Chapter 8

High-Throughput Automated Confocal Microscopy Imaging Screen of a Kinase-Focused Library to Identify p38 Mitogen-Activated Protein Kinase Inhibitors Using the GE InCell 3000 Analyzer

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Abstract

The integration of fluorescent microscopy imaging technologies and image analysis into high-content screening (HCS) has been applied throughout the drug discovery pipeline to identify, evaluate, and advance compounds from early lead generation through preclinical candidate selection. In this chapter we describe the development, validation, and implementation of an HCS assay to screen compounds from a kinase-focused small-molecule library to identify inhibitors of the p38 pathway using the GE InCell 3000 automated imaging platform. The assay utilized a genetically modified HeLa cell line stably expressing mitogen-activated, protein-activating protein kinase-2 fused to enhanced green fluorescent protein (MK2–EGFP) and measured the subcellular distribution of the MK2–EGFP as a direct readout of p38 activation. The MK2–EGFP translocation assay performed in 384-well glass bottom microtiter plates exhibited a robust Z-factor of 0.46 and reproducible EC_{50} and IC_{50} determinations for activators and inhibitors, respectively. A total of 32,891 compounds were screened in singlicate at 50 μM and 156 were confirmed as inhibitors of p38-mediated MK2–EGFP translocation in follow-up IC_{50} concentration response curves. Thirty-one compounds exhibited IC_{50}s less than 1 μM, and at least one novel structural class of p38 inhibitor was identified using this HCA/HCS chemical biology screening approach.

Keywords: High-content imaging, High-content analysis, High-content screening, Confocal microscopy, Kinase, p38, MAPKAP-k2, GFP, InCell.

1. Introduction

The mitogen-activated protein kinases (MAPK) sit at key nodes of the signaling pathways for extracellular stimuli that regulate the

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fundamental processes of cells in both normal and diseased states *(1–5)*. The p38 (reactivating kinases (RKs) or p40) kinase module is known to mediate stress responses activated by heat shock, ultraviolet light, bacterial lipopolysaccharide, and proinflammatory cytokines, and p38 MAPK has been a major target for drug discovery by the pharmaceutical industry *(3, 6–8)*. Mitogen-activated protein kinase-activating protein kinase-2 (MK2) is a substrate of the p38 MAPK, and phosphorylation of MK-2 by p38 induces a nucleus to cytoplasm translocation *(9–12)*.

The generation and characterization of a stable MK2–EGFP-expressing cell line that was subsequently utilized as an HCS assay to screen for inhibitors of the p38 MAPK signaling pathway has been described previously *(12, 13)*. Briefly, HeLa cells (ATCC, CCL-2) were infected with MK2-ehanced green fluorescent protein (EGFP) retrovirus, placed under selective antibiotic pressure for 2 days, then sorted by flow cytometry to isolate single-cell clones that were then expanded and characterized. Interestingly, the majority of the single-cell clones with very high fluorescent signal did not show optimal signal-to-noise ratio in the MK2–EGFP translocation assay (data not shown). Several clones displayed a MK2–EGFP translocation concentration response to anisomycin with EC_{50} values ranging from 23 to 35 nM (**Fig. 8.1**). However, a cell line expanded from a single-cell clone designated "A4" (MK2–A4) that exhibited a translocation response that was very sensitive to both anisomycin and TNF-α was used in a screening campaign to identify inhibitors of the p38 pathway.

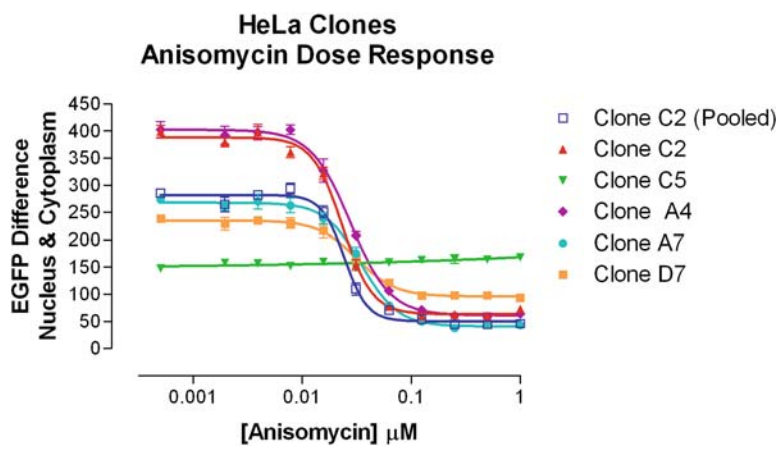


Fig. 8.1. **Comparison of HeLa MK2–EGFP clones induced by anisomycin**. Cells selected for clonal expansion were seeded in 96-well plates and treated with the indicated concentrations of anisomycin for 25 minutes to stimulate MK2–EGFP translocation. Cells were fixed with 3.7% formaldehyde and 2 μg/ml of Hoechst 33342 for 10 minutes, images were acquired on the Cellomics ArrayScan II (Cellomics (Thermo Fisher), Pittsburgh, PA), and analyzed for MK2–EGFP translocation using the nuclear translocation bioapplication.

There are several commercial turnkey HCA/HCS automated imaging platforms on the market but the GE InCell 3000 is a unique combination of several features, such as laser line scanning confocality, environmental control, on-board pipetting, and fast unorthodox algorithms to quantify fluorescent cellular objects. The InCell 3000 is a confocal line scanning imager that projects a line of illumination into the specimen using three independent water-cooled lasers with excitation wavelengths of 351–364, 488, and 635 nm and images the fluorescence emission simultaneously on three independent water-cooled CCD line cameras (blue, green, and red). The InCell 3000 is equipped with a fixed Nikon 40 × 0.6 NA ELWD objective, which allows a large field of view (0.75 × 0.75 mm) that at 0.6 mm pixilation provides 1280 × 1280 pixels, and 100–900 cells/frame depending upon the seeding density utilized and is capable of 10 μm resolution. It has a near-IR fiber-coupled laser tracking autofocus that is very fast, with a time to focus of between 100 and 150 msec for up to 40 μm and with z-position focus errors around <0.2 mm in glass plates and around 0.5 mm in plastic plates. The InCell 3000 has two peristaltic pumps for pipetting and an environmental chamber to control temperature, CO_2, and relative humidity, which provides kinetic live well imaging capability. The InCell 3000 system is capable of high resolution in X, Y, and Z. The InCell 3000 acquires and saves images that can be analyzed on the fly, or postacquisition, using the priority "Raven" software from GE Healthcare and the appropriate image analysis modules to produce feature sets appropriate to the assay being run. The InCell 3000 imaging platform has been designed to provide high-throughput image acquisition and analysis capability *(14–16)*. The multiple excitation lasers and CCD cameras of the InCell 3000 when combined with the appropriate selection of fluorescent probes and sample cell density enable the user to utilize short exposure times together with simultaneous parallel acquisition of multiple fluorescent channels from a single field of view to achieve fast scanning times of ≤10 minutes for a 384-well plate.

1.1. Image Analysis and Algorithm

The nuclear trafficking analysis module is dependent upon cells labeled with nuclear dyes such as Hoechst 33342, DAPI, DRAQ5, or any other dye that fluorescently stains cellular DNA. The nuclear dye fluorescent signal is used to identify the nuclear region and to define a nuclear mask overlay based on thresholding of object size and intensity of fluorescent probe inside the object (**Fig. 8.2A**). The nuclear mask overlay is eroded by a user-defined pixel threshold to reduce cytoplasmic contamination within the nuclear area (**Fig. 8.2B**), and the final reduced mask overlay can be used to quantify the amount of fluorescence inside the nuclear area in any selected channel. The nuclear mask overlay is then dilated by

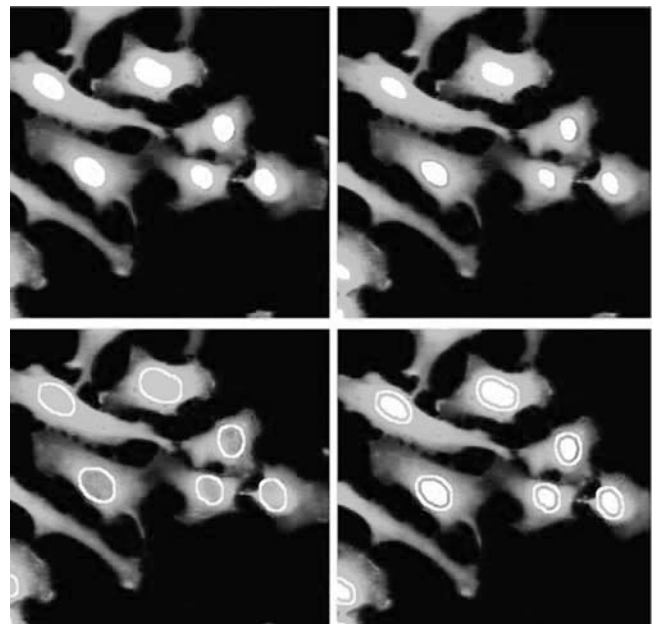


Fig. 8.2. **Nuclear trafficking analysis module**. (**A**) *Upper Left*: In images acquired on the InCell 3000, cells labeled with Hoechst 33342 nuclear dye were used to identify the nuclear region and to define a nuclear mask in white, based on thresholding, size filtering, and fluorescence intensity criteria. (**B**) *Upper Right*: The mask is eroded to reduce cytoplasmic contamination within the nuclear area and the final reduced mask is used to quantify the amount of target channel fluorescence within the nucleus. (**C**) *Lower Left*: The nuclear mask is dilated to cover as much of the cytoplasmic region as possible without going outside the cell boundary. Removal of the original nuclear region from this dilated mask creates a ring mask that covers the cytoplasmic region outside the nuclear envelope. (**D**) *Lower Right*: The nuclear trafficking analysis module calculates the ratio between the nucleus and cytoplasmic intensities in the target channel. For the MK2–EGFP translocation assay, the intensity of the EGFP fluorescence is measured in the eroded nuclear mask area and divided by the EGFP intensity in the cytoplasmic ring area to give a Nuc:Cyt ratio that is calculated on a per cell basis, which may also be reported as a well-averaged value.

a user-defined pixel threshold to cover as much of the cytoplasmic region as possible without going outside the boundary of the cell. When the original nuclear region is removed from this dilated overlay mask, this creates a ring mask that covers the cytoplasmic region outside the previously defined nuclear area (**Fig. 8.2C**). The nuclear trafficking analysis module calculates the intensity ratio between the nucleus and cytoplasmic regions in the target channel. For the MK2–EGFP translocation assay, the intensity of the EGFP fluorescence is measured in the eroded nuclear mask area and divided by the EGFP intensity in the cytoplasmic ring area (**Fig. 8.2D**) to give a Nuc:Cyt ratio that is calculated on a per cell basis, which may also be reported as a well-averaged value. In the MK2–EGFP translocation assay, "no translocation" readout is a

result of a high Nuc:Cyt ratio, while "positive translocation" is a result of a low Nuc:Cyt ratio numerical value. There are several data output features generated by the Raven software, which are summarized in **Table 8.1**.

