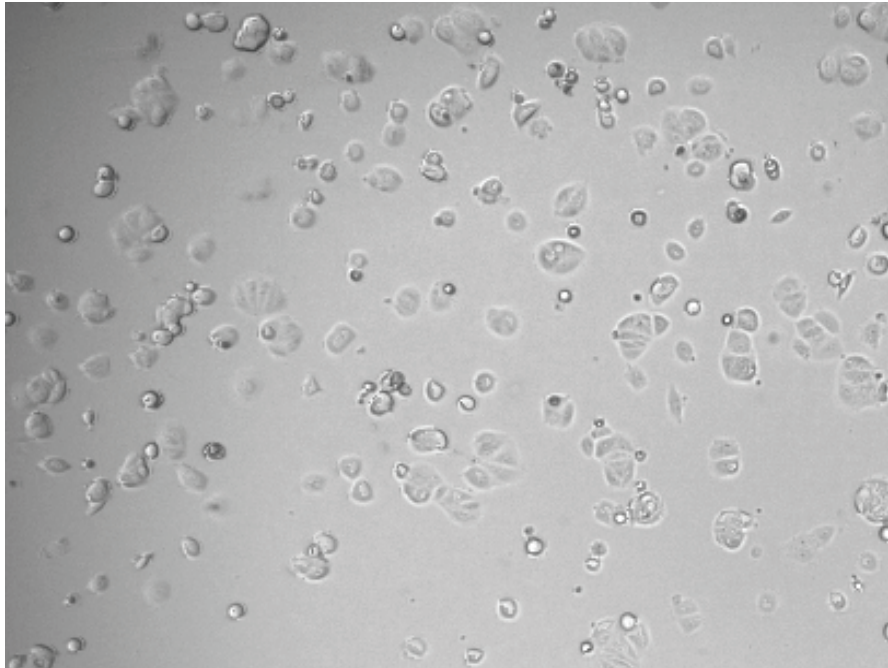


Multimode Detection

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Morphology and ATP-based Cytotoxicity Profiling of Cancer Cell Lines using the EnSight Multimode Plate Reader with Well Imaging Technology

Introduction

The imaging capabilities of the EnSight™ Multimode Plate Reader make it an ideal instrument for anti-cancer drug discovery and the determination of compound effects on cell

viability and proliferation. Unlike classical endpoint cell viability assays where cells may be lysed, fixed or stained, the “non-destructive” imaging readout of the EnSight brightfield imaging mode offers the capability to monitor cellular processes in a kinetic format with no fluorescence or similar reporter reagents needed. This can provide additional information on the kinetic development of cytotoxicity effects and has the potential to reduce assay cost and cycle times.

Demonstration of anti-cancer activity of test compounds can involve a range of assays monitoring proliferation, viability, apoptosis, angiogenesis and anti-tumor immunity as well as target specific effects, signal pathways and biomarkers¹ [Shoemaker, 2006]. Several cell viability assessments use ATP detection in luminescence-based assays as a surrogate readout for cell number. Such ATP detection methods can routinely be conducted with <500 cells per well – a relatively low number compared to fluorescence or colorimetric based methods. As an endpoint assay, ATP measurement requires a cell lysis step and use of appropriate detection reagents containing luciferase, luciferin and a variety of proprietary buffer components which serve to produce a stable luminescence signal lasting up to two hours² [Niles, 2009].

How can we improve the process of cytotoxicity determination?

- ATP detection is not a “universal” readout as some cell growth and toxicity mechanisms are not adequately reflected:
 - Compounds can increase the size and mitochondrial content of cells
 - Recent papers suggest discrepancies in quantification between cell number and ATP/MTS proliferation assays³ [Chan, 2013].
- Since ATP detection is an endpoint assay identifying the time dependence of cell proliferation and/or cytotoxicity is possible, but not straightforward. For example:
 - Membrane disrupting agent rapid onset in minutes
 - DNA cross linking – hours to days for effects to become apparent
 - Additional kinetic information can help in compound prioritisation.
 - Eliminate need for a separate detection step in the assay protocols which could:
 - Reduce reagent costs
 - Reduce detection related assay interference

In this application note, we compare the use of brightfield imaging on the EnSight system and ATP detection for routine profiling of two cancer cell lines against a panel of 4 cytotoxic and 2 non-cytotoxic compounds.

Table 1. Standard compounds.

