OMEGA^{AG}

User Guide

Contents

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v2.2

General Information

Unpacking

Thank you for purchasing eNUVIO's OMEGA^{AG} 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^{AG} starter kit (#eN-oag-001) contains:

- 4 x OMEGA^{AG} 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)

The OMEGA^{AG} starter kit (#eN-oag-002) contains:

- 4 x OMEGA^{AG} devices (individually packaged)
- 4 x 35 mm round culture dishes

OMEGA^{AG} 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

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 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. 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^{AG} 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

The OMEGA^{AG} 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 coating protocol should be selected and optimized for each culture/cell type that is being plated on the device. Some 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 10 - 100 µg/mL) followed by a secondary coating of laminin (at 5 µg/mL). Applying this combination of coatings on OMEGA devices **will not** result in the clogging or blocking of microchannels.

Flow Control

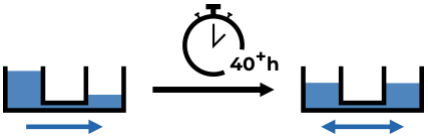
The OMEGA^{AG} device has 4 chambers that each adjoin to a common central viewing area via a series of microfluidic channels. The direction of the flow of fluid across these high-resistance microchannels can be controlled by adjusting the relative level of fluid contained within each of the chambers. 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 fluid 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,

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

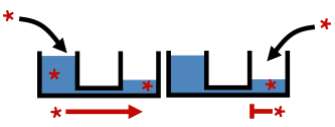
directional flow across the adjoining microchannels can be easily determined by directly comparing the fluid **volume** between each chamber (fluid will flow towards the chamber with a lower volume). However, in the case where two adjoining chambers do not have identical dimensions, the relationship 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 the chamber volumes accordingly.

For OMEGA^{AG} devices, all chambers except for the overflow chamber have identical dimensions. Therefore, the volumes added to the neuronal and source chambers can be used to intuitively determine the flow directionality across the microchannels. The overflow chamber is always kept at a relatively low volume since its purpose is to provide a destination (a sink) for all the fluids flowing from all the other chambers. Owing to the familiarity of scientists with liquid handlers which 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 with OMEGA devices, 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.

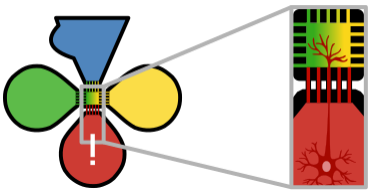
When to Apply Asymmetrical Volume Loading



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.

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.

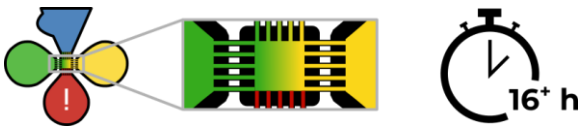


For performing axon guidance experiments such as growth cone turning assays, a larger level of fluid should always be maintained in the neuronal chamber (bottom, red) relative to the source (left and right, green and yellow) and the overflow (top, blue) chambers. This promotes axonal outgrowth into the viewing area and is necessary to avoid the backflow of chemotactic factors (or other molecules) from flowing from the viewing area towards the neuronal chamber (see Flow Schematic, page 7). Similarly, the fluid level in the overflow chamber must be kept at a relatively low level to promote flow from the viewing area towards the overflow chamber.

Chemical Gradient Formation

The OMEGA^{AG} device can be used to form a long-lasting and uniform chemical gradient in the central viewing area between chemotactic factors (or other molecules) that have been loaded or released into one or both source chambers (see Flow Schematic). A uniform gradient is formed in the viewing area when equal flow rates that have been established between the two source chambers. Since the source chambers have identical dimensions, this is achieved by loading equal volumes of fluid (containing molecules of interest) into each of the source chambers. When forming a gradient, it is important to promote the flow out of the viewing area and into the overflow chamber. This is achieved by maintaining a relatively low level of fluid in the overflow chamber. As mentioned above, keeping a large volume of fluid in the neuronal chamber (relative to all other chambers) is recommended to prevent backflow from the viewing area towards the neuronal chamber. This also serves to maintain the overall flow of the system towards the overflow chamber.

