

# Utilizing secondary high content readouts in cell-based assays as a tool to evaluate compound toxicity early in the drug discovery process

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## Abstract

Many users of high content screening assays do not take advantage of the breadth of possible readouts; instead the normal procedure is to reduce the raw data to one primary readout activity. That, coupled to the fact that many drug discovery screening units struggle with panels of assays to both get functional and toxicity data on test compounds, has prompted us to develop a simple generally applicable method for extracting cytotoxicity effects from high content assay data sets in addition to the primary activity readout. The method involves the use of a whole cell nuclear dye that stains the nucleus more than the cytoplasm, such as DRAQ5 and DAPI. Application of such dyes instead of nuclear stains, for example Hoechst, allows extraction of readouts for nuclear morphology, cell rounding, fluorescence, and cell number. We show that these secondary readouts are good indicators of compound toxicity and if used in combination with a primary readout, toxic compounds can be de-selected or down-prioritized early in the drug screening process.

## Introduction

### Using Redistribution@cell based assays

The use of cell-based assays in research and in drug discovery is becoming increasingly widespread. Redistribution assays are high content cell-based imaging assays where a target has been coupled to EGFP. Such assays can be used for profiling and screening of targets or pathways.

The use of a cellular system for analysis of a response allows the researcher to extract information about a pathway, protein-protein interactions, or modifications in a cellular context, as opposed to a cell-free in vitro assay. But even more information than the response of a given target can be extracted if high content analysis is performed.

### Cytotoxicity in High Content assays

In parallel to a compound-induced translocation of a target protein, the cell also responds to the compound. This can be both due to on-target effects of the compound, but also due to unwanted side effects of the compound such as cytotoxicity. Measurement of cell or nucleus shape, size, intensity, or number can give important indications about possible side effects of such a compound. For example, it is well known that compounds that induce apoptosis change chromatin structure, cause nuclear condensation, cell shrinkage and membrane blebbing. Since high content assays are image based, one can extract more information about these cellular features.

### False positives in High content assays

Take for example an assay that uses fluorescence intensity to monitor translocation of a protein from the cytoplasm to the nucleus. A cytotoxic compound will appear to be positive, when in fact all it is doing is causing the cell to round up. This rounding both lifts the nucleus out of the focal plane and increases the layer of cytoplasm that lies above and below the nucleus. On the images, this appears as translocation. An advantage of high content analysis is that it can help distinguish between true positives and false positives in an assay. By including secondary readouts that detect cell-rounding, such a compound can be flagged to distinguish it from compounds that have true effects on the translocation of the target protein.

To test these possibilities a panel of compounds were tested in four Redistribution assays for identification of activity, cytotoxicity and fluorescence.

## Methods

### Assay Selection

The 4 assays were selected to cover different translocations and different incubation times (see table 1).

**ATF6** is a sensor of ER stress. The cell line expresses GFP-ATF6 which is located in the ER under basal conditions. In response to ER stress it is cleaved and translocates to the nucleus. The positive control compound tunicamycin induces ER stress by inhibiting glycosylation. (1,2,3,4)

**CB1** is the receptor for cannabinoids. CB1-EGFP is expressed on the cell surface membrane and is internalized in response to agonists. WIN55212-2 is a CB1 agonist and used as control compound.(5,6)

**FKHR** is a transcription factor that translocates from the cytoplasm to the nucleus in response to activation of the PI3K pathway. The assay is performed as an antagonist assay with wortmannin as antagonist. (7)

**Akt2** is translocated to the cell membrane in response to activation of the PI3K pathway. The assay was performed in antagonist mode, using IGF-1 as agonist and wortmannin as antagonist. (8,9)

Assay	Pathway	Incubation time	Translocation type
ATF6	ER-Stress	5 hours	Nucleus-Cytoplasm
CB1	GPCR	2 hours	Spots
FKHR	PI3K	1 hour	Nucleus-Cytoplasm
Akt2	PI3K	4 minutes	Membrane to cytoplasm

Table 1. Redistribution assays selected

### Compound Selection

The 16 compounds were chosen to contain true positives (the reference compounds for the four assays), fluorescent compounds, and compounds that cover a large range of cytotoxic profiles, spanning from DNA damage inducers to protein kinase inhibitors. The panel is shown below.

