OMEGA^{ACE}

TRIPLE-CHAMBER NEURONAL CO-CULTURE DEVICE

User Guide

Contents

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v2.3

General Information

Storage and Shelf-life

OMEGA^{ACE} 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^{ACE}	OMEGA^{ACE} Devices (4)	Evaporation Minimizers (4) (reusable)	35 mm Dishes (4)	Microscope Adapter (1) (reusable)
Starter Kit (#eN-oace-001)	✓	✓	✓	✓
Refill Kit (#eN-oace-002)	✓		✓	
Refill Plus Kit (#eN-oace-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 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.**

Unpacking

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).

OMEGA^{ACE} 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.

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^{ACE} 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^{ACE} 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 common surface coating or 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.

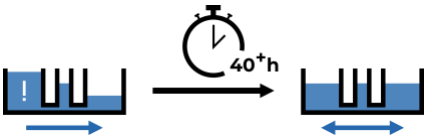
Flow Control and Asymmetrical Volume Loading

The OMEGA^{ACE} device has 3 chambers that are serially adjoined. 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.

* may require the use of a slide microscope stage adapter

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). However, in the case where two adjoined 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.

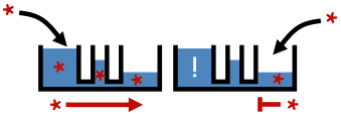
Importantly, chambers #1 and #3 of the OMEGA^{ACE} device are **not directly** joined by microchannels but are instead each adjoined to chamber #2. Chambers #1 and #3 have identical dimensions, whereas the surface area of chamber #2 is larger since it includes both upper and lower chambers, as well as the central 250 µm wide channel that links the upper and lower chambers. The total surface area of chamber #2 is approximately twice (2x) the surface area of chamber #1 or #3. Therefore, a level-to-volume ratio of approximately 2 should be implemented when calculating volume loading between chambers #1 and #2, or between chambers #2 and #3. For example, loading chambers #1 or #3 with 70 µL of fluid will result in a fluid level that is equivalent to loading chamber #2 (combined upper and lower chambers) with 140 µL (i.e. 70 µL x 2 = 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 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.

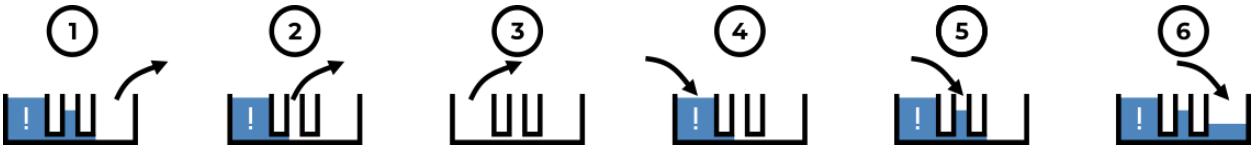
The upper and lower wells that comprise chamber #2 of the OMEGA^{ACE} device are separated by a 250 µm wide and 500 µm high channel. Unlike the microchannels that adjoin chambers #1 or #3 with chamber 2, and due to its relatively large dimensions, the main chamber #2 channel does not serve to fluidically isolate the upper and lower wells.

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^{ACE} device is approximately 30 mm^2. The total surface area of chamber #2 is approximately 60 mm^2. Optimal seeding 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 ~50 000 to 80 000 cells per 30 mm^2 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 30 mm^2 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 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