Chemotactic gradients set up as described in the protocol provided in this user guide can be maintained for at least 16 hours without the need to adjust chamber fluid volumes.



Cell Seeding

The surface area of the neuronal chamber of the OMEGA^{AG} device is ~0.28 cm^2. Optimal seeding density will depend largely on the type of neuronal 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 good starting point, seeding ~50 000 cells per chamber has been shown to yield good results using iPSC-derived neural progenitor cells (NPCs). For primary cultures, seeding density seems to vary by cell type, user, and lab. Some users have reported excellent results using a seeding density of as little as 30 000 cells per chamber, while others have had success seeding between 60 000 and 90 000 cells per chamber.

For co-culture applications, where cultures are being used as the sources of molecules of interest, the timing between the seeding of the source versus the neuronal culture(s) is important. Depending on the nature of the experiment, and the type of cultures being seeded, it may be necessary that source cultures be sufficiently established at the time that axons begin to project into the viewing area to affect growth cone turning. Consequently, it might be necessary to seed the source cultures before the neuronal culture, or vice versa. It is therefore recommended to first establish the time required for neuronal cultures to cross the microchannels into the viewing area. Similarly, and ideally, it would be very helpful to establish at what point after seeding do source cultures produce the factor of interest.

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 ("wettable") using a plasma or UV/ozone cleaner to facilitate fluid filling of the track.

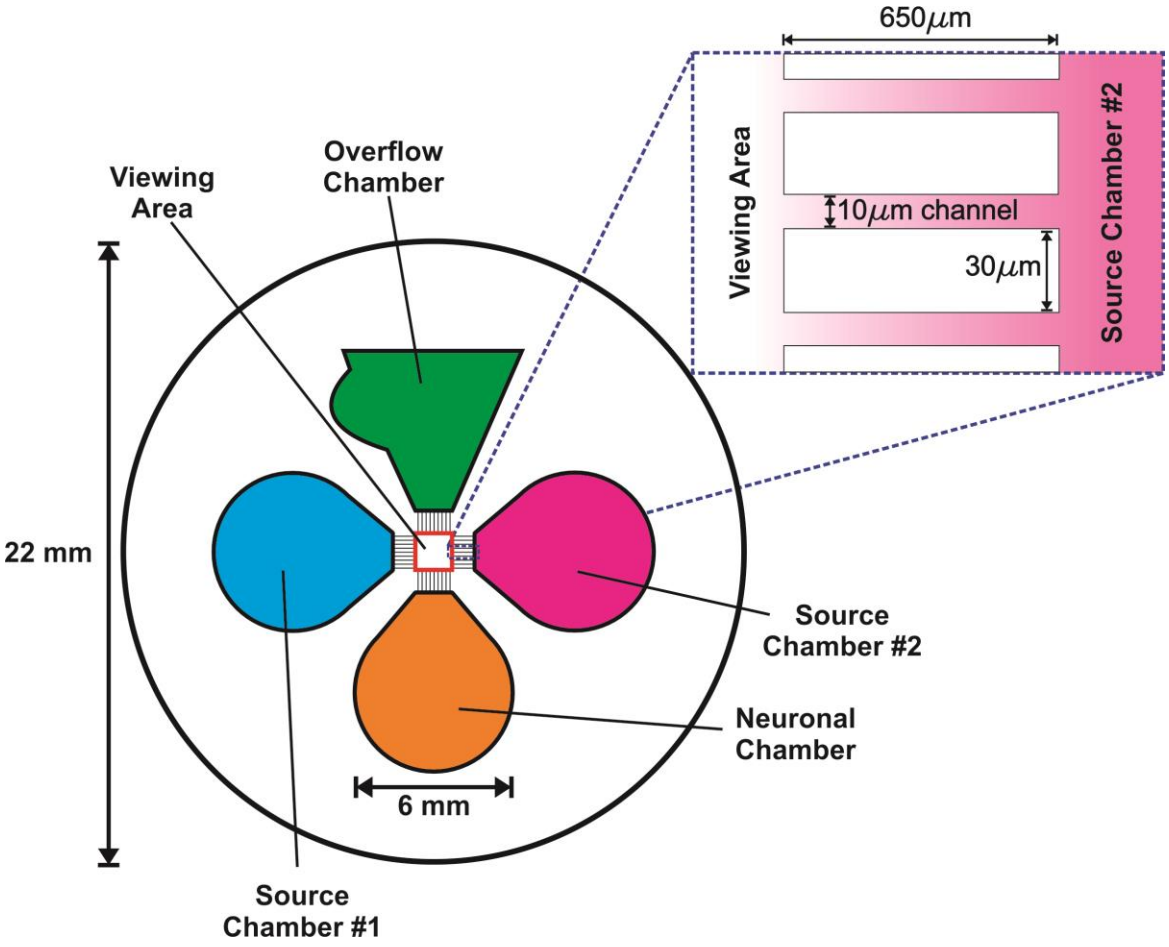
IMPORTANT: Although the culture evaporation minimizers do help to reduce evaporation rates during the incubation of cultures, they do not prevent evaporation. **Therefore, it is vital that the fluid level of each chamber of the device be verified and adjusted on a regular basis.** Verification frequency 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 them 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 setup for repeat live-cell imaging sessions using fluorescence markers, and/or fixed and immunolabeled with antibodies for immunohistochemical analysis. The OMEGA device is **permanently bonded** to

