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High Content Screening as a Screening Tool in Drug Discovery

Anthony Nichols

Summary

In most pharmaceutical and biotechnology companies there is a need to always improve the quality of lead candidates. This demand resulted in the use of cell-based screening as a method of choice in drug discovery *(1,2)*. High content screening (HCS) is multiplexed, functional cell-based screening *(3–6)*. HCS can be used in all aspects of drug discovery as an engine for driving lead discovery. The biological applications of HCS have been implemented in research in signaling, cell shape changes and toxicology. HCS has enabled an insight in the cellular effects of our clinical candidates in multiple cellular phenomena like dual reporter assay, subcellular target translocation and cellular morphology. Discovery of therapeutic protein and small molecule converge on diseases in therapeutic areas such neurological disorders and autoimmune diseases. HCS is used for assay development, primary, secondary screening and toxicology testing. In this chapter, the use of HCS assays in drug discovery is described and highlight the necessary step to set-up successfully these assays for screening.

Key Words: Cell-based assays; cell-membrane translocation; cell morphology assays; dual reporter assays; genotoxicity; high content screening (HCS); micronucleus; nuclear translocation.

1. Introduction

Traditional biochemical screening has not always delivered hits that can be followed up and that do not always yield the development of effective therapeutic agents. Cells are the smallest living complex entity. Biochemical screening formats do not address biological issues like cytotoxicity, complex biology or multicomponent target classes. Cell function is a complex interplay of signaling and feed back pathways lacking in isolated molecular or biochemical assays, moreover in vitro assays lack the possibility to provide information on the physicochemical properties of compound like cell permeability *(7–9)*. For these reasons cell-based assays are becoming more and more the preferred screening format to identify higher quality hits. Cell-based assays are the starting point for high information content screening (HCS) or multiple cellular event screening that provide access to major therapeutic target classes. HCS can perform multiple measurements per well or cell and allow screening for target function in a more physiological relevant setting. HCS provides more information than classical cell-free systems and can answer biological questions earlier in drug discovery. In the right format, cell-based screening can be target-based having the target in its more natural context. Cell-based screening and even more HCS for small molecule is well suited for orphan targets *(9)* or when it is not feasible to express and purify at the scale for high-throughput screening (HTS) a molecular target. Furthermore HCS may provide functional assay methodologies to identify compounds with a different mechanism of action, for example, allosteric modulator. Typically HCS can be divided into two categories

From: *Methods in Molecular Biology, vol. 356:*
High Content Screening: A Powerful Approach to Systems Cell Biology and Drug Discovery
Edited by: D. L. Taylor, J. R. Haskins, and K. Giuliano © Humana Press, Inc., Totowa, NJ

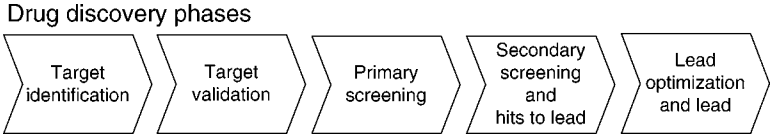

Fig. 1. Drug discovery phases.

1. Entire well measurement like dual reporter assays.
2. Multiparametric single cell measurements like cell shape, morphology, and target distribution changes within cells (*1,10*). HCS assays are used in early drug discovery process and can be described as the phase from target identification to leads. This phase can be divided as shown in **Fig. 1**. The purpose of this chapter is to review HCS methodologies used in drug discovery. Methods used for primary, secondary, and genotoxicity screening for potential therapeutic proteins and small molecules will be discussed. Drug discovery focusing on therapeutic protein discovery is well suited for the use of high content cell-based assay were the readouts are multiple phenotypic.

2. Materials

2.1. HCS Dual Reporter

1. HLR-cJun cells (Stratagene, La Jolla, CA).
2. Plasmids: pFC-MEKK, pBluescript II KS (+) (Stratagene), pRL-TK (Promega, Madison, WI).
3. Dual-Luciferase assay system (Promega), Tissue Culture Treated Microplate white 96-well clear well (PerkinElmer Life and Analytical Sciences, Boston, MA).
4. Dulbecco's modified Eagle's medium (DMEM) (high glucose, without L-glutamine, without sodium pyruvate), 2 m*M* L-glutamine, 10% fetal calf serum (FCS), 100 U of penicillin and streptomycin, Hygromycin B 100 µg/mL, and Gentamicin 250 µg/mL (Invitrogen, Carlsbad, CA).
5. FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN)
6. Microplate 2 injector luminometer Luminoskan Ascent (Labsystems, Finland).

