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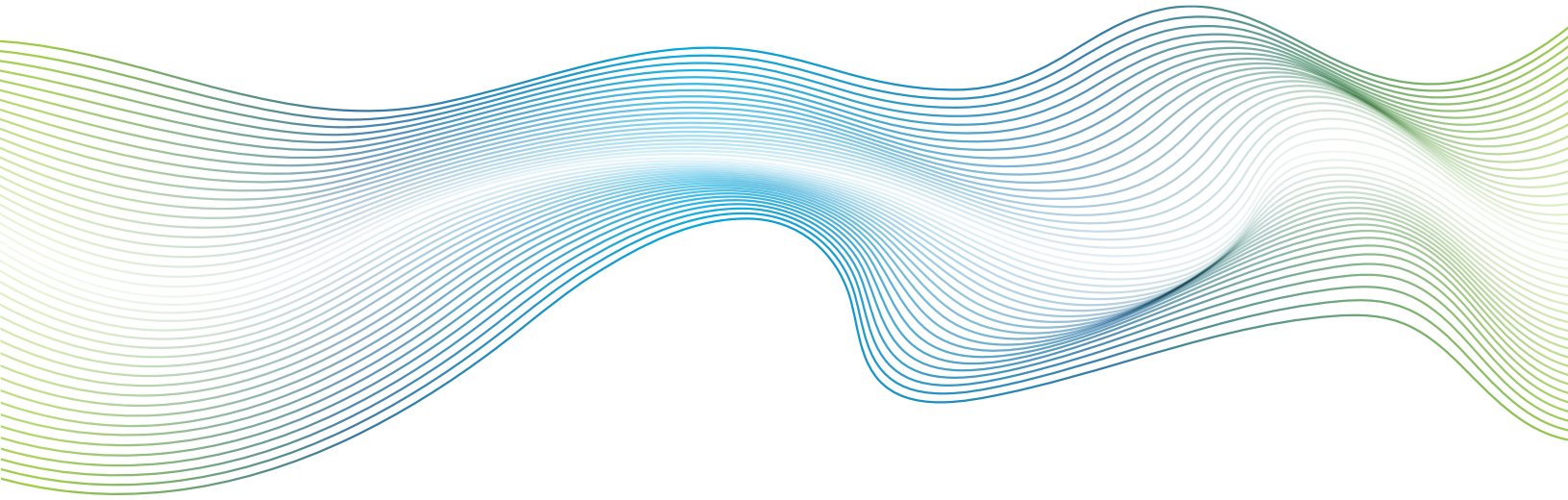
Axion MEA Protocol

Seeding and Maintenance of iN-Astrocyte Co-Cultures for MEA (Microelectrode Array) Applications



Seeding and Maintenance of iN-Astrocyte Co-Cultures for MEA (Microelectrode Array) Applications

Consumables from Other Suppliers

Item	Suggested Supplier and Catalog Number
CytoView MEA Plate (48-well)	Axion M768-tMEA-48W
Polyethylenimine (PEI) solution, 50% w/v	Sigma Aldrich P3143
Borate Buffer (pH 8.4)	Poly Scientific R&D S1605
Laminin	Gibco 23017015
PBS	Gibco 14190144
MACS® BSA Stock Solution (10%)	Miltenyi Biotec 130-091-376
Cell Counting Chamber Slides	Invitrogen C10283
Trypan Blue	Bio-Rad 1450022

Seeding of iN-Astrocyte Co-Cultures for MEA

Day –2: PEI Coating

1. Add 555.5 µl of a 9% PEI stock to 49.4 ml of borate buffer (pH 8.4) to prepare a 0.1% PEI solution.

   To make a 9% PEI stock, draw 1.8 ml of 50% PEI stock solution using a sterile 3-ml syringe and transfer to a 15-ml conical tube. Next, add 8.2 ml of borate buffer (pH 8.4) and mix thoroughly by pipetting up and down using the syringe. Store aliquots at –20°C.

2. Pass the 0.1% PEI solution through a 0.22 µM filter to sterilize.

   Important: The filtered 0.1% PEI solution can be stored for up to 1 week at 4°C.

3. Add 70 µl of 0.1% PEI solution to each well of a 48-well CytoView MEA plate.

   Important: Do not to touch the bottom of the MEA wells with the pipette and ensure that the PEI solution covers each well.