Compound
Paclitaxel
Gentian Violet
Colchicin
Trichostatin A
Pilocarpine (non-toxic)
Carbamylcholine Chloride (non-toxic)

Materials and Methods

Cancer cell lines (786-0 and MCF-7) were maintained in RPMI-1640 media containing 2 mM glutamine, 100 U/mL penicillin G, 100 µg/mL streptomycin and 10% FCS. At about 80% confluence in T-flasks, cells were washed, trypsinized, and re-suspended in RPMI-1640 medium. Cells from T-flask culture methods were then seeded (20 µL volume) at 500 to 2500 cells per well (depending on cell line requirements) onto 384-well ViewPlate™ microplates (clear bottom, white). Plates were incubated at 37 °C in the presence of 5% CO₂. The general protocol is shown in Figure 1. For plate 1 (P1), at 24 h post seeding, the growth of baseline control plates was assessed using the CellTiter-Glo® (CTG) protocol (Promega). At 24 h post seeding, P2 and P3 were dosed with compound (11 concentrations in duplicate) using the Echo® 550 (Labcyte). The compound panel (4 toxic and 2 non-toxic) is shown in Table 1. Final DMSO concentration in the assay plates was uniform at 0.5%.

Assays plates (P2 and P3) were read on the EnSight system at 24 h (P2 and P3), 32 h (P2), 42 h (P2), 56 h (P2) and 72 h (P2 and P3) post cell seeding (Figure 1). Plates were kept under controlled conditions (5% CO₂, 37 °C and 90% humidity) between each read and imaging took place at 37 °C using the temperature control facility of the EnSight reader, which eliminates potential water condensation effects. Readout cycle times were < 5 min per plate. Images were analyzed using the Kaleido™ Data Acquisition and Analysis Software and the derived results were fitted to 4-parameter logistic and plotted using TIBCO Spotfire®.

To verify the signal stability and comparable readout of different plate readers, the luminescence measurements were performed using the EnVision® Multimode Plate Reader and EnSight system.