2.2. HCS Nuclear Translocation

1. Neuro-2a (ATCC® CCL-131, Manassas, VA), HeLa cells (ATCC CCL-2, Manassas, VA), U373 (European Collection of Cell Cultures [ECACC]).
2. Minimum essential medium: with Earle's Salts, 2 m*M* L-glutamine, 1% nonessential amino acids, 1 m*M* sodium pyruvate, 10% FCS, 100 U of penicillin and streptomycin (Invitrogen).
3. c-Jun Activation HitKit HCS Reagent Kit, NF-κB Activation HitKit HCS Reagent Kit, STAT 2 Activation HitKit HCS Reagent Kits (Cellomics, Inc., Pittsburgh, PA).
4. Formaldehyde (37%), TNF-α, IL-1-α, IFN-β (Sigma, St. Louis, MO).
5. Tissue culture treated microplate black 96-well clear well (PerkinElmer Life and Analytical Sciences).
6. HCS reader ArrayScan® 3.1 and Nuclear Translocation bioapplication.

2.3. HCS Cytosol to Plasma Membrane Translocation

1. HeLa cells (ATCC CCL-2), U-2OS cells (ATCC HTB-96).
2. Weat Germ Agglutinin tetramethylrhodamine (TRITC) conjugate, TSA detection kit Alexa Fluor®488 conjugate and Hoechst 33342 dye (Molecular Probes, Eugene, OR), formaldehyde 37% and PMA (Sigma), IGF-1 (R&D Systems).
3. Goat antirabbit and antimouse coupled to Alexa Fluor 488 (Molecular Probes), PKCα Activation HitKit (Cellomics, Inc.), rabbit antiphospho-Akt (Ser473), (Cell Signaling Technology, Beverly, MA).
4. Minimum essential medium: with Earle's Salts, 2 m*M* L-glutamine, 1% nonessential amino acids, 1 m*M* sodium pyruvate, 10% FCS, 100 U of penicillin and streptomycin, McCoy's 5a medium, 2 m*M* L-glutamine, 10% FCS, 100 U of penicillin and streptomycin (Invitrogen).
5. HCS reader ArrayScan 3.1 and cytoplasm to cell membrane translocation bioapplication (Cellomics, Inc.).

2.4. HCS Morphology Assays

1. Neuroscreen-1 cells (NS-1) (Cellomics, Inc.).
2. RPMI-1640, 2 m*M* L-glutamine, 10% horse serum, 5% FCS, 100 U of penicillin and streptomycin (Invitrogen).
3. Neurite outgrowth HitKit (Cellomics, Inc.), 2.5S Nerve Growth Factor (NGF) (Promega), Tissue Culture Treated Microplate Black 96-well clear well Collagen I Coated (BD Bioscience, San Jose, CA).
4. HCS reader ArrayScan 3.1 and Neurite Outgrowth bioapplication (Cellomics, Inc.).

2.5. HCS in Genotoxicity Screening

1. CHO-K1 (ATCC).
2. F12K, 2 m*M* L-glutamine, 10% FCS, 100 U of penicillin and streptomycin (Invitrogen).
3. Micronuleus HitKit HCS Reagent Kit, (Cellomics, Inc.), Mytomycin C (Calbiochem), formaldehyde 37% (Sigma), tissue culture treated microplate black 96-well clear well collagen I coated (BD Bioscience).
4. HCS reader ArrayScan 3.1 and Micronucleus bioapplication (Cellomics, Inc.).

