

## User Guide

### Contents

General Information.....	2
Unpacking.....	2
Before Starting - IMPORTANT.....	2
Preparation for Use.....	3
Surface Coating.....	3
Flow Control and Asymmetrical Volume Loading.....	3
When to Apply Asymmetrical Volume Loading.....	5
Cell Seeding Density.....	5
Evaporation Minimizers.....	6
Microscopy.....	6
OMEGA <sup>ACE-2mm</sup> Schematic.....	7
OMEGA <sup>ACE-2mm</sup> Specifications.....	7
Protocol - Coating and Cell Seeding.....	8
Device Setup and Coating.....	8
General coating procedure.....	9
Seeding Cells.....	10
Single neuronal cultures.....	10
Establishing neuronal co-cultures (2 or 3 cultures).....	11
For seeding the first culture in chamber #1.....	11
For seeding a second culture in chamber #2.....	12
For seeding a second culture in chamber #3.....	13
For seeding a third culture in chamber #2.....	14
For seeding a third culture in chamber #3.....	15
Protocol - Fixation and Immunohistochemistry.....	16
Fixation.....	16
Immunohistochemistry.....	17
Blocking.....	17
Primary Antibody.....	18
Secondary Antibody.....	19
Nuclear Counterstaining.....	20
Protocol - Microscopy.....	21
Slide-size Microscopy Adapter.....	21
Live-cell Microscopy Adapter.....	22

## General Information

### Unpacking

Thank you for purchasing eNUVIO's OMEGA<sup>ACE-2mm</sup> devices. All the items contained within the shipping vial have been 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 (and label) is completely autoclavable and can be repurposed (it is also recyclable).

The OMEGA<sup>ACE-2mm</sup> starter kit contains:

- 4 x OMEGA<sup>ACE-2mm</sup> devices (individually packaged)
- 4 x circular cell culture evaporation minimizers (blue; reusable)
- 4 x 35 mm round culture dishes
- 1 x microscope stage adapter (reusable)

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

### Before Starting – IMPORTANT

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 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.**

## 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 (within minutes). 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>ACE-2mm</sup> devices are compatible with a variety of common downstream experimental procedures including:

- a) Cell fixation and immunohistochemical staining
- 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

OMEGA<sup>ACE-2mm</sup> devices are bonded to uncoated borosilicate glass. If required, steps should be taken to prepare this surface for culturing the desired cell type. The type of coating and the coating protocol should be selected and optimized for each culture/cell type that is being plated on the device. Some examples of surface coating or modifying reagents include (not a complete list): poly-D/L-lysine, poly-D/L-ornithine, laminin, fibronectin, collagen, and various hydrogels. Frequently, neuronal cultures require the sequential coating of poly-D/L-lysine or poly-D/L-ornithine followed by laminin. Applying this combination of coatings on OMEGA devices works without issue, and will not result in the clogging or blocking of microchannels.

## Flow Control and Asymmetrical Volume Loading

The OMEGA<sup>ACE-2mm</sup> device has 3 chambers that are serially joined. Chambers #1 and #3 are each adjoined to chamber #2 via a series of microfluidic channels. Consequently, chambers #1 and #3 are not directly connected via microchannels. The direction of the flow of fluid across the high resistance microchannels can be controlled by adjusting the relative level of fluid contained within 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 (height) and chamber volume, it is the fluid level that primarily contributes to the force applied across the microchannels. Consequently, differences in fluid levels will provide the force required to drive fluid to flow from one chamber with a relatively higher fluid level towards an adjacent 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 the fluid **volume** between each chamber (fluid will flow towards the chamber with a lower volume).

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\* may require the use of a 35 mm or slide microscope stage adapter, and optional device weight.

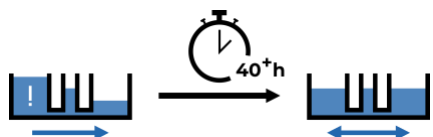
However, in the case where two adjoining chambers do not have identical dimensions, the relationship between fluid level and volume will not be identical for the two chambers. Given that the volumes of adjacent chambers are known, it is possible to determine the level-to-volume ratio (level/volume) between 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 correctly controlled.

