OMEGA⁴

User Guide

Contents

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General Information

Unpacking

Thank you for purchasing eNUVIO's OMEGA^4 devices. All the items contained within the shipping vial have been carefully packaged under sterile conditions. To maintain sterility, it is recommended to unpack the contents of the shipping vial in an aseptic environment (e.g. in a biological safety cabinet). The shipping vial (and label) is completely autoclavable and can be repurposed (it is also recyclable).

OMEGA^4	OMEGA^4 Devices (4)	Evaporation Minimizers (4)	35 mm Dishes (4)	Microscope Adapter (1)
Starter Kit (#eN-o4-001)	✓	✓	✓	✓
Refill Kit (#eN-o4-002)	✓		✓	
Refill Plus Kit (#eN-o4-003)	✓	✓	✓	

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

Before Starting – IMPORTANT

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

Owing to its thinness, the glass coverslip that has been bonded to each device is fragile and must be handled with care. We take great care in packaging each device for shipment, however if the product is mishandled or handled roughly during shipment, the glass bottom may arrive cracked or broken. Cracks in the glass can easily be seen through the individual device's plastic packaging, and therefore we strongly recommend that each device be inspected carefully **prior to opening the device's individual plastic packaging**. If any cracks within the glass coverslip are noticed, please send a photo of the damaged device in its unopened plastic sleeve including your order number to info@enuvio.com. 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. 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[4] devices 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) Patch-clamp electrophysiology

Surface Coating

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

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

Flow Control and Asymmetrical Volume Loading

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

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

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

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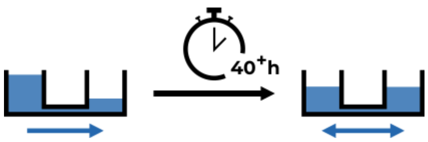
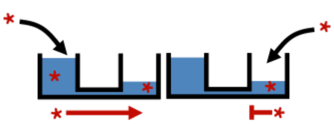
interconnected chambers in OMEGA[4] devices have identical dimensions, and therefore chamber fluid volume can be used to adjust the directionality of the flow across the microchannels.

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. With regular verification and adjustment of the chamber fluid volumes, the unidirectional flow can be maintained perpetually.

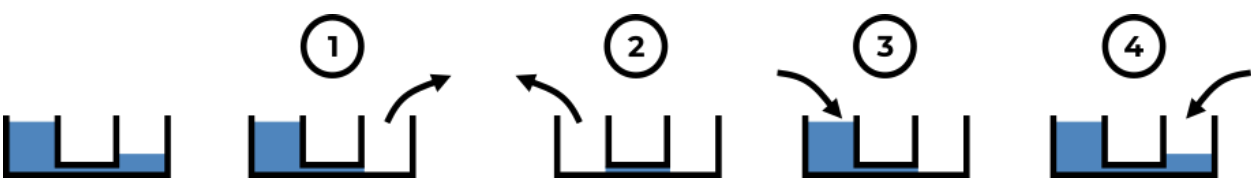


When to Apply Asymmetrical Volume Loading



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[4] device 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 plated 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 between ~40 000 - 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.

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 set up 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

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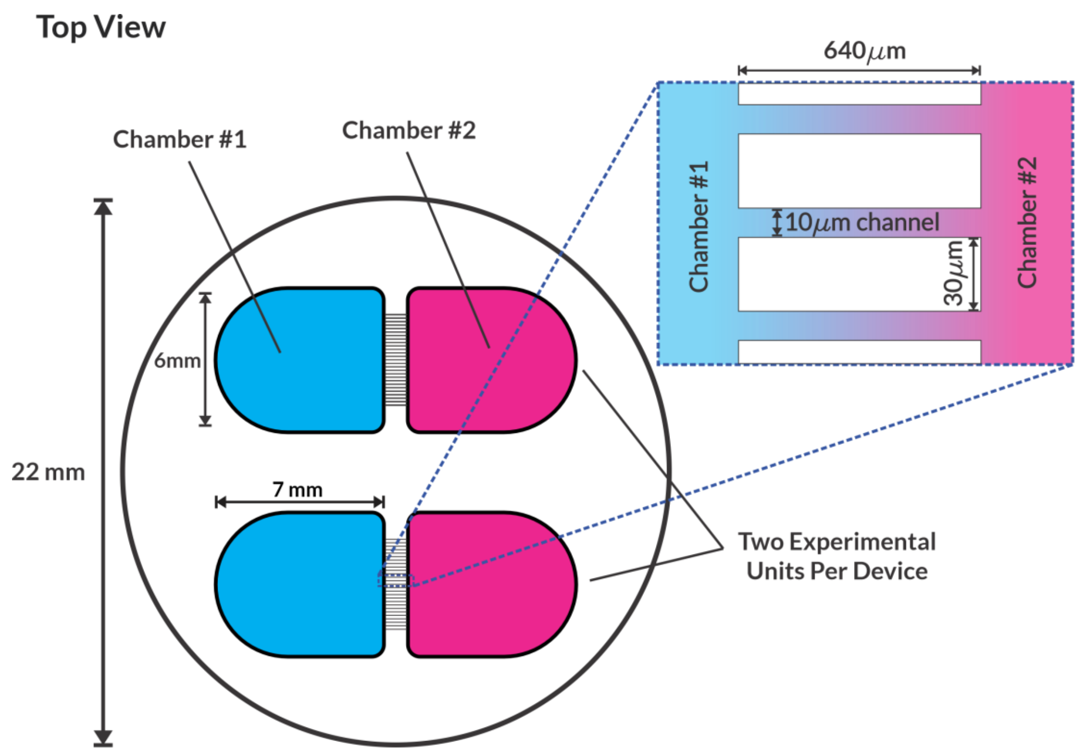
high-transmissive #1.5 thickness (0.16 mm - 0.19 mm) glass. T**he PDMS portion of the OMEGA device <u>cannot</u> be separated from bottom glass coverslip.** All processing for immunochemistry (for example) is easily performed with the device fully intact (see protocol below) and has the added benefit of protecting the delicate axonal processes from detaching from the surface during the labeling process. OMEGA devices are easily adapted to work with most fluorescence microscope stages using available stage holders (see protocol below).

