

Evaluate Compound Toxicity Early in the Drug Discovery Process Using High Content Analysis in Cell-Based Assays

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Abstract

Test compounds may have undesired side effects in addition to the desired modulation of a specific signaling pathway. Here we describe the use of high content cell-based assays to monitor such effects. In addition to the primary activity readout, many image analysis algorithms used for high content analysis report secondary readouts describing the cellular morphology, including number of cells, cell size and rounding, and nuclear morphology. Cell loss or changes in cell morphology as a consequence of compound treatment indicate acute compound cytotoxicity, and significant changes in the nuclear morphology are a sign of apoptosis and/or DNA damage. It is quite common that compounds which induce cytotoxic effects emerge as hits in small molecule screens and profiling studies. Such compounds can be discarded as false positives if secondary high content parameters are fully exploited. In this study, we show that this strategy may be applied to the Redistribution technology.

Introduction

The use of cell-based assays in research and in drug discovery is becoming increasingly widespread. The advantage of cellular systems over cell-free biochemical assays is the ability to collect information about target proteins, pathways, or protein-protein interactions in a cellular environment. Applying high content analysis to cell-based assays allows for more sophisticated cellular analysis. In parallel to the primary assay readout, for example the response of a target protein to a test compound, secondary changes in cellular morphology are often observed. Such changes in cell morphology may be

due to either on-target effects of the compound or to unwanted off-target effects of the compound such as cytotoxicity.

Measurement of cell or nuclear shape, size, intensity and number frequently results in valuable indications of possible side effects of a test compound. For example, it is well known that compounds that induce apoptosis change chromatin structure, cause nuclear condensation, cell shrinkage and membrane blebbing.

Another advantage that can be drawn from secondary parameters derived from high content analysis is the distinction between true and false positives. Take for example an assay that uses fluorescence intensity to monitor translocation of a protein from the cytoplasm to the nucleus. In this case a cytotoxic compound that causes the cell to round up will come out as a positive when in fact all it is doing is causing the cell to round up. Cell rounding both lifts the nucleus out of the focal plane and increases the layer of cytoplasm that lies above and below the nucleus. To imaging algorithms this will be interpreted as translocation. By including secondary

readouts that detect cell-rounding, such a compound can be flagged to distinguish it from compounds that have true activity.

To demonstrate the type of secondary readouts that can be obtained from high content analysis and the value of these to identify false-positives and cytotoxic effects, we ran four Redistribution assays using a range of toxic and non-toxic compounds. Redistribution assays are high content cell-based assays which monitor the cellular translocation of GFP-tagged proteins in response to drug compounds or other stimuli [1, 2, 3].

Materials and Methods

Redistribution Assays

Cells stably transfected with ATF6, CB1, FKHR/Foxo1, or Akt2 fused to EGFP were seeded in 96-well plates. The assays were performed according to the product manuals (Product IDs 084_01, 051_01, 008_01, and 011_02). Briefly, the cells were incubated with test compounds as indicated in Table 1.

The assays were run in triplicates using test compounds in nine-point

Table 1. Selected Redistribution assays and their characteristics

Assay	Description	Incubation time	Translocation event
ATF6	Sensor of ER-stress	5 hours	Endoplasmic Reticulum (ER) to Nucleus translocation. Located in the ER under basal conditions. In response to ER stress ATF6 is cleaved and translocates to the nucleus [4, 5, 6].
CB1	GPCR. Receptor for cannabinoids	2 hours	Receptor internalization (spots) Expressed on the cell surface membrane and internalized in endosomes in response to agonists [7, 8].
FKHR	Transcription factor in the PI3K pathway	1 hour	Cytoplasm to Nucleus translocation. Translocates from the cytoplasm to the nucleus in response to inhibition of the PI3K pathway [9].
Akt2	Ser/Thr kinase in the in the PI3K pathway	4 minutes	Membrane to Cytoplasm translocation. Translocates to the cell membrane in response to activation of the PI3K pathway by IGF. Wortmannin is used as reference inhibitor [10, 11].