Importantly, chambers #1 and #3 of the OMEGA<sup>ACE-2mm</sup> device are **not directly** joined by microchannels but are instead each adjoined to chamber #2. Chambers #1 and #3 have identical dimensions, whereas the surface area of chamber #2 is much larger since it includes the central 2 mm channel that links the upper and lower chambers. In order to calculate the level-to-volume ratio to determine flow directionality within the microchannels, it is important to note that the central channel of chamber #2 is not an open-top chamber, having an internal height of 2 mm. This effectively alters the level-to-volume relationship for chamber #2 at a specific volume threshold. Consequently, two different level-to-volume ratios should be employed depending on the volume used in chamber #2.

For a volume  $\leq 180 \mu\text{L}$  in chamber #2, a level-to-volume ratio of approximately 3 should be used to determine the directionality of the flow within the microchannels. For example, loading chambers #2 (combined upper and lower chambers) with  $150 \mu\text{L}$  of fluid will result in a fluid level that is equivalent to loading chambers #1 or #3 with  $50 \mu\text{L}$  (i.e.  $50 \mu\text{L} \times 3 = 150 \mu\text{L}$ ). For a volume  $\geq 180 \mu\text{L}$  in chamber #2, a level-to-volume ratio of approximately 1.7 should be used to determine the directionality of the flow within the microchannels. For example, loading chambers #2 (combined upper and lower chambers) with  $240 \mu\text{L}$  of fluid will result in a fluid level that is equivalent to loading chamber #1 or #3 with  $140 \mu\text{L}$  (i.e.  $140 \mu\text{L} \times 1.7 = \sim 240 \mu\text{L}$ ).

Since the aforementioned level-to-volume ratios are approximate, it is recommended to use chamber volumes that surpass these ratios when implementing unidirectional flow the microchannels. Using larger ratios will also ensure that chamber isolation will endure over as long a period as possible. For example, to isolate chamber #2 from both chambers #1 and #3 for a maximum duration, add  $240 \mu\text{L}$  to chamber #2 and  $40 \mu\text{L}$  to each chamber #1 and #3. Before the chamber volumes equate, the levels can be reestablished to maintain the unidirectional flow.

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 clarity and ease of use.

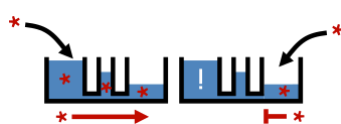


When adjacent chambers are loaded with different volumes of fluid for the purposes of driving a unidirectional flow across the adjoining microchannels, we refer to this as “**asymmetrical volume loading**” of the chambers. The unidirectional flow across the microchannels created by asymmetrically volume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

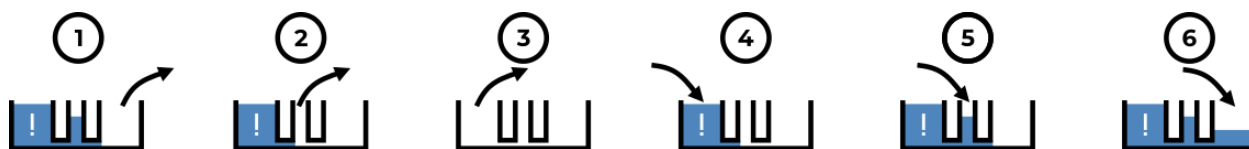
The upper and lower wells that comprise chamber #2 of the OMEGA<sup>ACE-2mm</sup> device are separated by a 2 mm wide and 2 mm high channel. Unlike the microchannels that adjoin chambers #1 or #3 with chamber 2, and due to its relatively large dimensions, the main chamber #2 channel cannot be used to fluidically isolate the upper and lower chambers.

