

# OMEGA<sup>4-2mini</sup>

## NEURONAL CO-CULTURE DEVICE

### User Guide

#### Contents

General Information .....	1
Storage and Shelf-life .....	1
Product Options & What's Included .....	1
Before Starting – IMPORTANT .....	1
Unpacking .....	1
Preparation for Use.....	2
Surface Coating .....	2
Flow Control and Asymmetrical Volume Loading.....	2
When to Apply Asymmetrical Volume Loading .....	3
Cell Seeding Density.....	4
Evaporation Minimizers .....	4
Microscopy.....	5
OMEGA <sup>4-2mini</sup> Schematic .....	6
OMEGA <sup>4-2mini</sup> Specifications.....	6
Protocol – Neuronal Cultures.....	7
General coating procedure .....	8
Single neuronal cultures seeded into the small chamber (compartmentalization purposes) .....	9
Establishing neuronal co-culture (seeding the large chamber first).....	10
Single neuronal culture seeded into the small chamber .....	11
Establishing neuronal co-culture (seeding the small chamber first) .....	12
Protocol – Fixation and Immunocytochemistry.....	13
Fixation (if large chamber is isolated) .....	13
Fixation (if small chamber is isolated).....	14
Immunocytochemistry .....	15
Blocking.....	15
Primary Antibody .....	16
Secondary Antibody.....	17
Nuclear Counterstaining .....	18
Protocol - Microscopy .....	19
Slide-size Microscopy Adapter .....	19
Live-cell Microscopy Adapter.....	20

## General Information

### Storage and Shelf-life

OMEGA<sup>4-2mini</sup> devices can be stored in their original packaging at room temperature and protected from direct sunlight/UV exposure for up to 6 months. Devices that have been removed from their packaging and placed in 35 mm dishes can also be stored prior to use. In this case, these should be stored in a sealed container (e.g. in a bag or parafilm-wrapped) at 4°C, making sure that all chambers contain a sterile solution. Verification of devices stored in this way should be performed periodically to ensure all chambers remain to be wet.

### Product Options & What's Included

OMEGA <sup>4-2mini</sup>	OMEGA <sup>4-2mini</sup> Devices (4)	Evaporation Minimizers (4) (reusable)	35 mm Dishes (4)	Microscope Adapter (1) (reusable)
Starter Kit (#eN-o4m2-001)	✓	✓	✓	✓
Refill Kit (#eN-o4m2-002)	✓		✓	
Refill Plus Kit (#eN-o4m2-003)	✓	✓	✓	

### Before Starting – IMPORTANT

Each device is double bagged to prevent loss of sterility during shipment. The inner-most bag containing the device is liquid filled, and this is placed in a sealed and sterile second bag. If devices have been handled roughly during shipping such that the inner bag may have been compromised, the sterile shipping PBS may leak and be trapped in the outer sealed bag. **Leaks of this kind will not affect the sterility or functionality of the device provided that (1) the outer bag has not been compromised, and (2) the device microchannels remain wet.**

Owing to its thinness, the glass coverslip that has been bonded to each device is fragile and must be handled with care. We take great care in packaging each device for shipment, however if the product is mishandled or handled roughly during shipment, the glass bottom may arrive cracked or broken. Cracks in the glass can easily be seen through the individual device's plastic packaging, and therefore we strongly recommend that each device be inspected carefully **prior to opening the device's individual plastic packaging**. If any cracks within the glass coverslip are noticed, please send a photo of the damaged device in its unopened plastic sleeve including your order number to [info@enuvio.com](mailto:info@enuvio.com). We will be happy to quickly send you a replacement device. **Please note that we cannot provide replacements for broken devices if they have already been removed from their individual plastic packaging.**

### Unpacking

All the items contained within the shipping vial have been carefully packaged under sterile conditions. To maintain sterility, it is recommended to unpack the contents of the shipping vial in an aseptic environment (e.g. in a biological safety cabinet). The shipping vial is autoclavable and can be repurposed (it is also recyclable).

OMEGA<sup>4-2mini</sup> devices are packaged in sterile filtered (0.1 micron) phosphate buffered saline (PBS; without divalents) solution and are ready to use in cell culture. Each device is packaged sterile and bonded to a 22 mm round #1.5 thickness glass coverslip.

### Preparation for Use

It is recommended to prepare all reagents and tools required to carry out the protocol in its entirety prior to opening and removing the device from its sealed packaging. **It is crucial to prevent the microchannels from drying** as this will cause the microchannels to lose their hydrophilic property. If the microchannels do dry, the device can be rejuvenated. This process involves thoroughly rinsing the device with deionized water, allowing it to dry completely, then oxidizing and sterilizing the device using a plasma or UV/ozone cleaner.

