

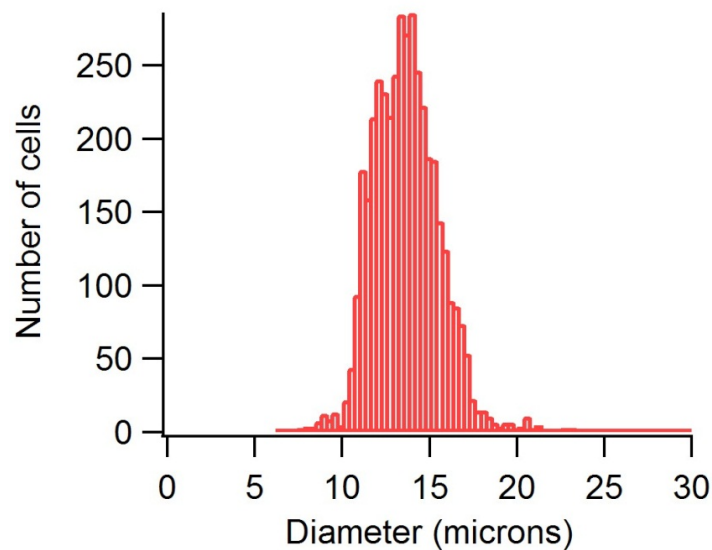


Figure 13: Diameter of CHO cells in suspension

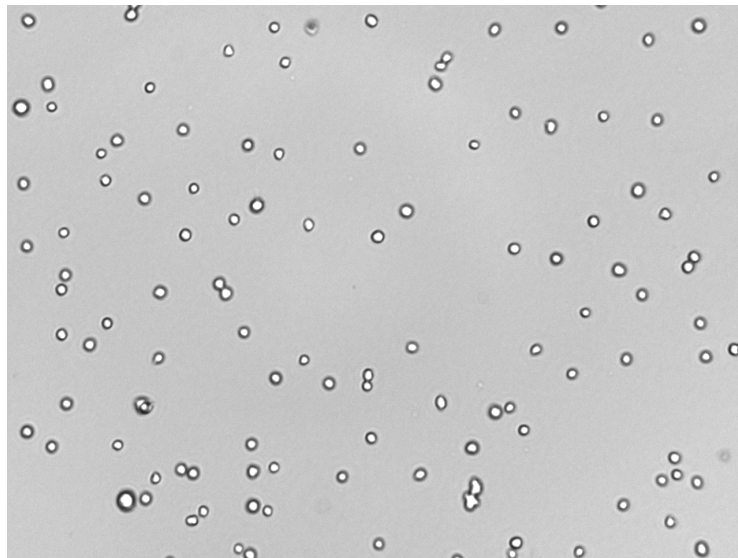


Figure 14: Image of CHO cells in suspension following detachment

5.8. Reduced temperature culture

In cases where limited channel expression is an issue, one approach that has been used successfully is to culture the cells at reduced temperature (27-30°C) for up to 3 days prior to use [2]. The lower temperature culture presumably results in a greater number of channels at the plasma membrane due reduced channel turnover. Typically, such an approach does not impact how the cells are prepared for automated electrophysiology. Growth at the reduced temperature is severely slowed such that the cell plating density will need to be increased to achieve suitable cell numbers for the assay. As a general rule, one can expect to harvest the same number of cells that are plated following a 2 day culture at 30°C. Cultures can also be transferred to the lower temperature once they reach the desired confluency.

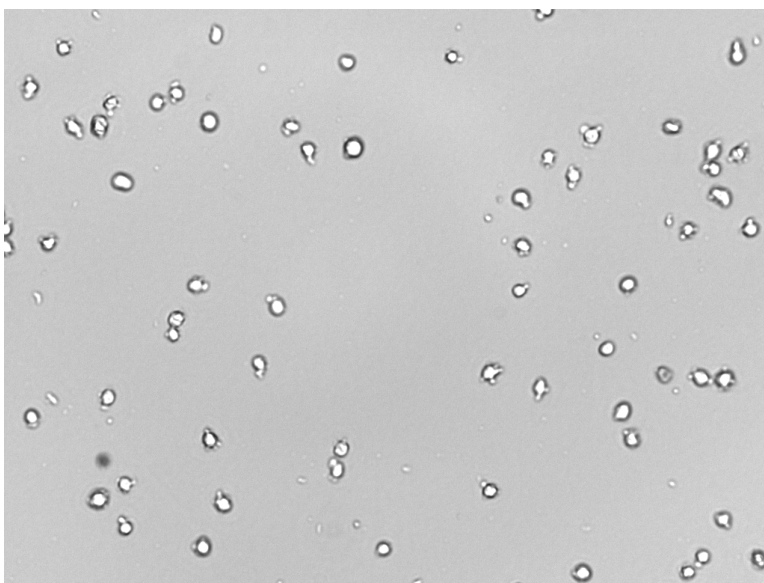


Figure 15: Image of HEK cells in suspension following detachment

5.9. Transient expression of channels

In general, transient expression of channels using lipid-based methods is not compatible with automated electrophysiology. Membrane integrity is likely compromised by the lipid-DNA micelle to such an extent that high resistance, stable recordings are unlikely across the entire cell population. Furthermore, non-transfected cells give rise to recordings without functional channels (or reduced total current amplitude in the case population recordings). Since such approaches are also fraught with high variability of transfection efficiency across experiments, this approach is generally not recommended.

There are now other technologies available that may be better suited for transient expression of channels for automated electrophysiology. For example, flow electroporation of cells has been used to transiently express ion channels for automated electrophysiology (MaxCyte, Inc.). Baculovirus-mediated expression of ion channels has been successfully used to express ion channels [7] and several channel expression kits are commercially available based on this technology [8] (Invitrogen). Both electroporation and baculovirus approaches are suitable for expression of large number cell numbers coupled with freezing in liquid nitrogen and single-use thawing.

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Automated Electrophysiology Assays

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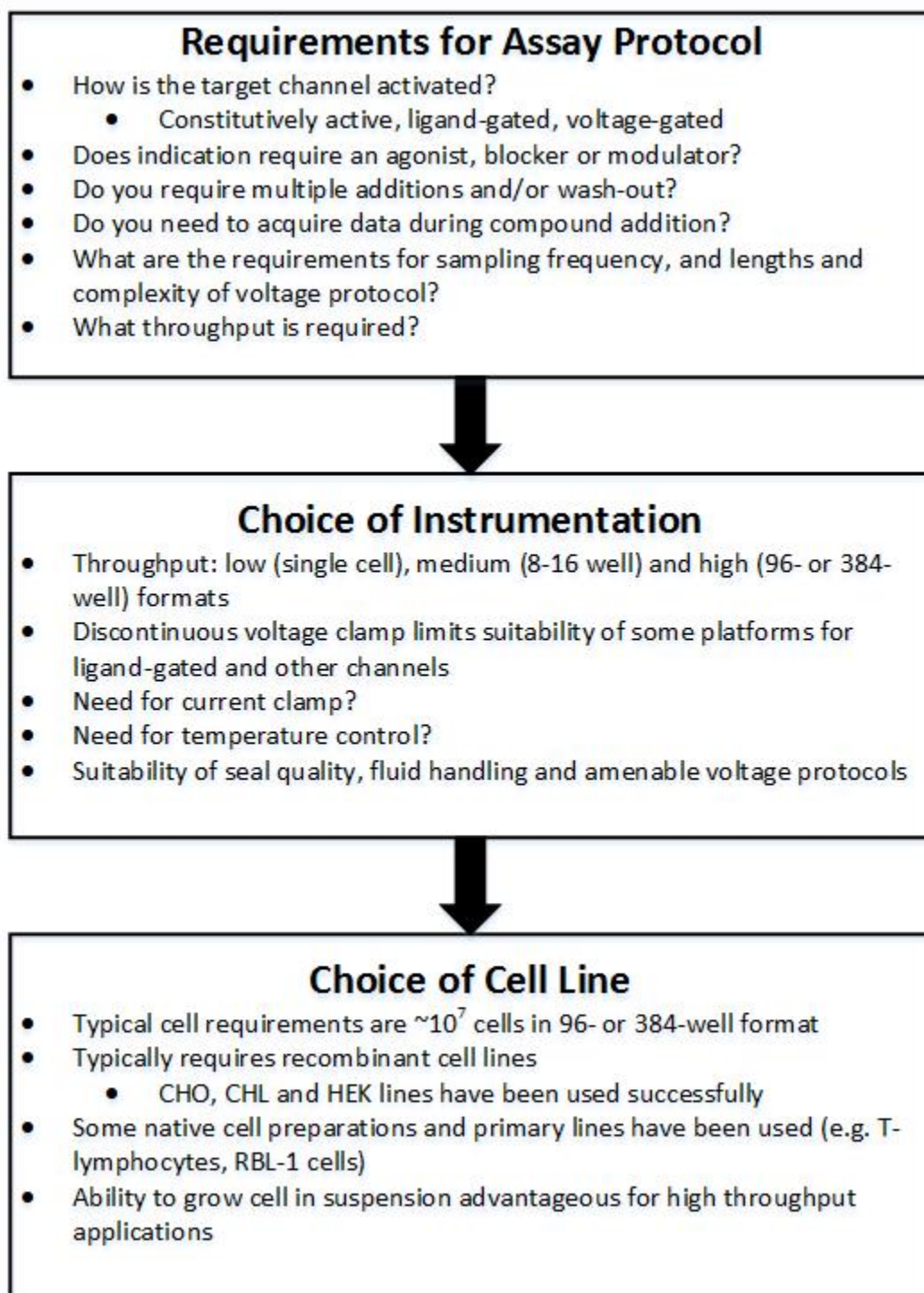
Introduction

Technological breakthroughs in electrophysiological techniques, such as development of voltage-clamp and patch clamp, have enabled direct measurements of membrane currents in single cells. The low throughput of traditional electrophysiology, however has limited its use in the drug discovery process. High-throughput, non-electrophysiology based primary assays were used successfully but lack key information content, such as voltage dependence and kinetics, and may not be suitable for all ion channels. Most importantly, non-electrophysiological assays lack the ability to accurately control membrane potential, preventing the selective targeting of disease relevant conformational states of voltage-gated channels. For these reasons, substantial efforts were devoted to the development of instruments capable of automated electrophysiological recording at moderate- to high-throughput.

Early on, a number of different approaches were investigated (1). The field quickly settled on planar substrates, and in 2002 two automated electrophysiology instruments came on the market: [PatchXpress®](#) and [IonWorks™](#) HT developed at Axon Instruments and Essen BioSciences, respectively, and later sold to Molecular Devices. Several other manufacturers launched their automated instruments in subsequent years; most notably [Sophion](#) (QPatch), [Nanion](#) (Patchliner®) and [Fluxion](#) (IonFlux). Based on learnings obtained with first generation devices, a new set of second generation instruments was developed in more recent years.

Instruments differ substantially in the composition of the patch plate, the configuration of the electrodes, liquid handling methods, and the software used to control the experiment and analyze data, and these features are discussed in greater detail below. Each platform has its strengths and weaknesses, and consideration should be given to the intended purpose before a device is purchased.

Flowchart



Choice of Instrumentation

Several excellent reviews of automated electrophysiology have been published (2-5) and may be consulted for a more detailed description of the available technologies. A general overview follows below.

Manual electrophysiology is enabled by the formation of a high resistance electrical seal between cell membranes and the glass used to manufacture patch pipettes. Most of the early attempts at automating electrophysiological recordings tried to replace the glass pipette with small holes in planar glass surfaces. Some medium-throughput

instruments (e.g. PatchXpress) still use this approach. Recordings on these instruments typically achieve high resistance gigaohm ($G\Omega$) seals, similar to manual electrophysiology. However, from a manufacturing standpoint, producing uniform holes of 1-2 μm diameter in planar glass represents a significant challenge. Attempts at increasing the throughput by increasing the number of parallel recordings or decreasing variability through ensemble recordings therefore employ alternative substrates. The consequence of using plastic substrates, however, is a lower seal resistance. For many applications, seal resistances in the 100-200 megaohm ($M\Omega$) range are sufficient to produce reliable data. Limitations may still exist for currents with low amplitude, rapid activation kinetics or very steep voltage dependence, or for currents lacking voltage-dependent gating where the channel current may be difficult to distinguish from background leak currents. Small currents may result in a poor signal-to-noise (S/N) ratio; and insufficient response speed and accuracy of voltage control may compromise accurate measurement of rapidly activating, steeply voltage-dependent currents.

Most platforms now offer the option of recording an “ensemble” current, referred to as population patch clamp (PPC) (6). In this configuration, each well in a seal chip/patch plate contains multiple (up to 64) holes for recording of an integrated total ensemble of currents across a number of individual cells. The advantage is greater uniformity, which typically equates to higher success rates (often as high as 100%). The uniformity afforded by PPC enables endpoint assays in which currents from wells receiving the test article are compared directly to control wells, without the need for normalization to pre-compound currents.

A consequence of ensemble recordings is the requirement for much larger currents to be passed across the electrodes, particularly if seal resistances are relatively low. Large current amplitudes can contribute to electrode instability, especially on high-throughput platforms with shared ground electrodes such as IonWorks Barracuda[®].

Problems with electrode instability are minimized by limiting the length of the voltage protocol, avoiding extreme voltage steps, especially in the hyperpolarizing direction, and reconditioning ground electrodes daily. For these reasons, early presentations on IonWorks Barracuda were often limited to recordings of ligand-gated channels, such as GABA-A channels. Including a silver chelator in the recording solutions may also help to avoid issues related to block of currents by silver ions emanating from the electrodes (7).

Most high-throughput electrophysiology assays for testing pharmacological agents are based on assessing whole cell currents, i.e. currents that flow across the entire cell membrane. The whole cell recording configuration can be established by two methods. Although both are technically whole cell recordings, the two methods are conventionally referred to as whole cell and perforated patch recordings, respectively. During whole cell recordings, the patch of membrane exposed to the intracellular solution, either in the patch pipette or across the hole in the planar seal chip/patch plate, is ruptured by applying negative pressure or a voltage transient. Typically the strength, duration and frequency of the negative pressure pulses can be varied and has to be optimized for each cell line such that good electrical access is achieved without damage to the cell or compromising the membrane-substrate seal. Good starting points for typically used cell lines (see below) can be obtained from the instrument manufacturers. Alternatively, perforated patch recordings establish electrical access by exposing the same membrane patch to agents that form non-selective cation pores within the lipid bilayer. More details on the choice of perforating agents follow below.

Choice of Cell Line

All automated electrophysiology instruments use a cell suspension that is pipetted into the recording chamber, where negative pressure facilitates the placement of single cells over the holes in the planar recording substrate. Requirements for cell numbers are typically on the order of 10^7 cells per 96- or 384-well recording. Given the requirement for preparing a cell suspension and for large numbers of cells per experiment, non-adherent cells offer advantages. However, historically, most ion channels have been stably expressed in either adherent Chinese Hamster Ovary (CHO) cells or in adherent Human Embryonic Kidney (HEK) cells, because of the limited

expression of endogenous channels, the propensity to form high resistance electrical seals on a glass surface, and the widespread use of these host cells for optical assays. Therefore, automated electrophysiology instrumentation has been optimized to work with these parental cell types. Good success rates have also been reported with Chinese Hamster Lung (CHL) and RBL-1 cells. If expression in another cell background is desired, it would be advantageous to first test the sealing properties of the parental cells on the automated electrophysiology instrument that will be used with the cell line. For other considerations regarding cell preparation and for a detailed protocol for preparing CHO and HEK cell suspensions for use on automated electrophysiology instruments, see the chapter on [Ion Channel Screening](#).

It is possible to use acutely dissociated native cells on automated electrophysiology devices; however, there are a number of important considerations and limitations (8). Cells have to be available in large numbers and the dissociation procedure has to produce a fairly homogeneous preparation of cells without a lot of cell debris. Because of these limitations, the native cell type used most successfully has been T-lymphocytes. Patchliner may offer an advantage for recording from native cells, because Nanion offers patch plates with different hole sizes and a low cell usage protocol, requiring as few as 300 cells per 16-well recording (9). Patchliner recordings from several native cell preparations have been reported. For some applications, induced pluripotent stem cells may offer a viable alternative to native cell preparations (10).

Choice of Solutions

The desire for higher resistance seals has led many researchers to employ intracellular solutions with high fluoride content. Intracellular solutions with F^- as the major anion lead to dramatically increased measured seal resistances; however, this may not be associated with an increase in the quality of the recording and may have unintended consequences such as changes in intracellular signaling and phosphorylation. For example, time dependent changes in sodium channel biophysics in the presence of high intracellular F^- were described in detail by Wang *et al* (11). These experiments were conducted using manual electrophysiology, but a similar lack of stability has been seen on the automated platforms. The reader is cautioned to explore alternatives and not to be persuaded by high resistance numbers alone.

High extracellular concentrations of divalent ion can also increase seal resistances, especially in the presence of high intracellular F^- . It may be helpful to temporarily expose cells to high extracellular Ca^{2+} concentrations during the sealing process, and change to a more physiological solution once seals are established. This is an approach recommended for Patchliner, which was designed with a space and protocol for use of a “seal enhancer” solution. Much of the increased seal resistance achieved by temporary exposure to a high divalent extracellular solution is maintained once the divalent concentration is lowered. While it is possible on Patchliner to exchange the intracellular solution, lowering the intracellular F^- concentration after seal formation leads to a dramatic loss of seal resistance (BTP, personal experience).

High-Throughput Automated Electrophysiology Assays

For the purpose of this discussion, high-throughput will be defined as the parallel recording from either 96 or 384 wells.

First Generation Instruments: IonWorks Quattro and IonWorks HT

IonWorks Quattro and IonWorks HT are distinguished by the use of single hole and PPC recording mode, respectively. Both instruments feature 48 movable electrodes and discontinuous voltage clamp and use the perforated patch configuration. The same voltage protocol is performed prior to and after a fixed incubation time with the test article.

Single Hole versus Population Patch Clamp (PPC)

On IonWorks instruments, cells can be supplied in a trough or in individual wells; this allows the user to test multiple different cell lines in the same experiment and makes the instruments well suited to selecting stable cell lines out of multiple clones. For this application, the Single Hole mode is ideal because it provides information on the distribution of current amplitudes across cells.

For other applications, the PPC mode is typically preferred (12). Because currents are effectively averaged across 64 cells, the PPC mode mitigates against cell-to-cell variability in parameters such as current amplitudes or fractional inactivation, and recordings are much more uniform. Often it is possible to achieve 100% success rate. However, if seal rates are poor, it may be possible to acquire data from a number of higher resistance wells in Single Hole mode, whereas recordings in PPC mode will fail altogether. For this reason, the Single Hole mode should be used to troubleshoot experiments when PPC recordings report “no seal” across the entire plate. If the reason for the failure is poor cell health, the Single Hole experiment should produce some wells with seal resistances that pass the intrinsic acceptance criteria (resistance $>20\text{ M}\Omega$ during seal test). If the Single Hole experiment shows no wells with acceptable seal resistances, the reason for the failure is most likely due to instrument failure or an error in setting up the experimental protocol.

Perforation Agent

The most commonly used perforation agent for IonWorks experiments is amphotericin B (from *Streptomyces*), although nystatin should be a viable alternative and is used more often for manual electrophysiology. There are important considerations when using amphotericin B. It is light sensitive and should be stored in the dark. Amphotericin B has limited solubility. It should always be prepared fresh on the day of the experiment, dissolved in 100% dry DMSO at approximately 30 mg/mL, sonicated for several minutes, and then diluted into the aqueous solution to a final concentration of 0.1 mg/mL. Amphotericin B of comparable quality is available from multiple vendors; however, lot differences exist and can affect various cell lines differently. Therefore it may be worthwhile to compare different lots of amphotericin B during assay development.

Although amphotericin B and nystatin form cation-selective pores, their permeability for chloride anions is not insignificant (13). This deserves some consideration when developing assays on the IonWorks platforms that employ the perforated patch method. In cases where the chloride concentration in the intracellular solution is high compared to typical cytoplasmic free chloride concentration, the cytoplasmic chloride concentration will most likely have reached steady-state at the time the actual data recording starts. However, this may not be the case; if the chloride concentration in the intracellular solution is low compared to the cytoplasmic free chloride concentration, this may lead to instability over time. Gramicidin may represent a viable alternative as a perforating agent and is much more selective for cationic currents (13).