OMEGA^4 Schematic



OMEGA^4 Specifications

Chamber working volume: 40 – 150 µL
Chamber surface area: ~0.35 cm^2
Glass coverslip diameter: 22 mm
Glass coverslip thickness: 0.16 mm - 0.19 mm (#1.5)
Microchannel number per interface: 70
Microchannel length: ~640 µm

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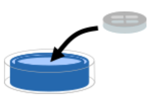
Protocol – Neuronal Cultures

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

2) Using a sterile blade or scissors, cut open the package of the OMEGA[4] device. This can be performed over a collection vessel to catch PBS that will drip during device removal.

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



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

5) Remove remaining PBS from each chamber 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 to avoid the microchannels from drying.**

6) If steps are required to coat/prepare a glass surface for culturing cells, proceed immediately with these steps. The working volume for each chamber is between 40 - 150 µL. When coating microchannels, maintain an excess fluid volume (30 - 60 µL) in **only one** of the interconnected chambers (asymmetric volume loading). It is recommended **not to use** less than 90 µL in volume. To help reduce evaporation from the chambers during incubation steps, add 500 - 600 µL of sterile water or PBS to the circular track of the evaporation minimizer.

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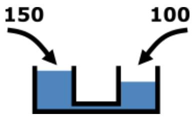
General coating procedure



i. Add 150 µL of coating solution to one chamber.

ii. Add 100 µL of coating solution to the adjacent chamber.

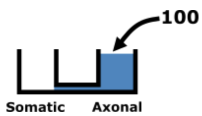
iii. Incubate the device for 2 – 16 hours, depending on the coating type and procedure being used. When using poly-D-lysine (PDL) or poly-L-ornithine (PLO), it is recommended to use a 100 µg/mL solution in sterile water and incubate the coating for 3 hours at room temperature.

iv. Remove coating solution. If required, the chambers can be washed with 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.

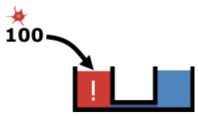
v. If a second coating is required, repeat the process with the second coating solution (**Step i.**). When using laminin (5 µg/mL in cold media) or basement membrane matrix (100x diluted in cold media), add the coating solution using asymmetric volume loading to a cold device and place the device at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

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

Single neuronal cultures (compartmentalization purposes)



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 device 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 150 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. 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.

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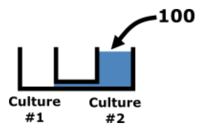
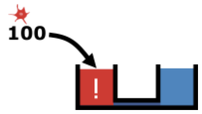
Establishing basic neuronal co-culture



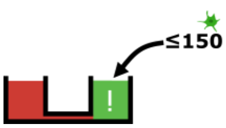
i. Add 100 µL of seeding media to the culture #2 chamber (to be seeded later). The volume to add should be equivalent to the planned seeding volume for culture #1. For example, if the seeding volume for culture #1 will be 100 µL, then add 100 µL to the culture chamber #2.



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 device for at least 1 hour before seeding a second culture. If the second culture is to be seeded several days later, top up both chambers to a maximum of 150 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.

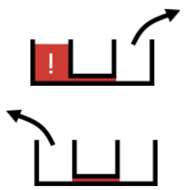
vii. The next day, top up each chamber as necessary for the intended experiment (e.g. for chamber isolation; up to a maximum of 150 µL of media in each chamber). If ROCK inhibitor was used in seeding culture #2, perform a full media change for culture chamber #2 with fresh media without ROCK inhibitor.

viii. 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.

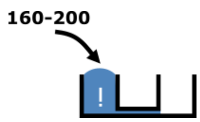
Protocol – Fixation and Immunohistochemistry

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 a high-volume ratio (e.g. 200 µL:50 µL) across adjacent chambers is required to efficiently immunolabel epitopes contained **within** the microchannels. In cases where immunolabeling within the microchannels is not required or desired, there is no need to use asymmetrical volume loading and equal volumes of solutions can be used in both chambers.

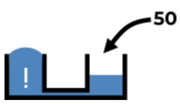
Fixation



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.