3. Methods

3.1. HCS Dual Reporter in Secondary Screening

One of the activation events of signaling pathways in cells is the phosphorylation of transcription factors, which subsequently activate transcription of genes that are dependent on the type of pathway that is stimulated. This method was used to measure the potential of c-Jun N-terminal kinase (JNK) inhibitors ***(11–13)*** in a cellular context. The cells used are cell line, which contain the expression plasmid for transcription factor c-Jun fused with the DNA binding domain of the yeast GAL4 and a plasmid that contains a synthetic promoter with five tandem repeats of the yeast GAL4 binding sites that control expression of the Firefly luciferase gene stably integrated into the HeLa cell. To activate the JNK pathway the upstream kinase MEKK1 ***(14)*** is transiently transfected with a constitutively active expression plasmid for Renilla luciferase. When c-Jun is phosphorylated by JNK, the fused DNA-binding domain binds the GAL4 binding sites and activates the transcription of the luciferase gene from the reporter plasmid. Expression (or activity) levels of Firefly luciferase reflect the activation status of JNK. The activity of Renilla luciferase reflects the transfection efficiency, the potential cytotoxicity of compounds and the presence of cells in individual wells.

In cell culture, the cells are routinely split when they reach 80–90% confluency.

3.1.1. Day 1 Morning

1. The cells from flasks of 90% confluent cultures are detached by treatment with trypsin-EDTA.
2. The cells are resuspended in culture medium and counted (*see* **Note 1**).
3. The cell suspension is diluted with medium at 3.5×10^6 cells/mL and 1 mL of cell suspension is seeded into a 10 cm culture dishes containing 9 mL of culture medium.
4. The plates are incubated at 37°C in a humidified atmosphere at 5% CO_2.

3.1.2. Day 1 Evening

The following plasmid mixtures were prepared:

1. Control: 0.2 µg pTK Renilla, 5.8 µg pBluescript KS 2. Induced: 0.1 µg pMEKK1, 0.2 µg pTK Renilla, 5.7 µg pBluescript KS (*see* **Note 2**).
2. The transfection mixture is prepared by mixing the above DNA with 18 µL of FuGENE 6 and 500 µL of OPTIMEM.
3. This mixture is added to the plated cells. The cells are incubated 12–18 h at 37°C in a humidified atmosphere of 5% CO_2.

3.1.3. Day 2

1. A 96-well plate containing100 µL of culture medium per well is prepared. Negative control: 2 µL of 100% dimethyl sulfoxide (DMSO) is added to the 100 µL (in triplicate). Compound: 2 µL of compound in 100% DMSO is added to the 100 µL (in triplicate).

2. The transfected cells are detached by treatment with trypsin-EDTA and resuspended in 12 mL of culture medium. 100 µL of cells are added to each well of the 96-well plate.
3. The plate is incubated 12–18 h at 37°C in a humidified atmosphere of 5% CO_2.

3.1.4. Day 3

1. The medium is removed from the plate and the cells are washed two times with 100 µL PBS and the solution is removed.
2. 50 µL of 1X PLB lysis buffer is prepared according to the manufacturer's instructions and is dispensed into each well.
3. The culture plates are shacked for 15 min at room temperature on an orbital shaker to ensure complete and even coverage of the cell monolayer with the lysis buffer.
4. Luciferase Assay Reageant II and Stop & Glow Reagent are prepared according to the manufacturer's instructions; 10 mL of each solution is used per 96-well plate.
5. Transfer 20 µL of the cell lysate into a white opaque 96-well plate.
6. Load the 96-well plate into the luminometer, for reading use the following sequence: (1) Inject 100 µL of Luciferase Assay Reagent II wait 5 s, read 10 s. (2) Inject 50 µL of Stop & Glo Reagent wait 5 s, read 10 s.
7. Determine normalized results: (Firefly Luciferase light units)/(Renilla light units × 1000).

3.2. HCS Nuclear Translocation in Primary and Secondary Screens for Therapeutic Proteins and Small Molecule

Signal transduction in cell is often generated by the translocation of macromolecules (such as transcription factors or protein kinases) or smaller molecules (second messengers) from one cellular compartment to another and play a fundamental role in almost all cellular physiological processes, such as cell division, differentiation, cell motility, immune system function, neuronal transmission, and apoptosis. The regulation of transcription factors, such as c-Jun, STAT2, and NF-κB is by the translocation of the factor from the cytoplasm to the nucleus *(10,15,16)*. This method was used to measure the potential of JNK inhibitors *(11–13)* in a cellular context and NF-κB pathway modulators *(17)*. This method was also used to screen for potential therapeutic proteins that activate the JNK, the STAT2, and the NF-κB pathway. The cells are Neuro-2a mouse neuroblastoma *(18)* for JNK inhibitors, HeLa cells for NF-κB pathway modulator and U373 human astrocytoma for therapeutic proteins screen. To stimulate JNK in Neuro-2a we used the superoxide generator menadione *(19)*, JNK inhibitors were tested for their ability to inhibit phosphorylated c-Jun to translocate to the nucleus of these cells. TNF-α was used to activate the NF-κB pathway to test for compounds that inhibit the nuclear translocation of this transcription factor. IL-1 α was used as positive control for U373 for c-JUN and NF-κB *(15,16)* translocation and IFN-β was used as control inducer for STAT2.

