Simplifying high throughput 3D spheroid growth and shrinkage assays using live content imaging

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Summary & Impact

- Creating spheroids using Corning® Ultra Low Attachment plates provides a technically simple, robust and economical approach to creating 3D spheroids in 96-well plates (equivalent to 2D).
- Using fluorescently labelled cells and IncuCyte ZOOM™, we have assembled and validated fully-kinetic, spheroid growth and shrinkage assays.
- IncuCyte ZOOM™ metrics (spheroid size, (area/confluence) and fluorescence intensity (Mean Image Fluorescence, MIF)) are informative, and can be readily gathered to monitor spheroid growth and shrinkage over time.
- Spheroid fluorescence intensity can be measured without masking, and may be a useful indicator of spheroid 3D nature and/or cell health.
- The images and time-lapse movies provide important information and validation regarding spheroid morphology and the effects of test samples.
- These assays should prove useful for medium throughput, quantitative pharmacology of test samples for effects on the growth and shrinkage of tumor cells in 3D.
- Cell proliferation/ spheroid growth can be directly compared in 2D and 3D.

96-well spheroid assay workflow – an integrated solution

DAY 0 — HARVEST & SEED CELLS
Cell seeding
Seed cells (100 µl/well, 1-5K/well, ± ECM) into 96W ULA plate (Corning 7007). Centrifuge (1000 rpm, 5 mins.)

DAY 0 -3 — FORM SPHEROIDS
Spheroid formation (0-3d)
Position plate in IncuCyte ZOOM and gather images (10x, every 6h, 72-96h)

DAY 3 — TREAT SPHEROIDS
Sample addition (3d)
Remove plate, add samples (100 µl/well) and return to IncuCyte ZOOM

DAY 7, 11 — RE-FEED SPHEROIDS (optional)
Spheroid growth & shrinkage (3-12d)
Monitor & quantify spheroid parameters over time
Re-feeds (remove 100 µl/well, & replace with treatments)

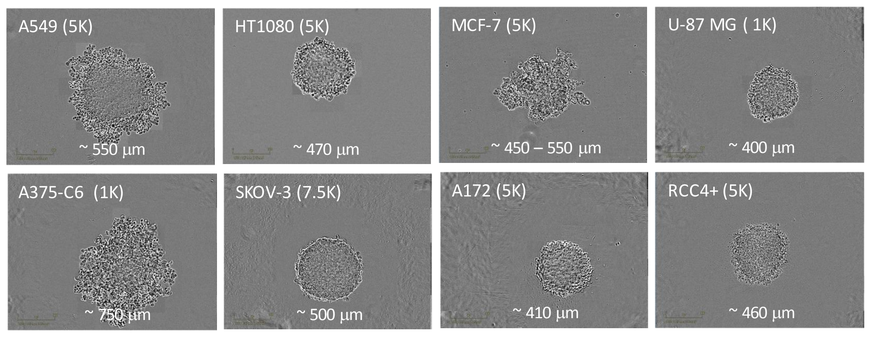
Spheroid formation protocol adapted from Vinci et al. BMC Biology 2012,10:29

- Cells seeded into each well on a ULA plate and placed within the IncuCyte ZOOM™ to monitor spheroid formation (72 – 96h)
- Plate removed, samples added, plate returned to the IncuCyte to monitor and quantify spheroid parameters (size, fluorescence)
- Option to re-feed spheroids every 96h: remove media from each well, replace with an equal volume of test samples in media

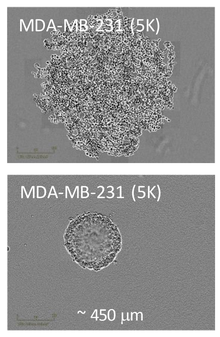
- Format compatible with 96- & 384-well ULA plates, manual or automated liquid handling
- Method compatible with a large selection of cell types (> 30 cell types tested)

Successful spheroid formation using multiple cell types

Protocol compatible with different cell types



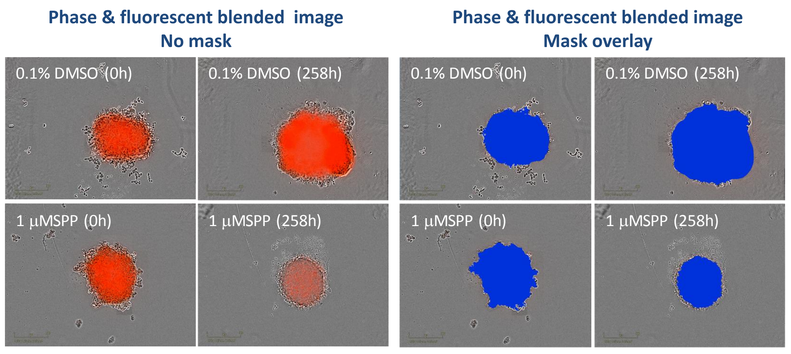
- Matrigel™



+ Matrigel™ (2.5%)