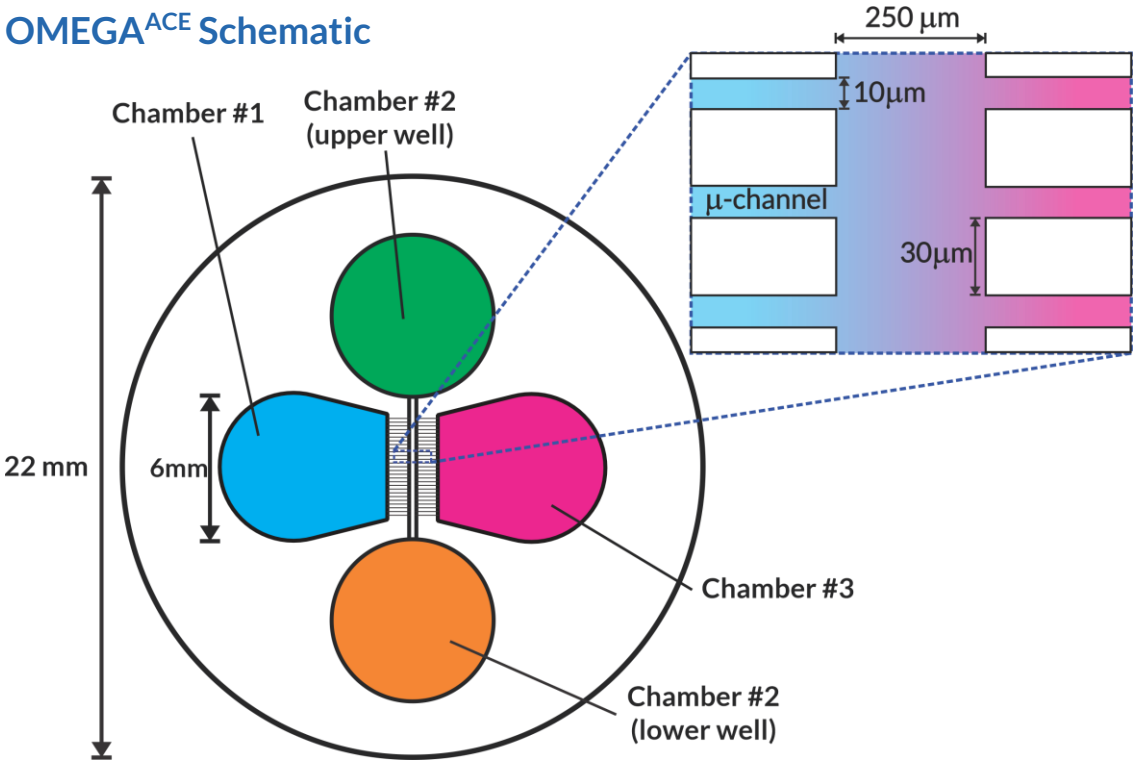
blue polydimethylsiloxane (PDMS) rings come packaged sterile and are designed to be reused (they can be sterilized using an autoclave or steam sterilizer for reuse). The inserts can be used as-is or can be rendered hydrophilic ("wettable") using a plasma or UV/ozone cleaner to facilitate fluid filling 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^{ACE} device is **permanently bonded** to high-transmissive #1.5 thickness (0.16 mm - 0.19 mm) glass. **The PDMS portion of the OMEGA^{ACE} 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^{ACE} Schematic



OMEGA^{ACE} Specifications

Chamber #1, #3 working volume: 30 – 140 µL
Chamber #1, #3 surface area: 29.6 mm^2
Chamber #2 working volume (combined): 60 - 280 µL
Chamber #2 combined surface area: 58.6 mm^2
Chamber #2 main channel width: ~250 µm
Chamber #2 main channel height: ~500 µm
Microchannel width: 10 µm
Microchannel length: >630 µm
Glass coverslip diameter: 22 mm
Glass coverslip thickness: 0.16 mm - 0.19 mm (#1.5)
Number of microchannels per interface: 70

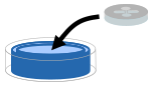
Protocol – Coating and Cell Seeding

Device Setup and Coating

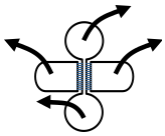
1) Under aseptic conditions, place a blue OMEGA cell culture evaporation minimizer into the bottom of the provided 35 mm culture vessel, ensuring the opening of the circular track is facing upwards.

2) Using a sterile blade or scissors, cut open the package of the OMEGA^{ACE} 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.

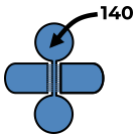


5) Remove remaining PBS from each of the chambers using a vacuum apparatus or manual pipette that has been fit with a fine tip (10 µL or 200 µL pipette tips work well). **Work efficiently to minimize the time chambers stay dry since the microchannels can quickly lose their hydrophilicity**.

6) Proceed immediately with coating/preparing the surface for culturing cells. To coat the microchannels, maintain an excess fluid level in **only one** of any adjoined chambers (i.e. asymmetric volume loading).

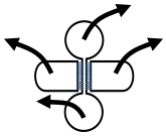
General coating procedure (this will coat all microchannels):

i. Add 80 µL of coating solution to chamber #1 and #3.



ii. Add 140 µL of coating solution to **only one** of the two wells of chamber #2. Alternatively, this volume can be split, and distributed between the upper and lower wells of chamber #2.