Key Words

- High Content Analysis
- Cell-Based Assays
- Secondary Readouts
- Flag Compound Toxicity
- Identify False Positives

concentrations at half log dilutions as indicated in Table 2. Cells were fixed and stained with 7 μ M DAPI instead of 1 μ M Hoechst. At this concentration DAPI gives an intense staining of the nuclei and weaker staining of the cytoplasm, which allows for identification of both the nucleus and cytoplasm by image analysis.

Image acquisition and primary analysis

The assay plates were imaged and analyzed on a Cellomics Arrayscan V^{TI} with Cellomics Toolbox software. The analysis of the primary data was performed as described in the product manuals. Briefly, the images were acquired using the XF100 filter cube with one image of the DAPI stain and one image of the GFP signal. The Molecular Translocation BioApplication was used for the ATF6 and FKHR assays, Cytoplasm to Cell Membrane BioApplication for Akt2, and SpotDetector Bioapplication for CB1. The percent activities of compounds were calculated relative to the positive and negative control wells present on all plates. All assay plates passed a quality criterion of $Z' > 0.3$ on the primary output parameter.

High Content Analysis

The acquired images were subjected to a secondary analysis in the ColocalizationV3 Bioapplication. The nucleic and cytoplasmic areas were defined and a number of output parameters related to nucleus and cell size, shape, and intensity were exported. These outputs were normalized to similar readouts derived from negative control wells within the assay plates. Note that it was not necessary to repeat imaging of the assay plates on the ArrayScan since the same set of images were used for both the primary analysis of GFP localization and the secondary analysis of cell morphology parameters.

Results

Selection of assays and compounds

To investigate whether Redistribution assays are suitable for extracting information about compound cytotoxicity and fluorescence based on cellular morphology readouts, four Redistribution assays were selected based on their differences in translocation events and incubation times

(Table 1). The selected Redistribution assays were Activator of Transcription Factor 6 (ATF6), Cannabinoid Receptor 1 (CB1), FOXO1A (FKHR), and Akt2. A panel of 16 test compounds was selected, including true positives (reference compounds), fluorescent compounds, and compounds displaying a range of cytotoxic profiles spanning from DNA damage inducers to protein kinase inhibitors (Table 2).

Primary data analysis

The primary readout of Redistribution assays is the translocation event of a GFP fusion protein and

compounds that generate a translocation event are classified as active in a given assay. To determine which compounds were active in the selected assays, concentration response curves were performed in triplicates. EC50 values and maximum activities relative to positive and negative control values were calculated. As expected, the reference compound for each assay was found to be active, for example WIN55212-2 was active in the CB1 assay and wortmannin in the Akt2 assay. Furthermore, several of the known toxic compounds were found to be active in the four assays (Table 3).

Table 2. Panel of test compounds

Compound	Cellular effect	Conc. range
Aphidicolin	G1/S cell cycle arrest	10 nM-100 μ M
Camptothecin	Topoisomerase inhibitor, DNA damage	3 nM-30 μ M
Anisomycin	Protein synthesis inhibitor, JNK activator	3 nM-30 μ M
Paclitaxel	Stabilization of microtubules, G2/M cell cycle arrest	1 nM-10 μ M
Doxorubicin	Fluorescent, DNA damage inducer	1 nM-10 μ M
CellTracker (CMFDA)	Fluorescent compound	1 nM-10 μ M
WIN55212-2	CB1 receptor reference agonist	1 nM-10 μ M
Tunicamycin	Induces ER stress, N-glycosylation inhibitor, ATF6 activation	1 nM-10 μ M
Rotenone	Inhibitor of mitochondrial function, inhibitor of ATP synthesis	1 nM-10 μ M
Valinomycin	Ionophore	0.1 nM-1 μ M
Thapsigargin	ATPase inhibitor, leads to release of Ca ²⁺ in cytoplasm	0.1 nM-1 μ M
PMA	Kinase activator	0.1 nM-1 μ M
Staurosporine	Broad kinase inhibitor, induces apoptosis	0.1 nM-1 μ M
Jasplakinolide	F-Actin stabilizer	0.1 nM-1 μ M
Wortmannin	Akt2 and Foxo1 reference compound, PI3K inhibitor	0.1 nM-1 μ M
Okadaic Acid	Phosphatase inhibitor	0.1 nM-1 μ M

Compound name, cellular effect and concentration range are indicated.

