Integrating an automated *in vitro* combination screening platform with live-cell and endpoint phenotypic assays to support the testing of drug combinations

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1. Introduction

To date, *in vitro* drug combination testing in the pharmaceutical industry is typically based around current standards of care, specific hypothesis-driven or opportunistic strategies, that are often initially validated in simplistic assay formats. Such *in vitro* studies have not been supported by the necessary assay development, statistical design/analysis and data storage components required to underpin a robust combination screening platform.

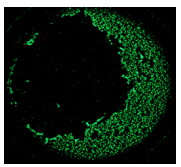
A suite of high content and live-cell imaging assays with associated image analysis algorithms have been developed to mechanistically profile the onset of apoptosis following exposure to a selection of proprietary targeted AstraZeneca compounds, commercially available targeted compounds and clinical standard of care agents for the purpose of supporting drug combinations discovery.

2. Cell death: *In vitro* assay bottle necks

The process of programmed cell death or apoptosis is characterised by distinct morphological changes that include:

- Cell membrane blebbing and loss of asymmetry;
- Loss of cell attachment and cell shrinkage;
- Nuclear fragmentation and chromatin condensation;

Apoptosis involves the activation of energy dependent biochemical mechanisms involving the mitochondria and a family of cysteine proteases commonly known as caspases, one of the main executors of the apoptotic process.



Cell loss is the hallmark of cell death and the major bottleneck in developing an assay that retains cells undergoing apoptosis.

Assay bottle necks:

- Lost information due to cell loss;
- Data inconsistency & irreproducibility;
- Lack of mechanistic apoptotic biochemical markers for kinetic imaging;
- Necessary throughput to enable large scale testing of potential combinations;

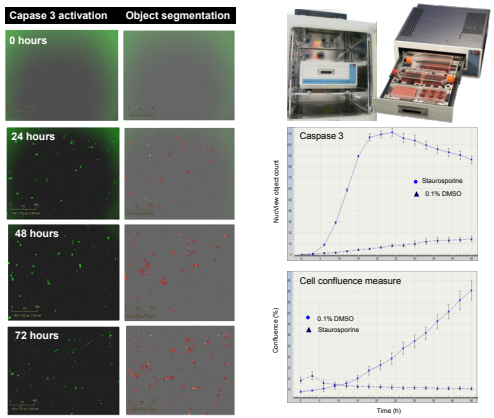
Traditional drug discovery strategies typically employ simplistic assays that monitor the activity of a single target or enzymatic pathway. While these approaches are amenable to high-throughput screening, they provide limited information on how therapeutics influence complex biological systems. Such limitations are a contributing factor to high attrition rates at later stages in the drug discovery process.

3. Automated live-cell imaging phenotypic assays: *Kinetic and end point apoptosis readouts*

We have designed kinetic and endpoint apoptosis cell based high-content assays with associated image analysis algorithms to generate as much mechanistic information as possible on how candidate therapeutics influence cellular phenotypes. We have employed a whole well imaging based approach in capturing apoptotic cells in real-time to support compound profiling.

1. NucView Assay: *Kinetic fluorescence imaging*

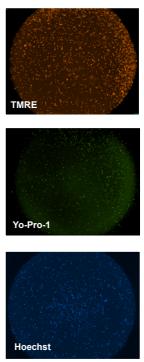
NucView™ (*developed by Biotium*) is a bi-functional fluorogenic enzyme substrate that detects Capase 3 activity in living cells. We have implemented the NucView capase detection system to measure apoptosis in real-time using the FLR IncuCyte™ (*developed by Essens Instruments*).



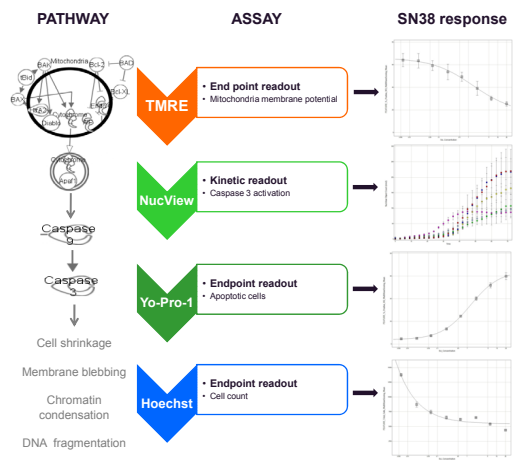
2. Endpoint assay: *Multiplexing TMRE, Yo-Pro-1 and Hoechst*

We have multiplexed biochemical markers in a live-cell assay to capture early and late stage apoptotic events . We have employed a cocktail of cell stains that include Tetramethylrhodamine, ethyl ester (TMRE), Yo-Pro-1 and Hoechst to capture endpoint information on cell health and organelle function.

- TMRE is a red- orange fluorescent dye that is sequestered by active mitochondria.
- Yo-Pro-1 is a carbocyanine nucleic acid stain that fluoresces green in apoptotic cells.
- Hoechst (a nucleic acid stain) is commonly used to stain for the cell nucleus.



