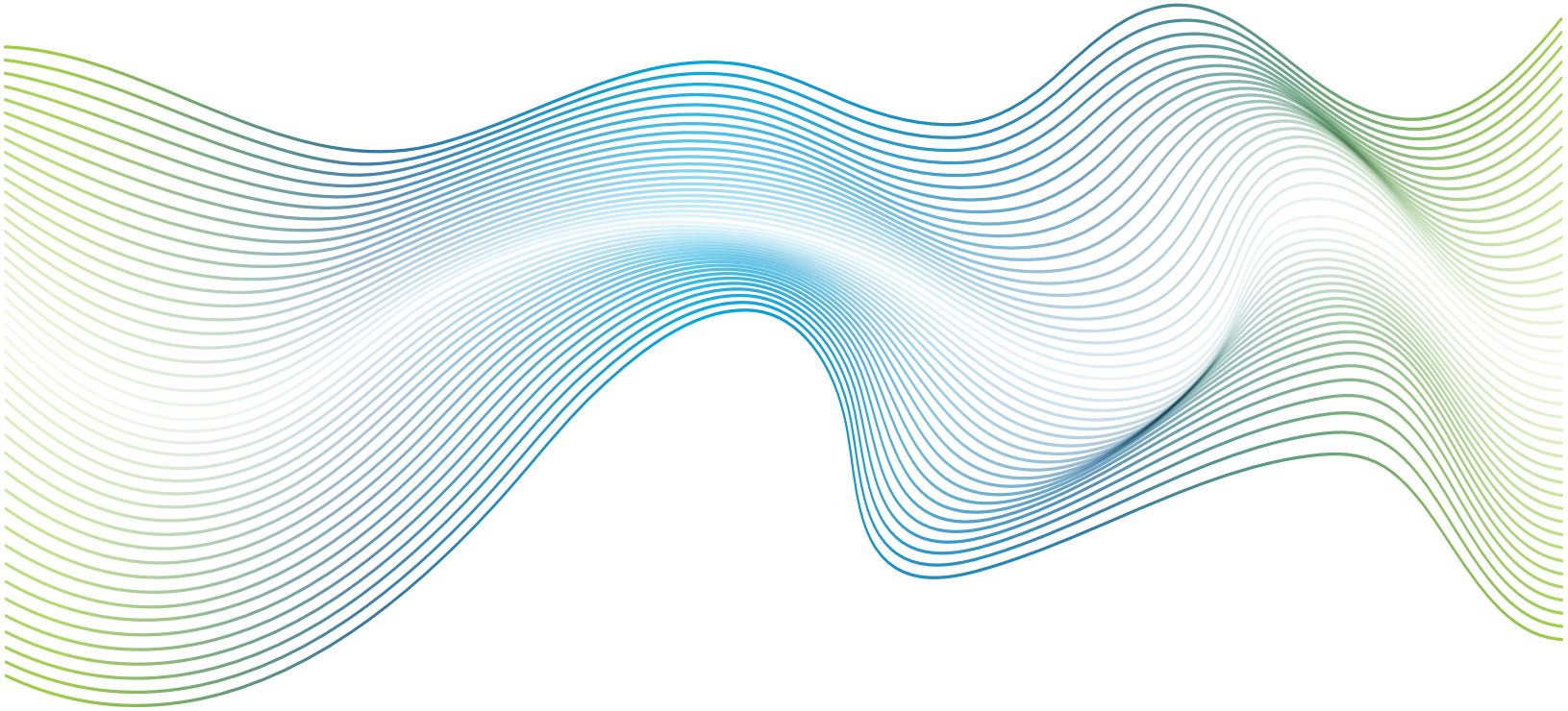
User Guide

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



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NeuCyte is a leading provider of human iPSC-derived neural cells. For our business practices, we strictly comply with the following policies:

- We are committed to operating under the highest ethical and legal standards in the acquisition of human and animal tissues used for the preparation of iPS cells and other biological materials, and only work with suppliers that operate under similar standards.
- We represent that our suppliers warrant that human tissues have been obtained in compliance with local, state, and federal laws and regulations.
- We strictly adhere to the guidelines for human tissue collection and distribution according to established protocols.
- We represent that our suppliers warrant that human tissue used for the isolation of primary cells is derived from donors who have provided informed consent, either personally or through an authorized agent acting on the donor's behalf.
- We protect the privacy and autonomy of all donors.
- We believe that human actions have social, economic, and environmental impacts and that they should promote good and avoid harm.
- We believe in the integrity of our activities in the field of biotechnology and that they should be conducted honestly, truthfully, lawfully, impartially, competently, and with transparency of process.
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Origin

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

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Revision History

Version 2 — November 2025

SynFire® iN Seeding and Maintenance of Neuron-Astrocyte Co-Cultures for Axion MEA (Multi-electrode Array) Applications

This protocol describes the procedure for thawing and seeding glutamatergic iNs, GABAergic iNs, and astrocytes as a co-culture in a defined ratio on a 48-well Axion multi-electrode array (MEA) plate.