Table 1
Description of plate image algorithmic module data analysis output parameter features

Plate	Plate Name
Cycle	Number of passes at this plate. Always zero for this MAPKAP project
Well	Well number, varies from A1 to P24
Msg	Errors such as dry well or focus error. 0 if no problems
NPasNc	Number of nuclei found passing both intensity and size filters
NPasSg	Number of cells found which pass above filters and cytoplasm intensity filter (see signal sampling threshold below), set at 100 cts for the original screen data
NPasAq	Number of cells passing both of the above filters and for which the fraction of pixels is above the threshold shown in the secondary analysis parameters. A significant difference between NPasSg and NPasAq is evidence of a toxic response
Nuc/Cyt	Ratio of the intensity in the eroded nuclear mask to the dilated cytoplasm ring, both measured in the signal channel (green)
Std Dev	Cell by cell std Dev of the above
Nuc Intsty	Average intensity over the well in the eroded nuclear mask measured in the signal channel (green)
Cyt Intsty	The well average intensity of the cytoplasm sample rings
Aqlity	A secondary analysis parameter. A threshold for cytoplasm intensity is chosen (see below). A cell by cell calculation is made of the fraction of the pixels above this threshold. The fraction of cells with their ring area above the quality threshold expressed as a percentage is Aqlity
Mrk Mode	The most probable intensity in the nuclear marker channel. This is a convenient measure of the background intensity in the marker channel (blue for the MAPKAP screen)
Sig Mode	The most probable intensity in the signal channel. This is a convenient measure of the background intensity in the signal channel (green for the MAPKAP screen)

1.2. MK2–EGFP Translocation Assay Development

Microscopic observation shows that the vast majority of MK2–EGFP fluorescence in the A4 HeLa cell clone appears localized within the nuclear boundaries with relatively little signal apparent in the cytoplasm of the cell (**Fig. 8.3A**). However, when the cells undergo treatment with an activator of the p38 MK2 pathway such as anisomycin or TNF-α, MK–EGFP redistributes from the nucleus into the cell's cytoplasm (**Fig. 8.3B**). In cells treated with a known inhibitor of the p38 pathway such as SB203580 in conjugation with anisomycin, stimulation fails to induce redistribution of MK2–EGFP from the nucleus to the cytoplasm (**Fig. 8.3C**). Inhibition of MK2–EGFP translocation by SB203580 is equally effective whether cells are pretreated with inhibitor or added simultaneously with an activator of the p38 MAPK pathway. By using the InCell 3000 Raven software nuclear trafficking analysis module, an anisomycin concentration response for translocation was derived from the calculated ratio of MK–EGFP fluorescence signal in the nucleus to the cytoplasm. Similarly, when the HeLa A4 cells were treated with the p38 inhibitor SB203580 and subsequently stimulated with anisomycin, an inhibition curve of MK2–EGFP translocation from the nucleus to the cytoplasm in cells was derived using the InCell 3000 Raven software nuclear trafficking analysis module (**Fig. 8.4**).

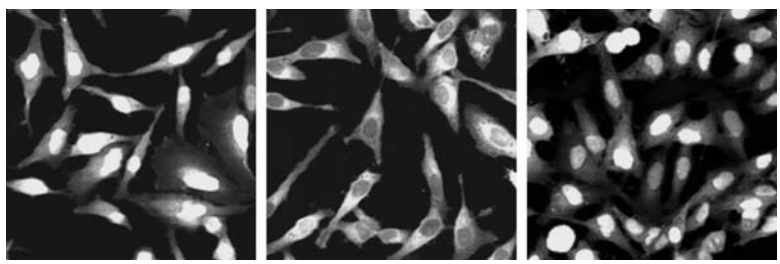
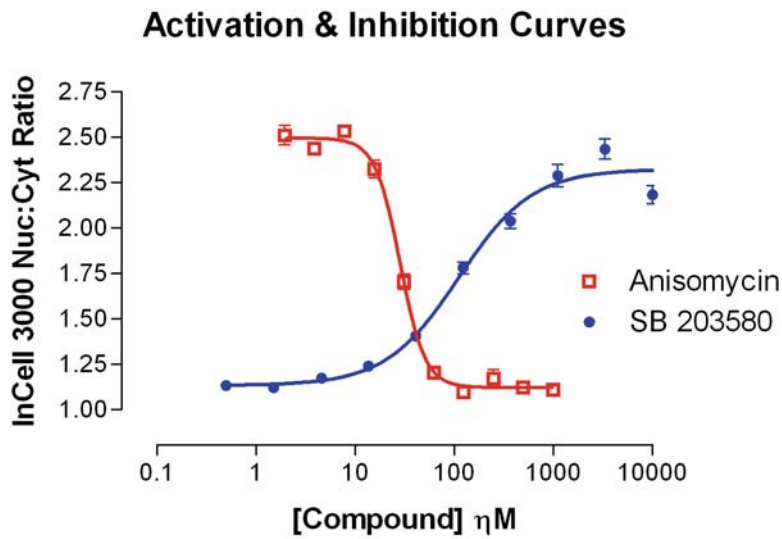


Fig. 8.3. **Images of MK2–EGFP translocation**. (**A**) *Left*: Unstimulated control cells displaying predominantly MK2–EGFP nuclear localization. (**B**) *Middle*: Cells stimulated with 100 nM anisomycin for 25 minutes display translocation of MK2–EGFP from the nucleus to the cytoplasm. (**C**) *Right*: Cells simultaneously treated with 10 μM SB203580 and 200 nM anisomycin for 40 minutes; the p38 inhibitor blocks MK2–EGFP translocation to retain MK2–EGFP predominantly in the nucleus, similar to images from untreated media controls.

The assay was originally developed and validated in 96-well plastic plate format, then transferred to 384-well glass plate format to accommodate the high-end optics and confocality and robustness of the InCell 3000 analyzer *(12)*. We followed the HTS guidelines for development and validation of this assay including development end points such as cell seeding density, time course of activation and inhibitor responses, 3-day EC_{50} of activator and 3-day IC_{50} of inhibitor, DMSO tolerance, and whole plate Z-factors, which were all optimized before progressing into screening compounds.



	Anisomycin	SB 203580
EC50	28.45	111.7

Fig. 8.4. **MK2–EGFP activation and inhibition curves in 384-well plates**. A total of 2.5 × 10^3 HeLa–MK2–EGFP A4 cells were seeded in 384-well Matrical glass plates in EMEM + 10% FBS and incubated overnight at 37°C and 5% CO_2. For activation of the response, HeLa A4 cells were treated with the indicated concentrations of anisomycin for 40 minutes, fixed in 3.7% formaldehyde + 2 μg/ml Hoechst dye and fluorescent images were collected on the InCell 3000. For inhibition of the response, the indicated doses of SB203580 were added simultaneously with the coaddition of 200 nM anisomycin (final) and plates were incubated for 40 minutes. Plates were fixed in 3.7% formaldehyde + 2 μg/ml Hoechst dye and fluorescent images were collected on InCell 3000. The nuclear trafficking analysis module was used to analyze the images captured on the InCell 3000 and quantify the Nuc:Cyt ratio response for anisomycin stimulation and SB203580 inhibition of anisomycin-induced MK2–EGFP translocation.

The optimal cell seeding density in 384-well Matrical plates was determined by testing cell densities at $2.5 \times 10^3, 5 \times 10^3, 10 \times 10^3$ cells per well. Cells were incubated overnight, then acutely treated with either media alone or 100 nM anisomycin for 40 minutes, or were pretreated with 1 μM of the p38 inhibitor SB203580 for 15 minutes followed by 40-minute treatment of 100 nM anisomycin. The translocation of MK2–EGFP was then quantified using the InCell 3000 analyzer with the nuclear trafficking analysis module. MK2–EGFP translocation was adequately measured at all three seeding densities (**Fig. 8.5**). Although the three cell seeding densities showed a "screenable" delta (max to min signal window), a seeding density of 2.5×10^3 cells/well was chosen for all further assay development for the best segmentation algorithm fit of cell objects and for reduction of the cell culture burden required in screening operation.

