Standard Operating Procedure

Title: CBE SOP0203 SOP Mantarray Platform for 3D muscle contraction

Location: CBE

1. PURPOSE

This Standard Operating Procedure describes the method to prepare and stimulate engineered muscle tissue using Mantarray system.

2. SCOPE

Creation of 3D muscle models using human primary myoblasts.

3. RESPONSIBILITIES

CBE Laboratory Users

Shall
1) Work in accordance with the instructions for the operation and maintenance detailed in this SOP, Risk Assessment and the operator manual.
2) Carry out the routine inspection, cleaning, and maintenance of the equipment, as required.
3) Record any adverse events and alarms that indicate non-conformance or malfunction on the Maintenance Record and notify the Laboratory Manager/Responsible Person

Responsible Person (RP)/Laboratory Manager (LM)

Shall
1) Ensure that authorized laboratory personnel are given suitable information, instruction, training and supervision in the correct use and maintenance of the equipment.
2) Investigate any reported problems, adverse event, alarms, or non-conformities associated with equipment usage.
3) Organise the maintenance, repair, or servicing of the equipment by trained and authorized contract/service personnel.

4. EQUIPMENT AND MATERIALS

4.1 Equipment

- Biological Safety Cabinet
- BD 6220 CO2 incubator
- Bench-top Centrifuge
- Water bath (37°C)
- NucleoCounter® NC-3000™ automatic counting system
- Microscope, inverted
- Mantarray Instrument: Magnetometric Analyzer for Engineered Tissue Arrays (MANTA-24-B1, Curi Bio)
- Mantarray Stimulation Kit, 24-well- Pack of 2 lids (MA-STM-2, Curi Bio)

4.2 Supplies

- Plastic Serological Pipettes: 1ml, 2 ml, 5,10 ml
- Sterile primary and secondary containers: 15 ml or 50 mL centrifuge tube with screw cap
- Collagen I coated T25 flasks (Fisher Sc, 10190103)
- Mantarray Plate Kit (12x), Pack of 2 (CuriBio, MA-12x-24-2)

4.3 Media and Chemicals

- DMEM, low glucose, pyruvate (Gibco, 11574446)
- Fetal Bovine Serum (Hyclone, HYC86)
- Penicillin-Streptomycin (5,000 U/mL) (Gibco, 11528876)
- Dulbecco's Phosphate Buffered Saline (DPBS), w/o Ca and Mg (Gibco, 12559069)
- TrypLE Select Enzyme (1X), no phenol red (Gibco, 10718463)
- DMSO (Thermo Scientific Chemicals, 11410843)
- Solution 13 AO-DAPI Staining Reagent (Chemometec 910-3013)
- Primary Human Skeletal muscle cells (Promocell, C-12530 and Cambridge Bioscience, SKB-F and SKB-F1)
- Fibrinogen (F3879-100MG, Sigma)
- Thrombin (T4648-1KU, Sigma)
- Aminocaproic acid (A0420000, Sigma)

- Polyethylenimine (PEI), Sigma, #P3143
- Glutaraldehyde, Fisher Scientific #BP25481

5. REAGENT PREPARATION

5.1 Polyethyleneimine and glutaraldehyde

a. Prepare 0.1% PEI solution by diluting pre-made 10% PEI solution with molecular biology grade DI H2O. 1.5mL of 0.1% PEI solution is needed per well. Both 50% and 10% solutions are stored at room temperature.

a. *Poly(ethyleneimine) solution (PEI) comes as a solution that is 50% w/v. It is highly viscous and difficult to pipette. Use a 50 mL conical to measure 5 mL of solution and top up to 25 mL with diH20 to yield a 10% stock solution. This highly concentrated stock solution cannot be sterile filtered. Store at room temperature. To make a 0.1% solution for cell culture, add 1 mL of 10% stock to 99 mL diH20. Sterile filter and use the same day. Adjust volumes as needed.

