

High Content Analysis of Cytotoxicity by High Throughput Imaging

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Introduction

Toxicological profiling is a key part of both drug discovery and drug safety. Cell-based assessment of toxicity is best served by a multi-parametric approach in order to establish both phenotypic and mechanistic information and ultimately, make decisions about drug development.

Using several ready-to-use kits and reagents developed for HCS (Invitrogen), we performed a variety of cytotoxicity assays using the Acumen® eX3 microplate cytometer (TTP LabTech). A number of key cytotoxic phenomena were analyzed including: plasma membrane integrity, DNA damage, DNA synthesis, mitotic arrest, and cytoskeletal disruption.

The Acumen eX3 used in this study uses a triple laser system to maximize multiplexing of fluorophores with optimal signal acquisition and scan times of less than ten minutes per plate. This is significantly faster than multi-channel scanning with camera-based automated imaging platforms. The multiplex capabilities and rapid plate scanning of the Acumen eX3 system combined with validated cytotoxicity kits from Invitrogen provide robust, reproducible assays with a simple workflow, enabling automated, relatively rapid high content cytotoxicity profiling.

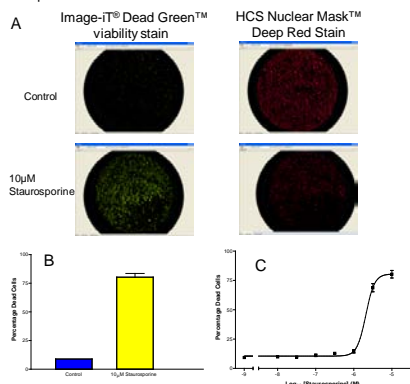
1. Acumen eX3 imaging cytometer

Combining proprietary cytometric analysis and state-of-the-art scanning equipment Acumen eX3 delivers high-content, cell-based screening at ultra-high throughputs. Triple laser excitation, coupled with simultaneous four colour detection, enable a wide range of common fluorophores to be combined in multicolour, multiplexed assays. This instrument combines whole well scanning with the ability to measure cell number as part of the same assay, which gives researchers the capability to normalise responses to total cell number.



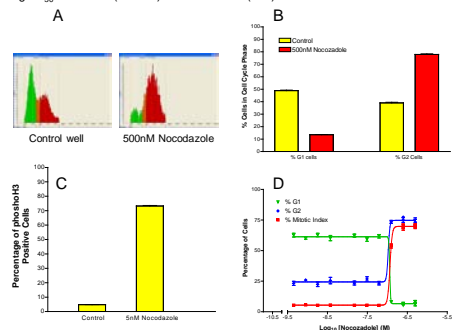
2. Cell Viability

The image-iT® DEAD™ Green viability and HCS NuclearMask™ Deep Red stains are amenable to fixation and permeabilisation enabling multiplex assays with other biomarkers of cytotoxicity. Panels A and B show Acumen well views and quantitation, respectively of untreated and staurosporine treated cells with clear increases in dead cell staining in treated cells. The dose response curve in panel C was used to calculate a log EC₅₀ value of -5.68 M (2 μM) (n=3) for staurosporine.



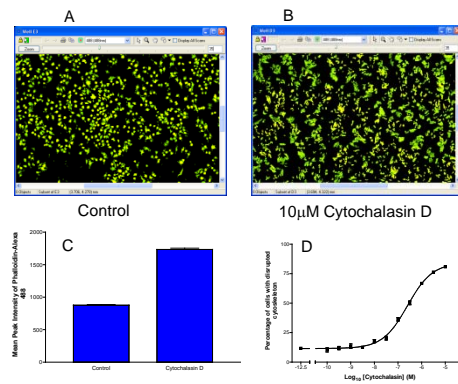
3. Mitotic Index and Cell Cycle: Phospho-Histone H3 and DNA Content

Histone H3 phosphorylation is required for proper segregation and condensation of chromosomes during mitosis and represents a marker for mitotic cells. Cells were treated with nocodazole and the HCS Mitotic Index Kit was used to assess DNA profile and mitotic index. The histograms in Panel A show control and treated cells in G1 (green) and G2/M (red) as reflected by DNA profiling (DAPI). Panel B displays control (yellow) and treated (red) cells as a percent distribution in G1 or G2 as determined from DAPI staining. Panel C shows strong nocodazole-induced pH3 with a high Z' score of 0.88, indicating the assay's excellent screenability. Panel D shows a nocodazole dose-dependent percentage of cells in M (pH3), G1 or G2 (DAPI) and a log EC₅₀ of -6.92 M (120 nM) was calculated (n=7).



