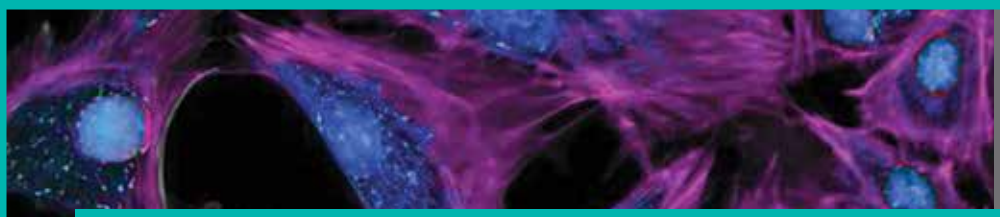

acumen® CELLISTA
application note

Eliminating false-negative hits in ATP-luminescence viability screens by use of an alternative phenotypic approach with acumen® Cellista

introduction

Cell viability and proliferation assays are a fundamental tool in the drug discovery process. They are used to evaluate both the potency of compounds, as well as their toxicity profiles for drug safety assessments. Many commonly used plate reader assays (e.g. ATP-luminescence measurements) assume a linear relationship between the assay signal, the number of cells and their viability. However, as this application note illustrates, this assumption is not always justified: ATP-luminescence measurements can significantly overestimate toxicity and underestimate potency, leading to false-negative viability/efficacy hits.

Here we show the development and implementation of an alternative no-wash high content (HCA) assay using acumen, which eliminates common problems associated with ATP-luminescence measurements. We compare the drug-responses of several well-characterised anti-cancer drugs on HeLa cells, measured by acumen and a commercial ATP-luminescence detection system, and highlight key differences between the measurements.

materials and methods

cell culture and drug addition

All plates are prepared as duplicates, one for imaging on the acumen Cellista and the other for ATP-luminescence determination.

day 1

Seed 500 HeLa cells in 25 µL of medium into the wells of a 384-well plate and culture overnight.

day 2

Prepare 2-fold serial dilutions of drugs in medium (2x concentrated), add 25 µL to each well and incubate for 48 hours.

day 4

HCA assay: add 5 µL of stain "master mix" in PBS to each well, to give final dye concentrations of 10 µM Hoechst 33342, 1 µM calcein-AM and 1.5 µM propidium iodide. Incubate the plate for 90 minutes at room temperature and then image on the acumen Cellista to determine the cell number, % live cells and cell cycle phase distribution for each well.

ATP-luminescence assay: add 40 µL of CellTiter-Glo reagent (Promega) to each well, agitate the plate for 60 minutes at room temperature and then read the luminescence signal on a multimode plate reader.

data analysis

Average and normalise the well readouts for each drug concentration to the control wells. Plot drug-response curves of the normalised values and fit to sigmoidal curves to derive EC_{50} values.

cost

reagent	unit price	unit size & stock concentration	cost per well
calcein-AM	£223	1 mL, 1 mM	1.12p
proidium iodide	£85	10 mL, 1.5 mM	1.12p
Hoechst33342	£82	5 mL, 20 mM	0.04p
CellTiter-Glo	£56	10 mL (sufficient for 1 x 384-well plate)	14.58p

results

phenotypic assay validation

% live determination

Control or doxorubicin-treated cells were classified as either live or dead based on the ratio of the green (calcein-AM, FL2) to the red (propidium iodide, FL3) fluorescence intensity per cell. The percentage of live cells was calculated from the total number of live and dead cells.

cell cycle determination

acumen cell cycle classification gates were derived from the total FL1 fluorescence intensity histograms of untreated cells, and of cells treated with 3 µM etoposide for 48h (Figure 1). The predominant cell cycle response for each drug is summarised in Table 2.

key benefits

TTP Labtech's acumen facilitates replacement of ATP-luminescence based cell health assays, resulting in better quality data with cheaper reagent costs:

- **multiplexed readouts including cell number, % live cells, cell cycle phase**
- **ready distinction between cytostatic and cytotoxic mechanism of action for higher quality hits**
- **simple, homogenous assay protocol @ 2.28 pence/well (acumen) versus 14.6 pence/well (CellTiter-Glo)**
- **compatible with high density 384-well plates, scanned in 8 minutes**
- **applicable to 1536-well plates for even higher throughputs**