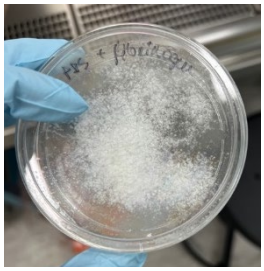
b. Use a 50mL conical tube to measure 5 mL of the off-the-shelf 50% w/v PEI solution
c. Add molecular biology grade water to the 50 mL conical to yield 25mL of 10%PEIstock solution
d. Note: Although moderately diluted, this 10% stock solution cannot be sterile filtered yet
e. Store the stock 10% solution at room temperature for up to 1 months
f. Per 24-well plate, prepare 40 mL of 0.1% PEI working solution by diluting 0.4 mL of the stock 10% PEI solution in 39.6 mL of molecular biology grade water
g. Note: Adjust the volume of the 0.1% PEI working solution according to your needs. You will need 1.5mL of 0.1% PEI solution per well of posts, or equivalently 36 mL per 24-well plate.
h. Sterile filter your 0.1% PEI working solution, and store at 4°C for up to 1 month.
i. Prepare 0.01% glutaraldehyde (GA) solution by diluting 25% glutaraldehyde stock solution with molecular biology grade DI H2O. 1.5mL of 0.01% glutaraldehyde solution is needed per well. Diluted GA solutions should be used same day. GA is not stable. Please aliquot and store in –20C
j. Prepare 40 mL of 0.01% working glutaraldehyde (GA) working solution by diluting 0.016 mL of the 25% GA stock solution with molecular biology grade water

Note: Adjust the volume of the 0.01% GA working solution according to your needs. You will need 1.5mL of 0.1% GA solution per well of posts, or equivalently 36 mL per 24-well plate.

Note: Make sure to store your 25% GA stock solution at 4°C following the manufacturer's expiration instructions. Store the 0.01% GA working solution at 4°C for up to 1 week.

5.2 Fibrinogen reconstituting and aliquoting

a. Reconstituting 1g in 20mL DPBS and aliquoting into 350µL. This will make roughly 50-55 aliquots of 50 mg/mL fibrinogen
b. Add 20 mL DPBS into 10cm dish
c. Place dish into a 37°C incubator for 60 minutes to warm DPBS and dish
d. Shake bottle vigorously to break apart fibrinogen crystals
e. Evenly distribute the entire bottle of fibrinogen on top of PBS by tapping and pouring
f. Incubate at 37°C for 3hrs until fully dissolved.
g. Pass fibrinogen through a 100µm filter and collect into a 50mL conical tube (if solution does not easily pour through the filter, it needs more time in the incubator)
h. Cap 10mL syringe with a luer lock 0.2µm sterile filter
i. Pour filtered fibrinogen into the syringe and sterile filter, collecting into a new 50mL conical tube
j. May need to change the sterile filter several times to filter the entire volume
k. Aliquot 350µL into autoclaved 1.5mL Eppendorf tubes (should make 50-55 tubes)
l. Label tubes and store at -20°C



To thaw this solution, leave it at 4°C. When using the thawed aliquot, always keep on ice. If the solution accidentally warms up, it might prematurely coagulate. In such a case, use a new aliquot.

5.3 Thrombin reconstituting and aliquoting

a. Add 6 mL DPBS to Thrombin bottle
b. Add 4 mL sterile water. Now total of 10mL
c. Cap bottle and mix by inverting
d. Pour contents into a 50 mL conical tube through 0.2µm sterile syringe filter
e. Aliquot 100uL into autoclaved 1.5mL Eppendorf tubes.
f. Label tubes and store at -20°C

6. PROCEDURES

6.1 Mantarray Post Lattice Preparation

a. Fill the wells of a second 24-well plate with 2 ml/well of sterile diH20 and transfer posts. Let sit for 1 minute.

