Monitoring cell health in real-time / time-lapse cell-based assays

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Introduction

The purpose here is to validate a novel far-red DNA binding viability probe, DRAQ7™, in real-time cell based assays for 2-D and 3-D models. This requires DRAQ7™ to neither synergise nor interfere with toxicants / anti-cancer compounds. It must be capable of addition at any stage of an assay to permit real-time monitoring of membrane integrity loss, retaining its functionality throughout.

As background, DRAQ7™'s DNA-specificity allows monitoring cell-by-cell while spectral properties permit incorporation in multi-colour experiments e.g. with vital probes, mitochondrial health probes or Annexin V. DRAQ7™ has previously been shown to be truly cell impermeant yet retains DNA binding and far-red fluorescence of the DRAQ chromophore which makes it compatible with HCS imagers, fluorescence microscopes and cytometers. The excitation/emission properties are particularly suited to penetrative imaging of multi-cellular structures and thick ex-plant tissue sections, whilst limiting risks of short wavelength DNA damage.
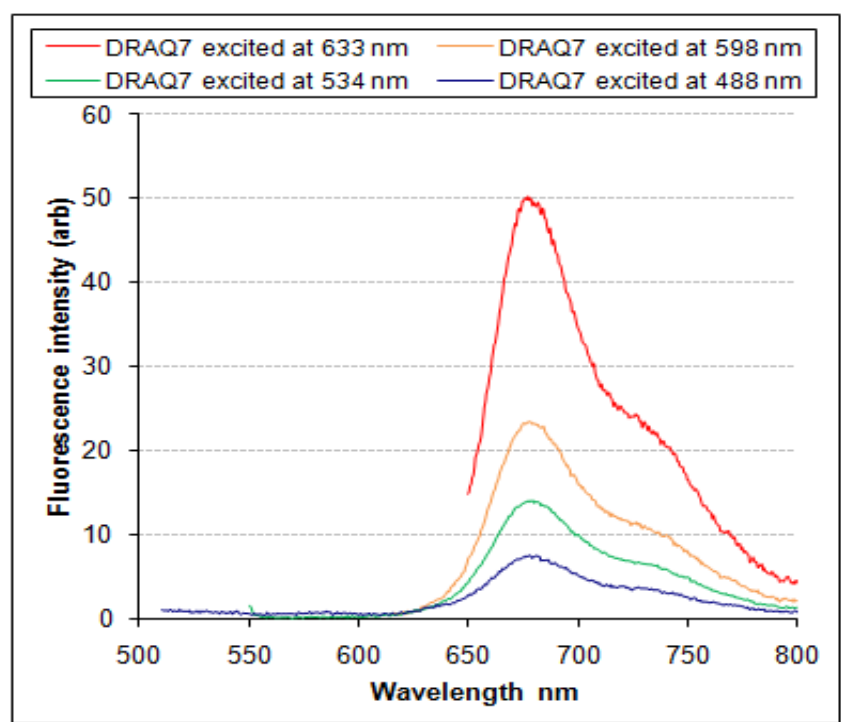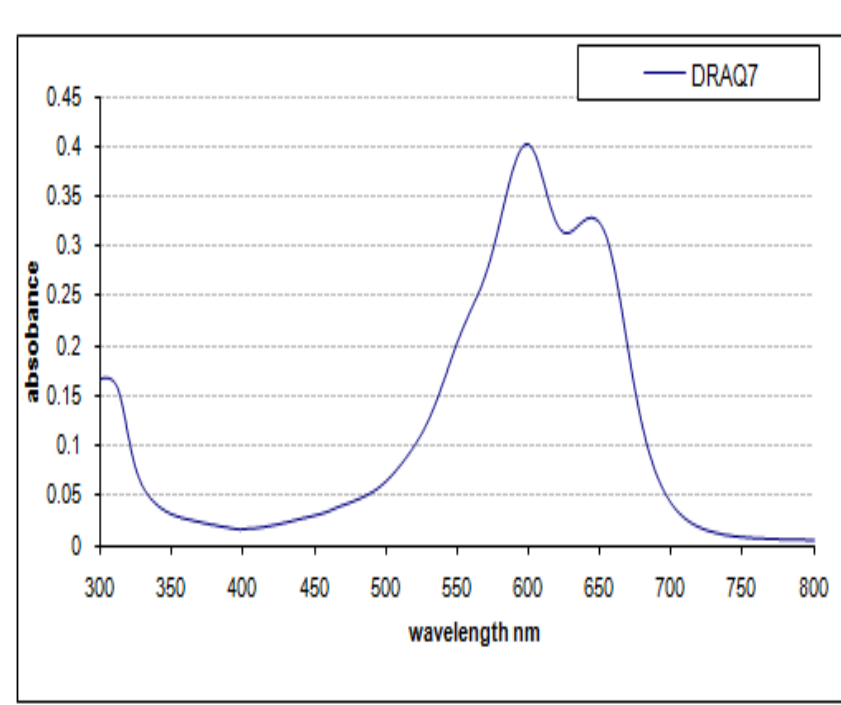
DRAQ7™ - Identity

Figure 1: absorbance spectrum for DRAQ7™. Useful excitation at 488nm by flow cytometry.