### 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 immunohistochemical staining procedures.

### Cell Seeding Density

The surface area of chambers #1 and #3 of the OMEGA<sup>ACE-2mm</sup> device is approximately 35 mm<sup>2</sup>. The total surface area of chamber #2 is approximately 72 mm<sup>2</sup>. Optimal plating density will depend largely on the nature and type of culture being plated in the device. **It is strongly recommended to conduct a series of optimization experiments to determine the ideal cell plating density.** As a good

starting point, seeding ~55 000 to 80 000 cells per 35 mm<sup>2</sup> chamber has been shown to yield good results using iPSC-derived neural progenitor cells (NPCs). For primary cultures, it is recommended to seed 80 000 cells per 35 mm<sup>2</sup> chamber.

### Evaporation Minimizers

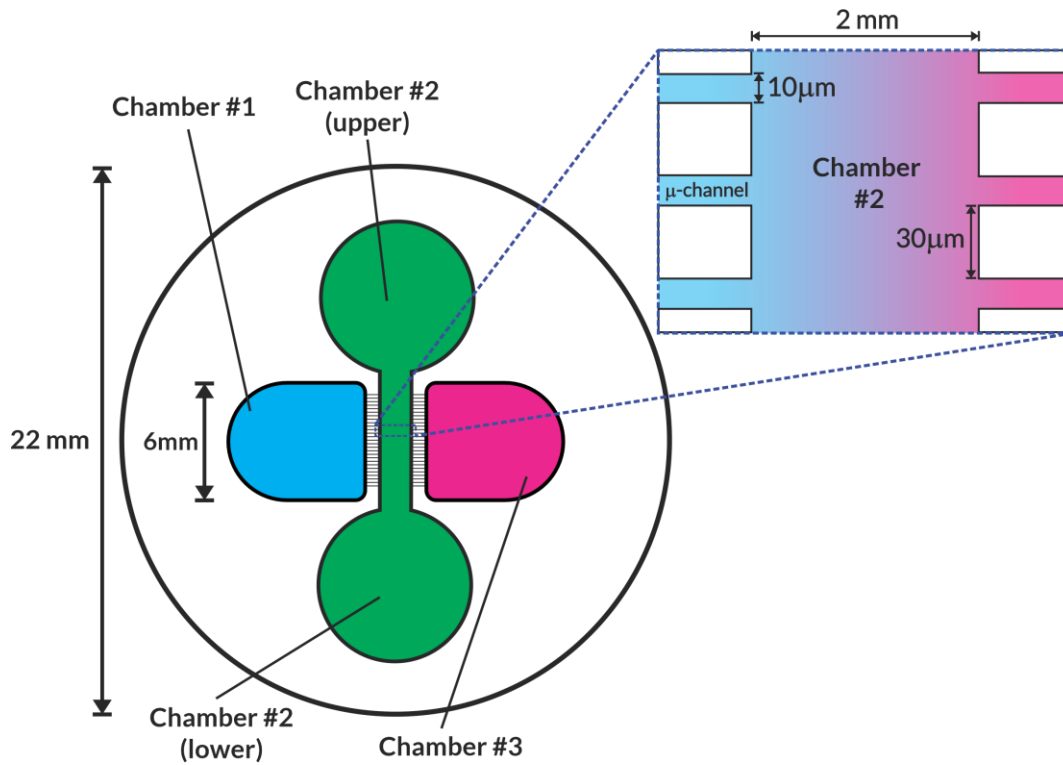
The osmotic pressure, pH and nutrient concentration of the culture media is critical for maintaining a healthy culture. This can be particularly problematic when having to maintain cultures for longer periods of time (weeks or months). Due to their size and the way these devices are generally used, the small chambers are particularly prone to evaporation. This can lead to the loss of the seeded culture (often to the surprise of the user) as the media gradually concentrates over time. For this reason, all OMEGA kits come with cell culture evaporation minimizers that are filled with fluid to reduce the evaporation rate from the OMEGA device chambers. These polydimethylsiloxane (PDMS) rings come packaged sterile and are designed to be reused (they can be sterilized using an autoclave or steam sterilizer). The inserts can be rendered hydrophilic (“wetable”) using a plasma or UV/ozone cleaner to facilitate fluid filling of the track. They can also be used as is.

