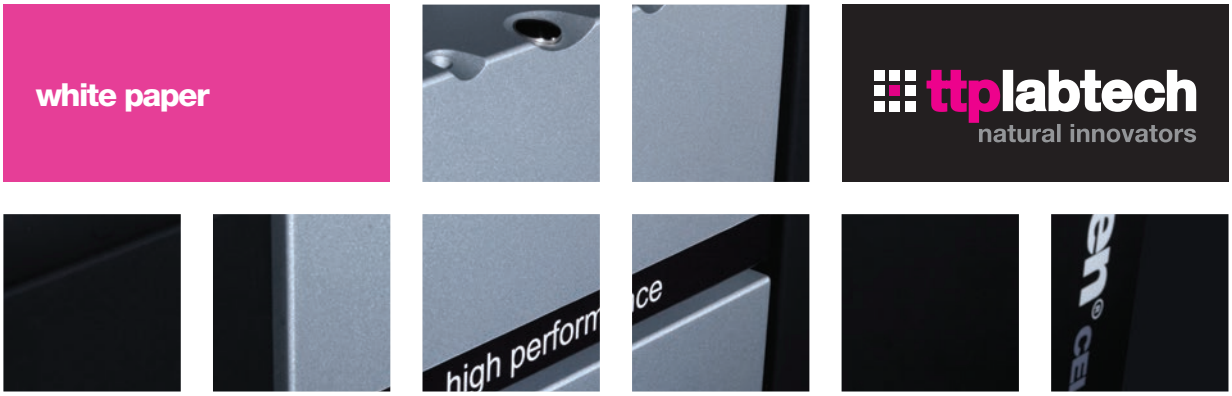
white paper



Accurate cytotoxicity and proliferation determination: advantages of a high-throughput phenotypic approach over ATP-luminescence assays

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Introduction

Cell viability and proliferation assays are a fundamental tool in the drug discovery process and are used to evaluate both the antiproliferative potency and the toxicity of compounds [1]. Some lead discovery groups generate cell viability data for up to 2 million compounds per screen [2] so any method used to assess these parameters needs to deliver not only on data quality, but also on throughput and assay cost per well. Most methods used to determine cell viability cannot deliver on all three of these requirements, so compromises have to be made.

High content analysis (HCA) can be used for direct cell enumeration by counting the number of cells or nuclei per well. Additionally, by multiplexing different fluorescent readouts in the same well more complex drug-response profiles can be obtained, for example the numbers of live or dead cells per well, changes in DNA content or cell morphology [3]. It is the multi-parametric nature that underpins the true power of this approach, which allows ready distinction between cytostatic and cytotoxic mechanisms of action, and further pathway sub-classification into cell cycle arrest. Understanding mechanisms of action is critical for optimising drug candidates, since off-target cytotoxicity is a frequent problem with many compounds. Despite their recognised utility, HCA assays have not been widely adopted in high-throughput screening (HTS), as they were traditionally complex to set up, required highly specialised instrumentation and operators, and were ultimately too slow to run in high-throughput environments.

By contrast, many biochemical viability assays involve a simple homogenous assay setup that lends itself to miniaturisation and automation, thus enabling very high-throughputs.

However, such assays only give indirect measurements of cell number and viability by determining the metabolic activity of all cells in the well with ATP-luminescence systems [4], or with MTS reagents that measure dehydrogenase activity [5]. A linear relationship between the assay signal, the number of cells and their viability is assumed, Chan *et al.* have shown that this assumption is not always justified: Many well-known cancer drugs lead to cell cycle arrest long before cytotoxic effects become apparent and ATP-luminescence measurements cannot distinguish between increases in average cell size and genuine cell proliferation [1]. Reliance on ATP measurements alone can therefore lead to significant underestimation of drug potency and overestimation of toxicity.