In cell culture, the cells are routinely split when they reach 80–90% confluency.

3.2.1. Day 1

1. The cells from flasks of 90% confluent cultures are detached by treatment with trypsin-EDTA.
2. The cells are resuspended in culture medium and counted (*see* **Note 1**).
3. The cell suspension is diluted with medium at 5×10^4 cells/mL (*see* **Note 3**) and 90 µL of cell suspension is seeded into a 96-well plate.
4. The plate is incubated 12–18 h at 37°C in a humidified atmosphere of 5% CO_2.

3.2.2. Day 2

1. For a small molecule inhibitor assay, a 96-well plate containing 98 µL of culture medium, 2 µL of 100% DMSO for negative controls and 2 µL of compound in 100% DMSO is prepared. 10 µL of these solutions are transferred to the 96-well plate containing the cells and incubate 15 min at 37°C in a humidified atmosphere of 5% CO_2.
2. For a small molecule inhibitor assay, a 96-well plate is prepared containing 50 µL of medium with 5X final concentration of stimuli or 50 µL of medium for nontreated wells. 25 µL of these solutions is

transferred to the 96-well plate containing the cells. For agonist protein screen a 96-well plate is prepared containing 50 µL of medium with 10X concentrated proteins and 10X concentrated protein dilution buffer for negative control in medium. 10 µL of these solutions is transferred to the 96-well plate containing the cells (*see* **Note 4**).

3. Incubate 30 min at 37°C in a humidified atmosphere of 5% CO_2 (*see* **Note 5**).
4. Add 75 µL of fixation solution (10% formaldehyde in PBS) and incubate at room temperature for 15 min in a fume hood.
5. Aspirate fixation solution and wash the plate once with 100 µL PBS per well.
6. Aspirate the PBS and add 100 µL 1X permeabilization buffer (PBS 0.5% Triton X-100) per well, and incubate for 90 s (*see* **Note 6**).
7. Aspirate permeabilization buffer and wash plate once with 100 µL PBS per well.
8. Aspirate wash buffer-M and add 50-µL primary antibody solution per well. Incubate for 1 h (*see* **Note 7**).
9. Aspirate primary antibody solution and add 100 µL 1X detergent buffer (PBS 0.01% Tween-20) per well. Incubate for 5 min (*see* **Note 7**).
10. Aspirate detergent buffer and then wash twice with 100 µL PBS per well.
11. Aspirate the PBS and then add 50 µL of staining solution (*see* **Note 8**) per well. Incubate for 1 h.
12. Aspirate staining solution and then add 100 µL 1X detergent buffer per well. Incubate for 5 min.
13. Aspirate detergent buffer and then wash twice with 100 µL PBS per well. Add 200 µL of PBS in wells.
14. Seal plate and run on ArrayScan HCS Reader using ×10 objective and the nuclear translocation bioapplication.
15. Store sealed plates in the dark at 4°C.

3.3. HCS Cytosol to Plasma Membrane Translocation in Small Molecule Secondary Screening

Stimulation of cells with growth factors initiates signal transduction cascades and subsequent intracellular activities that include recruitment to the cell membrane of macromolecules such as PKC and Akt/PKB. The cellular functions and regulation of these proteins, in most part, depend on specific subcellular localization ***(20)***. Membrane targeting is mediated mainly by two conserved cysteine-rich domains for protein kinase C (PKC) and the pleckstrin domains for Akt that bind to charged phospholipids ***(20)***. These cell-signaling events provide molecular targets for therapeutic intervention ***(21)***. This method was used to assess the cellular activity of inhibitors of protein–phospholipid interaction.

In cell culture, The cells are routinely split when they reach 80–90% confluency.

