C2C12 Myotube Formation Assay in LunaX™ ECM

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Introduction

This protocol describes an optimised procedure for differentiating C2C12 myoblasts into contractile myotubes within LunaX ECM hydrogels. The amorphous structure of LunaX ECM helps minimise construct shrinkage over the culture period compared with collagen-based substrates, while its tuneable stiffness enables closer replication of healthy and diseased skeletal muscle tissue mechanics for more physiologically relevant *in vitro* modelling.

This protocol has been optimised for the C2C12 mouse myoblast cell line. Parameters may require further optimisation when using alternative myoblast cell lines or primary myoblasts.

Expected Results

Differentiated myotubes expressing skeletal myosin heavy chain and other sarcomeric markers are expected to form within 7–12 days of initiating differentiation. Myotube formation should be readily visible by brightfield microscopy, with elongated, multinucleated structures becoming increasingly apparent over the differentiation period. Successful differentiation can be confirmed by immunofluorescent staining for skeletal myosin heavy chain and additional sarcomeric markers.

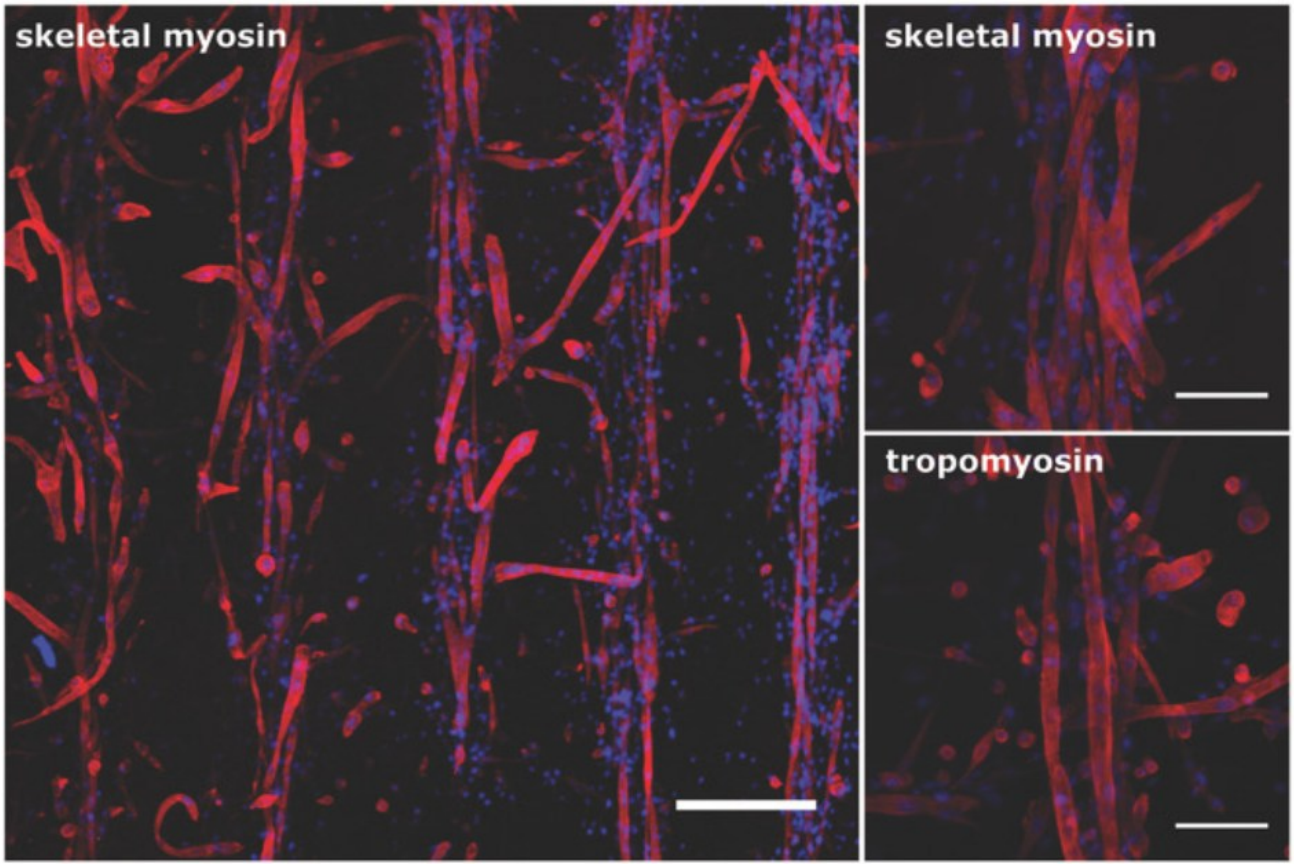

Figure 1. *Representative immunofluorescence images of C2C12-derived myotubes formed within LunaX ECM and stained for skeletal myosin and tropomyosin. Armstrong, J. P. K., et al. (2018). Engineering anisotropic muscle tissue using acoustic cell patterning. Advanced Materials.*