a high-transmissive #1.5 thickness (0.16 mm - 0.19 mm) glass. **The PDMS portion of the OMEGA 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 the process. OMEGA devices are easily adapted to work with most fluorescence microscope stages using available stage holders (see protocol below).

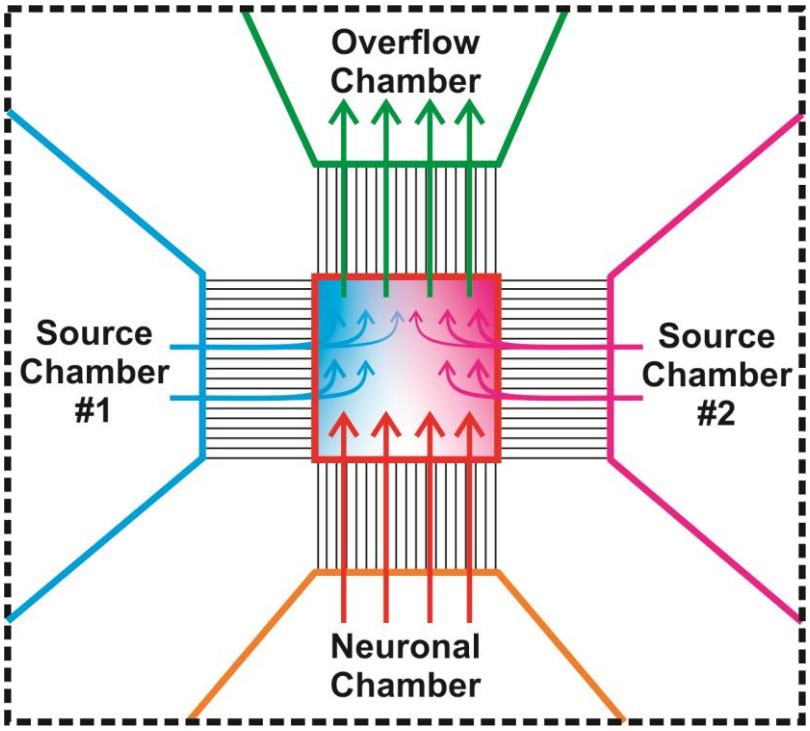
OMEGA^{AG} Schematic



OMEGA^{AG} Specifications

Chamber working volume: 30 – 100 µL
Neuronal, source chamber surface area: ~0.28 cm^2 each
Viewing area surface area: 1 mm^2
Overflow chamber surface area: ~0.23 cm^2
Glass coverslip diameter: 22 mm
Glass coverslip thickness: 0.16 mm - 0.19 mm (#1.5)
Number of interfaces: 4
Number of microchannels per interface: 20