• pure synthetic DNA-specific anthraquinone probe
• supplied in aqueous, ready-to-use, no DMSO
• ships ambient, stores at 2-8°C

• excitation: red - low photo-toxicity / DNA damage
• emission: far-red (>675 nm) - "DRAQ" chromophore

• compatible with culture medium, buffers
• low photobleach, chemically stable, no-wash

Figure 2: Emission spectra for DRAQ7™ at various excitations. Peak emission is red-shifted 15nm on DNA binding.

Core Performance – Viability Monitoring

As shown in figures 3 & 4, DRAQ7™ demonstrates the fundamental requirement of a viability or cell health reporter in both flow cytometric and image-based assays.

Figure 3: Lymphoma cells exposed to apoptosis-inducing Staurosporine.

Controls: with and without serum

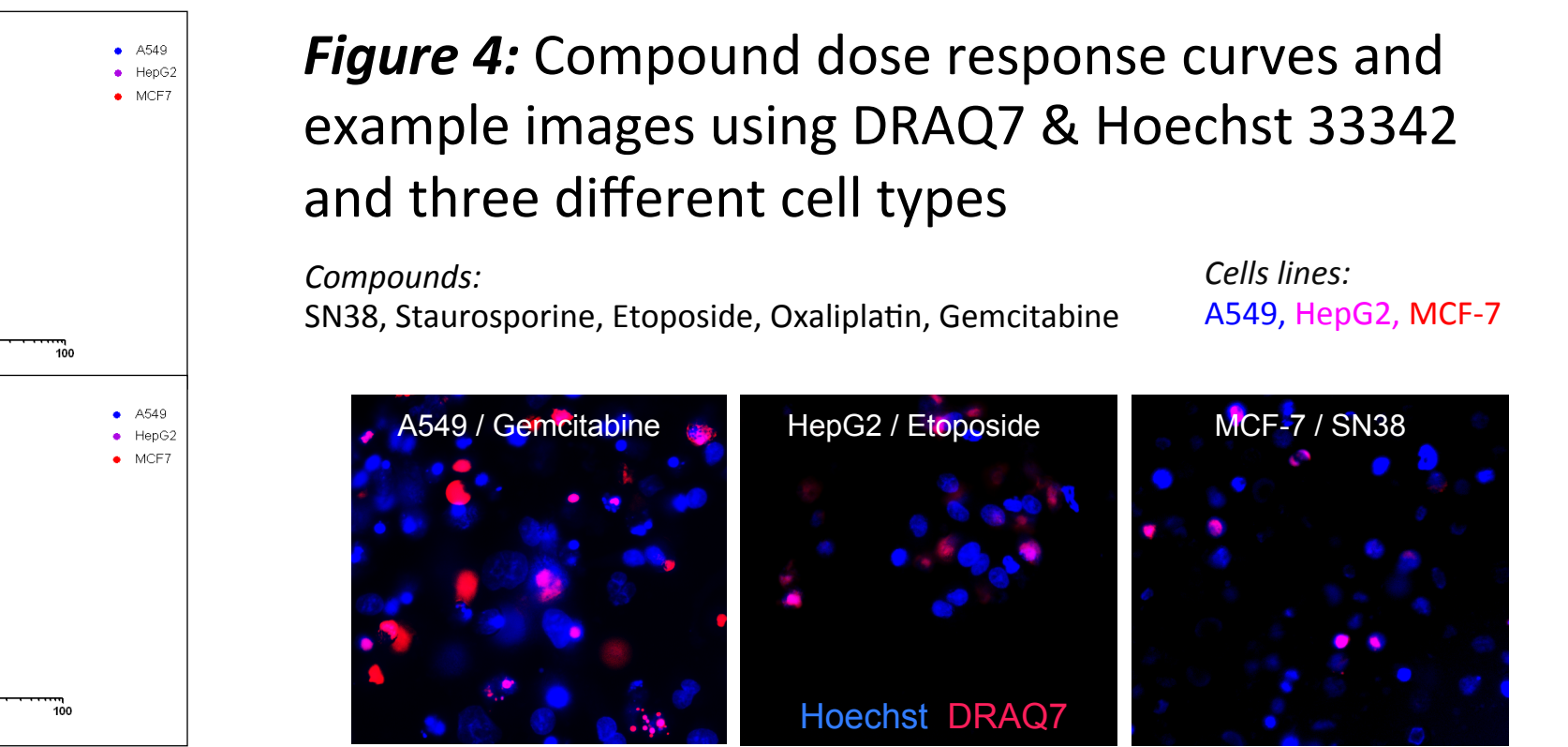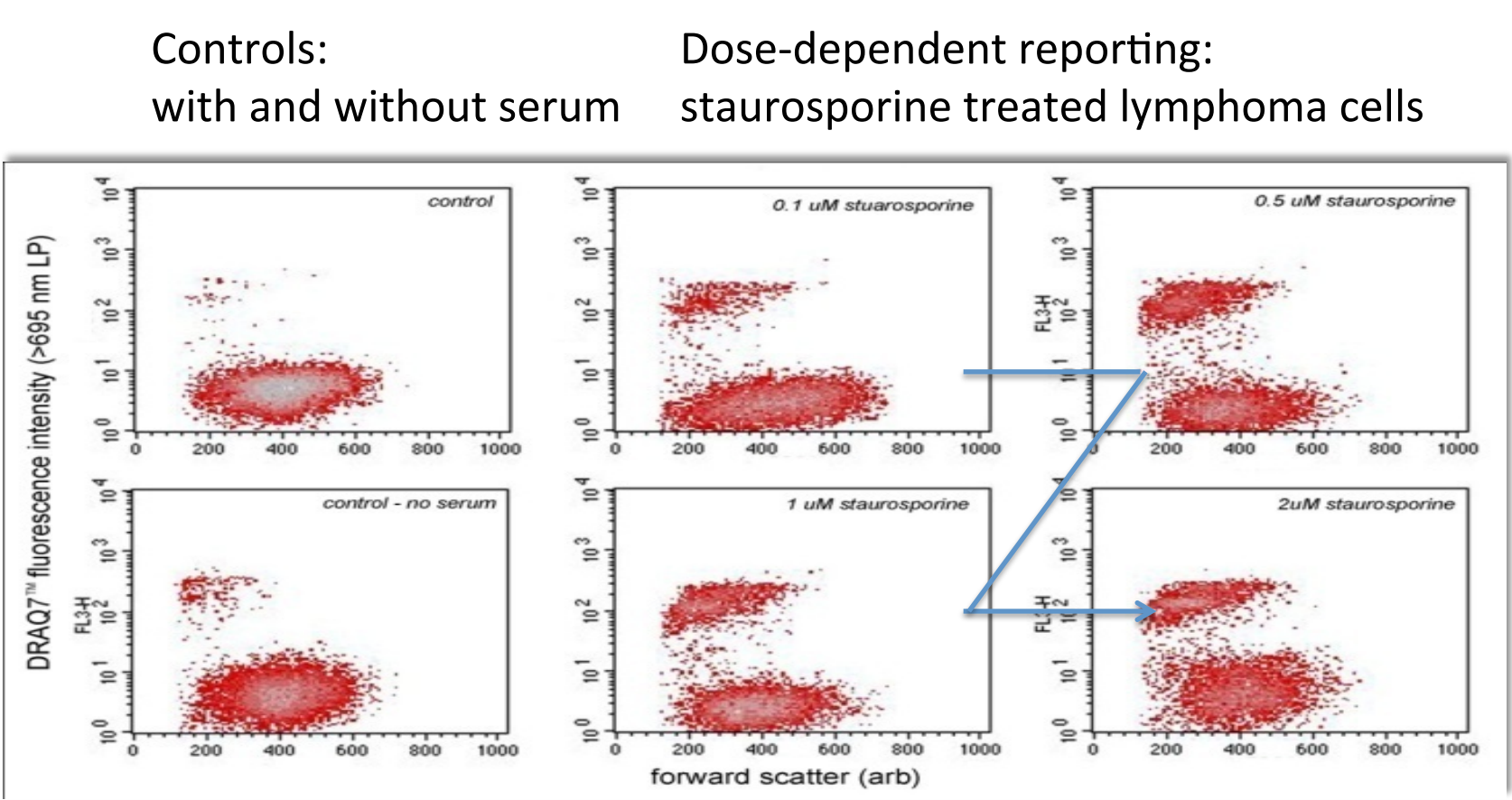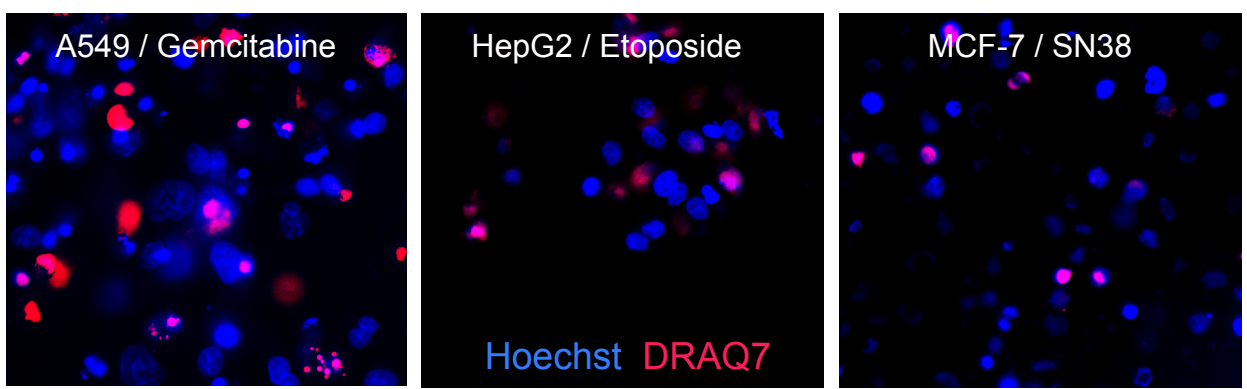Dose-dependent reporting: staurosporine treated lymphoma cells

