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**Title:** 3D Cell Viability Assay using Calcein AM, Propidium Iodide and Hoescht 33342.

**Version:** v3

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SOP History		
Number	Date	Reason for Change
v1	18/04/2016	Original
v2	18/04/2018	Update
v3	18/04/2020	Biennial Update

### 1.0 Purpose –

This SOP describes the current procedure for analysing cell viability on 3D Ultra Low Attachment (ULA) spheroids grown in 96R well trays (Costar 7007) using Calcein AM in lab 248 at the St Andrews School of Medicine (SASoM). Calcein AM is used as a measure of enzymatic activity in live cells, Propidium iodide is used as a measure of dead cells with compromised membrane integrity and Hoescht is used as a marker of all nucleated cells (alive or dead). Calcein AM was purchased from Fisher Scientific (C3100MP).

### 2.0 Scope –

The scope of this document is to describe the procedure for looking at cell viability of 3D Ultra Low Attachment (ULA) spheroids grown in 96-well trays using Calcein AM (live cells), Propidium iodide (dead cells) and Hoescht 33342 (all cells).

### 3.0 Responsibilities –

All staff involved in looking at cell viability of 3D Ultra Low Attachment (ULA) spheroids grown in 96-well trays using Calcein AM, Propidium iodide and Hoescht 33342 are responsible for ensuring that the methods are followed in accordance with this SOP. All staff must have read and signed the relevant risk assessment documents before performing this procedure.



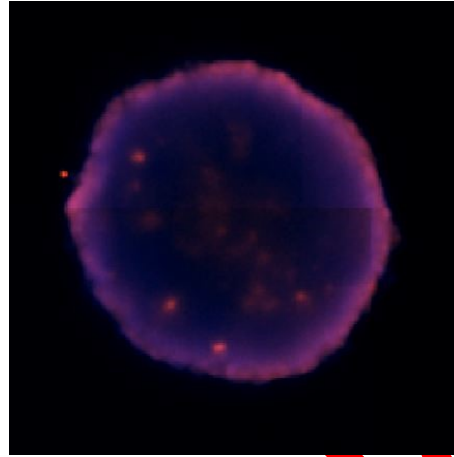
#### 4.0 Procedure – Cell Culture:

1. Set up and treat spheroids 'Celigo Supported' 96-well round bottom Ultra Low Attachment (ULA) trays in the usual manner. This assay is a sacrificial endpoint assay designed to measure (i) live cells using Calcein AM (Fisher Scientific; C3100MP), (ii) dead cells using Propidium Iodide (Sigma, P4864), and all nucleated cells (dead and / or alive) using Hoescht 33342 (Sigma H3570).
2. Reconstitute Calcein AM by adding 50.2 $\mu$ L of DMSO into one of the 50 $\mu$ g vials of lyophilized Calcein AM. Mix gently by inversion.
3. Prepare a 2X 'Mixed Dye Solution' of Calcein AM, Propidium Iodide, and Hoescht 33342 – 12mL of the 2x mixed dye solution will be enough for all the wells of a 96-well tray. Adjust accordingly if not all the wells are being used. Dyes should be made up in F12 media (with no FCS) in order to reduce autofluorescence on the green channel.
4. The following table can be used to prepare the **2X** mixed dye solution:

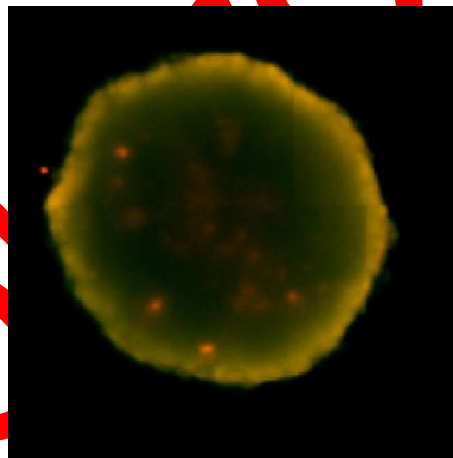
Dye	Stock Concentration	Recommended Concentration	Dilution Factor	Volume in 12mL media	Conc Range for other cell types
Calcein AM	1mM	1 $\mu$ M	1:1000	24 $\mu$ L	0.1 - 10 $\mu$ M
Propidium Iodide	1mg/mL	2 $\mu$ g/mL	1:500	48 $\mu$ L	0.1 - 5 $\mu$ g/mL
Hoescht 33342	10mg/mL	5 $\mu$ g/mL	1:2000	12 $\mu$ L	1 - 10 $\mu$ g/mL

5. At the endpoint of your growth assay, carefully remove 100 $\mu$ L of experimental media to leave 100 $\mu$ L in the well.
6. Carefully wash the spheroids by adding and then removing 200 $\mu$ L F12 media (x2). Total volume in well should now be 100 $\mu$ L.
7. Add 100 $\mu$ L of 2x mixed dyes solution.
8. Incubate spheroids for 30 min at 5% CO<sub>2</sub>, 37°C.
9. Since spheroids in the wells are still 'live', the plate should be read immediately on the Celigo.

10. All cells (nuclei) stained with Hoescht 33342 will appear blue whilst those stained with Propidium Iodide (the dead cells) will appear red as illustrated below:



11. All cells stained with Calcein AM (live cells) will appear green whilst those stained with Propidium Iodide (the dead cells) will again appear red as illustrated below:



12. All liquid waste from this assay should be placed inside a sealable bag containing paper towels or sawdust to soak up the liquid, securely sealed and then disposed of in the red 'Chemical Waste' bins.

### 5.0 Personal protection -

A Howie laboratory coat and lab gloves must be worn at all times.



## 6.0 Spillages -

Always clean up and disinfect any spills immediately after use, only you know what you have spilt and are aware of its hazard.

## 7.0 Training -

All staff should undergo training in this technique before performing procedure.

## 8.0 Related documents –

## 9.0 Approval and sign off –

### Author:

Name: Peter Mullen

Position: Research Fellow

Signature: Date:

### Management Approval:

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Name: Alex MacLellan

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Signature: Date:

