, add the entire volume of contained in each Supplement to the corresponding labeled Basal Media bottle as follows:

Note: Add supplement to basal media **immediately before** use. To prevent media from expiring prior to completion of your assays, do not prepare media too early in the workflow. Components in the supplements are light-sensitive, therefore, always protect the media bottles from light after supplement addition.

- **For Seeding Media.** Transfer the entire volume of the Seeding Supplement into the Seeding Basal Media bottle.
- **For Short-Term Media.** Transfer the entire volume of the Short-Term Supplement into the Short-Term Basal Media bottle.
- **For Long-Term Media.** Transfer the entire volume of the Long-Term Supplement into the Long-Term Basal Media bottle.
- When pre-warming media for media exchanges, **only pre-warm the volume of media needed** for **each exchange.** To prevent media from expiring before completion of the assay, do not pre-warm the entire bottle of media for every media exchange.

4

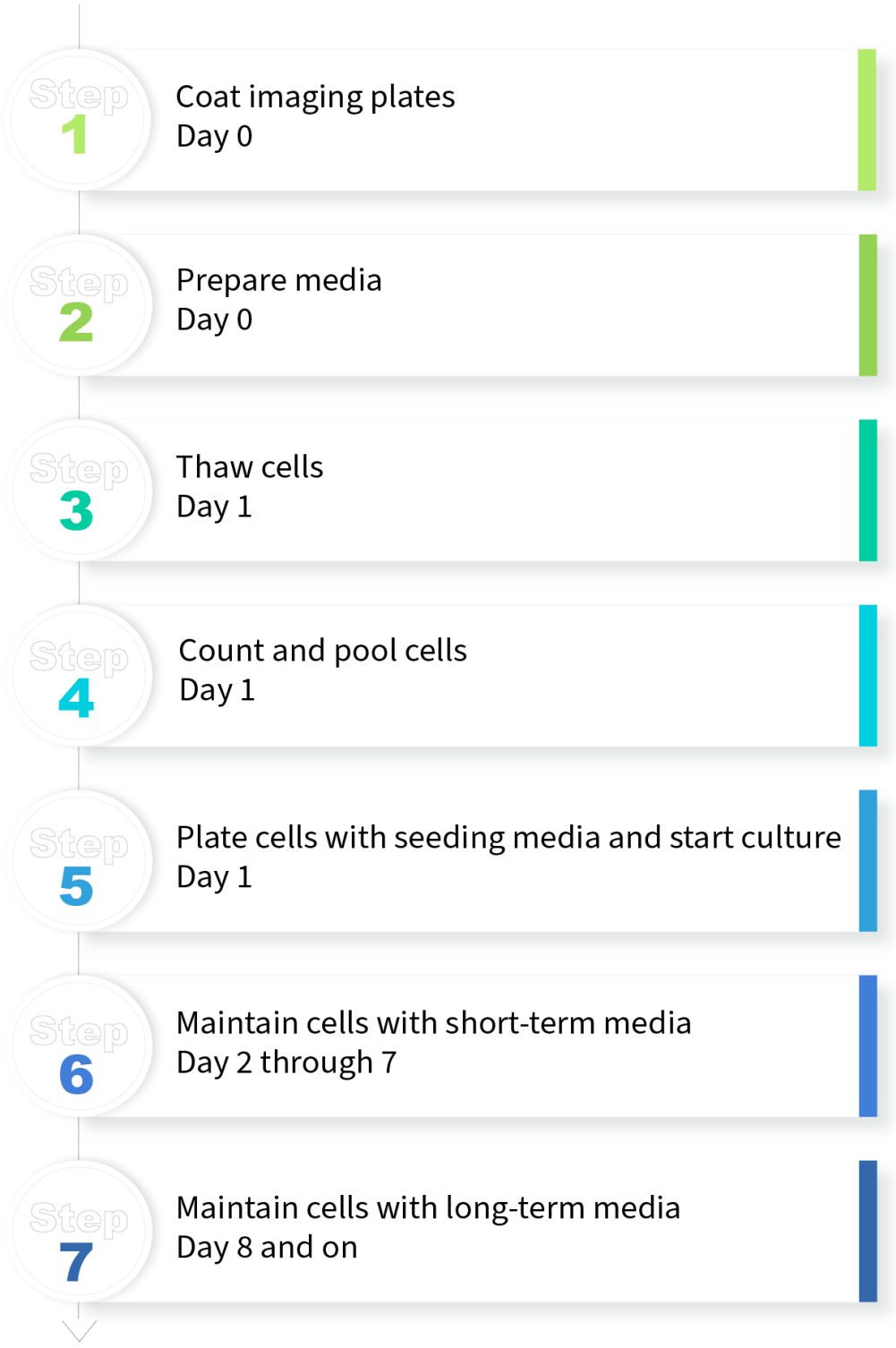
Seeding and Maintenance of iN-Astrocyte Co-Culture for Imaging