Voltage Protocols

The complexity of voltage protocols that can be used on IonWorks HT or IonWorks Quattro is limited. The software is best suited for single voltage steps or ramps or trains of voltage pulses at a constant frequency. Generally, the length of the voltage protocol is limited to 5 s. However, another limitation exists in the number of data points that can be acquired, and therefore the maximal length of recordings depends on the sampling frequency employed. Recordings can be performed at sample intervals ranging from 0.1 ms to 6.4 ms. A sampling interval of 0.1 ms allows for voltage protocols lasting up to 2.25 s. It is possible to include conditioning intervals or trains, during which cells are voltage clamped but no data are acquired. Adding conditioning segments can quickly extend the total time of the experiment, because recordings are performed in 8 banks of 48 recordings, and it will be important to determine the stability of currents over the time course of the experiment.

Data Analysis

Leak subtraction is performed based on a small voltage step at the beginning of the voltage protocol. Because of the error in extrapolating from a small voltage step to the entire voltage range of the experiment and the possibility of offset potentials, the use of leak subtraction should be combined whenever possible with an offset correction based on leak currents recorded during a voltage step to 0 mV. However, this may not be possible if a membrane potential of 0 mV produces active currents. A good alternative for fast inactivating channels is the measurement of currents based on subtracting current at the end of a voltage step from the peak current.

For non-inactivating currents that operate across the voltage range, leak subtraction is not an option, and a combination of a voltage step to 0 mV to minimize the contribution of leak currents combined with a voltage ramp has been used successfully (14).

After data has been acquired, it can be filtered based on acceptance criteria such as seal resistance or current amplitude. The user then chooses one or several metrics that will be used to analyze the current traces such as the peak or average current during a particular interval. These data are stored in the form of ASCII files that can be imported into a variety of software programs for further data analysis. Overall, the analysis of IonWorks recordings is rapid and user friendly.

Example Protocol

See Appendix 1 for a detailed protocol that can be used to evaluate block of voltage-gated sodium currents on the IonWorks platform. Other voltage-gated currents can be recorded using the same workflow with appropriate adjustments in the voltage protocols and potentially in the composition of recording solutions.

Second Generation Instruments: IonWorks Barracuda, Qube and SyncroPatch

A key limitation of the first generation instruments is the inability to voltage clamp during test article addition. In this regard, second generation high-throughput instruments represent a significant advantage by enabling continuous voltage clamp. Another advantage of the 384 amplifiers is the shorter time required to conduct an experiment, i.e. the run time of a typical experiment is reduced from approximately one hour on IonWorks Quattro to 15-20 minutes on the second generation instruments. All platforms offer a choice between Single Hole and PPC modes, and the same considerations apply that are discussed above for IonWorks Quattro and IonWorks HT. IonWorks [Barracuda](#) and [Qube](#) (Sophion) are free-standing instruments, whereas [SyncroPatch 384PE](#) (Nanion Technologies) is designed to be integrated into a liquid handling platform, such as [Biomek® FX](#) (Beckman Coulter).

Whole Cell Access

Like the original IonWorks instruments, IonWorks Barracuda uses the perforated patch configuration, typically resulting in low access resistance that is very stable over time. In contrast, Qube and SyncroPatch obtain whole cell access by applying negative pressure to rupture the cell membrane. Different cell lines can differ substantially with regard to the negative pressure required to rupture the membrane, and the associated parameters need to be optimized carefully during assay development. In general, the user can vary the number, length and amplitude of the pressure pulses. Options may also include pressure ramps. If no or very little negative pressure is required for whole cell access, the cell membrane may have been damaged by excessive enzyme treatment and a gentler dissociation procedure should be tried.

Voltage Protocols

Second generation instruments can handle fairly complex voltage protocols, including protocols that combine different holding potentials and sampling rates. All instruments feature graphic user interfaces for setting up

voltage protocols and experimental parameters. It is reasonable to assume that protocols commonly used to characterize voltage-gated channels can be implemented on these second generation instruments, and the reader is referred to the instrument manufacturer for questions concerning specific protocols.

Data Analysis

All second generation platforms include customized data analysis software that allow the user to subtract leak currents, set well-level criteria for data acceptance and define metrics that specify the data (such as peak current, average current during a specified time interval, etc.) that will be used for further analysis. Some software programs support further analysis of data across wells, such as IC₅₀ or EC₅₀ determination, whereas other platforms require well-level data to be exported and analyzed separately. Generally, the latter is not an impediment, because most users will already have access to analysis software that is integrated with compound management information and database publication tools. Nonetheless, real-time analysis can be a nice feature when deciding whether to repeat experiments.

Example Protocols

See Appendix 2 for a protocol that can be used to evaluate block of KCNQ1/KCNE1 voltage-gated potassium currents on the IonWorks Barracuda platform.

Appendix 3 contains an example of a protocol that can be used to record currents from ligand-gated ion channels on IonWorks Barracuda. The example given is for GABA_A currents; similar protocols can be used with other ligand-gated chloride and cationic currents, although fluidic handling on the instrument precludes accurate recording of the most rapidly activating and inactivating channels (NMDA and nAChR $\alpha 7$).

Medium-Throughput Automated Electrophysiology Assays

Medium-throughput instruments use 8- or 16-well chambers. Currently available commercial instruments include PatchXpress, Patchliner and Qpatch. PatchXpress and Qpatch are free-standing instruments, whereas Patchliner is a bench-top instrument. A difference between these platforms is the configuration of the electrodes that can be either movable (PatchXpress and Patchliner) or embedded into the patch plate (Qpatch). Movable electrodes involve the risk of carryover of test articles between experiments, but are less prone to electrical cross-talk between electrodes, potentially leading to recording artifacts.

The PPC mode is available for Patchliner and Qube instruments.

Scripting

The PatchXpress and Patchliner instruments offer the option of scripting, referring to protocols that are adjusted *ad hoc* based on criteria applied to the data. A commonly used example is the addition of test article once currents evoked by baseline pulses have stabilized and vary by less than 5%. Another commonly used application of scripting is to set the test pulse voltage based on the maximum current determined during a family of voltage pulses. Especially for cell lines with low expression, this type of script can help maximize the number of successful recordings. Most modulators of voltage-gated sodium and calcium channels are sensitive to the conformational state of the channel and typically interact preferentially with channels in open and inactivated states. To avoid potency differences based on cell-to-cell variability in the voltage-dependence of inactivation, scripting can be used to set the membrane potential on the fly to a voltage corresponding to a set fractional inactivation based on steady state inactivation data.

Data Analysis

PatchXpress and Qpatch come with analysis software that is flexible but time consuming. Analysis performed by Patchliner software is limited to all-points IC₅₀ values and more detailed analysis requires importing the data into another program.

Plate-based analysis software used by most pharmaceutical companies and contract research operations is incompatible with the data formats used by medium-throughput automated electrophysiology devices.

Example Protocol with Scripting

See Appendix 4 for a protocol designed to evaluate block of voltage-gated sodium currents on the PatchXpress.

Low-Throughput Automated Electrophysiology Assays

The **Port-a-Patch** offered by Nanion is comparable to manual electrophysiology in terms of throughput. Its advantages are the small footprint and ease of operation relative to manual electrophysiology. It may be a viable option for laboratories new to electrophysiology. The main disadvantage is that the experimenter does not choose the individual cells that will be voltage clamped. This limits the use with transiently transfected or acutely dissociated cells.

Cost of Operation

The cost of consumables for any automated electrophysiology platform can be substantial and should be considered prior to purchasing an instrument. Outside of the price per patch plate/seal chip, factors that affect the cost per data point relate to the options of multiple test article additions, wash-out of test article, cell reuse, and scripting.

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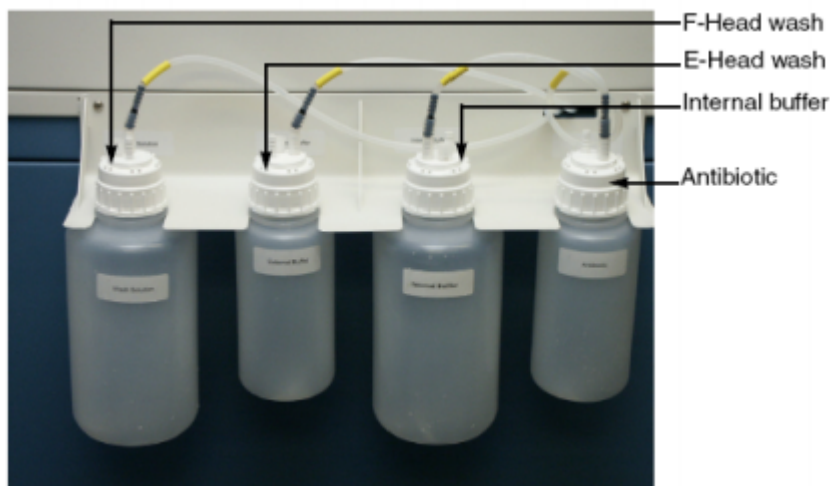
Appendix 1: Protocol for Assessment of Use-Dependent Sodium Channel Inhibitors on IonWorks Quattro

Overview

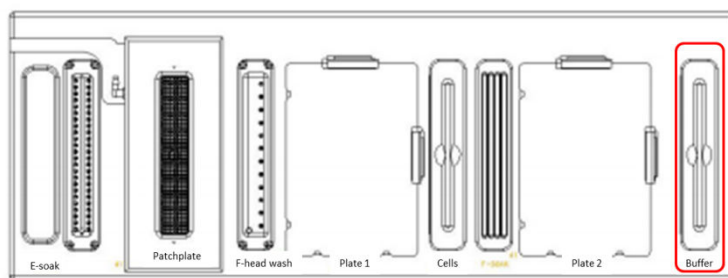
This protocol is designed to evaluate the type of use-dependent block of voltage-gated sodium channels that is seen with classic local anesthetics. After a brief period at a hyperpolarized voltage to allow recovery from inactivation, channels are subjected to a set of 26 depolarizing pulses. More pronounced inhibition of current during the later depolarizing pulses indicates use-dependent block, which typically arises from an increased affinity of test compounds for channels in open and inactivated states.

Start of Day

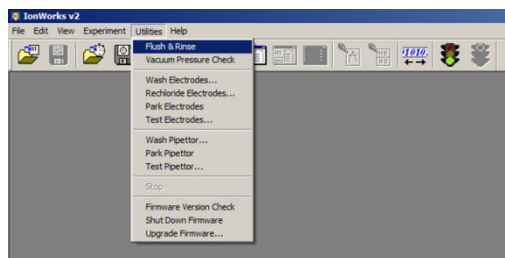
1. Turn on IonWorks Quattro using the green button located on the front of the instrument on the lower right.
2. Make sure waste bottles are empty.
3. Make sure main vacuum is on and apply vacuum to patch plate in **Patchplate** holder slot (use an old patch plate for these steps).
4. Place D-PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ in all buffer solution bottles on the left side of the instrument (leave waste bottles empty).



5. Place a buffer trough in the **Buffer** position and fill with 5-7 mL of D-PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$.



6. After instrument initialization, start IonWorks software.
7. From the **Utilities** menu, run the **Flush & Rinse** protocol twice.



8. Replace D-PBS with internal buffer solution (see below) in the bottles designated for the internal buffer solution and the antibiotic solutions. Make sure the correct lines are connected to each bottle.
9. Replace the buffer in the buffer trough with 5-7 mL external buffer solution (see below).
10. Run the **Flush & Rinse** protocol one time.

Buffer Solutions

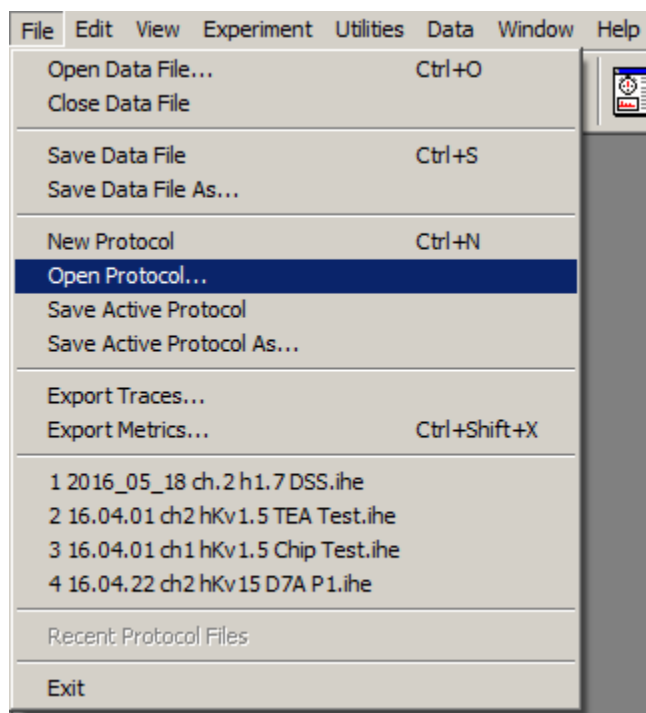
External Buffer Solution: 140 mM NaCl, 4 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, 10 mM Glucose, pH 7.4 with NaOH

Internal Buffer Solution: 70 mM KF, 70 mM KCl, 0.5 mM MgCl_2 , 5 mM HEDTA, 10 mM HEPES, pH 7.25 with KOH

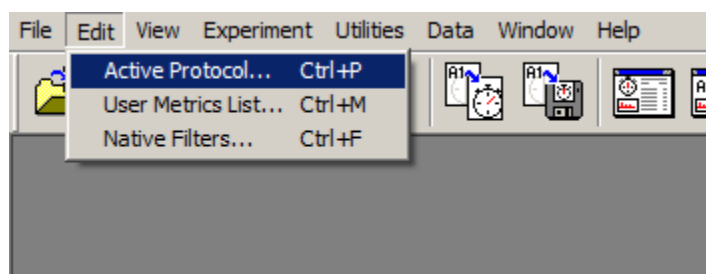
- Amphotericin B is added to internal buffer solution to a final concentration of 0.1 mg/mL to make the antibiotic solution on the day of an experiment.
 - i. Amphotericin B is weighed out and dissolved to a concentration of ~30 mg/mL in 100% anhydrous DMSO and then sonicated for 10 min.
 - ii. Amphotericin B is then added to the internal buffer solution with vigorous mixing.

Protocol Setup

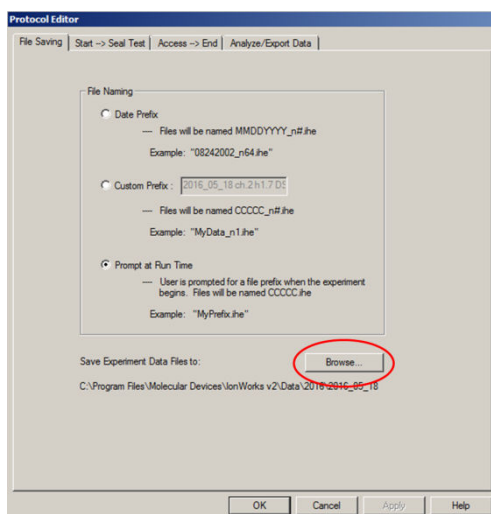
To load an existing protocol, select **Open Protocol** on the **File** menu.



Once the protocol has loaded, it may be edited from the Edit menu by selecting Active Protocol.



To set the location to save the data file, go to the **Protocol Editor** screen, select the **File Saving** tab and click the **Browse** button.



The user can also select a file format. Options are a standard **Date Prefix**, a **Custom Prefix**, or customizing the file name after the protocol has started.

File Naming

☐ **Date Prefix**
 --- Files will be named MMDDYYYY_n#.ihe
 Example: "08242002_n64.ihe"

☐ **Custom Prefix** :
 --- Files will be named CCCCC_n#.ihe
 Example: "MyData_n1.ihe"

☒ **Prompt at Run Time**
 --- User is prompted for a file prefix when the experiment begins. Files will be named CCCCC.ihe
 Example: "MyPrefix.ihe"

On the **Start >> Seal Test** tab of the **Protocol Editor**, the user has to enter the type of patch plate (Standard or PPC) that will be used, as well as the time used for the seal test.

Protocol Editor

File Saving | **Start -> Seal Test** | Access -> End | Analyze/Export Data

Start Prime Plate Add Cells Seal Test

Patch Plate Selection
☒ Standard Patch Plate ☐ Patch Plate PPC

Prime Plate
 Select Plate Coverage:
☒ Whole Plate
☐ 1/2 plate using [Test well]
☐ 1/4 plate using [Test quadrant]
☐ 1/8 plate using [Test eighth]
☐ Add Perforation Agent during Prime Plate

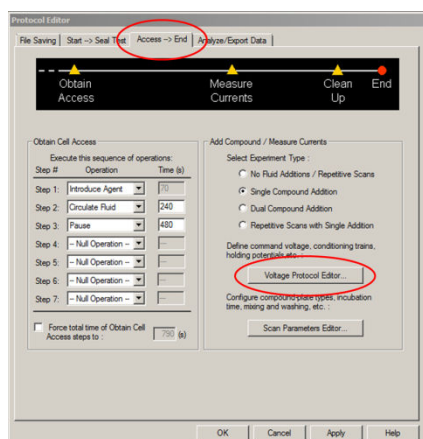
Adding Cells and Seal Test
☒ Resuspend Cells before Dispense Max cycles: 2
 Time to wait before Seal Test (s): 500
 Max (mV): -70
 Min (mV): -80
 Period (ms): 160

Hole Test
 Max (mV): 0
 Min (mV): -10

Enter Remarks
☒ Prompt on Run ☐ Enter Now

OK Cancel Apply Help

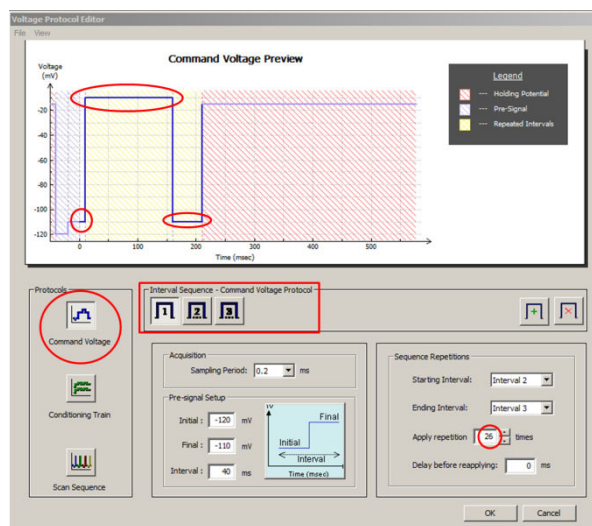
To set up/edit the voltage protocol, select the **Access >> End** tab of the **Protocol Editor** and click the **Voltage Protocol Editor**.



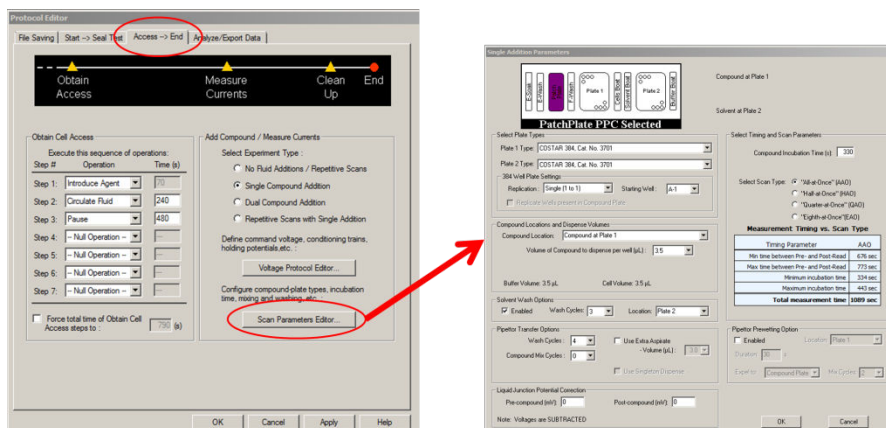
In the **Voltage Protocol Editor**, voltage protocols are generated by clicking **Command Voltage** and adding **Interval Sequences**. Each test pulse must be defined by an interval sequence. In the below example of a 200 ms step to -10 mV from -110 mV, the interval sequences consist of:

1. Holding at -110 mV for 20 ms
2. Holding at -10 mV for 200 ms
3. Holding at -110 mV for 50 ms

By selecting the **Apply repetition** function, the user can repeat any of the interval sequences up to the memory capacity of the IonWorks program. In the below example, interval sequence 2 and 3 are repeated a total of 26 times.