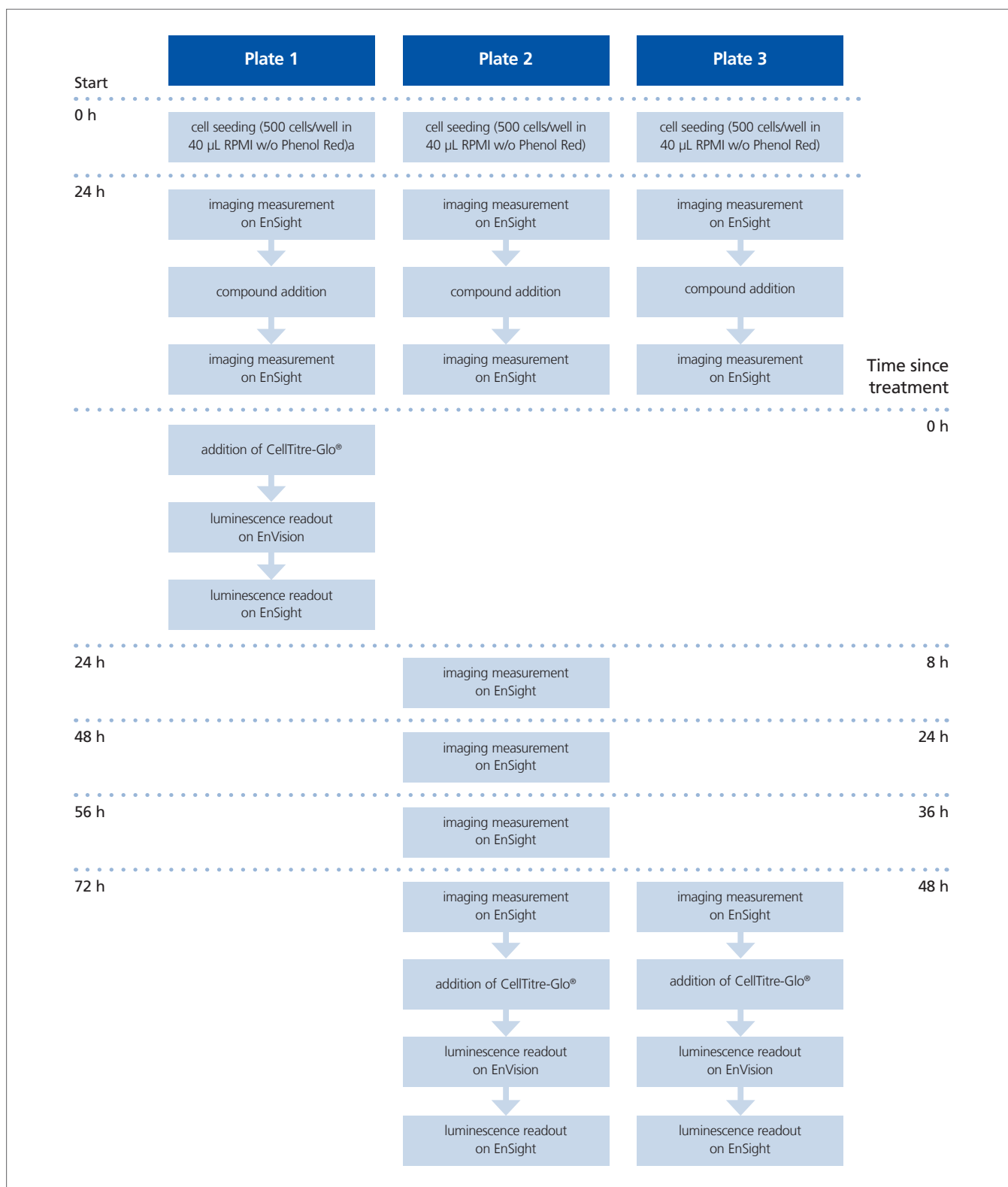


Figure 1. Protocol for combined CellTiter-Glo® and brightfield imaging readouts.

Results

Brightfield Images

Representative Brightfield images of the 786-0 and MCF-7 cells acquired by the EnSight system at 72 h post seeding (48 h post compound exposure) are shown Figure 2 (A-D). Gross visual inspection shows the morphological phenotype anticipated

following cell exposure to toxic compounds (Figure 2 B, D at 25 μ M Paclitaxel) compared to controls (Figure 2 A and C at 0.5% DMSO), i.e. fewer cells which are typically more rounded in appearance.