Table 3. Active compounds in selected assays

Assay	ATF6		FKHR		CB1		Akt2	
	EC50	Max	EC50	Max	EC50	Max	EC50	Max
Compound								
Aphidicolin								
Camptothecin	—	40%						
Anisomycin	—	50%						
Paclitaxel								
Doxorubicin	—	380%	—	25%				
CellTracker Green	—	120%	—	181%			1.03E-06	34%
WIN55212-2	2.89E-06	70%			1.71E-08	85%		
Tunicamycin	3.78E-07	107%					3.29E-06	31%
Rotenone					1.23E-06	39%		
Valinomycin								
Thapsigargin	3.41E-10	80%						
PMA								
Staurosporine	2.44E-08	146%	4.40E-08	134%			—	148%
Jasplakinolide	2.79E-08	232%	1.38E-07	65%			1.42E-07	22%
Wortmannin			1.08E-08	108%			3.40E-08	105%
Okadaic Acid	1.15E-07	367%						

Activities less than 20% compared to the relevant positive control compound were discarded. EC50 values are shown where curve fitting could be applied. Max = maximal activity relative to positive control (assay specific reference compound).

Figure 1 shows concentration response curves in the ATF6 Redistribution assay of the reference compound Tunicamycin and the known apoptosis inducer Staurosporine.

Secondary data analysis

To determine whether the active compounds were true positives or false positives due to cytotoxic side effects, a set of seven output parameters were selected and applied to all the assays. These parameters include cell number, nuclear intensity, nuclear area, variation in nuclear intensity, nuclear shape and cell area.

The values of the secondary output parameters were normalized to the negative control wells on each plate, enabling them to be compared in a concentration response graph. Any deviation from the normalization value of 1 indicates a change from control wells. Using the ATF6 assay as an example, it is immediately clear from the secondary data that the test compound Staurosporine affects the morphology of the cells (Figure 2B and 3).

Even at the lowest Staurosporine concentrations tested, there are clear signs of morphological changes (deviation from 1). At concentrations higher than 50 nM the magnitude of the changes increases dramatically. Thus, these data indicate that Staurosporine affects the cells already at the lowest concentrations, but there is a steep change in the extent of the cytotoxic response at around 50 nM and higher (Figure 2B). The detected effects on cell morphology caused by Staurosporine are evident when comparing to the reference compound Tunicamycin (Figure 2A) which has a clean profile in the ATF6 Redistribution assay.

Detection of false-positives and early indicators of cytotoxicity

Changes in cell morphology or fluorescence can be signs of compound cytotoxicity or fluorescence, respectively. The secondary readout data can be used to identify such changes and to determine whether these changes invalidate the primary readout data (activity in the assay) at a given concentration of the test compound.

For example, we know that cytoplasm to nucleus translocations are sensitive to cell rounding. A threshold to the average cell area output is a

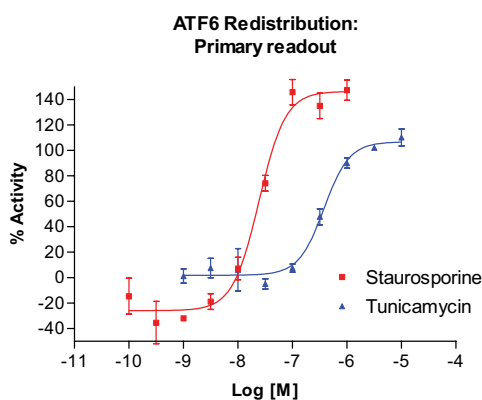


Figure 1. Comparison of concentration response curves for Staurosporine and Tunicamycin in the ATF6 Redistribution assay. Percent activity is calculated based on the negative and positive control well values. Each value represents the mean \pm standard deviation of three independent determinations.