OMEGA^{AG} Flow Schematic

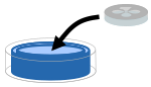


Protocol A – Growth cone turning assay (single neuronal culture)

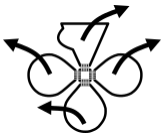
1) Under aseptic conditions, place the blue OMEGA cell culture evaporation minimizer into the bottom of the provided 35 mm culture vessel, ensuring the opening of the circular track faces upwards.

2) Using a sterile blade or scissors, cut open the package of the OMEGA^{AG} device. This can be performed over a collection vessel to catch PBS that will drip during removal of the device.

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.



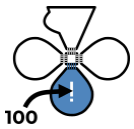
4) Place the device glass side down into the central opening of the evaporation minimizer.



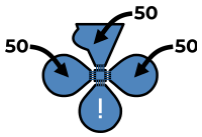
5) Remove the remaining PBS from all 4 chambers using a vacuum apparatus or manual pipette that has been fit with a fine tip (10 µL or 200 µL pipette tips work well). **Work efficiently to minimize the time chambers stay dry since the microchannels can quickly lose their hydrophilicity**.

6) If steps are required to coat/prepare the glass surface for culturing cells, proceed immediately with these steps. The working volume for each chamber is between 30 - 100 µL. Maintaining an excess fluid volume (30 - 60 µL) in the neuronal chamber (asymmetric volume loading) will permit the coating of microchannels. To minimize evaporation from the chambers during any incubation steps, carefully add ~500 µL of sterile water or PBS to the circular track of the evaporation minimizer.

Coating the neuronal chamber

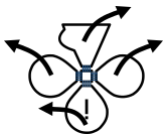


i. Add 100 µL of coating solution to the neuronal chamber.



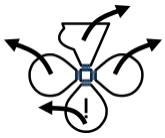
ii. Add 50 µL of sterile PBS or deionized water to all other chambers.

iii. Place in the incubator for the desired coating period (generally 2 – 16 hours depending on the coating type/procedure; it is recommended to incubate the coating for at least 1 hour).



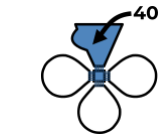
iv. Remove coating solution. If required, the chambers can be washed with 100 µL PBS or media. **Work efficiently to minimize the time chambers stay dry since the microchannels can quickly lose their hydrophilicity.**

v. If a second coating is required, repeat this process with the second coating solution (**Step i.**)



7) Prior to seeding cells in the neuronal chamber, remove all fluids from all chambers.

Seeding the neuronal chamber



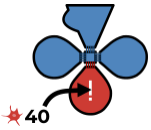
i. Add 40 µL of media to the overflow chamber.

ii. Add 30 µL of media to both source chambers **and** to the neuronal chamber.

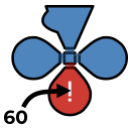
iii. Allow the viewing area and all microchannel interfaces to fill with media (allow ~10 minutes to ensure exchange).

iv. Add the appropriate number of neurons to at least 40 µL of media.

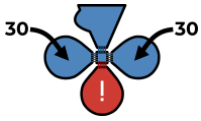
v. Remove media from the neuronal chamber and seed 40 µL of neurons into the neuronal chamber.



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

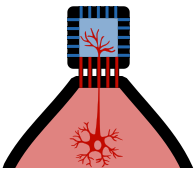


vii. Once the cells have settled, gently top up the neuronal chamber by adding 60 µL of media (final volume 100 µL).



viii. Add 30 µL of media to each source chamber (final volume 60 µL in each chamber).

