Whole-Mount Immunofluorescence Staining of LunaX™ Hydrogel Constructs

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Introduction

This protocol describes the preparation and immunofluorescence staining of intact 3D cell cultures encapsulated in LunaX hydrogels, for visualising target proteins and cellular structures by widefield or confocal fluorescence microscopy.

Applicable to LunaX ECM-encapsulated 3D cell cultures processed as intact (whole-mount) constructs without sectioning. Suitable for nuclear, cytoskeletal, and cell-surface or intracellular targets. For sectioned constructs, refer to LX-APR-007 (Cryosectioning) or LX-APR-008 (paraffin embedding and sectioning).

Expected Results

Successfully stained whole-mount constructs show distinct, low-background fluorescent signal for the target proteins or cellular structures, with nuclear (DAPI) and, where included, cytoskeletal (phalloidin) counterstains clearly resolved throughout the depth of the construct. LunaX hydrogels support efficient antibody diffusion throughout the construct making them well suited to whole-mount fluorescence imaging. Confocal imaging can be used to acquire z-stacks and generate clear maximum intensity projection images across a defined imaging depth.

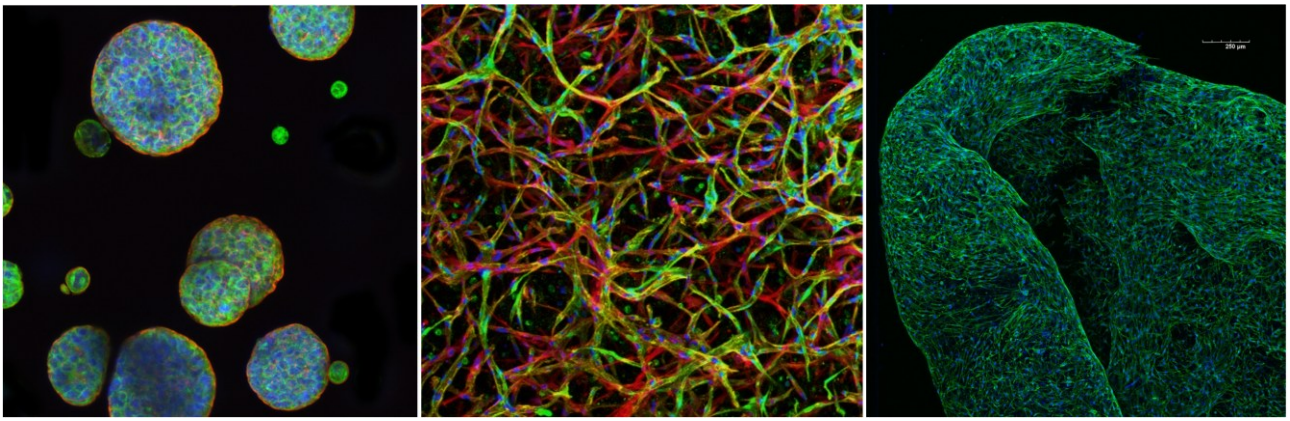

Figure 1. *Representative immunofluorescence images of whole-mount LunaX constructs showing target staining with nuclear counterstain and minimal background fluorescence.*

Materials and Equipment

Reagent / Item	Specification / Concentration
Paraformaldehyde (PFA)	4% solution in PBS for fixation; handle in a fume hood
Phosphate-buffered saline (PBS)	pH 7.4
Blocking buffer	5% goat serum (or BSA) + 0.1% Triton X-100 in PBS. Example (50 mL): 47.5 mL PBS + 2.5 mL goat serum + 50 µL Triton X-100 – or suppliers' recommended formulation.

Reagent / Item	Specification / Concentration
Wash buffer	20% v/v blocking buffer in PBS (e.g. 10 mL blocking buffer + 40 mL PBS) – or suppliers' recommended formulation
Primary antibody	Target-specific (e.g. anti-CD31 for endothelial cells); dilute in wash buffer
Secondary antibody	Fluorophore-conjugated (e.g. Alexa Fluor 488 goat anti-mouse); dilute in wash buffer
Phalloidin (optional)	Fluorophore-conjugated; for F-actin/cytoskeletal staining; 1:1,500 dilution
DAPI	For nuclear staining; 1:1,000 dilution in wash buffer
Fume hood	For PFA preparation, handling and disposal
Orbital shaker or rocker	4 °C
Fluorescence microscope	Widefield or confocal, with appropriate filter sets

Procedure

This procedure provides recommended starting conditions and an overview of the general whole-mount immunofluorescence workflow. Users should optimise staining parameters according to the target antigen, antibody, construct size and cell type, with reference to relevant manufacturer's instructions and published methods where applicable.

1. Fixation

1. Remove culture medium. In a fume hood, add 4% PFA (0.5–1 mL depending on well size) and incubate for 45 minutes at 4 °C.
2. Remove PFA (dispose as chemical waste per institutional guidelines) and wash samples 3× with PBS.

> ***Note:*** *Fixed constructs may be stored in PBS at 4 °C for several days before proceeding.*

2. Permeabilisation and Blocking

1. Add blocking buffer and incubate for ≥2 hours at 4 °C on a gentle shaker. This step simultaneously permeabilises the gel matrix and reduces non-specific antibody binding.

3. Primary Antibody Incubation

1. Prepare the primary antibody in wash buffer at the appropriate dilution (e.g. 1:200; 400 µL per well – or sufficient volume to fully submerge each construct).
2. Incubate overnight at 4 °C on a shaker.
3. Wash gels with wash buffer for a minimum of 8 hours, with a minimum of 3 buffer changes during the wash period.

4. Secondary Antibody and Phalloidin Staining

1. Prepare secondary antibody (e.g. Alexa Fluor 488 goat anti-mouse, 1:200) and, if required, phalloidin (1:1,500) in wash buffer.
2. Incubate for 2 hours at 4 °C on a shaker, protected from light.
3. Wash as above (≥8 hours with 3 buffer changes), protected from light.

5. Nuclear Staining

1. Prepare DAPI at 1:1,000 in wash buffer.
2. Incubate for 2 hours at 4 °C on a shaker, protected from light.
3. Wash as above (≥8 hours with 3 buffer changes).

4. Perform a final 3× wash with PBS, leaving PBS in the well for imaging.

6. Imaging

1. Image whole constructs using a widefield or confocal fluorescence microscope with appropriate filter sets.
2. For thick constructs, confocal z-stacks are recommended. Use identical acquisition settings when comparing experimental groups.
3. After staining, Image as soon as practical to minimise loss of fluorescence signal. If samples need to be stored, store in PBS at 4 °C protected from light.

Notes

- Antibody dilutions require optimisation for each target and cell type. Titration experiments are recommended before running full assays.
- For best results, keep staining steps sequential rather than combining antibody and DAPI incubations, to minimise background fluorescence.
- For cell-surface epitopes or fixation/permeabilisation-sensitive targets, reduce or omit Triton X-100 and optimise fixation and permeabilisation conditions.
- Once fluorophores are introduced, protect all samples from light during incubation and wash steps.

References

- LX-APR-007 – Immunofluorescence Staining of Cryosectioned LunaX Hydrogel Constructs (Gelomics)
- LX-APR-008 – Paraffin Embedding and Sectioning of LunaX Hydrogel Constructs (Gelomics)
- Protocols and suppliers' instructions for antibodies and other staining reagents.