Assay Protocol

C2C12 Myotube Formation Assay

gelomics
3D cell culture technologies

Introduction

This protocol outlines the use of LunaGel™ Photocrosslinkable Extracullar Matrix (ECM) as a 3D cell culture substrate for the differentiation of C2C12 cells into myotubes. In contrast to other commonly used substrates such as collagen I, the amorphous structure of LunaGel™ ECMs significantly reduces shrinkage of 3D cell culture samples over the duration of culture. LunaGel™'s unique photocrosslinking technology enables scientists to closely replicate mechanical proerpties of healthy and diseased muscle tissues and study cell biology in highly physiological conditions.

In the following procedure, we describe optimized assay conditions for the formation contractile myotubes formed by C2C12 cells which closely resemble key aspects of native tissue.

Materials

- Gelomics® LunaGel™ Photocrosslinkable Extracellular Matrix, Porcine Skin Gelatin, Low Stiffness (Gelomics Cat. No. SKU0002)
- 24 well flat-bottom non-treated tissue culture well plate
- C2C12 cell line (available from various vendors) cultured to 70-80% confluency
- Expansion Medium: High Glucose Dulbecco's Modified Eagle's Medium (HG-DMEM) with 20% Fetal Bovine Serum (FBS)
- Differentiation Medium: HG-DMEM with 1% (v/v) penicillin/streptomycin, 1% (v/v) non-essential amino acids, 1% (v/v) N-2, and 20 ng/µL recombinant human IGF-1 (PeproTech

Procedure

The protocol below outlines the procedures for a final volume of 1 mL of cell laden LunaGel™ ECM. Adjust volumes as required based on your desired sample volume (30 µl recommended) and replicate number.

1. Place the 2X LunaGel™ ECM solution into a water bath at 37 °C for < 15 min.
2. Reconstitute one vial of the photoinitiator with 1 ml sterile PBS and store protected from light.
3. Lift and count cells according to your standard protocol. Inhibit and remove trypsin/protease solution from the cells.
4. For each 1 ml of LunaGel™ Photocrosslinkable Extracellular Matrix, transfer 5×10^7 C2C12 cells into a reaction tube and centrifuge to pellet cells. Remove the entire supernatant, taking care to minimize liquid residues that may dilute the ECM solution in later steps.

   **Higher cell concentrations up to 1×10^8 C2C12/ml of LunaGel™ ECM can improve myotube formation.*
5. Add 500 µl of the LunaGel™ ECM solution to the cell pellet and gently pipette up and down to homogenously resuspend cells.
6. Add 500 µl of the photoinitiator solution to the cell suspension. Store the remaining photoinitiator solution at 4 – 8 °C, protected from light, for future use.
7. Mix thoroughly by pipetting up and down to ensure a homogenous cell suspension. Take care to avoid the introduction of air bubbles.
8. Dispense 30 µl of the LunaGel ECM™/cell suspension into the centre of each well of a 24 well plate to create dome-shaped samples.

   Tip: Use reverse pipetting to avoid introducing air bubbles when dispensing the mixture into well plates.
9. Carefully transfer the well plate to the LunaCrosslinker, taking care to avoid any disturbance/smudging of the samples before crosslinking.

10. Crosslink the cell laden LunaGel™ ECM by light exposure using the Luna Crosslinker™ for 2 minutes (~3.5 kPa elastic modulus).
11. Add 1 ml pre-warmed expansion media to each well and incubate in a humidified cell culture incubator at 37 °C and 5% CO_2 for 1 day.
12. After approximately 24 hours of culture, replace expansion medium with 1 ml of differentiation medium per sample. Refresh cell culture media every 2 days.

Expected results and downstream assays

Contractile myotubes expressing skeletal myosin and other markers should form within 7-12 days of culture. These structures can be observed using brightfield microscopy.

Recommended downstream assays include:

- Live/Dead viability staining using fluorescein diacetate and propidium iodide
- Immunofluorescence analysis (for example skeletal myosine or tropomyosine)
- Gene expression analyis

Troubleshooting guide

Problem	Solution
LunaGel™ ECM solution solidifies during protocol	Re-heat LunaGel™ ECM solution (with or without cells) to 37 °C in a water bath. Once liquid, mix by pipetting up and down. Do not heat above 37 °C.
Air bubbles in LunaGel™ ECM solution.	Centrifuge solution (with or without cells) at 300*g* for 1 min and mix by pipetting up and down.
The LunaGel™ ECM solution does not crosslink when exposed to light.	Ensure photoinitiator solution is prepared fresh and/or extend crosslinking time.
Myotubes not forming	Ensure healthy cells, fresh media and growth factors are used. Cell patterning can significantly improve myotube formation. Several protocols can be found in literature, for example Armstrong *et al,* Advanced Materials 2019.
LunaGel™ ECM samples dissolve following cell encapsulation.	Traces of trypsin or other proteases may degrade the hydrogel samples. Ensure complete removal of trypsin before cell encapsulation by washing the cells pellet with medium of buffer.

Please contact us at info@gelomics.com for questions or more information.