3.3.1. Day 1

1. The cells from flasks of 85% confluent cultures are detached by treatment with trypsin-EDTA.
2. The cells are resuspended in culture medium and counted (*see* **Note 1**).
3. The cell suspension is diluted with medium at 4×10^5 cells/mL for HeLa cells and 3×10^5 cells/mL for U-2OS cells and 100 µL of cell suspension is seeded into a 96-well plate.
4. The plate is incubated 12–18 h at 37°C in a humidified atmosphere of 5% CO_2.

3.3.2. Day 2

1. For Akt, carefully aspirate 100 µL of medium with multi channel and add 200 µL of medium without serum (*see* **Note 4**). Incubate at 37°C in a humidified atmosphere of 5% CO_2.
2. Remove medium and add 40 µL of prewarmed serum free medium to all wells.
3. Add 10 µL of medium with 5% DMSO in medium for controls or medium with compound at 5% DMSO final concentration.
4. Incubated 20 min at 37°C in a humidified atmosphere of 5% CO_2.
5. Add 50 µL of inducer 2 µ*M* of PMA for PKC translocation and 50 µL 600 ng/mL of IGF-1 for Akt, incubated 10 min for PKC and 5 min for Akt at 37°C in a humidified atmosphere of 5% CO_2.
6. Aspirate culture medium and add 100 µL of fixation solution (3.7% formaldehyde in PBS) to each well. Incubate in fume hood for 15 min at room temperature.
7. Aspirate fixation solution and wash wells once with 100 µL of PBS.
8. Aspirate PBS and add 100 µL membrane stain to each well. Incubate for 30 min (*see* **Note 9**).

9. Aspirate membrane stain and wash three times with 100 µL PBS.
10. Aspirate the PBS and fix by adding 100 µL fixation solution. Incubate for 5 min in fume hood.
11. Aspirate fixation solution and wash twice with 100 µL PBS.
12. Aspirate the PBS and add 100 µL of 0.2X permeabilization buffer (PBS 0.1% Triton X-100). Incubate for 15 min.
13. Aspirate permeabilization buffer and wash twice with 100 µL PBS.
14. For Akt aspirate the PBS and add 100 µL of blocking buffer (PBS 10% FCS, 1% BSA). Incubate 45–60 min.
15. For PKC aspirate the PBS and add 50-µL primary antibody solution (*see* **Note 10**). Incubate for 1 h.
16. For Akt aspirate blocking buffer and add 50-µL primary antibody solution (*see* **Note 10**). Incubate overnight at 4°C.
17. Aspirate primary antibody solution and wash three times with 100 µL PBS.
18. For Akt aspirate PBS and add 100 µL of PBS 1% H_2O_2. Incubate 30 min at room temperature. Wash three times with 100 µL of PBS.
19. For PKC aspirate the PBS and add 50 µL of staining solution. Incubate for 1 h (*see* **Note 11**).
20. For Akt aspirate the PBS and add 50 µL of staining solution (*see* **Note 12**). Incubate for 1 h.
21. Aspirate staining solution and wash three times with 100 µL PBS. For PKC go to 24.
22. For Akt aspirate the PBS and add 50 µL of tyramine staining solution (*see* **Note 3**). Incubate 10 min.
23. Aspirate the tyramine staining solution and wash three times with 100 µL PBS.
24. Aspirate PBS and add 200 µL of PBS. Seal plates and scan on ArrayScan HCS Reader using ×20 objective and cytoplasm to membrane Translocation bioapplication.
25. Store sealed plates in the dark at 4°C. Plates are stable for 48 h after preparation.

3.4. HCS Morphology Assays in Primary Screen for Therapeutic Proteins

Neurons assemble into functional networks by growing out axons and dendrites (collectively called neurites). Neuronal cell morphology, including neurite outgrowth, elongation, cell body hypertrophy, and growth cone behavior, is modulated by a variety of conditions such as trophic factors, electrical activity, synaptogenesis, and functional maturation and differentiation of neurons *(22,23)*. This method was used to screen for potential therapeutic proteins that activate the neurite outgrowth *(24)* of PC12 subclone NS-1. In cell culture, the cells are routinely split when they reach 70–80% confluency.