OMEGA<sup>4-2mini</sup> devices are compatible with a variety of common downstream experimental procedures including:

- a) Fixation and immunocytochemistry
- b) Brightfield and fluorescence microscopy\* (e.g. widefield, confocal, TIRF, etc...)
- c) Calcium imaging\*
- d) RNA/Protein extraction and analysis (e.g. Western blotting)
- e) Patch-clamp electrophysiology

### Surface Coating

The OMEGA<sup>4-2mini</sup> devices are bonded to uncoated borosilicate glass. If required, steps should be taken to render the surface suitable for culturing the desired cell type. The type of coating and protocol for coating should be selected and optimized for each culture/cell type that is being plated on the device. Examples of common surface coating/modifying reagents include (not a complete list): poly-D/L-lysine, poly-D/L-ornithine, laminin, fibronectin, collagen, as well as various hydrogels.

Frequently, neuronal cultures require a sequential coating of poly-D/L-lysine or poly-D/L-ornithine (applied at between 50 - 100 µg/mL) followed by a secondary coating of laminin (at 5 µg/mL) or a basement membrane matrix (e.g. Geltrex<sup>®</sup> or Matrigel<sup>®</sup>). If applied as directed, this combination of coatings on OMEGA devices **will not** result in the clogging or blocking of microchannels.

### Flow Control and Asymmetrical Volume Loading

The OMEGA<sup>4-2mini</sup> device has 2 pairs of interconnected chambers, where each pair of chambers is joined via a series of microfluidic channels. The direction of the flow of fluid across these high resistance microchannels can be controlled by adjusting the relative level of fluid in each of the chambers. It is the chamber fluid **level** that provides the force required to drive flow across the microchannels. Although there is a direct relationship between chamber fluid level (i.e. fluid height in the chamber) and fluid volume, it is the fluid level that primarily contributes to the force that will be applied across the microchannels. Consequently, it is differences in fluid levels that will

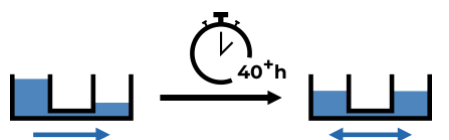
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\* may require the use of a slide microscope stage adapter

provide the force required to drive fluid to flow from a chamber with a relatively higher fluid level towards a chamber with a relatively lower fluid level.

When two adjacent chambers joined by microchannels have identical dimensions, the relationship between chamber fluid level and volume is identical for each of the chambers. Therefore, directional flow across the joining microchannels can be easily determined by directly comparing each chamber's fluid **volume** (fluid will flow towards the chamber with a lower volume). However, in the case where two adjacent chambers do not have identical dimensions, the relationship between fluid level and volume will not be identical for the two chambers. Given that the volumes of adjacent chambers are known, it is possible to determine the level-to-volume ratio (level/volume) between the two chambers by simply calculating the volume quotient between the two chambers, and subsequently using this ratio to adjust chamber volumes accordingly. In this way, the directionality of the flow across the microchannels can be controlled.

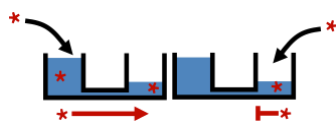
**The interconnected chambers in OMEGA<sup>4-2mini</sup> devices do not have identical dimensions.** The surface area of the small chamber is 0.20 cm<sup>2</sup> whereas the large chamber surface area of 0.35 cm<sup>2</sup>. Given equal depths of both chambers, a level-to-volume ratio of 1.75 should be implemented when calculating volume loading between small and large chambers. For example, loading the large chamber with 140 µL of fluid will result in a fluid level that is approximately equivalent to loading the small chamber with 80 µL (i.e. 80 µL x 1.75 = 140 µL). Since many scientists are familiar with liquid handlers that measure volume, the protocol provided in this user manual refers exclusively to chamber fluid volumes, and not levels, for simplicity and clarity.



When adjacent chambers are loaded with different volumes of fluid for the purposes of driving a unidirectional flow across the adjoining microchannels, we refer to this as “**asymmetrical volume loading**” of the chambers. The

unidirectional flow across the microchannels created by asymmetrically volume loading can serve to fluidically isolate the chamber with a relatively higher fluid level from any adjacent chambers containing relatively lower fluid levels. The flow will persist until the fluid levels (which supply the driving forces) in each of the chambers equalizes, at which point the directionality of flow will subside. Having reached an equilibrium, a slow bidirectional mixing of fluids will now occur between chambers. The duration of controlled unidirectional flow (e.g. for chamber isolation) depends on the **extent of the difference** in fluid levels between adjacent chambers. From the testing done on OMEGA devices, the unidirectional flow across the microchannels can be maintained for 40+ hours without adjusting chamber volumes. With regular verification and adjustment of the chamber fluid volumes, the unidirectional flow can be maintained perpetually.

