OMEGA^{96}

NEURONAL CO-CULTURE MICROPLATE
User Guide

Contents

eNUVIO
5524 Saint-Patrick, Office 460
Montreal, Quebec, Canada
H4E 1A8

CONTACT US AT INFO@ENUVIO.COM

v2.2

General Information

Storage and Shelf-life

Uncoated OMEGA^{96} microplates can be stored in their original packaging at room temperature and protected from direct sunlight/UV exposure for up to 6 months. Pre-coated microplates can be stored in their original packaging at room temperature and protected from direct sunlight/UV exposure for 1 month. Devices that have been removed from their packaging 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

eN-o96-001: 1 microplate, uncoated
eN-o96-002: 1 microplate, Poly-L-Ornithine coated
eN-o96-003: 1 microplate, Poly-D-Lysine coated

Before Starting – IMPORTANT

The OMEGA^{96} plates have 200µm glass bottoms that are fragile. To avoid cracking the glass, the plates should be handled with care, touching only the outer black plastic frame when manipulating or moving the plate. When manipulating the plate, it is recommended to grasp the black plastic frame at its corners to avoid compressing the anti-evaporation reservoirs during manipulation. Compressing the reservoirs will not lead to damage of the frame; however, this can cause fluids that have been added to the reservoirs to spill.

The tops of the wells of the OMEGA^{96} plate have been sealed to prevent fluid loss during shipment. The sealed plate is packaged in a sterile bag. If the plate has been handled roughly during shipping, some of the shipping PBS may escape the top sealer and may be found on the inside of the plate lid and/or on the plate frame. **Leaks of this kind do not affect the sterility or functionality of the plate, provided that (1) the bag has not been compromised, and (2) the device microchannels remain wet.**

Unpacking

Each plate has been carefully packaged under sterile conditions. To maintain its sterility, it is recommended to unpack the plate in an aseptic environment (e.g. in a biological safety cabinet). OMEGA^{96} plates are shipped "wet", that is their wells contain sterile filtered (0.1 micron) phosphate buffered saline (PBS; without divalents). The plates are **ready-to-use** for coating and/or seeding of cells.

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 plate from its sealed packaging. The wells of the OMEGA^{96} plates have been sealed to prevent liquid loss during shipping. The plate sealer is removed by gently pulling on the white tab located at one side of the plate. **NOTE: remove the sealer by exerting a gentle and horizontal force to prevent damage to the upper structure of the plate.**

OMEGA^{96} plates are compatible with a variety of common downstream experimental procedures including:

a) Fixation and immunohistochemistry
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) Classical patch-clamp or optical electrophysiology

Surface Coating

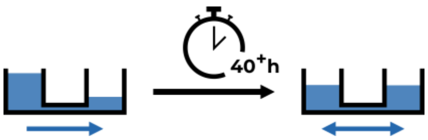
To render the growth surface of each OMEGA^{96} well suitable for culturing cells, they must first be modified with a suitable coating. If plates have been purchased uncoated, these will need to be coated before seeding cells. The type of coating should be optimized for each culture/cell type that is being seeded in the plate. 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 ECM 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 in **cold** media). Applying this combination of coatings on OMEGA devices **will not** result in the clogging or blocking of microchannels.

Flow Control and Asymmetrical Volume Loading

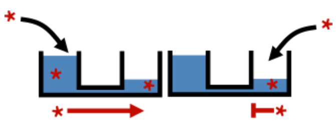
The OMEGA^{96} plate contains 48 individual chamber-pairs (for a total of 96 chambers), where each chamber-pair consists of 2 interconnected chambers that are joined by a series of 70 microfluidic channels. The direction of the flow of fluid across these high resistance microchannels can be controlled by adjusting the relative level of fluid in each of the chambers. It is the chamber fluid **level** that provides the force required to drive flow across the microchannels. Although there is a direct relationship between chamber fluid level (i.e. fluid height in the chamber) and fluid volume, it is the fluid level that primarily contributes to the force that will be applied across the microchannels. Consequently, it is differences in fluid levels that will provide the force required to drive fluid to flow from a chamber with a relatively higher fluid level towards a chamber with a relatively lower fluid level.

