

VIABILITY STAINING PROTOCOL

Calcein AM and Propidium Iodide

This is a suggested procedure, please adjust according to your experimental needs.

Protocol aim

This protocol describes how to perform Calcein AM and PI live/dead staining for imaging viable and non-viable cells in 3D constructs printed with CELLINK bioinks and photoinks.

Materials needed

- Calcein AM (Invitrogen™ eBioscience™, product number 15560597)
- Propidium iodide (Sigma-Aldrich, product number 81845-25MG)
- Cell-laden bioprinted constructs
- Hank's Balanced Salt Solution (HBSS+/-)
- 5 mL amber Eppendorf tubes
- Inverted fluorescence microscope with filter sets for FITC and Texas Red

KEEP STOCKS, WORKING SOLUTIONS AND STAINED CELLS AWAY FROM LIGHT SOURCES.

Protocol

This protocol is optimized for CELLINK's cell-compatible bioinks and photoinks when printing droplets or small constructs. For larger structures, the staining volumes and incubation times should be adjusted accordingly. Larger constructs require higher volumes of staining solution to ensure full coverage, as well as longer incubation times to allow the dyes to penetrate to the center of the construct and to ensure effective washing steps. Here, the workflow is tailored for a 96-well plate setup.

1. Preparing stock solutions

MATERIAL

Calcein AM

DMSO

PI

HBSS+/-

DESCRIPTION

- **Calcein AM stock solution (1 mM):** Add 50.3 μ L DMSO to one vial of Calcein AM, mix gently until fully dissolved, aliquot, and store at -20 °C.
- **PI stock solution (1 mg/mL):** Weigh 1 mg PI into an Eppendorf tube, add 1 mL HBSS+/-, mix thoroughly until completely dissolved, aliquot, and store at 2-10 °C.
- If both stock solutions are already prepared, proceed to the next step of the main protocol.

2. Pre-washing of printed constructs

MATERIAL

HBSS+/-

DESCRIPTION

- Remove a cell medium and wash printed constructs with 150 μ L HBSS+/- once for 15 min at 37 °C.
- Optional: For a positive dead control, remove HBSS+/- and add ethanol 70% to cover the constructs. Remove after 5 min and add HBSS+/-.

3. Preparing Calcein AM working solution and staining

MATERIAL

Stock solution of Calcein AM (1 mM)

4 mL HBSS+/-

5 mL amber Eppendorf tube

DESCRIPTION

- Thaw stock solution of Calcein AM.
- Prepare a Calcein AM working solution by adding 1 μ L of the stock solution to 4 mL HBSS+/- in an amber Eppendorf tube.
- Remove HBSS+/- and add 100 μ L of the Calcein AM working solution to each construct-containing well.
- Incubate at 37 °C for 20 minutes.

Notes:

- The Calcein AM working solution should be used within 2 hours after preparation.
- The required staining volume depends on a construct size; ensure that each construct is fully covered by the solution.
- Adjust the incubation time according to a construct size. For most constructs, an incubation range of 20–30 minutes is sufficient.

4. Preparing PI working solution and staining

MATERIAL

Stock solution of PI (1 mg/mL)

4 mL HBSS+/-

5 mL amber Eppendorf tube

DESCRIPTION

- Prepare the PI working solution by adding 400 μ L of PI stock solution to 4 mL HBSS+/- in an amber tube.
- Remove the Calcein AM working solution and add 100 μ L of the PI working solution to each construct-containing well.
- Incubate at 37 °C for 10 minutes

Notes:

- The PI working solution should be used within 2 hours of preparation.
- The required staining volume depends on construct size; ensure the solution fully covers each construct.
- Adjust the incubation time according to construct size. For most constructs, an incubation range of 10–25 minutes is sufficient.

5. Washing

MATERIAL

HBSS+/-

DESCRIPTION

- Remove the PI working solution and wash the constructs twice in 150 μ L HBSS+/- for 15 minutes each at 37 °C.
- Store the constructs in fresh HBSS+/- until imaging.
- Imaging should ideally be performed immediately.

6. Imaging

MATERIAL

Fluorescence microscope

Image analysis software (e.g. ImageJ)

DESCRIPTION

- Place the well plate containing the constructs on the fluorescence microscope for imaging.
- Acquire images in the green channel (FITC/488) and red channel (Texas Red/570), preferably as Z-stacks to capture the full depth of the constructs.
- Use image analysis software (e.g., ImageJ) to quantify live (Calcein AM–positive) and dead (PI-positive) cells and calculate the cell viability percentage.