OMEGA^{NMJ} 3D Neuromuscular Junction Device

User Guide

Contents

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General Information

Unpacking

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

The OMEGA^{NMJ} 3D Neuromuscular Junction Device Starter Kit contains:

- 3 x OMEGA^{NMJ} devices (individually packaged)
- 3 x circular cell culture evaporation minimizers (blue; reusable)
- 3 x 35 mm round culture dishes
- 1 x microscope stage adapter (reusable)

OMEGA^{NMJ} 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.

Before Starting -IMPORTANT

Each device is double bagged to prevent loss of sterility during shipment. The inner-most bag containing the device is filled with liquid (PBS) and is placed within 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 do 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.**

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^{NMJ} devices are compatible with a variety of downstream experimental procedures including:

a) Fixation and immunocytochemistry
b) Live-cell or fixed brightfield and fluorescence microscopy
c) Calcium imaging
d) RNA/Protein extraction and analysis (e.g., Western blotting)
e) Patch-clamp electrophysiology
f) Muscle contractility measurements

Surface Coating

All inner chamber surfaces of the OMEGA^{NMJ} are made from uncoated polydimethylsiloxane (PDMS). Surface coating/modification is required to prepare PDMS surfaces for the culture of both motoneurons as well as muscle tissue. For the neuronal chamber, the type of coating and coating protocol should be selected and optimized for adherence, outgrowth and survival of the neuronal culture. Motoneurons have been shown to be successfully grown on surfaces coated with a combination of poly-D/L-ornithine (or poly-D-lysine) and laminin. Basement membrane matrices such as Matrigel®*, Geltrex®* or other hydrogels can also be used as coating reagents. However, to obtain an even coating within the microchannels when using thermosensitive basement membrane matrices or hydrogels, it is important that the coating procedure be carried out on ice, using cooled pipet tips, and subsequently allowing the coating to incubate overnight at 4°C.

An anti-fouling (non-adherent) surface coating is required to prepare the PDMS surface of the muscle chamber for the formation of 3D muscle microtissues. This antifouling agent prevents seeded myoblasts from settling and adhering to the chamber base and walls during seeding. Several biocompatible antifouling coating options exist, many of which are based on the antifouling properties of polyethylene glycol (PEG) or polyethylene oxide (PEO) polymers. For example, poloxamer surfactants (e.g., Pluronic® F-127†), poly-L-lysine grafted PEG (PLL-g-PEG) and PEG-siloxane compounds can all be used as antifouling reagents for cell culture applications.

Flow Control and Asymmetrical Volume Loading

The OMEGA^{NMJ} 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 chamber 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.

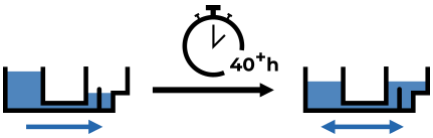
When two adjacent chambers joined by microchannels have identical dimensions, the relationship between chamber fluid level and volume is identical for each of the chambers. Therefore, directional flow across the joining microchannels can be easily determined by directly comparing the fluid volume between 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,

* *Matrigel® and Geltrex® are registered trademarks of Corning and Thermo Fisher Scientific, respectively*
† *Pluronic® is a registered trademark of BASF.*

and subsequently using this ratio to adjust chamber volumes accordingly. In this way, the directionality of the flow across the microchannels can be controlled.

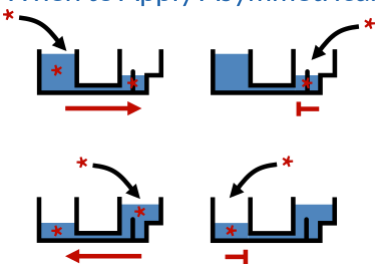
The adjoined chambers in OMEGA^{NMJ} devices have unequal dimensions. A level-to-volume ratio of approximately 1.5 should be implemented when calculating volume loading between the neuronal chamber and the **lower muscle seeding chamber** (oval seeding chamber). Loading the lower muscle chamber with 40 µL of fluid results in a fluid level equivalent to loading the neuronal chamber with ~60 µL (i.e. 40 µL x 1.5 = 60 µL). Importantly, this 1.5 level-to-volume ratio only applies when seeding the **lower** muscle seeding chamber. Due to the change in overall chamber shape moving toward the opening of the chamber, the level-to-volume ratio becomes ~1.05 as the fluid level exceeds the lower muscle seeding chamber's capacity (~40 µL). Consequently, loading a total of 190 µL into the muscle chamber will result in a chamber level that is equivalent to loading the neuronal chamber with 200 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.