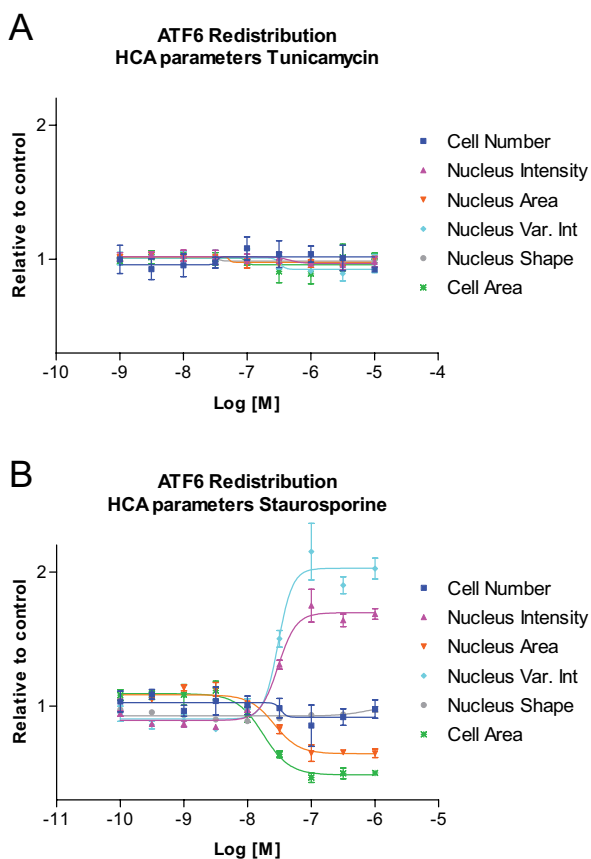


Figure 2. Comparison of Colocalization V3 high content output parameters derived from images of the ATF6 Redistribution assay treated with either Tunicamycin (A) or Staurosporine (B). Cells were stained with DAPI for cell morphology measurements. All data were normalized to negative control values. Each value represents the mean \pm standard deviation of three independent determinations.

method to alleviate such false activities, i.e. any images with average cell area smaller than this threshold value are excluded from the calculation of the nucleus to cytoplasm translocation. High cell loss is another common cause of invalidation of primary data, since accurate measurement of assay activity requires a certain number of cells or objects to be analyzed. An additional high content output is

whole cell fluorescence. This parameter can be exploited to flag wells where the cells have abnormal levels of fluorescence compared to cells in control wells.

When running many compounds in parallel it is inconvenient to examine data sets for all test compounds individually. For simplicity, thresholds were set for each secondary parameter based on inspection of the images,

variation in the secondary data and sensitivity of the primary readout to specific secondary readouts. For all assays a high cell loss was set to invalidate the primary data, since accurate measurement of assay activity requires a certain number of cells. From these values we constructed a heat map to clarify where significant changes in cell features were occurring and where these changes invalidated the primary data. In some cell assays a change in morphology is expected and the thresholds have to be adjusted accordingly.

By studying the heat maps for Tunicamycin and Staurosporine in the ATF6 Redistribution assay (Figure 4), we are able to remove four primary data points in the Staurosporine concentration response curve. At these concentrations, we observe a significant change in cell area due to cell rounding, which prevents a valid determination of the primary assay response. After removing these values we conclude that Staurosporine is a false positive due to toxic side effects.

Applying the heat map method to all tested compounds in each assay reveals that several of the positive hits could be discarded as false positives (Table 4). Some compounds are flagged to be associated with “high” cytotoxicity in which case any activity is invalid. Other compounds are associated with “low” cytotoxicity and any activity may be used bearing in mind that the compound may have potential cytotoxicity problems. An example of this is Thapsigargin which, as expected, has a positive activity in the ATF6 Redistribution assay, but also shows limited signs of cytotoxicity. This kind of indication can be used when ranking hit compounds if potential off-target effects are unwanted. The nature of a change in cell morphology may also guide researchers in further testing to clarify the mechanism of any off-target effects.

It is clear from the assays tested that the longer time the compounds are in contact with the cells, the more changes in secondary output parameters are detected. This simply means that short incubation times generally lead to fewer disturbances from compound off-target effects.

