96-Well live-cell assays for immune cell killing of 3D tumour spheroids

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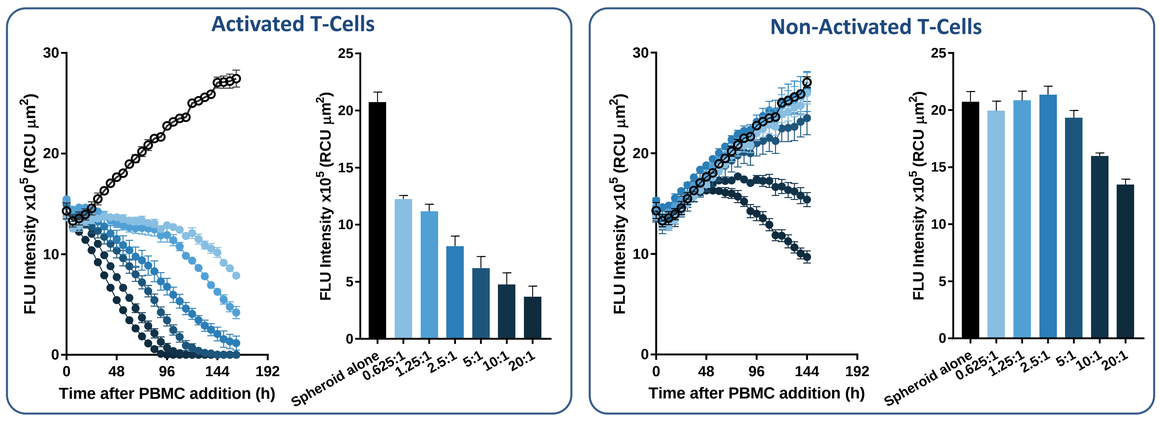
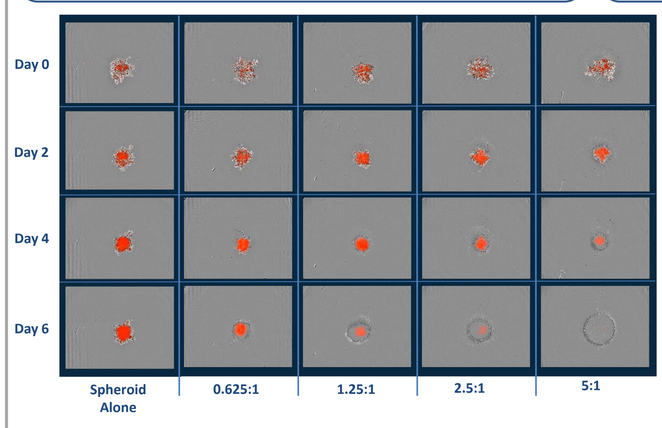
Summary & Impact

- Immunotherapies such as checkpoint inhibitors, CAR-Ts and immune-targeting Abs have great promise for cancer treatment. Translational cell-based assays are required to optimise these approaches.
- Here we describe image-based, immune cell-killing assays of 3D tumour spheroids, geared for assessing the efficacy of novel immune-modulators.
- Human tumour cell lines expressing RFP were used to form spheroids in 96-well ULA plates. Immune cells were then added and activated to kill. Spheroid viability was assessed over time (up to 10 days) by measuring the loss of RFP fluorescence using IncuCyte live-cell analysis.
- This method is exemplified with a range of immune cell types ((PBMCs, T-cells, NK-cells) and activators, including anti-CD3 & IL-2
- In an ADCC format, Herceptin induced a concentration-dependent specific killing of Her-2 expressing tumours. Higher concentrations of Herceptin were required in 3D vs 2D ADCC assays.
- These data demonstrate how immune-cell killing and ADCC assays can be extended from traditional 2D mono-cultures to 3D spheroid assays, providing the potential for greater translational relevance. These assays will be highly valuable in the search for novel immune-modulators.

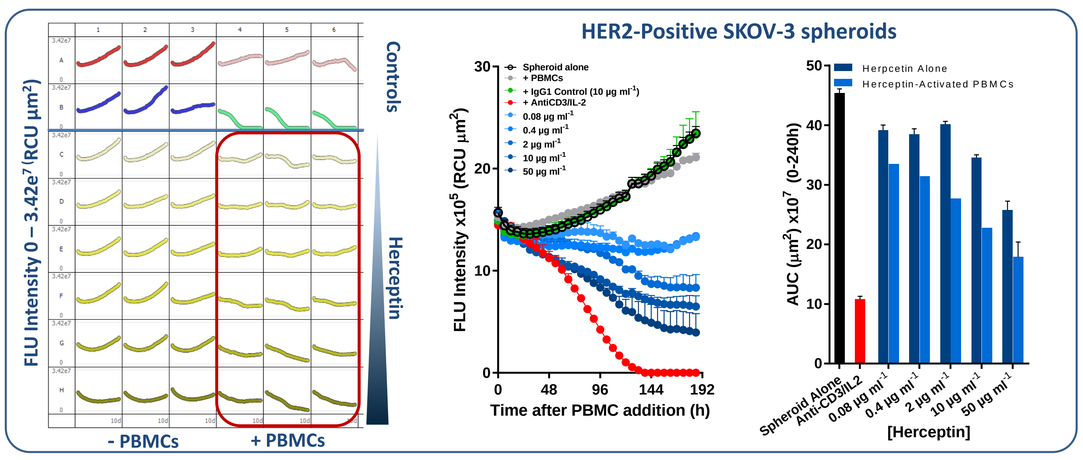
Effector-to-Target Ratio Dependent Cytotoxicity