b. In a third 24-well plate, fill the wells with 1.5 ml/well of 0.01% glutaraldehyde and transfer posts. Let sit for 30 minutes.
c. While the posts sit in glutaraldehyde, aspirate the diH20 wells, wash with 2 ml/well of sterile diH20, aspirate, and refill with 2 ml/well sterile diH20. You may also opt to use a fresh, 24-well plate instead of rinsing.
d. When 30 minutes are up, transfer posts to the 2 ml/well of sterile diH20. Let sit for 1 minute.
e. Aspirate the diH20 and add another 2 ml/well of sterile diH20. Let sit for 5 minutes.
f. Allow posts to dry in a BSC by putting the lattice with lid facing up for at least 15 minutes.
g. Once dried, reassemble Mantarray Kit. Parafilm lid, post lattice, and 24 well plate together and store at 4°C for up to two weeks prior to cell seeding

6.2 Human myoblast culture

a. Grow myoblasts in T75 flasks until 80% confluent
b. Aspirate medium and wash myoblasts using sterile DPBS. Aspirate DPBS with pasture pipette
c. Add 5ml TrypLE select (1x) in the flask and place into the incubator for 3-5min. Monitor trypsinization by microscopy
d. Add 5ml prewarmed medium to cell/TrypLE select solution to block trypsin reaction. Transfer cell suspension to a 15ml falcon tube
e. Centrifuge at 300 g for 5 minutes
f. Prepare skeletal muscle casting medium: add 0.25g of aminocaproic acid to 50ml growth medium (low glucose DMEM+20%FBS+1%pen/strep)
g. Resuspend the skeletal muscle cells at a density of 8.5×10^6 cells per mL and put on ice. Use 90µL of this suspension per construct (7.5×10^5 cells per tissue construct)
h. Calculate the total number of cells required and pipette in 15ml tube
i. Add 10µL fibrinogen solution per one construct to the cells suspension and keep on ice
j. Prepare 50µL thrombin solution per one casting well on ice: 3µL thrombin stock + 47µL casting medium (cell culture medium) on ice

Improvement 1: Addition of human primary young and old fibroblasts or alternatively DOXO induced senescent fibroblasts. Percentages are ~1/4 of myoblast cells. Adding fibroblasts generates 3D tissue that are resembling primary tissue more closely and also helps to generate “old” tissues. Please always mix female with female donors and male with males.

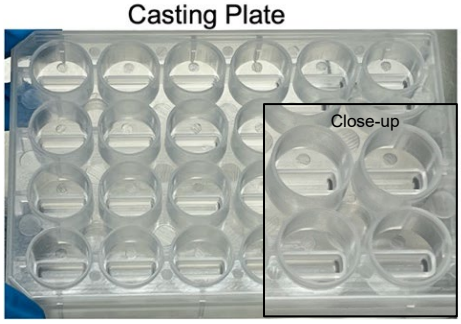
Improvement 2: Adding human serum instead of animal serum. The addition of human serum further develops the ageing phenotype. The same as above always add female to female.

6.3 3D muscle casting

a. Pre-chill the tissue casting kit at 4C

Casting Kit

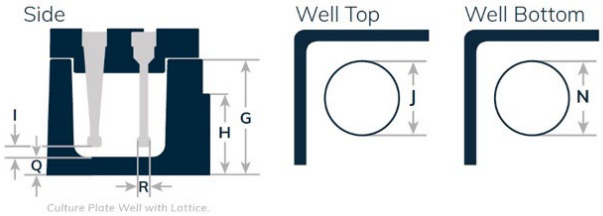
Casting Plate



Lattice top view

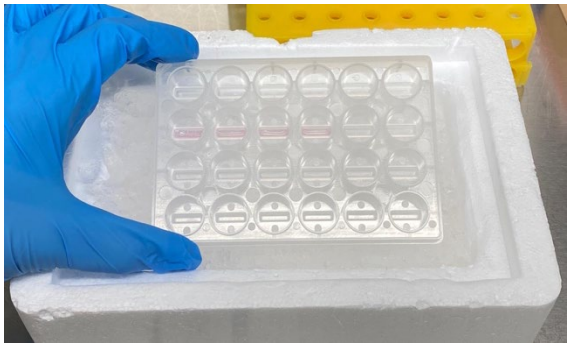
b. Casting Plate and Lattice is used together to cast 3D engineered muscle tissues.