3.4.1. Day 1

1. The cells from flasks of 80% confluent cultures are detached by treatment with trypsin-EDTA.
2. The cells are resuspended in culture medium and counted (*see* **Note 1**).
3. The cell suspension is diluted with medium at 2×10^4 cells/mL for NS-1 cells 90 µL of cell suspension is seeded into a collagen I coated 96-well microplate containing 10 µL of controls with or without 2000 ng/mL of NGF or 10 µL of test proteins.
4. Incubate 3 d at 37°C in a humidified atmosphere of 5% CO_2.
5. Aspirate medium and add 100 µL PBS 3.7% formaldehyde, 1/2000 diluted Hoechst Dye Solution to each well. Incubate 20 min in fume hood at room temperature.
6. Aspirate fixation/Hoechst solution and wash three times with 100 µL 1X neurite outgrowth buffer.
7. Aspirate neurite outgrowth buffer and add 50 µL primary antibody solution (*see* **Note 14**). Incubate for 1 h.
8. Aspirate primary antibody solution and wash three times with 100 µL 1X neurite outgrowth buffer.
9. Aspirate neurite outgrowth buffer and add 100 µL secondary antibody solution (*see* **Note 15**). Incubate 1 h.
10. Aspirate secondary antibody solution and wash twice with 100 µL 1X neurite outgrowth buffer.
11. Aspirate neurite outgrowth buffer and wash twice with 100 µL PBS.
12. Add 200 µL of PBS and seal plate and run on ArrayScan HCS Reader using a ×5 or ×10 objective and the neurite outgrowth bioapplication.
13. Store sealed plates in the dark at 4°C.

3.5. HCS in Genotoxicity Screening

The in vitro micronucleus assay is a genetic toxicology assays, in which cultured cells are treated with compounds and scored for micronucleus induction. Micronucleus (are pieces of

chromosomes or entire chromosomes that have failed to be included in daughter nuclei during cell division) formation can be because of clastogens, which cause chromosomal breaks, and/or aneugens, which affect the spindle apparatus. This method is used to screen compounds for there potential to form micronuclei ***(25)***. In cell culture, the cells are routinely split when they reach 70–80% confluency.

3.5.1. Day 1

1. The cells from flasks of 80% confluent cultures are detached by treatment with trypsin-EDTA.
2. The cells are resuspended in culture medium and counted (*see* **Note 1**).
3. The cell suspension is diluted with medium at 4×10^4 cells/mL and is seeded into a Biocoat Collagen I 96-well plates.
4. The plate is incubated 12–18 h at 37°C in a humidified atmosphere of 5% CO_2.

3.5.2. Day 2

1. Remove medium and add 100 µL cellular dye solution, prepared as specified by the supplier (*see* **Note 16**). Incubate 1 h at 37°C in 5% CO_2.
2. In a 96-wells round bottom plate, prepare compounds dilutions in DMSO from a 10 m*M* stock. Add DMSO in control wells and MMC solution (33.3 ng/mL; 100 µ*M*) in positive control wells.
3. Transfer 2.5 µL into a new plate containing 247.5 µL of medium per well.
4. Wash 1X the cells with medium.
5. Transfer 100 µL of the compound plate on the cells.
6. Incubate 20h at 37°C in 5% CO_2.

3.5.3. Day 3

1. Remove medium from cells.
2. Wash once with medium and remove medium.
3. Add cytokinesis blocking agent, prepared as specified by the supplier (*see* **Note 16**).
4. Incubate 28h at 37°C in 5% CO_2.

3.5.4. Day 4

1. Add 50 µL permeability dye solution to the cells, prepared as specified by the supplier (*see* **Note 16**).
2. Incubate 30 min, 37°C, 5% CO_2.
3. Remove medium and wash once with medium.
4. Discard medium and add 100 µL fixation solution (PBS 3.7% formaldehyde).
5. Incubate 20 min.
6. Remove medium and wash twice with 100 µL PBS.
7. Add 200 µL PBS and seal plate and scan using the Cellomics Arrayscan reader using ×20 objective and the Micronucleus bioapplication.