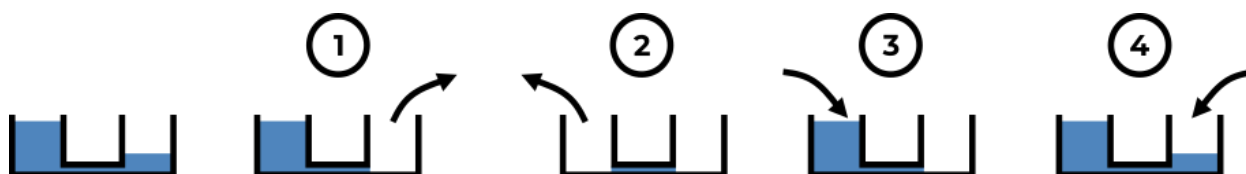
#### When to Apply Asymmetrical Volume Loading



Asymmetrical volume loading of chambers is particularly useful when it is desirable to fluidically isolate one chamber from its adjacent, interconnected partner. Since the flow across the microchannels will be towards the chamber with the relatively lower fluid level, the chamber with higher relative fluid level **will not be** exposed to molecules that have been

specifically added to the chamber with the lower fluid level. However, the chamber with lower fluid level **will be** exposed to molecules that have been specifically added to the chamber with the higher fluid level.

Chamber isolation can be maintained by simply maintaining the asymmetry of fluid levels between the chambers. However, care must be taken when exchanging media in each chamber to maintain the desired directionality of flow. Consequently, the order in which media is removed and replaced in each chamber needs to be considered when performing media exchanges. Media should be removed from the chamber with the lower level prior to removing the media from the chamber with the higher fluid level. Subsequently, media should be added to the chamber with the higher fluid level prior to adding the media in the chamber with the lower fluid level.



In addition to chamber isolation purposes, asymmetrical volume loading of chambers is useful when it is desirable to induce a flow through the microchannels. For example, this might be the case, when coating the microchannel surfaces or chemically fixing neuronal projections located within the microchannels. Also, asymmetrical volume loading is necessary to allow antibody access to epitopes located within the microchannels when performing immunocytochemical staining procedures.

### Cell Seeding Density

The surface area of the larger chamber of the OMEGA<sup>4-2mini</sup> device is ~0.35 cm<sup>2</sup> (approximately equivalent to the area of a single chamber of a standard 96-well plate), whereas the smaller adjacent chambers have a surface area of ~0.20 cm<sup>2</sup>. Optimal seeding density will depend largely on the nature and type of culture being seeded in the device. It is therefore strongly recommended to conduct a series of optimization experiments to determine the ideal cell seeding density. As a starting point, seeding between ~40 000 - 50 000 cells per large chamber or between ~23 000 - 28 500 cells per small chamber yields good results when using iPSC-derived neural progenitor cells (NPCs). For primary cultures, seeding density seems to vary significantly by cell type, user, and laboratory. Some users have reported excellent results using a seeding density of as little as 30 000 cells per large chamber, while others have had success seeding between ~60 000 and 90 000 cells per large chamber. When available, it is suggested to follow the cell provider's cell seeding density recommendation.

### Evaporation Minimizers

The osmotic pressure, pH and nutrient concentration of the culture media is critical for maintaining a healthy culture. This can be particularly problematic when having to maintain cultures for longer periods of time (weeks or months). Due to their size and the way these devices are generally used, the small chambers are particularly prone to evaporation. This can lead to poor

culture health and loss of the seeded culture (often to the surprise of the user) as the media gradually concentrates over time. For this reason, OMEGA starter kits come with cell culture evaporation minimizers that are filled with fluid to help reduce the evaporation rate from the OMEGA device chambers. These blue polydimethylsiloxane (PDMS) rings come packaged sterile and are designed to be reused (they can be sterilized using an autoclave or steam sterilizer for reuse). The inserts can be used as-is or can be rendered hydrophilic (“wetable”) using a plasma or UV/ozone cleaner to facilitate fluid filling of the track.