Compound	Effect	Highest Test Concentration
Aphidicolin	G1/S cell cycle arrest	100 $\mu$ M
Etoposide	Topoisomerase inhibitor, DNA damage	30 $\mu$ M
Antisomycin	Protein synthesis inhibitor, DNA activator	30 $\mu$ M
Paclitaxel	Stabilization of microtubules; G2M cell cycle arrest	10 $\mu$ M
Doxorubicin	Fluorescent and DNA damage inducer	10 $\mu$ M
CellTracker Green CMFDA	Fluorescent compound	10 $\mu$ M
WIN55212-2	Agonist CB1	10 $\mu$ M
Tunicamycin	Agonist ATF6, inhibition of N-glycosylation $\leftrightarrow$ ER stress	10 $\mu$ M
Rotenone	Inhibitor of mitochondrial function, inhibitor of ATP synthesis	10 $\mu$ M
Valinomycin	Ionophore	1 $\mu$ M
Thapsigargin	ATPase inhibitor, leads to release of Ca <sup>2+</sup> in cytoplasm	1 $\mu$ M
PMA	Kinase activator	1 $\mu$ M
Staurosporine	Broad kinase inhibitor – induces apoptosis	1 $\mu$ M
Jaspilkinolide	F-actin stabilizer	1 $\mu$ M
Wortmannin	PI3K inhibitor, antagonist Akt and Foxo1	1 $\mu$ M
Oxidac Acid	Phosphatase inhibitor	1 $\mu$ M

Table 2. Compound panel selected

### Assay procedure

Assays were performed as described in product manuals, except for the replacement of Hoechst with 7  $\mu$ M DAPI. The assay plates were imaged and analyzed on an ArrayScan VTI 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 for the DAPI stain and one image for GFP. 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 the quality criterion of Z > 0.3 on the primary output parameter.

## Results

### Primary analysis results

The results from the primary analysis is shown in table 3. Several hits were identified in the four assays – this would have been the result of a pure primary analysis with no secondary parameters. An example of two positive concentration response curves is shown in Figure 1.

Assay	ATF6		FKHR		CB1		Akt2	
	EC50	Max	EC50	Max	EC50	Max	EC50	Max
Aphidicolin	-	40%	-	-	-	-	-	-
Etoposide	-	50%	-	-	-	-	-	-
Antisomycin	-	-	-	-	-	-	-	-
Paclitaxel	-	380%	-	25%	-	-	-	-
Doxorubicin	-	120%	-	181%	-	-	1.03E-06	34%
CellTracker Green CMFDA	2.88E-08	70%	-	-	1.71E-08	85%	3.29E-06	31%
Tunicamycin	3.78E-07	107%	-	-	1.23E-06	39%	3.29E-06	31%
Rotenone	-	-	-	-	-	-	-	-
Valinomycin	3.41E-10	80%	-	-	-	-	-	-
Thapsigargin	-	-	-	-	-	-	-	-
PMA	2.44E-08	146%	4.40E-08	134%	-	-	-	148%
Staurosporine	2.79E-08	232%	1.38E-07	65%	-	-	1.42E-07	22%
Jaspilkinolide	1.15E-07	36%	1.08E-08	100%	-	-	3.45E-08	100%
Oxidac Acid	-	-	-	-	-	-	-	-

Table 3. Activity of compounds in ATF6, FKHR, CB1, and Akt2. Activity less than 20% was discarded. EC50 values are shown if a proper fit could be made. Max. indicates maximal activity.

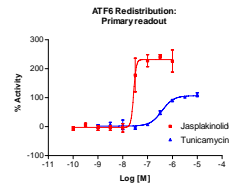


Figure 1. Concentration response curves of Jaspilkinolide and Tunicamycin in the ATF6 Redistribution assay. Percent activity was calculated on the basis of the reference compound response. Presented values are average of three determinations with standard deviation indicated.

### Secondary analysis

Using the images for a secondary analysis provided us with more information about the compound activities. The images were reanalyzed with ColocalizationV3 for parameters related to cell or nucleus size, shape, number, and intensities. A set of seven output parameters were selected and applied to all the assays. The values of the secondary output parameters were normalized to the negative control wells on each plate, enabling them to be plotted together on a concentration response graph. Secondary data for two compounds in the ATF6 assay are shown in Figure 3.