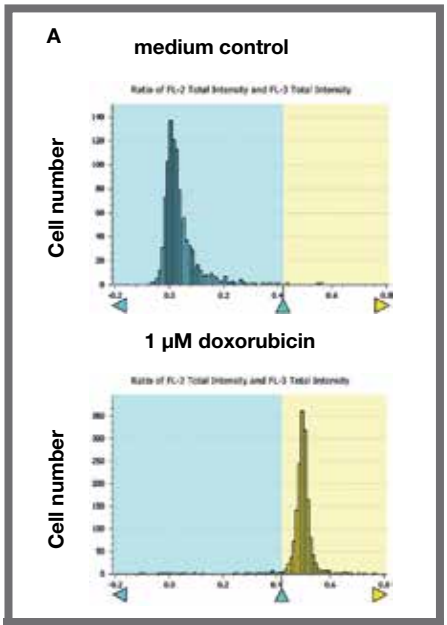

Fig 1. acumen ratio of FL2/FL3 intensity histograms used for live/dead cell classification

ttplabtech

drug	log EC_{50}			cell cycle response
	cell count	% live cells	CTG	
staurospine	-9.1	-6.7	-8.7 (monotonic) -6.8 (bipfasic)	G2/M, >4N
doxorubicin	-6.4	-6.3	-6.8	G1
etoposide	-5.9	-4	-4.8	G2/M
cycloheximide	-6.6	N.D.	-6.5	G1

Table 1. Summary of Log EC_{50} values

comparison of drug-responses across different assay formats

The drug-response curves (Figure 3) and EC_{50} values (Table 1) show that the agreement between the acumen Cellista cell number and % live cell readings, and the ATP-luminescence readings varies widely between the compounds.

For **doxorubicin**, a classic cytotoxic agent, there is very close agreement between all three readouts.

For **etoposide** and **cycloheximide** there is less agreement, with the EC_{50} values for the acumen % live cell readout differing from the cell count by more than 1 log unit. The discrepancy between the two acumen readouts immediately highlights the *cytostatic* potency of these compounds and the cell cycle profiles confirm that etoposide is indeed a cell cycle inhibitor, whereas cycloheximide (a protein synthesis inhibitor) by elimination must work via a different mechanism of action. Furthermore, the acumen Cellista Tiff images show an unusual enlarged morphological phenotype for the etoposide-treated cells. These conclusions cannot be drawn from the ATP-luminescence measurements: The cycloheximide drug-response curves mirror the acumen cell count, which taken in isolation would falsely suggest a highly toxic compound. The etoposide drug-response curve falls in between the acumen cell count and % live readouts, probably because the antiproliferative action of the drug is accompanied by an increase in average cell size (and mitochondrial mass). With etoposide, the ATP-luminescence measurements would also overestimate the toxicity of the compound.

With **staurosporine**, the acumen readouts show clear *monotonic* drug-response curves, with a cytostatic mechanism of action at lower drug concentrations, which becomes cytotoxic only at higher drug concentrations. The ATP measurements are more complicated, with a *bi-phasic* dose response showing the composite effect of cell health and number. In this example the *bi-phasic* nature of the dose response curve hints at different mechanisms of action operating at different drug concentrations, however, the effect is quite subtle and could easily be missed. As with etoposide and cycloheximide, staurosporine would be appear much more toxic at lower concentrations than it actually is.