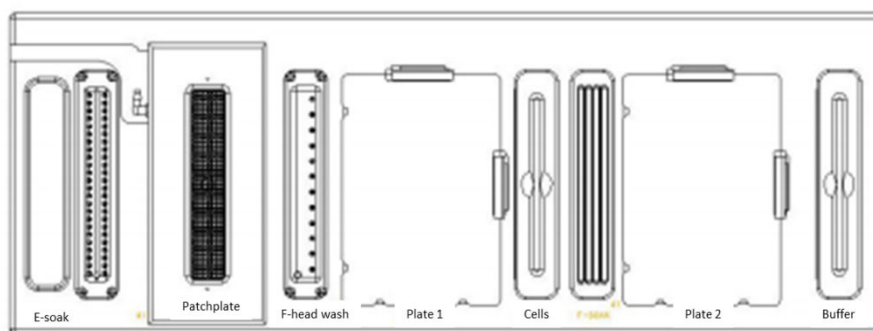


Also on the **Access >> End** tab, compound additions are controlled by using the **Scan Parameters Editor**.

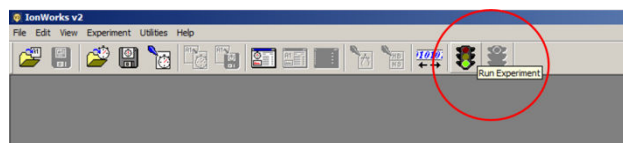


Start of Experiment

1. Place compound plate in **Plate 1** position (for guidelines on preparing compounds, see Compound Preparation below).



2. In **Plate 2** position, place a polypropylene 384-well plate with 50 μ l per well of a 50% DMSO/water mixture to wash F-head in between compound additions.
3. In the **Buffer** position, place a buffer trough filled with fresh 5-7 mL of external buffer solution.
4. Remove patch plate used during the **Flush & Rinse** protocols. With a piece of lint-free tissue, carefully dry out the **Patchplate** area, making sure not to touch the ground electrode.
5. Place a new patch plate into the **Patchplate** area, replace the aluminum lid, and apply the vacuum. You should not be able to lift the lid after the vacuum is applied.
6. Follow Cell Preparation directions below and place cells into the instrument.
7. From main menu, click the green traffic light button to start program.



Cell Preparation

1. Obtain one 70-80% confluent T-75 flask of HEK cells stable expressing the Nav1 channel of interest.
2. Aspirate media from flask.
3. Add 10 mL D-PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$, tilt gently to wash the cell monolayer.
4. Remove the D-PBS wash and add 2 mL trypsin (0.25%), tilt gently to wet the cell monolayer.

- Place flask in 37°C, 5% CO₂ incubator undisturbed until cells start to round up and dislodge.
- Tilt flask to dislodge cells and/or tap sides of flask.
- Add 10 mL of cell media containing 10% FBS, washing the cells down the wall of the flask.
- Gently triturate the cells against the bottom of the flask four times.
- Place cell suspension into a 15 mL conical centrifuge tube and centrifuge @ 1300 rpm for 3 min.
- Aspirate supernatant and resuspend cells in 4 mL external buffer solution.
- Gently triturate the cells using a 200 µl pipette to break up the cell pellet. It is important that cell dispersion is carefully performed.
- Perform cell count.
- Cell concentration should be 2-2.5M cells/mL.
- Dilute with external buffer solution if needed.
- Carefully add entire contents of tube to trough located in **Cells** slot.

Compound Preparation

For concentration response curves (CRC), create a DMSO dilution plate in a 384-well plate following the plate map below to minimize effects from potential carryover of compounds on the E-head pins.

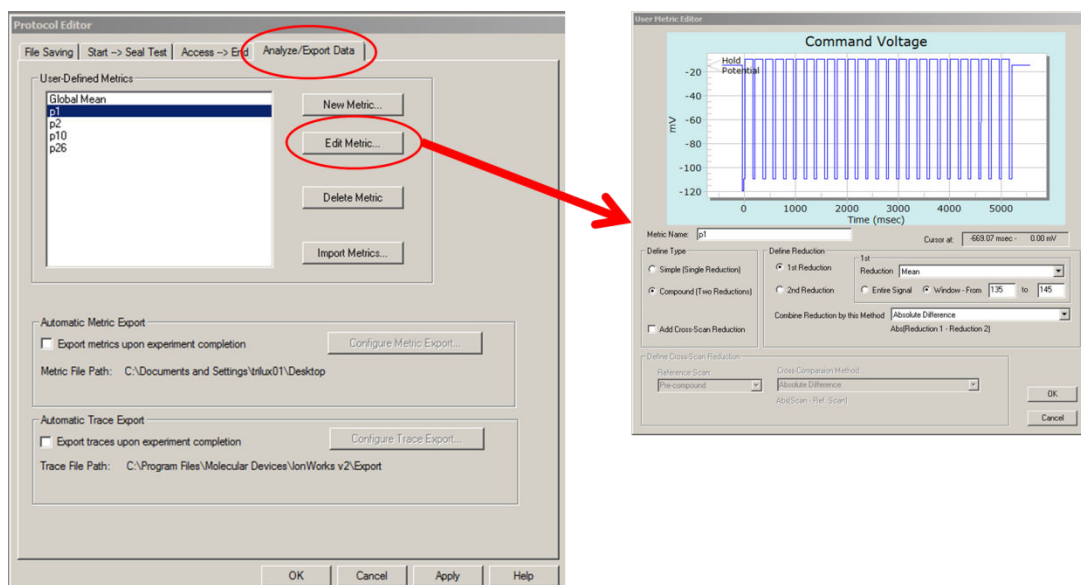
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	High Control		1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	High Control	
B			1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39		
C			2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40		
D			2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40		
E	High Control		1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	High Control	
F			1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39		
G			2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40		
H			2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40		
I	DMSO Control		1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	DMSO Control	
J			1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39		
K			2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40		
L			2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40		
M	DMSO Control		1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39	DMSO Control	
N			1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39		
O			2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40		
P			2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40		

Numbers listed in the plate map correspond to individual test compounds. Concentrations for each test compound should start with the lowest concentration in row A or C and go up in concentration as one moves down the plate (i.e. the highest concentration should be in row N or P).

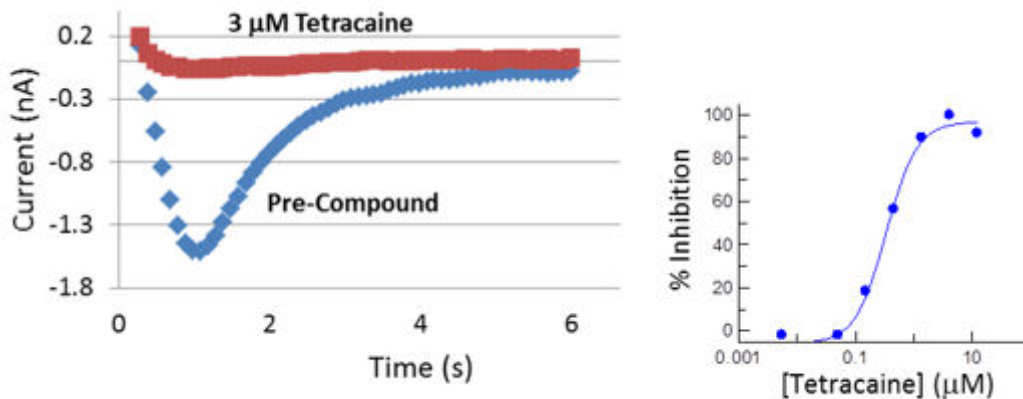
For single point testing, compounds can be placed in any order.

Data Analysis

During an experiment, the IonWorks software generates an ASCII file based on one or more user defined analysis metrics. The metrics are defined under the **Analyze/Export Data** tab in the **Protocol Editor**. Examples of simple metrics, called **Simple (Single Reduction)** include peak current within a voltage interval and average current during a user defined time interval. Simple calculations, such as the difference or ratio between two currents, can also be applied to the data by selecting **Compound (Two Reductions)**.



Nav1 current traces recorded on IonWorks Quattro are shown below before (blue) and after (red) addition of 3 μM tetracaine. For the protocol described, a typical concentration-response curve (CRC) is shown for tetracaine. To generate this CRC, block during the last depolarization was determined at the well level by comparing Nav1 current amplitude before and after compound application, and data were exported out of the IonWorks software as % block, defined as $(\text{current in compound} - \text{current in control}) / \text{current in control} \times 100$. Data were further analyzed by fitting a standard Hill equation to the data using Excel Fit.



End of Day

1. Replace external and internal buffer solutions with 70% ethanol.
2. Place a buffer trough in the **Buffer** position and fill with 5-7 mL of 70% ethanol.
3. On the **Utilities** menu, run **Flush & Rinse** protocol once.
4. Close IonWorks Software and turn off IonWorks Quattro.

Overview

Buffer Solutions (prepared in advance and stored at 4°C)

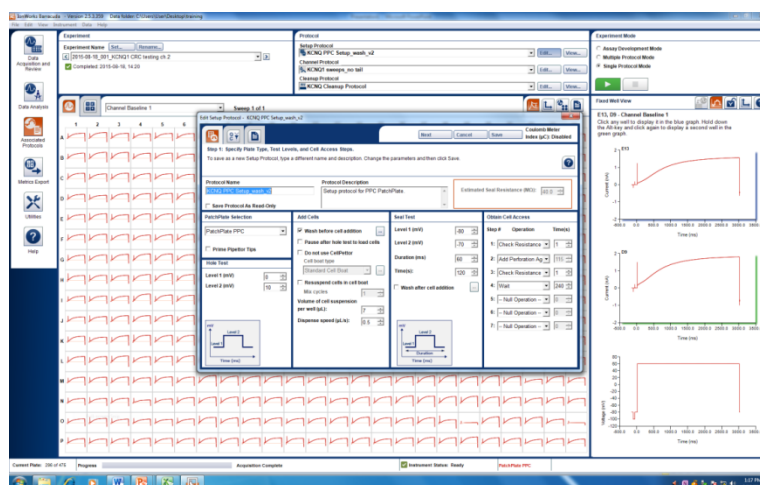
Internal Buffer Solution: 90 mM KGluconate, 40 mM KCl, 3.2 mM MgCl₂, 3.2 mM EDTA, 5 mM HEPES, pH 7.2 with KOH. Osmolarity 290-300 mOsm

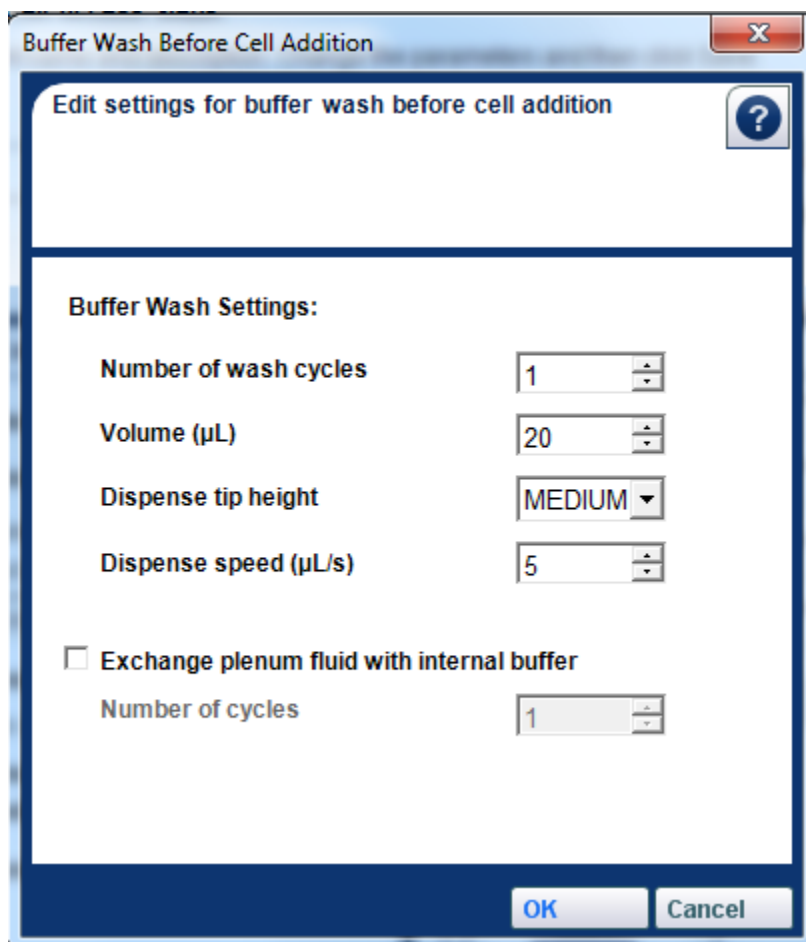
- Amphotericin B is added to internal buffer solution to a final concentration of 0.1 mg/mL to make the antibiotic solution on the day of an experiment.
 - i. Amphotericin B is weighed out and dissolved to a concentration of ~30 mg/mL in 100% anhydrous DMSO and then sonicated for 10 min.
 - ii. Amphotericin B is then added to the internal buffer solution with vigorous mixing.

Building Protocols

Setup Protocol

On the **Edit Setup Protocol** screen, input the following criteria into each of the different folder tabs:





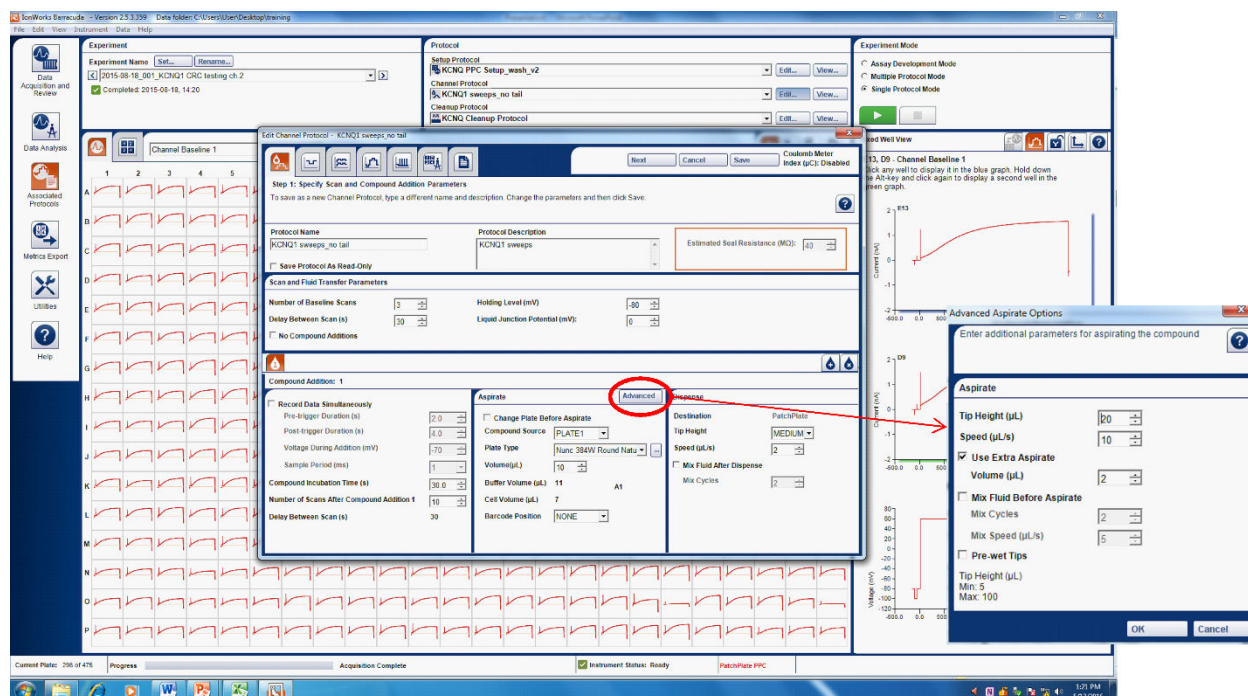
The image shows a software dialog box titled "Buffer Wash Before Cell Addition". It has a standard Windows-style title bar with a close button (X) in the top right corner. Below the title bar, there is a header area with the text "Edit settings for buffer wash before cell addition" and a help icon (question mark) on the right. The main content area is titled "Buffer Wash Settings:" and contains four rows of settings, each with a label and a control element: "Number of wash cycles" with a numeric spinner set to 1, "Volume (μL)" with a numeric spinner set to 20, "Dispense tip height" with a dropdown menu set to "MEDIUM", and "Dispense speed (μL/s)" with a numeric spinner set to 5. Below these settings is a checkbox labeled "Exchange plenum fluid with internal buffer", which is currently unchecked. Under this checkbox is another setting, "Number of cycles", with a numeric spinner set to 1. At the bottom of the dialog box are two buttons: "OK" and "Cancel".

Setting	Value
Number of wash cycles	1
Volume (μL)	20
Dispense tip height	MEDIUM
Dispense speed (μL/s)	5
Exchange plenum fluid with internal buffer	☐
Number of cycles (if checked)	1

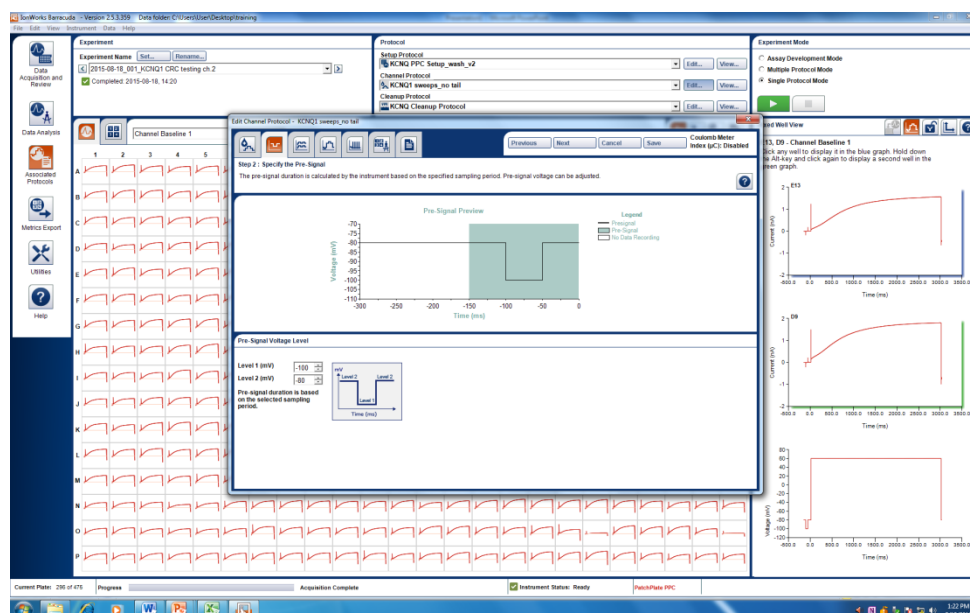
Channel Protocol

On the **Edit Channel Protocol** screen, input the following criteria into each of the different folder tabs:

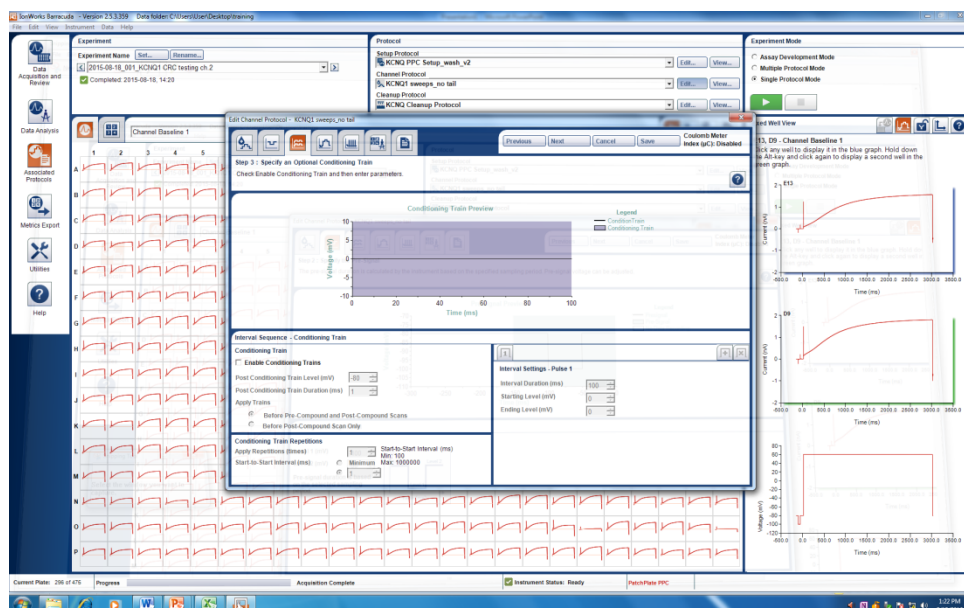
Step 1 specifies the scan and compound addition parameters. Please note the **Advanced** aspirate options as indicated.