When two adjacent chambers joined by microchannels have identical dimensions, the relationship between chamber fluid level and volume is identical for each of the chambers. Therefore, directional flow across the joining microchannels can be easily determined by directly comparing each chamber's fluid **volume** (fluid will flow towards the chamber with a lower volume). **All of the chambers in OMEGA^{96} plates have identical dimensions, and therefore chamber fluid volume can be used to simply adjust the directionality of the flow across the microchannels.**



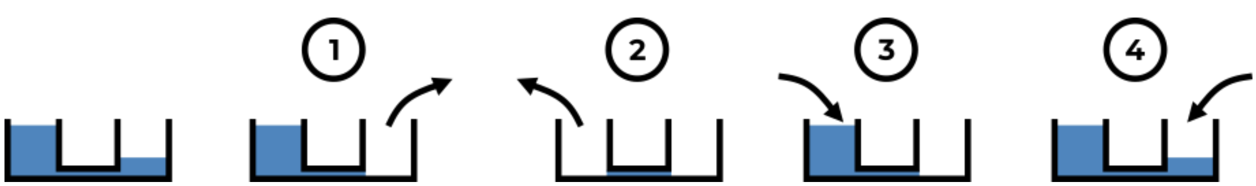
When adjacent chambers are loaded with different volumes of fluid for the purposes of driving a unidirectional flow across the adjoining microchannels, we refer to this as **"asymmetrical volume loading"** of the chambers. The unidirectional flow across the microchannels created by asymmetrically volume 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. Accordingly, 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 **<u>will not be</u>** exposed to molecules that have been specifically added to the chamber with the lower fluid level. However, the chamber with lower fluid level **<u>will be</u>** 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 each chamber of the OMEGA^{96} plate is ~0.35 cm^2 (approximately equivalent to the area of a single chamber of a standard 96-well plate). Optimal seeding density will depend largely on the nature and type of culture being seeded in the device. It is therefore strongly recommended to conduct a series of optimization experiments to determine the ideal cell plating 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.

Anti-Evaporation Reservoirs

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). Microplate wells are known to be particularly prone to evaporation, especially those located around the outer border of the plate. This can lead to the loss of the seeded culture (often to the surprise of the user) as the media gradually concentrates over time and can introduce unwanted variability in the data collected for the experiment. Unfortunately, these edge effects are so problematic that wells located along the outer edge of 96-well microplates are often left unused. To combat evaporation, all OMEGA^{96} plates have fluid reservoirs built into their black outer structure. When these are filled with liquid, they serve to reduce evaporation that occurs from the outermost chambers of the plate. In this way, these outer chambers can become available to be used in the experiment.

IMPORTANT: Although the culture evaporation reservoirs do help to reduce evaporation rates during the incubation of cultures, they do not outright prevent evaporation. **Therefore, it is vital that the fluid levels 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 anti-evaporation reservoirs 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.