- High quality HD phase images of spheroids formed from a range of tumor cell lines (72h post seeding)
- A single spheroid is centrally located in each well of ULA plate
- Different 3D morphologies are observed (tight spheroids, compact and loose aggregates)
- Note, the 3D structure of loose aggregates can be improved by the inclusion of a basement membrane extract (e.g. 2.5% Matrigel™) at the stage of spheroid initiation

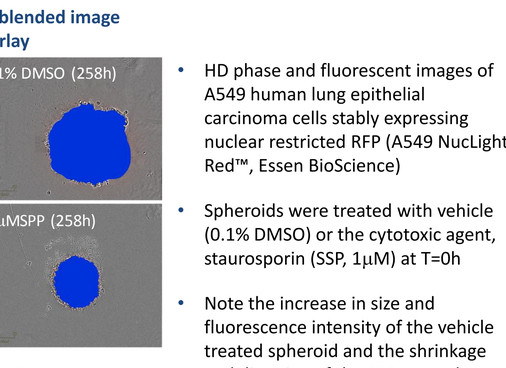
Quantifying spheroid growth and shrinkage over time



- HD phase and fluorescent images of A549 human lung epithelial carcinoma cells stably expressing nuclear restricted RFP (A549 NucLight Red™, Essen BioScience)
- Spheroids were treated with vehicle (0.1% DMSO) or the cytotoxic agent, staurosporin (SSP, 1µM) at T=0h
- Note the increase in size and fluorescence intensity of the vehicle treated spheroid and the shrinkage and dimming of the SPP treated spheroid
- Note the IncuCyte software's ability to accurately mask fluorescent images
- Spheroid growth and shrinkage can be quantified kinetically using the IncuCyte size metrics (fluorescence area and fluorescence confluence) which require masking of the fluorescent spheroid

Validation of size metric

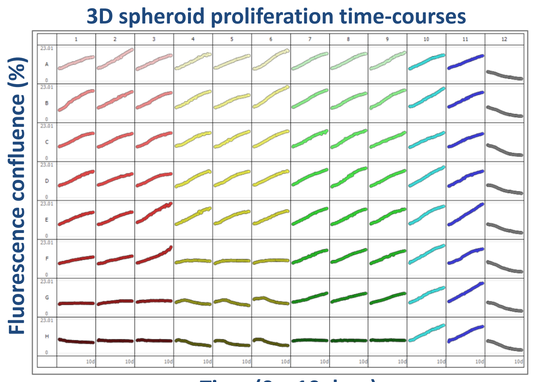
- Spheroid area is proportional to cell seeding density
- IncuCyte area metric tracks mean image fluorescence (MIF) metric



- HD phase and fluorescent images of A549 human lung epithelial carcinoma cells stably expressing nuclear restricted RFP (A549 NucLight Red™, Essen BioScience)
- The use of MIF may better represent cell health, alongside spheroid depth