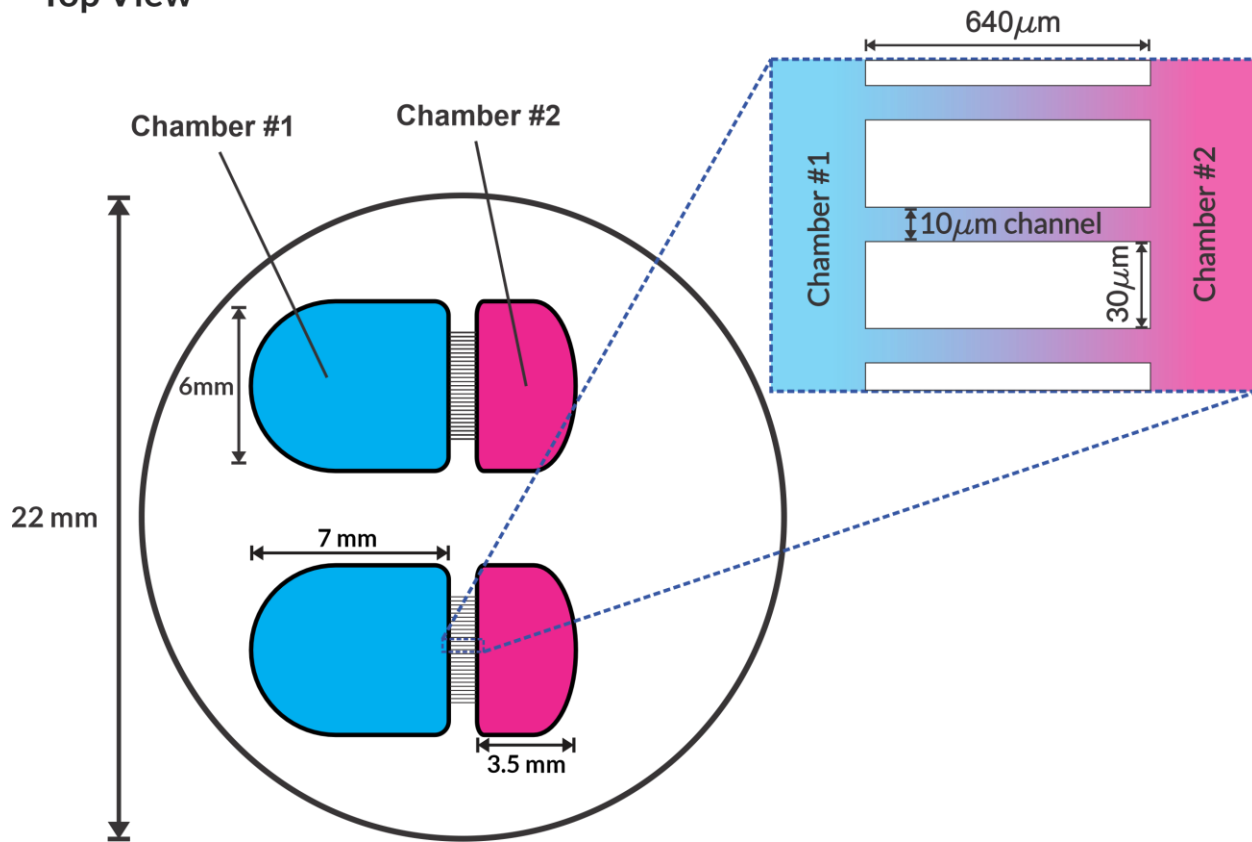
**IMPORTANT:** Although the culture evaporation minimizers do help to reduce evaporation rates during the incubation of cultures, they cannot completely prevent evaporation. **Therefore, it is vital that the fluid level of each chamber of the device be verified and adjusted on a regular basis.** Verification frequency will depend on culture type, the number of times the culture is removed from the incubator, and on the environmental conditions (especially the humidity level) within the incubator. It is strongly recommended that the fluid level in both the evaporation minimizers and device chambers be verified every 2 days, exchanging culture media (e.g. 1/3 or 1/2 volume changes) and refilling as needed.

### Microscopy

Once cultures have been seeded, they can be examined over time in their culture dish using common microscopy techniques (e.g. brightfield or phase contrast). The devices can also be set up for repeat live-cell imaging sessions using fluorescence markers, and/or fixed and immunolabeled with antibodies for immunocytochemical analysis. Each OMEGA device is **permanently bonded** to high-transmissive #1.5 thickness (0.16 mm - 0.19 mm) glass. **The PDMS portion of the OMEGA device cannot be separated from the bottom glass coverslip.** All processing for immunocytochemistry (for example) is easily performed with the device fully intact (see protocol below) and has the added benefit of protecting the delicate axonal processes from detaching from the surface during the labeling process. OMEGA devices are easily adapted to work with most fluorescence microscope stages using available stage holders (see protocol below).

## OMEGA<sup>4-2mini</sup> Schematic

### Top View



## OMEGA<sup>4-2mini</sup> Specifications

- Large chamber working volume: 40 – 150 μL
- Small chamber working volume: 40 – 80 μL
- Large chamber surface area: ~0.35 cm<sup>2</sup>
- Small chamber surface area: ~0.20 cm<sup>2</sup>
- Glass coverslip diameter: 22 mm
- Glass coverslip thickness: 0.16 mm - 0.19 mm (#1.5)
- Microchannel number per interface: 70
- Microchannel length: ~640 μm

## Protocol – Neuronal Cultures



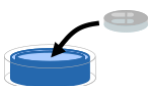
- i. Under aseptic conditions, place the blue cell culture evaporation minimizer into the bottom of the provided 35 mm culture vessel, ensuring the opening of the circular track reservoir is facing upwards.