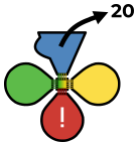
ix. Return the device to the incubator.



8) Incubate the neurons until axons have extended into the viewing area (this can take several days depending on the culture/cell type). Over the course of incubation, monitor and maintain the culture by performing media exchanges as required by the culture. Ensure that the relative fluid volumes in each chamber is maintained to preserve the direction of the flow through the microchannels. Verify and refill the fluid in the evaporation minimizers as needed.

9) Once axons have grown into the viewing area, replace the media in one or both source chambers (depending on the experiment) with 40 µL of fresh media supplemented with molecules of interest (e.g. chemotactic factor). In order to establish a uniform chemical gradient within the central viewing area, it is important that source chambers each contain equivalent volumes of fluid (they should each contain 40 µL). Ensure that the neuronal chamber contains a volume of at least 90 µL.

10) Immediately remove 20 µL of fluid from the overflow chamber (final volume 20 µL) and return the device to the incubator.



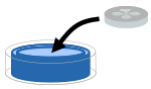
11) Axons in the viewing area can now be monitored for growth cone turning behavior.

Protocol B – Growth cone turning assay (co-culture applications)

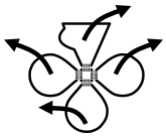
1) Under aseptic conditions, place the blue OMEGA cell culture evaporation minimizer into the bottom of the provided 35 mm culture vessel, ensuring the opening of the circular track faces upwards.

2) Using a sterile blade or scissors, cut open the package of the OMEGA^{AG} device. This can be performed over a collection vessel to catch PBS that will drip during removal of the device.

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.



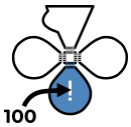
4) Place the device glass side down into the central opening of the evaporation minimizer.



5) Remove the remaining PBS from all 4 chambers a vacuum apparatus or manual pipette that has been fit with a fine tip (10 µL or 200 µL pipette tips work well). **Work efficiently to minimize the time chambers stay dry since the microchannels can quickly lose their hydrophilicity.**

6) If steps are required to coat/prepare the glass surface for culturing cells, proceed immediately with these steps. The working volume for each chamber is between 30 - 100 µL. Maintaining an excess fluid volume (30 - 60 µL) in the neuronal chamber (asymmetric volume loading) will permit the coating of microchannels. To minimize evaporation from the chambers during any incubation steps, carefully add ~500 µL of sterile water or PBS to the circular track of the evaporation minimizer.

Coating all chambers (for co-culture applications)

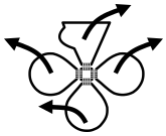


i. Add 100 µL of coating solution to the neuronal chamber.

ii. Add 70 µL of coating solution to each source chamber.

iii. Add 40 µL of sterile PBS to the overflow chamber.

iv. Place the device in the incubator for the coating incubation period (generally 2 – 16 hours depending on the coating type).

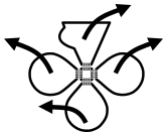


v. Remove the coating solution. If required, the chambers can be washed with 70 - 100 µL PBS or media.

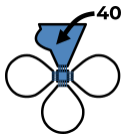
vi. If a second coating is required, repeat this process starting at **Step i.**

Depending on the timing for seeding the neuronal and source cultures, follow the relevant procedure below:

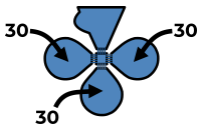
Seeding the neuronal culture first



i. Remove all fluids from all chambers.



ii. Add 40 µL of media to the overflow chamber.

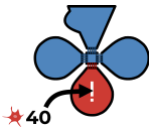


iii. Add 30 µL of media to each source chamber **and** to the neuronal chamber.

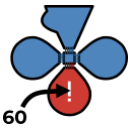
iv. Allow the viewing area and all microchannel interfaces to fill with media (~10 minutes).

v. Add the appropriate number of neurons to at least 40 µL of media.

