Surface-Seeded 3D Spheroid Invasion Assay

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Introduction

This assay quantifies invasive outgrowth from preformed cancer cell spheroids seeded onto the surface of LunaX™ ECM hydrogels in a 96-well plate format. The model enables assessment of tumour cell invasion into a defined matrix environment and can be used to compare matrix conditions, treatment effects, or intrinsic invasive potential across 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. Passage number is generally not critical for established immortalised cell lines, although passage limits should be followed where applicable. Seeding density, spheroid formation time, matrix stiffness, and assay duration should be optimised for the specific cell type and experimental objective.

Expected Results

Cancer spheroids seeded on LunaX CoreMatrix hydrogels typically exhibit progressive invasion and outgrowth over several days, 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.

These observations are consistent with stiffness-dependent modulation of cellular contractility and integrin-mediated adhesion signalling, where stiffer ECMs promote enhanced migratory and invasive behaviour.

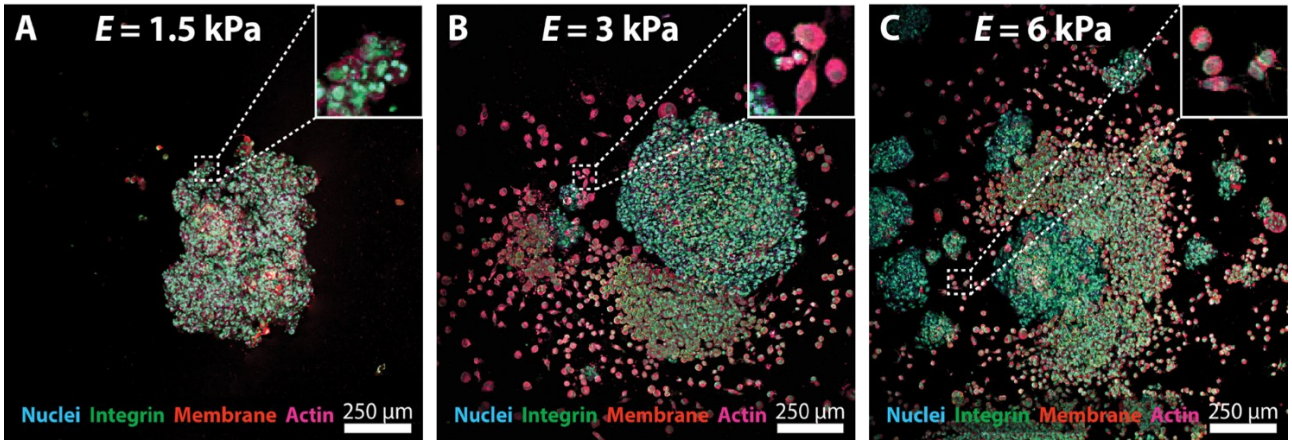

Figure 1. *Representative immunofluorescent images showing spheroid invasion after 7 days on low, intermediate, and high LunaX ECM stiffness using MDA-MB-231 spheroids.*

Materials and Equipment

Reagent / Item	Specification / Concentration
LunaX ECM Low Stiffness Kit (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
96-well flat-bottom well plate	Compatible with LunaX Crosslinker and imaging
Calibrated pipettes and sterile tips	Including wide-bore or cut tips for spheroid transfer
Sterile PBS	Without calcium and magnesium
Trypsin or dissociation reagent	As per cell-specific SOP
CO_2 incubator	37 °C, 5% CO_2, humidified
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 in a ULA plate before seeding.
- LunaX ECM formulation: CoreMatrix or MultiMatrix; stiffness range 0.5 – 6 kPa.
- Recommended 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 medium and wash cells once with serum-free medium or 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 and confirm viability is above 90%.

8. Adjust the cell concentration to $5 \times 10^3 – 1 \times 10^4$ cells/mL, equivalent to 1,000–2,000 cells per 200 µL. Mix gently to ensure a uniform suspension.

3. Spheroid Formation in ULA Plate

1. Dispense 200 µL of 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.

4. Preparation of LunaX ECM Surface

1. Prepare LunaX ECM precursor solution according to Gelomics' instructions. Record batch number, composition, and preparation time. Protect from light.
2. Pipette 60 µL of cell-free LunaX ECM into each well of a sterile flat-bottom 96-well plate. Avoid bubbles and ensure even coverage of the well base.
3. Transfer the plate to the LunaX Crosslinker and crosslink to the required stiffness using validated exposure settings.
4. Allow the crosslinked ECM to swell to an equilibrium state by adding 150–200 µL of pre-warmed culture medium per well. Incubate for 30 minutes at 37 °C. Gently aspirate, leaving a thin film to prevent drying.

5. Spheroid Transfer and Surface Seeding

1. Using a P1000 fitted with a wide-bore or cut tip, gently aspirate a single spheroid from the ULA plate, minimising shear stress.
2. Deposit one spheroid at the centre of each ECM surface. Avoid touching the gel surface with the pipette tip.
3. Allow spheroids to settle for 3–5 minutes without disturbance.
4. Carefully add 150–200 µL of pre-warmed culture medium per well by dispensing slowly against the side wall to avoid displacing the spheroid.

6. Post-seeding Culture

1. Return the plate to the incubator at 37 °C, 5% CO_2.
2. Maintain culture for 10–14 days; replace 50–100% of medium every 48–72 hours by aspirating and dispensing slowly against the well wall.

7. Imaging and Quantification

1. Observe spheroid outgrowth and surface invasion daily by inverted brightfield microscopy.
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 – the day spheroids are seeded onto gel substrates.

Recommended Controls

- Negative control: non-invasive or low-invasive cell line.
- Matrix-only wells (no cells) to identify gel artefacts.
- Positive control: include a known pro-invasion stimulus (e.g. growth factor supplementation).

Acceptance Criteria

- Spheroids appear compact and centred on the gel surface after seeding.
- LunaX ECM appears uniform and is free of visible bubbles.

- Exclude wells containing displaced or damaged spheroids.

Troubleshooting

Problem	Solution
Spheroid is disrupted during transfer	Use a cut P1000 tip; pipette slowly to minimise shear force.
Spheroid floats or moves after seeding	Allow 3–5 minutes settling; add medium slowly against the wall; ensure a residual liquid film remains on the gel before adding medium.
Uneven ECM surface or gel detaches	Ensure 60 µL is dispensed centrally; avoid bubbles; confirm LunaX Crosslinker exposure settings. Some meniscus formation is expected.
Inadequate gel formation	Verify LunaX ECM preparation procedure, including that freshly reconstituted photoinitiator was used. Increase crosslinking durations if required.
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).
- Institutional protocols for the specific cell line used.