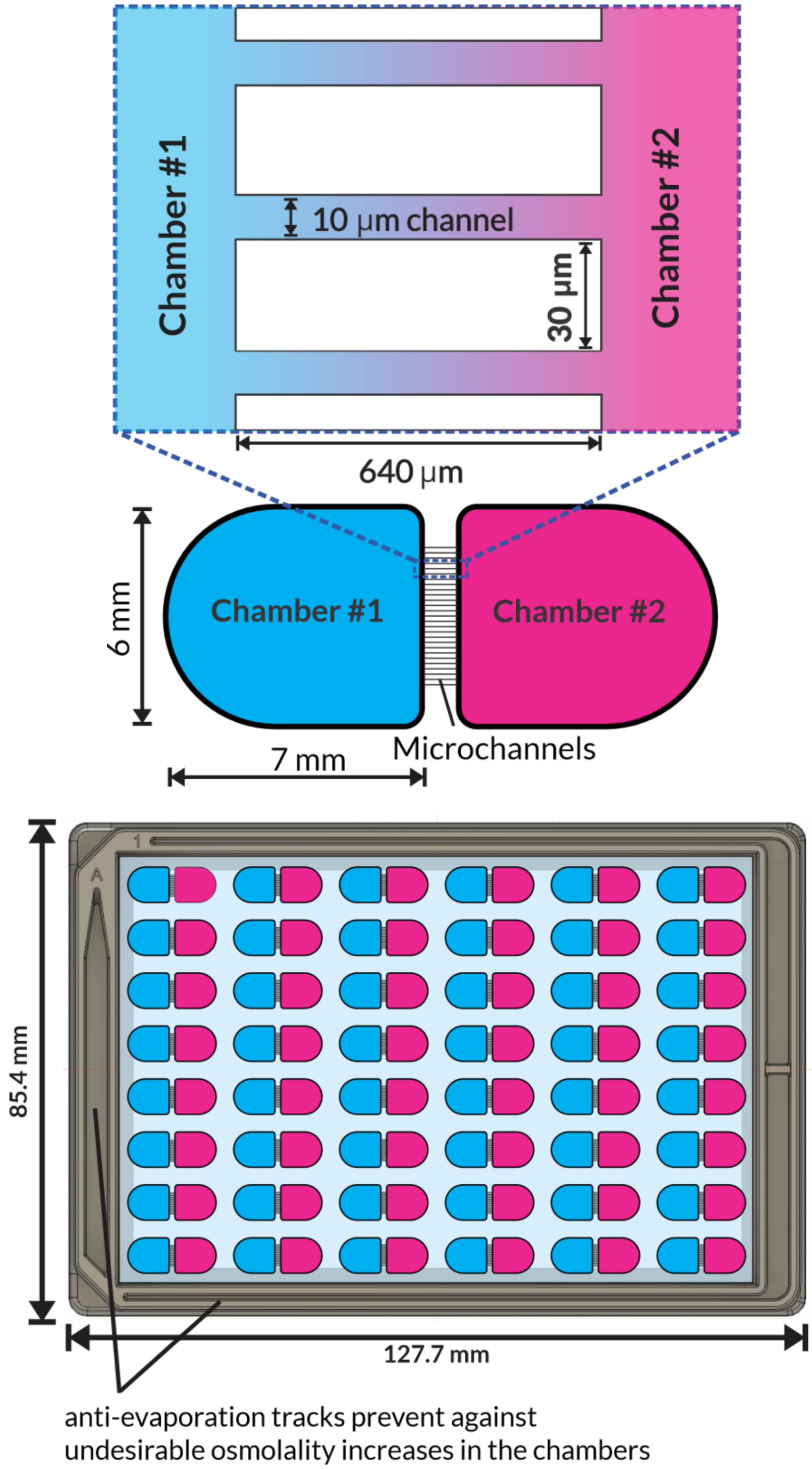
Microscopy

Once cultures have been seeded, they can be examined over time using common microscopy techniques (e.g. brightfield or phase contrast). For example, OMEGA^{96} plates can be used for repeated live-cell imaging sessions using fluorescence markers, and/or fixed and immunolabeled with antibodies for immunohistochemical analysis. Immunochemistry processing, for example, can be performed by following the protocol provided in this user guide. The procedure can be used to label axonal processes located in the microchannels. Purposefully designed to conform to

eNUVIO
5524 Saint-Patrick, Office 460
Montreal, Quebec, Canada
H4E 1A8

ANSI/SLAS microplate dimensional standards, OMEGA[96] plates are adapted to fit in most fluorescence microscope stages using universal microplate stage holders. Similarly, the pitch and relative position of each well conforms to 96-well standards, ensuring compatibility with automated machinery such as robotic liquid handlers, plate hotels/incubators, and high-content screening systems that are designed to function with standard microplate formats.