This procedure will guide you through thawing, seeding, and maintenance of glutamatergic iNs, GABAergic iNs, and primary astrocytes as a co-culture in a defined ratio for imaging applications.

CONSUMABLES FROM OTHER SUPPLIERS

Item	Suggested Supplier	Suggested Catalog Number
Laminin	Gibco	23017015
PBS	Gibco	14190-144
PhenoPlate™ 96-well imaging plate	PerkinElmer	6055300
PhenoPlate™ 384-well imaging plate	PerkinElmer	6057300

Workflow


Step 1
Coat imaging plates
Day 0
Step 2
Prepare media
Day 0
Step 3
Thaw cells
Day 1
Step 4
Count and pool cells
Day 1
Step 5
Plate cells with seeding media and start culture
Day 1
Step 6
Maintain cells with short-term media
Day 2 through 7
Step 7
Maintain cells with long-term media
Day 8 and on

Seeding of iN-Astrocyte Co-Culture

Coating Imaging Plates

1. Prepare a 20 µg/ml laminin solution by adding laminin stock to PBS. Repeat as needed to ensure sufficient volume for all wells of the microplate.
   Important: Always prepare this solution fresh at the time of use.
2. For a 96-well plate, add 70 µl of prepared laminin solution to each well; for a 384-well plate add 50 µl of diluted laminin solution to each well.
3. Incubate plate for at least 3 hours at 37°C.
   Important: Make sure you have thawed the material you will need for pooling cells prior to starting.
   See Prepare media for more information.

Thawing and Plating Cells

- Important: This section is time sensitive. Perform these steps as quickly as possible to minimize a decrease in cell viability

1. Thaw cryopreserved cell vials in a 37°C water bath with gentle agitation until only very small ice crystals are visible (~2 mins). Keep the cap above the water line to minimize the risk of contamination.
2. Upon removal from the water bath, spray each vial with the ethanol (70%), then let air dry in the cell culture hood.
3. Add 1 ml of pre-warmed Seeding Media to each vial of cells, bringing the total volume to 2 ml. (By exposing the cells to this small amount of Seeding Media, the risk of the cells suffering effects of osmotic shock is greatly reduced).
4. Separately, by each cell type, use a P1000 pipette to transfer the cells from the vial to the 15 ml conical tube containing the pre-warmed Seeding Media.
5. With a 5 ml serological pipette, gently mix the cell suspension (up and down several times) to ensure a uniform single-cell suspension.
6. Gently invert each conical tube several times, then count cells (See appendix).
   Note: To calculate the total number of cells needed, use this equation:

   Cells per well x Number of wells to seed x 1.125 pipetting error

Item	96-well plate	384-well plate
Glutamatergic iNs	140 K	42 K
GABAergic iNs	60 K	18 K
Astrocytes	70 k	21 K
Seeding Media*	50 µl	25 µl

*Use the number of need-to-seed wells x 1.125 to calculate the total volume of Seeding Media needed.