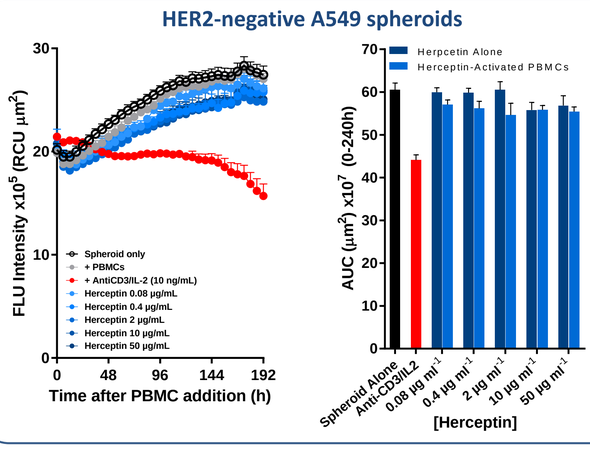




- Blended phase and fluorescent images of A549 NucLight Red™ spheroids in the presence and absence of immune cells.
- A549 cells (2.5K/well) seeded with PBMCs activated with anti-CD3 (10 ng/ml) and IL-2 (10 ng/ml). Cytotoxicity was quantified based on the red fluorescent intensity.
- Data demonstrates an E:T ratio-dependent destruction of tumour spheroids by the activated T-cell population.
- Note that E:T ratio optimisation is required as non targeted cell death was observed at E:T ratios >5:1.

Herceptin Induced ADCC in HER2-Positive SKOV-3 cells

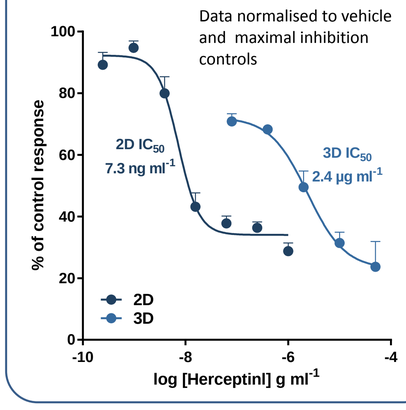




- HER2-positive SKOV-3 or HER2-negative A549 NucLight Red™ spheroids (2.5K/well) were seeded with PBMCs (6.25K/well) and treated with Herceptin (mAb targeting HER2 receptors).
- Herceptin induced concentration-dependent inhibition of SKOV-3 spheroid growth.
- Herceptin-induced cytotoxicity was measured in SKOV-3 but not A549 spheroids.

- A similar assay was conducted in a 2D culture model. SKOV-3 cells (1.6K/well) were seeded overnight prior to the addition of PBMCs (8K/well) and subsequent treatment with Herceptin.
- SKOV-3 tumour spheroids appear to exhibit ~300-fold lower Herceptin sensitivity in comparison to 2D.
- Note the apparent 34% inhibition of the 3D spheroid at the lowest test concentration (0.08 µg ml^{-1}). This suggests that a biphasic concentration response curve may exist, where the outermost cells behave as in the 2D model, whereas the spheroid centre has lower sensitivity.
- Additional experimentation is required to further understand the differential effects of Herceptin in 2D vs 3D models.

2D vs. 3D Herceptin Response



Continuous Live-Cell Analysis: Methodology

IncuCyte® S3 Live-Cell Analysis System

A flexible assay platform that sits inside a standard tissue culture incubator. IncuCyte automatically and continuously acquires and analyzes HD phase and fluorescent images of living cells cultured in microplates, dishes, or flasks.

IncuCyte® Software

Fast, flexible, and powerful control hub for continuous live-cell analysis comprising image acquisition, processing, and date visualization.

IncuCyte® Reagents & Consumables

A suite of non-perturbing cell labeling and reporter reagents. Includes nuclear-targeted GFP and RFPs for cell counting, no-wash caspase 3/7 substrate for apoptosis, and cell kits for angiogenesis.

96-Well 3D Immune Cell Killing Assay Workflow

1 SEED TARGET CELLS (Day 0)

Seed fluorescently labelled target cells of interest (100 µL/well, 1000-5000 cells/well) into a 96W Ultra Low Attachment plate (Corning or BRAND). Centrifuge plate (125 g, 10 minutes) at room temperature.

2 MONITOR SPHEROID FORMATION (Day 0-3)

Place plate inside an IncuCyte® and scan every six hours (4x). Monitor spheroid formation to ensure that by Day 3, spheroids form with desired size (e.g. 200-500 µm after 3 days).