- ii. Using a sterile blade or scissors, cut open the package of the OMEGA<sup>4-2mini</sup> device. This can be performed over a collection vessel to catch PBS that will drip during device removal.



- iii. Use sterile flat-tipped tweezers or another suitable tool to carefully remove the device from its package. Take note of the device orientation. With the chamber openings facing up, gently dab the glass bottom coverslip with a wipe to remove residual PBS. **Removing the residual PBS from the bottom of the glass is critical to avoid the device from adhering to the dish surface due to the PBS crystallizing as it dries.**



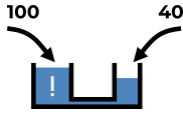
- iv. Place the device glass side down into the central opening of the blue evaporation minimizer.



- v. Remove remaining PBS from each chamber using a vacuum apparatus or manual pipette that has been fit with a fine tip (10  $\mu$ L or 200  $\mu$ L pipette tips work well). **Work efficiently to minimize the time chambers stay dry to avoid the microchannels from drying.**

- vi. If steps are required to coat/prepare a glass surface for culturing cells, proceed immediately with these steps. The working volume for the large chamber is between 40 - 150  $\mu$ L, and between 40 - 80  $\mu$ L for the small chamber. When coating microchannels, maintain an excess fluid volume (30 - 60  $\mu$ L) in **only one** of the interconnected chambers (asymmetric volume loading). To help reduce evaporation from the chambers during incubation steps, add 500 - 600  $\mu$ L of sterile water or PBS to the circular track of the evaporation minimizer.

## General coating procedure



- i. Add 100  $\mu\text{L}$  of coating solution to the large chamber.
- ii. Add 40  $\mu\text{L}$  of coating solution to the adjacent small chamber.
- iii. Incubate the device for 2 – 16 hours, depending on the coating type and procedure being used. When using poly-D-lysine (PDL) or poly-L-ornithine (PLO), it is recommended to use a 100  $\mu\text{g}/\text{mL}$  solution in sterile water and incubate the coating for 3 hours at room temperature.



- iv. Remove coating solution. If required, the chambers can be washed with 70 - 100  $\mu\text{L}$  PBS or media. **Work efficiently to minimize the time chambers from completely drying.** For PDL or PLO coatings, perform three 20-minute washes with sterile water at room temperature using asymmetric volume loading. **If a secondary coating of laminin or basement membrane matrix is to be employed, perform the washes with cold sterile water and incubate at 4°C between washes.**

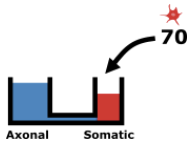
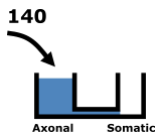


- v. If a second coating is required, repeat the process with the second coating solution (**Step i.**). When using laminin (5  $\mu\text{g}/\text{mL}$  in cold media) or basement membrane matrix (100x diluted in cold media), add the coating solution using asymmetric volume loading to a cold device and place the device at 4°C for 12 - 16 hours (i.e. overnight).



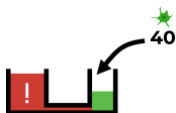
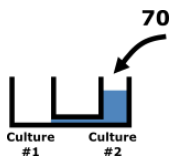
- vi. **Just prior to seeding cells**, remove all fluids from each chamber. If starting with a cold device, place the device in a 37°C incubator for 1 hour before removing all fluid. Follow the correct appropriate protocol below for your intended experiment.

### Single neuronal cultures seeded into the small chamber (compartmentalization purposes)



- i. Add 140  $\mu\text{L}$  of seeding media to the axonal (non-seeded) large chamber. (The added volume should be 1.75x or more than the planned seeding volume. Doing so will prevent the cells to travel in the microchannels before they adhere).
- ii. Seed cells in 70  $\mu\text{L}$  of seeding media into the somatic small chamber.
- iii. Place the device in the 37°C / 5% CO<sub>2</sub> incubator overnight to allow cells to settle and adhere to the surface.
- iv. The next day, top up chambers as necessary for the intended experiment (e.g. chamber isolation; up to a maximum of 150  $\mu\text{L}$  of media in the large chamber and 80  $\mu\text{L}$  in the small chamber). If a rho-kinase inhibitor (ROCK inhibitor) was used when seeding, perform a full media change with fresh media without ROCK inhibitor.
- v. Over the course of incubation, monitor the fluid volumes of the culture chambers, exchanging the media as required by the culture. Ensure that each chamber contains the correct volume of media to maintain the desired experimental conditions (e.g. chamber isolation). Verify and refill the fluid in the evaporation minimizers as needed. Axonal outgrowth can require several days to weeks to fully establish.