OMEGA[96] Schematic



OMEGA^{96} Specifications

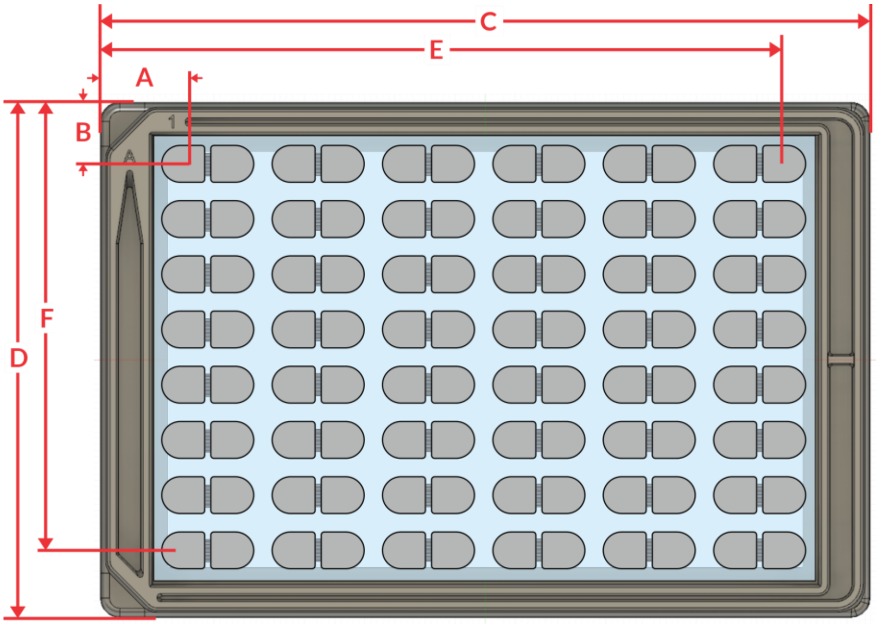
48 chamber pairs per microplate
Outer dimensions (w/ lid; *l* x *w* x *h*): 127.7 x 85.4 x 16.9 mm
Glass bottom thickness: 200 µm
Chamber dimensions: 6 x 7 mm
Chamber working volume: 100 - 325 µL
Max. chamber volume: 350 µL
Chamber surface area: ~0.35 cm^2
Number of microchannels per chamber-pair: 70
Microchannel length: ~640 µm
Microchannel width: 10 µm
Anti-evaporation reservoir (each): Recommended working volume: 1.6 mL (max. vol. 2 mL)

OMEGA^{96} Dimensions to Calibrate Automated Equipment

<u>Please note it is strongly recommended to test and verify the calibration with your equipment.</u>

General Plate Dimensions

(A) A1 to side offset: 15.5 mm
(B) A1 to top offset: 11.4 mm
(C) Length: 127.76 mm
(D) Width: 85.48 mm
(E) Well A12 to side offset: 112.5 mm
(F) Well H1 to top offset: 74.15 mm
Plate height: 14.2 mm
Glass bottom height from frame base: 1.19 mm
Glass bottom thickness: 0.2 mm



Revvity (formerly Perkin Elmer) Opera Phenix HCS Instrument Plate Type Definition

(A) Column offset: 18.47 mm
(B) Row offset: 11.4 mm
(C) Length: 127.76 mm
(D) Width: 85.48 mm
(E) Last column: 107.45 mm
(F) Last row: 74.15 mm
(G) Total plate height: 14.2 mm
(H) Bottom plate to bottom glass height: 1.19 mm
(I) Glass bottom thickness: 0.2 mm
(L) Well top (X) 15.24 mm
(M) Well top (Y) 6 mm
(O) Well bottom (X) 15.24 mm
(P) Well bottom (Y) 6 mm

Columns: 6
Rows: 8
Top Shape: Rectangle
Bottom Shape: Rectangle

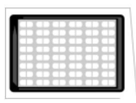
eNUVIO
5524 Saint-Patrick, Office 460
Montreal, Quebec, Canada
H4E 1A8

Protocol – Neuronal Cultures

General Information

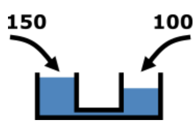
OMEGA^{96} microplates are shipped sterile. To maintain sterility, perform all relevant cell culture steps in a biological safety cabinet. Prepare all necessary reagents and consumables (media, PBS, tips, etc.) to complete the protocol prior to starting. If performing these steps manually, the use of a multichannel repeater pipette is recommended. **The following protocols are intended as a guideline only and the user is encouraged to modify this to best suit their experimental needs.**

General coating procedure



i. Under aseptic conditions, remove the OMEGA^{96} from its plastic packaging. Remove the plate lid, and then the plate sealer. Apply a constant force to peel off the plate sealer by gently pulling on the white sealer tab.

ii. Remove the shipping PBS from each well using a vacuum apparatus. **Work efficiently to minimize the time the wells stay dry since the microchannels can quickly lose their hydrophilicity.**

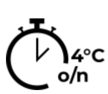


iii. Add 150 µL of coating solution to one chamber of each chamber-pair.

iv. Add 100 µL of coating solution to the adjacent chamber of each chamber-pair.

v. Incubate the microplate 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.