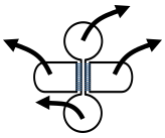
iii. Incubate the device for 2 – 16 hours, depending on the coating type and procedure being used. When using poly-D-lysine (PDL) or poly-L-ornithine (PLO), it is recommended to use a 100 µg/mL solution in sterile water and incubate the coating for 3 hours at room temperature. To minimize evaporation from the chambers during incubation steps, add ~500 µL of sterile water or PBS to the circular track of the evaporation minimizer.



iv. Remove coating solution. If required, the chambers can be washed with 70 - 100 µL 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 µg/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).**

Seeding Cells

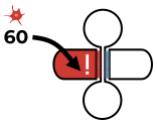


1) Prior to seeding cells, **remove all solutions from all chambers**. If starting with a cold device, place the device in a 37°C incubator for 1 hour before removing all fluid.
Follow the correct appropriate protocol below for your intended experiment. The protocols assume the user has prepared and counted their cells prior to proceeding with seeding.

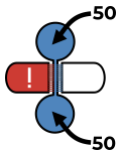
Single neuronal cultures

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

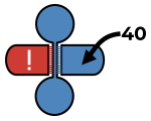
a. Add the appropriate number of cells into at least 60 µL of media.



b. Seed 60 µL of cells into chamber #1.

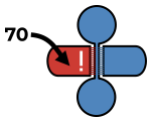


c. Add 50 µL of media to **each (upper and lower)** well of chamber #2 (total 100 µL).

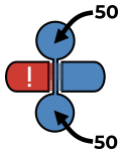


d. Add 40 µL of media to chamber #3.

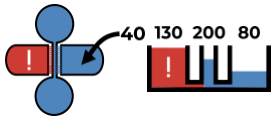
e. Cover the dish and place it in the incubator. Allow the cells to settle and adhere to the surface (~10 minutes).



f. Once the cells have adhered, gently top up chamber #1 by adding 70 µL of media (final volume 130 µL).



g. Add 50 µL of media to **each (upper and lower)** well of chamber #2 (final volume 200 µL in chamber #2).



h. Add 40 µL of media to chamber #3 (final volume 80 µL).

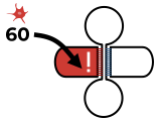
i. Return the device to the incubator.

Establishing neuronal co-cultures (2 - 3 cultures)

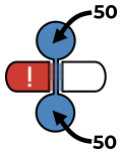
Note: The following protocol provides example fluid volumes for the described culture setup (i.e. direction of flow across microchannels, compartmental isolation). These volumes can be adjusted accordingly to suit your specific experimental needs.

For seeding the first culture in chamber #1

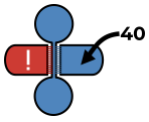
a. Add the appropriate number of cells in at least 60 µL of media. Start by plating the neuronal culture (the culture destined for outgrowth).



b. Seed 60 µL of cells into chamber #1.

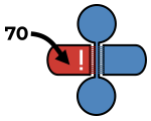


c. Add 50 µL of media to **each (upper and lower)** well of chamber #2.



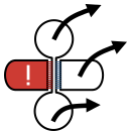
d. Add 40 µL of media to chamber #3.

e. Place the device in the incubator to 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 µL of media (final volume 130 µL).

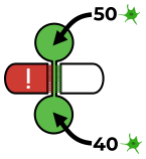
g. Incubate the device for at least 1 hour before seeding the second culture. If waiting an extended period of time (i.e. days) before seeding the second culture, add 50 µL of media to **each (upper and lower)** well of chamber #2 and 40 µL of media to chamber #3 and return to the incubator.



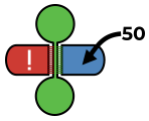
h. When the **second culture** is ready to be seeded, remove all media from all unseeded chambers. Ensure chamber #1 has 130 µL of fluid.

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 µL of media.

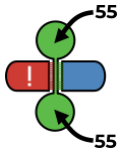


b. Seed 90 µL of cells into chamber #2, splitting the volume **unevenly** between the upper and lower wells. Note: Asymmetrical loading will promote the flow of cells into the central channel of chamber #2. The flow rate, and therefore the cell seeding rate, between the upper and lower well of chamber #2 is controlled by extent of difference in the fluid level between these two wells. Therefore, seeding 50 µL into the upper well, and then quickly seeding 40 µL into the lower well will result in cells flowing from the upper to the lower well through the central channel.