Consumables from Other Suppliers

Item	Suggested Supplier	Catalog Number
CytoView MEA Plate (48-well)	Axion	M768-tMEA-48W
Poly-Ornithine (PLO) solution, 0.01%	Sigma Aldrich	P4957
Laminin Mouse Protein	Thermo Fisher Scientific	23017015
DPBS, no calcium, no magnesium	Thermo Fisher Scientific	14190144
Neurobasal-A Medium	Thermo Fisher Scientific	10888022
MACS® BSA Stock Solution (10%)	Miltenyi Biotec	130-091-376
Cell Counting Chamber Slides or Hemocytometer	Thermo Fisher Scientific	C10283
Trypan Blue Stain	Thermo Fisher Scientific	T10282

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Seeding of iN-Astrocyte Co-Cultures for MEA

Day -2: PLO Coating

1. Add 70 µL of 0.01% Poly-L-ornithine (PLO) solution to each well of a 48-well CytoView MEA plate.

Important: Avoid touching the bottom of the MEA wells with the pipette tip. Gently add the PLO solution to fully cover the well bottom, ensuring no bubbles form.

2. Incubate overnight at 4°C.

Day -1: Laminin Preparation and Coating

3. Aspirate the PLO solution. Rinse each well **three times** with 800 µL of sterile water using a P1000 multichannel pipette.

Important: Avoid touching the bottom of the MEA wells with the pipette tip. Gently add the PLO solution to fully cover the bottom of the well, taking care to not introduce bubbles.

4. Aspirate any residual liquid, then place the MEA plate(s) in a biosafety cabinet to air-dry for at least 4 hours.
5. Prepare a 20 µg/mL laminin working solution.
6. Thermo Fisher Scientific laminin 1 mg stocks are received at variable concentrations. Use the formula below to calculate the volume of laminin needed to prepare 100 µg aliquots. Thaw laminin on ice, prepare aliquots, and store at -80°C until use.

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

7. After pre-chilling sterile PBS on ice, thaw one laminin aliquot on ice and prepare a 20 µg/mL laminin working solution by diluting the stock with ice cold sterile PBS (total volume 5 mL).

Important: Always prepare laminin solution fresh and keep on ice until use.

8. Add 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 tip or introduce bubbles.

9. Incubate overnight at 4°C.
10. Before seeding, bring the MEA plate(s) to room temperature.

Day 0: Media Preparation

11. For a tri-culture of glutamatergic neurons, GABAergic neurons, and astrocytes, label three 15-mL conical tubes, one for each cell types.
12. Prepare Thawing Medium for each cell type by mixing 3.5 mL of Neurobasal-A Medium with 0.5 mL of 10% BSA Stock Solution, and add 4 mL of the mixture to each labeled conical tube.
13. Prepare Seeding Medium by combining 18.0 mL of Seeding Basal Medium with 2.0 mL of Seeding Supplement.
14. Pre-warm all media tubes (from **Steps 12 and 13**) in a 37°C incubator for 30 minutes.

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

15. For each cell type, prepare one Cell Counting Chamber Slide (or hemocytometer) and two microcentrifuge tubes for viability assessment. Add 10 µL of Trypan Blue to each microcentrifuge tube.

    Tip: Use Cell Counting Chamber Slides with the Countess II Automated Cell Counter for optimal accuracy. If using a different cell counting platform, follow the manufacturer's protocol. Sterile-filter the Trypan Blue to remove particulates that may interfere with cell counting accuracy, and label all counting slides and microcentrifuge tubes with the corresponding cell type.

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

Important: This section is time-sensitive. Perform all steps as quickly as possible to maximize cell viability. Keep cell suspensions on ice throughout the procedure.

16. Thaw cells by gently swirling each cryovial in a 37°C water bath for 2 minutes, keeping the vial cap above water. 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 it in the biosafety cabinet to air dry.
17. Transfer the thawed cell suspension to the labeled conical tube containing pre-warmed Thawing Medium using a 5-mL serological pipette. Draw up 1 mL of Thawing Medium first, then the cell suspension, and dispense into the tube.
18. Gently pipette the suspension up and down 10 times to obtain a homogenous single-cell suspension.