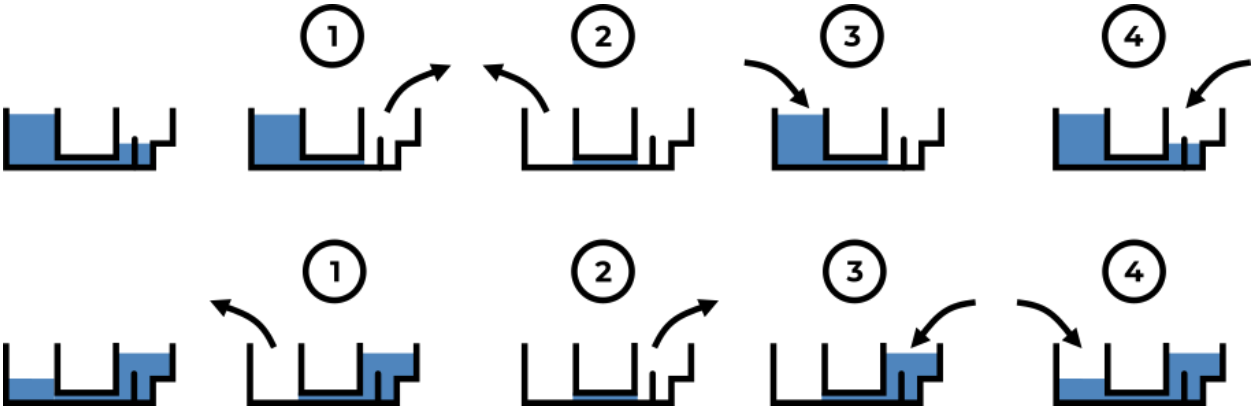
When adjacent chambers are loaded with different volumes of fluid for the purposes of driving a unidirectional flow across the adjoining microchannels, we refer to this as **"asymmetrical volume loading"** of the chambers. The unidirectional flow across the microchannels created by asymmetrically volume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

When to Apply Asymmetrical Volume Loading



Asymmetrical volume loading of chambers is particularly useful when it is desirable to fluidically isolate a chamber from its adjoined 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 level of fluid. Importantly, the chamber with the lower fluid level **will be** exposed to molecules that have been 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 the neuronal chamber of the OMEGA^{NMJ} device is ~0.35 cm^2. Optimal plating 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 starting point, seeding ~50 000 cells in the large chamber yields good results using iPSC-derived neural progenitor cells (NPCs). For primary cultures, seeding density seems to vary significantly by cell type, user, and laboratory. 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. When available, it is suggested to follow the cell provider's seeding density recommendation.

The surface area of the lower muscle chamber of the OMEGA^{NMJ} device is ~0.25 cm^2. For the successful formation and maturation of 3D skeletal muscle microtissue, optimal seeding density should be determined empirically for different sources of myoblasts (e.g., primary versus iPSC-derived). A seeding density of between 7.0 x 10^6 - 10 x 10^6 cells/mL of myoblasts or myogenic progenitor cells is suggested.

Evaporation Minimizers

The osmotic pressure, pH and nutrient concentration of the culture media is critical for maintaining a healthy culture. Controlling these factors can be particularly problematic especially when having to maintain cultures for longer periods of time (weeks or months). Due to their size and the way

OMEGA devices are generally employed, the chambers are prone to evaporation during incubation. **Excessive evaporation is a common issue that results in seemingly unexplained poor culture health or complete loss of the seeded culture 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). These 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.

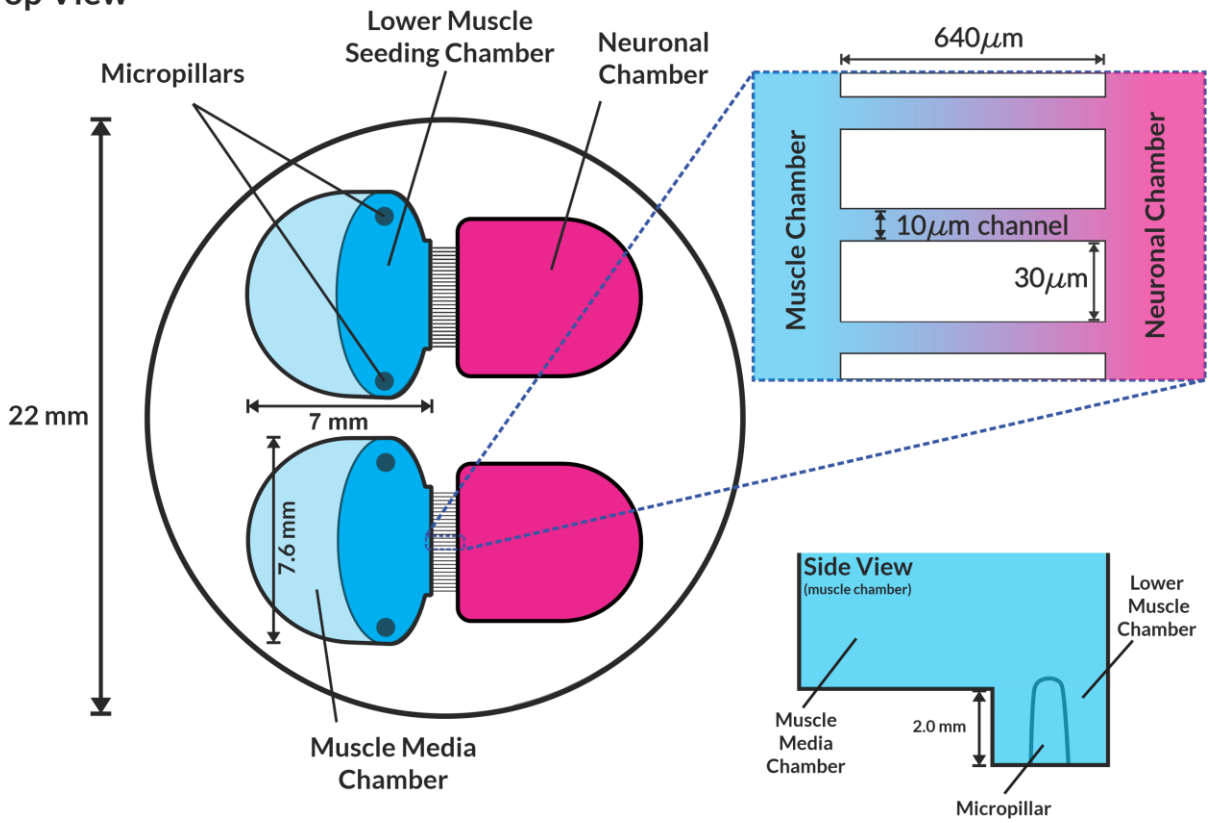
IMPORTANT: Although the culture evaporation minimizers do help to reduce evaporation rates from OMEGA device chambers during the incubation of cultures, they do not completely prevent evaporation. **Therefore, it is vital that the fluid level of each chamber of the device be verified on a regular basis and adjusted accordingly.** 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 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^{NMJ} device is **entirely made of PDMS and the base cannot be removed from the top.** All processing for immunochemistry (for example) can be easily performed with the device fully intact (see protocol below) and has the added benefit of protecting the delicate axonal processes from detaching from the surface during processing. OMEGA devices are easily adapted to work with most fluorescence microscope stages using available stage holders (see protocol below).