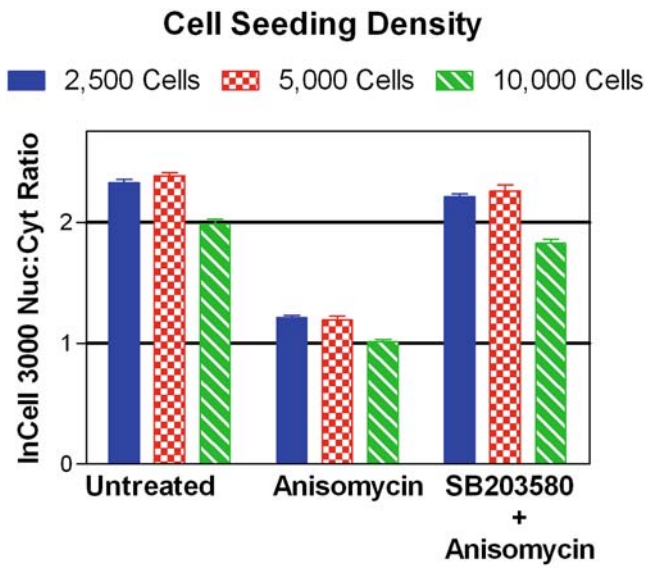


Fig. 8.5. **Cell seeding density**. The indicated numbers of HeLa–MK2–EGFP–A4 cells were seeded into each of the 384 wells of Matrical glass plates in EMEM + 10% FBS and incubated overnight at 37°C and 5% CO_2. Cells were treated with ±200 nM anisomycin for 25 minutes, fixed in 3.7% formaldehyde + 2 μg/ml Hoechst 33342 dye, fluorescent images were acquired on the InCell 3000, and the nuclear trafficking analysis module was used to quantify the Nuc:Cyt ratio translocation response.

Kinetic time course experiments to measure activation and inhibition of MK2–EGFP translocation were conducted to select the "ideal time" for an automated screening assay. In the activation time course when cells were either left untreated or stimulated with 100 nM anisomycin, cells showed an abrupt MK2–EGFP cytoplasmic translocation within 10 minutes and a peak response recorded after 20 minutes, and this remained unchanged for more than 40 minutes. After 60 minutes and up to 120 minutes there appears to be a slight increase in MK2–EGFP Nuc:Cyt ratio, suggesting a modest redistribution of MK2–EGFP back into the nucleus, although the Nuc:Cyt ratio never reestablishes pretreatment measurements (**Fig. 8.6A**). For compound screening logistics a 40-minute incubation time for anisomycin activation was selected for further assay development. In the inhibition time course, experiment cells were preincubated with SB203580 for the indicated times before anisomycin addition or SB203580 was added simultaneously with anisomycin. The effects on MK2–EGFP translocation were then quantified on the InCell 3000 by measuring the Nuc:Cyt ratio. Simultaneously adding SB203580 and anisomycin was as effective at blocking the MK2–EGFP Nuc:Cyt translocation event as preincubating with SB203580 prior to the anisomycin treatment (**Fig. 8.6B**). We elected to run the screen by simultaneously adding compounds and anisomycin to save time in screening operations.

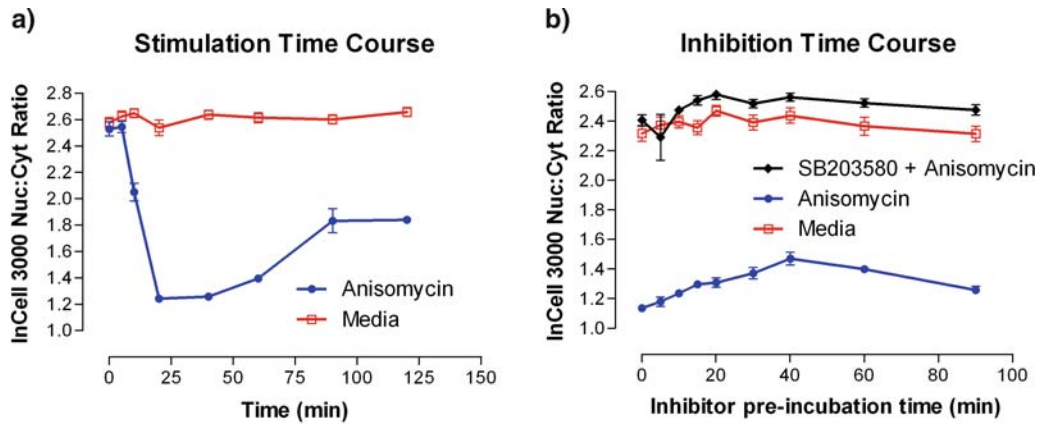


Fig. 8.6. **Time course experiments**. (**A**) **Stimulation time course:** 2.5 $\times 10^3$ HeLa–MK2–EGFP–A4 cells were seeded into each of the 384 wells of Matrical glass plates in EMEM + 10% FBS and incubated overnight at 37°C and 5% CO_2. Cells were treated with ±200 nM anisomycin for the indicated times, fixed in 3.7% formaldehyde + 2 μg/ml Hoechst dye, fluorescent images were collected on the InCell 3000, and the nuclear trafficking analysis module was used to quantify the Nuc:Cyt ratio translocation response. (**B**) **Inhibition time course**: 2.5 $\times 10^3$ HeLa–MK2–EGFP–A4 cells were seeded into each of the 384 wells of Matrical glass plates in EMEM + 10% FBS and incubated overnight at 37°C and 5% CO_2. The p38 inhibitor SB203580 was preincubated with the cells for the indicated times prior to the addition of 200 nM anisomycin. Plates were incubated for 40 minutes, fixed in 3.7% formaldehyde + 2 μg/ml Hoechst dye, fluorescent images were collected on the InCell 3000 and the nuclear trafficking analysis module was used to quantify the Nuc:Cyt ratio translocation response.

Since the compound library was dissolved in dimethyl sulfoxide (DMSO) for screening we tested the amount of DMSO that cells can tolerate before significantly altering the assay window. MK2–EGFP–A4 cells were treated with the indicated amounts of DMSO with and without 100 nM anisomycin for 40 minutes at 37°C, 5% CO_2, and 95% relative humidity. DMSO less than 0.625% did effect MK2–EGFP Nuc:Cyt translocation ratio (**Fig. 8.7A**). In contrast, DMSO concentrations greater than 0.625% altered MK2–EGFP Nuc:Cyt ratio as evident not only by the numerical data but also by altered cell morphology including nuclear swelling or shrinkage at very high DMSO concentrations, which resulted in a nuclear mask that is not proportional to the cytoplasmic mask as observed in normal untreated cells (**Fig. 8.7B**).

To further validate the MK2–EGFP translocation assay performance using the InCell 3000 imager and Raven software nuclear trafficking analysis module, we ran activation and inhibition concentration response experiments to determine the EC_{50} for anisomycin and the IC_{50} for SB203580 in the presence of anisomycin. About 2.5 $\times$ 10^3 cells/well were seeded in 384-well glass bottom Matrical plate overnight. Cells were treated with indicated concentrations of anisomycin or SB203580 plus a twofold increase in anisomycin (200 nM) for 40 minutes at 37°C, 5% CO_2, and 95% relative humidity. Using GraphPad Prism (San

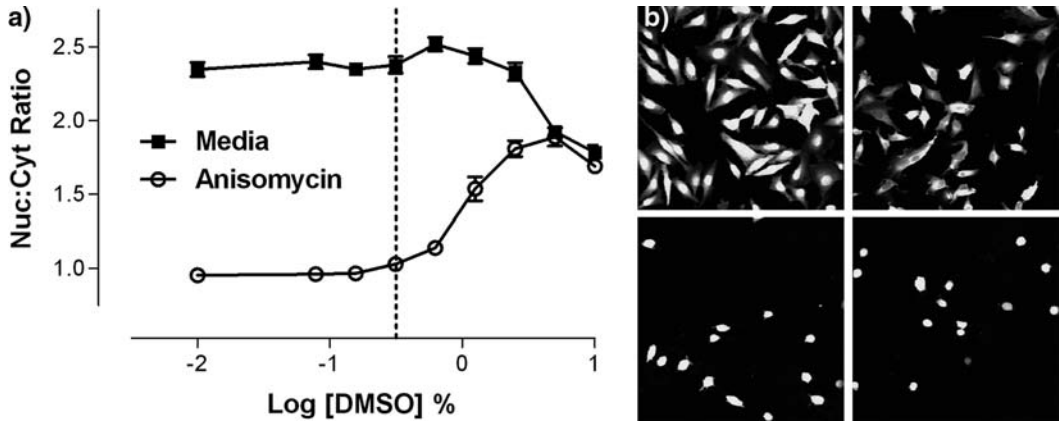


Fig. 8.7. **DMSO tolerance**. (**A**) A total of 2.5 $\times 10^3$ HeLa–MK2–EGFP–A4 cells were seeded in 384-well Matrical glass plates in EMEM + 10% FBS and incubated overnight at 37°C and 5% CO_2. Cells were treated with ±200 nM anisomycin containing the indicated concentrations of DMSO for 40 minutes, fixed in 3.7% formaldehyde + 2 μg/ml Hoechst dye, and fluorescent images were collected on the InCell 3000. The nuclear trafficking analysis module was used to analyze the images captured on the InCell 3000 and quantify the translocation response. (**B**) Images from untreated media control cells and 100 nM anisomycin treated cells in the presence or the absence of 5% DMSO.

Diego, CA) curve fitting and analysis software we obtained an EC_{50} for anisomycin stimulation of 28.45 nM and an IC_{50} for SB203580 of 112 nM, both had R2 curve fits greater than 0.95. It is clearly evident that the InCell 3000 can robustly measure changes in the Nuc:Cyt ratio in the HeLa–MK2–EGFP–A4 cell model using these assay conditions.