Figure 4: Compound dose response curves and example images using DRAQ7 & Hoechst 33342 and three different cell types

Compounds:
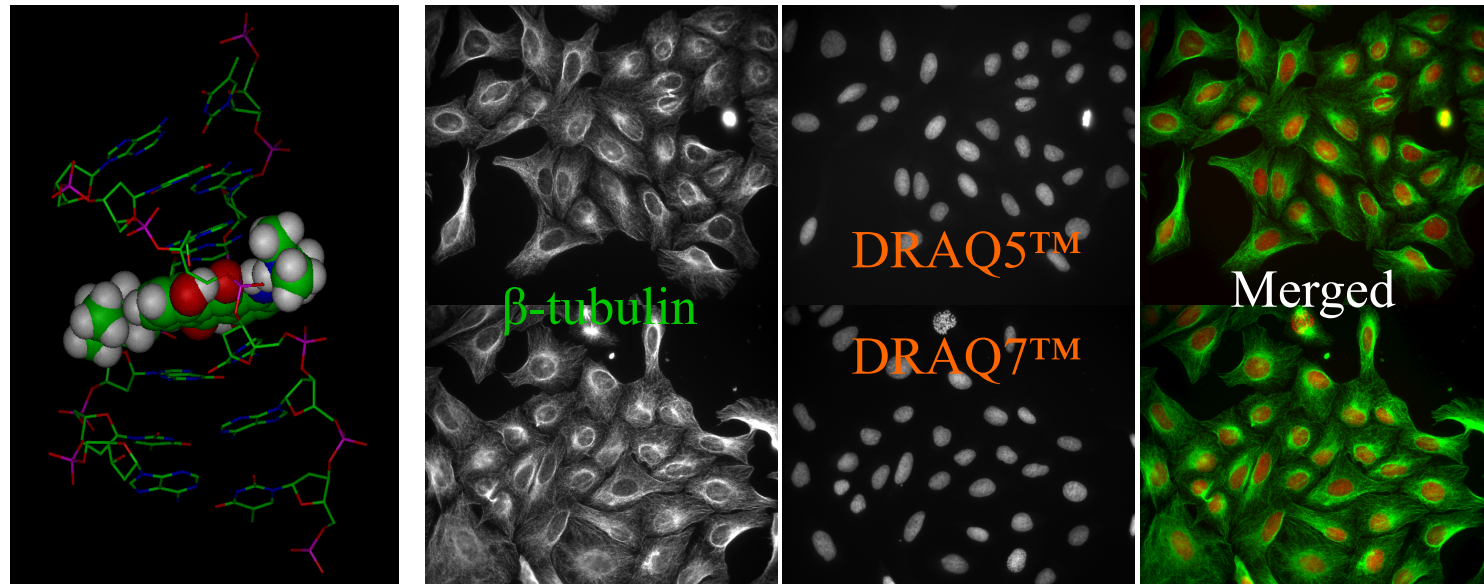SN38, Staurosporine, Etoposide, Oxaliplatin, Gemcitabine