Here we show the development and implementation of a cost-effective, no-wash HCA assay to simultaneously report the cell number, percentage of live cells and cell cycle phase distribution as markers of proliferation and viability. We demonstrate that this assay can be applied to high density plate formats, and be imaged and analysed in only 8 minutes per plate using the acumen Cellista laser scanning imaging cytometer from TTP Labtech. By comparing the drug-responses of several well-characterised anti-cancer drugs on HeLa cells, we highlight the key differences between a phenotypic assay enabled by acumen, and a biochemical ATP-luminescence approach.

Materials and methods

1. cell culture and drug addition

All plates were prepared in duplicate, one for HCA and the other for ATP-luminescence determination.

For the cell number validation experiments, HeLa cells were grown in T75 flasks to approximately 80% confluency, lifted off the plate using trypsin and the cell suspension was diluted with complete cell culture medium. 0, 125, 250, 500 or 1000 cells were seeded into the wells of a 384-well plate (Corning #3712, n=9 replicate wells) in 50 µL of medium, and analysed by HCA assay or ATP-luminescence determination immediately.

For the drug-response experiments, a suspension of HeLa cells (500 cells in 25 µL in complete cell culture medium) was added to the wells of a 384-well plate (Corning #3712) and cultured at 37ºC/5% CO_2 in a humidified incubator overnight to allow the cells to adhere. All compounds were prepared as two-fold serial dilutions in complete cell culture medium (2x concentrated). 24 hours after seeding, 25 µL of compound was added to each well and the plate was cultured for a further 48 hours.

2. high content analysis on the acumen Cellista laser scanning imaging cytometer

5 µL of a staining "master mix" containing 100 µM Hoechst 33342 (Thermo Fisher Scientific #H3570), 10 µM calcein-AM (Thermo Fisher Scientific #C3099) and 15 µM propidium iodide (Thermo Fisher Scientific #P3566) in PBS was added to each well of the assay plate and incubated for 90 minutes at room temperature. The plate was then transferred to the acumen Cellista laser scanning imaging cytometer for imaging. The scan and analysis time on the acumen Cellista was 8 minutes per plate.

For the cell cycle analysis, single nuclei were identified based on their size and roundness (Gaussian fit). The cell cycle phase was then determined from the total DNA content, which is proportional to total FL-1 (blue fluorescence intensity). The Cellista software reports the total cell number, as well as the percentage of cells in each phase of the cell cycle for each well.

For the cell viability determination, single red or green fluorescent cells were identified based on their size. For each cell, the ratio of the calcein-AM (FL-2-green) to propidium iodide (FL-3-red) total intensity was taken and used to classify the cell as either live or dead. From this classification, the percentage of live cells was determined.

3. ATP quantification using the Promega CellTiter-Glo® (CTG) kit

The CTG reagent (Promega #G7570) was reconstituted in the supplied reagent buffer according to the manufacturer's instructions. Promega recommend adding 25 µL of the CTG reagent to 25 µL of cells in cell culture medium, however, such a low volume of media would have been incompatible with a 72 hour cell incubation period. In order to avoid removing dead/dying cells from the well by buffer exchange, the cells remained in 50 µL of culture medium and the amount of CTG reagent was increased to 40 µL per well. After addition of the CTG reagent, the plate was gently rocked for 10 minutes to mix the reagents and to allow cell lysis to proceed. The luminescence signal was measured after 1 hour on a Tecan plate reader, using auto exposure and gain settings.

4. data Analysis

For each assay parameter (percentage live cells, cell count, luminescence intensity) the well reading was normalised to the medium control wells. All data were plotted in GraphPad Prism 3.0 and fitted to sigmoidal concentration-response curves, from which EC_{50} values were derived.