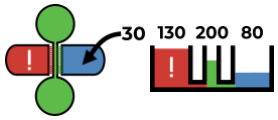


c. Add 50 µL of media to chamber #3.

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 55 µL of media to each (upper and lower) wells of chamber #2 (final volume 200 µL).



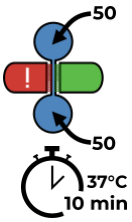
f. Add 30 µL of media to chamber #3 (final volume 80 µL).

g. Return the device to the incubator.

For seeding a second culture in chamber #3

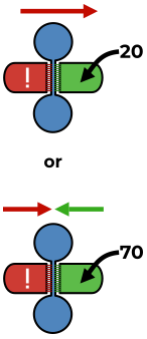
a. Add the appropriate number of cells into 60 µL of media.

b. Seed 60 µL of cells into chamber #3.

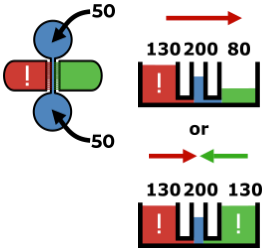


c. Add 50 µL of media to **each (upper and lower)** well of chamber #2 (total 100 µL).

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, adjust the volume in chamber #3. If overall flow is desired to be towards chamber #3, add 20 µL of media (final volume 80 µL). If flow is desired away from chamber #3 (towards chamber #2), add 70 µL of media (final volume 130 µL).

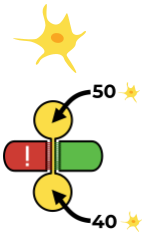


f. Add 50 µL of media to **each (upper and lower)** well of chamber #2 (final volume 200 µL).

g. Return the device to the incubator

For seeding a third culture in chamber #2

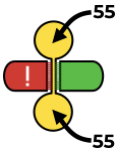
If a **third culture** is to be seeded, remove all media from the unseeded chamber. Ensure chamber #1 has 130 µL of fluid



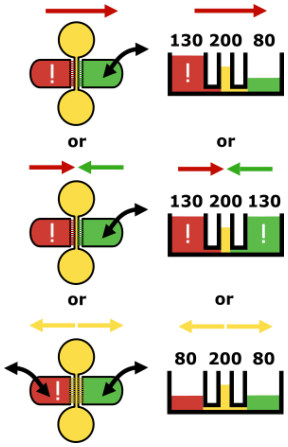
a. Add the appropriate number of cells into at least 90 µL of media.

b. Seed 90 µL of cells into chamber #2, splitting the volume unevenly between the upper and lower chambers. Note: Asymmetrical loading will promote the flow of cells into the central channel of chamber #2. The flow rate, and therefore the cell seeding distribution, between the upper and lower well of chamber #2 is controlled by extent of difference in the fluid level between these upper and lower chambers. Consequently, seeding 50 µL into the upper well, and then quickly seeding 40 µL into the lower well will result in cells flowing from the upper to the lower chambers through the central channel.

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 55 µL of media to **each (upper and lower)** well of chamber #2 (final volume 200 µL).



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

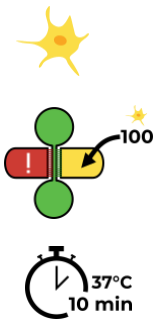
f. Return the device to the incubator.

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

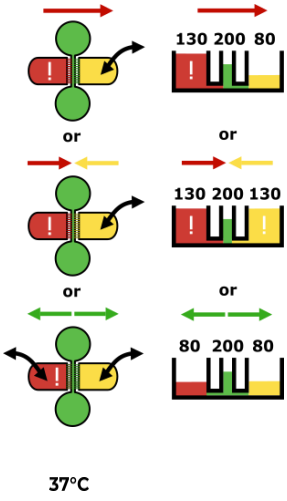
If a **third culture** is to be seeded, remove all media from the unseeded chamber. Ensure chamber #1 has 130 µL of fluid



a. Add the appropriate number of cells into at least 100 µL of media.

b. Seed 100 µL 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 volume in chambers #1 and/or #3 to determine the desired direction of flow access the microchannels.

