

## VIABILITY STAINING PROTOCOL

# Calcein AM and Propidium Iodide

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

### Protocol aim

The aim of this protocol is to provide instructions for performing live and dead staining using Calcein AM and propidium iodide (PI) for imaging of live respectively dead cells in 3D constructs of all CELLINKs bioinks.

### 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 CELLINKs bioinks, printed as droplets in 96-well plates. For larger structures, please adjust volumes and incubation times. Larger constructs need larger staining solution volumes to fully cover the construct and longer incubation times for the dye to penetrate to the center of the construct.

### 1. Preparing stock solutions

#### MATERIAL

Calcein AM

DMSO

PI

HBSS+/-

#### DESCRIPTION

- If stock solutions already are prepared, move to Step 2.
  - To prepare 1 mM of Calcein AM stock solution: add 50.3  $\mu$ L DMSO into a vial of Calcein AM and mix gently. Aliquot and store stock solutions at -20°C.
  - To prepare 1 mg/mL of PI stock solution: weigh 1 mg of PI in an Eppendorf tube. Add 1 mL of HBSS+/- to the tube with PI and mix properly. Make sure that all PI powder is dissolved. Aliquot and store stock PI solution at 4°C.

### 2. Pre-washing

#### MATERIAL

HBSS+/-

#### DESCRIPTION

- Transfer the constructs (triplicates of each condition is preferred) to a separate well plate with wells filled with HBSS+/-, wash for 5 min at 37°C.
- Optional: For a dead control, remove HBSS+/- and add ethanol to cover the construct. Remove after 5 min and add HBSS+/-.

### 3. Calcein AM working solution

#### MATERIAL

Stock solution of Calcein AM

4 mL HBSS+/-

5 mL amber Eppendorf tube

#### DESCRIPTION

- Thaw stock solution of Calcein AM.
- Add 1  $\mu$ L Calcein AM stock solution into the Eppendorf tube with 4 mL of HBSS+/- to make the Calcein AM working solution.

Note: Staining solution should be used within 2 hours from preparation.

Note: Alternatively dilute Calcein AM in medium instead of HBSS+/-.

- Remove the HBSS+/- from constructs and add 100  $\mu$ L Calcein AM working solution to each well, incubate at 37°C for 20 min.

Note: The amount of staining solution required per construct depends on sample size. Add enough staining solution to cover the construct.

## 4. PI working solution

### MATERIAL

Stock solution of PI

4 mL HBSS+/-

5 mL amber Eppendorf tube

### DESCRIPTION

- Add 400  $\mu$ L of PI stock solution into an amber tube with 4 mL HBSS+/- to make the PI working solution.  
Note: Staining solution should be used within 2 hours from preparation.
- Remove Calcein AM working solution and add 100  $\mu$ L PI staining solution, incubate constructs for 6 min at 37°C.

Note: Required amount of staining solution needed depends on sample. Add enough staining solution to cover construct.

## 5. Washing

### MATERIAL

HBSS+/-

### DESCRIPTION

- Remove PI working solution and wash twice for 15 min in HBSS+/- at 37°C.
- Store in HBSS+/- until imaging.

Note: Wash by adding and removing HBSS+/- once.

## 6. Imaging

### MATERIAL

Fluorescent microscope

Image analysis software

### DESCRIPTION

- Bring cell-laden constructs to fluorescent microscope for imaging.
- Capture images in the green (FITC/488) and red (TexasRed/570) channels as Z-stacks.
- Assess the viability percentage using a software for image analysis, e.g. Image J to count the number of live and dead cells per image.