Lattice Side view



c. Remove the chilled casting kit from the refrigerator and place it on ice inside the cell culture hood.
d. Lay casting plate flat on ice and move the lattice top from the casting plate to a new, sterile 24-well plate.

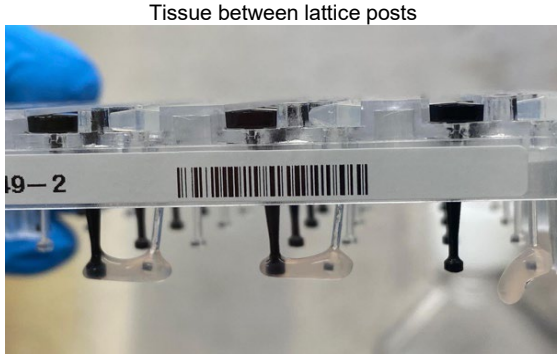
Casting plate on ice in the hood

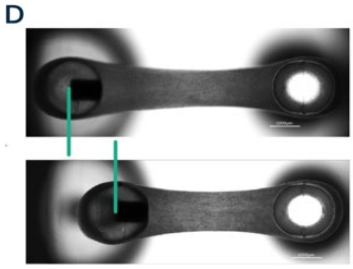


e. Pipette 50µL thrombin solution into each pre-chilled well of the casting plate.
f. Reassemble the kit and return to refrigerator until tissue casting (within 3h of thrombin addition to the wells).
g. Transfer the tissue casting kit to cell culture hood and remove the lid (post lattice containing flexible and rigid posts should remain on the casting plate).
h. Mix the cell/fibrinogen mixture and draw up 100µL and add to the wells prepared with 50µL thrombin solution and triturate 5x to mix well (avoid bubbles).
i. Avoid the movement of the lattice.
j. Repeat until all the tissues are cast and transfer the seeded kit to the incubator, making sure not to move the lattice. Incubate 80min at 37C to allow polymerization of the hydrogel and attachment of the proteins to the posts.

k. Meanwhile, prepare fresh 24-well plate with 2ml/well casting medium containing 5g/L aminocaproic acid (0.2mm sterile filtered). Incubate the plate with medium at 37C.
l. After 80min, add 1mL of the medium to the edge of the casting wells and incubate for 10min.
m. Lift the post lattice and transfer the tissues from the casting plate to a pre-pared 24-well plate with medium. Return to incubator.
n. One day after plating, engineered muscle tissues are transferred to a low-serum (2%) medium supplemented with 5 g/L aminocaproic acid (ACA) to induce myoblast fusion.
o. Cultures are maintained in the low-serum medium for the remainder of the culture period and are fed with fresh medium every 2–3 days from this point onward.
p. Measurement times depend on experiment (e.g., at day 7, 10 and 14). We expect that the model will contract like in the picture seen here.