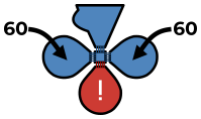
vi. Remove media from the neuronal chamber and seed 40 µL of neurons into the neuronal chamber.



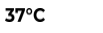
vii. Place the device in the incubator to allow the cells to settle and adhere to the surface (~10 minutes).



viii. Once the cells have adhered, gently top up the neuronal chamber by adding 60 µL of media (final volume 100 µL).

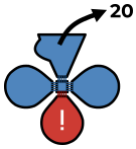


ix. Add 60 µL of media to each source chamber (final volume 90 µL).

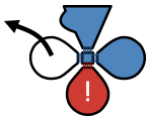


x. Return the device to the incubator.

Seeding the first source culture (can be days later)

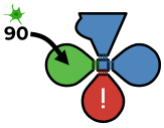


xi. Remove 20 µL of media from the overflow chamber.

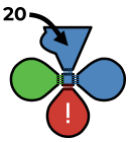


xii. Select one of the source chambers for seeding and remove the fluid from this chamber.

xiii. Add the appropriate number of cells (second culture) to at least 90 µL of media.



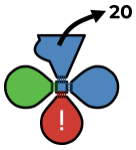
xiv. Seed 90 µL of cells into the selected source chamber.



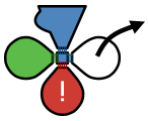
xv. Add 20 µL of media to the overflow chamber.

xvi. Return the device to the incubator.

Seeding the second source culture

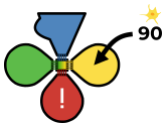


xvii. Remove 20 µL of media from the overflow chamber.

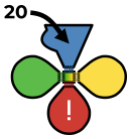


xviii. Remove all fluid from the unseeded source chamber.

xix. Add the appropriate number of cells (third culture) to at least 90 µL of media.



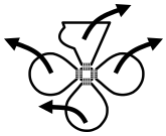
xx. Seed 90 µL of cells into the empty source chamber.



xxi. Add 20 µL of media to the overflow chamber.

xxii. Return the device to the incubator.

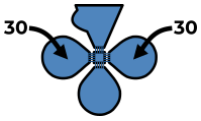
Seeding a source culture first



i. Remove fluids from all chambers.

ii. Add 40 µL of media to the neuronal chamber.

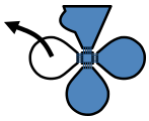
iii. Add 40 µL of media to the overflow chamber.



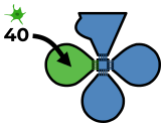
iv. Add 30 µL of media to **both** source chambers.

v. Allow the viewing area and all microchannel interfaces to fill with media (~10 minutes).

vi. Add the appropriate number of cells to at least 40 µL of media.

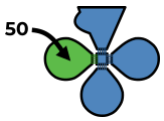


vii. Select one of the source chambers for seeding and remove the fluid from this chamber.

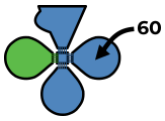


viii. Seed 40 µL of cells into this source chamber (the other source chamber can be seeded later).

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



x. Once the cells have adhered, gently top up the seeded source chamber by adding 50 µL of media (final volume 90 µL).

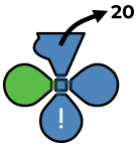


xi. Add 60 µL of media to the **unseeded** source chamber (final volume 90 µL).

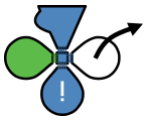
xii. Add 70 µL of media to the neuronal chamber (final volume 100 µL).

xiii. Return the device to the incubator.

Seeding the second source culture (optional)

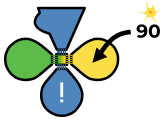


xiv. Remove 20 µL of media from the overflow chamber.

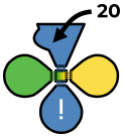


xv. Remove all fluid from the unseeded (empty) source chamber.

xvi. Add the appropriate number of cells (second culture) to at least 90 µL of media.



xvii. Seed 90 µL of cells into the unseeded source chamber.