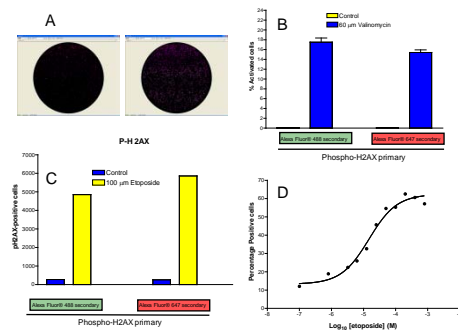
4. Cytoskeleton disruption: Alexa Fluor® 488 Phalloidin

Alexa Fluor® 488 phalloidin is a high-affinity probe for F-actin, which can be used to detect cytoskeletal disruption on the Acumen® eX3. Panels A and B show a significant difference in cytoskeletal organization between untreated and cytochalasin D treated cells. The dose response curve in Panel C was used to calculate a log EC₅₀ of -6.56 M (275 nM) (n=5) for cytochalasin D.



5. Genotoxicity: Phospho-H2AX

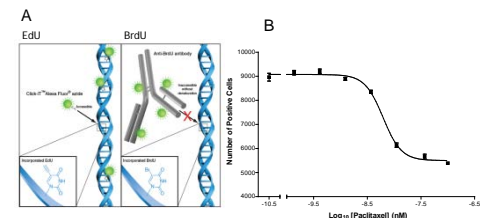
Phosphorylation of histone variant H2AX has been identified as an early response of cells to double stranded DNA breaks. A549 cells were treated with 60 μM valinomycin or 100 μM etoposide for 24 hours before performing the DNA damage assay. Figure 5A shows the Acumen well views of untreated and valinomycin-treated cells. Figures 5B and 5C show quantitative representations of pH2AX-positive cells detected with secondary antibodies conjugated to either Alexa Fluor® 488 dye or Alexa Fluor® 647 dye. The dose response curve in Figure 5D was used to calculate a log EC₅₀ value of -4.89 M (13μM) for etoposide.



6. Nascent DNA Synthesis and Proliferation: The Click-iT® Edu Assay

Traditionally, cell proliferation is performed by incorporating the nucleoside analog bromodeoxyuridine (BrdU) into DNA, followed by detection with an anti-BrdU antibody. Although effective, this method requires DNA denaturation (using HCl, heat, or DNase) to expose the BrdU to the antibody—a step that can be lengthy and difficult to perform consistently, and can adversely affect the sample.

The Click-iT® Edu Cell Proliferation Assay removes the need to denature DNA, providing a superior alternative to the standard BrdU antibody-based method for measuring nascent DNA synthesis and cell proliferation. Panel A shows the differences between the Click-iT® method and the BrdU method. Panel B shows a dose response curve for paclitaxel plotted against Edu-positive cells which was used to calculate a log IC₅₀ of -8.24 M (6 nM) (n=5).



Conclusion

With its high throughput capabilities, the Acumen eX3 provides a means for performing high content analysis at an earlier stage in drug screening than is traditional. Each of the Invitrogen assays described were performed using protocols that:

- Analyse every cell contained within each well
- Capture, normalise, and export data rapidly
- Are amenable to multiplexing with other markers and targets of interest

The new kits highlighted in this study add to the list of Invitrogen assays which have been validated for the Acumen eX3 microplate cytometer. These assays are robust and suitable for fixed and permeabilized cells, enabling multiplex interrogation of off- and on-target effects of compounds for toxicological profiling.

- HCS LIVE/DEAD® Green Kit (H10290)
- HCS Mitotic Index Kit (H10293)
- HCS DNA Damage Kit (H10292)
- Image-iT® DEAD Green™ viability stain (I10291)
- Click-iT® Edu Assay Kits (A10028, C10081, C10082)