### Establishing neuronal co-culture (seeding the large chamber first)



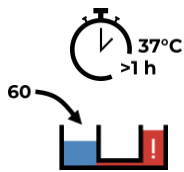
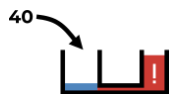
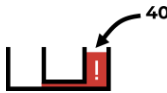
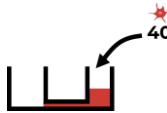
- i. Add 70  $\mu\text{L}$  of seeding media to the culture #2 small chamber (to be seeded later). The volume to add should consider the 1.75 level-to-volume ratio with respect to the planned seeding volume for culture #1. For example, if the seeding volume for culture #1 will be between 100-120  $\mu\text{L}$ , then add 70  $\mu\text{L}$  to the small chamber.
- ii. Seed cells in 100  $\mu\text{L}$  of seeding media into culture chamber #1.
- iii. Incubate the device for at least 1 hour before seeding a second culture. If the second culture is to be seeded several days later, top up both chambers to a maximum of 150  $\mu\text{L}$  for the large chamber and 80  $\mu\text{L}$  for the small one. Employ asymmetrical volume loading where necessary for the intended experiment. If a rho-kinase inhibitor (ROCK inhibitor) was used when seeding cells, perform a full media change with fresh media without ROCK inhibitor the day after seeding.
- iv. When the second culture is ready to be seeded, remove all media from the culture chamber #2.
- v. Seed the second culture into culture chamber #2 in an equivalent or less volume of media compared to culture chamber #1. For example, if the first culture is maintained with 150  $\mu\text{L}$  of media, seed the second culture in  $\leq 150$   $\mu\text{L}$  of media. If ROCK inhibitor is used when seeding the second culture, it is recommended to use less volume when seeding compared to the culture chamber #1 chamber to isolate the ROCK to culture chamber #2.
- vi. Place the device in a 37°C / 5%  $\text{CO}_2$  incubator overnight.
- vii. The next day, top up each chamber as necessary for the intended experiment (e.g. for chamber isolation; up to a maximum of 150  $\mu\text{L}$  of media in each chamber). If ROCK inhibitor was used in seeding culture #2, perform a full media change for culture chamber #2 with fresh media without ROCK inhibitor.
- viii. Over the course of incubation, monitor the fluid volumes of the culture chambers, exchanging the media as required by the culture. Ensure that each chamber contains the correct volume of media to maintain the desired experimental conditions (e.g. chamber

isolation). Verify and refill the fluid in the evaporation minimizers as needed. Axonal outgrowth/co-cultures can require several days to weeks to fully establish.



- ix. Gently add 40  $\mu\text{L}$  of media (80  $\mu\text{L}$  total volume) to top up the **small chamber** and return the device to the incubator. **The volume of media in each chamber should be adjusted according to the desired experimental conditions (e.g., chamber isolation).**
- x. Monitor the fluid volumes of the culture chambers, exchanging the media as required by the culture. Ensure that each chamber contains the correct volume of media to maintain the desired experimental conditions (e.g., chamber isolation). Verify and refill the fluid in the evaporation minimizers as needed.

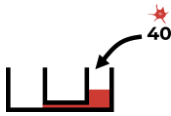
### **Single neuronal culture seeded into the small chamber**



- i. Add the appropriate number of cells to 40  $\mu\text{L}$  of media, and pipette this into the **small chamber**.
- ii. Place the device in the incubator to allow the cells to settle and adhere to the surface (~10 minutes).
- iii. Once the cells have adhered, gently top up the **small chamber** by adding 40  $\mu\text{L}$  of media (final volume 80  $\mu\text{L}$ ).
- iv. Add 40  $\mu\text{L}$  to the **large chamber** to prevent drying.
- v. Incubate the device for 1 hour.
- vi. Add 60  $\mu\text{L}$  of media to the **large chamber** (final volume 100  $\mu\text{L}$ ).
- vii. Return the device to the incubator. Monitor the fluid volumes of the culture chambers, exchanging the media as required by the culture (half- or third-volume media changes are recommended for neuronal cultures). Ensure that each chamber contains the correct volume of media to maintain the desired experimental conditions (e.g., chamber isolation). Verify and refill the fluid in the evaporation minimizers as needed.

### Establishing neuronal co-culture (seeding the small chamber first)

Note: If two different cell types will be co-cultured, start by plating the neuronal culture (the culture destined for outgrowth).



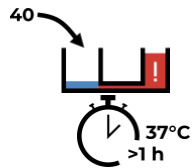
- i. Add the appropriate number of cells to 40 µL of media, and pipette this into the **small chamber**.



- ii. Place the device in the incubator to allow the cells to land and adhere to the surface (~10 minutes)



- iii. Once the cells have adhered, gently top up the **small chamber** by adding 40 µL of media (final volume 80 µL).