Cells lines:
A549, HepG2, MCF-7

A549 / Gemcitabine   HepG2 / Etoposide   MCF-7 / SN38

Hoechst DRAQ7

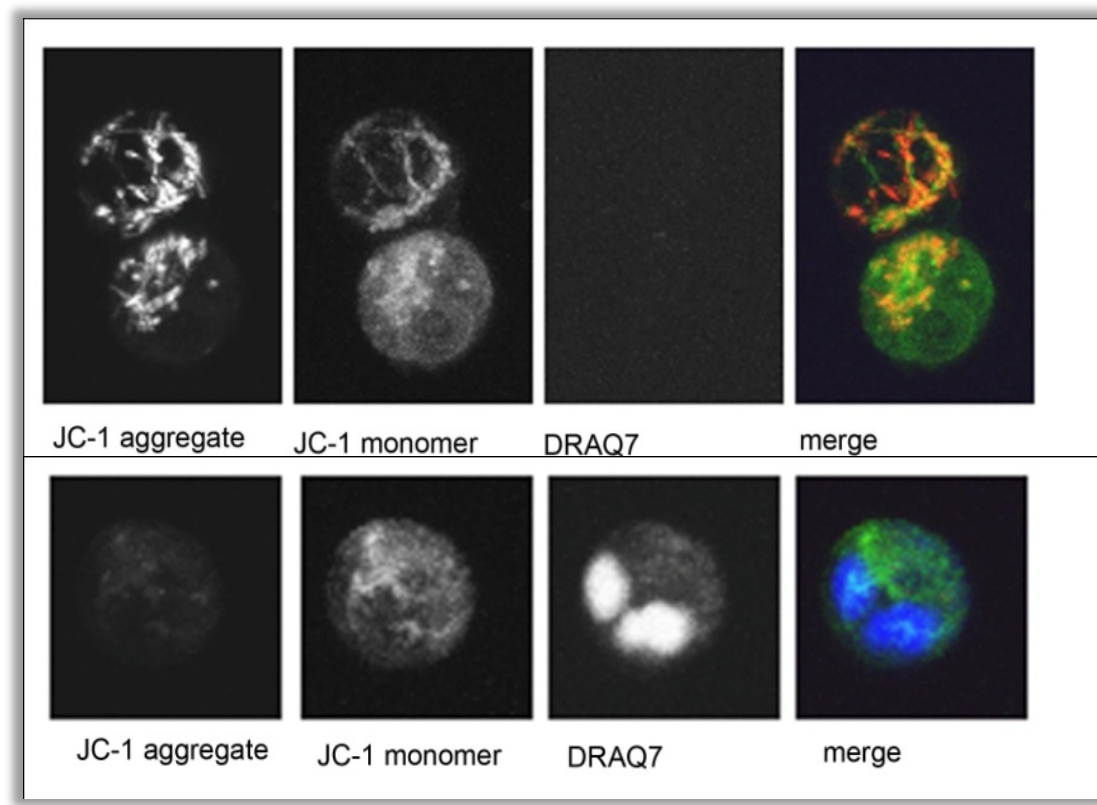## Cell-by-Cell and Spatio-Temporal Reporting

DRAQ7™ utilises the DRAQ chromophore, a DNA intercalator (figure 5a), evidenced by equal performance as a counterstain in fixed cell imaging vs. DRAQ5™ (figure 5b).

Figure 5a (l): Molecular modelling of the DNA intercalation of the parent DRAQ5 chromophore. Figure 5b (r): U2-OS cells fixed & permeabilised for anti- β-tubulin staining (AlexaFluor® 488) and DRAQ5 / DRAQ7 as counterstain (equimolar)

β-tubulin   DRAQ5™ DRAQ7™   Merged

DRAQ7™ reports, cell-by-cell, membrane failure (unlike bulk ATP) and can be combined with other indicators of cell failure e.g. mitochondrial membrane potential (e.g. JC-1 in figure 6). Cells in the upper panel are DRAQ7™-, differing in mitochondrial health (orange vs. green); lower panel shows mitochondrial collapse 9green) and failed membranes – DRAQ7™+ (blue), separating events in both time & space.

JC-1 aggregate   JC-1 monomer   DRAQ7   merge

Figure 6: U2-OS cells were stained with both DRAQ7 (blue) and the mitochondrial potential probe JC-1 (orange - aggregate / green - monomer).
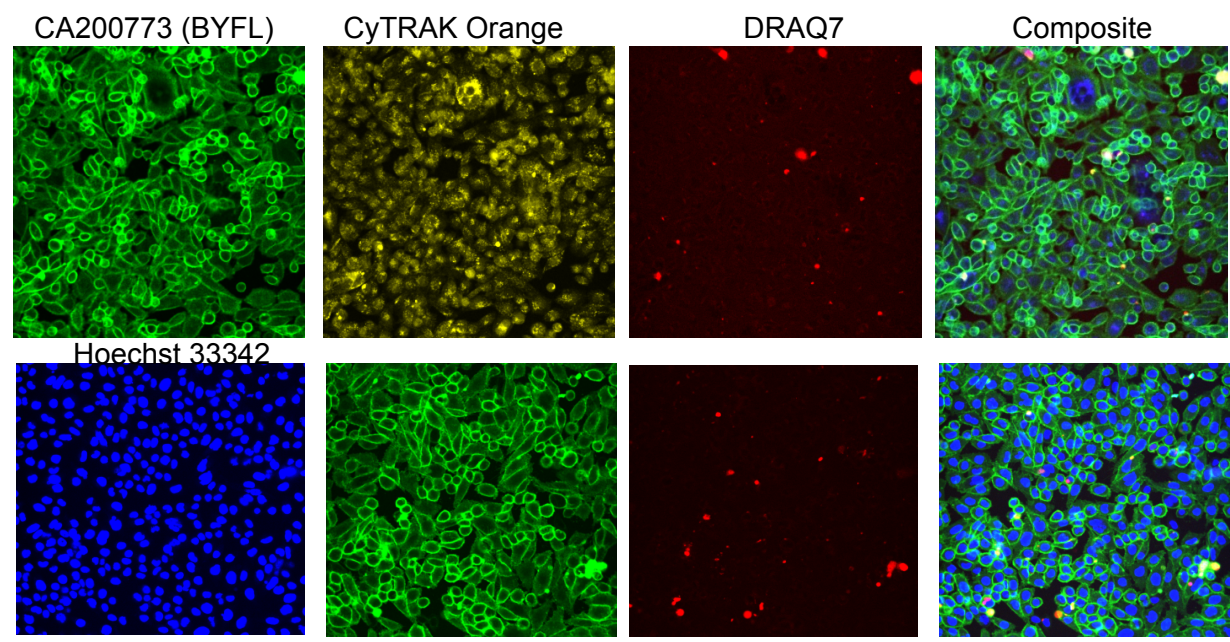
Spectral (and Cross-Platform) Compatibility

