ECM-Embedded 3D Spheroid Invasion Assay

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Introduction

This assay quantifies invasive outgrowth from preformed cancer cell spheroids embedded within LunaX™ ECM hydrogels in a 96-well plate format. The model enables assessment of tumour cell invasion through a surrounding 3D matrix environment and can be used to evaluate matrix-dependent invasion, treatment response, or differences in invasive behaviour between cell types.

This protocol is suitable for metastatic cancer cell lines expanded *in vitro*, such as MDA-MB-231 breast cancer cells, and may also be adapted for primary cancer cells where appropriate. Spheroid size, seeding density, matrix stiffness, hydrogel volume, and assay duration should be optimised for the specific cell type and experimental objective.

Expected Results

Following embedding and culture, invasive cancer spheroids exhibit progressive outgrowth modulated by matrix stiffness:

- Softer matrices (~1.5 kPa): compact morphology with limited radial migration.
- Intermediate stiffness (~3 kPa): collective outgrowth with cell protrusions extending from the spheroid surface.
- Stiffer matrices (~6 kPa): pronounced invasive spreading with increased dissemination of single cells from the spheroid core.

Quantitative image analysis typically shows a time-dependent increase in total invasion area, consistent with mechanotransduction-driven regulation of cell motility and ECM remodelling.

Materials and Equipment

Reagent / Item	Specification / Concentration
LunaX ECM Low Stiffness (0.5–6 kPa)	CoreMatrix (Gelomics Cat. No. SKU0007) or MultiMatrix (Gelomics Cat. No. SKU0002)
LunaX Crosslinker	Gelomics Cat. No. SKU0028
Cancer cell line of interest	Metastatic line (e.g. MDA-MB-231); primary cancer cells optional
Basal medium and supplements	As per cell-line-specific SOP
Tissue culture treated flasks	For cell expansion
96-well ULA round-bottom plate	For spheroid formation and embedding
96-well flat-bottom non-TCT plate	Optional; for improved confocal imaging (transfer spheroids post-aggregation)
Calibrated pipettes and sterile tips	pH 7.4, sterile
PBS	Without calcium and magnesium
Trypsin or dissociation reagent	As per cell-line-specific SOP
CO_2 incubator	37 °C, 5% CO_2
Benchtop centrifuge with plate carrier	—
Automated or manual cell counter	—
Inverted microscope	For daily observation and brightfield imaging

Key Parameters - Define Before Starting

- Cell line and passage range: metastatic (e.g. MDA-MB-231); passage not critical for immortalised cells unless otherwise specified.
- Spheroid aggregation: 1,000–2,000 cells per 200 µL per well; culture undisturbed for 3 days before embedding.
- LunaX ECM and target stiffness: CoreMatrix or MultiMatrix; 0.5–6 kPa.
- LunaX Crosslinker exposure settings at 15 mW/cm²: 0.5 kPa = 60 s; 1.5 kPa = 90 s; 2.5 kPa = 120 s; 4 kPa = 180 s; 6 kPa = 300 s.
- Imaging schedule: brightfield images every 24 hours, typically for up to 10 days.

Procedure

1. Cell Expansion

1. Thaw and expand cells in tissue culture flasks until they reach approximately 70–80% confluency.
2. Confirm normal morphology and viability by microscopy before proceeding.

2. Cell Harvest and Counting

1. Aspirate spent medium and wash cells once with sterile PBS.
2. Add sufficient trypsin or dissociation reagent to cover the cell monolayer.
3. Incubate at 37 °C until cells detach (typically 3–5 minutes, depending on cell line).
4. Neutralise trypsin by adding an equal volume of complete medium containing serum.
5. Transfer the suspension to a centrifuge tube and centrifuge at 200 × g for 5 minutes.
6. Aspirate the supernatant and resuspend the pellet in a small volume of fresh medium.
7. Count cells; confirm viability above 90%.
8. Adjust to $5 \times 10^3 – 1 \times 10^4$ cells/mL (1,000–2,000 cells per 200 µL per well).

3. Spheroid Formation in ULA Plate

1. Dispense 200 µL of the single-cell suspension into each well of a 96-well ULA round-bottom plate.
2. If required, briefly centrifuge the plate at 150 × *g* for 1 minute to assist aggregation.
3. Incubate undisturbed for 3 days to allow compact spheroid formation.
4. If evaporation is noticeable, carefully top up with pre-warmed medium.

> ***Note:*** *Standard practice is to embed spheroids directly in the ULA plate. For improved confocal imaging, spheroids may be carefully transferred to a flat-bottom imaging-compatible plate after aggregation.*

4. Preparation of LunaX ECM

1. Prepare LunaX ECM precursor solution according to the manufacturer's instructions. Record batch number, composition, and preparation time. Protect from light.

5. Embedding Spheroids in LunaX ECM

1. On day 3, confirm that compact spheroids have formed in each well.
2. Carefully remove the culture medium, leaving minimal residual liquid to prevent drying.
3. Add 30 µL of LunaX ECM precursor solution to each well, ensuring the spheroid is fully submerged. Avoid introducing air bubbles.
4. Immediately centrifuge the plate at approximately 150 × *g* for 15 seconds to centralise spheroids at the well base.

6. Photocrosslinking

1. Transfer the plate to the LunaX Crosslinker.

2. Set light intensity and photocrosslinking duration according to the required stiffness (refer to Key Parameters above).
3. Crosslink all samples and record the program name, irradiance, and duration for documentation.

7. Post-crosslink Culture

1. After photocrosslinking, gently add 200 μL of pre-warmed culture medium to each well.
2. Return the plate to the incubator at 37 °C, 5% CO_2.

8. Imaging and Quantification

1. Observe spheroid invasion daily by inverted brightfield microscopy. In softer matrices (0.5–1.5 kPa), invasion typically begins within 1–3 days.
2. Capture brightfield images at identical acquisition settings every 24 hours.
3. For quantification in ImageJ/Fiji: (i) open the image sequence; (ii) convert to 8-bit and subtract background; (iii) calibrate the image scale; (iv) threshold to segment spheroid core and invasion area; (v) measure total area and core area, and calculate invasion area = total – core; (vi) optionally measure invasion front radius by radial profiling; (vii) normalise all metrics to day 0.

Troubleshooting

Problem	Solution
Spheroids lost during medium removal	Reduce aspiration speed; leave a small residual liquid volume.
Spheroids not centred in the gel	Confirm centrifuge reaches ~150 × g; extend spin to 20–30 seconds if necessary.
Inadequate gelation	Verify LunaX ECM preparation procedure.
No observable invasion	Check cell line invasive capacity, serum concentration in medium, and LunaX ECM stiffness selection.

References

- LunaX ECM and LunaX Crosslinker manufacturer's instructions (Gelomics).
- Internal SOPs for the specific cell line used.