Tissue between lattice posts





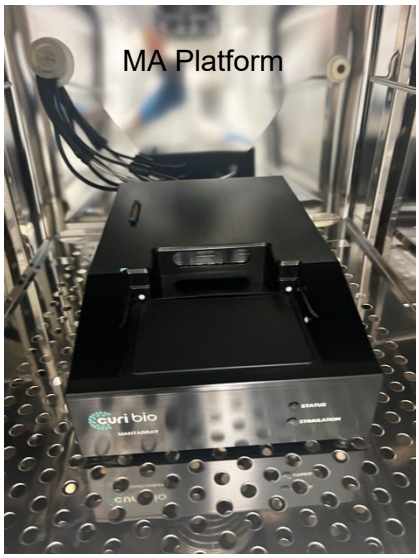
6.4 Starting up Mantarray System

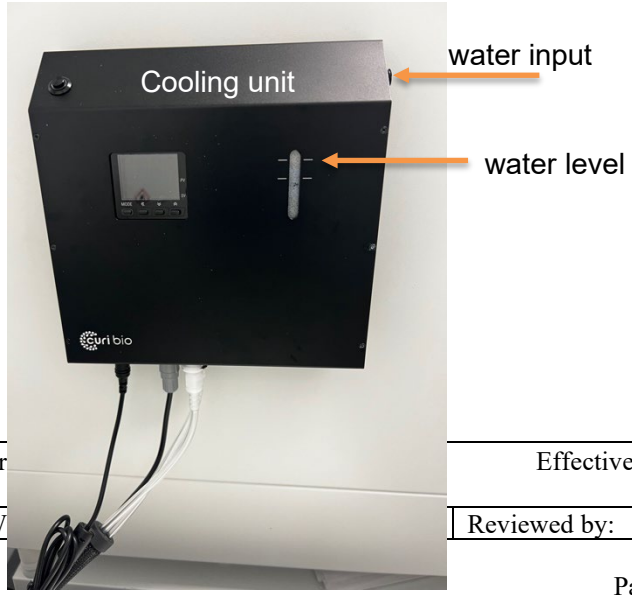
6.4.1 Cooling unit

a. Add the cooling unit to the outside of your incubator (it is magnetic) so that the cable can go through the back of the incubator
b. Add roughly 350ml distilled water until the indicator is at the second mark
c. This only needs to be done once unless you have not used the unit for a long time. If you move the unit just keep the cooling unit up-right

6.4.2 Switching on Mantarray system

a. Switch on the power brick that also switches on the Mantarray platform in the incubator
b. Switch on the cooling unit
c. Switch on the laptop with software





6.4.3 Starting software:

a. Start software
b. Add labels to wells according to use
c. Select or deselect wells according to the experiment
d. Select what should be recorded (data is saved locally and all automated analysis is done in the cloud, make sure to download regularly

6.4.4 Using the stimulation lid

a. Remove the stimulation lid from it's packaging when ready for use in a biological safety cabinet following standard sterile techniques.
b. Replace the normal culture plate lid on the desired plate of Mantarray tissue with the stimulation lid.

The stimulation lid can only fit in one orientation. Make sure to follow the appropriate marking on the 24-well plate and the lid to ensure the accurate orientation.

c. Plug the lid into the instrument by securely holding the stimulation lid and then connecting it with counter pressure to prevent dislodging it from the 24 well tissue culture plate.
d. Failure to securely attach the ribbon cable will lead to an inability to stimulate your tissues.
e. Engage the preferred stimulation protocol. This step may be done inside or outside the incubator.

6.4.5 Electrode cleaning stimulation lid

a. Stimulation Lid carbon electrodes will absorb by products from your tissue culture/ drug treatment, and it is recommended they are cleaned every 24-48h.
b. Prepare a clean container with distilled water and a stir-bar on a stir-plate.
c. Clean electrodes only with distilled water. Do NOT use any detergents
d. Place electrodes in a waterproof container with the water level below the electrode socket and begin stirring

The water level should NOT reach the body of the stimulation lid, maximum level should be several millimeters below the point at which the electrodes enter the sockets.

e. The stir bar should either be below the electrodes or adjacent to the electrode sin order to agitate the water. Use of lower rotation settings is recommended to avoid any damages to the stimulation lid.

f. The water should be changed at least every 24h for at least 2 days. More frequent water changes (every 12h) are recommended. Following this cleaning the water should contain no pink color from residual pH indicator (phenolred) leaching from the carbon electrode

Cleaning should last about two days but care should be given to observing no discoloration or debris in the water prior to autoclave

g. CuriBio every 3rd rinse, CuriBio will autoclave the stimulation lid using a cycle duration of 15min at 112C. It is important that the lid is removed immediately after the cycle and is not left in the autoclave to cool down. Failure to do this could result in lid warping