Although the culture evaporation minimizers do reduce evaporation rates during incubation of cultures, it is strongly recommended that the fluid level of each chamber of the device be verified 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 within the incubator. We recommend verifying the fluid level in evaporation minimizers and device chambers every 2 days, and refilling when required.

### 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 setup for repeat live-cell imaging sessions using fluorescence markers, and/or fixed and immunolabeled with antibodies for immunohistochemical analysis. The OMEGA<sup>ACE-2mm</sup> device is **permanently bonded** to high-transmissive #1.5 thickness (0.16 mm - 0.19 mm) glass. **The PDMS portion of the OMEGA<sup>ACE-2mm</sup> device cannot be separated from bottom glass coverslip.** All processing for immunochemistry (for example) can be 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 processing. OMEGA<sup>ACE-2mm</sup> devices are easily adapted to work with most fluorescence microscope stages using available stage holders (see protocol below).

## OMEGA<sup>ACE-2mm</sup> Schematic



## OMEGA<sup>ACE-2mm</sup> Specifications

- Chamber #1, #3 working volume: 30 – 145  $\mu\text{L}$
- Chamber #1, #3 surface area:  $\sim 0.35 \text{ cm}^2$
- Chamber #2 working volume (combined): 60 - 240  $\mu\text{L}$
- Chamber #2 combined surface area:  $\sim 0.72 \text{ cm}^2$
- Chamber #2 main channel width: 2 mm
- Chamber #2 main channel height: 2 mm
- Microchannel width: 10  $\mu\text{m}$
- Microchannel length:  $>630 \mu\text{m}$
- Glass coverslip diameter: 22 mm
- Glass coverslip thickness: 0.16 mm - 0.19 mm (#1.5)
- Number of microchannels per interface: 70

## Protocol – Coating and Cell Seeding

### Device Setup and Coating



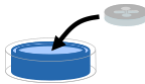
1) Under aseptic conditions, place a blue cell culture evaporation minimizer into the bottom of the provided 35 mm culture vessel, ensuring the opening of the circular track is facing upwards.



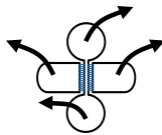
2) Using a sterile blade or scissors, cut open the package of the OMEGA<sup>ACE</sup>-2<sup>mm</sup> device. This can be performed over a collection vessel to catch PBS that may drip during device removal.



3) Using sterile flat-tipped tweezers or another suitable tool, carefully remove the device from its package, taking note of its orientation. With the chamber openings facing up, gently dab the bottom of the glass coverslip with a wipe to remove any 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 over time.



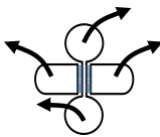
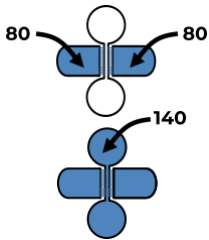
4) Place the device glass side down into the central opening of the evaporation minimizer.



5) Remove remaining PBS from each of the chambers 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 since the microchannels can quickly lose their hydrophilicity.**

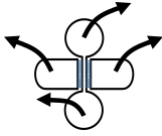
6) Proceed immediately with coating/preparing the surface for culturing cells.



**General coating procedure (this will coat all microchannels):**

- i. Add 80  $\mu\text{L}$  of coating solution to chamber #1 and #3.
- ii. Add 140  $\mu\text{L}$  of coating solution to **only one** of the two wells of chamber #2.
- iii. Place the device in the incubator for the coating incubation period (generally 2 – 16 hours depending on the coating type). To minimize evaporation from the chambers during incubation steps, add  $\sim 500 \mu\text{L}$  of sterile water or PBS to the circular track of the evaporation minimizer.
- iv. Remove the coating solution. If required, the chambers can be each washed with 70 - 100  $\mu\text{L}$  PBS or media.
- v. If a second coating is required, repeat this process starting at **Step i**.

## Seeding Cells



- 1) Prior to seeding cells, **remove all solutions from all chambers**. Follow the correct protocol below for your intended experiment. The protocols assume the user has prepared and counted their cells prior to proceeding with seeding.