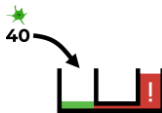


- iv. Add 40 µL of media to the **large chamber** to prevent drying.

- v. Incubate the device for at least 1 hour before seeding the second culture.



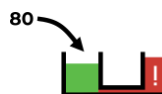
- vi. When ready to seed the second culture (this could be days later if required), first remove all media from the **large chamber**.



- vii. Add the appropriate number of cells to 40 µL of media, and pipette this into the **large chamber**.



- viii. Place the device in the incubator to allow the cells to settle and adhere to the surface (~10 minutes).



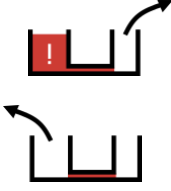
- ix. Gently add 80 µL of media (120 µL total volume) to top up the **large chamber** and return the device to the incubator. **The volume of media in each chamber should be adjusted according to the desired experimental conditions (e.g., chamber isolation).**

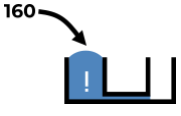
- x. Monitor the fluid volumes of the culture chambers, exchanging the media as required by the culture. Ensure that each chamber contains the correct volume of media to maintain the desired experimental conditions (e.g., chamber isolation). Verify and refill the fluid in the evaporation minimizers as needed.

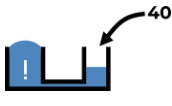
## Protocol – Fixation and Immunocytochemistry


The following protocol is designed to fix and immunolabel culture within the chambers **including** processes located within the adjoining microchannels. Microchannel labelling is achieved by simply employing asymmetrical volume loading, in the same way that it may have been used for chamber isolation. Maximizing the fluid level difference between adjacent chambers will increase microchannel fluid flow and will in turn maximize the immunolabelling of epitopes contained **within the microchannels**. In cases where immunolabeling within the microchannels is not required or desired, there is no need to use asymmetrical volume loading.

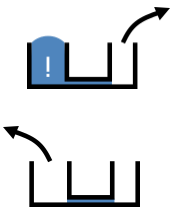
### Fixation (if large chamber is isolated)


- 


1) Remove all solution from both chambers. Ensure chamber isolation (flow directionality) is maintained by removing solution from the **non-isolated chamber** (the chamber with the lower fluid level; in this case the **small chamber**) before removing solution from the **isolated large chamber**.
- 

2) Carefully add 160 µL of fixative (e.g. 4% formaldehyde in PBS) to the **large chamber** (isolated). Note that this volume of solution may overflow the chamber and slightly “balloon out” of the top of the respective chamber.
- 

3) Add 40 µL of fixative to the **small chamber**.
- 

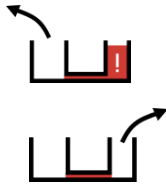
4) Incubate the device at room temperature for 20 minutes.
- 

5) Remove fixative from both chambers. As in **Step 1**, begin by removing the solution from the **small chamber** first.
- 

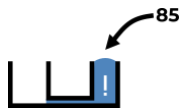
6) Wash the chambers by repeating **Steps 1 - 4** with **PBS**, observing the order in which chambers are emptied and refilled to maintain chamber isolation.
- 

7) Repeat **Step 6** twice more, so that all chambers have been washed a total of three times.

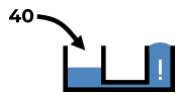
## Fixation (if small chamber is isolated)



- 1) Remove all solution from both chambers. Ensure chamber isolation (flow directionality) is maintained by removing solution from the **non-isolated chamber** (the chamber with the lower fluid level; in this case the **large chamber**) before removing solution from the **isolated small chamber**.



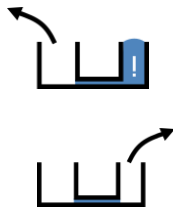
- 2) Carefully add 85 µL of fixative (e.g. 4% formaldehyde in PBS) to the **small chamber** (isolated). Note that this volume of solution may overflow the chamber and slightly “balloon out” of the top of the respective chamber.



- 3) Add 40 µL of fixative to the **large chamber**.



- 4) Incubate the device at room temperature for 20 minutes.



- 5) Remove fixative from both chambers. As in **Step 1**, begin by removing the solution from the **large chamber** first.



- 6) Wash the chambers by repeating **Steps 1 - 4** with **PBS**, observing the order in which chambers are emptied and refilled to maintain chamber isolation.

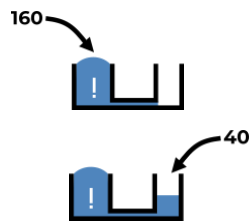


- 7) Repeat **Step 6** twice more, so that all chambers have been washed a total of three times.

## Immunocytochemistry

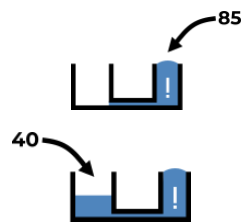
**Blocking**

8) Remove all solution from both chambers (maintain isolation where necessary).