DRAQ7™ is optimally red excited (fig.1) emitting in the far-red / NIR (fig. 2). Spectral compatibility with a broad range of vis. range fluors is described in figure 7. Utility in imaging is shown in figure 8, showing DRAQ7™ with supravital stains and fluorescein-ated GPCR ligands.

Figure 7: fluorescence spillover from named chromophores into PMT detectors on a standard 5 colour clinical flow cytometer

Beckman Coulter FC500

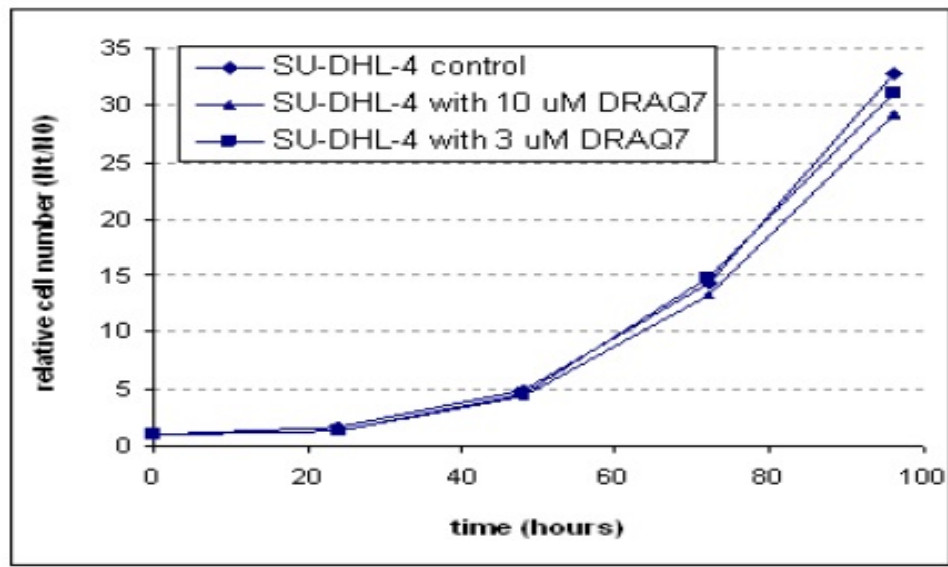| FL	FITC	PE	ECD	PE-Cy5	DRAQ
1 (433V)		2.3	3.8	3.5	0.3
2 (467V)	16.8		12.1	4.2	0.3
3 (556V)	11.5	62.8		3.9	0.3
4 (481V)	2.4	7.5	24.1		10.1
5 (692V)	1.9	3.1	12.8	57.1	

Figure 8: 3-colour assays for live cell imaging of GPCR ligand binding to receptor pos. cells with supravital Hoechst 33342 or CyTRAK Orange (to label all cells) & DRAQ7™ to exclude non-viable cells

CA200773 (BYFL)   CyTRAK Orange   DRAQ7   Composite

Hoechst 33342

Real-Time / Long-Term Cell Health Monitoring

DRAQ7™ at both excess or standard concentration shows no impact on cell growth compared to untreated controls making DRAQ7™ an ideal read-out for cell death in real-time viability and toxicity assays (Figure 9).

Figure 9: SU-DHL-4 cells were cultured in the presence of DRAQ7™ at both the standard concentration or 3.3X excess and untreated controls. Cultures were sampled at intervals over 96 h and analysed by flow cytometry.

3D Imaging of Cell Health (in real-time)

Based on this accumulated data Imagen-Therapeutics have utilised DRAQ7™ to monitor cell viability in real-time 2-D and 3-D spheroid/micro-tissue assays including a study on glioblastoma (GBM)-derived stem Cell lines in response to a library of chemo-therapeutic agents (as shown in figure 10; Thermo Arrayscan; detailed elsewhere).