### Single neuronal cultures

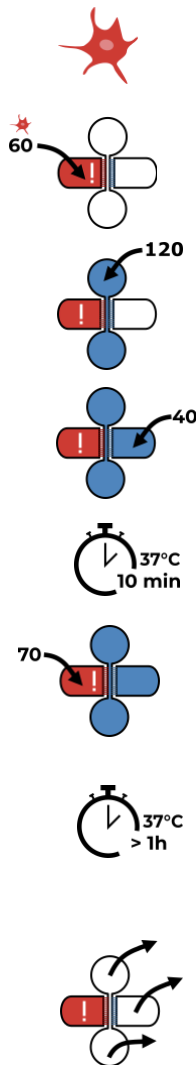
(Compartmentalization purposes; growth from chamber #1 towards chambers #2 and #3)

- 
- a. Add the appropriate number of cells into at least 60  $\mu\text{L}$  of media.
  - b. Seed 60  $\mu\text{L}$  of cells into chamber #1.
  - c. Add 120  $\mu\text{L}$  of media to chamber #2.
  - d. Add 40  $\mu\text{L}$  of media to chamber #3.
  - e. Place the device in the incubator. Allow the cells to settle and adhere to the surface (~10 minutes).
  - f. Once the cells have adhered, gently top up chamber #1 by adding 70  $\mu\text{L}$  of media (final volume 130  $\mu\text{L}$ ).
  - g. Add 80  $\mu\text{L}$  of media to chamber #2 (final volume 200  $\mu\text{L}$  in chamber #2).
  - h. Add 30  $\mu\text{L}$  of media to chamber #3 (final volume 70  $\mu\text{L}$  in chamber #3).
  - i. Return the device to the incubator.

### Establishing neuronal co-cultures (2 or 3 cultures)

**Note:** The following protocol provides example fluid volumes for the described culture setup (i.e. direction of flow across microchannels; compartmental isolation). These volumes can be adjusted accordingly to suit your specific experimental needs.

#### *For seeding the first culture in chamber #1*



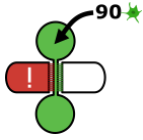
- Add the appropriate number of cells in at least 60  $\mu\text{L}$  of media. Start by plating the neuronal culture (the culture destined for outgrowth).
- Seed 60  $\mu\text{L}$  of cells into chamber #1.
- Add 120  $\mu\text{L}$  of media to chamber #2.
- Add 40  $\mu\text{L}$  of media to chamber #3.
- Place the device in the incubator to allow the cells to settle and adhere to the surface (~10 minutes).
- Once the cells have adhered, gently top up chamber #1 by adding 70  $\mu\text{L}$  of media (final volume 130  $\mu\text{L}$ ).
- Incubate the device for at least 1 hour before seeding the second culture. If waiting an extended period of time (i.e. days) before seeding the second culture, add 80  $\mu\text{L}$  of media to chamber #2 and 30  $\mu\text{L}$  of media to chamber #3, and return to the incubator.
- When the **second culture** is ready to be seeded, remove all media from all **unseeded** chambers. Ensure chamber #1 has 130  $\mu\text{L}$  of fluid.

**For seeding a second culture in chamber #2**

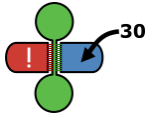
(Go to page 13 if seeding in chamber #3 instead)



- a. Add the appropriate number of cells into at least 90  $\mu\text{L}$  of media.



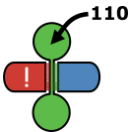
- b. Seed 90  $\mu\text{L}$  of cells into chamber #2.



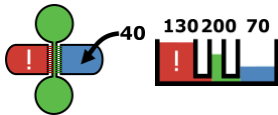
- c. Add 30  $\mu\text{L}$  of media to chamber #3.



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



- e. Once the cells have adhered, gently top up chamber #2 by adding 110  $\mu\text{L}$  of media (final volume 200  $\mu\text{L}$ ).