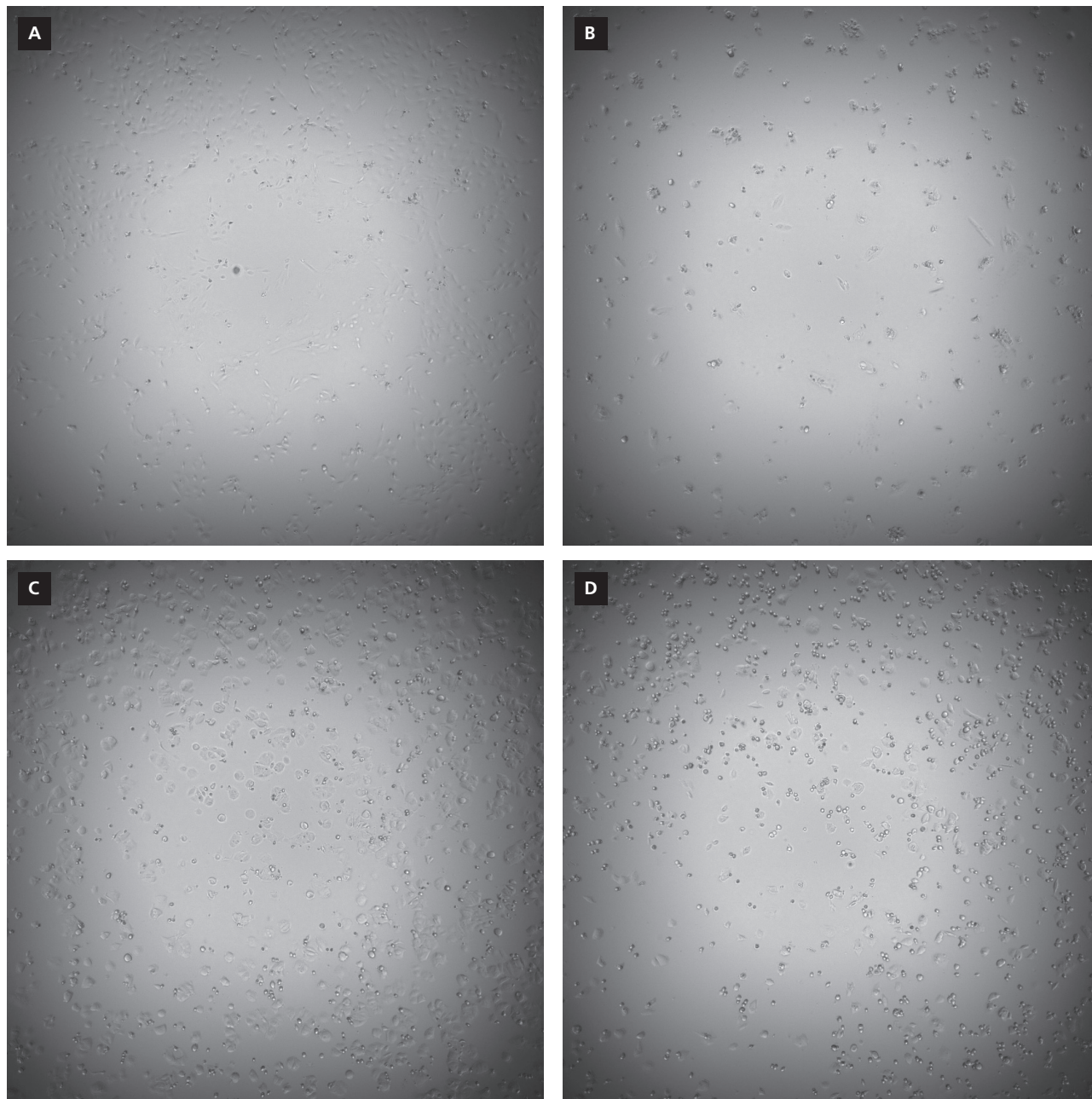


Figure 2. Brightfield example images of 786-0 (top) and MCF-7 (bottom) cells without (left) and with toxic compounds (right). Images have been acquired 72 h after cell seeding. Cells have been exposed 48 h to DMSO control or toxic substance.

A: 786-0 cells exposed to 0.5% DMSO. B: 786-0 cells exposed to 25 μ M Paclitaxel.
C: MCF-7 cells exposed to 0.5% DMSO. D: MCF-7 cells exposed to 25 μ M Paclitaxel.

Image Analysis Results

Advanced image analysis methods available within the Kaleido software were applied to the brightfield images in order to identify relevant morphological and texture based parameters. From these lists, a group of key image descriptors were identified as being strongly associated with the underlying cytotoxicity and cytostatic responses of the cells. Roughness is a texture parameter designed to characterize areas covered by cells. Increasing roughness indicates rounding up of cells or other effects that increase cellular contrast in brightfield imaging. Using the change in confluency values accounts for the any variations in the starting cell number in each well. Over time, decreasing roughness usually indicates continuing cell growth where the relative impact of the high roughness of some dead (rounded up) cells decreases over time. Biological information which can be inferred from these parameters is described in Table 2.

Assay Results

Dose response curves for MCF-7 cells using the change in confluency parameter (%) are shown for the cytotoxic compound Colchicin (Figure 3, top) at 3 time points post-exposure to compound. The starting confluency was relatively low (5%) and it can be seen that small changes in overall confluency can be detected as the cells proliferate at low Colchicin concentrations, whilst cytostatic effects can be seen at higher concentrations. Texture based readouts of roughness (Foreground Roughness Mean) (Figure 3, bottom) show this parameter is inversely correlated with cell confluency at lower Colchicin concentrations. At higher concentrations of the cytotoxic compounds, the roughness parameters increase with time and compound concentration. Interestingly, "healthier" cells, for instance those exposed to lower concentrations of Colchicin ($< 0.005 \mu\text{M}$), show time-dependent reductions for the roughness parameter. The relative assay window and variability observed in the Foreground Roughness Mean parameter is similar to that seen for the "orthogonal" confluency parameter.

Table 2. Readout parameters of brightfield image evaluation method.