Control   Staurosporine   Etoposide
Oxaliplatin   Gemcitabine   Taxol

Figure 10: micro-tissues generated from patient-derived GBM stem cell lines were challenged with anti-cancer agents, including examples shown. Micro-tissues were stained with DRAQ7 (red) and subsequently with Hoechst 33342 (blue).

Real-time Cell Health – HCS Case Study

To explore this further a HCS screen was established with Imagen Therapeutics on Nexcelom Biosciences' Celigo S.

Study: test a panel of 56 compounds on MCF-7 cell viability. 500 cells/well were plated in 20 µl DMEM at Day -2 in a 384-well tissue culture plate. Medium was replenished and compounds were added on day 0. Plates were read on the Celigo S using the *Dead + Total Viability* application on Day 1, 2 and 3. Total cells were identified via the brightfield channel and dead cells via the Far-Red channel using DRAQ7™.

Figure 11: Plate map view of 56 test compounds, at 5 concentrations

Figure 12: Plate view heatmap, at 72 h after compound addition. Column 12 of the plate remains negative for cell death, confirming DRAQ7 does not cause cumulative toxicity over time.

BF +ve Control   DRAQ7 +VE Control   BF 50% Dead   BF 90% Dead
BF -VE Control   DRAQ7 -VE Control   DRAQ7 50% Dead   DRAQ7 90% Dead

Figure 13: whole well zoom of +ve and –ve controls using fluorescence and bright field

Figure 14: whole well zoom of test compounds showing representative cell death

24 hours   48 hours   72 hours

Figure 15: Heatmap of 56 cmpnds. measured at 24 h, 48 h & 72 h showing comparative cell death

Study summary: As shown in figures 11-15 the 2-channel Dead + Total application provides for HTS, generating quantitative, reproducible results in under 15 min. in 384-well format, 19 candidate compounds induced ≥ 60% cell death in MCF-7 cells with 2 compounds eliciting effects at 100nM. DRAQ7™ provides a new approach to monitor cell health in real-time culture. Pairing of brightfield imaging and DRAQ7™ is a powerful tool for compound screening, providing analysis of cell death over time.

Importantly, unlike ATP measurement (due to unwanted residual ATP carryover from dead cells) there is no practical upper limit to DRAQ7™ cell death reporting, that is essential in the testing of cancer cell killing.

Discussion / Future Work

Real-time or time-lapse cell health monitoring offers a new paradigm for cell biology and drug development. The performance characteristics of DRAQ7™ make it an ideal choice for this, and importantly it is amenable to automation.