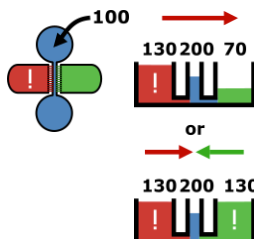
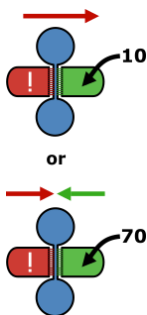
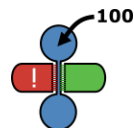
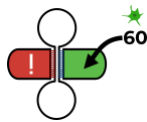


- f. Add 40  $\mu\text{L}$  of media to chamber #3 (final volume 70  $\mu\text{L}$ ).

37°C

- g. Return the device to the incubator.

### For seeding a second culture in chamber #3



37°C

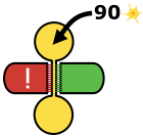
- Add the appropriate number of cells into 60  $\mu\text{L}$  of media.
- Seed 60  $\mu\text{L}$  of cells into chamber #3.
- Add 100  $\mu\text{L}$  of media to chamber #2.
- Place the device in the incubator to allow the cells to settle and adhere to the surface (~10 minutes).
- Once the cells have adhered, adjust the volume in chamber #3. If overall flow is desired to be towards chamber #3, add 10  $\mu\text{L}$  of media (final volume 70  $\mu\text{L}$ ). If flow is desired away from chamber #3 (towards chamber #2), add 70  $\mu\text{L}$  of media to chamber #3 (final volume 130  $\mu\text{L}$ ).
- Add 100  $\mu\text{L}$  of media chamber #2 (final volume 200  $\mu\text{L}$ ).
- Return the device to the incubator

### For seeding a third culture in chamber #2

If a **third culture** is to be seeded, remove all media from the unseeded chamber.



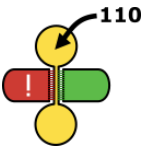
a. Add the appropriate number of cells into at least 90  $\mu\text{L}$  of media.



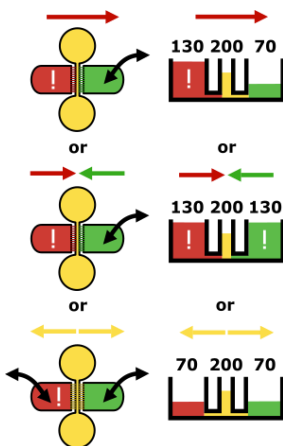
b. Seed 90  $\mu\text{L}$  of cells into chamber #2.



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



d. Once the cells have adhered, gently top up fluid by adding 110  $\mu\text{L}$  of media to chamber #2 (final volume 200  $\mu\text{L}$ ).



e. If preferred, adjust the fluid level in chambers #1 and/or #3 to determine the desired direction of flow across the microchannels.

37°C

f. Return the device to the incubator.

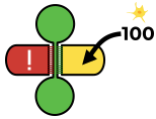
- 2) Over the course of incubation, monitor the fluid volumes of each of the culture chambers, exchanging the media as is required by the culture (half-volume changes are common). Ensure that each chamber contains the correct volume of media to maintain the desired experimental conditions (e.g. chamber isolation/directionality of flow). Verify and refill the fluid in the evaporation minimizers as needed. Axonal outgrowth/co-cultures may require several days to weeks to fully establish.

### For seeding a third culture in chamber #3

If a **third culture** is to be seeded, remove all media from the unseeded chamber.



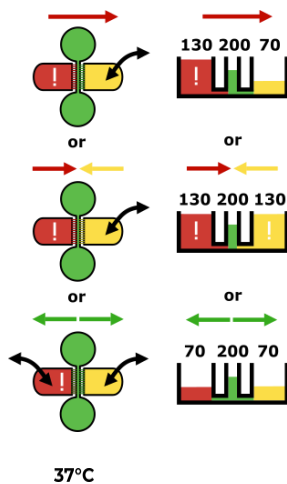
a. Add the appropriate number of cells into at least 100  $\mu$ L of media.



b. Seed 100  $\mu$ L of cells into chamber #3.



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



d. Once the cells have adhered, adjust the fluid level in chambers #1 and/or #3 to determine the desired direction of flow across the microchannels.

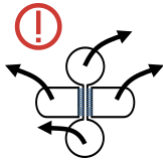
e. Return the device to the incubator.