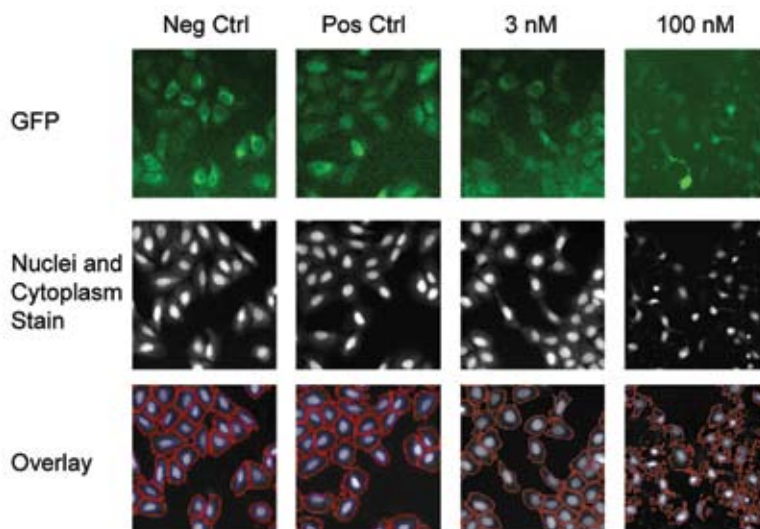


Figure 3. Images and analysis of ATF6 cells treated with Tunicamycin (Positive control) and Staurosporine. It is clear that the response to Tunicamycin is “clean” whereas the response to Staurosporine is accompanied with cytotoxicity. The degree of cytotoxicity results in an invalid analysis of the translocation at 100 nM Staurosporine. Below is an overlay based on the ColocalizationV3 bioapplication used for the secondary parameters. The nuclei and cell areas are calculated on the basis of the whole cell stain.

	Staurosporine					Tunicamycin				
	Log(M)					Log(M)				
Cell Number										
Nuc. Intensity										
Nuc. Area										
Nuc. Var. Int										
Nuc. Shape										
Cell Area										
Cell fluorescence										

Figure 4. Heat map of secondary parameter values. Each column represents a concentration of compounds, each row a secondary parameter. Yellow label indicates a value outside the set thresholds determined as deviation from normal. If a value is expected to invalidate the primary response, it is labeled red.

Table 4. Active compounds in selected assays after correction for false-positives

Assay	ATF6		FKHR		CB1		Akt2	
	EC50	Max	EC50	Max	EC50	Max	EC50	Max
Aphidicolin								
Camptothecin	—	40%						
Anisomycin								
Paclitaxel								
Doxorubicin			—	25%				
CellTracker Green CMFDA								
Win55212-2	2.89E-06	70%			1.71E-08	85%		
Tunicamycin	3.78E-07	107%					3.29E-06	31%
Rotenone					1.23E-06	39%		
Valinomycin								
Thapsigargin	3.41E-10	80%						
PMA								
Staurosporine							—	148%
Jasplakinolide							1.42E-07	22%
Wortmannin			1.08E-08	108%			3.40E-08	105%
Okadaic Acid								

Activities less than 20% compared to the relevant positive control compound were discarded. EC50 values are shown where curve fitting could be applied. Corrections are indicated as follows: False positives (red), significant signs of morphological or fluorescence changes (yellow), and strong morphological or fluorescence changes that could have caused a false positive (orange). Max. = maximal activity relative to the positive control.

Conclusion

The advantage of high content analysis is the ability to collect multiparametric information about how the cells are affected by test compounds or other stimuli. In this study, we show that high content data collected during screening or profiling studies represents valuable information for selection and de-selection of compounds for further testing. We show that high content data can identify changes in cellular morphology and fluorescence and that this data can be used to flag compounds with potential cytotoxicity problems. We were able to remove false-positive hits by studying changes in cell morphology and whether these had detrimental influence on the primary data. Furthermore, we were able to flag compounds that caused minor changes in cell morphology, suggesting these may be associated with cytotoxicity although they did not have direct consequences for the primary assay readout.

This study makes a compelling reason for performing cell-based assays in a high content manner and including the type of analysis shown here. This way, researches can avoid spending time and resources on validating false positive compounds, and instead concentrate on ranking true positive compounds based on cytotoxicity indications.

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