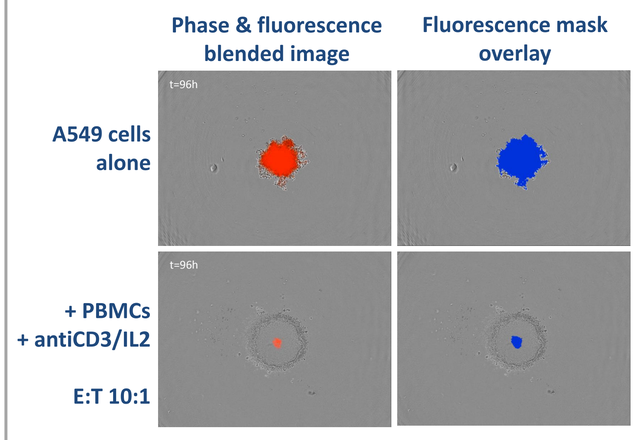
3 ADD IMMUNE CELLS (Day 3)

Add chosen immune cells (e.g. T-cells, PBMCs, NK cells) at an optimized effector-to-target cell ratio (50 µL/well). Add appropriate treatments, 50 µL/well (e.g T-cell stimuli, antibodies or cytokines) to achieve a final assay volume of 200 µL.

4 LIVE CELL IMAGING

Place plate inside an IncuCyte® and continue to capture images every six hours (4x) to monitor spheroid proliferation and immune-mediated cytotoxicity. Analyse using integrated software.

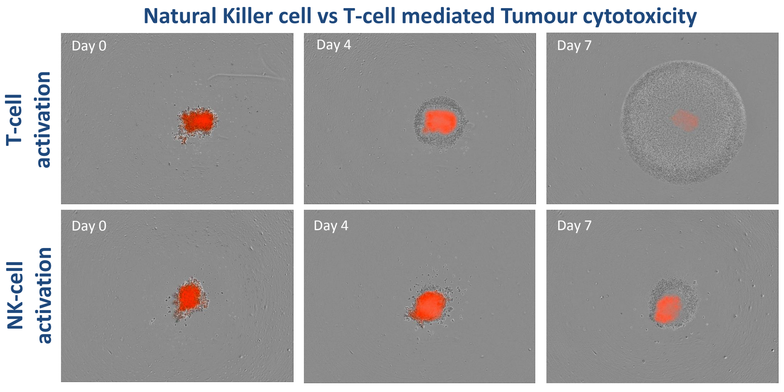
Fluorescence as a Measure of Spheroid Cytotoxicity



- Blended phase and fluorescent images, with corresponding masks, of A549 human lung epithelial carcinoma cells stably expressing RFP (A549 NucLight Red™, Essen BioScience).
- Note the increase in fluorescence intensity of the spheroid alone and the decline of fluorescence in the presence of immune cells.
- Spheroid proliferation and immune cell-mediated cytotoxicity can be quantified kinetically using the IncuCyte® size metrics (fluorescence intensity and fluorescence area) which require masking of the fluorescent spheroid.

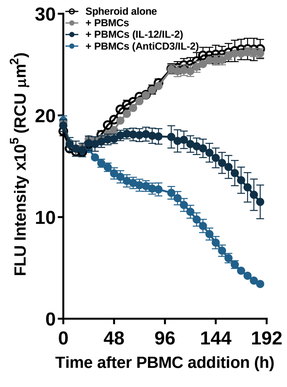
Activator-Dependent Tumour Cytotoxicity

Natural Killer cell vs T-cell mediated Tumour cytotoxicity

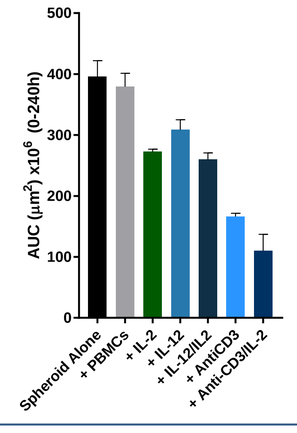


- A549 NucLight Red™ cells were treated with isolated PBMCs activated with Anti-CD3/IL-2 (10 ng ml^{-1}) or IL-12/IL-2 (10 ng ml^{-1}) targeting T-cell or NK-cell populations, respectively.
- Activating different populations of PBMCs, resulted in differential effects on tumour spheroid destruction.
- In contrast to activated NK-cells, T-cell activated populations exhibit increased proliferation.
- T-cell mediated spheroid destruction occurred more rapidly compared to NK-cell mediated death.
- Data illustrates that T-cell mediated spheroid destruction is significantly driven by the presence of anti-CD3 antibody.
- The frequencies of cell types within PBMC populations vary from donor to donor. Typically CD3+ T-cells account for 45-70% of PBMCs, while only 5-20% of are NK cells. This variability in occurrence could explain the differential effects observed in tumour spheroid cytotoxicity.

Spheroid death Time-course



Profile of Activators



A549 NucLight Red™ cells (2.5K/well). Optimised E:T ratios of 2.5:1

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