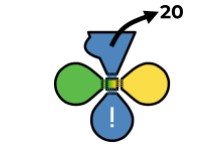


xviii. Add 20 µL of media to the overflow chamber.

37°C

xix. Return the device to the incubator.

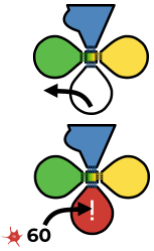
Seeding the neuronal culture (may be days later)



xx. Remove 20 µL of media from the overflow chamber.

xxi. Remove 40 µL of media from **each** source chamber.

xxii. Add the appropriate number of neurons to at least 60 µL of media.



xxiii. Seed 60 µL of neurons into the neuronal chamber.

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

xxv. Once the cells have settled, gently top up the neuronal chamber by adding 40 µL of media (final volume 100 µL).

xxvi. Add 40 µL of media to **each** source chamber (final volume 90 µL).

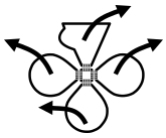
7) Over the course of incubation, monitor and maintain the cultures by performing media exchanges as required for each culture. Ensure that the relative fluid volumes in each chamber is maintained to preserve the established direction of the flow through the microchannels. It is important that source chambers each contain equivalent volumes of fluid in order to establish a uniform chemical gradient within the central viewing area. Verify and refill the fluid in the evaporation minimizers as needed.

8) Incubate the neurons until axons have extended into the viewing area (this may take several days depending on the culture/cell type). Axons can then be monitored for growth cone turning behavior.

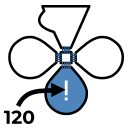
Protocol – Fixation and Immunohistochemistry

The following protocol is designed to fix and immunolabel culture within the chambers **as well as** processes located within the adjoining microchannels and viewing area. Microchannel labelling is achieved by simply employing asymmetrical volume loading, in the same way that it may have been used for chamber isolation and gradient formation. Please note that using the highest volume difference possible (e.g. 120 µL:40 µL) across chambers has been shown to be optimal for immunolabelling epitopes contained **within** the channels. If immunolabeling within microchannels and viewing area is not required, there is no need to use asymmetrical volume loading (equal volumes can be used in all adjacent chambers).

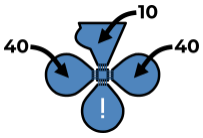
Fixation



1) Remove all solution from all chambers.

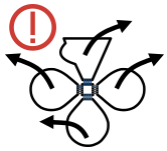


2) Carefully add 120 µL of **fixative** (e.g. 4% formaldehyde in PBS) to the neuronal chamber. Note: adding this volume of solution will overfill the chamber and slightly "balloon out" of the top of the chamber.



3) Add 40 µL of fixative to adjacent source chambers and 10 µL to the overflow chamber. Even though chambers may not contain cultures, it is necessary to perform these steps to ensure the viewing chamber is exposed to fixative.

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



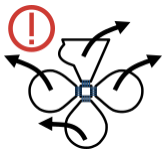
5) Remove fixative from all chambers.

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.

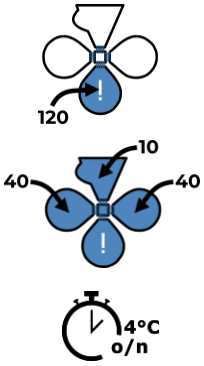
7) Repeat **Step 6** twice more, so that all chambers have been washed a total of three times

Immunohistochemistry

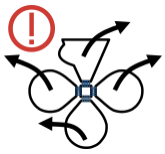
Blocking



8) Remove all solution from all chambers.



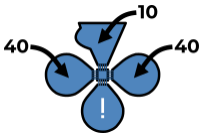
9) Repeat **Step 2 – 3** with **blocking solution** (e.g. 5 % normal serum, 0.2 % Triton X100, 0.05 % BSA), and incubate overnight at 4°C.



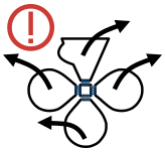
10) Remove blocking solution from all chambers and proceed with primary antibody application.