- 2) Over the course of incubation, monitor the fluid volumes of each of the culture chambers, exchanging the media as is required by the culture (half-volume changes are common). Ensure that each chamber contains the correct volume of media to maintain the desired experimental conditions (e.g. chamber isolation/directionality of flow). Verify and refill the fluid in the evaporation minimizers as needed. Axonal outgrowth/co-cultures may require several days to weeks to fully establish.

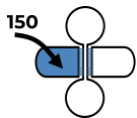
## Protocol – Fixation and Immunohistochemistry

The following protocol is designed to fix and immunolabel culture within the chambers **as well as** 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. Please note that using a high-volume ratio (i.e. 150:50  $\mu$ L) across adjacent chambers has been shown to be optimal for immunolabelling epitopes contained **within** the microchannels. If immunolabelling within microchannels is not required, there is no need to use asymmetrical volume loading, and equal volumes of fluid can be used in adjacent chambers.

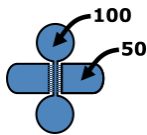
### Fixation



- 1) Remove all solution from all chambers. If necessary, ensure chamber isolation (flow directionality) is maintained by removing solution from the **non-isolated chamber** (the chamber with the lower volume) before removing solution from the isolated chamber.



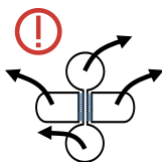
- 2) Carefully add 150  $\mu$ L of **fixative** (e.g. 4% formaldehyde in PBS) to the isolated chamber. Note: this volume of solution may overflow the chamber and slightly “balloon out” of the top of the chamber – this is normal.



- 3) Add 100  $\mu$ L to chamber #2, and 50  $\mu$ L of fixative to chamber #3.



- 4) Incubate the device at room temperature for 20 minutes. The duration and temperature of fixation should be adjusted as needed.



- 5) Remove fixative from all chambers. As in **Step 1**, begin removing solution from the non-isolated chamber first.



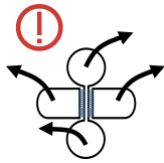
- 6) Wash the chambers by repeating **Steps 1 - 4** with **PBS**, observing the order in which chambers are emptied and refilled to maintain chamber isolation (if necessary).



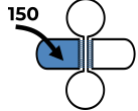
- 7) Repeat **Step 6** twice more, so that all chambers have been washed a total of three times



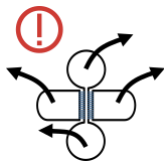
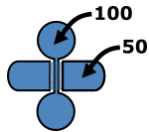
## Immunohistochemistry

**Blocking**

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

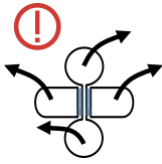


9) Repeat **Step 2 - 3** (above) with **blocking solution** (e.g. 5 % normal serum, 0.2 % Triton X100, 0.05 % BSA), and incubate for at least 1 hour at room temperature (this can be also done overnight if desired).

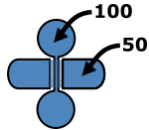
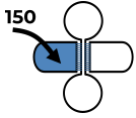


10) Remove blocking solution from all chambers. As in **Step 1 above**, begin removing solution from the non-isolated chamber first.

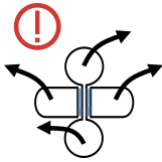
## Primary Antibody



11) Repeat **Step 1 - 3** (above) with **primary antibody solution** (dilution ratio(s) to be optimized).



12) Incubate overnight at 4°C.

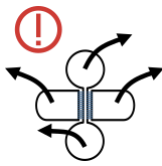


13) Remove primary antibody solution from all chambers. As in **Step 1 above**, begin removing solution from the non-isolated chamber first.

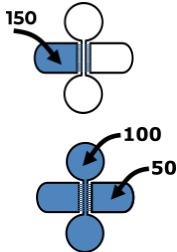


14) Wash the chambers three times with PBS as described in **Steps 6 - 7 above**.

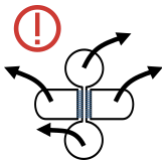
## Secondary Antibody



- 15) Repeat **Step 1 – 3** (above) with **secondary antibody solution** (dilution ratio(s) to be optimized).