5. costs

reagent	unit price	unit size and stock concentration	cost per 384 well
calcein-AM	£223	1 mL, 1mM	1.115p
propidium iodide	£85	10 mL, 1.5 mM	0.04p
Hoechst 33342	£82	5 mL, 20 mM	0.04p
CellTiter-Glo®	£56	10 mL (sufficient for 1 x 384-well plate)	14.58p

Table 1: cost comparison between the acumen Cellista HCA assay dyes and an ATP-luminescence system

Results

assay validation

1. cell count and ATP content determination

In order to establish if a linear relationship exists between the number of healthy cells dispensed per well, the number of nuclei counted on acumen and the ATP-luminescence signal, HeLa cells were seeded into the wells of a 384-well plate at densities between 0-1000 cells per well and stained/analysed with either Hoechst 33342 or CellTiter-Glo® immediately. Figure 1 shows that there is an excellent correlation between the acumen cell count, the magnitude of the ATP-luminescence signal and the number of cells seeded. These data demonstrate that the two methods generate equivalent assay readouts for healthy HeLa cells.

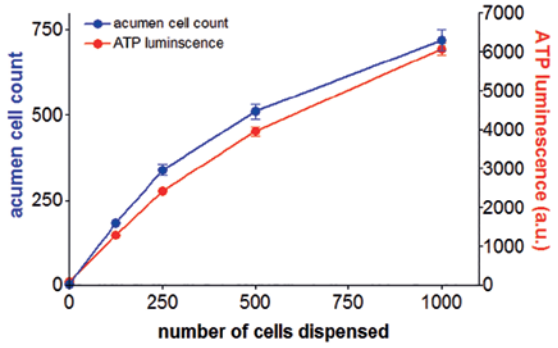

Fig 1: the relationship between the number of cells seeded per well, the acumen cell count and the ATP-luminescence signal

2. acumen percentage live readout

The cells for this assay were treated either with 1 µM doxorubicin (a well-known cytotoxic agent) or cell culture medium (control) for 48 hours, and then stained with two complimentary dyes: calcein-AM as a metabolic viability stain and propidium iodide as a dead cell stain. Figure 2 shows the "intensity ratio" histograms of doxorubicin-treated cells and control cells, which display two distinct populations from which the live/dead gates were set. The number of cells in each population were counted and from this the percentage of live cells was calculated.

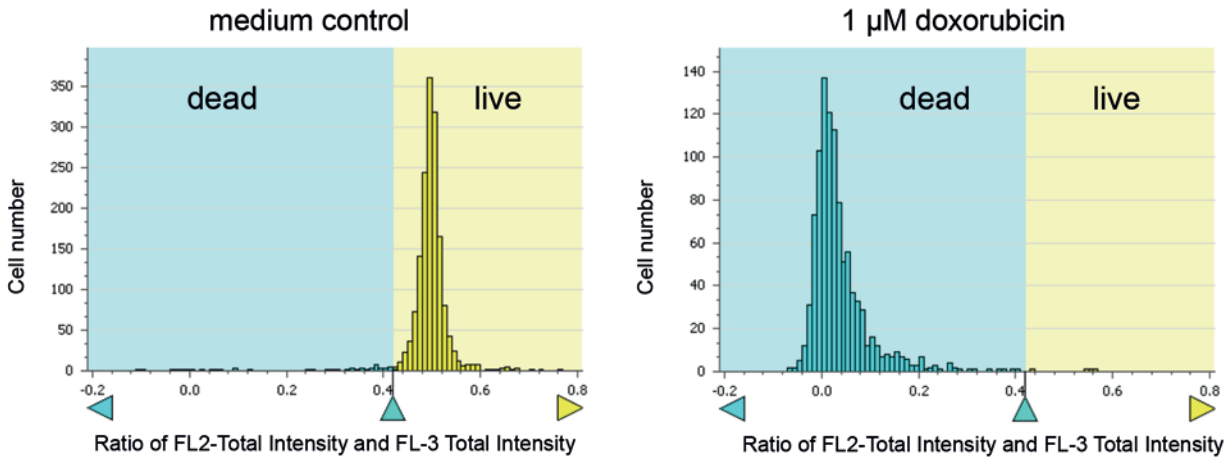

Fig 2: acumen live/dead classification of cells stained with calcein-AM and propidium iodide