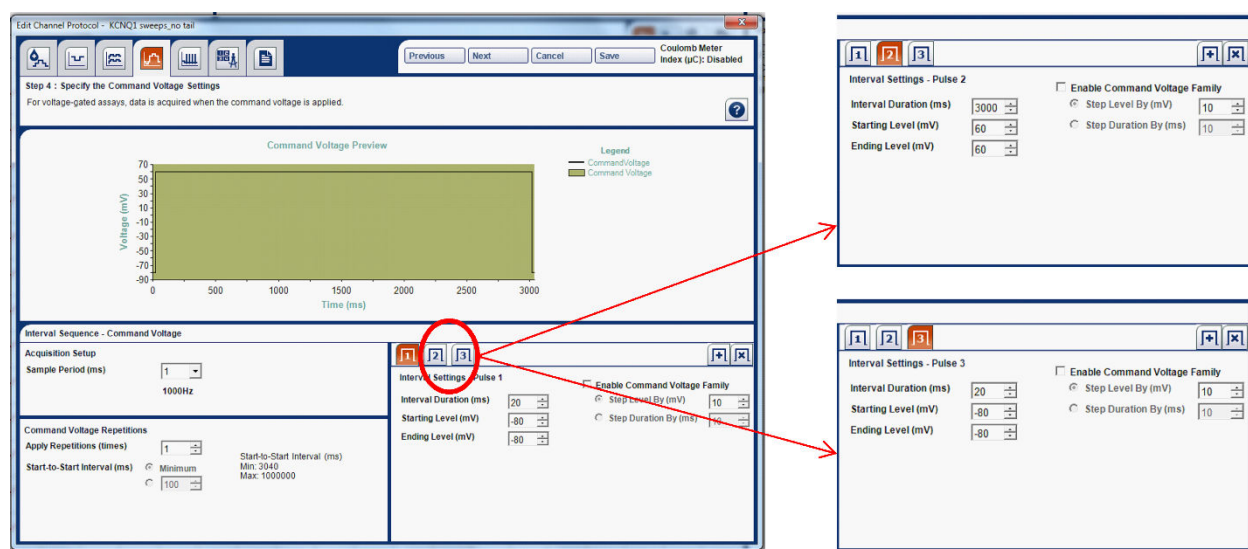
Step 2 specifies the pre-signal criteria.



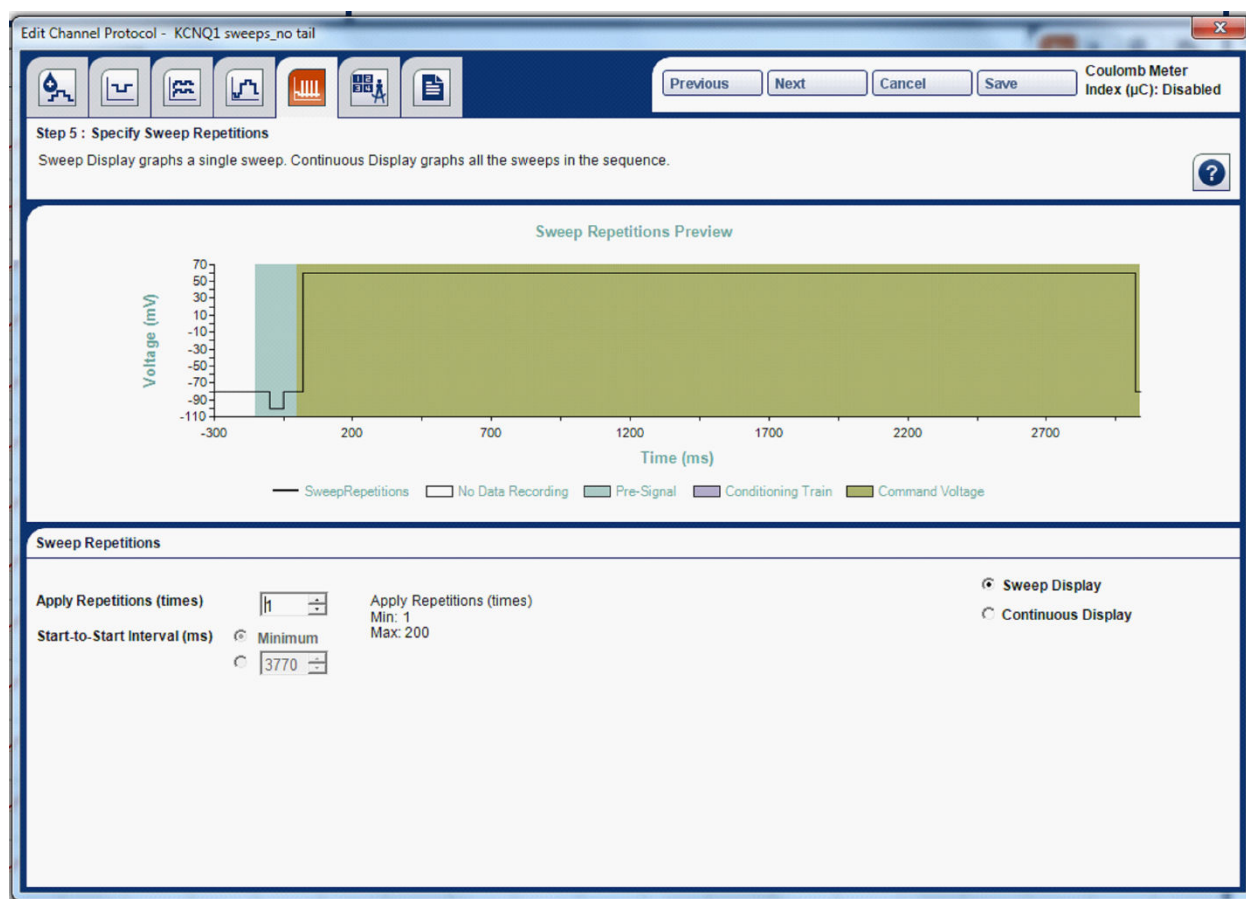
Step 3 specifies the conditioning train criteria. Please note that a conditioning train is not utilized for this particular protocol example.



Step 4 specifies the different command voltage settings available.



Step 5 specifies the sweep repetitions and timeline display of the command voltage protocol (by sweep or continuous display).



Step 6 specifies the metric definitions. Please note that automatic export of metrics is turned off so as to allow manual inspection and rejection/acceptance of well traces before committing to metrics export.

Running Experiments

Start of Day

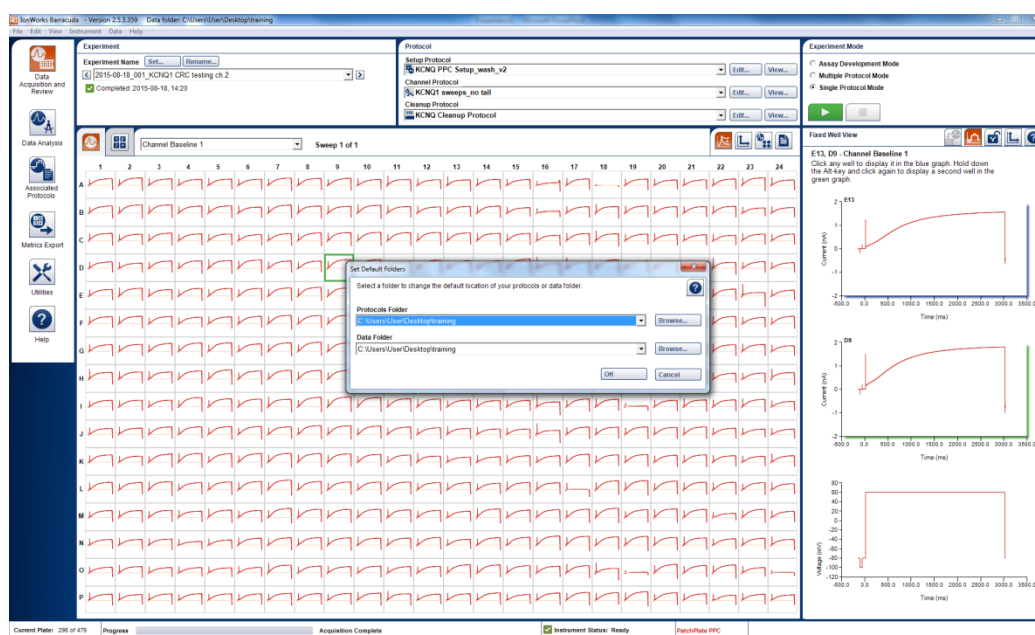
1. Turn on IonWorks Barracuda Instrument/computer using the switch located on right side of instrument.
2. Open the IonWorks Barracuda Control Software.
3. The instrument will go through an initialization.
4. Make sure all waste containers are empty.
5. Make sure there is 0.9% NaCl in both A & B wash containers.
6. External solution bottle should be filled with DPBS (not external solution) that does not contain Mg and Ca.
7. On the **Utilities** menu, select **Load Cell Pipettor** and follow the prompts.
8. Fill both amphotericin B and Internal Solution bottles with internal solution containing 0.1 mg/mL amphotericin B.
9. Place Model Cell on support pins above wash station.
10. Insert ground electrodes into plenum (they must have soaked for at least 20 min in internal solution).
11. Make sure a blank patch plate is inserted over the plenum and click **Start of Day Flush** from the **Utilities** menu.

Cell Preparation

1. Start with >70% confluent T-150 flask of CHO cells stably expressing KCNQ1/KCNE1.
2. Aspirate media, rinse with DPBS, and add 3 mL TrypLE™.
3. Incubate 8 min at 37°C.
4. Resuspend in 9 mL of growth media lacking selection factors.
5. Spin cells at 1000 rpm for 3 min and resuspend in external solution at a density of 2-3 million cells/mL. A minimum of 5 mL of cell suspension are required.
6. Place cells on IonWorks Barracuda.

Start of Experiment

1. In **Set Default Folders** window, select the folders where the data and protocol files will reside.

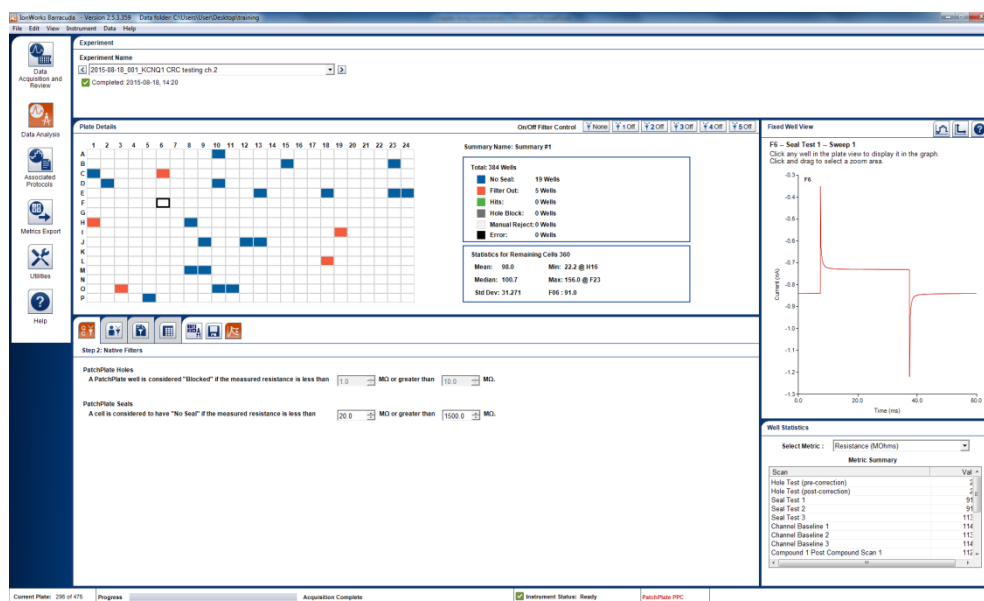


2. Select the desired **Setup**, **Channel**, and **Cleanup Protocols** (as previously described).

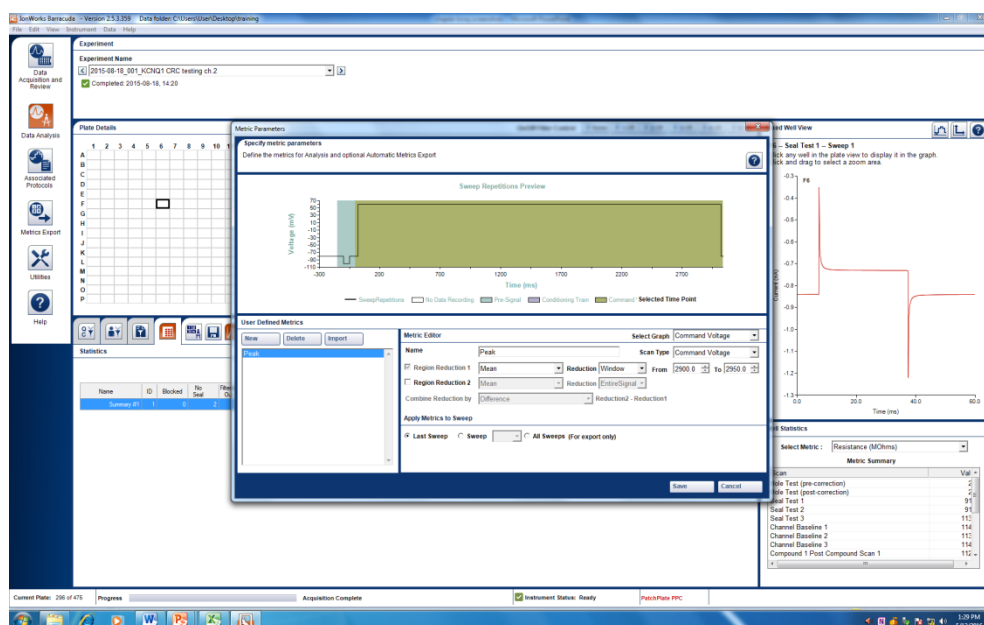
3. Replace patch plate used for **Start of Day** protocol with a fresh patch plate.
4. Replace Model Cell with proper recording E-Head.
5. Make sure tips, buffer boats, and compound plates are seated correctly on the instrument deck.
6. Make sure cells and empty cell dispense plate are seated correctly.
7. Set the experiment name.
8. Click the green **Play** button to start the experiment.

Data Analysis

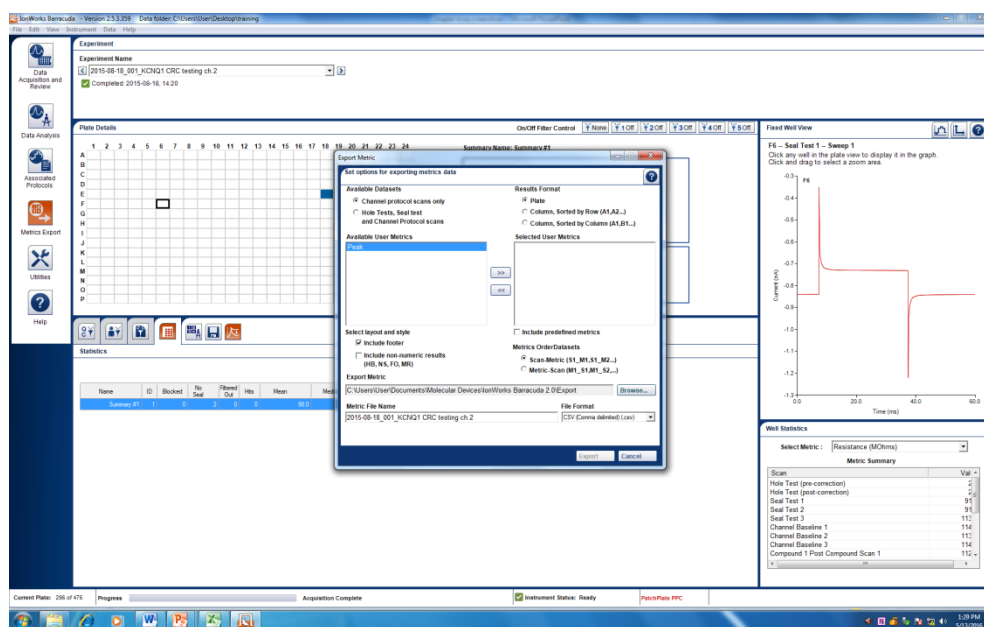
1. From the **Data Analysis** button on the left, see below for an example of all the tabs that can be opened to configure data analysis and export.



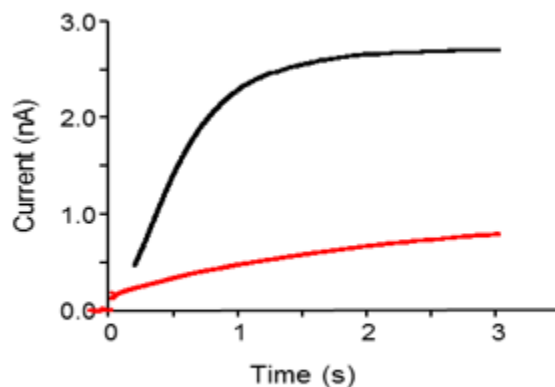
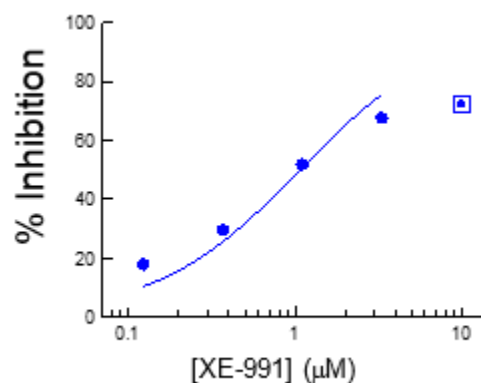
2. On the **Define Filters** tab, set your desired hole/seal resistance, peak current amplitude, and/or other criteria for removing well data from the analysis.
3. Open up the **Metrics Editor** and set the desired time and value measurements (an example is below):



4. Click the **Metric Exports** button on the left and define how and which metrics should be exported.



KCNQ1/KCNE1 current traces recorded on IonWorks Barracuda are shown below before (black) and after (red) addition of XE-991. For the protocol described, a typical concentration-response curve (CRC) is shown for XE-991. Percent inhibition was calculated by averaging the last 10 ms of the current traces during the baseline depolarizations and during the last depolarizing step following the compound addition. Data were further analyzed by fitting a standard Hill equation to the data using Excel Fit.



End of Day Flush

1. Make certain 50% EtOH is in the alcohol wash bottle.
2. Disconnect external solution bottle, amphotericin B bottle, and internal solution bottle and replace with empty 50 mL conical vials.
3. Run **End of Day** protocol from the **Utilities** menu.
4. Remove ground electrodes and place them back into internal solution soak.
5. Shut everything off.

