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 dead staining using Calcein AM and Propidium iodide (PI) for imaging of live respectively dead cells in 3D constructs of CELLINK bioink, GelMA and GelX variations.

Material needed

- Stock solution of 1 mM Calcein AM*
- Stock solution of 2 mg/ml PI**
- Scale
- Cell-laden printed constructs
- Cell culture medium without Fetal Calf Serum (FCS)
- Hank's Balanced Salt Solution (HBSS+/+)
- 2x 15 ml Falcon tubes
- Inverted fluorescence microscope with filter sets for FITC and Texas Red

* Calcein AM stock solution is prepared by dissolving 1 vial of 50 µg of Calcein AM in 50,3 µl DMSO. Store stock solution at -20°C. Calcein AM can be purchased from Invitrogen™ eBioscience™, product number 15560597.

** PI stock solution is prepared by dissolving 2 mg of PI in 1 ml HBSS+/+. Store stock solution at 4°C. PI can be purchased from Sigma-Aldrich®, product number 81845-25MG. Note that this chemical should be treated as a Hazardous chemical and proper precautions should be taken.

KEEP STOCK, WORKING SOLUTION AND STAINED CELLS FROM LIGHT SOURCES.

Protocol

This protocol is optimized for CELLINK bioink, GelMA and GelX variations, printed 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 centre of the construct.

TIME: ~110 min if stock solutions already prepared, otherwise ~130 min.

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Step	Title	Material	Description
1	Prepare stock solutions	- Calcein AM - DMSO - PI - HBSS+/+	If stock solutions already are prepared– move to step 2. - Add 50,3 µl DMSO into a vial of Calcein AM and mix gently. - Weigh 2 mg of PI in an Eppendorf tube. - Add 1 ml of HBSS+/+ to the tube with PI and mix gently. - Store Calcein AM solution at -20 °C and PI solution at 4 °C. TIME: ~20 min.
2	Pre-Wash	- HBSS+/+	- Remove medium from constructs and add HBSS+/+, wash for 15 min at 37°C. - For a dead control, remove HBSS+/+ and add ethanol to cover the construct. Remove after 5 min and add HBSS+/+. Note: Constructs to be imaged can be moved to a separate well plate for easier handling. TIME: ~15 min
3a	Prepare green staining solutions	- Stock solution of Calcein AM (1 mM) - 6 ml cell culture medium without FCS	- Thaw stock solution of Calcein AM at room temperature (RT). - Pre-warm cell culture medium without FCS to 37°C. TIME: ~8 min
3b	Preparation of green staining solution	- 6 ml pre-warmed cell culture medium without FCS - 1,5 µl Calcein AM (1 mM)	- Add 1,5 µl Calcein AM (1 mM) into the 6 ml of the pre-warmed cell culture medium in a 15 ml Falcon tube. Note: Staining solution should be used within 2 hours from preparation. Store the stock of Calcein AM in the freezer directly after use. Note: Alternatively dissolve Calcein AM in HBSS+/+ instead of medium. TIME: ~10 min

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4	Live staining	- Calcein AM staining solution	- Remove HBSS+/+ from constructs and add 100 µl green staining solution, 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. TIME: ~25 min
5a	Prepare red staining solutions	- Stock solution of PI (2 mg/ml) - 5 ml cell culture medium without FCS	- Allow stock solution of PI (2 mg/ml) to reach RT. - Pre-warm cell culture medium without FCS to 37°C. TIME: ~2 min
5b	Preparation of red staining solution	- 5 ml pre-warmed cell culture medium without FCS - 50 µl PI (2 mg/ml)	- Add 50 µl PI (2 mg/ml) into 5 ml of the pre-warmed cell culture medium in a 15 ml Falcon tube. Note: Staining solution should be used within 2 hours from preparation. Store the PI in the fridge after use. Note: Alternatively dissolve PI in HBSS+/+ instead of medium. TIME: ~5 min
6	Dead staining	- PI staining solution	- Remove green staining solution and add 100 µl red PI staining solution, incubate 5 min at RT. Note: Required amount of staining solution needed depends on sample. Add enough staining solution to cover construct. TIME: ~5 min
7	Wash	- HBSS+/+	- Remove staining solution and wash twice for 15 min in HBSS+/+ at 37°C. Note: Dispose any liquids containing PI to Hazardous waste. - Store in HBSS+/+ until imaging. Note: Wash by adding and removing HBSS+/+ once. TIME: ~40 min
8	Imaging	- Fluorescent microscope	- Bring cell-laden constructs to fluorescent microscope for imaging.

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			- Capture images in the green (FITC/488) and red (TexasRed/570) channels as Z-stacks.
9	Imaging analysis	- Image analysis software	- Assess the viability percentage using a software for image analysis, e.g. Image J.

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