3D Pharmacology

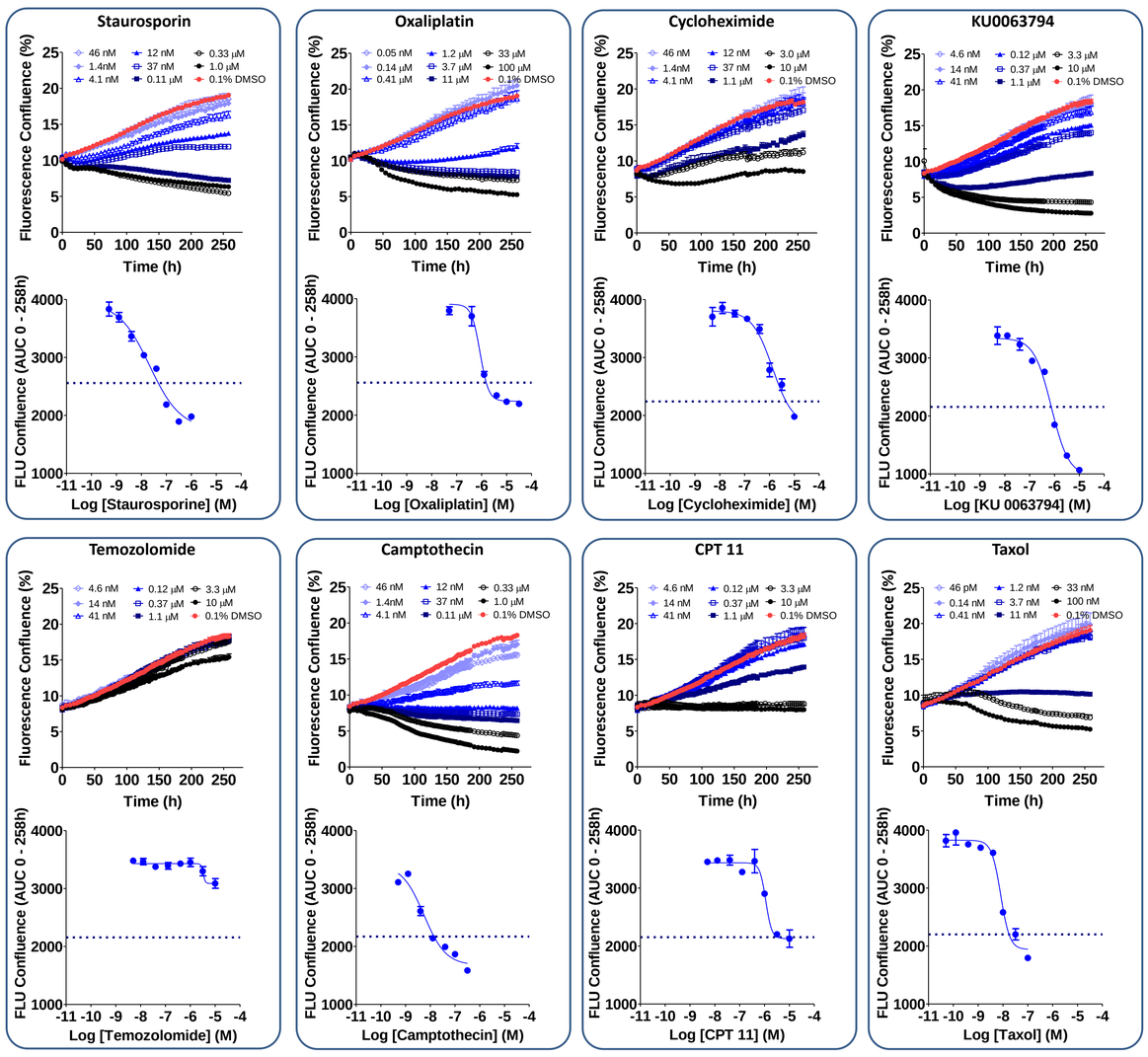
3D spheroid proliferation time-courses



96-well plate map

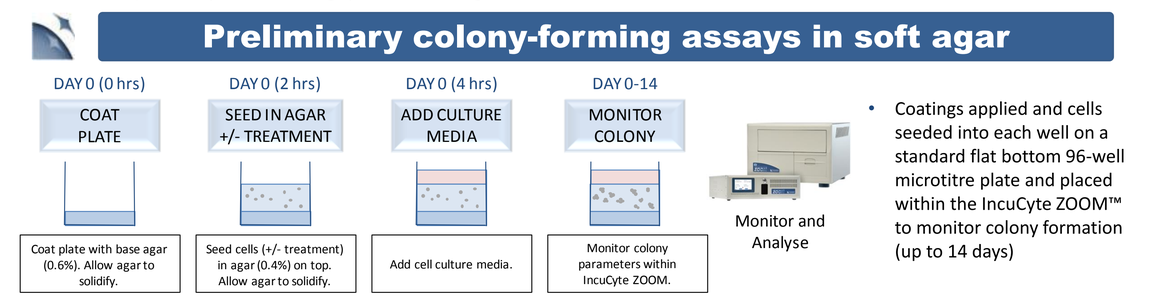
	1	2	3	4	5	6	7	8	9	10	11	12
A	CPT 11 4.57e-3 µM A549 NucRed (11) 2.5K / well			Taxol 0.05 nM A549 NucRed (11) 2.5K / well			Doxorubicin 0.05 nM A549 NucRed (11) 2.5K / well			A549 NucRed (11) 2.5K / well Media	A549 NucRed (11) 2.5K / well 0.1 % DMSO	Camptothecin 1 µM A549 NucRed (11) 2.5K / well
B	CPT 11 0.01 µM A549 NucRed (11) 2.5K / well			Taxol 0.14 nM A549 NucRed (11) 2.5K / well			Doxorubicin 0.14 nM A549 NucRed (11) 2.5K / well					
C	CPT 11 0.04 µM A549 NucRed (11) 2.5K / well			Taxol 0.41 nM A549 NucRed (11) 2.5K / well			Doxorubicin 0.41 nM A549 NucRed (11) 2.5K / well					
D	CPT 11 0.12 µM A549 NucRed (11) 2.5K / well			Taxol 1.23 nM A549 NucRed (11) 2.5K / well			Doxorubicin 1.23 nM A549 NucRed (11) 2.5K / well					
E	CPT 11 0.37 µM A549 NucRed (11) 2.5K / well			Taxol 3.7 nM A549 NucRed (11) 2.5K / well			Doxorubicin 3.7 nM A549 NucRed (11) 2.5K / well					
F	CPT 11 1.11 µM A549 NucRed (11) 2.5K / well			Taxol 11.11 nM A549 NucRed (11) 2.5K / well			Doxorubicin 11.11 nM A549 NucRed (11) 2.5K / well					
G	CPT 11 3.33 µM A549 NucRed (11) 2.5K / well			Taxol 33.33 nM A549 NucRed (11) 2.5K / well			Doxorubicin 33.33 nM A549 NucRed (11) 2.5K / well					
H	CPT 11 10 µM A549 NucRed (11) 2.5K / well			Taxol 100 nM A549 NucRed (11) 2.5K / well			Doxorubicin 100 nM A549 NucRed (11) 2.5K / well					

