

Simultaneous measurement of cytotoxicity, proliferation and cell cycle progression using a simple two dye method

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Cell cycle analysis – modulation of cell cycle progression can alter cell growth rates. Cell cycle analysis requires accurate determination of DNA content, and is routinely performed using flow cytometry. The latter offers no information on cytotoxicity. None of the simple cytotoxicity or proliferation methods are suitable.

2. Multiplexed High Content Analysis

Population Manager

- Add Population
- Delete Population
- Data
 - 405 (Hoechst) (405nm) Data
 - Nuclei
 - G1
 - S
 - G2/M
 - >4N
 - 488 (PI) (488nm) Data
 - Dead

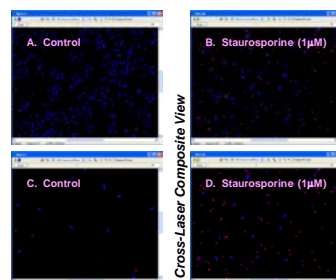
Proliferation – cell count based on nuclear staining with Hoechst 34580 (405nm laser).

Cell cycle analysis – Hoechst 34580 staining of nuclei permits assignment of cells to the main phases of the cell cycle. Can be performed on live cells, *in situ* allowing multiplexing with other dyes.

Cytotoxicity – dead cells identified by positive staining with cell-impermeable DNA stain propidium iodide (488nm laser).

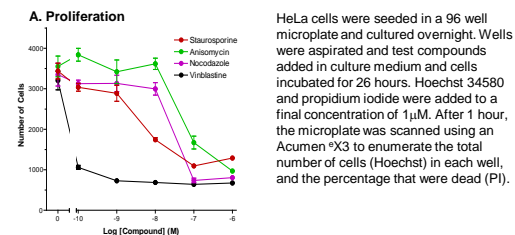
Laser-scanning fluorescence microplate cytometers, such as the Acumen[®]X3 (TTP LabTech Ltd) can scan with up to 3 lasers (405, 488 and 633nm), ensuring compatibility with a wide variety of fluorescent probes. The lasers scan sequentially to reduce crosstalk and permit high levels of multiplexing. Here, two common DNA stains, Hoechst 34580 and propidium iodide (PI), were applied to live cells and subjected to high content analysis on an Acumen[®]X3. The instrument's cytometric software permitted determination of cell number, cytotoxicity and cell cycle analysis in a single dual laser read. Plate cycle time was 14 minutes per plate with whole well analysis.

3. Phenotype Characterisation

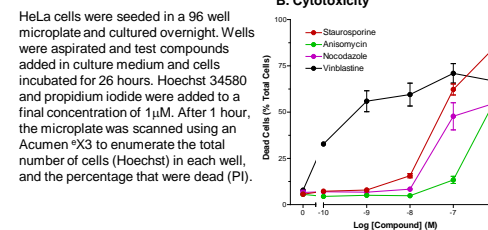


Acumen[®]X3's cytometric software identifies fluorescent objects 'on-the-fly' allowing immediate assessment of labelling between treatments. **A & B**, overlay of Hoechst 34580 and propidium iodide staining – note greater incidence of dual-positive cells in B indicating staurosporine-induced cytotoxicity. **C & D**, cross-laser composite view in which only dead cells (dual-stained) are displayed.

4. Cytotoxicity and Proliferation

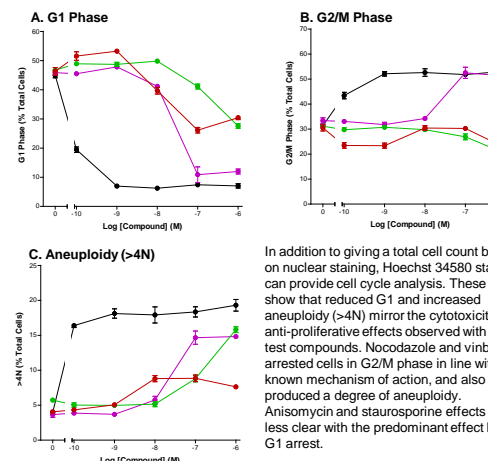


HeLa cells were seeded in a 96 well microplate and cultured overnight. Wells were aspirated and test compounds added in culture medium and cells incubated for 26 hours. Hoechst 34580 and propidium iodide were added to a final concentration of 1 μ M. After 1 hour, the microplate was scanned using an Acumen[®]X3 to enumerate the total number of cells (Hoechst) in each well, and the percentage that were dead (PI).



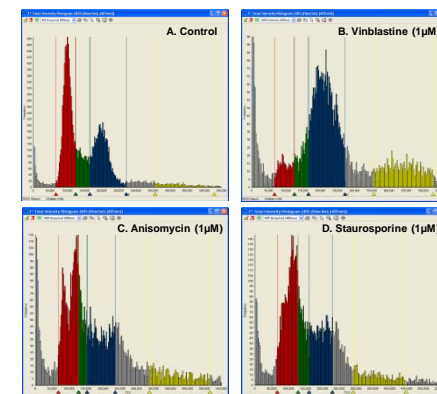
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5. In situ Cell Cycle Analysis



In addition to giving a total cell count based on nuclear staining, Hoechst 34580 staining can provide cell cycle analysis. These data show that reduced G1 and increased aneuploidy (>4N) mirror the cytotoxicity and anti-proliferative effects observed with the test compounds. Nocodazole and vinblastine arrested cells in G2/M phase in line with their known mechanism of action, and also produced a degree of aneuploidy. Anisomycin and staurosporine effects were less clear with the predominant effect being G1 arrest.

6. Cell Cycle Analysis – Histograms



A. Histograms of Hoechst 34580 labelling allowed definition of cell cycle phases. **B.** Vinblastine arrested cells in G2/M phase and caused aneuploidy. **C.** Anisomycin disrupted the cell cycle histogram, appearing to arrest cells in G1 and cause apoptosis seen as a pre-G1 peak. **D.** Staurosporine arrested cells in G1 phase with no evidence of apoptosis.

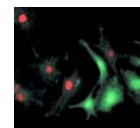
Together, these data differentiate all three compounds and their mechanisms of action.

Conclusion

We have developed a rapid, toxicity screening assay with the following features:

- Simple dye-based method using two low-cost DNA stains.
- Simultaneously reports cytotoxicity, cell proliferation and cell cycle analysis in a single plate read.
- Applicable to single and clustered cell cultures.
- Compatible with microplate cytometers enabling 40,000 tests per day in 384 well format.
- Due to its simplicity, the method could be enhanced by multiplexing with other markers of interest.

1. Background to Method Development



Cytotoxicity – routinely measured using MTT or LDH release assay. MTT assay issues can result from presence of antioxidants and variable cell number. LDH release is a good measure of cytotoxicity but provides no information on cell number. For high content analysis, calcein AM is a common viability indicator. At high cell densities, calcein AM can underestimate cell number due to clumping.



Proliferation – alteration of cell growth rates is a simple predictor of cell modulation. Accurate determination of cell number is paramount for data normalisation in multiplexed protocols. Traditionally, DNA content was measured using [³H] thymidine incorporation but this has been largely superseded by the MTT assay where cytotoxicity is minimal.