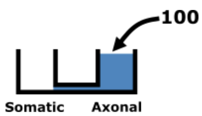
vi. Remove the coating solution. If required, the chambers can be washed with 100 -150 µL PBS or media. **Work efficiently to minimize the time chambers from completely drying.** For PDL or PLO coatings, perform three 20-minute washes with sterile water at room temperature using asymmetric volume loading. **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.**



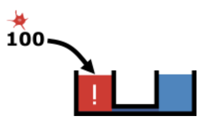
vii. If a second coating is required, repeat the process with the second coating solution (**Step iii.**). When using **laminin** (5 µg/mL in **cold media**) or **basement membrane matrix** (100x diluted in **cold media**), add the coating solution using asymmetric volume loading to a **cold microplate**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

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

<u>Single neuronal cultures (compartmentalization purposes)</u>



i. Add 100 µL of seeding media to the axonal (non-seeded) chamber. The volume to add should be equivalent to the planned seeding volume. For example, if the seeding volume in the somatic chamber will be 100 µL, then add 100 µL to the axonal chamber.



ii. Seed cells in 100 µL of seeding media into the somatic chamber. The volume of media to seed with should be equivalent to the volume of media in the axonal chamber from **Step i**.

iii. Place the microplate in the 37°C / 5% CO_2 incubator overnight to allow cells to settle and adhere to the surface.

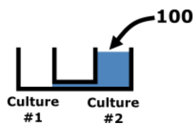
iv. The next day, top up chambers as necessary for the intended experiment (e.g. chamber isolation; up to a maximum of 325 µL of media in each chamber). If a rho-kinase inhibitor (ROCK inhibitor) was used when seeding, perform a full media change with fresh media without ROCK inhibitor.

v. Fill each anti-evaporation reservoir with 1.6 mL of water or PBS, and return the plate to the incubator.

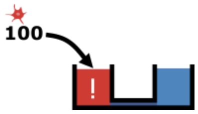
vi. Over the course of incubation, monitor the fluid volumes of the culture chambers, exchanging the media as required by the culture. Ensure that each chamber contains the correct volume of media to maintain the desired experimental conditions (e.g. chamber isolation). Verify and refill the fluid in the evaporation minimizers as needed. Axonal outgrowth can require several days to weeks to fully establish

eNUVIO
5524 Saint-Patrick, Office 460
Montreal, Quebec, Canada
H4E 1A8
CONTACT US AT INFO@ENUVIO.COM

Establishing neuronal co-culture



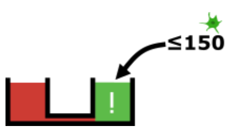
i. Add 100 µL of seeding media to the culture #2 chamber (to be seeded later). The volume to add should be equivalent to the planned seeding volume for culture #1. For example, if the seeding volume for culture #1 will be 100 µL, then add 100 µL to the culture chamber #2.



ii. Seed cells in 100 µL of seeding media into culture chamber #1. The volume of media to seed with should be equivalent to the volume of media in the culture chamber #2 from **Step i**.

iii. Incubate the microplate for at least 1 hour before seeding a second culture. If the second culture is to be seeded several days later, top up both chambers to a maximum of 325 µL per chamber employing asymmetrical volume loading where necessary for the intended experiment. If a rho-kinase inhibitor (ROCK inhibitor) was used when seeding cells, perform a full media change with fresh media without ROCK inhibitor the day after seeding.

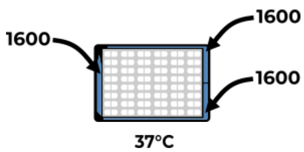
iv. When the second culture is ready to be seeded, remove all media from the culture chamber #2.



v. Seed the second culture into culture chamber #2 in an equivalent or less volume of media compared to culture chamber #1. For example, if the first culture is maintained with 150 µL of media, seed the second culture in ≤150 µL of media. If ROCK inhibitor is used when seeding the second culture, it is recommended to use less volume when seeding compared to the culture chamber #1 chamber to isolate the ROCK to culture chamber #2.

vi. Place the device in a 37°C / 5% CO_2 incubator overnight.

vii. The next day, top up each chamber as necessary for the intended experiment (e.g. for chamber isolation; up to a maximum of 325 µL of media in each chamber). If ROCK inhibitor was used in seeding culture #2, perform a full media change for culture chamber #2 with fresh media without ROCK inhibitor).



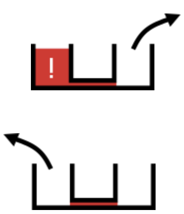
viii. Fill each anti-evaporation reservoir with 1.6 mL of water or PBS, and return the plate to the incubator.