OMEGA^{NMJ} Schematic

Top View



OMEGA^{NMJ} Specifications

Neuronal chamber working volume: 35 – 200 µL
Muscle lower chamber volume (max): 40 µL
Muscle chamber volume (total, max.): 200 µL
Neuronal chamber surface area: ~0.35 cm^2
Muscle seeding chamber surface area: ~0.25 cm^2
Pillar height: 2.7 mm
Pillar width (avg.): 1 mm
Microchannel width: 10 µm
Microchannel length: ~640 µm
PDMS bottom thickness: ~200 µm
Number of interfaces per device: 2
Number of microchannels per interface: 70

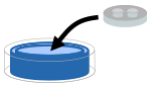
Protocol – Cell Culture

Device Setup

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

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

3) Use a sterile flat-tipped tweezers or another suitable tool to carefully remove the device from its package. Take note of its orientation. With the chamber openings facing up, gently dab the bottom with a wipe to remove residual PBS.

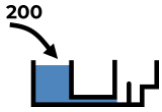


4) Place the device into the central opening of the evaporation minimizer, ensuring chamber opening are facing up.

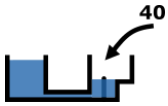
5) Remove remaining PBS from each chamber using a vacuum apparatus or manual pipette that has been fit with a fine tip (10 µL or 200 µL pipette tips work well). **Work efficiently to minimize the time chambers stay dry since the surface and the microchannels can quickly lose their hydrophilicity.**

6) Proceed immediately with coating/preparing the chamber surfaces for culturing cells. Generally, myoblast cultures are seeded several days before neuronal cultures (see **Seeding Cultures** section below). To minimize evaporation from the chambers during any incubation steps, add 500 µL of sterile water or PBS to the circular reservoir of the evaporation minimizer.

Coating the OMEGA^{NMJ} Device

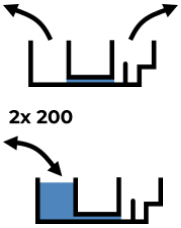


7) Add 200 µL of a 10 µg/mL poly-D/L-ornithine (or poly-D-Lysine) solution to the neuronal chamber.

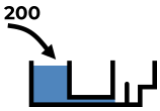


8) Add 40 µL of PBS to the muscle chamber.

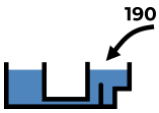
9) Incubate for at least 2 hours at 37°C.



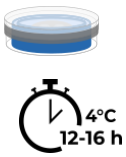
10) Remove all solution from both chambers and wash the neuronal chamber twice with 200 µL PBS.



11) Add 200 µL of PBS to the neuronal chamber.



12) Add 190 µL of 5% Pluronic® F-127* (in PBS; see **Reagents and Solutions** section below) to the muscle seeding chamber.



13) Cover the dish with its lid and seal with parafilm. Place the dish at 4°C for 12 -16 hours.

* *Pluronic® F-127 is a registered trademark of BASF*

Cell Seeding

Seeding cultures: General Information

The following protocol was designed to use primary myoblasts to generate the 3D skeletal muscle microtissue. Importantly, the general methodology presented below is also compatible with other sources of skeletal muscle progenitors (e.g. iPSC-derived skeletal muscle, immortalized myoblast lines, and FACS-sorted primary myogenic progenitors). Successful differentiation and maturation of 3D skeletal muscle microtissues depends heavily on the initial seeding density and ECM environment. Therefore, it is strongly recommended that plating densities be optimized for each cell type/source and ECM type to be used with these devices.