2. Materials

1. HeLa–MK2–EGFP–A4 stable clone cell line (*see* **Note 1**).
2. Cell culture maintenance medium: EMEM (Invitrogen/Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 2 mM l-glutamine (Sigma-Aldrich, St. Louis, MO), 10 mM HEPES (Biowhittaker, Walkersville, MD), 800 μg/ml G418 (Sigma-Aldrich, St. Louis, MO).
3. Trypsin-versene (Biowhittaker, Walkersville, MD).
4. Dulbecco's phosphate-buffered saline (DPBS) (Biowhittaker, Walkersville, MD).
5. Dimethyl sulfoxide (DMSO) (JT Baker, Phillipsburg, NJ).
6. 384-well black clear glass bottom plates (Matrical, Spokane, WA).
7. 37% formaldehyde (Sigma-Aldrich, St. Louis, MO).

8. Hoechst 33342 (Invitrogen/Molecular Probes, Eugene, OR).
9. Microplate Seals (Perkin-Elmer, Boston, MA).
10. MAPK stimulus – anisomycin (Sigma-Aldrich, St. Louis, MO).
11. p38 inhibitor SB203580 (Calbiochem, San Diego, CA).
12. p38 inhibitor RWJ68354 (synthesized in-house).
13. JNK inhibitor SP600125 (Calbiochem, San Diego, CA).
14. LOPAC collection (Sigma-Aldrich, St. Louis, MO).
15. Alexa-carboxylic acid (Invitrogen/Molecular Probes, Eugene, OR).
16. Oregon Green 488 (Invitrogen/Molecular Probes, Eugene, OR).

2.1. Working Solution

1. Cell plating medium: EMEM, 10 mM HEPES, 0.5% FBS, 800 μg/ml G418.
2. Fixation solution: 3.7% formaldehyde in PBS containing 2 μg/ml Hoechst 33342.
3. Flat field dye solution: combine Alexa-carboxylic acid or Alexa-succinimidyl ester with Oregon Green 488 in one or more wells in microwell plate. Seal top of plate and protect from light.

3. Methods

3.1. Cell Plating Procedure

1. HeLa–MK2–EGFP–A4 cells in EMEM media supplemented with 10% FBS, 2 mM l-glutamine, 10 mM HEPES, and 800 μg/ml G418 less than passage 20 are grown to 70–80% confluence in tissue culture flasks at 37°C, 5% CO_2, and 95% relative humidity.
2. Detach cell monolayer from tissue culture flasks using trypsin-versene rinse. Resuspend cells in complete media, count cells, and adjust cell density concentration to 6.25×10^4 cells/ml. If cells are clumpy, depending on your method of detachment, filter cells through 70 μm strainer that fits on top of the 50-ml conical tube (BD Falcon).
3. Plate 2500 cells/well (40 μl) using the Multidrop (Thermo Electron, Boston, MA) or other liquid dispenser in Matrical 384-well glass bottom plates (*see* **Note 2**). Allow plates to set at room temperature on flat surface for approximately 20 minutes before placing in incubator to minimize edge effects created by temperature fluctuations (*see* **Note 3**). Incubate overnight at 37°C, 5% CO_2, and 95% relative humidity. To

reduce edge effects and enhance equivalent cell monolayer, prewet bottom of Matrical 384-well glass plates with cell plating media prior to plating cells (*see* **Note 4**).

3.2. Assay Development for Stimulation Dose–Response

1. Make 4 × final concentration of anisomycin (100 nM) in cell plating media. Warm to 37°C in incubator. Add 20 μl of media, DMSO, or compound. Incubate for 15 minutes at 37°C. At desired time intervals, add 20 μl of prewarmed 4 × anisomycin to wells (*see* **Note 5**).
2. Remove media and fix cells by adding 20 μl of prewarmed formaldehyde (3.7% final) in PBS containing 2 μg/ml Hoechst 33342. Incubate at room temperature for 10–15 minutes. Remove formaldehyde and replace with 100 μl of PBS (*see* **Note 6**). It is important to remember to follow safety procedures when working with formaldehyde fixation. Always prepare stock solutions in fume hood and follow safety guidelines for use and proper disposal at your institution.
3. Cover tops of wells with plate seals (*see* **Note 7**).
4. Analyze on the InCell 3000 (*see* **Section 3.4**).

3.3. Automated Screening Protocol for MK2–EGFP Translocation

1. Prepare compounds and plate controls in 384-well plastic plates. Transfer 20 μl of prewarmed (37°C) plate controls and/or test compounds using the Beckman-Coulter Multi-Mek device or equivalent. Incubate plates at 37°C, 5% CO_2, and 95% relative humidity for approximately 40 minutes (*see* **Note 8**).
2. In a fume hood immediately add 20 μl/well of prewarmed formaldehyde and Hoechst 33342 fix solution using the Multidrop device. Incubate for 10–15 minutes at room temperature (*see* **Note 9**).
3. Remove fixation solution and wash cells twice leaving the last wash in wells with 100 μl of PBS using MultiMek device or equivalent.
4. Cover tops of plates with plate seals.
5. Analyze plate on the InCell 3000 platform (*see* **Section 3.4**).

3.4. Instrument Setup

Although we used the GE InCell 3000 confocal instrument in this study, any high-content imaging platform that is capable of two-color acquisition with adequate imaging resolution could be used such as the BD Pathway, Cellomics ArrayScan, Evotec Opera, InCell 1000, MDC MicroXpress, Yokogawa and PMT-laser based units such as the Acumen Explorer and Blueshift Isocyte.

1. Turn on InCell 3000 instrument and allow to warm up. Launch the Raven software and choose the nuclear trafficking algorithms in the online mode. Adjust the Enterprise-II

488-nm argon laser (Coherent, Santa Clara, CA) at 50% full power (~90 mW) and collect EGFP light emission through the 535/45-nm bandpass filter set and capture on the independent green CCD cameras (*see* **Note** 11).

2. Adjust the multiline UV (MLUV) argon Enterprise laser producing 351–364 nm light at approximately 10% full power (~5 mW). Hoechst 33342 excited light is captured through 450/65-nm emission filter and detected on the independent blue CCD camera.
3. Image scans sequentially, 488 nm first, then MLUV line to reduce photobleaching of fluors. Bin the image capture 2× to 280 pixels per line and adjust the laser lines (488-nm and MLUV) at an appropriate exposure time (usually less than 1-2s) for an optimally capture image without saturating the CCD camera chip with bright "hot-spot" pixels. At 640 × 640 pixels the resolution is about 1.2 μm pixels. Binning at 2× will increase throughput speed of plates on the InCell 3000.
4. In the online mode, set up the collection stops at a minimum of 100 cells/well or a maximum of 2 frames/well, whichever came first (*see* **Note** 12). Double check the instrument parameters and make any additional adjustments.
5. Prepare flat-field solution for calibrating the unevenness of fluorescence in wells. Place calibration solution in same type of plates used for cell plating. In the plate setup window in Raven, be sure that the correct flat-field correction wells are selected. Flat-field solution is used as a calibration procedure to correct nonuniform fluorescent intensities measured in the captured image across the well.
6. Begin acquiring images on the InCell 3000 controlled by the Raven software following procedures outlined by the user's manual.
7. Quantify captured images using the nuclear trafficking analysis module and determine if data from the population table are acceptable as compared and correlated with observed images (*see* **Note** 10). Make any necessary algorithm adjustments and reanalysis if appropriate.

3.5. Assay Validation

To determine the likelihood the assay can be used in screening operation over a period of days or weeks; it is necessary to perform day-to-day experiments to demonstrate the reproducibility and validity of the assay. We have outlined steps to run DMSO tolerance, 3-day validation on EC_{50} stimulation, 3-day validation on IC_{50} inhibition in the presence of stimulus, and Z-factor score to determine the signal window as it relates to variation in measured signal.

3.6. Guidelines for DMSO Tolerance Procedure

Use the maximum tolerable amount of DMSO possible without irreversibly affecting the assay performance (*see* **Note 13**). Test DMSO from maximum of 8% final, twofold dilution, 8 points to determine if and when the assay performance is altered with increasing DMSO concentrations (*see* graph X-7).

1. Plate cells (40 µl/well) in multiwell plates as previously described.
2. Add 20 µl (4 × final concentration) of DMSO to wells containing 40 µl of cells and media.
3. Add either 20 µl media or 20 µl anisomycin stimuli to induce MK2 signaling.
4. Stop reaction by fixation as previously described.
5. Analyze on InCell 3000 using Nuc:Cyt translocation module algorithm to determine the amount of redistribution of MK2–EGFP protein from the cytoplasm to the nucleus.

3.7. MK2–EGFP Translocation Assay Reproducibility and Signal Window Evaluation

Run three independent experimental EC_{50} dose–response assays on different days to assess the reproducibility of the MK–EGFP translocation response in HeLa–A4 cells after 40 minutes stimulation with the indicated concentrations of anisomycin (**Fig. 8.8A**). Run three independent IC_{50} dose–response assays using known inhibitors of the p38 pathway and at least one off-target selective compound to validate the assay model. The p38 inhibitor compounds SB203580 and RWJ68354 from three different runs produced an average IC_{50} of 101 and 84 nM, respectively (**Fig. 8.8B**,C). A selective inhibitor of the JNK pathway, SP600002, showed no evidence of MK2–EGFP translocation inhibition in the presence of anisomycin (**Fig. 8.8D**).

3.8. Procedure for 3-Day Reproducibility of Anisomycin EC_{50} Dose–Response

1. Plate cells as described in the cell plating procedure. Allow cells to attach overnight at 37°C.
2. Add 20 µl of media to wells. This is used to mimic compound addition and correct the volume for addition of stimuli. Use automated liquid handling device such as a MultiMek or equivalent.
3. Make 4 × final concentration of anisomycin starting at 1 µM (therefore make 4 µM concentration). Dilute twofold in media and at least 10 points.
4. Add 20 µl of anisomycin or other stimuli (*see* **Note 14**) to wells using automated liquid handling device such as a MultiMek or equivalent.
5. Incubate at 37°C, 5%CO_2 for 40 minutes.
6. Fix cells with formaldehyde as previously described.