- 16) Incubate for 2 hours at room temperature.

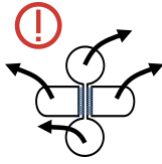


- 17) Remove secondary antibody solution from all chambers. As in **Step 1** above, begin removing solution from the non-isolated chamber first.

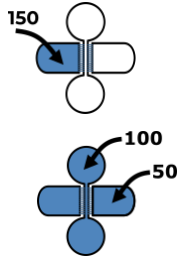


- 18) Wash the chambers three times with PBS as described in **Steps 6 – 7** above.

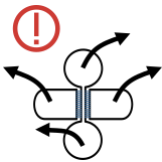
## Nuclear Counterstaining



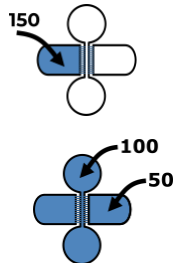
19) Repeat **Step 1 - 3 (above)** with **nuclear counterstain solution** (e.g. Hoechst or DAPI; dilution ratio to be optimized).



20) Incubate for 5 minutes at room temperature.



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

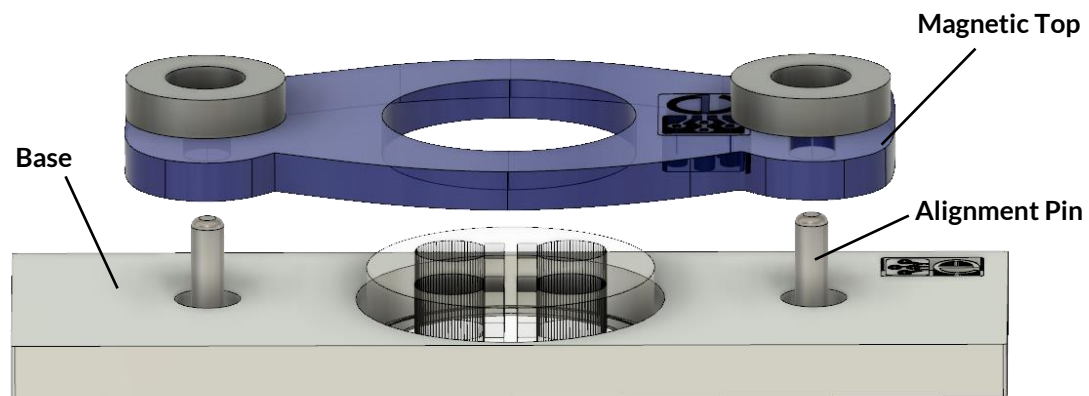


22) Add PBS solution to each chamber as described in **Steps 2 - 3**.

## Protocol - Microscopy

### Slide-size Microscopy Adapter

This adapter is suitable for end-point imaging of OMEGA<sup>ACE-2mm</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>ACE-2mm</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>ACE-2mm</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 (see below) under aseptic conditions (i.e. in a biological safety cabinet). To protect the culture during microscopy, use the lid from the 35 mm culture dish once the device has been placed in the imaging adapter. 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.

After establishing and maintaining the culture, carefully remove the OMEGA<sup>ACE-2mm</sup> device from the 35 mm plastic culture dish with tweezers or forceps and place it in the 35 mm round microscope stage adapter (see schematic below). Position the device in the center of the adapter to expose the bottom glass, ensuring the device is level and flat. The assembly is designed to be used with microscope stage adapters that accommodate round 35 mm culture dishes.

Where additional stability is required, a microscopy weight can be incorporated into the assembly (see schematic below). The weight serves as an interface between the top of the OMEGA<sup>ACE-2mm</sup> device and the bottom of the 35 mm dish lid, such that stage clips can be placed on top of the lid to stabilize the entire adapter assembly to a universal 35 mm stage holder. For live-cell applications, make sure to sterilize the weight using 70 % ethanol, and assemble the chamber under aseptic conditions using a sterile 35 mm culture dish lid to maintain sterility of the sample during imaging sessions (as described above).