After counting cells and collecting the appropriate number of cells by centrifugation to be used for seeding the muscle chambers, it is necessary to extract as much medium as possible from the cell pellet to prevent media carryover into the ECM-based seeding mixture.

Seeding cultures: Timing

The optimal timing for seeding each of the cultures will strongly depend on the origin and type of cells that are being used in the co-culture. Depending on their origin, seeded ECM-embedded myogenic progenitors can start to form microtissues within 1 - 2 days, mature over the course of ~14 days, and can be kept for >4 weeks in culture with proper culture maintenance. iPSC-derived MN-NPCs differentiate within 5 to 7 days after seeding and can continue to mature over the course of 2 to 3 weeks. Seeded MN-NPCs can project across the microfluidic channels in as little as 3 days. Due to these different rates of maturation, it is important to consider the seeding timing between the neuronal culture and muscle culture. In many cases, it is desirable to first seed skeletal muscle, about 1 – 2 days prior to the motoneuron progenitors.

Troubleshooting Cultures

1) Seeded cells form visible clumps or aggregates within the chamber

   3D skeletal muscle microtissues form best when they are seeded in a homogenous manner. That is, the cells are spread evenly within the seeding ECM and throughout the chamber. If this is not the case, there is a tendency for cells to clump, aggregate and form spheroid-type structures. This type of observed aggregation can also occur if the ECM is not sufficiently homogenous due to the seeding ECM mixture not being thoroughly mixed or if it has polymerized during mixture preparation. To avoid this, it is highly recommended to keep all plasticware, including pipette tips, that contact temperature-sensitive ECM mixture cold during preparation. The device should be kept on ice during plating to ensure seeding homogeneity within the chamber.

2) Cells float from the seeded ECM mixture when media is added

   Seeded cells will separate from the ECM mixture when media is added if the ECM mixture has not fully polymerized or is insufficiently homogenous. In the case of the former, the device can be incubated at 37°C for longer than 10 minutes to ensure complete polymerization of the ECM.

3) Muscle microtissue fails to form

There are several reasons why skeletal muscle microtissue might fail to form. The most common cause of microtissue formation failure is due to low cell density. Seeding the correct number of cells per chamber is critical for successful formation of the microtissue. Microtissue formation can also fail if the physical ECM properties are incompatible with microtissue formation. The ECM scaffold is essential for the myoblasts to rearrange correctly to form the muscle microtissue. Usually, this is due to one of the components of the seeding mixture being erroneously omitted from the seeding mixture.

4) Muscle microtissue detaches from one of the pillars

a) Detachment usually occurs after the microtissue has formed and begins to create tension between the pillars. Under tension, the pillars bend and the microtissue slips off the top (usually only from one side). This type of detachment tends to occur because the seeding volume is too high, leading to the formation of a thicker muscle microtissue that sits closer to the top of the pillars. Reducing the mixture seeding volume, usually by 3 – 5 µL, solves this problem. Note that the seeding volume should not be reduced to lower than 25 µL, as this will likely result in failure to form the microtissue.

b) Pillar detachment is also observed if the microtissue is allowed to remodel for too long. Remodeling should be monitored 16 – 24 hours after seeding, and the differentiation process started as soon as remodeling is observed.

c) Pillar detachment can occur if too much volume of ECM is added post-remodeling. The ECM will push the microtissue off the top of the pillars. Adding less volume will avoid this issue, however adding too little volume will not sufficiently fill the space between the base of the muscle chamber and the bottom of the microtissue. In this case, projecting motor neurons will not readily be able to reach the muscle.

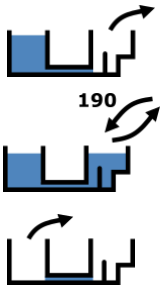
Seeding of Myogenic Progenitors using Thrombin/Fibrinogen-based ECM

1) Prepare the fibrinogen/ECM seeding mixtures on ice using pre-cooled pipette tips. The OMEGA^{NMJ} device should already be cool from the previous poloxamer coating step and should be kept cool during seeding by keeping the device on ice.

2) Prepare 100 µL of fibrinogen/ECM mixture per device (2 chambers per device; each lower muscle chamber has a seeding volume of ~25 - 30 µL). Prepare this ECM mixture **on ice without thrombin.**

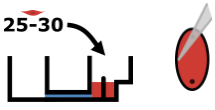
3) In a 1.7 mL microcentrifuge tube, pellet the appropriate number of cells required for seeding by centrifugation at 300 x *g* for 3 minutes. The final seeding density should be between 7.0 x 10^6 and 10 x 10^6 cells/mL. For example, to seed **two chambers** each with 30 µL at 10 x 10^6 cells/mL, pellet 600,000 cells. After centrifugation, make sure to remove as much media as possible to minimize carryover into the muscle chambers. **Place the cell pellet on ice.**

4) Add 50 µL (i.e. for seeding 2 chambers, and using 30 µL seeding volume per chamber) of the final ECM mixture to the cell pellet and mix the resulting 60 µL volume **thoroughly, ensuring not to introduce bubbles**.