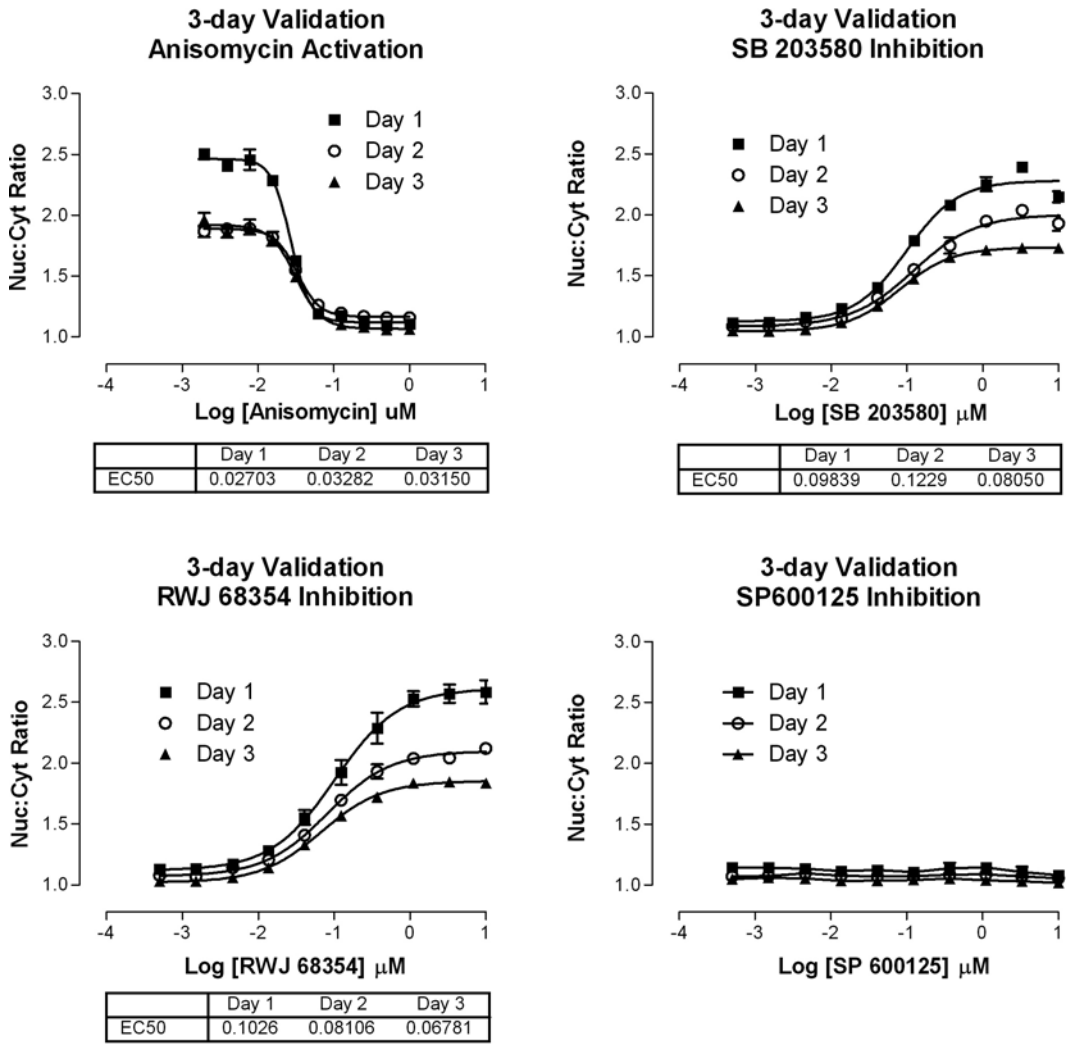


Fig. 8.8. **Three-day activation and inhibition curves. Three-day EC_{50} curves.** (**A**) A total of 2.5 $\times 10^3$ HeLa–MK2–EGFP–A4 cells were seeded in 384-well Matrical glass plates in EMEM + 10% FBS and incubated overnight at 37°C and 5% CO_2. For activation of the response, cells were treated with the indicated doses of anisomycin for 40 minutes, fixed in 3.7% formaldehyde + 2 μg/ml Hoechst dye, and fluorescent images were collected on the InCell 3000. Data are presented from three independent experiments, each performed in triplicate wells and run on separate days. **Three-day IC_{50} curves**. A total of 2.5 $\times 10^3$ HeLa–MK2–EGFP–A4 cells were seeded in 384-well Matrical glass plates in EMEM + 10% FBS and incubated overnight at 37°C and 5% CO_2. For inhibition of the response, the indicated doses of (**B**) SB203580, (**C**) RWJ68354, or (**D**) SP600125 were added simultaneously with the addition of 200 nM anisomycin (final) and plates were incubated for 40 minutes. Plates were fixed in 3.7% formaldehyde + 2 μg/ml Hoechst dye, and fluorescent images were collected on InCell 3000. Data are presented from three independent experiments, each performed in triplicate wells and run on separate days. The nuclear trafficking analysis module was used to analyze the images captured on the InCell 3000 and quantify the Nuc:Cyt ratios of anisomycin-stimulated MK2–EGFP translocation and the compound-mediated inhibition of anisomycin-induced MK2–EGFP translocation.

3.9. Procedure for 3-Day Reproducibility of Inhibitors of the p38 Pathway

1. Plate cells as described in the cell plating procedure.
2. Prepare inhibitor compounds at 4 × the final concentration starting at 10 μM, diluting threefold for a 10-point IC_{50} dilution series in media. Prewarm to 37°C
   a. SB203580
   b. RWJ68354, another inhibitor of the p38 pathway and
   c. SP600125 (this compound is used as a negative control). It is well documented *(17, 18)* to inhibit the JNK pathway and should not alter the translocation of MK2.
3. Prepare 200 nM final concentration of anisomycin. Make 4 × concentration (800 nM) and prewarm to 37°C.
4. Add 20 μl of inhibitors to wells using automated liquid handling device such as a MultiMek or equivalent.
5. Add 20 μl of anisomycin to wells.
6. Incubate at 37°C, 5%CO_2 for 40 minutes.
7. Fix cells with formaldehyde as previously described.
8. Analyze plates on InCell 3000.

3.10. Z-Factor Determination Procedure

To measure the robustness and variability of the automated assay signal window for the MK2–EGFP translocation assay in the HeLa–A4 cell line we determined the assay Z-factor *(19)*. HeLa–MK2–EGFP–A4 cells were treated with media to determine the minimum baseline response, 200 nM anisomycin to determine the maximum of the assay signal window, and SB203580 inhibitor in the presence of 200 nM anisomycin to determine a reference range for p38 inhibitor compounds (**Fig. 8.9**). The assay signal window of MK2–EGFP translocation was approximately 2.16-fold, based on an average Nuc:Cyt ratio media control of 2.374 and an average anisomycin-stimulated ratio of 1.1. A Z-factor of 0.46 indicated that the assay was compatible with HTS.

1. Plate cells as described in the cell plating procedure. Allow cells to attach overnight at 37°C.
2. Prepare three separate 384-well plates to contain (1) media, (2) 200 nM anisomycin, (3) p38 inhibitor reference compound plus 200 nM anisomycin. Prewarm plates to 37°C.
3. Add 20 μl of media, 200 nM of anisomycin, or reference compound with 200 nM anisomycin to the cell plate.
4. Incubate at 37°C for 40 minutes.
5. Fix cell plate with formaldehyde as previously described.
6. Analyze on the InCell 3000.