Materials and Equipment

Reagent / Item	Specification / Concentration
LunaX MultiMatrix, Low Stiffness	Gelomics Cat. No. SKU0002, includes photoinitiator
LunaX Crosslinker	Gelomics Cat. No. SKU0028
C2C12 cell line	Available from life sciences suppliers; expand to 70–80% confluency before use
Expansion Medium	High-glucose DMEM (HG-DMEM) + 20% v/v foetal bovine serum (FBS) + 1% penicillin/streptomycin
Differentiation Medium	HG-DMEM + 1% penicillin/streptomycin + 1% non-essential amino acids + 1% N-2 supplement + 20 ng/mL recombinant human IGF-1
24-well plate	Clear, flat bottom
Sterile PBS	1x concentration for photoinitiator reconstitution
Trypsin or dissociation reagent	As per standard protocol
Class II biosafety cabinet	-
CO_2 incubator	37 °C, 5% CO_2, humidified
Water bath	37 °C
Centrifuge	-
Automated or manual cell counter	-
Inverted brightfield microscope	-

Procedure

The procedure below is described for a final volume of 1 mL of cell-laden LunaX ECM (approximately 33 domes at 30 μL each). Adjust all volumes and cell numbers proportionally based on your desired replicate number. Culture parameters, such as media compositions and cell density, are provided as a recommended starting point and can be adjusted based on your protocol and requirements.

1. Prepare ECM and Photoinitiator

1. Warm the 1.5× LunaX ECM solution to 37 °C in a water bath for approx. 15 minutes.
2. Determine the volume of precursor solution required. Precursor solution can be prepared in 1.5 mL increments as follows:
   - Reconstitute one 2.7 mg vial of photoinitiator in 0.6 mL PBS. Protect from light.
   - In a sterile tube, combine 1 mL of 1.5× LunaX ECM solution with 0.5 mL of photoinitiator solution.
3. Reconstitute the required number of photoinitiator vials using 0.6 mL sterile PBS per vial. Protect from light.
4. Prepare the required volume of gel precursor solution by mixing the 1.5× LunaX ECM solution with photoinitiator solution as described above. Place the precursor solution in a water bath at 37 °C.
5. If you use part of a vial of photoinitiator, the remaining reconstituted photoinitiator can be stored at 2–8 °C, protected from light, for use on the same day. Only use photoinitiator

solutions prepared on the day of use. For experiments on subsequent days, prepare a fresh photoinitiator solution.

2. Cell Preparation

1. Lift cells according to your standard protocol. After enzymatic detachment, wash cells with serum-containing medium to inactivate trypsin or protease activity.
2. For each 1 mL of LunaX ECM, transfer 5 × 10^6 C2C12 cells to a tube and pellet by centrifugation. Remove the entire supernatant, minimising residual liquid that could dilute the ECM.

> ***Note:*** *Higher cell densities (for example 1 × 10^7 C2C12 cells/mL) may improve myotube formation and alignment.*

3. Encapsulation and Dome Formation

1. Add 1 mL of LunaX ECM precursor solution to the cell pellet.
2. Mix gently but thoroughly by pipetting to ensure a homogeneous suspension. Important: take care to avoid introducing air bubbles.
3. Using reverse pipetting, dispense 30 µL domes of the cell–ECM suspension into the centre of each well of a 24-well plate.
4. Carefully transfer the plate to the LunaX Crosslinker, avoiding any disturbance or smudging of the domes.

4. Photocrosslinking

1. Crosslink in the LunaX Crosslinker at 10 mW/cm² for 120–180 seconds to achieve an elastic modulus of approximately 2.5–4 kPa. For softer gels, use a shorter crosslinking duration, and for stiffer gels, increase crosslinking duration.

5. Culture and Differentiation

1. Add 1 mL of pre-warmed Expansion Medium to each well and incubate at 37 °C, 5% CO_2 for approximately 24 hours.
2. After 24 hours, replace Expansion Medium with 1 mL of Differentiation Medium per well.
3. Refresh Differentiation Medium every 2 days.
4. Continue differentiation for 7–12 days, or until elongated multinucleated myotubes are visible and the intended experimental endpoint is reached.

Troubleshooting

Problem	Solution
LunaX ECM solidifies during protocol	Return ECM (with or without cells) to the 37 °C water bath until re-liquefied. Mix by gentle pipetting. Do not heat above 37 °C.
Air bubbles in the LunaX ECM suspension	Centrifuge the ECM suspension at 300 × g for 1 minute. If cells have been added, gently mix by pipetting to resuspend the cells.
LunaX ECM does not crosslink on light exposure	Confirm the photoinitiator was prepared fresh and reconstituted correctly.
Poor myotube formation or alignment	Confirm cell health and use of fresh, complete Differentiation Medium with all growth factors. Cell alignment strategies (e.g. micropatterning) can improve myotube formation; refer to published protocols.

Problem	Solution
ECM domes dissolve after encapsulation	Residual trypsin or protease may be degrading the hydrogel. Ensure complete removal by washing the cell pellet with serum-containing medium before encapsulation.

References

- LunaX ECM and LunaX Crosslinker manufacturer's instructions (Gelomics)
- LX-APR-006 – Whole Mount Immunofluorescence (Gelomics)
- Institutional or cell-specific protocols for the cell types used.