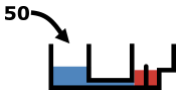


5) Aspirate the coating solution from the muscle chambers first. Wash the muscle chamber once with **cold PBS**, then aspirate the PBS. Be sure to remove all PBS from the muscle chambers. Then, aspirate all solution from the neuronal chambers.

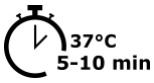
6) Add thrombin (at 0.2 U/mg of fibrinogen) to the fibrinogen/ECM/cell mixture and **mix immediately** using a cold pipette tip (use of a 200 µL tip is recommended). **When mixing, be careful not to introduce bubbles into the mixture**.



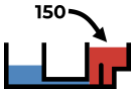
7) Promptly seed 25 - 30 µL of the final ECM/cell mixture into each muscle chamber. Ensure the seeding volume distributes uniformly within the chamber and **does <u>not</u> completely submerge the pillars.** If necessary, a pipet tip can be used to distribute the seeding volume evenly in the chamber**.**



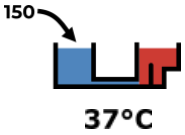
8) Add 50 µL of PBS to each neuronal chamber.



9) Incubate the device for 5 - 10 minutes at 37°C to allow the final ECM/cell suspension to polymerize. During this incubation, prepare the myoblast seeding media.



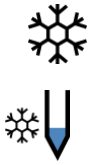
10) Gently top up the muscle chamber by adding 150 µL of myoblast seeding media to each muscle chamber.



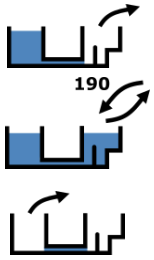
11) Add 150 µL PBS to each neuronal chamber and return the dish to the incubator. Proceed to **Step 21.**

Seeding of Myogenic Progenitors using Collagen-based ECM

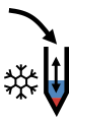
12) In a 1.7 mL microcentrifuge tube, pellet the appropriate number of cells required for seeding by centrifuging at 300 x g for 3 minutes. The final seeding density should be between 7.0 x 10^6 and 10 x 10^6 cells/mL. For example, to seed **two chambers** each with 30 µL at 10 x 10^6 cells/mL, pellet 600,000 cells. After centrifugation, make sure to remove as much media as possible to minimize carryover into the muscle chambers. **Place the cell pellet on ice**.



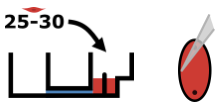
13) In a separate 1.7 mL microcentrifuge tube, promptly prepare 100 µL of the final collagen/ECM (1:4 ratio) mixture **on ice** using pre-cooled pipette tips by adding 20 µL of basement membrane matrix to 80 µL of neutralized collagen/Phenol red solution. **This final solution must be prepared <u>just prior</u> to seeding and must be well mixed. Ensure that mixing does not introduce bubbles.**



14) Aspirate all coating solution from the well containing the OMEGA MP device. Wash the device once with **cold PBS,** then aspirate the PBS. Be sure to remove any PBS from the device chambers. Then aspirate all solution from the neuronal chambers.

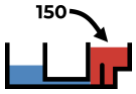


15) Add 50 µL (i.e., for 2 chambers at 30 µL seeding volume per chamber) of the collagen/ECM mixture to the cell pellet (~10 µL volume) and mix the total 60 µL volume **thoroughly, ensuring not to introduce bubbles**.

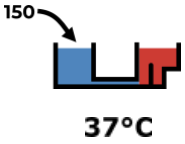


16) Promptly seed 25 - 30 µL of the ECM mixture into each muscle chamber. Ensure the seeding volume distributes uniformly within the chamber and **does <u>not</u> completely submerge the pillars.** If necessary, a pipet tip can be used to distribute the seeding volume evenly in the chamber.

17) Incubate the plate for 5 - 10 minutes at 37°C to allow the collagen/ECM/cell suspension to polymerize. During incubation, prepare the myoblast seeding media.

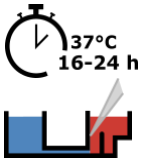


18) Gently top up the muscle chamber by adding 150 µL of myoblast seeding media to each muscle chamber.

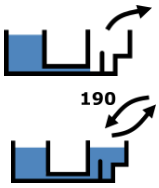


19) Add 150 µL PBS to each neuronal chamber and return the dish to the incubator.

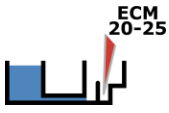
Differentiation and Maintenance



20) After 16 - 24 hours, verify that the culture has not adhered to the inner walls of the chamber. If so, these can be gently detached using a small pipette tip.

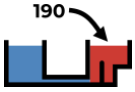


21) When cultures have remodeled, aspirate all myoblast seeding media and wash the well once with PBS. Be sure to remove all residual PBS.

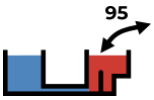


22) Using a pre-cooled pipet tip, carefully add 20 - 25 µL of basement membrane matrix to the bottom of each muscle chamber without disturbing the remodeled muscle microtissue. The volume of basement membrane matrix is inversely related to the initial seeding volume (i.e. if the initial seeding volume was 30 µL, add 20 µL of basement membrane matrix). **Note:** adding too much ECM will cause the microtissue to detach over the top of the pillars.

23) Place the device in the incubator for 5 minutes to allow the ECM to solidify.



24) Add 190 µL of **freshly prepared myoblast differentiation medium** and return the device to the incubator.

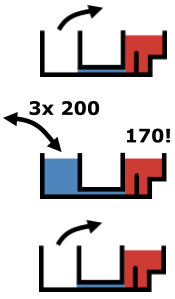


25) Over the course of incubation, perform half-media changes every 2 days with myoblast differentiation medium.

Seeding Motoneuron Neural Progenitor Cells

1) Coat the neuronal chamber with laminin 12 – 16 hours prior to seeding of the MN-NPCs.

Coating the neuronal chamber with laminin



i. Remove all solution from the neuronal chamber and wash the neuronal chamber three times with 200 µL DMEM/F12. Adjust volume in muscle chamber to 170 µL.

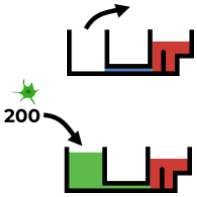
ii. Add 200 µL of 5 µg/mL laminin (in DMEM/F12) to the neuronal chamber.

iii. Replace dish in the 37°C incubator for 12 - 16 hours.

2) Prepare motoneuron final differentiation medium (see **Reagents and Solutions**).

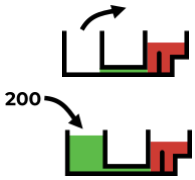
3) Add the appropriate number of MN-NPCs in a 450 µL volume (or at least 200 µL per neuronal chamber to be seeded) of motoneuron final differentiation media containing 10 µM Y-27632. Recommended starting MN-NPC seeding density is ~375 000 cells/mL (~75 000 cells/chamber).

4) Adjust volume of media in the muscle chamber to 170 µL.

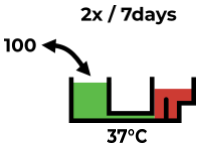


5) Remove the solution from each neuronal chamber and replace with 200 µL of the neuronal media/cell mixture.

6) Place device into the incubator to allow the cells to settle and adhere to the surface.



7) After 24 hours, exchange the media in the neuronal chamber with 200 µL fresh motoneuron final differentiation media **without** Y-27632.



8) Perform half-media changes (100 µL) with motoneuron final differentiation medium at least twice a week.

9) Incubate the neurons, monitoring for axons that extend across the microchannels into the muscle chamber.

10) Over the course of incubation, maintain the correct fluid volume in the chambers, ensuring that the level of media in the neuronal chamber promotes a small directional flow of fluid towards the muscle chamber. Verify and refill the fluid in the evaporation minimizers as needed.

Reagents and Solutions

(all solutions should be sterile)

Final Hydrogel Mixture (thrombin/fibrinogen-based)

- DMEM
- 4 mg/mL bovine fibrinogen
- 20 % v/v of basement membrane matrix (e.g., Geltrex® or Matrigel®)*
- 0.2 units of thrombin/mg of fibrinogen

Final Hydrogel Mixture (collagen-based)

- 1.6 mg/mL Collagen (Type I; neutralized pH)
- 20 % v/v of basement membrane matrix (e.g., Geltrex® or Matrigel®)*

Myoblast Seeding Medium

- Ham's F-10 nutrient mix
- 20 % fetal bovine serum (FBS)
- 1.5 mg/mL 6-aminocaproic acid (for thrombin/fibrinogen-based ECM only; omit for collagen-based ECM)
- 1X Antibiotic-antimycotic or Pen/Strep (optional)

Myoblast Differentiation Media

- DMEM (1 g/L glucose)
- 2 % horse serum
- 10 µg/mL insulin
- 2 mg/mL 6-aminocaproic acid (for thrombin/fibrinogen-based ECM only; omit for collagen-based ECM)
- 1X Antibiotic-antimycotic or Pen/Strep (optional)

Insulin

Available as a sterile solution at 10 mg/mL.

6-Aminocaproic acid

Prepare a 50 mg/mL solution in sterile water.

Basement Membrane Matrix (Geltrex®/Matrigel®)*:

Follow manufacturers' recommendation for preparation and storage. Aliquot in usable quantities and store frozen until use.

Pluronic® F-127†

Prepare a 5 % w/v solution in PBS. Filter sterilize with a 0.1 or 0.2 µm filter and store at 4°C.

Geltrex® and Matrigel® are registered trademarks of Corning and Thermo Fisher Scientific, respectively

†*Pluronic® F-127 is a registered trademark of BASF*

Fibrinogen (bovine)
Prepare a 10 mg/mL (can be up to 33 mg/mL) solution in sterile 0.9% w/v NaCl (saline) by dissolving slowly over several hours at 37°C. Filter-sterilize by gently passing through a 0.2 µm filter (do not apply too much force, **do not use a 0.1 µm filter**). Aliquots of 1 mL can be stored for 6 months at -20°C. Thaw on ice before use.