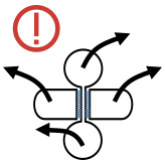
e. Return the device to the incubator.

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.

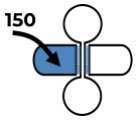
Protocol – Fixation and Immunohistochemistry

The following protocol is designed to fix and immunolabel cultures 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 the highest volume difference possible (e.g. 150 µL:50 µL) across adjacent chambers is critical to efficiently immunolabel epitopes contained **within** the microchannels. In cases where immunolabeling within microchannels is not required or desired, there is no need to use asymmetrical volume loading, and equal volumes can be used in both chambers.

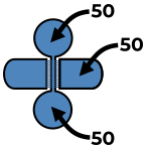
Fixation



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.

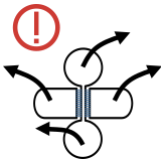


2) Carefully add 150 µL of **fixative** (e.g. 4% formaldehyde in PBS) to the isolated chamber. Note: this volume of solution may overfill the chamber and slightly "balloon out" of the top of the chamber – this is normal.



3) Add 50 µL to **each of the wells** of chamber #2, and 50 µL of fixative to chamber #3.

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



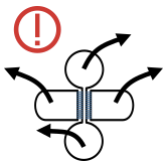
5) Remove fixative from all chambers. As in **Step 1**, begin removing solution from the non-isolated chamber first.

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).

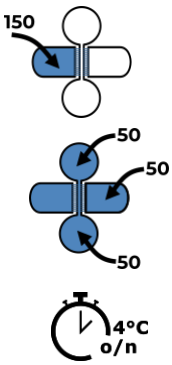
7) Repeat **Step 6** twice more, so that all chambers have been washed a total of three times

Immunohistochemistry

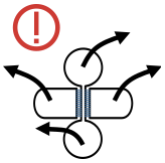
Blocking



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

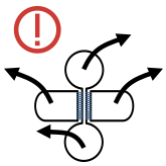


9) Repeat **Step 2 – 3** with **blocking solution** (e.g. 5 % normal serum, 0.2 % Triton X100, 0.05 % BSA), and incubate overnight at 4°C.

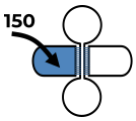


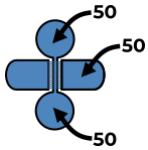
10) Remove blocking solution from all chambers. As in **Step 1**, begin removing solution from the non-isolated chamber first.

Primary Antibody

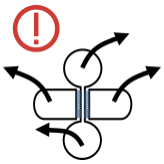


11) Repeat **Step 1 – 3** with **primary antibody solution** (dilution ratio(s) to be optimized).





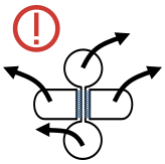
12) Incubate for 24 – 72 hours at 4°C.



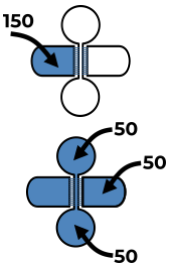
13) Remove primary antibody solution from all chambers. As in **Step 1**, begin removing solution from the non-isolated chamber first.

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

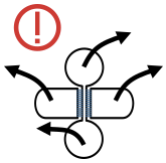
Secondary Antibody



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



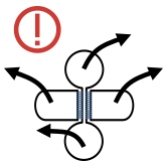
16) Incubate for 24 – 72 hours at room temperature.



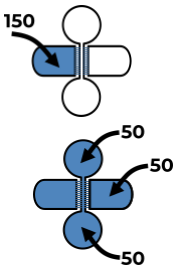
17) Remove secondary antibody solution from all chambers. As in **Step 1**, begin removing solution from the non-isolated chamber first.

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

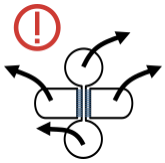
Nuclear Counterstaining



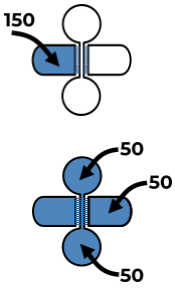
19) Repeat **Step 1 – 3** with **nuclear counterstain solution** (e.g. Hoechst or DAPI; dilution ratio to be optimized).



20) Incubate for 5 minutes at room temperature.



21) Remove nuclear counterstain solution from all chambers. As in **Step 1,** 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^{ACE} 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^{ACE} 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^{ACE} 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^{ACE} 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.