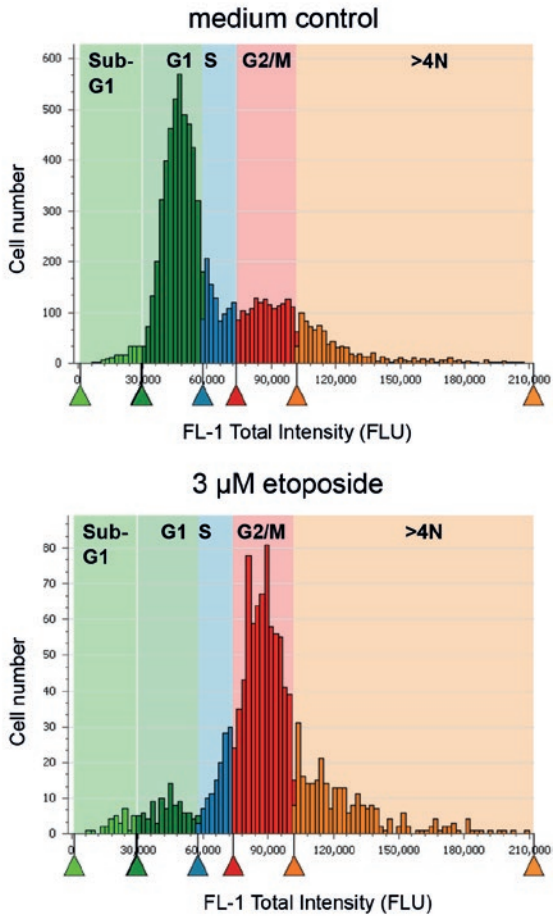

Fig 3: acumen cell cycle phase classification

3. acumen cell cycle phase determination

The DNA content of cells stained with Hoechst 33342 is proportional to their total FL1 fluorescence intensity. Figure 3 shows histogram plots of total FL1 fluorescence intensity versus cell number for untreated cells, and cells treated with 3 µM etoposide for 48 hours. Etoposide is a well known anti-cancer drug that causes cell cycle arrest in the G2/M phase, whereas untreated cells are expected to be found mostly in the G1 phase. Figure 3 shows the cell cycle classification gates derived from these histograms, which match the expected distribution of phases.

4. comparison of drug-responses across different assay formats

Replicate assay plates were treated with serial dilutions of different drugs for 48 hours and analysed by either high content analysis on the acumen Cellista or by ATP-luminescence determination. The results were averaged and normalised to the control wells to facilitate comparison across the different assay formats. Concentration-response curves of the normalised values were plotted and fitted to sigmoidal curves (Fig 4-7), from which EC_{50} values (Table 2) were derived. In addition, the cell cycle phase distribution for each drug concentration was determined (Fig 5-7), and representative TIFF images of the well (Fig 8) were obtained to highlight any morphological changes. Together, these data show that differences between the acumen Cellista and ATP-luminescence readings are due to compound mechanism of action.

With **doxorubicin** (Fig 4), a classic cytotoxic agent that causes cell death, the concentration-response curves show a steep decline for all three readouts in the high nanomolar concentration range. This result demonstrates that for this particular drug the acumen high content assay and the ATP-luminescence assay are equally suitable methods for cell health determination.