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

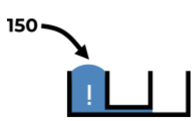
Protocol – Fixation and Immunohistochemistry

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. 350 µL:50 µL) across adjacent chambers will facilitate immunolabelling 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.

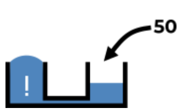
Fixation



1) Remove all solution from both 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.

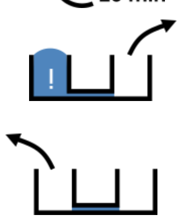


2) Carefully add at least 150 µL of **fixative** (e.g. 4% formaldehyde in PBS) to the isolated chamber. Note: this volume can be increased to as much as 350 µL, however this volume of solution may overfill the chamber and slightly "balloon out" of the top of the chamber.



3) Add 50 µL of fixative to the adjacent chamber.

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



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

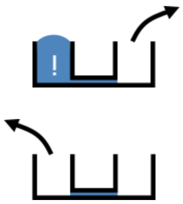
6) Wash the chambers by repeating **Steps 1 - 4** with **PBS**, observing the order in which chambers are emptied and refilled to maintain chamber isolation.

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

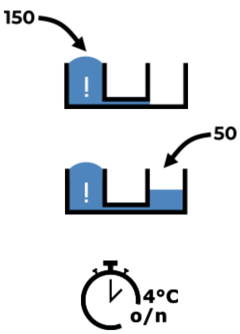
eNUVIO
5524 Saint-Patrick, Office 460
Montreal, Quebec, Canada
H4E 1A8

Immunohistochemistry

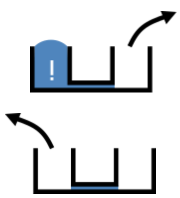
<u>Blocking</u>



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

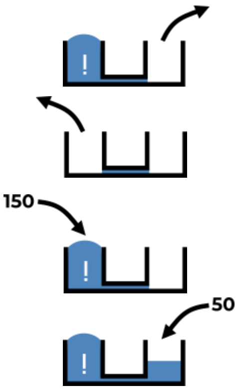


9) Repeat **Step 2 – 3** with **blocking solution** (e.g. 5 % normal serum, 0.2 % Triton X100, 0.05 % BSA), and incubate overnight at 4°C.



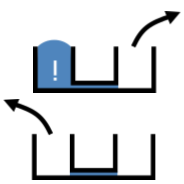
10) Remove blocking solution from both chambers. As in **Step 1**, begin removing solution from the non-isolated chamber first.

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.



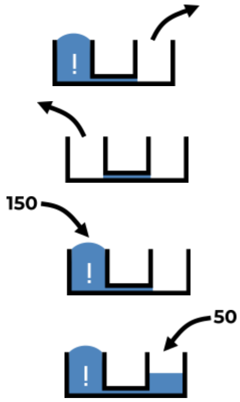
13) Remove primary antibody solution from both chambers. As in **Step 1**, begin removing solution from the non-isolated chamber first.

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

eNUVIO
5524 Saint-Patrick, Office 460
Montreal, Quebec, Canada
H4E 1A8

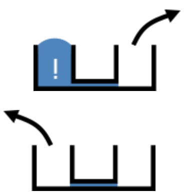
CONTACT US AT INFO@ENUVIO.COM

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.



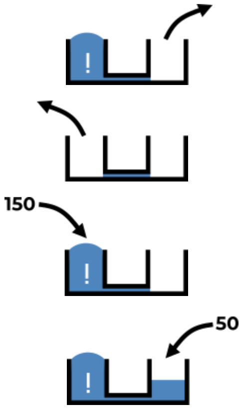
17) Remove secondary antibody solution from both chambers. As in **Step 1**, begin removing solution from the non-isolated chamber first.

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

5524 Saint-Patrick, Office 460
Montreal, Quebec, Canada
H4E 1A8

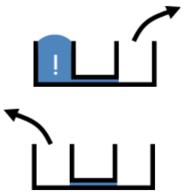
CONTACT US AT INFO@ENUVIO.COM

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 both 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.**

5524 Saint-Patrick, Office 460
Montreal, Quebec, Canada
H4E 1A8

CONTACT US AT INFO@ENUVIO.COM