7. Calculate the volume of each cell solution needed. (For example, each well of a 96-well plate should have 1.4 x 10^5 glutamatergic iNs, 6 x 10^4 GABAergic iNs, and 7 x 10^4 astrocytes).
8. Gently invert each cell source tube prior to dispensing the calculated volume needed for each cell type into a new conical tube.
9. Centrifuge the tube containing the pooled cells for 4 minutes at 300 g at room temperature.
10. Carefully aspirate as much of the supernatant, without disturbing the cell pellet.

11. Resuspend the pooled cell pellet in pre-warmed Seeding Media (37°C)
12. Use the pipette to mix up and down several times to ensure a single-cell suspension.

Plating Cells Onto Imaging Plates

1. Aspirate laminin solution thoroughly from each well of a 96-well plate.
2. For 96-well plates, add 50 µl of pooled cell mix to each coated well; for 384-well plates, add 25 µl of pooled cell mix to each coated well.
3. Store seeded plates in an incubator at 37°C, 5% CO_2, and 95% humidity.

iN-Astrocyte Co-Culture Maintenance for Imaging

The day of seeding is DPP (Days Post Plating) 0. To maintain the co-culture, the media must be changed periodically starting from DPP 1. The media volumes are specific for each plate format, as follows:

- **For 96-well plates.** Half media change = remove 150 µl of old media, then add 150 µl new media.
- **For 384-well plates.** Half media change = remove 45 µl of old media, then add 45 µl new media.
- **For cover slips in 24-well plates.** Half media change = remove 400 µl of old media, then add 400 µl of new media.

Important: Media must be pre-warmed to 37°C before use. Media are light- and temperature-sensitive. Do not warm up the entire bottle of media; only take the amount needed for pre-warming. Leave the remainder in the dark and store at 4°C.

Maintenance Schedule

# of Days Post Platting (DPP)	Task	Configuration	Action
1	Add freshly made Short-Term Media	96-well plates	Add 250 µl of new Short-Term Media to each well
		384-well plates	Remove 10 µl of old media from wells; add 75 µl of new Short-Term Media to each well
		Cover slips	Remove 400 µl of old media; add 200 µl of new Short-Term Media to each well
3	Change Short-Term Media	96-well plates	Do a half media change of Short-Term Media
		384-well plates	Do a half media change of Long-Term Media
		Cover slips	Remove 2000 µl of old media; add 400 µl of new Short-Term Media to each well
5	Change Short-Term Media	All	Do a half media change of Short-Term Media
7	Change Long-Term Media	All	Do a half media change of Long-Term Media
After 7- Assay Completion	Change Long-Term Media	All	Do a half media change of Long-Term Media every 2-3 days until your assay is complete

For example, the following table could represent your maintenance schedule for the remainder of your assay when doing experiments on Days Post Plating (DPP) 23:

DPP	Action
9/10	Half media change: Long-Term Media
11/12	Half media change: Long-Term Media
14	Perform an ontogeny recording, then do a full media change: Long-Term Media
16/17	Half media change: Long-Term Media
18/19	Half media change: Long-Term Media
21	Perform an ontogeny recording, then do a full media change: Long-Term Media
23	Completing experiment

For example, the following table could represent your maintenance schedule for the remainder of your assay when doing experiments on DPP 35:

DPP	Action
9/10	Half media change: Long-Term Media
11/12	Half media change: Long-Term Media
14	Perform an ontogeny recording, then do a half media change: Long-Term Media
16/17	Half media change: Long-Term Media
18/19	Half media change: Long-Term Media
21	Perform an ontogeny recording, then do a full media change: Long-Term Media
23/24	Half media change: Long-Term Media
25/26	Half media change: Long-Term Media
28	Perform an ontogeny recording, then do a half media change: Long-Term Media
30/31	Half media change: Long-Term Media
32/33	Full media change: Long-Term Media
35	Completing experiment