4. Incubate overnight at 37°C.

Day –1: Laminin Stock Preparation and Coating

5. Prepare a 20 µg/ml laminin stock solution.

6. Gibco laminin 1 mg stocks are received at various concentrations. Use the calculator to determine the volume of laminin per 100 µg aliquot. Thaw laminin on ice, prepare 100 µg stocks, and store at –80°C until ready to use.

   $$\frac{100}{\text{Gibco Laminin concentration}} = \boxed{\quad\text{µl}\quad}$$ volume of stock to aliquot and snap freeze

7. After pre-chilling sterile PBS on ice, thaw one aliquot of laminin on ice to prepare a 20 mg/ml stock by diluting laminin with ice cold sterile PBS (total volume 5 ml).

   Important: Always prepare laminin solution at the time of use and store on ice.

8. Aspirate the PEI solution from each well and rinse each well with 800 µl of sterile water **three times.**

   Important: Do not touch the bottom of the wells when rinsing and aspirating sterile water. Aspirate all the water from each well after the last rinse. .

9. Pipette 100 µl of the prepared ice-cold laminin solution to the center of each well of the MEA plate.

   Important: Do not touch the bottom of the wells with the pipette or introduce bubbles during the application of the laminin solution. Incubate overnight at 4°C.

10. Incubate MEA plate(s) overnight at 4°C.

11. Prior to seeding MEA plate(s), pre-warm plate(s) to room temperature.

Day 0: Media Preparation

12. For a tri-culture of glutamatergic neurons, GABAergic neurons, and primary astrocytes, label three 15-ml conical tubes, one for each of the corresponding cell types.

13. Prepare Thawing Media (3.5 ml of Neurobasal A and 0.5 ml 10% BSA Stock Solution for each cell type) and add 4 ml to each of the labeled conical tubes.

14. Prepare Seeding Media by combining Seeding Media Supplement with Seeding Basal Media according to the volumes below.
    a. Seeding Basal Media: 18.3 ml
    b. Seeding Supplement: 1.7 ml

    Important: Seeding Media must be used on the day of preparation.

15. For each cell type to be seeded, prepare one Cell Counting Chamber Slide or hemocytometer and two microcentrifuge tubes for cell counting. Pipette 10 µl of Trypan Blue into each microcentrifuge tube.

    Tip: Use Cell Counting Chamber Slides with the Countess II Automated Cell Counter. If using a different cell counting platform, adhere to the relevant manufacturer's protocol. Sterile filter the Trypan Blue to remove particulates which can affect cell counting accuracy. Label counting slides and microcentrifuge tubes with the corresponding cell type to be counted.

Day 0: Thawing, Counting, and Combining Cells for iN-Astrocyte Co-cultures

Important: This section is time sensitive. Perform these steps as quickly as possible to maximize cell viability. Keep cell suspensions on ice to maximize cell viability.

16. Thaw frozen cells by gently swirling each cryovial in a 37°C water bath for 2 minutes. Keep the cap above the water surface to minimize the risk of contamination. Upon removing the vials from the water bath, a small ice crystal should be observed within each cryotube. Spray each vial with 70% ethanol and place into a cell culture hood to air dry.

17. Remove the cap from each vial of cells and transfer the cell suspension from each cryovial to its labeled tube containing pre-warmed Thawing Media. Transfer cells with a 5-ml serological pipette by drawing up 1 ml of pre-warmed Thawing Media from the target tube, and then drawing up the cell suspension from the cryovial.

18. Using the same 5-ml serological pipette, gently pipette the cell suspension up and down 10 times to ensure a homogenous mixture of the single-cell suspension.

    Tip: If not thawing all the vials concurrently, thaw cells starting with the astrocytes, then glutamatergic neurons, and finally GABAergic neurons. Complete Steps 16–18 for each cell type before proceeding to Step 19. In the meantime, store resuspended cells on ice.

19. Count cells by gently inverting the conical tube to evenly re-mix each cell suspension and pipetting 10 µl of the cell suspension into the corresponding labeled microcentrifuge tubes containing 10 µl Trypan Blue. Mix counting sample with trypan blue thoroughly by gently pipetting up and down 10 times. Dispense 10 µl of the cell mixture into each chamber of a cell counting slide.

20. Measure cell number and viability of each cell type using an automated cell counter. Record the live cell number and cell viability. Repeat count for each cell type and calculate the average live cell number for each cell type.

21. Using the average of the **live** cell numbers, calculate the volumes of each cell solution needed for each well of a 48-well MEA plate **(1.4 x 10^5 glutamatergic neurons, 6 x 10^4 GABAergic neurons, and 7 x 10^4 astrocytes)**.

    Tip: We recommend preparing enough cells for 50 wells **(7 x 10^6 glutamatergic neurons, 3 x 10^6 GABAergic neurons, and 3.5 x 10^6 astrocytes)** to take into account pipetting error.

22. Using the volumes calculated in **Step 21**, combine the glutamatergic neurons, GABAergic neurons, and primary astrocytes needed for 50 wells into a newly labeled 15-ml conical tube.