Primary Antibody

11) Repeat **Step 2 – 3** with **primary antibody solution** (dilution ratio(s) to be optimized).



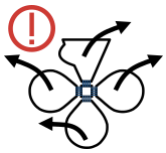
12) Incubate for 24 – 72 hours at 4°C.



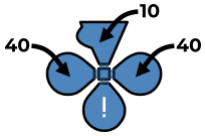
13) Remove primary antibody solution from all chambers.

14) Wash the chambers three times with PBS as described in **Steps 6 – 7** and proceed with secondary antibody application.

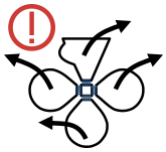
Secondary Antibody



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



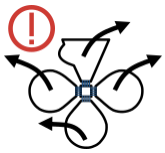
16) Incubate for 24 – 72 hours at room temperature.



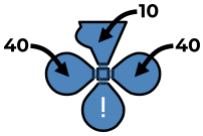
17) Remove secondary antibody solution from all chambers.

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

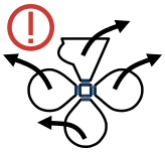
Nuclear Counterstaining



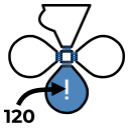
19) Repeat **Step 1 – 3** with **nuclear counterstain solution** (e.g. Hoechst or DAPI; dilution ratio to be optimized).



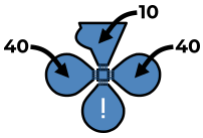
20) Incubate for 5 minutes at room temperature.



21) Remove nuclear counterstain solution from all chambers.



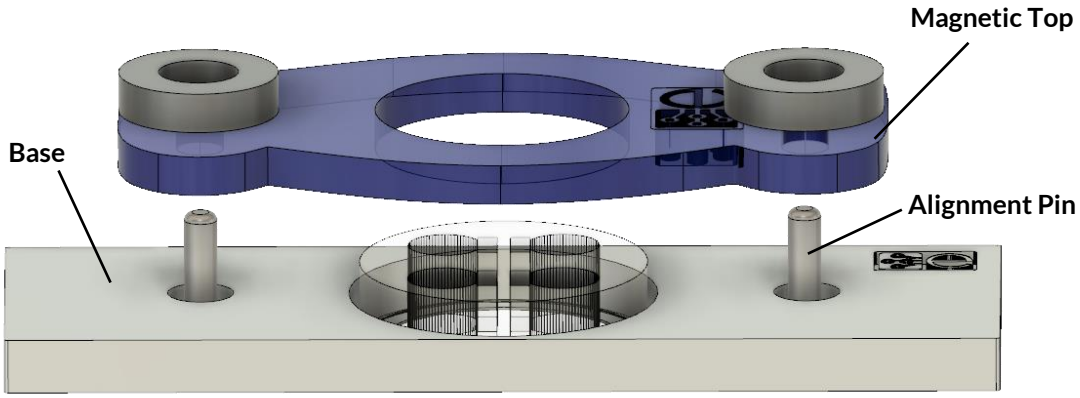
22) Add PBS solution to each chamber as described in **Steps 2 - 3**.



Protocol - Microscopy

Slide-size Microscopy Adapter

This adapter is suitable for end-point imaging of OMEGA 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 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 device into the central opening of the base, then slide the magnetic top onto the alignment pins. 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 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 the culture, carefully remove the OMEGA device from the 35 mm plastic culture dish with tweezers or forceps and place it in the 35 mm round microscope stage adapter (see figure below). Position the device in the center of the adapter to expose the bottom glass,

ensuring the device is level and flat. This assembly is designed to be used with microscope stage adapters that accommodate round 35 mm culture dishes.

Where additional stability is desired, a microscopy weight can be incorporated into the assembly (see schematic below). The weight serves as an interface between the top of the OMEGA 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 the 35 mm culture dish lid to maintain sterility during imaging sessions (as described above).