    Tip: If not thawing all the vials concurrently, thaw in the following order: astrocytes first, then glutamatergic neurons, and finally GABAergic neurons. Complete Steps 16–18 for each cell type before proceeding to Step 19. In the meantime, keep resuspended cells on ice until use.
19. Gently invert each conical tube to mix the cell suspension evenly. Pipette 10 µL of the cell suspension into the corresponding labeled microcentrifuge tubes containing 10 µL Trypan Blue. Mix by gently pipetting up and down 10 times. Dispense 10 µL of the mixture into each chamber of a cell counting slide.
20. Measure cell number and viability for each cell type using an automated cell counter. Record live cell number and viability, then calculate the average live cell number for each cell type.
21. Using the average **live** cell counts, 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: Prepare enough cells for 50 wells to account pipetting error: **7 x 10^6 glutamatergic neurons, 3 x 10^6 GABAergic neurons, and 3.5 x 10^6 astrocytes**.
22. Using the calculated volumes, combine the required numbers of glutamatergic neurons, GABAergic neurons, and astrocytes for 50 wells into a newly labeled 15-mL conical tube.

    Tip: Gently invert each cell tube before pipetting to ensure uniform cell distribution.
23. Centrifuge the tube containing the combined cell mixture at 300 x g for 5 minutes at room temperature.
24. Carefully aspirate the supernatant without disturbing the cell pellet. Gently re-suspend the pellet in pre-warmed Seeding Medium (2.5 mL for 50 wells) to obtain homogenous single-cell suspension.

Day 0: Seeding MEA Plates

25. Transfer room temperature-acclimated laminin-coated MEA plate(s) into the biosafety cabinet. Spray the outside of plate with 70% ethanol and allow it to air dry before opening.
26. Aspirate the laminin solution **thoroughly**, taking care **NOT** to touch the bottom of the wells (electrodes) with the pipette.

    Tip: To prevent the laminin coating from drying out, aspirate and seed only half of the plate at a time. Complete seeding of the first half before aspirating laminin from the remaining wells.
27. Dispense 50 µL of the cell mixture into each well. Re-mix the suspension by gently pipetting up and down every 8 wells to maintain a homogeneous cell distribution across all wells.

    Tip: Dispense only to the first pipette stop to avoid introducing bubbles. Apply each droplet carefully to the center of the well, ensuring that the entire electrode area is covered by the cell suspension in Seeding Medium.
28. Incubate the MEA plate in a humidified incubator at 37°C, 5% CO_2, and 95% humidity.
29. On the following day (1 Day Post Plating; 1DPP), begin the feeding schedule by adding 250 µL per well of Short-Term Medium, as described in the maintenance schedule below.

Warning: Electrodes are located at the bottom of each MEA well. Avoid touching the well bottoms with the pipette when adding or removing media to prevent damage to the electrodes.

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, perform media changes every 2-3 days using one of the two methods below:

- **Half-media change:** Remove 150 µL of conditioned medium from each well, then add 150 µL of fresh medium.
- **1:1 media change with volume correction:** Remove all the conditioned medium from each well, then add 150 µL of fresh medium followed by 150 µL of the conditioned medium**.**

Tips and Techniques

- **Media Preparation**: Prepare Short-Term or Long-Term Medium by combining Media Supplement and Basal Medium as shown below:

Medium Type	Basal Medium	Media Supplement	Expiration after preparation
Short-Term	38 mL	2 mL	1 week
Long-Term	114 mL	6 mL	2 weeks

- **Pre-warming**: Pre-warm only the volume of medium required for each change to 37°C. Do not warm up the entire bottle. Store unused medium at 4°C, protected from light.
- **Media Changes**: During feeding, angle the pipette tip along the side of the wall of the well, and gently aspirate or dispense media to minimize disturbance to the co-culture.
- **MEA Recordings**: Perform MEA recordings 48 hours after a media change to minimize effects on network activity. For media changes that fall on the same day as an ontogeny recording, perform the media change after the recording.
- **Routine Inspections (at each media change)**:
    a. Inspect the humidity reservoir water levels; refill with sterile water if low.
    b. Inspect the media volume in each well. If the volume for any wells becomes noticeably low, add 150 µL of fresh medium without removing existing media.
    c. Inspect each well for contamination by removing the lid in a biosafety cabinet. Ensure 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 Platting (DPP)	Task	Action
1	Add fresh Short-Term Medium	Add 250 µL of new Short-Term Medium to the 50 µL seeding volume (final volume = 300 µL per well)
3	Change Short-Term Medium	Perform a half-media change using Short-Term Medium
5	Change Short-Term Medium	Perform a half-media change using Short-Term Medium
7	Ontogeny recording and Short-Term Medium Change	Perform ontogeny recording, then perform a half-media change with Short-Term Medium.
Beyond 7 DPP until Assay Completion	Change Long-Term Medium	Perform a half-media change with Long-Term Medium every 2-3 days, replacing 150 µL per well with fresh medium. For dosing, perform a full media change (300 µL) 2 days before dosing to ensure uniform well volumes for dosing and MEA recordings.

