Eliminating the false negatives in ATP-luminescence cell health assays by the use of a phenotypic assay approach

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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 poster 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 HCA assay, 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 both the HCA assay and a commercial ATP-luminescence detection system, and highlight key differences between the measurements.

1. 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 48h

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.

costs (Table 1)

reagent	unit price	unit size and stock concentration	cost per 384 well
calcein-AM	£223	1 mL, 1 mM	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 1x 384-well plate)	14.58p

2. phenotypic assay validation

cell cycle phase determination

acumen cell cycle classification gates were derived from the total FL1 (Hoechst) 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**.

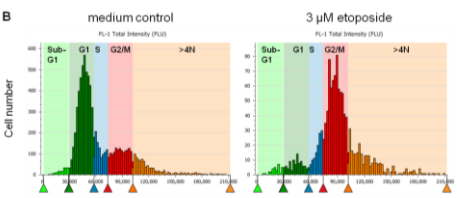

Fig 1. acumen cell cycle profiles

% 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.

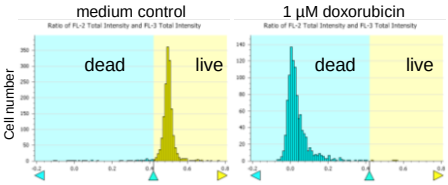

Fig 2. acumen ratio of FL2/FL3 intensity histograms used for live/dead cell classification

3. results

The drug-response curves (Figure 3) and EC_{50} values (Table 2) show that the agreement between the acumen Cellista cell number and % live cell readings, and the ATP-luminescence readings varies widely between the compounds.

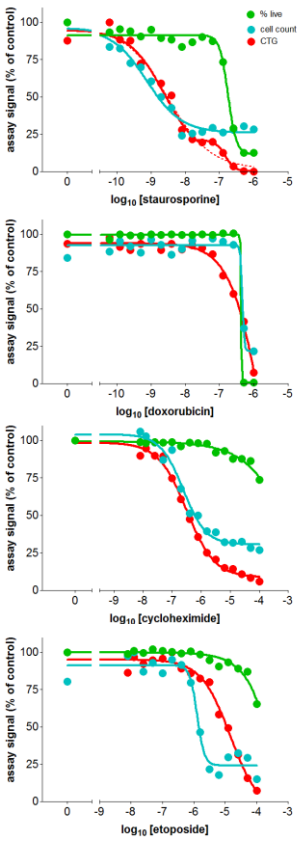

Fig 3. Drug response curves comparing acumen phenotypic readouts (% live; cell) to an ATP-luminescence measurement (CTG). Log EC_{50} values derived from these curves are summarised in **Table 2** (below)

drug	log EC_{50}			cell cycle response
	cell count	% live cells	CTG	
stauros-porine	-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
cyclo-heximide	-6.6	N.D.	-6.5	G1

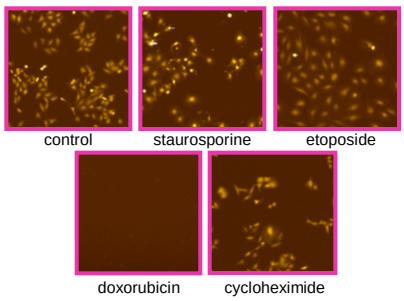

Fig 4. Representative Tiff images of drug treated cells stained with calcein-AM and propidium iodide. The Hoechst signal is not shown.

4. discussion

comparison of drug-responses across different assay formats

The drug-response curves (Figure 3) and EC_{50} values (Table 2) show that the agreement between the acumen Cellista cell number and % live cell readings, and the ATP-luminescence readings varies widely between the compounds and was strongly influenced by the compound mechanism of action.

With **doxorubicin**, a classic cytotoxic agent that causes cell death, there is very close agreement between all three readouts.

With **etoposide** and **cycloheximide,** both cytostatic agents, there is less agreement between the three measurements: The ATP luminescence measurements for cycloheximide 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 DNA content measurements confirmed that cycloheximide (a protein synthesis inhibitor) does not affect the cell cycle, as expected.

For etoposide, the ATP luminescence EC_{50} value differs from both acumen readouts by more than 1 log unit, again highlighting the fact that ATP luminescence measurements give unreliable results for cytostatic drugs. The acumen cell count and % 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. In addition, the acumen Cellista Tiff images (Figure 4) show an unusual enlarged morphological phenotype for the etoposide-treated cells, which could explain the higher than expected ATP measurements.

With **staurosporine**, the ATP measurements show a subtle bi-phasic drug response, which presents problems to accurate data fitting and also overstimates 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. In addition, the DNA content measurements indicate that there is cell cycle arrest in the G2/M and >4N phases.

conclusions

- ATP-luminescence measurements alone give misleading data for cytostatic drugs, by overestimating toxicity and underestimating potency
- acumen Cellista provides content rich data that includes cell number and cell health, thereby allowing better distinction between cytotoxic and cytostatic compound mechanisms of action
- a multiplexed total DNA content readout allows rapid identification of drugs affecting the cell cycle, while the Tiff export function can highlight unusual cellular phenotypes
- the simple and homogenous assay format lends itself to automation and miniaturisation. With entire 1536-well microplates imaged and analysed in only 4.5 minutes, full deck viability screens of whole compound libraries are feasible

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