9) Repeat **Step 2 - 3** with **blocking solution** (e.g., 5 % normal serum, 0.2 % Triton X100, 0.05 % BSA), and incubate overnight at 4°C.

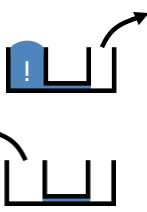
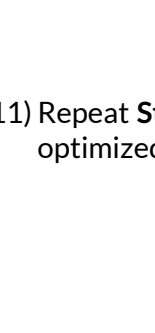
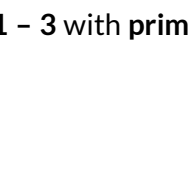

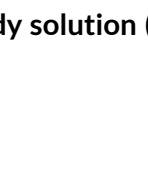

or



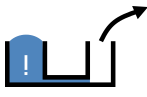









10) Remove blocking solution from both chambers. As in **Step 1**, begin by removing the solution from the non-isolated chamber first.



## Primary Antibody

- 
- 11) Repeat **Step 1 – 3** with **primary antibody solution** (dilution ratio(s) to be optimized).
- 
- 160
- 40
- or
- 
- 85
- 40
- 
- 12) Incubate for 24 – 72 hours at 4°C.
- 
- 13) Remove primary antibody solution from both chambers. As in **Step 1**, begin by removing the solution from the non-isolated chamber first.
- 
- 14) Wash the chambers three times with PBS as described in **Steps 6 - 7**.

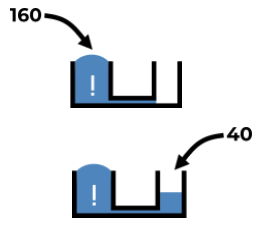
## Secondary Antibody

- 
- 15) Repeat **Step 1 – 3** with **secondary antibody solution** (dilution ratio(s) to be optimized).
- 
- 160
- 
- 40
- 
- or
- 85
- 
- 40
- 
- 16) Incubate for 24 – 72 hours at room temperature.
- 
- 17) Remove secondary antibody solution from both chambers. As in **Step 1**, begin by removing the solution from the non-isolated chamber first.
- 
- 
- 18) Wash the chambers three times with PBS as described in **Steps 6 - 7**.
- 

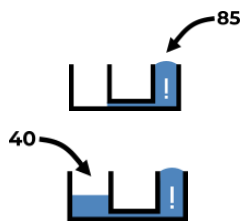
## Nuclear Counterstaining



19) Repeat **Step 1 - 3** with **nuclear counterstain solution** (e.g. Hoechst or DAPI; dilution ratio to be optimized).



or



20) Incubate for 5 minutes at room temperature.



21) Remove nuclear counterstain solution from both chambers. As in **Step 1**, begin by removing the solution from the non-isolated chamber first.



22) Add PBS solution to each chamber as described in **Steps 2 - 3**.

## Protocol - Microscopy

### Slide-size Microscopy Adapter

This adapter is suitable for **end-point** imaging of OMEGA<sup>4-2mini</sup> devices at low- and high-magnification imaging (5x - 100x). This low-profile adapter is compatible with oil immersion objectives since it provides adequate clearance for the relatively large size and shallow taper angles of many oil immersion objectives. The adapter properly stabilizes the OMEGA<sup>4-2mini</sup> device on a level plane using magnets to “sandwich” the device between the magnetic top and the base. It has a 75 x 25 mm footprint which fits microscope stages which accommodate standard-sized glass slides. To use it, simply place the OMEGA<sup>4-2mini</sup> device into the central opening of the base. Optionally, the magnetic top can be slid onto the alignment pins to stabilize the device. Place the entire assembly into a universal standard glass slide accommodation commonly available on microscope stages.



### Live-cell Microscopy Adapter

(suitable for 4x – 20x magnification)

When performing repeated live-cell imaging, it is important to **sterilize** the live-cell microscopy adapter (autoclave or using 70% ethanol) and to carry out the assembly steps under **aseptic conditions** (i.e. in a biological safety cabinet).

After establishing and maintaining the culture, carefully remove the OMEGA<sup>4-2mini</sup> device from the 35 mm plastic culture dish with tweezers or forceps and place it in the Live-cell Microscopy Adapter (see photo below). Position the device in the center of the adapter to expose the bottom glass, ensuring the device is level and flat. To protect the culture during microscopy, cover the assembly with the sterilized lid. The assembly is designed to be used with microscope stage adapters that accommodate standard-sized glass slides.

After imaging, return to the safety cabinet and replace the device into the original 35 mm culture dish (containing the evaporation minimizer). Check the volume levels of each chamber (adding if necessary), replace the lid, and continue to incubate the culture in the incubator.