Appendix 3: Protocol for Assessment of GABA_A Current Block on IonWorks Barracuda

Overview

This protocol is designed to evaluate inhibition of GABA-induced currents in a two-addition protocol. Small modifications allow this protocol to be used for agonists or allosteric modulators.

Buffer Solutions (prepared in advance and stored at 4°C)

External Buffer Solution: 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM Glucose, pH 7.4 with NaOH. Osmolarity 310-320 mOsm

Internal Buffer Solution: 90 mM KGluconate, 40 mM KCl, 3.2 mM MgCl₂, 3.2 mM EDTA, 5 mM HEPES, pH 7.2 with KOH. Osmolarity 265-275 mOsm

- Amphotericin B is added to internal buffer solution to a final concentration of 0.1 mg/mL to make the antibiotic solution on the day of an experiment.
 - i. Amphotericin B is weighed out and dissolved to a concentration of ~30 mg/mL in 100% anhydrous DMSO and then sonicated for 10 min.
 - ii. Amphotericin B is then added to the internal buffer solution with vigorous mixing.

Cell Suspension (prepared immediately prior to experimental run)

1. Start with 80-90% confluent T-150 flask of HEK cells stably expressing GABA_A (same protocol is used for all GABA_A isoforms).
2. Aspirate media, rinse with DPBS, and add 3 mL TrypLE.
3. Incubate 8 min at 37°C.

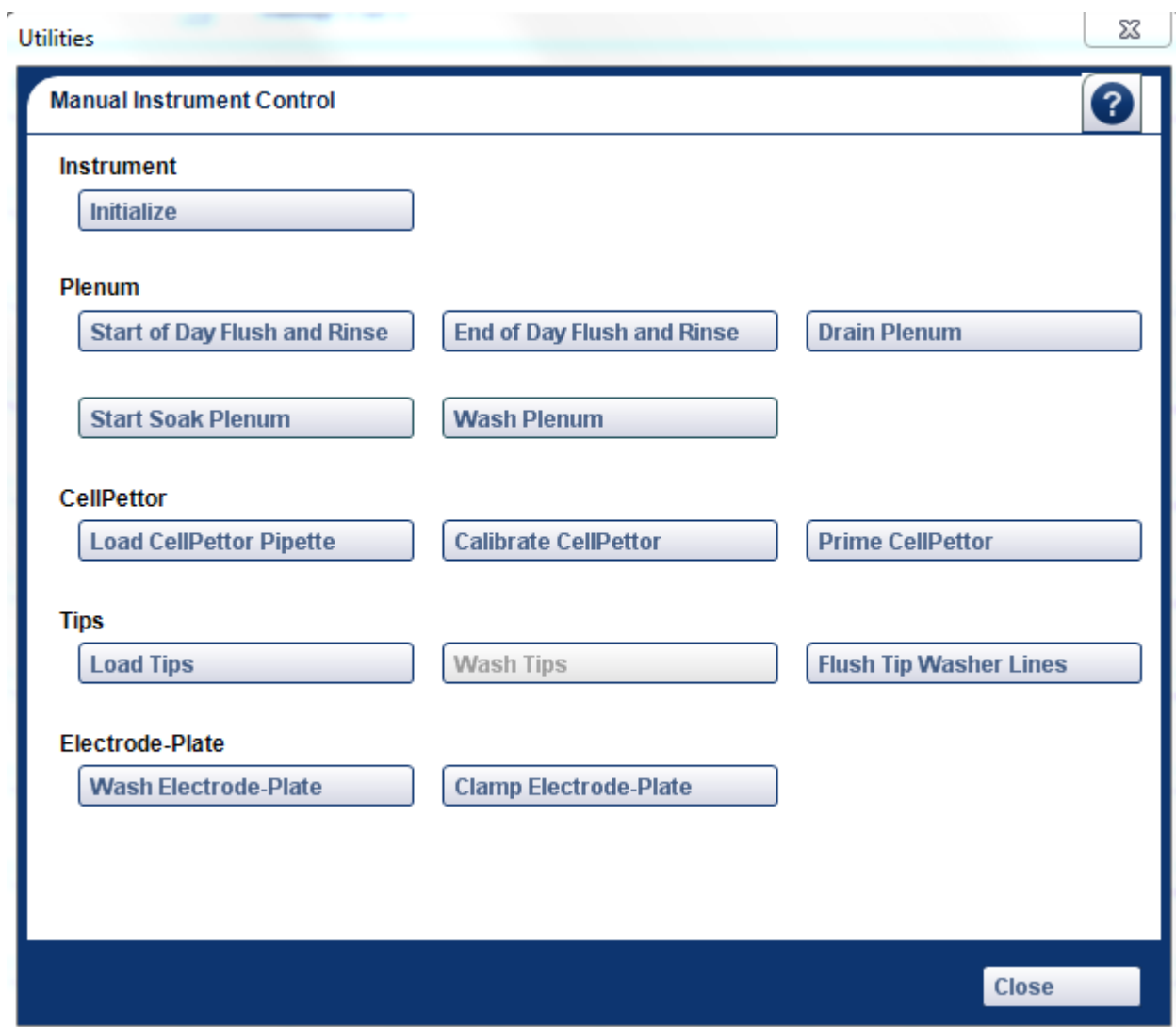
4. Transfer to 15 mL conical tube, add 9 mL of growth media lacking selection factors and triturate (7-10 times).
5. Spin cells at 1000 rpm for 3 min, aspirate supernatant and resuspend in external solution at a density of 3 million cells/mL.
6. There should be at least 5 mL of cell suspension ready to be placed on IonWorks Barracuda.

Test Compounds

1. Prepare a Mother Plate:
 - a. Plate 50 μ L of sample dissolved in 100% DMSO in columns 3 and 13 of 384-well plate.
 - b. Serially dilute (10 point CRC with 1:3 steps) using liquid handler robot.
 - c. Spin plate at 600 rpm for 1 min to collect all solution in the bottom of the well.
 - d. Seal under argon and store at room temperature for up to one month (may not be suitable for all samples).
2. Prepare Daughter Plates. On the day of experiment prepare compound and agonist plates:
 - a. Dispense 100 μ L of external solution into each well of a 384-well plate (Compound Plate).
 - b. Dispense 100 μ L of 1mM GABA containing external solution into each well of a 384-well plate (Agonist Plate).
 - c. Add positive and negative controls to columns 1, 2, 23 and 24 of the compound plate.
 - d. Immediately prior to the experiment, transfer 1 μ L of DMSO stock solution from Mother Plate to Compound Plate and to Agonist Plate.
 - e. Mix three times and spin plate at 600 rpm for 1 min.

Start IonWorks Barracuda (Start of Day)

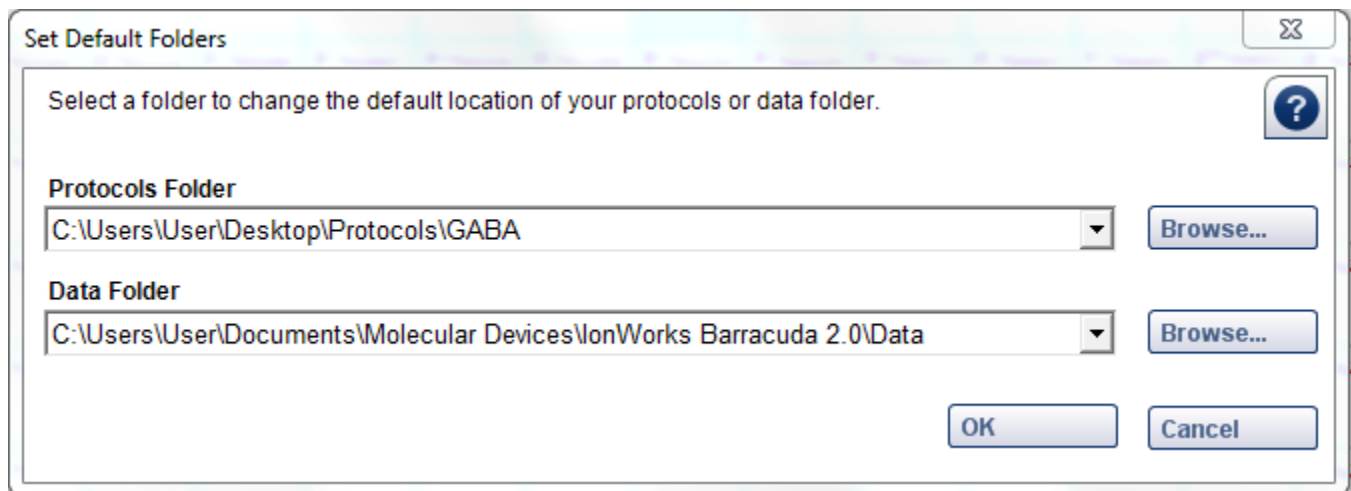
1. Turn on IonWorks Barracuda instrument/computer using the switch located on the right side of instrument.
2. Open IonWorks Barracuda control software (the instrument will initialize automatically).
3. Make sure all waste containers are empty.
4. Make sure there is 0.9% NaCl in both A & B wash containers (approximately 4 L).
5. External solution bottle should be filled with DPBS without Mg^{2+} and Ca^{2+} (prevents deposits).
6. On the Utilities menu, select **Load Cell Pipettor** and follow the prompts:



7. Fill both amphotericin B and Internal Solution bottles with internal solution containing 0.1 mg/mL amphotericin B.
8. Place Model Cell on support pins above wash station.
9. Insert ground electrodes into plenum (they must have soaked for at least 20 min in internal solution).
10. Make sure that a blank (uncontaminated with compounds) patch plate is correctly positioned in the **Patch Plate** position on the plenum.
11. On the **Utilities** menu, select **Start of Day Flush**.

Select Data and Protocol Folders

On the **File** menu, select **Set Data Folders**:

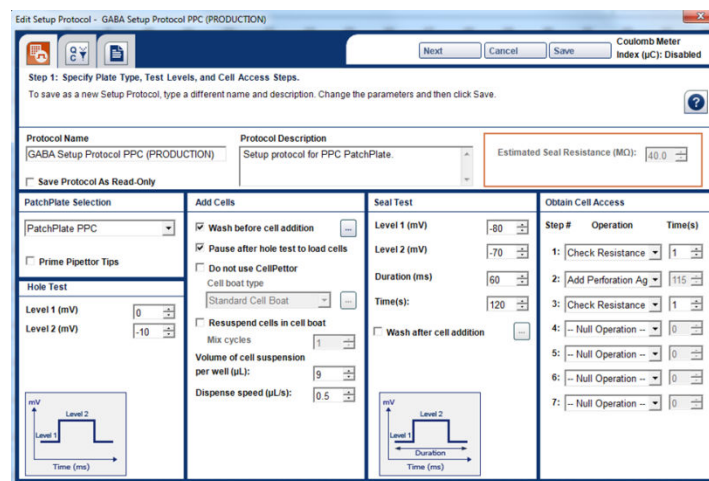


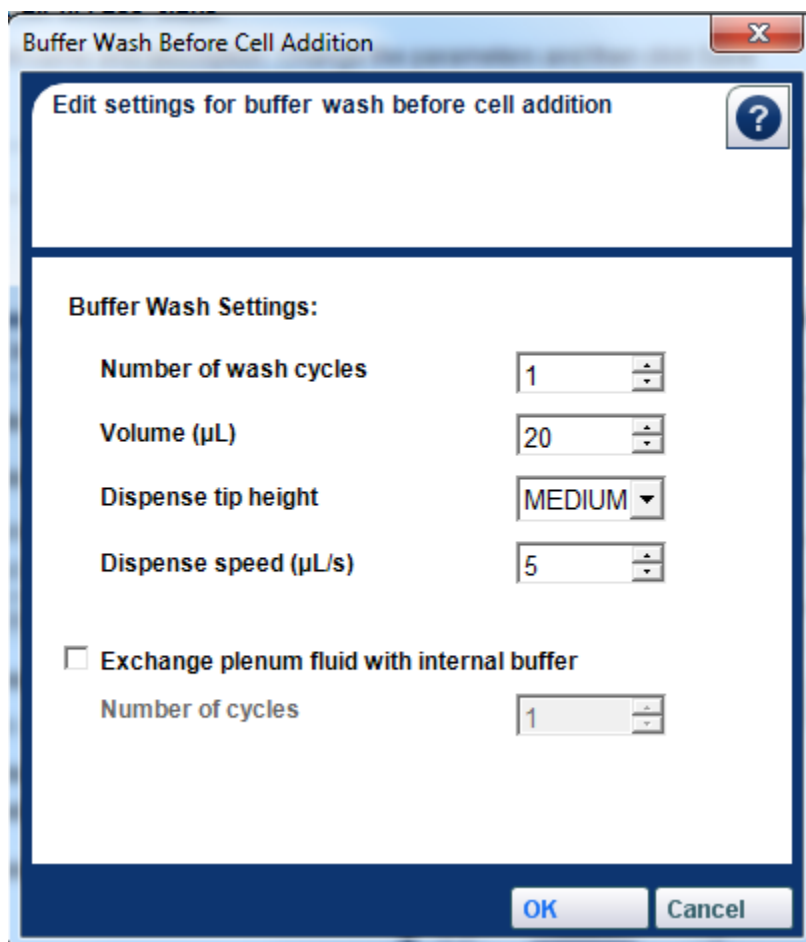
Select IW-Barracuda Protocols for GABA_A

On the **Protocol** window select **Setup**, **Command Voltage** and **Cleanup** protocols:

Setup Protocol

From the **Setup Protocol** menu, input the following criteria into each of the different folder tabs:





1. In the **Add Cells** panel select **Wash before cell addition** and specify the wash criteria
2. In case that amphotericin B is not added to Internal Solution bottle, then **Create Cell Access** needs to be modified by the addition of **Wait 240 s** following the **Add Perforation Agent**

Channel Protocol

From the drop down “Channel Protocol” menu, select appropriate protocol.

1. First compound addition:
 - a. Set holding potential to -80 mV.
 - b. Aspirate 10 µL of solution from **Compound Plate** (position **Plate 2**). Dispense at medium height at 5 µL/s.
 - c. Record current 2 s prior and 30 s after compound addition with sampling frequency at 1 kHz.
 - d. Follow with 3 min compound incubation.
 - e. No scans are recorded prior or after compound addition.

Edit Channel Protocol - GABA Ag-Antag Protocol (3min Incubation)

Next Cancel Save Coulomb Meter Index (μC): Disabled

Step 1: Specify Scan and Compound Addition Parameters
To save as a new Channel Protocol, type a different name and description. Change the parameters and then click Save.

Protocol Name: GABA Ag-Antag Protocol (3min Incubation) Protocol Description: GABA channel protocol with compound first and agonist second addition. Estimated Seal Resistance (MΩ): 40

☐ Save Protocol As Read-Only

Scan and Fluid Transfer Parameters

Number of Baseline Scans: 0 Holding Level (mV): -80
Delay Between Scan (s): 10 Liquid Junction Potential (mV): 0
☐ No Compound Additions

Compound Addition: 1

☒ Record Data Simultaneously

Pre-trigger Duration (s): 2.0
Post-trigger Duration (s): 30.0
Voltage During Addition (mV): -80
Sample Period (ms): 1
Compound Incubation Time (s): 180.0
Number of Scans After Compound Addition 1: 0
Delay Between Scan (s): 10

Aspirate Advanced

☐ Change Plate Before Aspirate
Compound Source: PLATE2
Plate Type: Nunc 384W Round Natsu
Volume (μL): 10
Buffer Volume (μL): 11 A1
Cell Volume (μL): 9
Barcode Position: NONE

Dispense

Destination: PatchPlate
Tip Height: MEDIUM
Speed (μL/s): 5
☐ Mix Fluid After Dispense
Mix Cycles: 2

2. Second compound addition:

- Maintain holding potential at -80 mV
- Aspirate 10 μL of solution from “Agonist Plate” (position **Plate 1**). Dispense at medium height at 5 μL/s
- Record current 2 s prior and 30 s after compound addition with sampling frequency at 1 kHz
- No scans are recorded prior or after compound addition

Edit Channel Protocol - GABA Ag-Antag Protocol (3min Incubation)

Next Cancel Save Coulomb Meter Index (μC): Disabled

Step 1: Specify Scan and Compound Addition Parameters
To save as a new Channel Protocol, type a different name and description. Change the parameters and then click Save.

Protocol Name: GABA Ag-Antag Protocol (3min Incubation) Protocol Description: GABA channel protocol with compound first and agonist second addition. Estimated Seal Resistance (MΩ): 40

☐ Save Protocol As Read-Only

Scan and Fluid Transfer Parameters

Number of Baseline Scans: 0 Holding Level (mV): -80
Delay Between Scan (s): 10 Liquid Junction Potential (mV): 0
☐ No Compound Additions

Compound Addition: 2

☒ Record Data Simultaneously

Pre-trigger Duration (s): 2.0
Post-trigger Duration (s): 30.0
Voltage During Addition (mV): -80
Sample Period (ms): 1
Compound Incubation Time (s): 35.0
Number of Scans After Compound Addition 2: 0
Delay Between Scan (s): 10

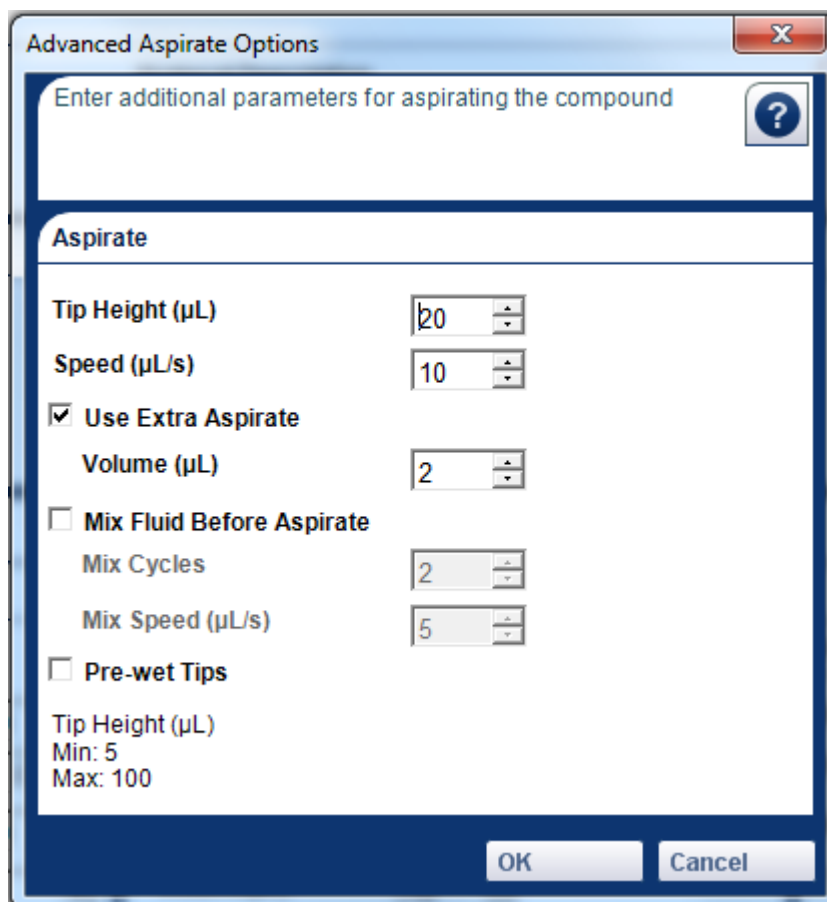
Aspirate Advanced

☐ Change Plate Before Aspirate
Compound Source: PLATE1
Plate Type: Nunc 384W Round Natsu
Volume (μL): 10
Buffer Volume (μL): 11 A1
Cell Volume (μL): 9
Barcode Position: NONE

Dispense

Destination: PatchPlate
Tip Height: MEDIUM
Speed (μL/s): 5
☐ Mix Fluid After Dispense
Mix Cycles: 2

For both, first and second dispense, adjust the **Advanced Aspirate Option** to the tip height of 20 μL, speed of 10 μL/s and extra volume to 2 μL. This setting minimizes the introduction of bubbles on dispense.