Fluorescent imaging technologies such as the ImageXpress 5000 offer the potential to leverage mechanistic information from simplistic and complex biological models.



The Nucview and endpoint apoptosis assays complement each other by providing mechanistic information detailing the stages of cell death that a cell undergoes. Mitochondria health and Capase 3 readouts together with plasma membrane integrity and cell number parameters provide the robust capture of the sequence of events contributing to apoptosis.

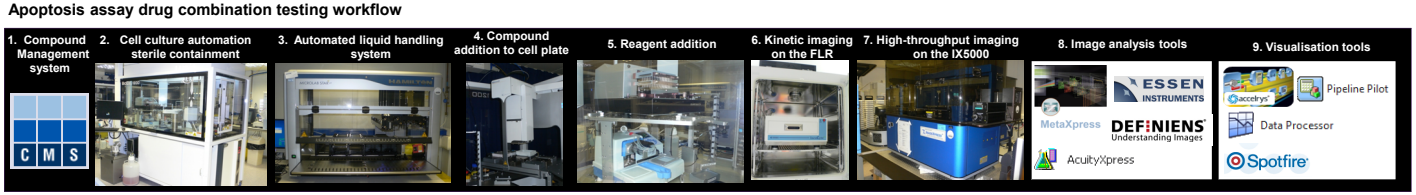
4. Automating drug combinations testing

Systematically testing drug combinations in a semi-hypothesis and hypothesis-free manner presents a number of challenges, particularly when considering sequential dosing and beyond pairwise combinations. The ASTL are currently engaged in the early stage development of an integrated combinations drug testing platform.

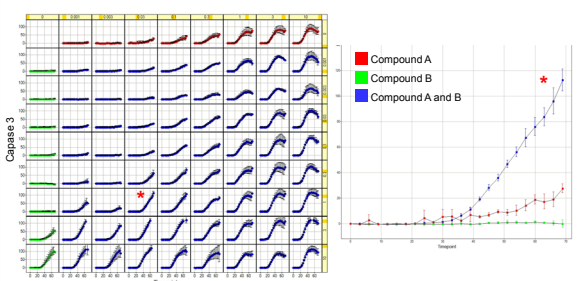
Compound testing workflow

1. Compound plates are designed with statistical support;
2. Our internal compound management system delivers solubilised compounds which are tracked by barcodes.
3. A drug combinations work-list is exported into the Hamilton software and used to generate unbiased randomised combinations (to eliminate plate effects).
4. Cell plating, compound addition and assay reagent addition is fully automated on Agilent Biocel 1200.
5. Our advanced automated microscopy facilities are equipped for imaging 2D and 3D complex *in vitro* models with customised image-based algorithms capable of capturing informative phenotypic changes.
6. Custom developed software and commercially available data processing and visualisation tools assist in highlighting novel compound pathways and relationships.

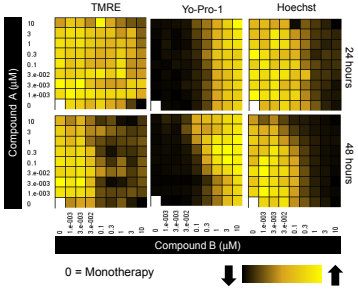
Apoptosis assay drug combination testing workflow



A. NucView assay: kinetic combination plot



B. Endpoint assay: combination readouts



Summary

We have developed phenotypic cell death assays that monitor both early (pathway driven events) and late stage apoptotic events (driven by morphological changes) *in vitro*. The assays are supporting the identification of rationale drug combinations in addition to semi-hypothesis or hypothesis-free screening.

We have specifically used biochemical markers to monitor mitochondria health and caspase 3 activation in order to understand those cells driven to self destruct with novel therapeutic targets as a monotherapy or in combination. These early mechanistic pathway events are crucial in identifying novel selective agents targeting malignant neoplasm for personalised healthcare.
Furthermore, Yo-Pro-1 and Hoechst biochemical markers provide further information on late stage apoptotic events associated with lost cell membrane integrity and eventually cell loss. The assays are able to provide a robust mechanistic assessment of cell death in both simplistic and complex *in vitro* cell based models.

Conclusion

- We have aimed to develop an automated infrastructure to predict which combinations of drug targets will be most efficacious in complex multigenic disease such as cancer and which patients will respond optimally to a selection of such therapeutics.
- We have developed two cell death assays for the purpose of capturing real time pathway information and endpoint cell health readouts.
- The ASTL have developed an automated HTS combinations testing platform utilising advanced imaging instruments, image intelligence analysis and sophisticated statically driven visualisation tools.
- The apoptosis assays have supported the combination testing and the prediction of targets that have showed early synergy in different models of cancer.

Future work

The ASTL are currently engaged in incorporating an automated statistical tool to calculate combination index to identify synergy, antagonism or additive effects. We also look to predict novel targets in complex multicellular disease models such as angiogenesis (see poster D404).

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