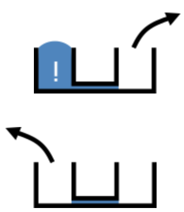


2) Carefully add 160 - 200 µL of **fixative** (e.g. 4% formaldehyde in PBS) to the isolated chamber. Note: adding this volume of solution will overfill the chamber and slightly "balloon out" of the top of the chamber.



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.

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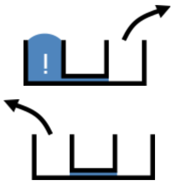
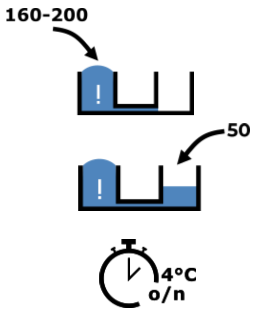
Immunohistochemistry

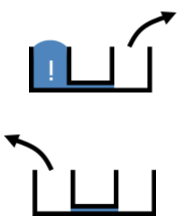
Blocking



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



9) Repeat **Step 2 – 3** with **blocking solution** (e.g. 5 % normal donkey 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.

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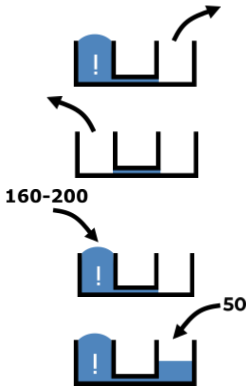
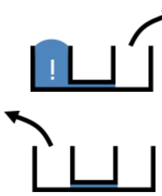
Primary Antibody



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.**

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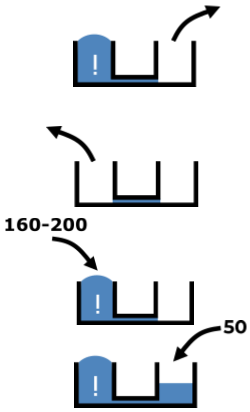
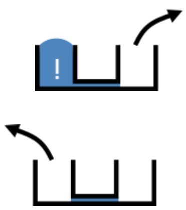
Secondary Antibody



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.**

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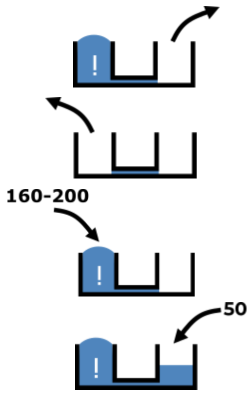
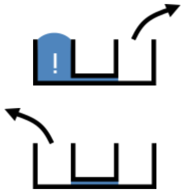
Nuclear Counterstaining



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.**

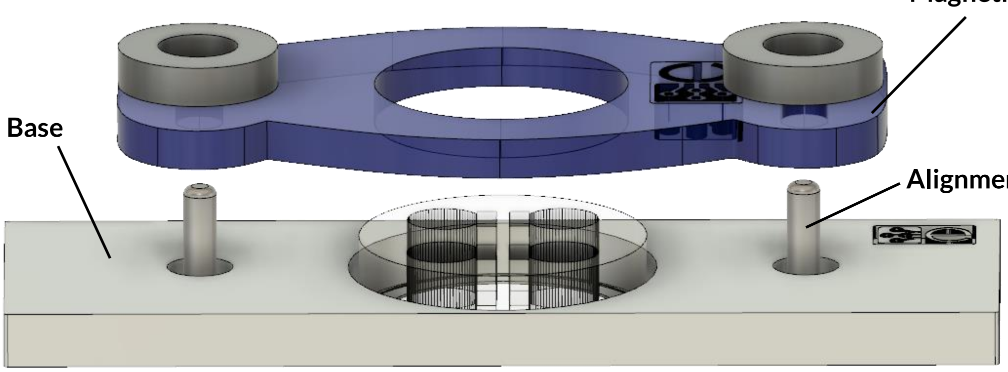
Protocol - Microscopy

Slide-size Microscopy Adapter and Live-cell Microscopy Adapter

These adapters are suitable for live-cell or end-point imaging of OMEGA devices at low- and high-magnification imaging (5x – 100x). The low-profile adapter base is compatible with oil immersion objectives by providing adequate clearance for the relatively large size and shallow taper angles of many oil immersion objectives. The opening in the adapter base positions the OMEGA device on a level plane and uses magnets to effectively stabilize the device between the top and the base.

The adapter has a 75 x 25 mm footprint which is designed to fit microscope stages that accommodate standard-sized glass slides. Setting up is simple: place the OMEGA device using tweezers into the central opening of the base and slide the magnetic top onto the alignment pins. Position the eNUVIO logo on each the top and base towards the same side such that the magnets attract. In the case of the live-cell microscopy adapter, sterilize the adapter with 70% ethanol under a biological safety cabinet. Place the OMEGA device using sterile tweezers into the central opening of the base and use the lid of a standard 35 mm culture dish to cover the device. The adapter can then be transported to the microscope for imaging.

The entire assembly can then be placed into a standard glass slide stage holder commonly available with most microscope setups.



Live-cell Microscopy Adapter

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