4. Notes

1. Add 10 µL of cell suspension to 90 µL of 0.4% Trypan Blue and count living cells using a hemocytometer.
2. The amount and ratio between the standardizing plasmid pTK Renilla and the stimulation plasmid should be at least 1:1 or 2.1 and the total DNA content 6 µg for the FuGENE 6 ratio used.
3. Cell seeding density for nuclear translocation should be set so that cells are sufficiently separated and that at least 100 cells per field of view can be observe using a microscope with a ×10 objective. Seeding density can vary from cell type to another.
4. For some stimulation or cell types, one can obtain better pathway activation by serum starving the cells for 2 h. A dose–response for concentration of nuclear translocation inducer for each cell type and each translocating macromolecule should be tested.
5. Using the top dose determined in **Note 4** a time-course of nuclear translocation should be established for each cell type and each translocating macromolecule.
6. The time and the concentration of permeabilization buffer can be adapted if the staining is note optimal. An alternative to using PBS 0.2% Triton X-100 incubated for 90 s can be PBS 0.1% Triton X-100 incubated for 15 min or any permutations of these conditions.

7. The primary antibody from the HitKits: c-Jun, NF-κB, and STAT2 are diluted in PBS 1:200, 1:100, and 1:200, respectively. For other cell type, if background is a problem other dilution can be tested, 1% BSA can be added to the diluted antibody solution, a preincubating the cells with blocking buffer (PBS 10% FCS, 1% BSA) for 1 h and also the Detergent Buffer can be adapted from PBS 0.01% Tween-20 to PBS 0.1% Tween-20.
8. The staining solution for the HitKits: c-Jun, NF-κB and STAT2 contains secondary antibody diluted in PBS 1/100 (antimouse Alexa Fluor 488), 1/100 (antirabbit Alexa Fluor 488) and 1/100 (antirabbit Alexa Fluor 488), respectively, with Hoechst Dye diluted at 1/2000. For other cell type, if background is a problem other dilution can be tested, 1% BSA can be added to the diluted antibody solution and also the detergent buffer can be adapted from PBS 0.01% Tween-20 to PBS 0.1% Tween-20.
9. The membrane stain is prepared by diluting in PBS the membrane marker (Alexa Fluor 488) 1/120, if the secondary antibody used is conjugated to Alexa Fluor 555. If the Secondary antibody used is conjugated to Alexa Fluor 488 or the TSA amplification is with Alexa Fluor 488 then the membrane marker used is Weat Germ Agglutinin TRITC conjugate 1 mg/mL stock solution diluted 1/170 in PBS.
10. The primary antibody for the HitKit PKC is diluted in PBS 1/100 and for Akt the antibody is diluted 1/250 in PBS 1% BSA. For other cell type and if background is a problem other dilution can be tested.
11. The Staining Solution for the HitKit PKC contains the secondary antibody diluted in PBS 1/200 (antimouse Alexa Fluor 555) and if the Membrane stain used is conjugated to TRITC, the secondary antibody is diluted in PBS 1/200 (antimouse Alexa Fluor 488), the solution contains also Hoechst Dye diluted at 1/2000. For other cell type and if background is a problem other dilution can be tested and 1% BSA can be added to the diluted antibody solution.
12. The staining solution for Akt contains the secondary antibody (HRP conjugated antirabbit) diluted in PBS 1% BSA 1/400; the solution contains also Hoechst Dye diluted at 1/2000.
13. The tyramide amplification solution is prepared by diluting the tyramide stock solution 1/150 in amplification buffer/0.0015(H_2O_2.
14. The primary antibody for the Neurite outgrowth HitKit is diluted in 1X Neurite outgrowth buffer 1/800.
15. The secondary antibody for the Neurite outgrowth HitKit is diluted in 1X Neurite outgrowth buffer 1/200.
16. The cellular dye is prepared by diluting 5.5 µL of cellular dye stock solution in 11 mL of cell culture medium. The cytokinesis blocking agent is prepared by diluting 6.6 µL of cytokinesis blocking agent stock solution in 11 mL of cell culture medium. The permeability dye is prepared by diluting 3.6 µL of permeability dye stock solution in 11 mL of cell culture medium.

Acknowledgments

The author would like to thank Karen Yeow, Paul Lang, and Alexander Scheer for fruitful discussions. The author thanks the members of the Serono Pharmaceutical Research Institute, especially the members of the Biomolecular Screening and Cellular Pharmacology department and especially François Duval for his technical help and discussions.

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