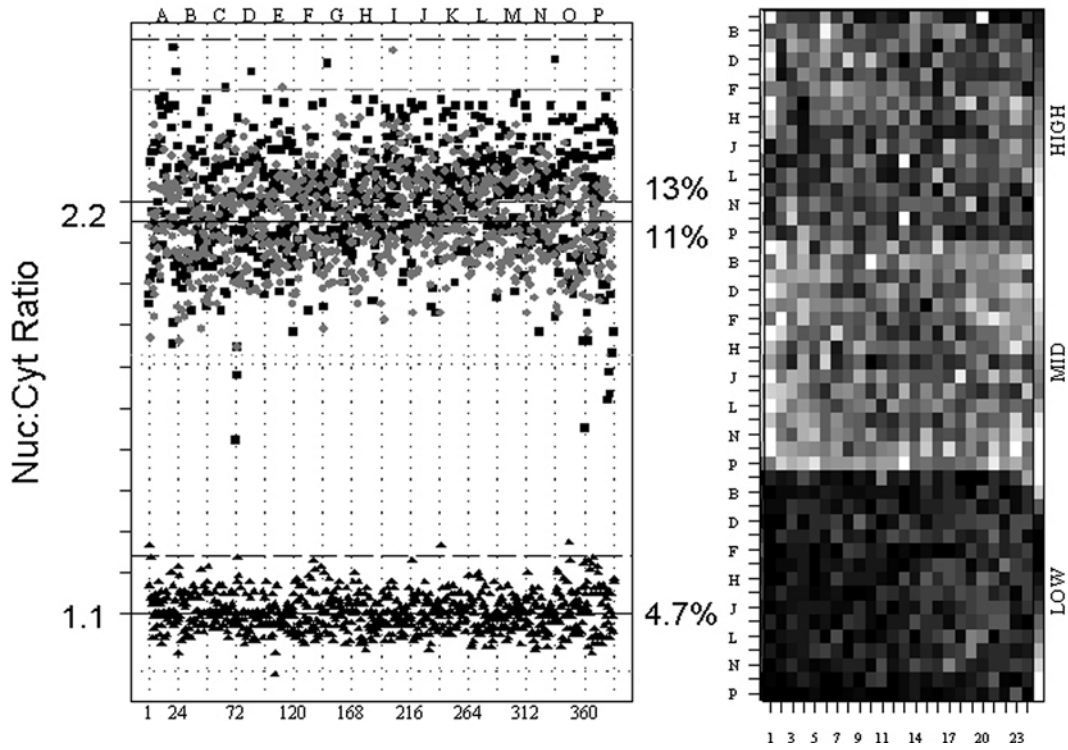


Fig. 8.9. **Assay signal window and variability assessment**. A total of 2.5 $\times$ 10^3 HeLa–MK2–EGFP–A4 cells were seeded in 384-well Matrical glass plates in EMEM + 10% FBS and incubated overnight at 37°C and 5% CO_2. Two full 384-well plates each were treated for 40 minutes under the following conditions; media alone (blue squares), 200 nM anisomycin (red triangles), and the p38 inhibitor SB203580 + 200 nM anisomycin (green circles). Plates were fixed in 3.7% formaldehyde + 2 μg/ml Hoechst dye and fluorescent images were collected on the InCell 3000. The nuclear trafficking analysis module was used to analyze the images captured on the InCell 3000 and quantify the anisomycin induction and/or SB203580 inhibition of the translocation response. The Z-factor was calculated according to the method of *(19)*. The Nuc:Cyt ratios from all the wells on the six 384-well plates are presented in (**A**) a scatter plot or (**B**) a plate heat map view.

3.11. LOPAC Screening MK2–EGFP Translocation HTS/HCS Assay for p38 Inhibitors

Prior to initiating the kinase-focused library screening campaign, test the MK2–EGFP assay model by screening the Library of Pharmacologically Active Compounds (LOPAC) (Sigma – RBI, St. Louis, MO) cassette in two separate runs.

3.11.1. Procedure to Test LOPAC and Compound Library

1. Plate cells as previously described.
2. Prepare compound plates using automated liquid handling robotics. Make working compound solution equal to 4 $\times$ the final concentration of 50 μM by diluting compound stocks (10 mM) into media containing less than 0.5% DMSO final.

3. Using liquid handling automation, add compounds from the LOPAC library cassette in single-well determinations. For the 4 × concentration, transfer 20 µl of compound to the cell plate.
4. Fix cell plate with formaldehyde as previously described.
5. Analyze on the InCell 3000.
6. Use an activity threshold of 50% inhibition active criterion to identify compounds that modified the MK2–EGFP Nuc:Cyt translocation ratio. If available, use data analysis and visualization software to review an overlay of the entire LOPAC screening run to cluster compounds with similar MK2 Nuc:Cyt translocation ratio.
7. If possible run the LOPAC compound library on a separate day to confirm reproducibility and correlate runs. For "hits" or "actives," run an IC_{50} dose–response curve to confirm activity.
8. Once satisfied with assay performance, begin screening compound libraries following the approach used in the LOAPC screen.

We identified at least nine active compounds in the single-point determination screen with MK2 Nuc:Cyt translocation ratios >1.8 and approaching the 2.4 ratio of the assay signal maximum plate controls (**Fig. 8.10**). There was good correlation between the two independent LOPAC screens with eight active

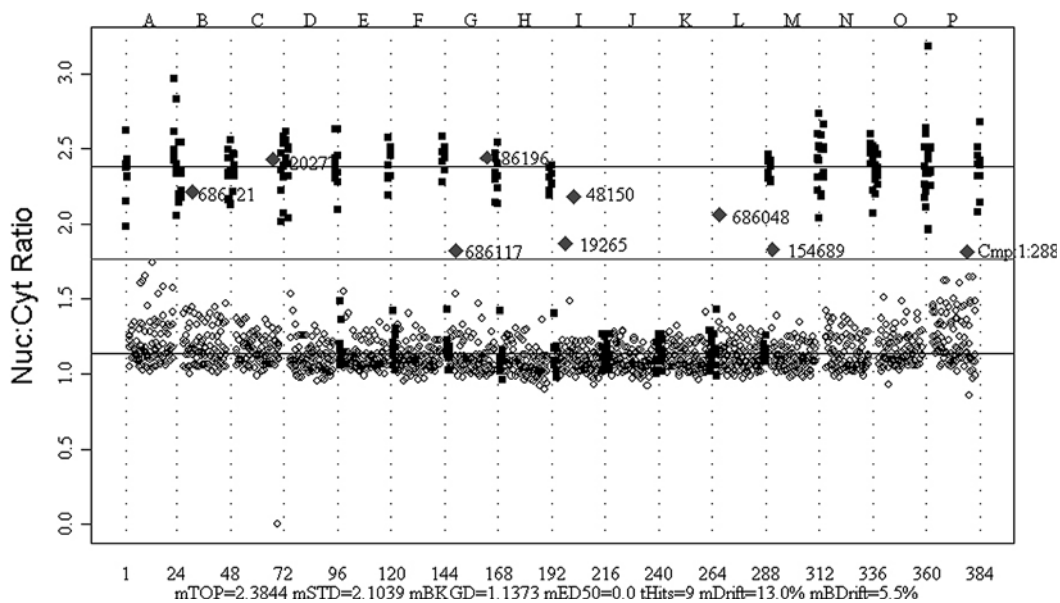


Fig. 8.10. **MK2–EGFP translocation assay LOPAC activity assessment**. Scatter plot overlay of three 384-well plate from the LOPAC screen plotted on a single graph. *X*-axis represents the well position from 384-well plate and the *y*-axis represents the Nuc:Cyt ratio activity translocation response. The diamonds indicate active or "hits" above the 50% inhibition threshold in the MK2–EGFP assay; the black squares represent maximum and minimum plate controls from the three plates; and the black open-round circles represent inactive compounds below 50% inhibition threshold.

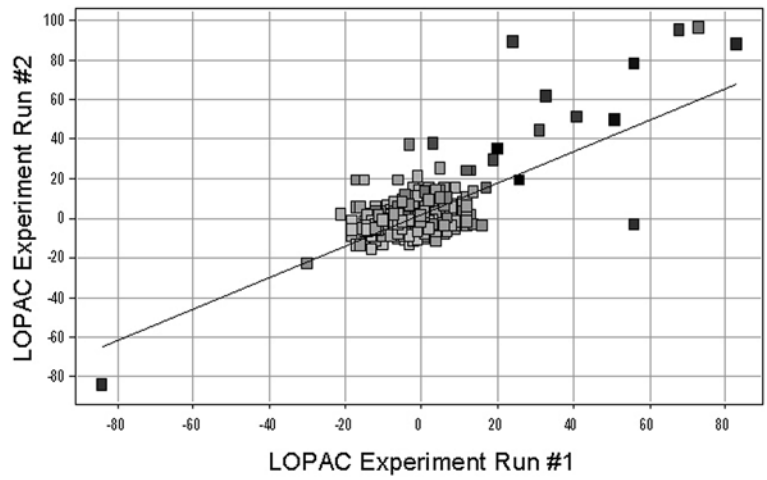


Fig. 8.11. **MK2–EGFP translocation assay – correlation between two independent LOPAC screening runs.** Correlation plot of 854 compounds run in two different LOPAC screening runs. *X*-axis represents screening run #1 and *y*-axis represents screening run #2.

compounds (0.91%) in screening run 1 and six active compounds (0.69%) in screening run 2 (**Fig. 8.11**). The active compounds were followed up in IC_{50} concentration response assays, which confirmed the six active compounds identified in both LOPAC runs. Interestingly, the six confirmed active compounds were from five different pharmacological classes and disease indications including the cognitive enhancer tacrine hydrochloride, the antipsychotic thioproperazine dimesylate, the vasodilator dilazep dihydrochloride, the antidepressant doxepin hydrochloride, and the antihypertensive compounds protoveratrine A and hexamethonium dibromide (**Fig. 8.12**). Upon review of the images from the wells of the six confirmed active compounds, it was apparent that five of the compounds significantly affected cell adherence or induced cytotoxicity at 50 μM, and one compound significantly increased the nuclear fluorescent signal, similar to nuclei intercalators such as Hoechst or propidium iodide. Additionally, the Raven software provided tools that allowed us to rapidly visualize wells with active compounds and assess the performance of the plate controls to provide a quick QC review of the screening data prior to uploading into the internal database (**Fig. 8.13**).