5

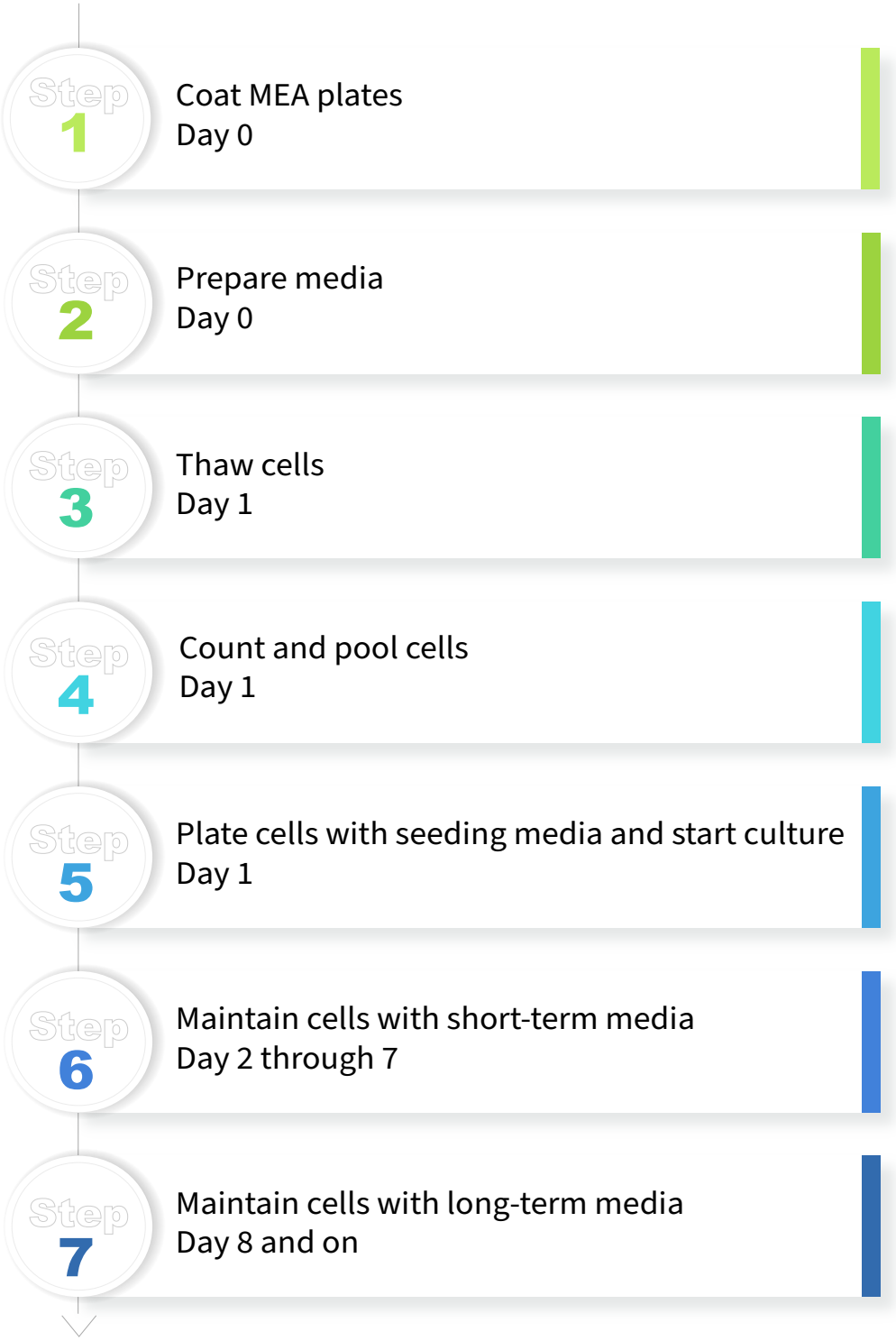
Seeding and Maintenance of iN-Astrocyte Co-Culture for MEA (Microelectrode Array) Assays

This procedure will guide you through thawing and seeding glutamatergic iNs, GABAergic iNs, and primary astrocytes as a co-culture in a defined ratio on a 48-well Axion MEA.