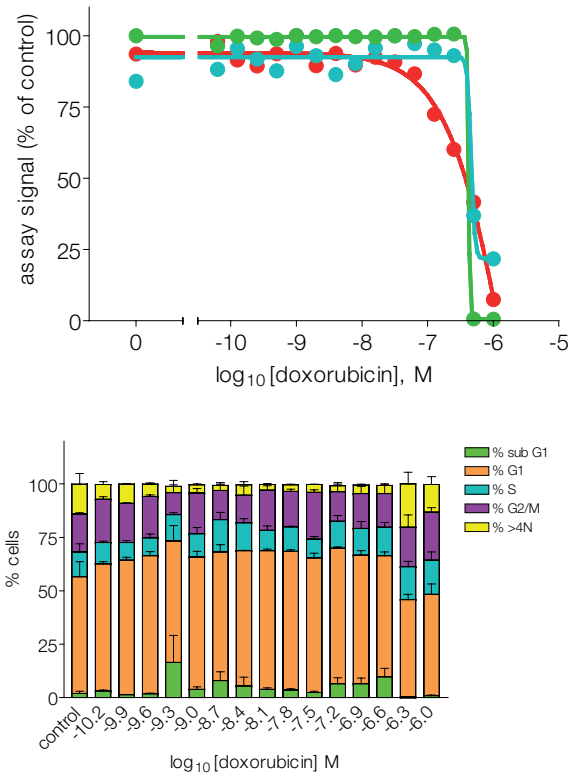

Fig 4: concentration-response curves and cell cycle classification chart of HeLa cells treated with doxorubicin

With **cycloheximide** and **etoposide**, both cytostatic agents but with different mechanisms of action, there is less agreement between the three measurements (Fig 5 & 6):

The ATP-luminescence measurements for **cycloheximide** (Fig 5) would suggest a compound that is active in the micromolar concentration range, however, the acumen readouts give a more accurate picture: While cycloheximide stops cell proliferation in the micromolar concentration range, it only affects cell health in the millimolar range. acumen Cellista DNA content measurements confirm that the antiproliferative action of cycloheximide (a protein synthesis inhibitor) is not due to cell cycle arrest, as expected.

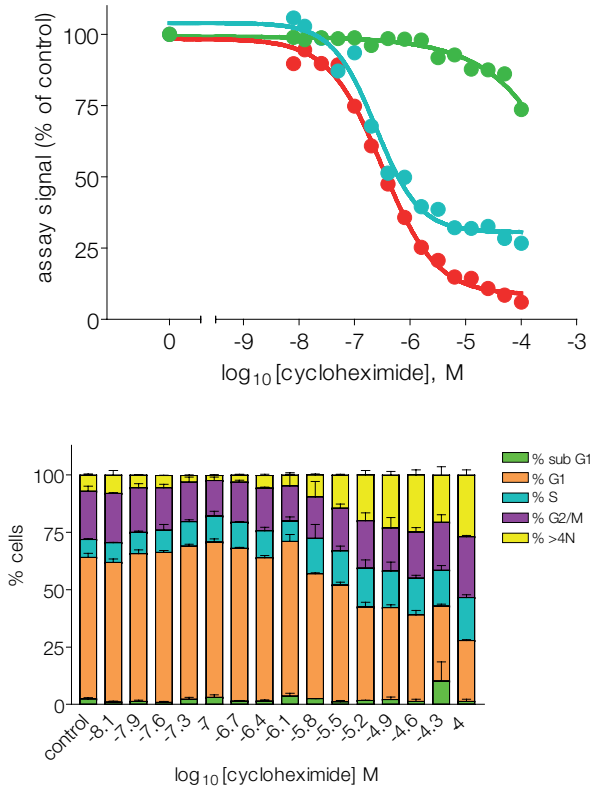

Fig 5: concentration-response curves and cell cycle classification chart of HeLa cells treated with cycloheximide

drug	Log EC_{50}			cell cycle phase
	cell count	% live cells	CTG	
staurosporine	-9.1	-6.7	-8.7 (monotonic) -8.7 & -6.8 (bi-phasic)	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 2: Log EC_{50} values determined from the concentration-response curves in Figures 4-7

For **etoposide** (Fig 6), the ATP-luminescence EC_{50} value differs from both acumen readouts by more than one log unit, highlighting the fact that ATP-luminescence measurements give unreliable results for cytostatic drugs. The acumen Cellista cell count and percentage live cell readouts clearly show that this drug has potent antiproliferative properties in the micromolar range *without* concomitant toxicity, and furthermore that etoposide causes cell cycle arrest in the G2/M phase. Interestingly, the decline in cell health at the very highest drug concentration is accompanied by an increase in the number of cells found in the sub G1 phase of the cell cycle, suggesting that these cells could have undergone apoptosis. Finally, the acumen Cellista TIFF images (Fig 6) show an unusual enlarged morphological phenotype for the cells treated with 1 µM etoposide, which could explain the higher than expected ATP measurements.