In a primary screen of 32,891 compounds, 110 × 384-well assay plates were processed over a 5-day period. An average of one frame or field of view per well was acquired by the InCell 3000 HCS imager, which required approximately 47.5 hours to scan and analyze all plates, with an average scan time of 25.84 minutes per 384-well plate or 4.02 seconds/well. Using 50% inhibition as a threshold criterion for activity, the majority of the compounds

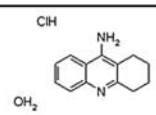
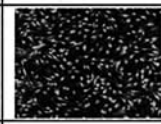
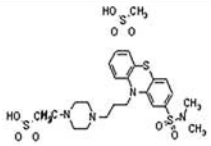
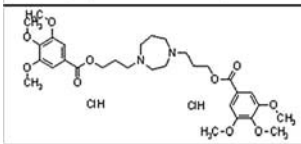
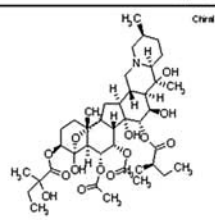
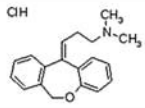
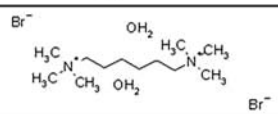
LOPAC Cassette from Sigma RBI			Rapid Data				
Structure	Compound Name	Pharmacological Class	Screening Run-1	Screening Run-2	IC50 (uM)	Comments	Images
	Tacrine hydrochloride hydrate	Cognitive enhancer	88	83	11.16	Nuclear fluorescent localization	
	Thioproperazine dimesylate	Antipsychotic	95	68	10.43	Cytotoxic or cell adherence	
	Dilazep dihydrochloride	Vasodilator	78	56	39.49	Cytotoxic or cell adherence	
	Protoveratrine A	Antihypertensive	62	33	33.97	Cytotoxic or cell adherence	
	Doxepin hydrochloride	Antidepressant	51	41	11.16	Cytotoxic or cell adherence	
	Hexamethonium dibromide dihydrate	Antihypertensive	44	31	16.06	Cytotoxic or cell adherence	

Fig. 8.12. **Active compound from LOPAC screening run**. Confirmation of active compounds from two independent LOPAC screening runs. Tacrine hydrochloride showed evidence of nuclear fluorescent localization at 50 μM. Some compounds shown were active but image data showed evidence that compounds tested at 50 μM were either cytotoxic and/or effected cell adherence.

exhibited no activity in the primary screen as shown in the results of frequency distribution (**Fig. 8.14A**). However, 474 compounds (1.44% of the library) produced ≥50% inhibition of the anisomycin-stimulated MK2–EGFP translocation in HeLa–A4 cells (**Fig. 8.14A**). Only 270 compounds were available for follow-up IC_{50} assays and these compounds were tested in a five-point, threefold concentration response, starting at a maximum of 50 μM. One hundred and sixty-three of the compounds (60.37%) were confirmed active with $IC_{50} < 50$ μM in follow-up assays that required only 1 day of screening operations to perform, with 3.57 hours scanning time on the InCell 3000. One hundred and fifty-six (95.71%) of the compounds confirmed in the 5-point IC_{50} assays were subsequently confirmed in 10-point, threefold

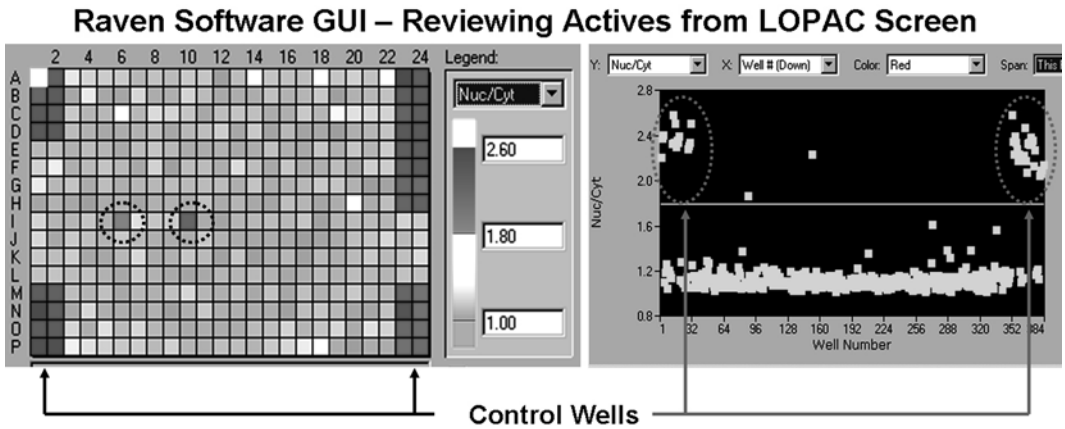


Fig. 8.13. **Reviewing LOPAC screen using Raven software.** The heat map (*left*) and scatter plot (*right*) visualizations demonstrate the GUI interfaces in the Raven software for displaying data from a 384-well plate. Columns 1, 2, 23, and 24 represent control wells. With two exceptions, all the compound wells in the heat map (*left side*) are green with a Nuc:Cyt ratio ~1.2 indicating that the compounds failed to inhibit translocation. There are two active compounds above the 50% inhibition threshold that are brighter in the heat map and outlined with circles in the scatter plot (*right*).

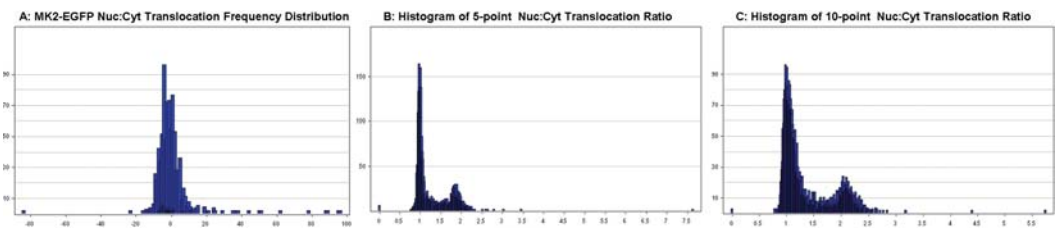


Fig. 8.14. **MK2–EGFP translocation assay screen performance data from 32 K biased kinase library**. (**A**) **Primary screen data**. All the calculated results (percentage inhibition) for the 32,891 compounds in the screen were exported to Spotfire® for visualization in a results frequency distribution plot. The median percentage inhibition was –4, and the mean percentage inhibition was –3.19 ± 16.26. The active criterion was set at ≥50% inhibition of anisomycin-induced MK2–EGFP translocation. (**B**) **Five-point IC_{50} data**. All the calculated data (Nuc:Cyt ratios) for compound wells from the five-point IC_{50} run were exported to Spotfire® for visualization in a results frequency distribution plot. (**C**) **Ten-point IC_{50} data**. All the calculated data (Nuc:Cyt ratio's) for compound wells from the 10-point IC_{50} run were exported to Spotfire® for visualization in a results frequency distribution plot.

dilution series IC_{50} assays, starting at a maximum concentration of 50 μM (**Table 8.2**). The 10-point IC_{50} assays were completed in 1 day of screening operations with an average of 1.03 frames or fields of view per well and a total scan time of 4.01 hours or 26.8 minutes per 384-well plate or 4.08 seconds mean time per well As expected, both the 5-point and the 10-point IC_{50} data exhibited two distinct bimodal histogram distributions of activity with one population representing wells that were inhibited by compounds at higher concentrations and the other population representing wells receiving compound concentrations that did not inhibit anisomycin-induced MK2–EGFP translocation (**Fig. 8.14B** and C).

Table 2
Summary of active hits from the primary screen and IC50 follow ups

MK2–EGFP InCell 3000			% of total
Rapid MTS	Number Screened >50%	32,891	100
		474	1.44
5-point IC_{50}s	Number tested Number Confirmed	270	100
		163	60.37
10-point IC_{50}s	Number tested Number Confirmed	163	100
		156	95.71

The InCell 3000 Raven software like many HCS imaging software provides a means to "post hoc" analyze potential artifacts such as noise, debris, fluorescent compound interference, and cell loss from suspected adherence issues and/or cytotoxicity as a result of morphological alterations in the cell. The Raven software provides a method to assess how captured images correlate with the numerical metadata from the images for the output parameters shown in **Table 8.1** such as Nuc:Cyt ratio, cell number, and the NPasNC parameter, which indicates the number of nuclei that were found to pass the intensity and size filters. Since the InCell 3000 was set up to capture a minimum of 100 cells/well or a maximum of two frames/well, whichever came first, compounds that generated data from wells of cell objects less than 100 were flagged as either cytotoxic or effecting cell adherence. We found the NPasNC parameter to be useful in determining cytotoxic or cell adherence issues in the IC_{50} dose–response data sets at higher compound concentrations such as 50 and 16.7 μM. After reviewing the images we in fact confirmed that compounds with an abnormal NPasNC parameter showed a reduction in cell number as a result of dose-dependent cell adherence or cytotoxicity (**Fig. 8.15A**). There are two distinct populations from data scatter plot of the mean cytoplasmic intensity per well versus average nuclear intensity per well. The plate control well data from anisomycin-treated cells and untreated or media-only wells are clearly separated into the two populations. Neither control populations exhibited cytoplasmic intensity thresholds above 2000 and/or nuclear intensity thresholds above 3000. Although the majority of compound-treated wells were also within these defined threshold ranges, there were a considerable number of compounds that