Parameter	Description	Notes
Confluency [%]	Fraction of the evaluated area that is covered by cells	
Foreground roughness	Average "strength of texture" within the area covered by cells	The rounding up of cells leads to an increase in this parameter.
Average roughness within well	Average "strength of texture" within the well	Independent of object segmentation. Mainly used for quality control
Roughness range within well	95% percentile of texture divided by 5% percentile	Quality control parameter revealing biologically relevant disturbances like infection with bacteria. Will drop for wells affected by bacterial contamination

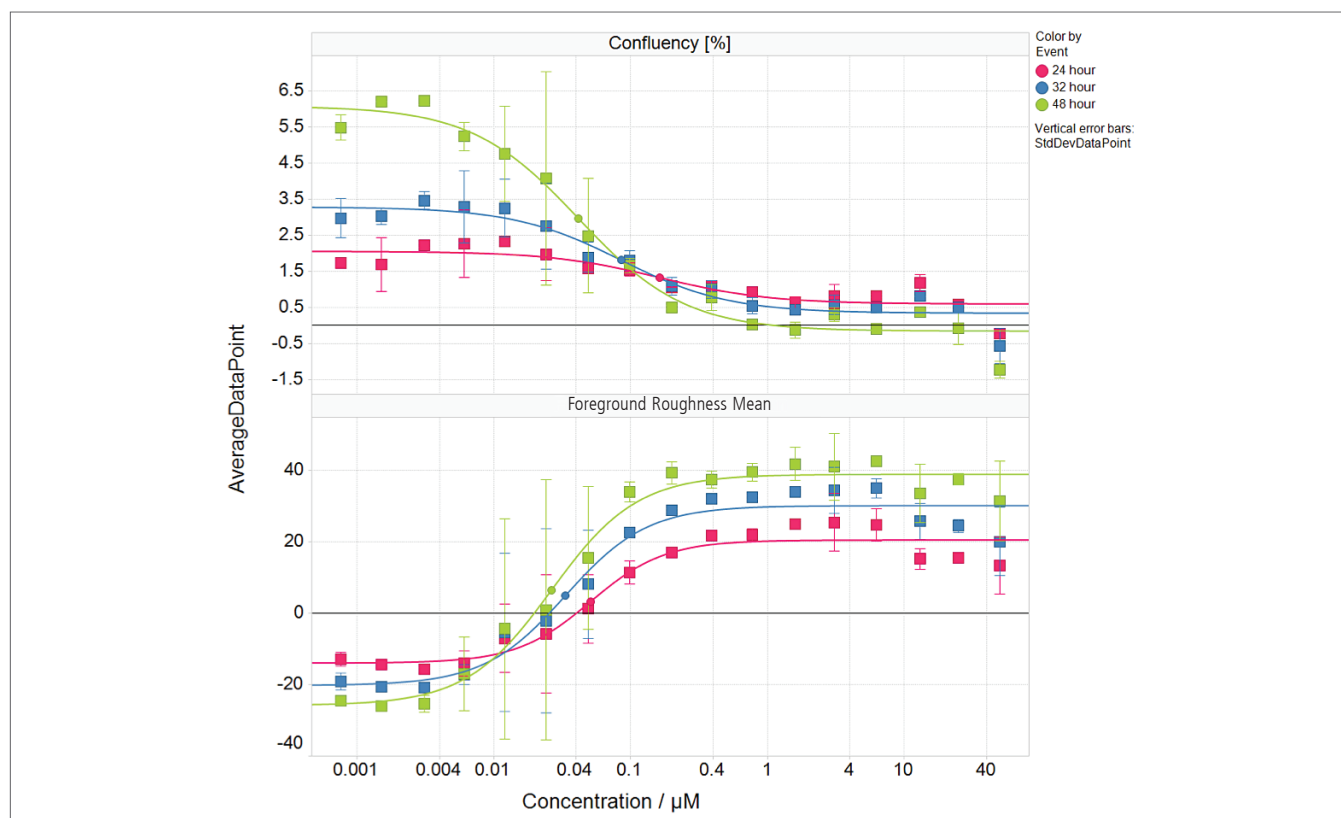


Figure 3. Dose and time dependent effect of Colchicin on MCF-7 cells using different readout parameter of brightfield imaging. Cells seeded 24 h previously and then baseline images taken immediately after compound addition ("treatment") and then at subsequent 24, 32 and 48 h time points after treatment. Top: Change of cellular confluency (%) since treatment versus compound concentration (μM). Bottom: Change of Foreground Roughness Mean versus compound concentration (μM).