Consumables from Other suppliers

Item	Suggested Supplier	Suggested Catalog Number
Cytoview MEA Plate (48-well)	Axion	M768-tMEA-48B or M768-tMEA-48W
Polyethylenimine (PEI)	Sigma Aldrich	P3143
Borate Buffer	Sigma Aldrich	89273
Laminin	Gibco	23017015
PBS	Gibco	14190144

Workflow


Step 1
Coat MEA plates
Day 0
Step 2
Prepare media
Day 0
Step 3
Thaw cells
Day 1
Step 4
Count and pool cells
Day 1
Step 5
Plate cells with seeding media and start culture
Day 1
Step 6
Maintain cells with short-term media
Day 2 through 7
Step 7
Maintain cells with long-term media
Day 8 and on

Seeding of iN-Astrocyte Co-Culture for MEA

PEI Coating

1. Prepare a 0.1% PEI solution by adding 555.5 µl of 9% PEI to 49.4 ml of borate buffer.
2. Pass the 0.1% PEI solution through a 0.22 µM filter to sterilize.
   Important: This 0.1% solution can be stored for up to 2 weeks at 4°C.
3. Add 70 µl of 0.1% PEI solution to each well of a 48-well Cytoview MEA plate.
   Important: Be cautious to not touch the bottom of the wells with the pipette.
4. Add approximately 6 ml of sterile water to fill the humidity reservoirs on the sides of the plate to reduce media evaporation, that is, 3 ml on each side.
5. Incubate overnight at 37°C.

Laminin Coating

1. Prepare a 20 µg/ml laminin solution by adding laminin stock to PBS.
   Important: Always freshly prepare this solution at the time of use.
2. Aspirate PEI solution and thoroughly rinse each well 3x with 800 µl of sterile water using a P1000 multichannel pipette.
   Important: Be cautious to not touch the bottom of the wells with the pipette. Be sure to aspirate all water droplets from every well after the last rinse.
3. Add 70 µl of the prepared laminin solution to each well of the MEA plate and incubate for at least 1 hour at 37°C. Leave laminin until ready to plate the cells. Use plates within 48 hours after laminin coating.

Preparing media

1. Label a 15 ml conical tube for each cell type to be thawed.
2. Add 3 ml of Seeding Basal Media to each tube for each vial of cells to be thawed, then place the tubes in a 37°C incubator to warm for more than 30 minutes.
3. Make fresh seeding media by adding 1 full tube of Seeding Media Supplement (stored at –80°C) into 18.272 ml of Seeding Basal Media.
4. Transfer the exact amount of Seeding Media needed (2.5 ml/plate) to a new 15 ml conical tube, then place in a 37°C incubator to warm for 30 minutes.
5. Store the remaining Seeding Media Supplement at 4°C.
   Important: Supplements in Seeding Media are temperature- and light-sensitive. Once they are combined with the basal media, they need to be used immediately, or stored in the dark at 4°C up to 2 days.
6. Prepare for cell counting.

Thawing and Pooling Cells

Important: This section is time sensitive. Perform these steps as quickly as possible to reduce cell death!

1. Thaw vials containing cells in a 37°C water bath with gentle agitation. Incubate until only a small ice crystal remains (~2 mins). Keep the cap above the water line to minimize the risk of contamination. Upon removing the vials from the water bath, spray them with ethanol (70%), then dry the vials with a Kimwipe before placing

them under the cell culture hood.