Thrombin (human plasma)
Dissolve between 25 - 100 U/mL in 0.1 µm-filtered 0.1% BSA in PBS. Mix the solution thoroughly. Prepare aliquots of 50 µL and store at -80°C. Thaw on ice before use.

Collagen (rat tail, Type I)
Neutralize collagen by first diluting collagen stock to ~2 mg/mL with a PBS/phenol red working solution (see below) and then titrating with a NaOH solution. For example, using a 3 mg/mL stock collagen solution (e.g. Gibco Cat. # A1048301), mix 660 µL collagen with 340 µL of PBS/phenol working solution to obtain a 1.98 mg/mL solution (this solution will have a yellow tint). Titrate with a 10 % NaOH solution dropwise until a sudden pink colour change is observed (~10 – 15 µL of NaOH will usually be required). Preparation of this solution should be on ice, and only when ready to seed the device. **Even when kept cool, this solution will begin to gel within 7-10 minutes of preparation.**

PBS/Phenol Red mixture
Prepare a 5mg/mL stock solution of phenol red in sterile PBS (1X). Add 4.7 µL of this 5mg/mL the phenol red stock solution to 995.3 µL of sterile PBS (1X) to make a 0.0235 mg/mL working solution.

Motoneuron Final Differentiation Medium:
- 50 % v/v DMEM/F12 nutrient mix
- 50 % v/v Neurobasal Medium
- 1X N-2 Supplement
- 1X B-27 Supplement
- 1X GlutaMAX (or L-alanine/L-glutamine dipeptide)
- 1X Antibiotic-antimycotic
- Ascorbic acid (100 µM)
- Compound E (0.1 µM)
- Retinoic acid (0.5 µM)
- Purmorphamine (0.1 µM)
- BDNF (10 ng/µL)
- CNTF (10 ng/µL)
- IGF-1 (10 ng/µL)
- GDNF (10ng/µL) (optional)

Y-27632 (Rho Kinase Inhibitor)
Prepare at 10 mM in water. Stored at -20°C, thaw at 4°C before use.

Poly-L-ornithine

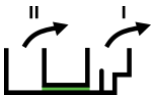
Prepare a stock at 1 mg/mL in divalent-free PBS. Can be stored at 4°C.

Laminin
Available at 1 – 2 mg/mL stock concentrations that are stored at -80°C. Thaw at 4°C just before use. Dilute to 5 µg/mL in DMEM/F12 to coat chamber surfaces.

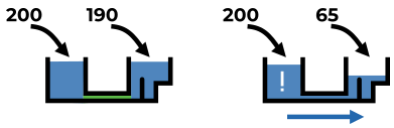
Protocol – Fixation and Immunocytochemistry

The following protocol is designed to fix and immunolabel the 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 at least a 3:1 volume ratio (e.g., 200:65 µL) across adjacent chambers has been shown to be optimal for immunolabelling epitopes contained **within** the microchannels. If immunolabeling within microchannels is not required, there is no need to use asymmetrical volume loading (equal volumes can be used in adjacent chambers).

Fixation

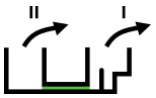


1) Remove all solution from both chambers. If it is desirable to label processes in the microchannels, ensure flow directionality is maintained throughout the procedure by using asymmetrical volume loading with a 3:1 volume ratio. Note: antibodies will be crossing the microchannels into the adjacent chamber in this case.



2) Carefully add 200 µL of **fixative** (e.g., 4% formaldehyde in PBS) to the neuronal chamber and 190 µL to the muscle chamber. If microchannels are to be labelled, add only 65 µL to the muscle chamber to promote flow through the microchannels.

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



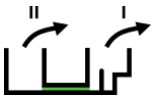
4) Remove fixative from both chambers, beginning with the muscle chamber.

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

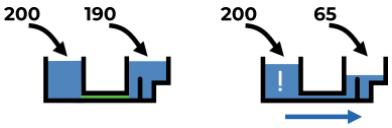
6) Repeat **Step 5** twice more, so that all chambers have been washed a total of three times.

Immunocytochemistry

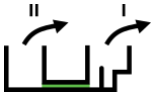
Blocking



7) Remove all solution from both chambers (observe the order in which chambers are emptied and refilled).

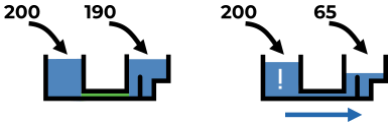


8) Repeat **Step 2** (above) with **blocking solution** (e.g., 5 % normal serum, 0.2 % Triton X100, 0.05 % BSA), and incubate for at least 1 hour at room temperature (this can be also done overnight if desired).



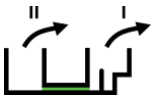
9) Remove blocking solution from both chambers.

Primary Antibody



10) Repeat **Step 2** (above) with **primary antibody solution** (dilution ratio(s) to be optimized).