With **staurosporine** (Fig 7), the ATP measurements show a subtle bi-phasic drug response, which presents problems accurately fitting the data, and also overestimates the toxicity of this compound. By contrast, 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. When non-monotonic curves are observed in ATP-luminescence assays without appreciation of the underlying mechanisms of action, not only is the quality of EC_{50} data compromised (Table 2), but also valuable mechanism-of-action information discarded. There is also significant risk of false-negative results when using ATP-luminescence assays to screen compounds for antiproliferative activity, especially if the compound mechanisms of action and effects on cell cycle, metabolic activity, and survival are not well understood.

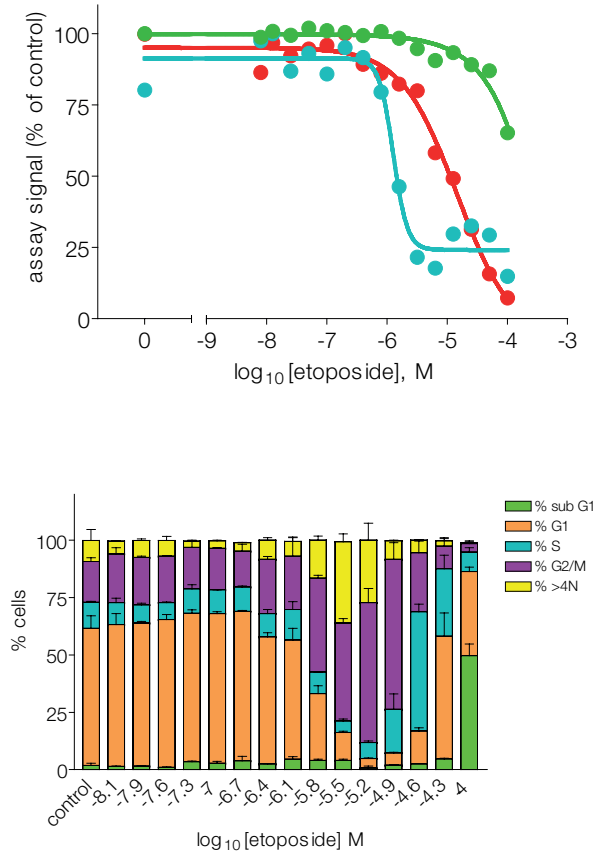

Fig 6: concentration-response curves and cell cycle classification chart of HeLa cells treated with etoposide