2. Add 1 ml of pre-warmed Seeding Basal Media to each vial of cells, which will bring the total volume in the vial to 2 ml. (By exposing the cells to this small amount of Seeding Basal Media, the risk of the cells suffering effects of osmotic shock is greatly reduced).
3. Separately, by each cell type, use a P1000 pipette to transfer the cells from the vial to the 15mL conical tube containing the pre-warmed Neurobasal A media.
4. Use a 5 ml serological pipette to mix up and down gently several times to ensure a single-cell suspension.
5. Gently invert each conical tube several times. Take 10 µl of cells for cell counting according to SOP 2.001.
6. Calculate the volume of each cell solution needed. Each well of a 48-well MEA plate should have 1.4 x 10^5 glutamatergic iNs, 6 x 10^4 GABAergic iNs, and 7 x 10^4 astrocytes. Use 50 wells (48 + 2 extra) per plate for your calculations. That is, 7 x 10^6 glutamatergic, 3 x 10^6 GABAergic, and 3.5 x 10^6 astrocytes are needed for each plate.
   Important: Keep track of these calculations for your record.
7. Pool the volumes calculated into a new 15 ml conical tube, then gently invert each source tube before pipetting from it.
8. Centrifuge the tube containing the pooled cells for 4 minutes at 300 g at room temperature.
9. Carefully aspirate all the supernatant, then re-suspend the cells in pre-warmed Seeding Media. For 50 wells, re-suspend in 2.5 ml of Seeding Media.
10. Pipette mix several times to ensure a single-cell suspension.

Seeding Plates

1. Aspirate the laminin solution thoroughly from all wells of the MEA. Aspirate all droplets of laminin but be careful **not** to touch the bottom of the wells with the pipette.
2. Add 50 µl of cell mixture to each well. Gently mix the cell solution in the tube up and down with the pipette in between seeding each well. Only dispense to the first pipette stop to prevent introduction of bubbles. Make sure the droplet falls within the center part of the well where the electrode array is, and that the entire electrode array is covered by the liquid.
3. Store the MEA plate in an incubator at 37°C, 5% CO_2, and 95% humidity.

Warning: Electrodes are located at the bottom center of an MEA well. Do not touch the bottom of the wells with the pipette when adding liquid, including cells! Aim the pipette tip precisely so that the liquid drops into the center of the wells to cover the electrodes.

MEA Maintenance

This procedure will guide you through maintaining a seeded 48-well Axion MEA plate to support neural network formation.

Maintain the Co-Culture

To maintain the co-culture, the media must be changed every 2-3 days.
There are two types of media changes:

- **Half-media change.** Remove 150 µl of old media from each well, then add 150 µl of new media.
- **Full-media change.** Remove 300 µl of old media from each well, then add 300 µl of new media.

Tips and Techniques

- Media should be pre-warmed to 37°C before use. Media are light- and temperature-sensitive. Do not warm up the entire bottle of media. Only take the amount needed to pre-warm. Leave the remaining in the dark at 4°C.
- When performing media changes, point the pipette tip at the side of the wall in a well, and pipette slowly so that the cells at the bottom are not disturbed.
- We do not recommend changing the media within 48 hours prior to any recording as medium change may disturb the network behavior. For media changes that fall on the same day as an ontogeny recording, do the media change after the recording.
- During every media change, inspect the plate for following:
    a. Inspect the water level of the humidity reservoirs. Replenish with sterile water if the water level is low. This reduces media evaporation.
    b. Inspect the media level in each well. If the volume for any wells becomes noticeably low due to evaporation, add 150 µl media and do not remove any media.
    c. Inspect each well by eye for signs of contamination. With the lid off (in the hood), the gold circuitry at the bottom of each well should be clearly visible and shiny. If the media in any of the wells is cloudy, the plate must be immediately discarded.

Maintenance Schedule

# of Days Post Platting (DPP)	Task	Action
1	Add freshly made Short-Term Media	Add 250 µl of new Short-Term Media to each well
3	Change Short-Term Media	Do a half media change of Short-Term Media
5	Change Short-Term Media	Do a half media change of Short-Term Media
7	Ontogeny reading Change Short-Term Media	Do a half media change of Short-Term Media
After 7- Assay Completion	Change Long-Term Media	Do Long-Term Media changes every 2-3 days until your assay is completed. Change a half volume (150 µl of old media each time except for the change occurring 2 days before a dosing experiment on which the change should be full volume (300 µl) of media