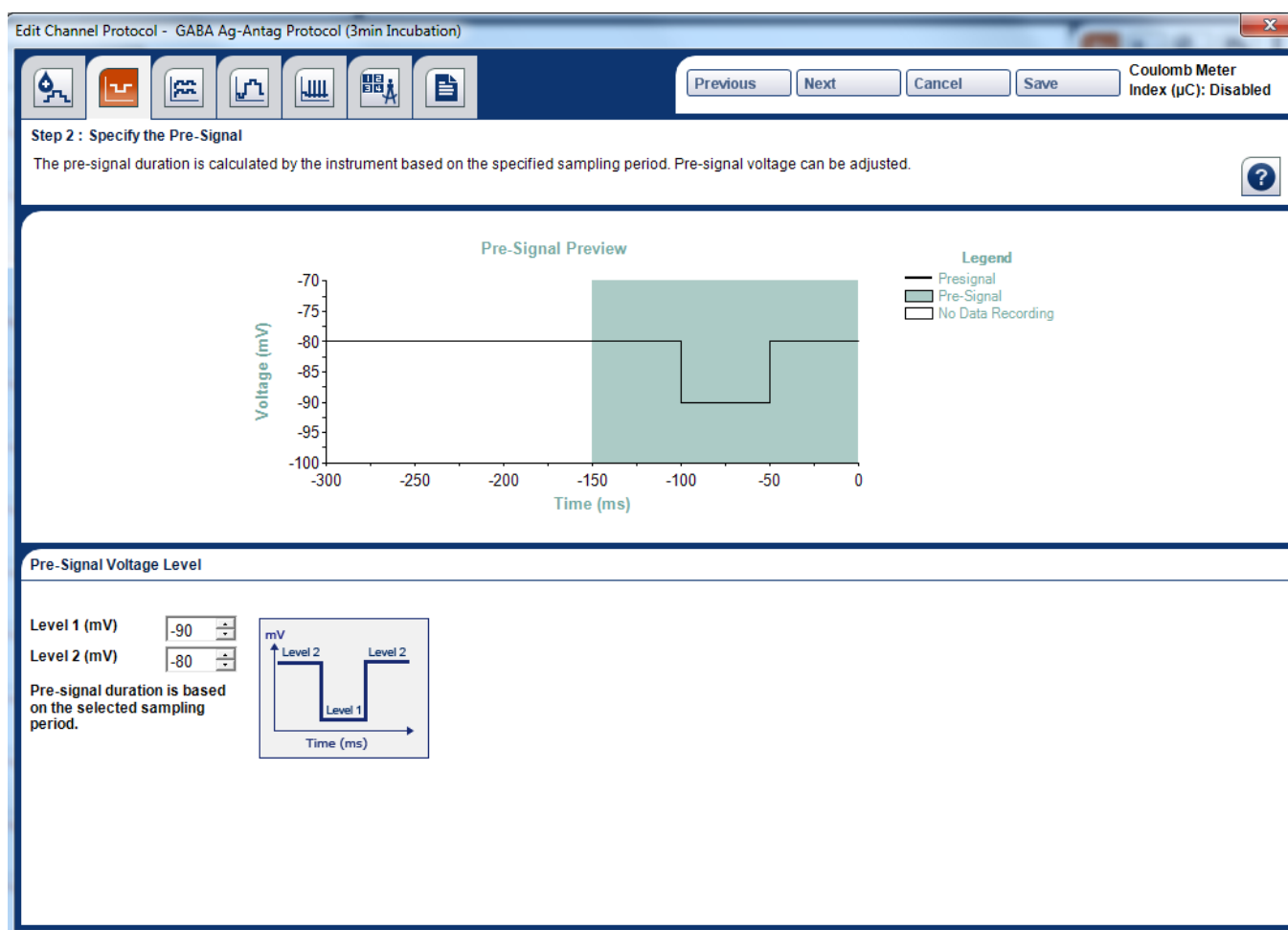


The image shows a software dialog box titled "Advanced Aspirate Options". At the top, there is a text field with the placeholder "Enter additional parameters for aspirating the compound" and a help icon (question mark). Below this is a section header "Aspirate". The settings are as follows:

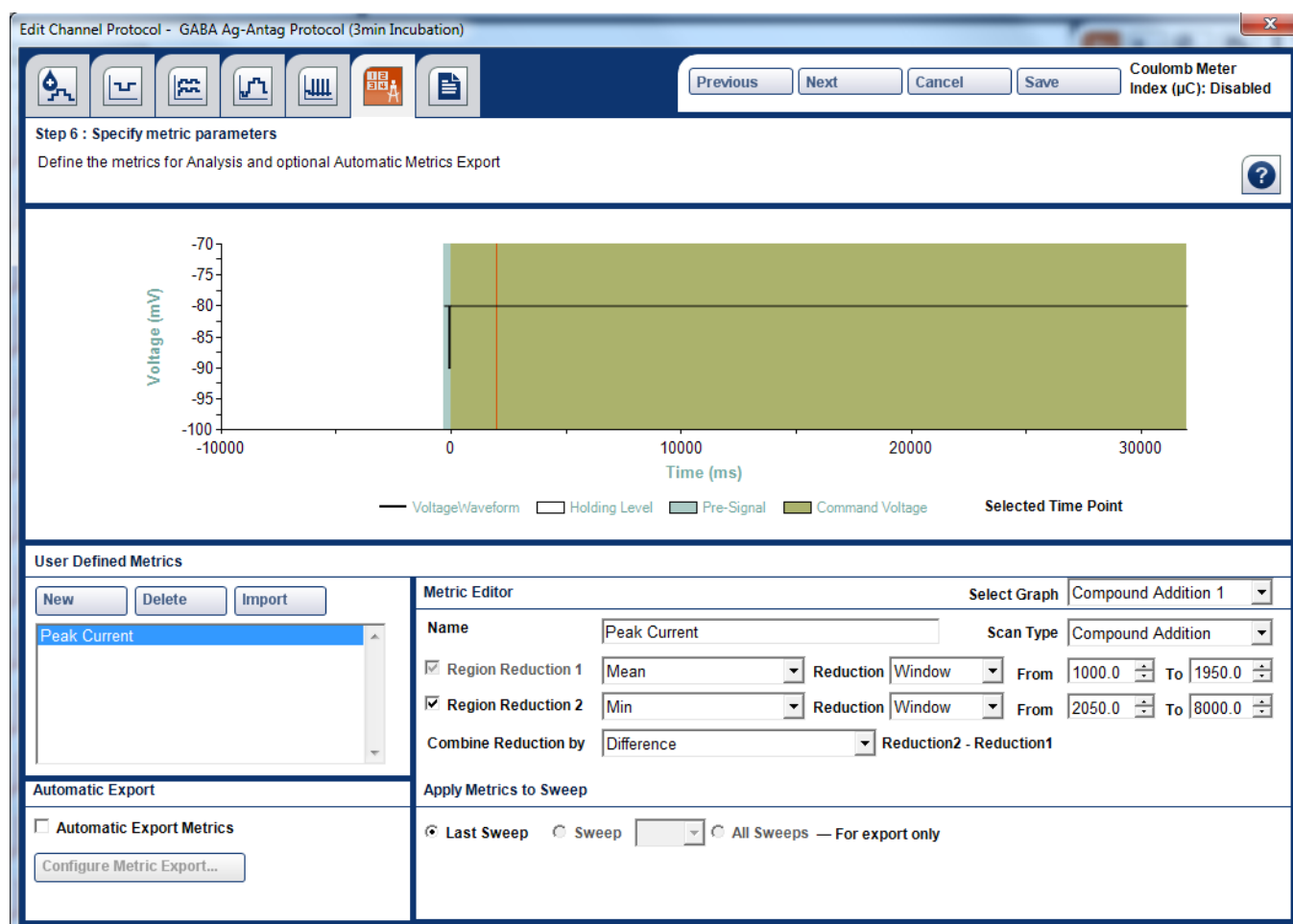
Parameter	Value
Tip Height (μL)	20
Speed ($\mu\text{L/s}$)	10
☒ Use Extra Aspirate	
Volume (μL)	2
☐ Mix Fluid Before Aspirate	
Mix Cycles	2
Mix Speed ($\mu\text{L/s}$)	5
☐ Pre-wet Tips	
Tip Height (μL)	
Min:	5
Max:	100

At the bottom right are "OK" and "Cancel" buttons.

3. Set the resistance pulse (pre-signal):



4. Set the metric parameters:



NOTE: In this metric, 'automated export metrics' is not used. This permits manual inspection and rejection of traces. The only measured parameter is peak current. Alternative measurements such as AUC or absolute current amplitude are not measured.

Cleanup Protocol

From the **Cleanup Protocol** menu, input the following criteria into each of the different folder tabs:

Edit Cleanup Protocol - GABA Cleanup Protocol (PRODUCTION)

Step 1: Specify Instrument Cleanup

One Wash is required. Choose the solution from Source A or Source B. Ultrasonic cleaning is recommended for the Electrode-Plate.

Protocol Name: GABA Cleanup Protocol (PRODUCTION)

Protocol Description: GABA cleanup protocol. Ultrasonic cleaning applied for E-Plate washing (2x0.5min). F-head tips are discarded.

☐ Save Protocol As Read-Only

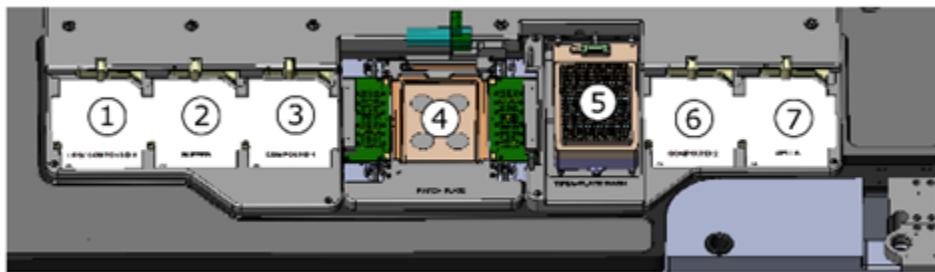
Plenum	Tip Washing	Electrode-Plate
Drain Plenum ☐ Wash plenum Soak Duration (m): 30 Number of cycles: 1 Soak Duration (m): Min: 5 Max: 1440	☒ Wash Fluid 1 ☐ Enable ultrasonic cleaning Wash Source: A Number of strokes per wash cycle: 10 Number of wash cycles: 1 ☐ Wash Fluid 2 ☐ Enable ultrasonic cleaning Wash Source: B Number of strokes per wash cycle: 5 Number of wash cycles: 1	☒ Wash Fluid 1 ☒ Enable ultrasonic cleaning Wash Source: A Dwell time per wash cycle (min): 0.5 Number of wash cycles: 2 ☐ Wash Fluid 2 ☐ Enable ultrasonic cleaning Wash Source: B Dwell time per wash cycle (min): 1.0 Number of wash cycles: 1
Wash Tip Options ☐ Wash Tips and Electrode-Plate ☒ Discard tips		

NOTE: This protocol discards tips and sonicates the electrode plate in aqueous solution. To further minimize compound carryover, an additional manual wash of the e-plate in 70% ethanol was performed off the instrument.

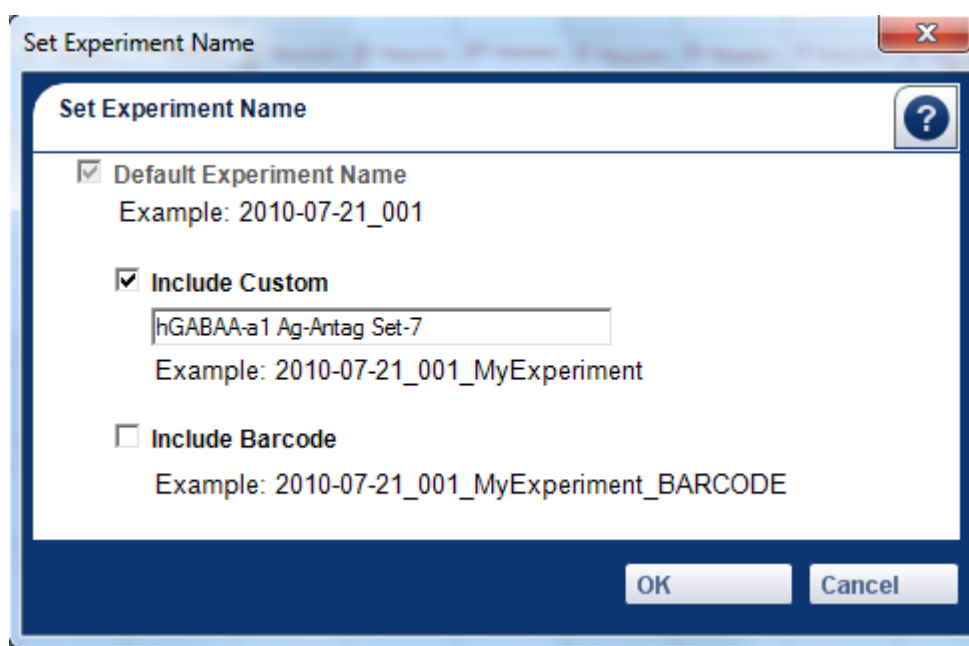
Run Experiment

1. On **Set Default Folders** window, select the proper default folders for where the data and protocol files will reside.
2. Select the desired **Setup**, **Channel**, and **Cleanup Protocols** (as previously described) from the drop down menus.
3. Load the instrument deck. Make sure tips, boats, and plates are seated correctly:
 - a. Pipette tips are in position **Tips** (position labeled 1 in the diagram below)
 - b. External buffer boat is in position **External Buffer** (position 2)
 - c. Agonist plate is in position **Compound 1** (position 3)
 - d. Appropriate new patch plate (SH or PPC) is in position **Patch Plate** (position 4)
 - e. Correct electrode plate is positioned on **Wash Station** (make sure that the plate is lying flat and in correct orientation) (position 5)

- f. Compound plate is in position **Compound 2** (position 6)
- g. Clean cell boat is in position **Cell Boat** (position 7)



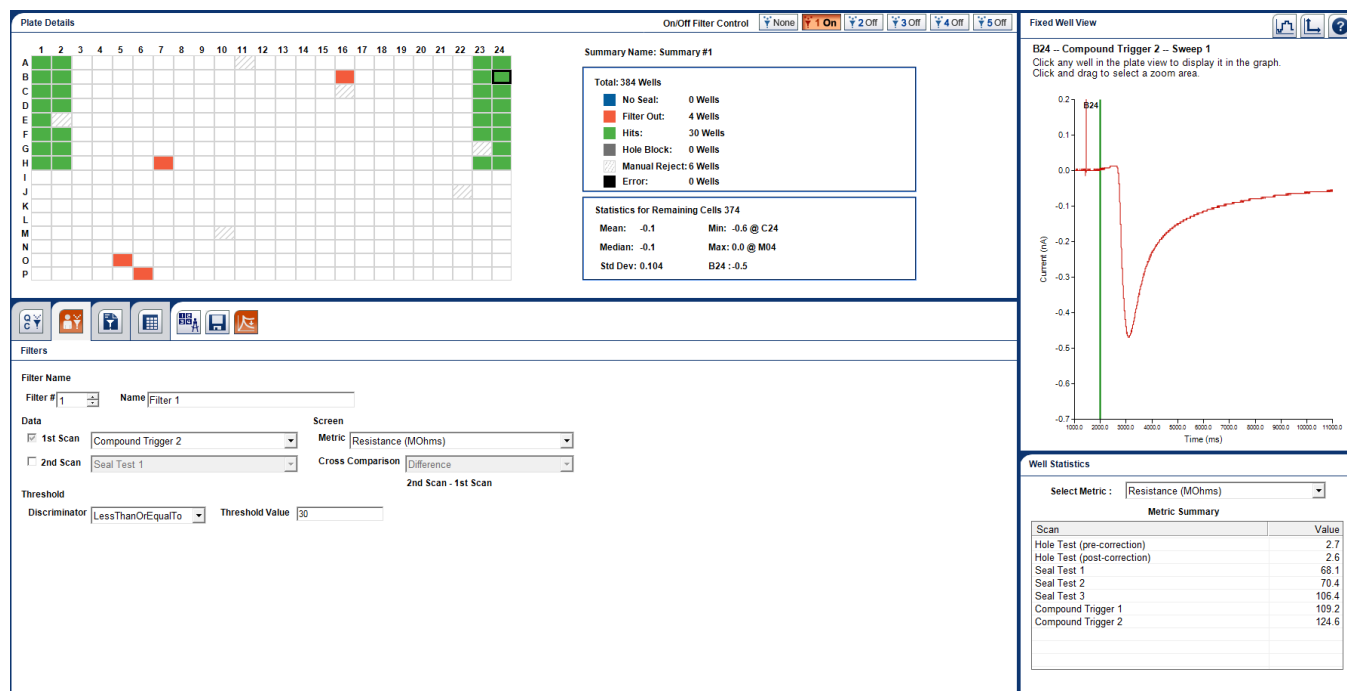
- 4. Make sure that the cells position is empty (conical vial has been removed).
- 5. Set the experiment name.



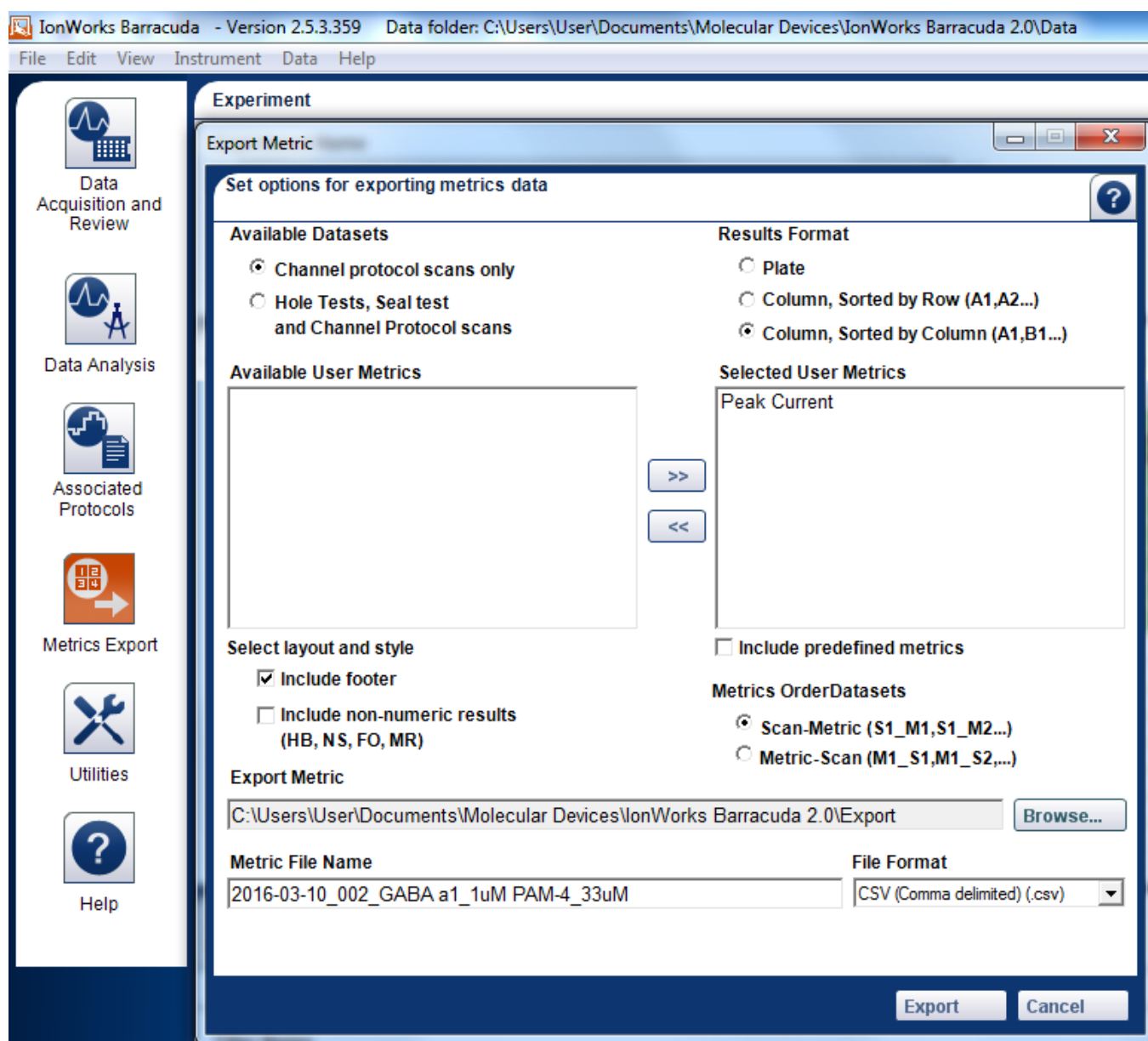
- 6. Click the green **Play** button to start the experiment.
- 7. When the instrument pauses to load cells (after Hole Test) add the conical vial with cell suspension.
- 8. At the end of the run, remove and discard conical vial, tip box, agonist plate and compound plate. Remove the cell boat, wash it with H₂O and wipe dry. Remove the electrode plate and wash by dipping in 70% ethanol followed by 0.9% NaCl solution. Buffer reservoir can be left on the deck if additional runs are planned.