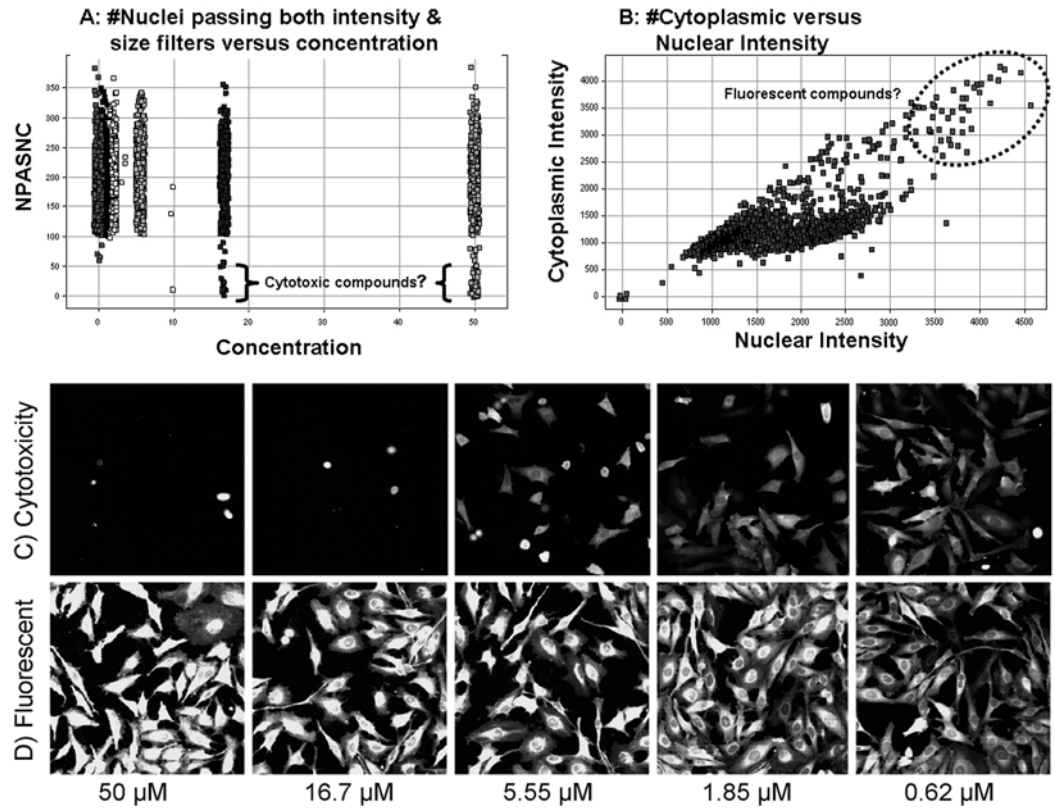


Fig. 8.15. **Nuclear trafficking module secondary analysis for cytotoxicity and fluorescence.** The nuclear trafficking analysis module provides data on a number of parameters that can be used to identify potential interference due to compound fluorescence or off-target compound effects such as cytotoxicity or disruption of cell adherence (**Table 8.1**). These data were exported from the Raven software to Spotfire® and visualized in a variety of scatter plots: (**A**) the NPasNC parameter was plotted for the different plate controls versus compound concentration to assess cytotoxicity or a reduction in cell adherence; (**B**) a scatter plot of the average cytoplasmic intensity/well versus average nuclear intensity/well in the target channel (EGFP) was used to identify fluorescent compounds. (**C**) Representative images from compound wells exhibiting a dose-dependent cytotoxicity or reduction in cell adherence; (**D**) representative images from compound wells exhibiting a dose-dependent fluorescence.

exceeded the cytoplasmic intensity threshold above 2000 and/or nuclear intensity threshold above 3000. After reviewing the images we confirmed that at high concentrations (50 or 16.7 µM), the majority of these compounds were either fluorescent and/or affected the MK2–EGFP fluorescence signal (**Fig. 8.15B**). Only 13 (8.33%) of the 156 active compounds from the 10-point IC_{50} dose–response curve showed NPasNC data with less than 100 cells/well, which may be a result of reduced cell adherence and/or cytotoxicity. However, a higher number (25, 16%) of the 10-point IC_{50} dose–response compounds were considered fluorescent as a result of the cytoplasmic intensity threshold output parameter above 2000 and/or nuclear intensity threshold above 3000 (**Table 8.3**).

Table 3
Summary of follow up active hit compounds with secondary image analysis parameters measured to determine false positive from high-levels of fluorescence in nucleus and/or cytoplasm, and number of cytotoxic or cytotoxic-like compounds

Secondary Analysis Parameters

Total number tested	163	%
>50 μM	7	4.29
<50 μM	156	95.71
Cytox	13	8.33
CYT INT NUC INT CYT + NUC INT	8 14 3	5.13 8.97 1.92
<50 μM	118	75.64

In summary, there were 59 (36%) confirmed inhibitors of the p38 MAPK pathway with $IC_{50} < 5$ μM and 31 (19%) with $IC_{50} < 1$ μM (**Table 8.4**). At least one new structural class of p38-α MAPK inhibitor that was identified in the screen was confirmed after additional secondary hit characterization assays.

Table 4
Breakdown of confirmed compound IC50 values

	Number Of Compounds	%
Number tested Number Confirmed	163 156	100 95.71
<1.0 1–10 μM 10–50 μM	31 73 52	19.02 44.79 31.90
>50 μM	7	4.29

4. Notes

1. HeLa–MK2–EGFP–A4 is a stable cell line derived from wild-type HeLa cells retrovirally infected with MAPKAP-k2 DNA fused with EGFP at 5′-end. Single-cell clones were deposited by flow cytometry sorting and clone A4 was selected based on

the expression and homogeneous of EGFP fluorescent intensity in the cell clone. It is critical to insure expression of stably transfected cell line after several passages in culture. For this cell line we did not observe substantial loss of expression since the cell line is under selective pressure with 800 μg/ml G418 to maintain stability. Further information can be found in a detailed description of the generation of the HeLa MK2–EGFP–A4 stable clone cell line *(12)*. Also if unable to create a genetically modified cell line, BioImage (Thermo Fisher Scientific) offers the MK2–EGFP in U2OS cell line for screening.

2. Please note at the time this research was conducted we washed 384-well Matrigel glass bottom plates with isopropanol to remove a residue that was suspected of causing cytotoxicity. Matrigel now offers residue-free glass bottom plates; however, keep this in mind if cells do not behave the same way as plastic and coating plates with poly-d-lysine or other extracellular matrix, proteins may be beneficial.
3. It is important to use a level surface as much as possible so that cells do not pile up on one side of the well. This trick to allow cells to settle at RT works well in all microwell plates that we tested. As soon as cells begin to adhere to the plastic or extracellular matrix, they can be placed in incubation.
4. By prewetting plates with small-volume wells such as 384- and 1536-well plates, we have found cells are more uniform and distrust throughout the well versus cells plated directly on to a dry surface. Very low volumes of liquid cell culture medium or buffer are needed to accomplish this, i.e., 10 μl/well for 384-well plates. Also, it is important for each assay and cell line used to optimize the number of cells plated per well. Although not discussed in this chapter, we previously optimized the cell number to 2500 cells/well using calculated anisomycin EC_{50} dose–response after extensive investigation of cells plated per well from 1250, 2500, 5000, 7500, and 10000 cells/well.
5. Alternatively add stimulus at the same time and fix selected wells at desired time intervals; however, we found this method to be more cumbersome.
6. Fixing cells expressing fluorescent proteins such as GFP and mutants of GFP decreases the fluorescent intensity. Unpublished work suggests that lowering formaldehyde or paraformaldehyde solution is beneficial and alcohol-based fixation tends to be worse than formaldehyde-based fixation.
7. Plate seals can be problematic in some plate handling robotics systems if there is evidence of hanging tabs. This can result in plates sticking to one another in plate stackers and causing problems.

8. For screening purposes we found that a coaddition of both the agonist stimuli and the inhibitor compound was effective. Alternatively we performed many experiments where we pretreated cells with inhibitor compound for 15 minutes at 37°C before adding anisomycin stimuli.
9. For "live cell" experiments, Hoechst dye is recommended to label the nucleus for the algorithm module to identify objects. Cells may pump Hoechst out of the nucleus, thus a higher concentration may be required. For fixed cells, other nuclear dyes such as DAPI work well in the blue channel; DRAQ5 is an alternative for both live and fixed cells in the red channel. *See* Molecular Probes web site for other fluorescent nuclear protein choices.
10. Measuring and defining the appropriate mask overlay and segmentation can be complex. For optimal measurement of translocation and best fit, compare known controls with untreated media. Make necessary adjustments based on image, not the output values. Once set, recheck the recorded measurements of the cell population. At the time of study, there were three nuclear trafficking algorithms; we used the nuclear trafficking 2 algorithm, which gave additional information to allow us to sort out cell populations based on fluorescent intensity in the nucleus, cytoplasm, or both. Nuc:-Cyt is the primary output feature to record and is used to optimize the algorithm. Additional secondary output parameters are also very useful in helping identify unusual morphology or modification in the fluorescent distribution. "NPasNc" is the number of nuclei found passing both intensity and size filters; "NPasSg" is the number of cells found passing above filters and cytoplasm intensity filter (see signal sampling threshold below), set at 100 cts for the original screen data; and "NPasAq" is the number of cells passing both of the above filters and for which the fraction of pixels is above the threshold shown in the secondary analysis parameters. A significant difference between NPasSg and NPasAq is evidence of a toxic response.
11. There are three independent water-cooled CCD cameras on the InCell 3000 Analyzer: red, green, and blue. The red camera was not used in this study.
12. Be sure to collect enough valid cellular objects to be statistically significant. Use the "50/500" as a rule of thumb. Fifty objects is minimum, 500 is more than enough for statistical significances if the window is at least twofold or greater with CV less than 8%. During screening process of libraries you will encounter a number of false-positive compounds; many of these are considered "toxic" to the assay. If the cell number is low, the number of "fields" output parameter feature is useful in

decision process. For example, if the count is set to 100 objects or 2 fields, whichever comes first, and then if the cells are healthy, typically one field is all it takes; however, if the number of fields exceeds 1, then it is likely an issue with cellular toxicity, cell adherence, or a result of uniformed plating of cells.

13. In most cell-based assays with short incubation times, 0.5% DMSO is commonly used for high compound concentrations. For longer incubation times, 0.1% DMSO may be appropriate. Although uncommon it may be necessary to use 1% or higher DMSO concentrations in some assays. There may be times when compound solubility is an issue that requires higher DMSO concentration in the assay; therefore, it is critical to know the limitation of the cell model. As long as you use an internal DMSO control at higher desired level, it is acceptable to compare to the compound treatment wells with appropriate DMSO control. However, keep in mind the original DMSO tolerance curve that indicates when the assay begins to alter, so this information is reported. Loss of cell adherence is the biggest side effect of high DMSO concentration, which can have a major impact on the assay.
14. Alternative stimuli include proinflammatory cytokines such as TNF-α and IL-1β. It is important to test all stimuli related to the investigating target and biology. Be sure to measure the kinetic time course for each stimulus independently.

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