For example, the following table could represent your maintenance schedule for the remainder of your assay when doing experiments on Days Post Plating (DPP) 23:

DPP	Action
9/10	Half media change: Long-Term Media
11/12	Half media change: Long-Term Media
14	Perform an ontogeny recording, then do a full media change: Long-Term Media
16/17	Half media change: Long-Term Media
18/19	Half media change: Long-Term Media
21	Perform an ontogeny recording, then do a full media change: Long-Term Media
23	Completing experiment

For example, the following table could represent your maintenance schedule for the remainder of your assay when doing experiments on DPP 35:

DPP	Action
9/10	Half media change: Long-Term Media
11/12	Half media change: Long-Term Media
14	Perform an ontogeny recording, then do a half media change: Long-Term Media
16/17	Half media change: Long-Term Media
18/19	Half media change: Long-Term Media
21	Perform an ontogeny recording, then do a full media change: Long-Term Media
23/24	Half media change: Long-Term Media
25/26	Half media change: Long-Term Media
28	Perform an ontogeny recording, then do a half media change: Long-Term Media
30/31	Half media change: Long-Term Media
32/33	Full media change: Long-Term Media
35	Completing experiment

Appendix

Counting cells with trypan blue and countess ii

This section provides an optional method for cell counting as a reference in case you haven't established your own method.

Materials and Reagents

Product/ Reagent:

- Trypan Blue stain (0.4%), Thermo Fisher
  - Countess cell counting chamber slide, Thermo Fisher
  - Countess ll Automated Cell Counter, Thermo Fisher

Safety Concerns

Trypan Blue stain (0.4%): no physical hazards, possibly carcinogenic, may cause eye or skin irritation and be harmful if inhaled or swallowed.

Procedure

PREPARATION FOR COUNTING CELLS

1. Depending on the number of cell types needed for your experiment (i.e., excitatory, inhibitory, astrocytes), prepare the appropriate number of 1.5 ml Eppendorf tubes, labeling each tube appropriately.
2. Add 10 µl of Trypan Blue dye to each prepared tube. May leave until ready to count.

PREPARATION OF CELL MIXTURE

There are various ways to prepare cell mixture, but use this general guideline:

Note the following:

- Type of cell
- Total volume
- Total amount of cells needed

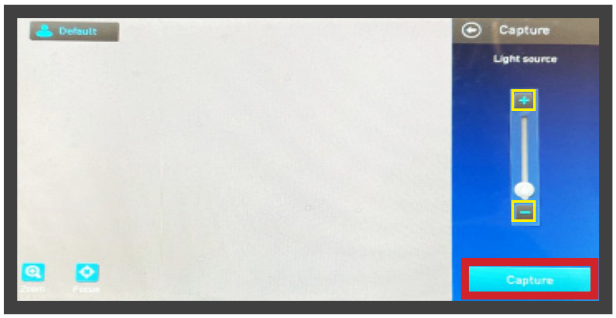
PREPARATION FOR COUNTING CELLS

1. When cell suspension is prepared and thoroughly mixed add 10 µl of cell suspension mixture to the 1.5 ml Eppendorf tube already containing the Trypan Blue dye.
2. Mix the cell suspension and Trypan blue dye thoroughly.
3. Using the Countess cell counting chamber slide, pictured below, label sides A and B with the appropriate cell name.

4. Pipette 10 µl to the appropriate sides for counting.

5. Turn on Countess II Automated Cell Counter (ON/OFF switch is located in the back of the instrument).
6. Insert slide into the Countess II Automated Cell Counter, pictured below.

7. An image (similar to the one pictured below) will pop up. Adjust the light source using the + or - buttons to best visualize cells. Wait until cells are in focus and press the Capture button.



8. A results page similar to the one depicted below should pop up.

*** Note the following information:

- Total concentration (given in cells/ ml)
- Live (%)

9. Remove the slide from the cell counter and if necessary, do the alternate side. Dispose.
10. Turn off the Countess II Automated Cell Counter.