- Plate views represent a facile visualisation the kinetic temporal data for spheroid growth and shrinkage (fluorescence confluence %) collected from a single 96-well microtitre plate

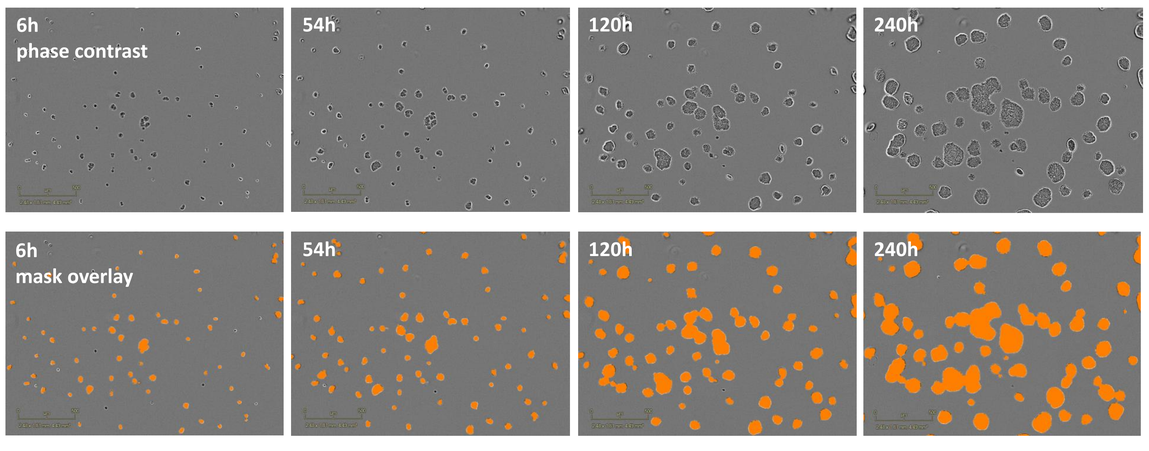


- A panel of compounds with different mechanisms of action were profiled in a 3D spheroid culture of human A549 cells stably expressing a red fluorescent label (A549 NucLight Red™)
- Time-course plots with the corresponding concentration response curves derived from the calculated area under the curve (AUC)
- The dotted line on the CRC plots represents the FLU confluence at time = 0 h, thus compounds yielding inhibition below this level (e.g. KU0063794) are likely to be inducing spheroid shrinkage and cell death

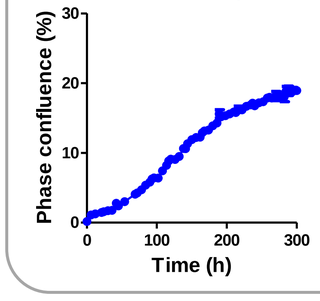
Preliminary colony-forming assays in soft agar



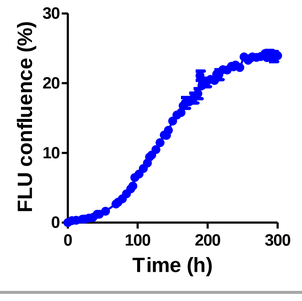
- Coatings applied and cells seeded into each well on a standard flat bottom 96-well microtitre plate and placed within the IncuCyte ZOOM™ to monitor colony formation (up to 14 days)



HD Phase analysis



Fluorescence analysis



- HD phase images of MCF-7 breast carcinoma cells stably expressing cytoplasmic RFP (MCF-7 CytoLight Red™, Essen BioScience)
- Note the increase in colony size and the ability to accurately mask phase images using the integrated IncuCyte software
- Colony growth can be quantified kinetically using the IncuCyte confluence metrics (phase or fluorescence confluence)
- Current challenges:
  - Meniscus of agar can lead to distorted focusing at well edge
  - Merged colonies require segmentation for accurate counting