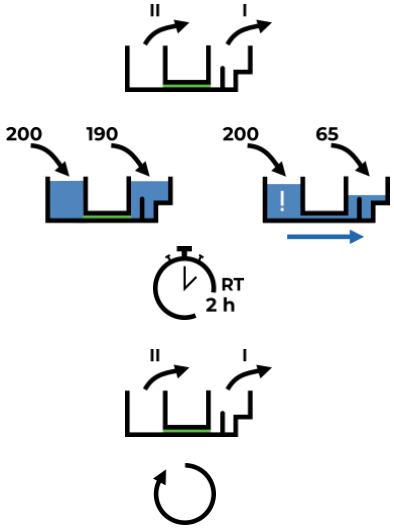
11) Incubate overnight at 4°C.



12) Remove primary antibody solution from both chambers, beginning with the muscle chamber.

13) Wash the chambers three times with PBS as described in **Steps 5 – 6** (above).

Secondary Antibody



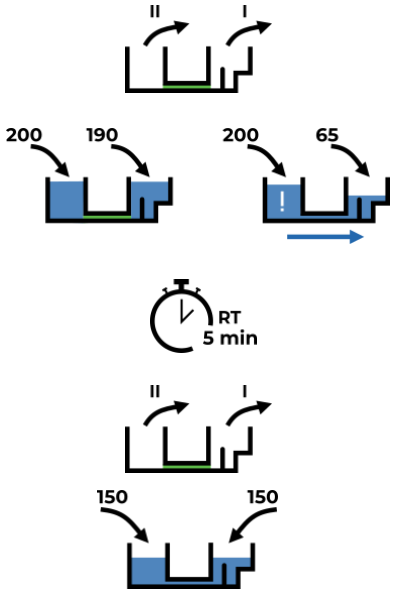
14) Repeat **Step 1 - 2** (above) with **secondary antibody solution** (dilution ratio(s) to be optimized).

15) Incubate for 2 hours at room temperature.

16) Remove secondary antibody solution from both chambers, beginning with the muscle chamber.

17) Wash the chambers three times with PBS as described in **Steps 5 – 6** (above).

Nuclear Counterstaining



18) Repeat **Step 1 – 2** with **nuclear counterstain solution** (e.g., Hoechst or DAPI; dilution ratio to be optimized).

19) Incubate for 5 minutes at room temperature.

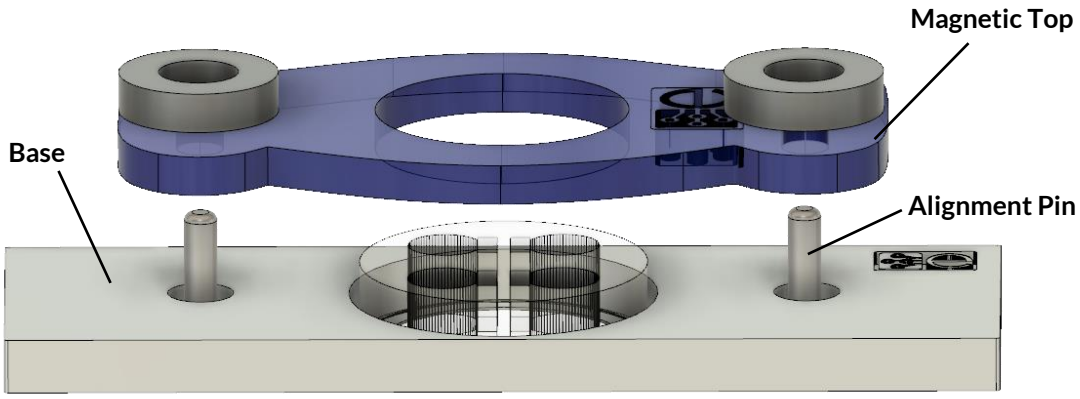
20) Remove nuclear counterstain solution from both chambers, beginning with the muscle chamber.

21) Add 100 – 150 µL of PBS solution to each chamber.

Protocol - Microscopy

Slide-size Microscopy Adapter

This adapter is suitable for end-point imaging of OMEGA devices at low- and high-magnification imaging (5x – 100x). This low-profile adapter is compatible with oil immersion objectives since it provides adequate clearance for the relatively large size and shallow taper angles of many oil immersion objectives. The adapter properly stabilizes the OMEGA^{NMJ} 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^{NMJ} device into the central opening of the base, then slide the magnetic top onto the alignment pins. Place the entire assembly into a universal standard glass slide accommodation commonly available on microscope stages.



Live-cell Microscopy Adapter
(suitable for 4x – 20x magnification)

When performing repeated live-cell imaging, it is important to sterilize the microscopy adapter (autoclave or using 70% ethanol) and to carry out the assembly steps (see below) under aseptic conditions (i.e., in a biological safety cabinet). After establishing the culture, carefully remove the OMEGA^{NMJ} device from the 35 mm plastic culture dish with tweezers or forceps and place it in the 35 mm round microscope stage adapter (see figure below). Position the device in the center of the adapter to expose the bottom glass, ensuring the device is level and flat. This assembly is designed to be used with microscope stage adapters that accommodate round 35 mm culture dishes.

To protect the culture during microscopy, use the lid from the 35 mm culture dish once the device has been placed in the imaging adapter. After imaging, return to the safety cabinet and replace the device into the original 35 mm culture dish (containing the evaporation minimizer). Check the volume levels of each chamber (adding or removing fluid if necessary), replace the lid, and continue to incubate the culture in the incubator.

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