Calculating total number of cells and volume needed

- To calculate the Total Number of Cells in the total volume:

  Total Concentration (# of cells/ ml) X Total Volume (ml) = Total # of cells

  For example, if the Total volume of the Cell suspension was 4 ml and the total concentration of cells was found to be 2.00 x 10^6 cells/ ml, then the total number of cells should be

  (2.00 x 10^6 cells/ ml) x (4.00 ml) = 8.00 x 10^6 cells

- To calculate the Volume of the Cells needed for the experiment:

  Number of Cells Needed (# of cells) / Total Number of Cells in Total Volume (# of cells/ # ml) = Volume needed

  For example, if the total number of cells needed for the experiment is 6.50 x 10^6 cells and you have a total of 8.00 x 10^6 cells in 4 ml, then the volume to be used for the experiment should be:

  (6.50 x 10^6 cells) / (8.00 x 10^6 cells) / (4.00 ml) = 3.25 ml

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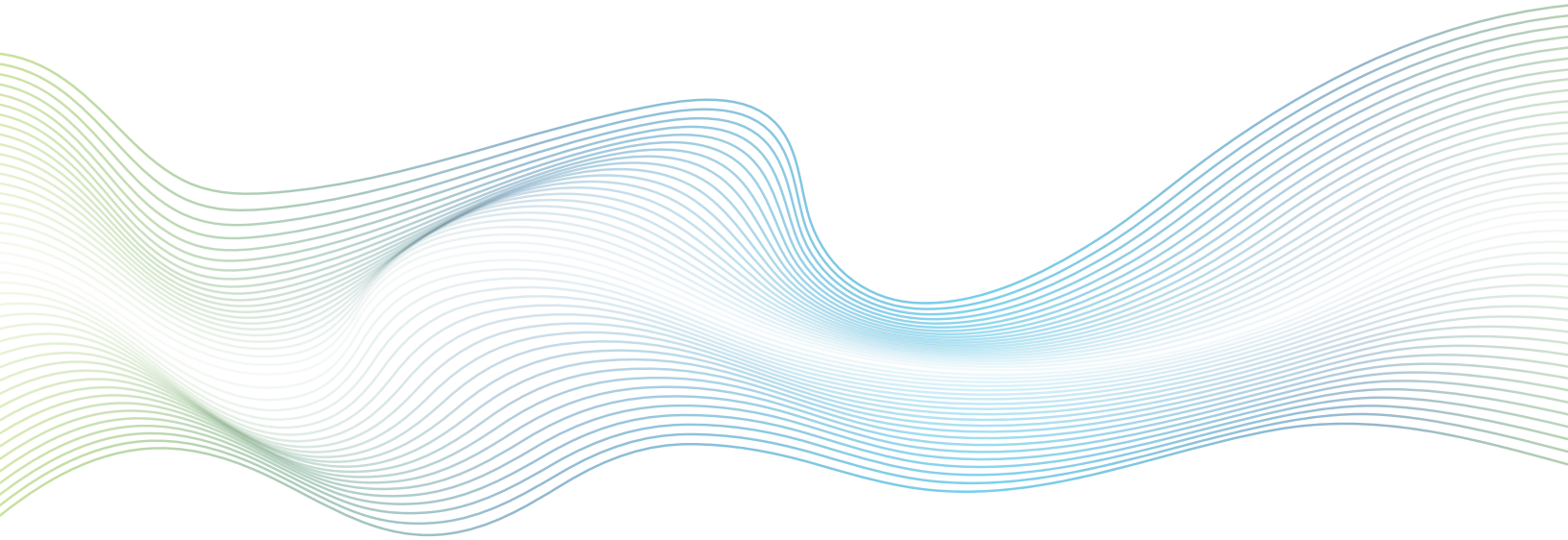
Human iPSC-Derived Induced Neurons and Co-Culture System



Technical Support: inquiries@neucytelabs.com

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