It is immediately clear that the test compound jaspilkinolide affects the morphology of the cells. At low concentrations the changes are minor (deviation from the value 1), but at higher concentrations the changes in cell and nuclei area increase. This indicates that jaspilkinolide is cytotoxic. The difference is clear when comparing to tunicamycin, which has a clean profile in this assay (ATF6).

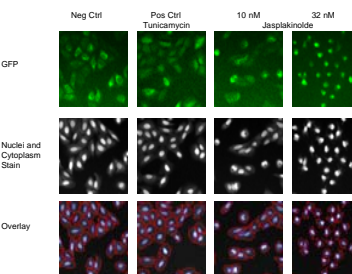


Figure 2. Images and analysis of ATF6 cells treated with tunicamycin and Jaspilkinolide. It is clear that the response to tunicamycin is clear, whereas the response to jaspilkinolide is accompanied with cytotoxicity. The degree of cytotoxicity results in a invalid analysis of the translocation. Below is an overlay image of the ColocalizationV3 bioapplication used for the secondary parameters. The nuclei and cell area are calculated on the basis of the whole cell stain.

### Simplifying the data for easy analysis

The secondary readout data can be used in two ways.

- To flag changes in cell morphology or fluorescence that indicate cytotoxicity or compound fluorescence.
- To determine whether these changes are sufficient for the primary readout data (activity in the assay) to be invalid for a given concentration of the test compound.

For simplicity, thresholds were set for each secondary parameter based on inspection of the images and on sensitivity of the primary readout in the individual assays to cell rounding, etc. For all assays a high cell loss also invalidated 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.

### Removal of false positives - Indications of cytotoxicity

We can then use the heat maps to remove false positives (marked red in Table 4) and flag morphological/fluorescence changes that are indications of cytotoxicity/fluorescence. As is evident from the figure below, we have now removed the false positives and we can rank the hit compounds based on the cytotoxicity information presented.

It is clear from the assays tested that the longer time the compound is in contact with the cells, the more sensitive the secondary output parameters of that assay are in detecting cell changes. However, since most of the false positives are found because of changes in cell morphology, the need for detection goes hand in hand with the sensitivity.

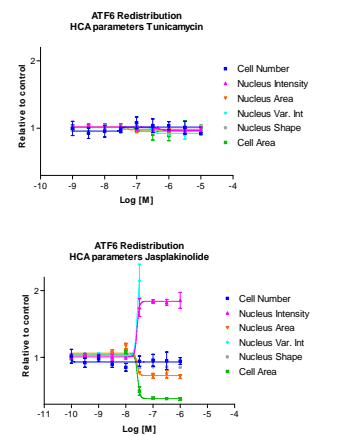


Figure 3. Concentration response curves in secondary parameters of Jaspilkinolide and Tunicamycin in the ATF6 Redistribution assay. Values are normalized to the values of the negative control wells on the same plate. Presented values are average of three determinations with standard deviation indicated.

Assay	ATF6		FKHR		CB1		Akt2	
	EC50	Max	EC50	Max	EC50	Max	EC50	Max
Compound								
Aphidicolin								
Etoposide		42%						
Antisomycin								
Paclitaxel								
Doxorubicin				25%				
CellTracker Green CMFDA								
WIN55212-2	4.68E-08	70%	-	-	1.71E-08	85%	3.29E-06	31%
Tunicamycin	3.78E-07	107%	-	-	1.23E-06	39%	3.29E-06	31%
Rotenone								
Valinomycin								
Thapsigargin	3.41E-10	80%						
PMA								
Staurosporine								
Jaspilkinolide							1.42E-07	22%
Wortmannin							3.45E-08	100%
Oxidac Acid								

Table 4. Activity of compounds in ATF6, FKHR, CB1, and Akt2. Activity less than 20% was discarded. EC50 values are shown if a proper fit could be made. Max. indicates maximal activity. Red background indicates the response to be a false positive, yellow background indicates significant signs of morphological or fluorescence changes.

## Summary

- We set out to show that the high content data collected during screening or profiling studies can be used to identify false positive hits.
- We can remove data points both because of an effect of cell morphology on the primary data, but also because of compound fluorescence.
- In addition we can flag changes in cell morphology that can be signs of cytotoxicity.
- Although this is not a comprehensive testing of many compounds, it shows that these features can be measured and used. We are currently using high content parameters on a larger set of assays and compounds.
- This kind of analysis should be performed on all high content assays to get the most information per well.

## References

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