    Tip: Gently invert the tubes of the three cell types before dispensing from each. Centrifuge the tube containing the cell mixture for 5 minutes at 300g at room temperature.

23. Centrifuge the tube containing the combined cell mixture (5 min, 300 x g, room temperature).

24. Carefully aspirate the supernatant, being careful not to aspirate the cell pellet. Gently re-suspend the cells in pre-warmed Seeding Media (2.5 ml for 50 wells) and to ensure a single-cell homogeneous suspension.

Day 0: Seeding MEA Plates

25. Transfer room temperature acclimated laminin-coated MEA plate(s) into the cell culture hood by first spraying the outside of plate with 70% ethanol and allowing the plate(s) to air dry.

26. Aspirate the laminin solution thoroughly; take care to NOT touch the bottom of the wells (the electrodes) with the pipette.

    Tip: To prevent the laminin coating from drying out, only aspirate half of the plate and proceed to seeding half the plate in **Step 27**. Complete the seeding of the first half of a plate before aspirating the laminin from the other half.

27. Dispense 50 µl of the cell mixture to each well. After seeding 8 wells, resuspend the cell solution by gently pipetting the cell mixture up and down to ensure a homogeneous cell solution is applied to each well while seeding.

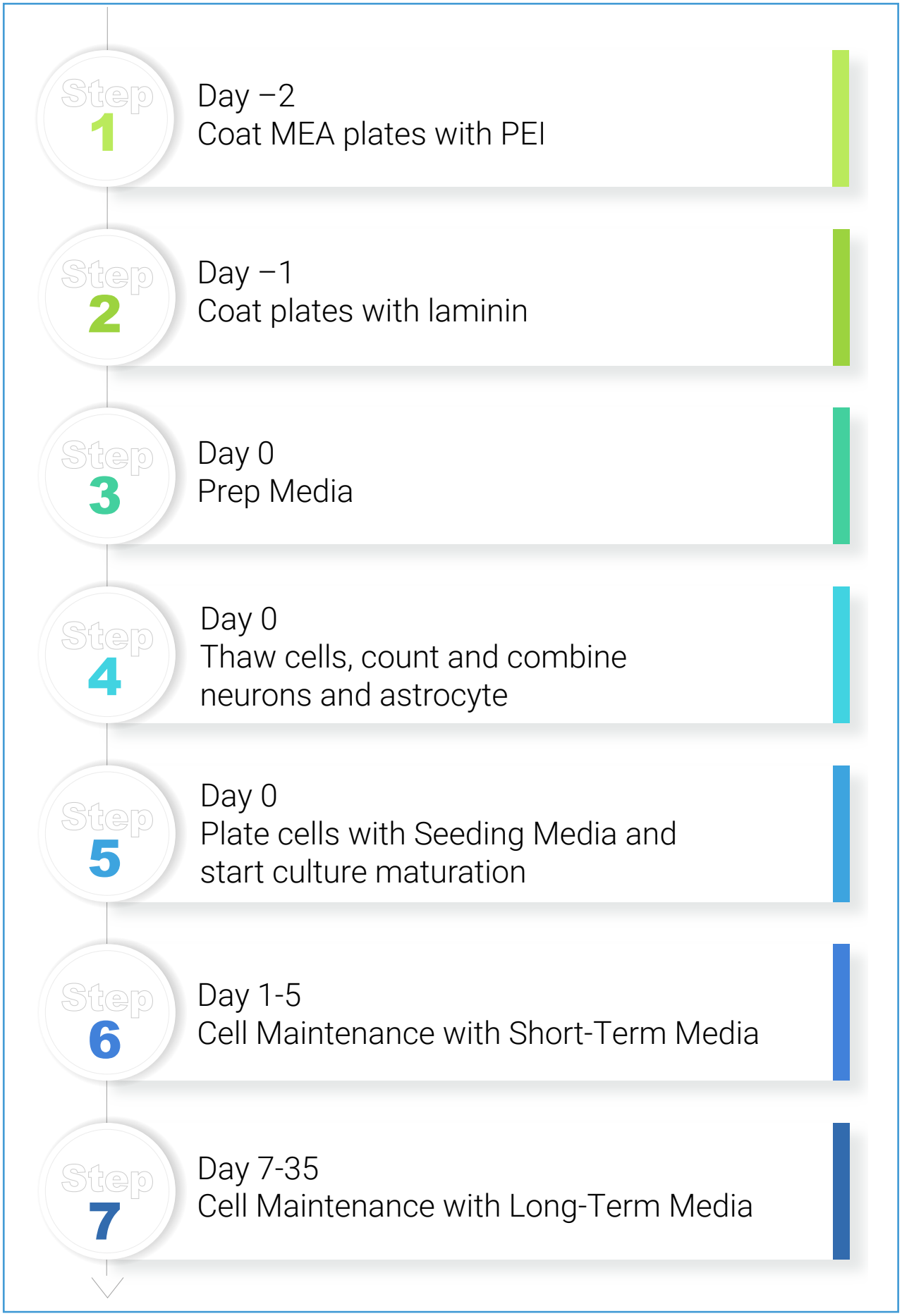
    Tip: Only dispense the cell suspension to the first pipette stop to prevent introducing bubbles. Ensure that the cell suspension droplet is applied to the center of each well where the electrodes are located, and that the entire electrode array is covered with the cell suspension in Seeding Media.