Time-dependent dose response data for the “Change in Foreground Roughness” are shown in Figure 4 for the panel of test compounds screened against the MCF-7 cell line. At all except the higher concentrations, the non-toxic compounds Carbamoylcholin and Pilocarpine show decreases in the Foreground Roughness parameter indicating no substantial toxic effects on the cells. A similar lack of toxicity was observed in the ATP endpoint point assay (data not shown) for these two compounds. Colchicine, Paclitaxel and Trichostatin A (TSA) all showed dose dependent changes in roughness parameters, but

the onset of the effect is markedly slower for TSA (compare 48 h dose response curves). This slower onset may reflect that TSA acts in the cell by altering of gene expression through its inhibition of epigenetic enzymes. Both Colchicine and Paclitaxel bind tubulin and disrupt mitosis, albeit via different paths. Interestingly, Gentian Violet does not show dose dependent changes in “Foreground Roughness”, but there is dose-dependent behaviour in the ATP assay (data not shown). One explanation is that Gentian Violet a colored compound which may influence the readouts.

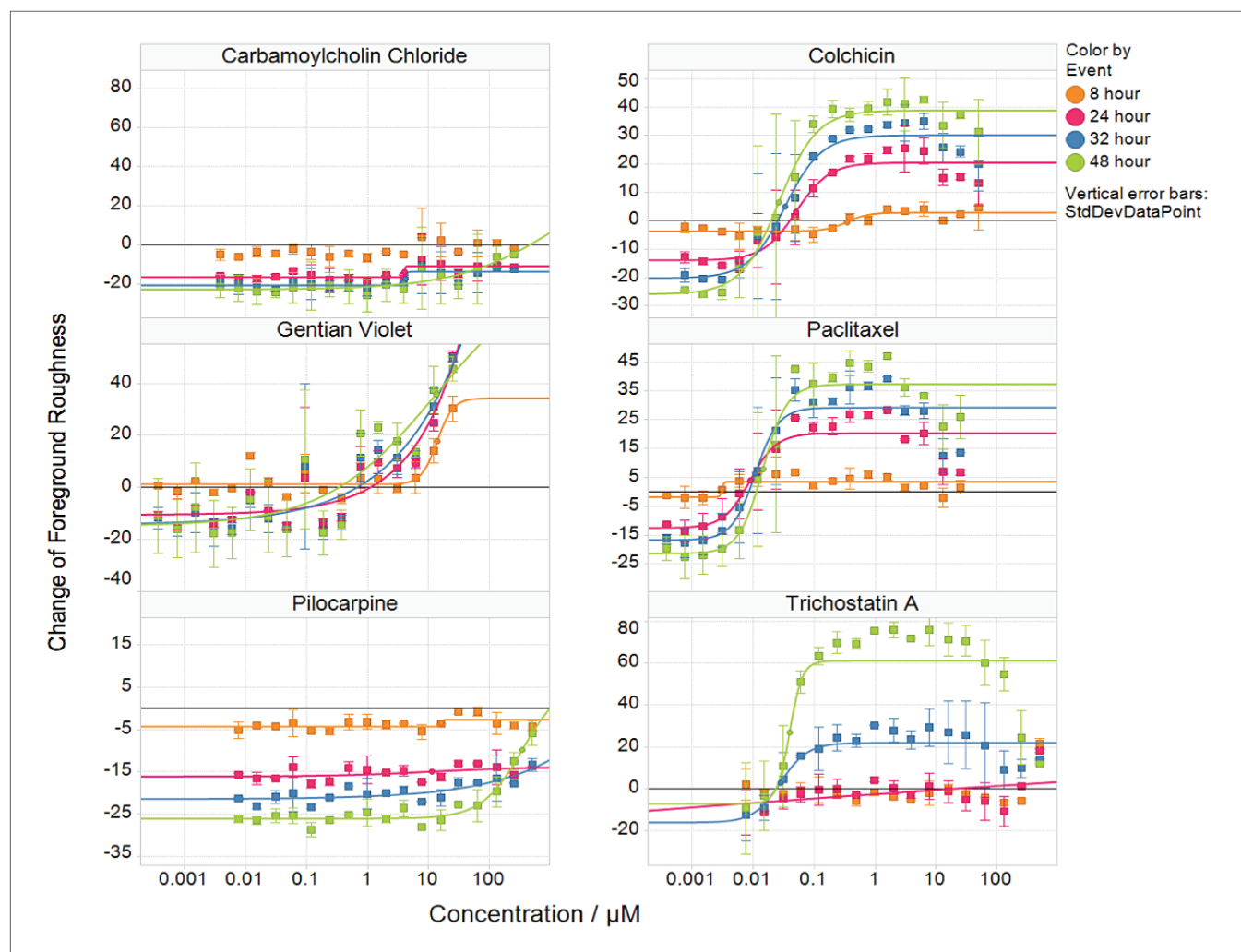


Figure 4. The change in roughness parameter versus compound concentration in μM for compound panel treated MCF-7 cells. Cells seeded at $T=0$ and compounds added at 24 h later. Imaging of plates in EnSight reader at 24 h (immediately before compound addition as reference) and at 8 h, 24 h, 32 h and 48 h after treatment.

The fitted results for the 4 cytotoxic compounds dose responses are summarised in Table 2 for the MCF-7 and 786-0 cell lines. Data were fitted to a four parameter logistics with no constraints using TIBCO Spotfire® and “Nfit” indicates that a robust data fit

was not possible. It can be seen that, with the exception of Gentian Violet, there is good concordance between the IC_{50} values determined using the ATP detection and the texture based analysis.

Table 3. RIC values (in μM) for cytotoxic test compounds against MCF-7 and 786-0 cell lines. Luminescence readout (ATP) was at 72 h after seeding (48 h after treatment). Derived image parameters: Change in Confluency and Foreground Roughness. Cells seeding at 0 h and compound added at 24 h. Robust fitting could not be performed on 0 h and 8 h data after treatment (results not shown) as at these points in time changes in cell viability were not apparent.