7. MAINTENANCE/SPECIFICATIONS

Instrument Physical Specifications:

a. **Length:** 315.8 mm (12.43 in)
b. **Width:** 200 mm (7.87 in)
c. **Height:** 85.0 mm (3.35 in) - including feet 91.5 mm (3.60 in)
d. **Instrument Weight:** 4.8 kg (10.5 lbs)
e. **Color:** Black
f. **Enclosure Material:** Anodized aluminum

Power Brick Physical Specification:

a. **Length:** 189.6 mm (7.46 in)
b. **Width:** 124.5 mm (4.90 in)
c. **Height:** 47.5 mm (1.87 in) - including feet 51.6 mm (2.03 in)
d. **Weight:** 1.2 kg (2.54 lbs)
e. **Color:** Black
f. **Enclosure Material:** Anodized aluminum

Connectivity:

a. **Ports:** USB-3 (cable included)
b. **Storage:** Onboard memory for saved protocols, external computer for data.

Power:

a. **Input Voltage:** 110-240 VAC, single phase, 50-60 Hz, <1 Amp.
   Power Adapter (Power Brick) included.
b. **Outlet type:** Plug type can be customized for different regions (e.g, North America, Europe, Asia, Australia)
c. **Recommendation:** Use of a surge protector (not included) is strongly recommended.

Software Requirements:

a. **Operating System:** Windows 10 and Windows 11. The designated laptop sold with the instrument includes Windows 11 Pro.
b. **Controller Application:** Included in the designated laptop sold with the instrument.

Incubation Conditions:

The instrument can operate at room conditions, and at incubator conditions as listed below:

a. **Temperature:** 37°C
b. **CO2 Level:** 5%
   Humidity: 95%

Power Connection & Surge Protection Guidelines:

The Mantarray instrument relies on sensitive electronic components to provide high-quality magnetic based data. These components are vulnerable to power disturbances, which can occur unexpectedly andmay impact functionality. To safeguard your Mantarray instrument, Curi Bio strongly recommends using a CE-marked surge protector between the instrument´s power cable and your facility´s power outlet. Ensure that the surge protector is connected directly to the outlet, without connecting multiple cords in series (daisy-chaining). Failure to meet these guidelines might lead to data distortion and critical failure of your instrument.

Compliance & Safety:

To ensure the utmost quality in our products, Curi Bio has pursued conformity with critical industry standards. This section serves as a Declaration of Conformity with the CE mark and ensures that Curi Bio products adhere to the essential safety requirements set forth by the European Union, highlighting product safety and performance. Curi Bio Inc. declares under its sole responsibility that the Mantarray Instrument described in this manual complies with the requirements of the following Directives and is eligible to bear the CE mark:

European Union Directives:

a. 2014/30/EU – Electromagnetic Compatibility (EMC) Directive Assurance of conformity with the provisions of this directive is achieved through compliance with the following standards:

Safety Standards:

a. EN 61326-1:2013 | EN 61326-2-3:2013
b. IEC 61326-1:2013 | IEC 61326-2-3:2013

Warranty:

The Curi Bio Mantarray is sold with a one year warranty, effective from the date of purchase, for any defects or failures in parts or workmanship. Additional warranty coverage may be purchased on a per-year basis by contacting sales@curibio.com. If the Mantarray fails or breaks within one year of purchase, Curi Bio will repair it at no charge to the customer. The customer is responsible for the cost of shipping the Mantarray to Curi Bio’s headquarters in Seattle, WA, USA. Curi Bio will pay for the return shipment of the repaired equipment. All warranty returns must be accompanied by an RMA number. This warranty does not cover cosmetic damage or wear.

SOP Version History

Version Reviewed	Date Revised/ Reviewed	Revision Summary	New Version Number
		[Insert specific changes from previous SOP] < e.g. changes in accountabilities, process steps, deviation actions, or records>.	