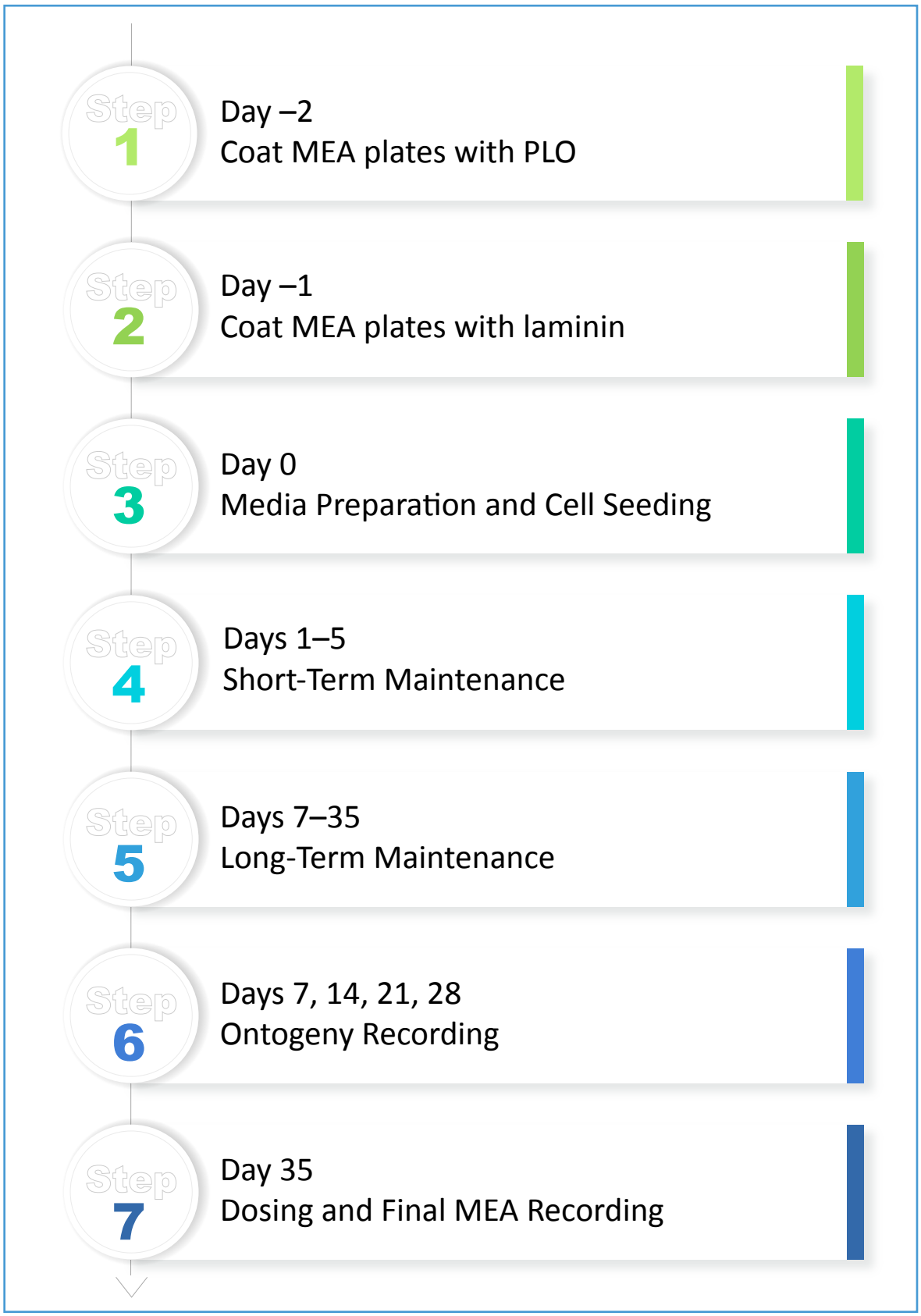
Example schedule: the following table represents your maintenance schedule for the remainder of your assay when your experimental endpoint is Days Post Plating (DPP) 23:

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

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

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

Workflow


Step 1
Day –2
Coat MEA plates with PLO
Step 2
Day –1
Coat MEA plates with laminin
Step 3
Day 0
Media Preparation and Cell Seeding
Step 4
Days 1–5
Short-Term Maintenance
Step 5
Days 7–35
Long-Term Maintenance
Step 6
Days 7, 14, 21, 28
Ontogeny Recording
Step 7
Day 35
Dosing and Final MEA Recording

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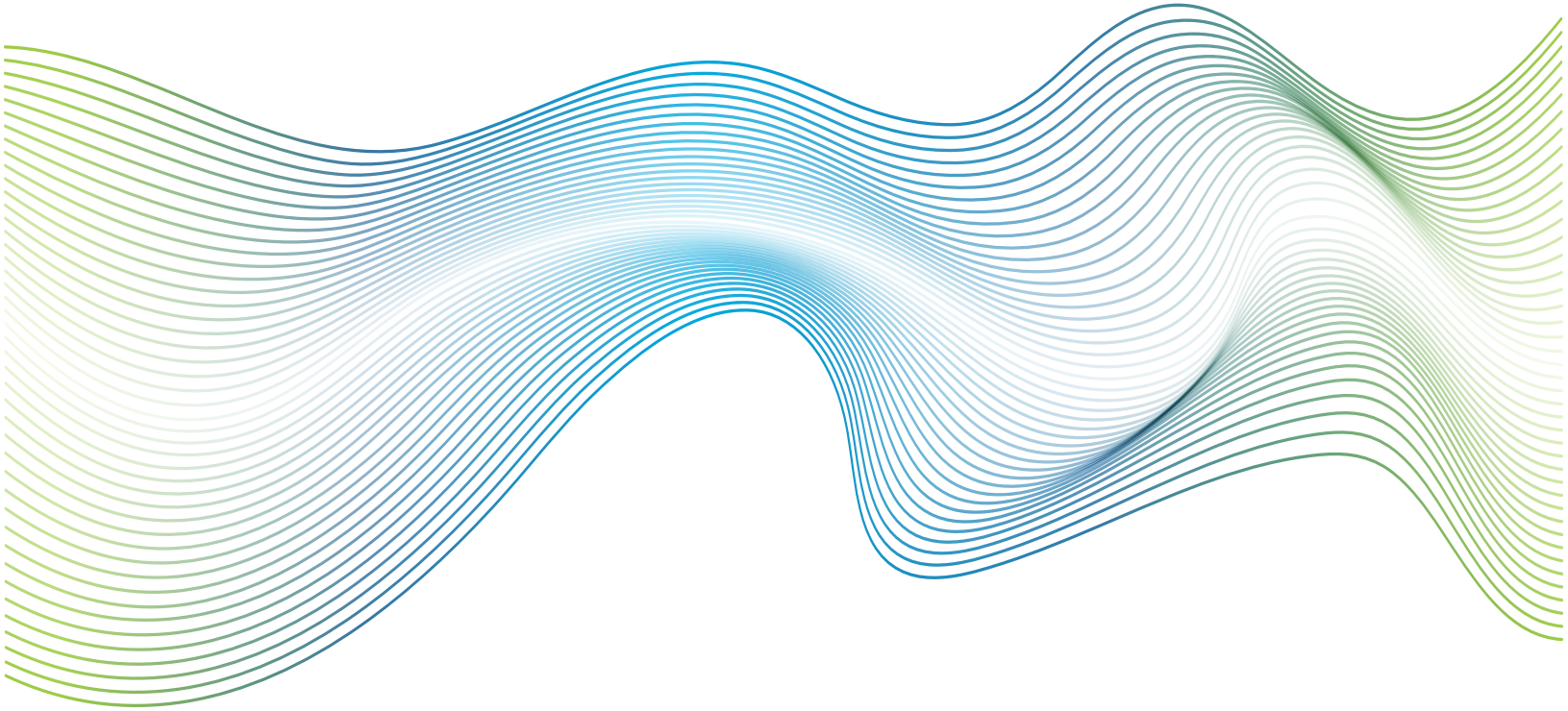
Axion MEA Protocol



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