28. Incubate the MEA plate in a humidified incubator (37°C, 5% CO_2, and 95% humidity).

29. The next day {(1 Day Post Plating (1DPP)}, begin the feeding schedule with 250 µL per well of Short-Term Media as described in the maintenance schedule below.

    Warning: Electrodes are located at the bottom of each MEA well. Take care not to touch the bottom of the wells (electrodes) with the pipette when adding media.

Workflow


Step 1
Day –2
Coat MEA plates with PEI
Step 2
Day –1
Coat plates with laminin
Step 3
Day 0
Prep Media
Step 4
Day 0
Thaw cells, count and combine neurons and astrocyte
Step 5
Day 0
Plate cells with Seeding Media and start culture maturation
Step 6
Day 1-5
Cell Maintenance with Short-Term Media
Step 7
Day 7-35
Cell Maintenance with Long-Term Media

MEA Maintenance

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

Maintaining Co-Cultures

To maintain neuron-astrocyte co-cultures, change the media 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

- Pre-warm media to 37°C before use. Media are light- and temperature-sensitive. Do not warm up the entire bottle of media; only remove and pre-warm the volume of media needed for each media change. Store the remainder protected from light at 4°C.
- When performing media changes, angle the pipette tip to the side of the wall within a well, and gently aspirate or dispense media with a pipette slowly to minimize disturbance to the co-culture.
- MEA recordings should be performed at least 48 hours after media changes to minimize the effects of media change on network behavior. For media changes that fall on the same day as an ontogeny recording, perform the media change after the recording.
- Add sterile water to humidity reservoirs to minimize media evaporation.
- During every media change, inspect the plate for the following:
    a. Inspect the humidity reservoir water levels and if low, replenish with sterile water.
    b. Inspect the media level in each well and if the volume for any of the wells is noticeably low, add 150 µl of fresh media without removing any media.
    c. Inspect each well for contamination by removing the lid in a biologic safety cabinet and ensuring that the gold circuitry at the bottom of each well is clearly visible and shiny. If cloudy, contamination may be present.

Maintenance Schedule

# of Days Post Plating (DPP)	Task	Action
1	Add fresh Short-Term Media	Add 250 µl of new Short-Term Media to to 50 µl day 0 seeding volume (final volume of each well is 300 µl)
3	Short-Term Media Change	Perform a Short-Term Media half-media change
5	Short-Term Media Change	Perform a Short-Term Media half-media change
7	Ontogeny reading and Long-Term Media Change	Remove 150 µl of conditioned Short-Term Media and replace with Long-Term Media (150 µl)
Beyond 7 DPP until Assay Completion	Long-Term Media Change	Perform a Long-Term half-media change every 2-3 days. Replace half the volume (150 µl) each time with fresh media. For dosing experiments, perform a full-media change (300 µl) 2 days prior to ensure uniform well volumes at dosing and MEA measurements.

Example schedule: maintenance table for the remainder of your assay when your experimental endpoint is 23DPP:

DPP	Action
9	Perform a Long-Term half-media change
12	Perform a Long-Term half-media change
14	Perform ontogeny recording, then perform a Long-Term half-media change
17	Perform a Long-Term half-media change
19	Perform a Long-Term half-media change
21	Perform ontogeny recording, then perform a Long-Term **full**-media change
23	Perform dosing experiment

Example schedule: maintenance table for the remainder of your assay when your experimental endpoint is 35DPP:

DPP	Action
9	Perform a Long-Term half-media change
12	Perform a Long-Term half-media change
14	Perform ontogeny recording, then perform a Long-Term half-media change
17	Perform a Long-Term half-media change
19	Perform a Long-Term half-media change
21	Perform ontogeny recording, then perform a Long-Term half-media change
24	Perform a Long-Term half-media change
26	Perform a Long-Term half-media change
28	Perform ontogeny recording, then perform a Long-Term half-media change
31	Perform a Long-Term half-media change
33	Perform a Long-Term **full**-media change
35	Perform dosing experiment

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