Analyze Data

- 1. From the **Data Analysis** button on the left, see below for an example of all the tabs that can be opened to configure data analysis and export.



- In the **Define Filters** tab, set your desired hole/seal resistance, peak current amplitude, and/or other criteria for removing well data from the analysis. Additional filtering can be performed by visual inspection of traces and manual removal of wells.
- Click the **Metric Exports** button on the left and define how your metrics should be exported.



End of Day Flush and Rinse

1. Make certain 50% EtOH is in the alcohol wash bottle.
2. Disconnect external solution bottle, amphotericin B bottle, and internal solution bottle and replace with empty 50 mL conical vials.
3. Replace the electrode plate with model cell.
4. Replace the contaminated patch plate with an un-contaminated (has not been exposed to compounds) used patch plate.
5. Run **End of Day Flush & Rinse** protocol from the **Utilities** menu.
6. Remove ground electrodes and place them back into internal solution soak.
7. Power off the IonWorks Barracuda.

Modifications of IW-Barracuda Protocols for Assessment of Agonists and Allosteric Modulators of GABA_A Channels

Agonist

Use the same protocol as described for assessment of current block. Compound plate should contain 1 mM GABA in position designated as maximum control (Col 1, 2, 23 and 24). In the analysis, select scan 1 instead of scan 2.

Positive Allosteric Modulator (PAM)

Use the same protocol as described for assessment of current block. Adjust the agonist concentration in “agonist Plate” to GABA EC₂₀. Add appropriate positive and negative controls to “Agonist Plate” (Col. 1, 2, 23 and 24). Analyze scan 2.

Negative Allosteric Modulator (NAM)

Use the same protocol as described for assessment of current block. Adjust the agonist concentration in “agonist Plate” to GABA EC₆₀. Add appropriate positive and negative controls to “Agonist Plate” (Col. 1, 2, 23 and 24). Analyze scan 2.

Appendix 4: Protocol for Assessment of State-Dependent Sodium Channel Inhibitors on PatchXpress

Overview

This protocol is designed to evaluate block of voltage-gated sodium channels at depolarizing voltages that favor interactions of test compounds with inactivated states of the channel.

Voltage Protocol using scripting

Cells are sealed and whole cell configuration attained according to defined patch protocols that can be obtained from Molecular Devices. Sampling rate is adjusted to the maximal available rate of 31.25 kHz (32 μ s sampling interval). Once a stable whole cell configuration is achieved, cells are held at -120 mV and currents are monitored with short (5-20 ms) test pulses to a voltage near the peak of the current-voltage curve until currents reach stable amplitude. At this point, a standard protocol is executed to determine the voltage dependence of steady-state inactivation, for example this protocol may consist of a set of 1 s conditioning pulses to voltages ranging from -120 mV to -20 mV followed by a short test pulse to the same voltage used during the previous test pulses. A software script is used to select a new holding potential that corresponds to approximately 40% channel inactivation. Current amplitudes are again allowed to stabilize at the new inactivated potential for approximately 2-3 minutes while applying test pulses at 0.1 Hz. Following current stabilization at the inactivated potential, a 10 pulse train at 5 Hz is applied. Following the pulse train, currents are again allowed to stabilize at 0.1 Hz prior to the first compound addition. Two compound additions occur approximately 15 s apart, and effects on current amplitude are monitored by test pulses at 0.1 Hz. Once the software script determines that stable current amplitudes have been reached, a second 5 Hz pulse train determines use-dependent effects. Wash-out occurs at the inactivated potential for approximately one minute before the holding potential is returned to -120 mV with continued wash-out until current recovers to 80% of its initial control amplitude. Assuming satisfactory wash-out has occurred, the cell may be used for further compound additions until cell health and/or current amplitude do not remain stable.

Typical Solutions

External Buffer Solution: 137 mM NaCl, 4 mM KCl, 1.8 mM CaCl₂, 0.2 mM MgCl₂, 10 mM HEPES, 10 mM D-mannitol, adjust pH to 7.4 using NaOH. Final osmolality: 300-310 mOsm

Internal Buffer Solution: 60 mM CsF, 70 mM CsCl, 5 mM NaCl, 10 mM EGTA, 10 mM HEPES, adjust pH to 7.4 using CsOH. Final osmolality: 290-300 mOsm

Notes:

- Slightly lower osmolality in the internal solution compared to the external solution often helps with sealing.
- Some F⁻ in the internal solution helps to maintain seal resistances; however, higher F⁻ concentrations can lead to time-dependent shifts in the voltage dependence of inactivation.
- At room temperature the Na⁺ reversal potential for this solution is approximately +84 mV.

Start of Day

1. Allow external and internal solutions to come to room temperature, if stored refrigerated.
2. Turn on PatchXpress 7000A Instrument using switch located on left side of instrument.
3. The associated CPU will need to be restarted to initialize settings between CPU and Hardware.
4. Open the PatchXpress Control Software.
5. When prompted by PatchXpress Commander, log on to the server housing the database.
6. After initialization, the instrument will prompt you to begin **Start of Day** procedure.
7. Make sure there is H₂O in the wash (thin) container and that the waste (large) container inside the PatchXpress cabinet is empty.
8. Load external and internal solutions onto the PatchXpress Instrument.
9. Begin **Start of Day** procedure - this replaces the H₂O in the lines with the internal and external assay buffers- using the blank seal chip stored in the instrument.
10. Prime wash station (hardware fluidics).
11. Add pipette tips.

Cell preparation

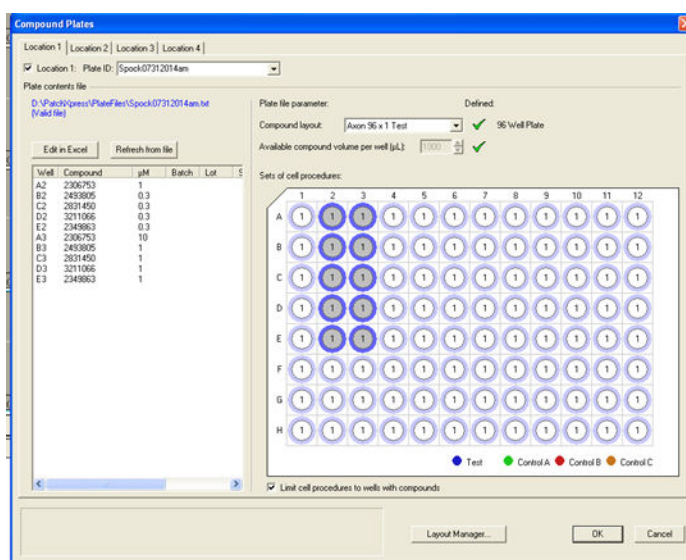
1. Start with >70% confluent T-75 flask (or equivalent) of HEK cells expressing the Nav1 channel of interest.
2. Pour off media, and add 2 mL TrypLE .
3. Incubate with TrypLE for 30-120s, tap flask to dislodge cells and add 3 mL of media.
4. Triturate with 5 mL pipette.
5. Transfer to 15 mL conical tube and spin at 1300-1500 rpm for 3 min.
6. Aspirate supernatant and resuspend cell pellet in 4-8 mL of media.
7. Triturate with 1 mL pipette and add 1 mL each of the cell suspension to 4-6 small Eppendorf tubes.
8. Store uncapped in incubator for at least 30 min and up to 3 h (optimal timing may differ between cell lines).
9. At the start of an experiment, spin cells at 2000 rpm for 2 min, remove supernatant, resuspend in 200 µL external buffer solution, triturate lightly and place the tube into the PatchXpress instrument

Start of Experiment

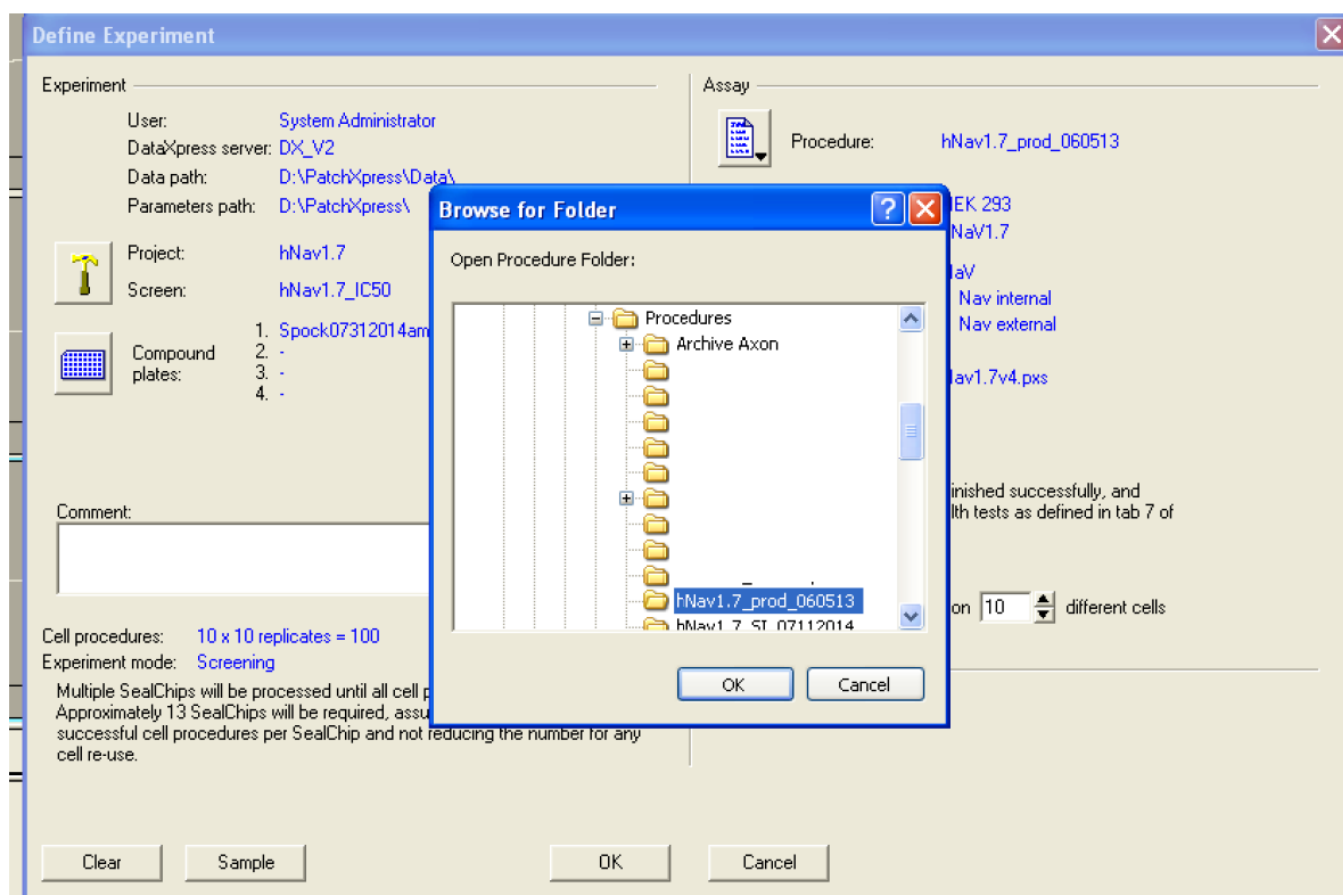
The following settings must be established to define the experiment on the PatchXpress prior to loading the Seal chip:

1. In **Define Experiment** window, select the proper project from the menu.

2. Create a table using Excel that specifies the location and concentration of each compound to be tested in the compound plate and select this table in the PatchXpress software.



3. Select the proper procedure from the dropdown box in **Procedure** window:



4. Check **Re-use cells** with 10 replicates.
5. Under **Select Cell**, select cell, i.e. the parental cell background – typically HEK293 or CHO, and channel, e.g. Nav1.x, from the drop down boxes.
6. Select the appropriate internal and external solutions.

7. Select the appropriate patch settings. Molecular Devices provides standard settings for HEK293 and CHO cells that can be modified for optimal results with a particular cell line of interest.
8. Select **Screening mode** which means that the software will keep track of which compounds and concentrations have been tested successfully.

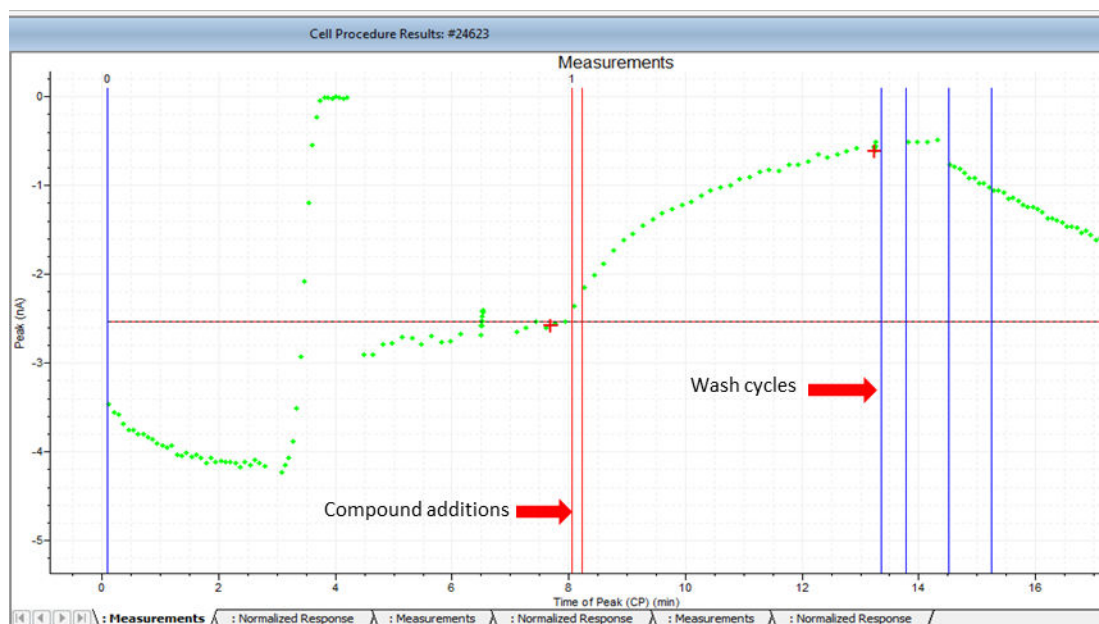
Once the experiment has been defined:

- Place test compounds onto the deck of the PatchXpress instrument. Test compounds need to be dissolved in external buffer solution and can be presented in a 96-well plate or in glass vials inserted into a plastic rack provided by Molecular Devices.
- Wash station may need to be primed again, if too much time has passed since this step was performed last.
- Resuspend an aliquot of cells in External Buffer Solution (see last step of Cell Preparation protocol) and place into the PatchXpress instrument.
- Remove a seal chip from the glass vial and flick out the remaining water. Blow the seal chip dry using the tool inside the PatchXpress cabinet and place the seal chip into the receiver on the deck of the PatchXpress with the straight edge to the back.
- Click the green **Play** button to start the experiment. Once the instrument initializes, it will request a barcode. Enter the barcode from the seal chip vial using the barcode reader (this step is optional).
- After the seal chip is loaded, the instrument will “call for cells”, requiring the user to enter information regarding the cell line for future reference (examples include cell line, passage number and time after dissociation).

Analysis

Aggregate data from multiple single concentration additions are plotted and fit using the Hill equation. Use-dependence based on the last pulse of the 5 Hz pulse trains may also be reported. Run down correction may be employed on a limited basis.

This procedure overview is summarized below for a compound which demonstrates inhibition:



End of Day

1. Replace external and internal buffer solutions with water.

2. Run **End of Day** protocol.
3. Turn off the computer and PatchXpress instrument.

Assay Development Guidelines for Image-Based High Content Screening, High Content Analysis and High Content Imaging

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Abstract

Automated microscope based High Content Screening (HCS, or HCA, HCI) has gained significant momentum recently due to its ability to study many features simultaneously in complex biology systems. HCS can be used all along the preclinical drug discovery pipeline, it has the power to identify and validate new drug targets or new lead compounds, to predict *in vivo* toxicity, and to suggest pathways or molecular targets of orphan compounds. HCS also has the potential to be used to support clinical trials, such as companion diagnostics. In this chapter, state of the art HCS approaches are detailed, and challenges specific to HCS are discussed. It should serve as an introduction for new HCS practitioners. More chapters will follow on specific assay examples and on high level informatics analysis.

1. Introduction

1.1. What is High Content Screening (HCS)?

High Content Screening (HCS) or automated microscope-based screening measures biological activity in single cells or whole organisms following treatment with thousands of agents, such as compounds or siRNAs, in multi-well plates. Typically, multiple features of the cell or organism are measured with one or more fluorescent dyes leading to the term High Content. At times, HCS has been called high content analysis (HCA) high content imaging (HCI) or image cytometry (IC). Generally, HCA, HCI and IC refer to lower throughput automated microscope based assays (<100,000 samples or data points), although HCA sometimes refers to the analysis portion of HCS. The term HCS was first used in a 1997 paper by Giuliano et al (1). It appears to be the natural successor to the automation of clinical histology (2) and an extension of HTS plate reader systems, with early references to the automation of the analysis of microscope images dating back to the advent of the desk top computing (3). Examples of early systems were the Oncor Videometric150, the BDS chromosome painting and the Meridian ACIS Ca²⁺ image tracker, among others. Our modern automated analysis solutions owe their origins to Metamorph and ImagePro as well as a 1969 publication by Rosenfeld (4).

In contrast to traditional HTS, which has a single read out of activity, HCS allows a scientist to measure many properties or features of individual cells or organisms at once. The ability to study many features and multiplex

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† Editor

‡ Editor

simultaneously is both what gives HCS tremendous power and challenging complexity. Like its predecessor technologies such as standalone low throughput automated image analysis systems and screening instruments including the FMAT (5), HCS can be quite effectively used simply to provide improved signal to background or signal to noise. But it can also enable both targeted and phenotypic assays that measure movement within a cell or between cells or allow analysis of specific sub-populations of cells in a heterogeneous mix that would be difficult or impossible to run with other techniques. Most powerfully, HCS can be used to help predict the efficacy of potential drugs in unique cellular niches when applied to physiologically relevant cellular systems. The predictive power of such systems can often be enhanced by working with primary cells or differentiated stem cells and in 3-D culture rather than with cell lines in a traditional 2-D culture where many unique aspects of cellular physiology have been lost (6,7). Bickmore focused on this critical problem when she noted “the exquisite cell-type specificity of regulatory elements revealed by the ENCODE studies emphasizes the importance of having appropriate biological material on which to test hypotheses” in the 2012 publication of the data from the ENCODE project (8).

