OMEGA^{MP} 3D Skeletal Muscle Device

User Guide

Contents

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v2.4

General Information

Unpacking

Thank you for purchasing eNUVIO's OMEGA^{MP} 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^{MP} device kit contains:

- 4 x OMEGA^{MP} devices (individually packaged)
- 1 x 12-well microplate (individually packaged)

OMEGA^{MP} devices are packaged sterile and are ready to use in cell culture.

Before Starting

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. If the sterility of the device is suspected to be compromised, the device can be re-sterilized using a plasma cleaner, UV/ozone cleaner or autoclave. OMEGA^{MP} devices are designed to fit tightly into the bottom of the wells of the included 12-well microplate.

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

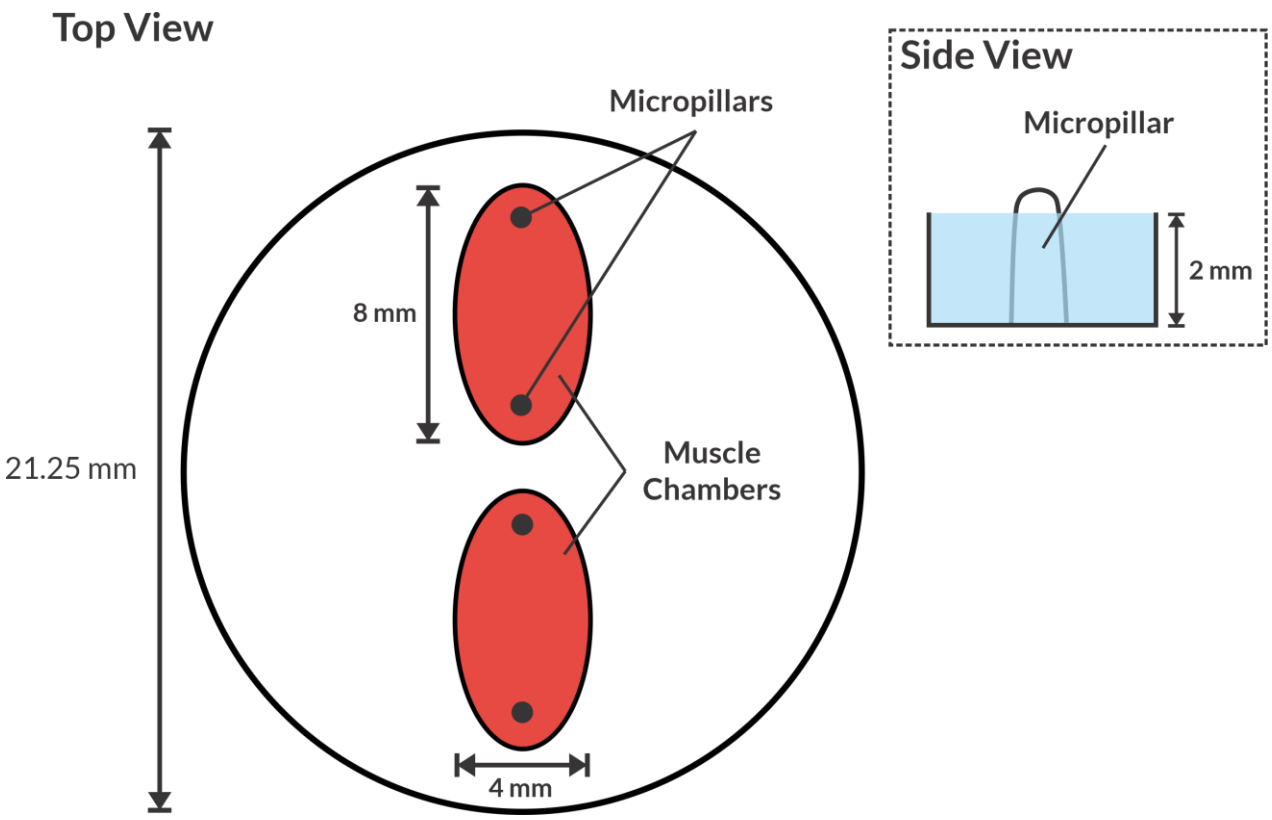
a) Cell fixation and immunohistochemical staining
b) Brightfield and fluorescence microscopy
c) Calcium imaging*
d) RNA/Protein extraction and analysis (e.g. Western blotting)
e) Patch-clamp electrophysiology
f) Muscle contractility measurements

Surface Coating

All surfaces of the OMEGA^{MP} are made from uncoated polydimethylsiloxane (PDMS). An anti-fouling (non-adherent) surface coating is required to prepare the PDMS surface 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.

* *Pluronic® is a registered trademark of BASF*

OMEGA^{MP} Schematic



OMEGA^{MP} Specifications

Chamber volume (max): 40 µL
Seeding volume: 25 – 30 µL
Chamber surface area: ~0.25 cm^2
Pillar height: 2.7 mm
Pillar width: 1 mm
Device diameter: 21.25 mm
PDMS base thickness: ~200 µm
Number of chambers per device: 2

Cell Seeding Information

Cell Seeding Density

The surface area of the chamber of the OMEGA^{MP} device is ~0.25 cm^2. For the successful formation and maturation of 3D skeletal muscle microtissue, optimal seeding density should be determined. A seeding density of between 7.0 x 10^6 - 10 x 10^6 cells/mL of myogenic progenitor cells is suggested.