Future investigations may include the testing of DRAQ7™ in more complex tissue models, embryogenesis and potentially *in vivo*.
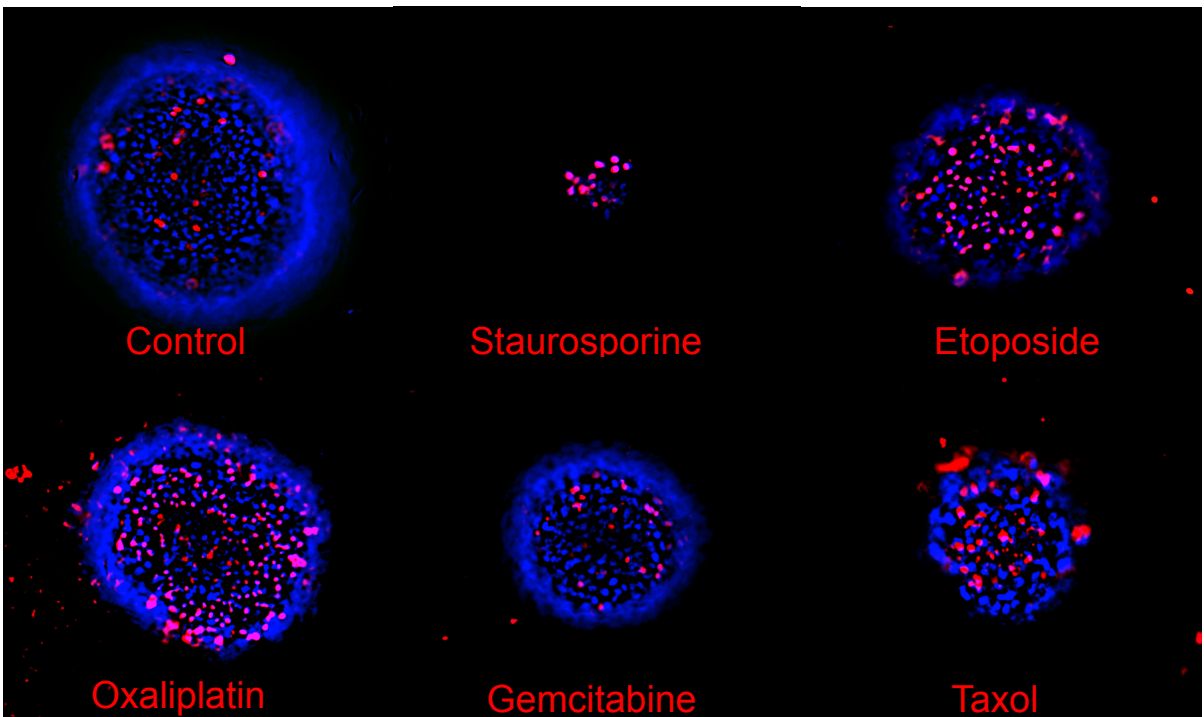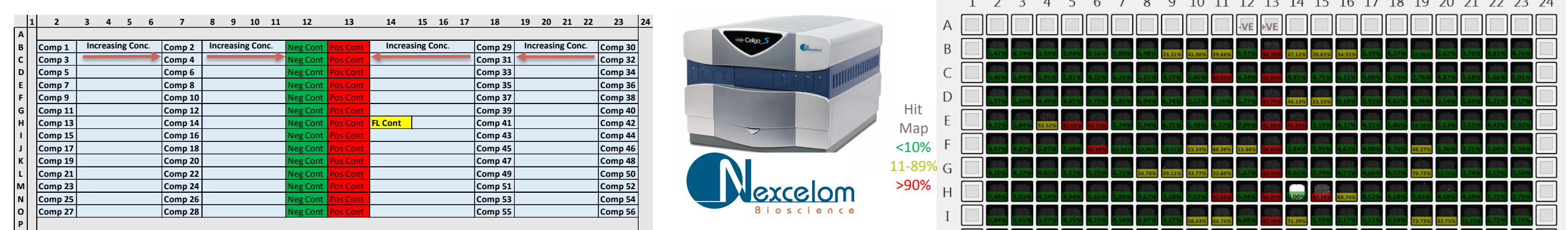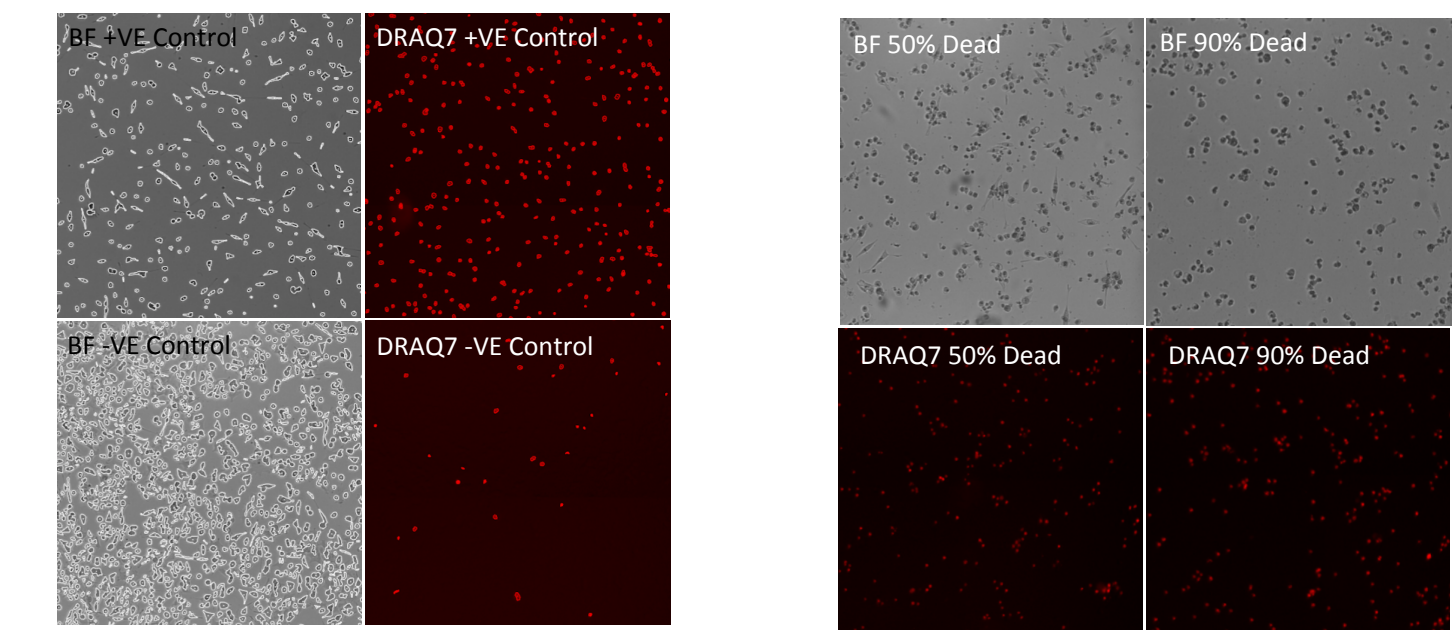
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Data in figs. 4, 10-15 courtesy of Dr Gareth Griffiths