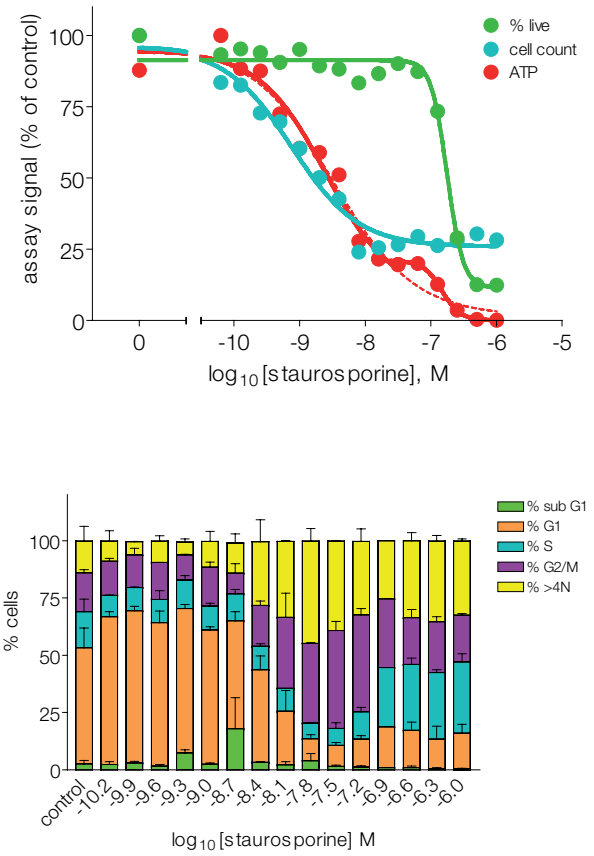

Fig 7: concentration-response curves and cell cycle classification chart of HeLa cells treated with staurosporine

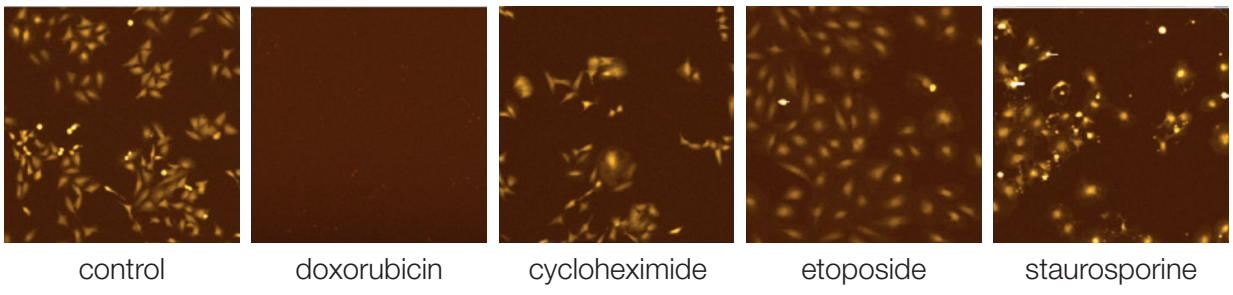

Fig 8: representative TIFF images from acumen of drug-treated HeLa cells stained with calcein-AM and propidium iodide

Conclusions

We set out to develop a high content cell viability assay that would deliver on data quality, throughput and cost compared with established technologies. Our data show that acumen Cellista is suitable to report several markers of viability and toxicity, including cell count, the percentage of live cells and cell cycle phase distribution. These features allow ready distinction between cytostatic and cytotoxic mechanisms of action, while the TIFF export function highlights morphological changes of the cells. The ability to distinguish between different mechanisms of action is particularly relevant for drugs affecting the cell cycle, which frequently prevent cell proliferation without causing toxicity. Our results highlight that ATP-luminescence measurements for such compounds are unsuitable, as they significantly overestimate the toxicity of such compounds, yet fail to recognise their antiproliferative properties.

Historically, the large amount of time required to prepare and analyse a single HCA plate used to limit the applicability of these assays in terms of throughput. Most phenotypic screens to date are still carried out on limited library subsets, rather than the full deck screens commonly used with biochemical assays [6]. In order to bridge the gap between the two screening approaches, we developed an automation-friendly homogenous assay, which is compatible with high density plate formats for ease of setup. The assay uses cost-effective generic reagents for reduced running costs. With the read times of acumen Cellista now being close to that of bulk fluorescence readers (typically < 5 minutes per plate), over 400,000 wells can be read and analysed in a single day.

Taken together, these features make the acumen Cellista ideally placed for practical high-throughput, full-deck toxicity screens. By assessing multiple viability markers simultaneously the risk of generating false-positive toxicity hits is minimised and confidence in the quality of the data is increased. The importance of accurate toxicity assessments should not be underestimated: Far too many drugs in recent years have failed in clinical trials due to safety/efficacy concerns, incurring vast financial losses. Therefore the earlier problematic compounds can reliably be flagged and removed from the pipeline, the better.

References

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About acumen

TTP Labtech's acumen is a laser scanning imaging cytometer designed to provide single scan, 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.

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