Titration of YOYO®-1

In order to determine if YOYO-1 affected the cell biology (cell proliferation) of treated cells, dilutions of YOYO®-1 were prepared. A 7-point concentration curve of YOYO®-1 demonstrated that a 0.1 µM YOYO®-1 concentration did not affect cell proliferation as measured by the IncuCyte FLR confluence algorithm (Supplemental Figure 1).

Supplemental Figure 1. YOYO®-1 titration curve. HT 1080 and MDA-MB-231 cells were treated with two-fold dilutions of YOYO®-1. Graphs illustrate inhibition of cell proliferation at concentrations above 0.16 µM, as measured by cell confluence. Each data point represents the mean ± SE in N=3 wells.

Determining Triton X-100 Concentration for Permeabilization

Triton X-100 was serially diluted in PBS and added directly to the wells containing cells to allow nuclear DNA staining by YOYO®-1. Fluorescent and phase-contrast images were acquired at 15 minutes and 2 hours. Triton X-100 at 0.0625% did not affect adherence of the cell lines tested (Supplemental Figure 2).

Supplemental Figure 2. Triton X-100. To correct for differential proliferation of cells, the total number of DNA containing objects was counted at the final time point using Triton X-100 to permeabilize the cells, allowing YOYO®-1 to stain the nuclear DNA. Data illustrates that a Triton X-100 concentration of 0.0625% permeabilizes the cells without affecting cell adherence. Each data point represents the mean ± SE in N=3 wells.
Using the IncuCyte FLR Confluence Metrics to Measure Cytostatic Compounds

No statistical induction of cytotoxicity, as measured by YOYO®-1 staining, is observed when either cell type was treated with any of the tested concentrations of Cycloheximide (CHX). However, a clear concentration-dependent inhibition of cell proliferation was observed as measured by the IncuCyte FLR confluence algorithm (Supplemental Figure 3).

Supplemental Figure 3. Using IncuCyte FLR confluence metrics to measure the cytostatic effect of cycloheximide (CHX). MDA-MB-231 and HT 1080 cells were treated with several concentrations of cycloheximide. Graphs illustrate inhibition of cell proliferation, as measured by cell confluence. Cell morphology did not significantly differ from untreated cells as illustrated in Figure 1C. Each data point represents the mean ± SE in N=3 wells.

Cell Density-Dependent and Concentration-Dependent Response Using HeLa Cells

In order to determine if the assay accurately quantifies cell death via YOYO®-1 DNA staining, HeLa cells were seeded at multiple seeding densities (5K, 2.5K, 1K, and 0.5K cells/well) and treated with varying concentrations of Staurosporine (SSP) Results showed a quantifiable relationship between SSP concentration and YOYO®-1 fluorescent objects illustrating a cell density-dependent response (Supplemental Figure 4A). The resulting R² values for data analyzed at 12, 24 and 48 hours, treated with 1000 nM SSP, are 0.9968, 0.9999, and 0.9997 respectively. This indicates a strong correlation between cell seeding density and the YOYO®-1 fluorescent object count (Supplemental Figure 4B).

Figure 4. Concentration-dependent and cell-dependent qualification. 96-well microplate graph showing the kinetic measurement of the number of YOYO®-1 positive HeLa cells in response to SSP and a cell seeding, density-dependent response (A). Data from HeLa cells treated with 1000 nM SSP shows a linear relationship between cell seeding density and object count at 12, 24, and 48 hours (B).
Using YOYO®-1 to Kinetically Measure Cell Viability in Cell Types Lacking Apoptotic Cell Death Pathway Factors

YOYO®-1 was further characterized as a live cell reagent using the breast adenocarcinoma cell line, MCF-7, in response to SSP treatment. The MCF-7 cell line lacks caspase-3 expression [9], a primary executioner in the apoptotic pathway, but remains sensitive when treated with SSP. MCF-7 cells were monitored using either YOYO®-1 or the Essen BioScience Caspase-3/7 apoptosis reagent after exposure to 333 nM SSP. End point normalization was performed by adding Vybrant DyeCycle Green DNA dye to a final concentration of 1 µM to the wells containing either YOYO®-1 or caspase-3/7 reagent, and the cytotoxic or apoptotic indices were calculated (Supplemental Figure 5). We did not observe a statistically significant induction of caspase 3/7 positive objects, whereas a significant increase in the cytotoxic index was observed in SSP treated MCF-7 cells.

Whole Plate Reproducibility

Plates containing 5,000 cells/well (MDA-MB-231 or HT 1080) were treated with either 250 nM CMP or 100 nM SSP and placed in an IncuCyte FLR and scanned every 2 hours using phase-contrast and fluorescent imaging. End point analysis was completed at the 48-hour time point and an unpaired t-test analysis was performed between 32 interior wells and the outer 32 wells (excluding corners). As shown in Figure 6, these data show that there was no significant difference detected between the cytotoxic indices measured in the outer wells compared to identically treated inner wells for either cell type.

Supplemental Figure 5. YOYO®-1 measures cell wall integrity regardless of the biochemical pathway used to initiate cell death. SSP induced cell death in MCF-7 (CASP-3-) cells measured by YOYO®-1 fluorescent staining of DNA compared to the Essen BioScience Caspase-3/7 reagent. End point normalization was calculated by dividing the number of YOYO®-1 fluorescent objects (cytotoxic index) or the number of caspase fluorescent objects (apoptotic index) by the total number of DNA containing objects.

Supplemental Figure 6. Whole plate intra-assay reproducibility. 96-well plates were seeded with MDA-MB-231 cells and treated with 100 nM SSP (A) or HT 1080 cells treated with 250 nM CMP (B). End point normalization was performed, followed by statistical analysis of the inner and outer 32 wells of each plate.
IncuCyte™ ZOOM Dual Fluorescence Analysis for Cytotoxicity

NucLight Red HT 1080 cells were seeded at 5,000 cells/well and treated with serially diluted concentrations of SSP, Camptothecin (CMP), or CHX. The ZOOM basic analyzer was used to mask the green fluorescent nuclear signal to quantitate cell death (YOYO-1® positive cells) as well as the red fluorescent nuclear signal to monitor cell proliferation (NucLight Red). Kinetic dose response curves for both YOYO®-1 positive events as well as nuclear counts of NucLight Red HT 1080 cells were exported to GraphPad Prism. The area under the curve (AUC) was analyzed for each well and replicate AUC values were used to calculate EC_{50} and IC_{50} values. Figure 7 shows the inverse relationship between cell proliferation (nuclear count) and membrane permeability (YOYO®-1 positive objects) over time for each compound.

Figure 7. Dual fluorescence calculations for IC_{50} and EC_{50} values. 96-well plates were seeded with NucLight Red HT 1080 cells and treated with log dilutions of either SSP, CMP, or CHX. AUC analysis was performed on the kinetic data for both YOYO-1 positive cells and cells retaining the NucLight Red marker. Each data point represents the mean ± SE in N=6 wells.