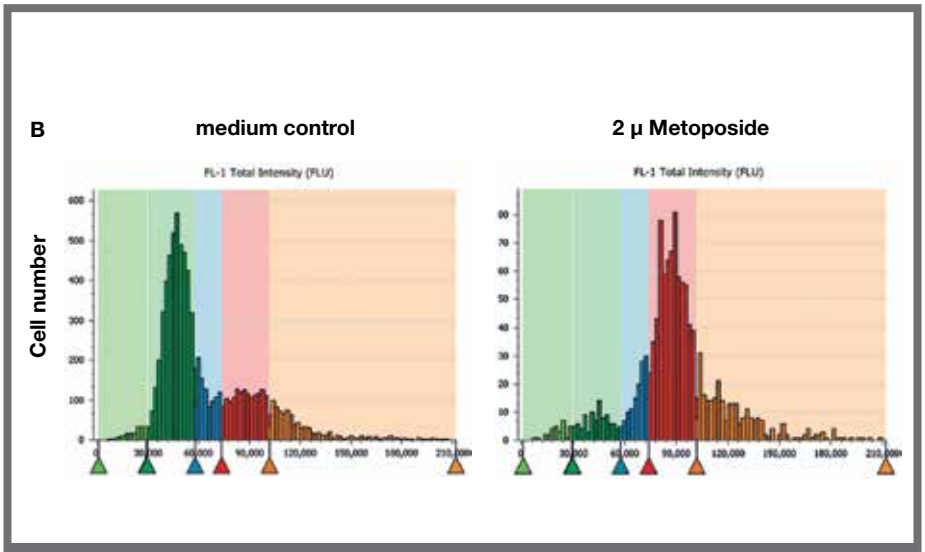

Fig 2. acumen cell cycle profiles

about acumen

TTP Labtech's acumen is a laser scanning imaging cytometer designed to provide single-shot, whole well, content-rich cytometric and image-based analysis. Its F-theta lens gives a uniform illumination across the field of view with a large focussed depth of field, which enables high throughput, whole well image acquisition across a range of plate types. acumen enables a wide range of fluorescent reagents to be combined in multicolour, multiplexed assays. Its easy-to-use, template-driven software offers an industry-proven route for quick adoption across a wide range of applications.

summary

- acumen Cellista provides content rich cell viability data in a simple and homogenous assay format that utilises cost-effective reagents.
- Whereas ATP measurements alone can significantly overestimate the toxicity of cytostatic drugs, acumen Cellista viability assays can readily distinguish between cytostatic and cytotoxic mechanisms of action.
- Cellular DNA content measurements by acumen Cellista can easily identify drugs affecting the cell cycle, whilst the Tiff export function can highlight morphological changes of the cells.
- acumen Cellista assays are amenable to miniaturisation and automation, offering improved process efficiencies.

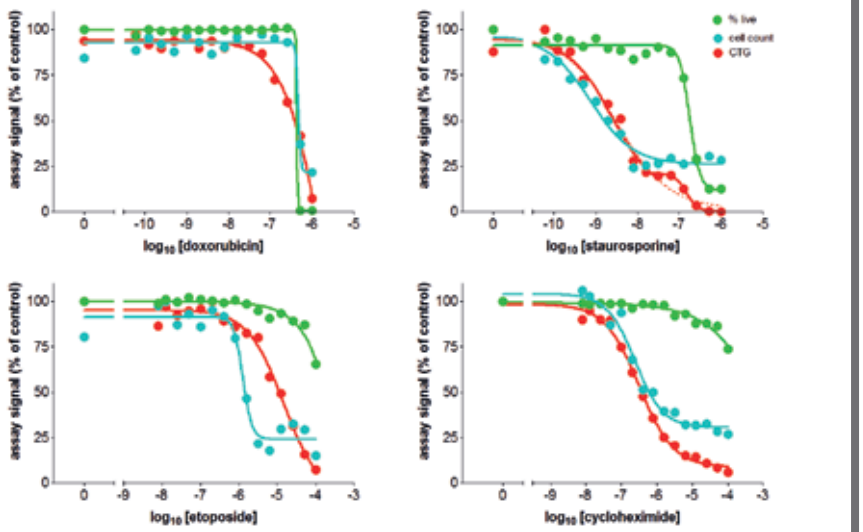

Fig 3. Normalised drug-response curves

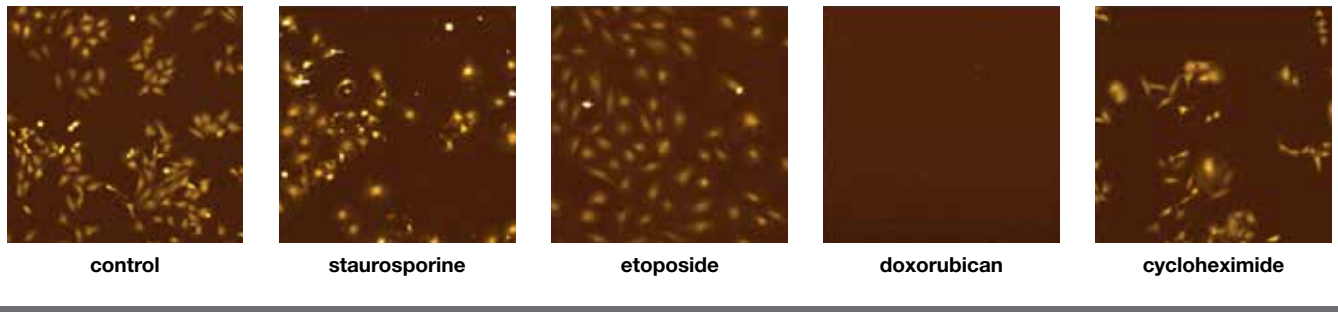

Fig 4. Representative Tiff images of drug treated cells stained with calcein-AM and propidium iodide. The Hoechst signal is not shown.

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