MCF7 Compound	ATP 48 h	Change of Confluency			Change of Foreground Roughness		
		24 h	34 h	48 h	24 h	34 h	48 h
Colchicin	0.079	0.162	0.087	0.042	0.048	0.032	0.026
Gentian Violet	4.119	Nfit	Nfit	Nfit	Nfit	18.929	5.875
Paclitaxel	0.004	0.016	0.017	0.019	0.009	0.011	0.015
Trichostatin A	0.037	Nfit	Nfit	Nfit	0.002	0.032	0.039

786-0 Compound	ATP 48 h	Change of Confluency			Change of Foreground Roughness		
		24 h	34 h	48 h	24 h	34 h	48 h
Colchicin	0.056	0.215	0.269	0.145	0.026	0.021	0.015
Gentian Violet	0.608	7.677	6.971	7.486	Nfit	Nfit	Nfit
Paclitaxel	0.036	0.037	0.069	0.082	0.021	0.025	0.011
Trichostatin A	0.537	Nfit	Nfit	Nfit	Nfit	0.094	0.125

Conclusion

The following benefits of image-based methods for determining cellular cytotoxicity were observed during this study:

1. Brightfield imaging on the EnSight system produces cell viability assessments which are consistent with data from ATP determination.
2. Brightfield imaging represents a non-destructive readout which, unlike endpoint methods as ATP determination, allows for gathering of data on the kinetics of possible toxicity effects.
3. By performing baseline evaluation of the cell numbers before compound addition, it is possible to distinguish between cytotoxic and cytostatic effects on a well-by-well basis.
4. Extracted morphological features such as "Cell Roughness" provide useful secondary readouts which complement the basic confluency readouts.
5. Rapid imaging time (< 5 min per plate) means unnecessary perturbations to cells are minimised.
6. Initial results show that optimisation of image analysis for individual cell lines is likely to be straightforward.
7. Set-up of the image analysis methods for new cell lines is straightforward and in addition to the 2 lines discussed here, protocols have been put in place for a further 10 lines (HeLa, CHO, HEK, NIH3T3, MCF-7, A431, A549, 786-0 and MDA-NB).

References

1. Shoemaker RH (2006): The NCI60 human tumour cell line anticancer drug screen. *Nature Rev Cancer*; 6, 813-823.
2. Niles AL, Moravec RA, Riss TL (2009): In Vitro Viability and Cytotoxicity Testing and Same-Well Multi-Parametric Combinations for High Throughput Screening. *Curr Chem Genomics*, 3, 33-41.
3. Chan GKY, Kleinheinz TL, Peterson D, Moffat JG (2013): A Simple High-Content Cell Cycle Assay Reveals Frequent Discrepancies between Cell Number and ATP and MTS Proliferation Assays. *PLoS ONE*, 8(5), e63583. doi:10.1371/journal.pone.0063583.