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 hydrogel-ECM seeding mixture.

Seeding cultures: Timing

The differentiation and maturation rate of each culture will strongly depend on the origin and type of cells that are being utilized. Depending on their source, seeded hydrogel-embedded myogenic progenitors generally start to form microtissues within 1 - 2 days, continue to mature over the course of 14 days, and can be kept for >4 weeks in culture.

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 hydrogel 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 hydrogel-ECM is not sufficiently homogenous due to the hydrogel 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 hydrogel-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 hydrogel mixture when media is added

   Seeded cells will separate from the hydrogel mixture when media is added if the hydrogel 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 hydrogel.

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 hydrogel properties are incompatible with microtissue formation. The hydrogel 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

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.

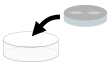
Pillar detachment is also observed if the microtissue is allowed to remodel for too long. Remodeling should be monitored 16 – 24 hours after seeding, and the differentiation process started as soon as remodeling is observed.

Protocol

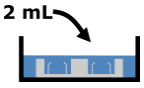
Device Setup and Coating

1) Under aseptic conditions and using a sterile blade or scissors, cut open the package of the OMEGA^{MP} device.

2) Using sterile flat-tipped tweezers or another suitable tool, carefully remove the device from its package, taking note of its orientation.



3) Place each device into a well of the included 12-well microplate or other suitable container. When using another vessel, note that the inner well diameter must properly accommodate the diameter of the device such that it fits snugly into the bottom of the well. This is to prevent the device from floating during subsequent incubation.



4) Proceed with coating the entire device by adding 2 mL of 5% Pluronic® F-127 in PBS to each chamber.

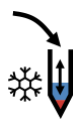
5) Place the lid on the microplate and seal with parafilm. Incubate the sealed plate at 4°C for at least 12 hours.

6) Proceed with seeding of myogenic progenitors in either thrombin/fibrinogen- (**Step 7**) or collagen-based hydrogel mixtures (**Step 16**)

Seeding of Myogenic Progenitors using Thrombin/Fibrinogen-based ECM

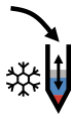
7) Prepare the fibrinogen/hydrogel seeding mixture on ice using pre-cooled pipette tips. Prepare 100 µL of fibrinogen/hydrogel mixture per device (2 chambers per device; each chamber has a seeding volume of ~25 - 30 µL). Prepare this hydrogel mixture **on ice without thrombin.**

8) 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**.



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

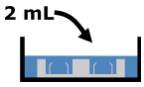
10) Aspirate all coating solution from the well containing the OMEGA MP device. Wash the device once with **cold PBS,** then aspirate the PBS. Be sure to remove any PBS from the device chambers.



11) Add thrombin (at 0.2 U/mg of fibrinogen) to the fibrinogen/hydrogel/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.**

12) Promptly seed 25 - 30 µL of the final hydrogel/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.

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

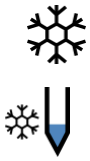


14) Gently add 2 mL of myoblast seeding media to the well to completely submerge the entire device.

15) Return the plate to the incubator. Proceed to **Step 24**.

Seeding of Myogenic Progenitors using Collagen-based ECM

16) 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**.



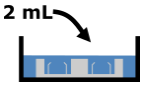
17) In a separate 1.7 mL microcentrifuge tube, promptly prepare 100 µL of the final collagen/hydrogel (1:4 ratio) mixture **on ice** using pre-cooled pipette tips by adding 20 µL of 10X basement membrane matrix to 80 µL of neutralized collagen/Phenol red solution. **This final solution must be prepared <u>just prior</u> to seeding and must be well mixed. Ensure that mixing does not introduce bubbles.**

18) 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.

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

20) Promptly seed 25 - 30 µL of the hydrogel 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.

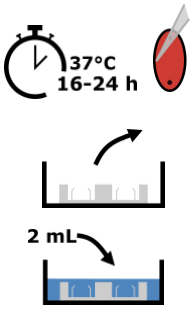
21) Incubate the plate for 5 - 10 minutes at 37°C to allow the collagen/hydrogel/cell suspension to polymerize. During incubation, prepare the myoblast seeding media.



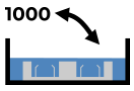
22) Gently add 2 mL of myoblast seeding media to the well to completely submerge the entire device.

23) Return the plate to the incubator.

Differentiation and Maintenance



24) 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. If cultures have remodeled, aspirate all myoblast seeding media, wash the well once with PBS, and replace with 2 mL of **freshly prepared myoblast differentiation medium.**



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

Reagents and Solutions

(all solutions must be sterile)

Final Hydrogel Mixture (thrombin/fibrinogen-based)

- DMEM
- 4 mg/mL bovine fibrinogen
- 20 % v/v of a 10X basement membrane matrix (e.g., Geltrex® or Matrigel®*, 2X final concentration)
- 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 a 10X basement membrane matrix (e.g., Geltrex® or Matrigel®*; 2X final concentration)

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.

ECM (Geltrex®/Matrigel®)*:

Follow manufacturers' recommendation for preparation and storage. Prepare a 10X stock solution in DMEM that can be aliquoted and stored 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.