1.2. Uses of HCS

The most obvious applications of HCS are primary screens of potential leads, molecules that can be further optimized into drug candidates, for cellular activities that cannot be easily measured by a single endpoint, such as spatially localized proteins or measurements of cellular morphology. In almost all instances, there are alternative assay formats that can be used for primary screening, but HCS increases the power of the experiment by measuring. For example, HCS can be used to measure the formation of gap junctions by mixing cells that have been preloaded with fluorescent dye with non-loaded cells, and measuring the spread of the fluorescent dye between cells over time in culture (9). This is a very robust way to measure the establishment of functional gap junctions; however, alternatively, a binding assay could be established that looked for an increase in the expression of the gap junction proteins on the cell surface as a primary screen. That kind of HTS approach could not, however, assess functional gap junctions, the way HCS can. Likewise, cellular differentiation can be monitored by measuring an increase in the expression of a marker of the differentiated cell type and/or a decrease in the expression of a marker of the undifferentiated cell type. However, coupling these measurements with a visual measure of differentiation can increase confidence in the outcome.

Cellular morphology changes, such as neurite outgrowth can only be measured in a microscopic image, with or without a molecular marker to confirm the relevance of observed morphology changes. Cell morphology can also be an important measure of cellular differentiation (10) such as the differentiation of epithelial cells to mesenchymal cells (11) or of precursor cells into oligodendrocytes, astrocytes and neurons (12). Here too, the concomitant use of a differentiation marker can help confirm initial analyses and clarify the lineage. Intracellular morphological changes such as protein expression, trafficking or translocation can also be studied. An example of protein translocation, NF- κ B translocation will be discussed in a separate chapter. Movement between the cytoplasm and the nucleus or other organelles can be measured or trafficking between organelles and the cell surface can be studied. Likewise, one can measure internalization of proteins or vesicles. Similarly, movement of cells across a well, such as models of wound healing, migration, or chemotaxis, can all be evaluated with HCS (13, 14).

In addition, there are many useful lead identification applications that could be done with more traditional HTS techniques but where HCS provides an advantage. Because HCS provides more than just the endpoint, off-target effects such as cytotoxicity or fluorescence from the test compounds are easily identified. Additionally, signal to background can often be improved; for example, cell surface binding can be seen in the presence of background noise by using confocal imaging and co-localizing the signal with a cellular surface marker. Alternatively, there are assays where the statistical power of a single read out is not sufficient to enable screening in singlicate but is enabled by combining multiple features to improve the predictive power of the assay.

1.3. Applications of HCS beyond lead identification

The utility of HCS goes well beyond the identification of lead compounds for the development of pharmaceuticals. HCS can be used all along the drug discovery pipeline for the identification or validation of appropriate drug targets, for predicting the pathway or molecular target of compounds identified in phenotypic screens, for lead optimization and toxicity prediction or for the analysis of clinical data. Outside of biological assays, HCS systems have also found utility in chemistry and material science, for instance for screening of crystallization conditions, corrosion resistance or ceramic formation/structure.

Target identification and validation is a very common application of HCS. Potential drug targets can be identified by assaying the effect of increasing or knocking-down RNA expression in an assay that could later be used for compound screening. The cell used in the assay must be amenable to transfection and the cell culture conditions will often need to be modified to allow for translation, transcription and expression of the desired protein to be over expressed or alternatively for the degradation of the existing mRNA of a protein to be knock-down. Alternatively, new targets and pathways can be identified by screening compounds with known mechanisms of action against a phenotype. Not surprisingly, the potential targets identified by these two approaches are often complementary rather than confirmatory since there is a bias in the timing of the effects (15, 16). Overexpression of proteins can occur very quickly, within minutes of transfections. In contrast, RNAi mediated knockdown can take 48 hours or longer to reduce protein levels. These same techniques can also be used for validating suspected targets although clinical or human genetic data is needed for full validation.

HCS can be used for target prediction or pathway profiling. Pathway profiling is an approach used to identify the pathway or target of an orphan compound coming from a phenotypic screen where assays known to be sensitive to the regulation of a pathway or target are used to suggest the mechanism of action, MOA, of an orphan compound. For example, Lonza-Odyssey Thera (17) and the Broad Institute's Metabolite profiling platform (<http://www.broadinstitute.org/scientific-community/science/platforms/metabolite-profiling-platform/metabolite-profiling-platform>) both have existing platforms for assigning MOA to compounds with unknown MOA.

HCS can also be used to improve our understanding of the on and off target effects of compounds identified by traditional HTS as they progress through lead optimization. For instance, a cellular enzyme activity assay can be used to confirm that a compound known to affect enzyme activity in a biochemical assay retains activity in the cellular environment. Alternatively, compounds identified by traditional screens can be clustered by cellular phenotype in an imaged based screen to suggest both selectivity and toxicity issues. More directly, *in vitro* micronuclei formation assays (18) use HCS to predict which compounds are likely to lead to DNA damage *in vivo* and developmental toxicity assays run on whole embryos can warn of potential reproductive toxicity liabilities that will need to be further evaluated (19).

Even further down the pharmaceutical pipeline, HCS is used to evaluate both *ex vivo* and clinical samples for relevant activity. For instance, both preclinical and clinical blood samples can be assayed for biomarker activity of particular cell types by HCS (20).

1.4. HCS Challenges

Even with the tremendous processing capacity of modern computers, high speed inter-connections, and relatively cheap data storage available with today's computers, the application of HCS has significant technical challenges that need to be considered. Although many instruments come with powerful, easy to use image analysis software, HCS practitioners often find that third party analysis software is needed to improve the performance of an occasional assay. In these cases, fluid partnership between the assay developer and an image analysis expert can greatly reduce assay development time by balancing the effort put into optimizing each. At the same time, due to lack of image and data format standards, many solutions for data storage, data transfer,

and data annotation, do not translate well between platforms. In addition, the total size of data collecting and manipulated in HCS can be daunting. Storage needs for a single academic lab are typically a few terabytes per year. The costs for on-line storage with RAID and off-line backup are not trivial (between \$20K - \$100K in 2012) and decisions will eventually have to be made about what data to keep and what to discard. Anticipating data and image transfer and sharing needs during the installation of your HCS solution will ultimately make your solution much more satisfying.

The other area that needs careful contemplation before embarking on an HCS campaign is the consistency and reproducibility of your cellular model. It is common for variables that would not be noticeable in a traditional HTS to become a major source of variance. Consider the effects of mechanical forces or thermal fluctuation on cellular stress responses (21, 22). Differentiation assays are acutely sensitive to changes in proliferation rates that vary with donor age (23, 24). Likewise, proliferation assays are sensitive to differentiation. Gene translation rates may be affected by what phase of the cell cycle cells begin in. The power of HCS to measure basic biological phenomenon, means that the practitioner also needs to be acutely aware of controlling the assay conditions and of interpreting results, with the latter being one of the most important aspect of HCS.

1.5. Summary

HCS has the power to identify new drug targets or new lead compounds, help predict cellular *in vivo* toxicity, suggest molecular targets of orphan compounds, and assess *in vivo* activity among other yet to be explored uses. HCS can be used to measure the effects of compounds and biological molecules such as plasmids carrying cDNAs, or RNAs on subpopulations of cells and specific cell types in mixed cultures. It can be used to measure movement, be it intracellular, cellular or intercellular. Any phenomenon that can be seen reproducibly in a microscope can ultimately be assayed with HCS. Important considerations and state of the art approaches will be detailed in rest of the chapter.

2. Image Technologies and Instruments

2.1. Introduction to Image Technologies

In theory, any instruments that produce multiparametric analysis of cellular or organismal phenotypes can produce high content data. Fluorescent Activated Cell Sorters (FACS) with multiple lasers can do this, as can MALDI-TOF mass spectrophotometers. But the term is most conventionally applied to automated microscopes using fluorescent and transmitted light to image cells, tissues, or small organisms such as Zebrafish embryos, *C. Elegans* or *Drosophila* larva followed by sophisticated image and data analysis. There are many commercial vendors of instruments in the High Content Analysis (HCA) arena and it is impossible to describe them all as the market and models are constant changing. Nonetheless, it is possible to divide the most widely used instruments into just three categories: wide-field imagers, confocal imagers and laser scanning cytometers. Basic descriptions of features that define each category will be provided along with representative examples.

2.1.1. High Content Screening requires speed

Effective screening campaigns required that the instrumentation have as high a throughput as possible. In the high content arena many choices have to be made to enable fast data acquisition. Lower magnification allows larger image fields that can capture larger numbers of cells which is needed to obtain statistically meaningful results. Similarly, cameras with larger chips (2048×2048 pixel vs 1040×1400 pixel) can image larger fields. Use of high intensity light sources such as lasers or optimizing fluorescence staining or fluorescent reporter expression so the fluorescent signals are bright will reduce integration exposure time and therefore the image acquisition time, as does using an objective with relatively high numerical aperture. If essential phenotypic features can be recognized with two channels as opposed to three, four or more channels, large savings in screening times can be achieved. During the assay development phase selecting the appropriate features that discriminate positive

and negative controls can dramatically speed the final screen design to achieve the highest throughput possible. It is important to keep in mind that the goal is not necessarily to produce attractive images of individual cells, of the sort acquired with high magnification on a confocal microscope. Rather the goal is to detect and quantify critical features from the captured image that define a large phenotypic space from hundreds of cells in a particular treatment condition in as short a time as possible.

2.1.2. High Content Imagers versus Microscopes

High content imagers (HCI) at the present time typically consist of an automated microscope or components of a microscope in a box supplied with image analysis software and commonly have image management packages to store images on a server/data storage system. They generate very large image sets and associated meta-data that can be greater than a 0.5 TB per day at full capacity in screening campaigns. Compared to research microscopes, the configuration choices are much more limited. For example, a particular instrument may have only one fluorescent light source option, one or two camera options and no choices regarding objectives. But they have rapid autofocus and very precise stages optimized for multi-well plates and most systems offer environmental control for live cell imaging.

2.1.3. High Content Analysis versus High Content Screening

HCI can be used in two contexts. In a research environment, HCA is often a medium throughput activity where a few hundred or a few thousand perturbagens (compounds, drugs, siRNAs, cDNAs) are tested and scores of parameters are recorded from each individual cell using multiple imaging channels. The readouts can be kinetic or single endpoint using live or fixed cells respectively. The images are retained and perhaps reanalyzed with the goal of getting a very complex assessment of different subpopulations of cells in each well. The purpose could be basic research or to serve as a tertiary screen to study the toxicological properties of hits from a target based screen. In contrast, HCS is like other HTS methods aimed at screening 100,000s of perturbagens. The goal is to identify hits for additional testing. The readout is typically a fixed endpoint to reduce noise and facilitate automation. A smaller number of parameters are often selected during the assay development phase to speed screening and reduce the data storage requirements.

2.1.4. Background Information on Microscopy

Fluorescence imaging is widely used in biomedical research so a detailed introduction is not appropriate here. Several commercial and non-commercial websites provide extremely detailed information about microscopy and readers are encouraged to explore them to learn details about different fluorescent techniques listed in the links provided in below:

- Florida State University
 - Home: <http://micro.magnet.fsu.edu/primer/index.html>
 - Microscopy: <http://micro.magnet.fsu.edu/primer/index.html>
 - Fluorescence microscopy: <http://micro.magnet.fsu.edu/primer/index.html>
 - Objectives: <http://micro.magnet.fsu.edu/primer/anatomy/objectives.html>
 - Filters: <http://micro.magnet.fsu.edu/primer/lightandcolor/filtershome.html>
 - Confocal: <http://micro.magnet.fsu.edu/primer/techniques/confocal/index.html>
 - Numerical Aperture: <http://micro.magnet.fsu.edu/primer/anatomy/numaperture.html>
 - Airy Disc: <http://micro.magnet.fsu.edu/primer/java/imageformation/airydiskbasics/index.html>
 - Nipkow: <http://micro.magnet.fsu.edu/primer/techniques/fluorescence/spinningdisk/index.html>
 - Laser light sources: <http://micro.magnet.fsu.edu/primer/lightandcolor/lasersintro.html>
 - Fluorescent Proteins: <http://micro.magnet.fsu.edu/primer/techniques/fluorescence/fluorescentproteins/fluorescentproteinshome.html>
 - FRET: <http://micro.magnet.fsu.edu/primer/techniques/fluorescence/fret/fretintro.html>
- Olympus

- Microscopy: <http://www.olympusmicro.com/primer/anatomy/anatomy.html>
- Fluorescence microscopy: <http://www.olympusmicro.com/primer/techniques/fluorescence/fluorhome.html>
- Objectives: <http://www.olympusmicro.com/primer/anatomy/objectives.html>
- Filters: <http://www.olympusmicro.com/primer/lightandcolor/filter.html>
- Confocal: <http://www.olympusmicro.com/primer/techniques/confocal/index.html>
- Numerical Aperture: <http://www.olympusmicro.com/primer/anatomy/numaperture.html>
- Airy Disc: <http://www.olympusmicro.com/primer/techniques/confocal/resolutionintro.html>
- Nipkow: <http://www.olympusmicro.com/primer/techniques/confocal/confocalscanningsystems.html>
- Bleed-through: <http://www.olympusmicro.com/primer/techniques/confocal/bleedthrough.html>
- Non-laser light sources: <http://www.olympusmicro.com/primer/techniques/confocal/noncoherentsources.html>
- Laser light sources: <http://www.olympusmicro.com/primer/techniques/confocal/confocallaserintro.html>
- Fluorescent Proteins: <http://www.olympusmicro.com/primer/techniques/confocal/applications/opticalhighlighters.html>
- FRET: <http://www.olympusmicro.com/primer/techniques/fluorescence/fret/fretintro.html>
- Nikon:
 - Home: <http://www.microscopyu.com>
 - Microscopy: <http://www.microscopyu.com/articles/optics/components.html>
 - Fluorescence microscopy: <http://www.microscopyu.com/articles/fluorescence/fluorescenceintro.html>
 - Objectives: <http://www.microscopyu.com/articles/optics/objectiveintro.html>
 - Filters: <http://www.microscopyu.com/articles/fluorescence/filtercubes/filterindex.html>
 - Confocal: <http://www.microscopyu.com/articles/confocal/index.html>
 - Numerical Aperture: <http://www.microscopyu.com/articles/formulas/formulasna.html>
 - Airy Disc: <http://www.microscopyu.com/articles/optics/mtfintro.html>
 - Nipkow: <http://www.microscopyu.com/articles/confocal/confocalintrobasics.html>
 - Bleed-through: <http://www.microscopyu.com/tutorials/java/cubeprofiles/triple/index.html>
 - Fluorescent Proteins: <http://www.microscopyu.com/articles/livecellimaging/fpintro.html>
 - FRET: <http://www.microscopyu.com/articles/fluorescence/fret/fretintro.html>
- Zeiss:
 - Home: <http://zeiss-campus.magnet.fsu.edu/index.html>
 - Microscopy: <http://zeiss-campus.magnet.fsu.edu/articles/basics/index.html>
 - Fluorescence microscopy: <http://zeiss-campus.magnet.fsu.edu/articles/basics/fluorescence.html>
 - Objectives: <http://zeiss-campus.magnet.fsu.edu/articles/basics/objectives.html>
 - Filters: <http://zeiss-campus.magnet.fsu.edu/tutorials/matchingfiltersets/index.html>
 - Confocal: <http://zeiss-campus.magnet.fsu.edu/tutorials/opticalsectioning/confocalwidefield/index.html>
 - Numerical Aperture: <http://zeiss-campus.magnet.fsu.edu/print/basics/resolution-print.html>
 - Airy Disc: <http://zeiss-campus.magnet.fsu.edu/tutorials/basics/airydiskbasics/index.html>
 - Nipkow: <http://zeiss-campus.magnet.fsu.edu/articles/spinningdisk/index.html>
 - Dual-spinning disc confocal: <http://zeiss-campus.magnet.fsu.edu/articles/spinningdisk/introduction.html>
 - Bleed-through: <http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/introduction.html>
 - Non-laser light sources: <http://zeiss-campus.magnet.fsu.edu/articles/lightsources/index.html>
 - Laser light sources: <http://micro.magnet.fsu.edu/primer/lightandcolor/lasersintro.html>
 - Fluorescent Proteins: <http://zeiss-campus.magnet.fsu.edu/articles/probes/index.html>

- FRET: <http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/spectralfret.html>

2.1.5. Excitation Sources for High Content Imaging

There are three basic types of light sources for microscopy: lamps, lasers and light emitting diodes. Lamps provide a relatively broad excitation source from UV to IR. Lamps are likely to be able to excite many fluorescent dyes and proteins but the power in a given region may be low. Lamps may need to be replaced frequently and be realigned to give optimal excitation. Xenon lamps have a broad spectrum but relatively little UV and substantial IR that is beyond the region used to excite most dyes and fluorescent proteins. Halogen lamps also have a broad spectrum but, like Xenon lamps, give off large amounts of useless IR and heat. Mercury lamps have sharp and narrow emission bands that require a careful selection of filters. They offer strong UV excitation but have relatively short useful lifetimes, compared to other light sources. Conventional lasers provide a fixed monochromatic wavelength per laser. Power is substantial but may not offer optimal excitation for certain targets. White light and tunable lasers are entering the market but have not yet been used in commercially available HC imagers. Lasers have long lifetimes but are relatively expensive to replace, although this is changing. LEDs are now bright enough to be used in microscopy and have made a rapid entry into the HC arena. LEDs offer long-life and are much more stable light output. They have much less fluctuations on the second time scale compared to lamps and also do not slowly dim in intensity on the week/month timescale the way lamps do. This can substantially reduce noise in HC assays as images are acquired across a plate.

2.1.6. Objectives

Anyone using microscopy as a primary tool needs to be familiar with microscope objectives. The imaging sites listed above provide outstanding introductions to this important knowledge area (Figure 1). Some key facts to know include the following:

1. Objectives are designed with a certain working distance and plate or coverglass thickness in mind. To get the best image there should not be a mismatch.
2. Objectives are designed to be used dry (an air gap between the objective and the specimen) or use water or oil between the objective and the plate/coverglass to enhance numerical aperture multiplier. A dry objective cannot be used with fluid and vice versa.
3. As a rule of thumb, for a given magnification, for example 20x, the objective with the higher numerical aperture (N.A.) will collect more light, reducing exposure times and increasing throughput. Similarly, water and oil objectives collect more light than dry lenses. In fluorescence applications, more light is better and in HCS, since speed is important, anything that can be done to reduce imaging time will speed screening. But using immersion objectives (water or oil) makes microscopy much more difficult, especially in a screening environment. Therefore, most HCI uses dry objectives but with as high a N.A. as possible (Figures 2 and 3).

2.1.7. Airy Discs

When light comes from a point in a specimen and goes through an object to form a part of an image, the light from the point does not form a point in the image. Rather it forms a diffraction pattern with a central maximum surrounded by a few rings (Figure 4). The central maximum, usually called an Airy Disc, contains most (>80%) of the energy. The minimum distance between airy discs then can be resolved defines the resolution of the objective and varies with the wavelength of the light ($r_{\text{Airy}} = 0.61 \times (\lambda_{\text{Ex}} / \text{NA}_{\text{Obj}})$). The higher the N.A. of the objective, the smaller the airy disc and the better the resolution (Figure 5).



Figure 1: Important properties of objectives are indicated on the barrel of the objective, these include magnification, numerical aperture, working distance, immersion medium and coverslip thickness. Taken from <http://www.olympusmicro.com/primer/anatomy/specifications.html>

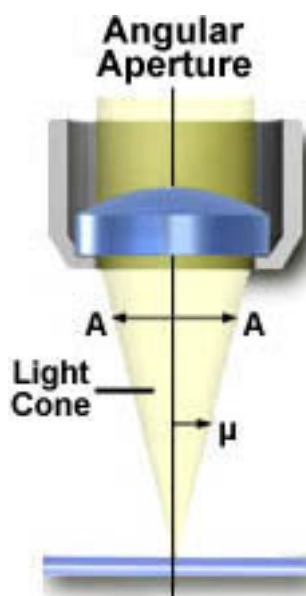


Figure 2: Numerical aperture is dependent on the half angle of the aperture (μ) and the refractive index of the medium (n) between the objective and the specimen. Taken from <http://www.olympusmicro.com/primer/anatomy/numaperture.html>

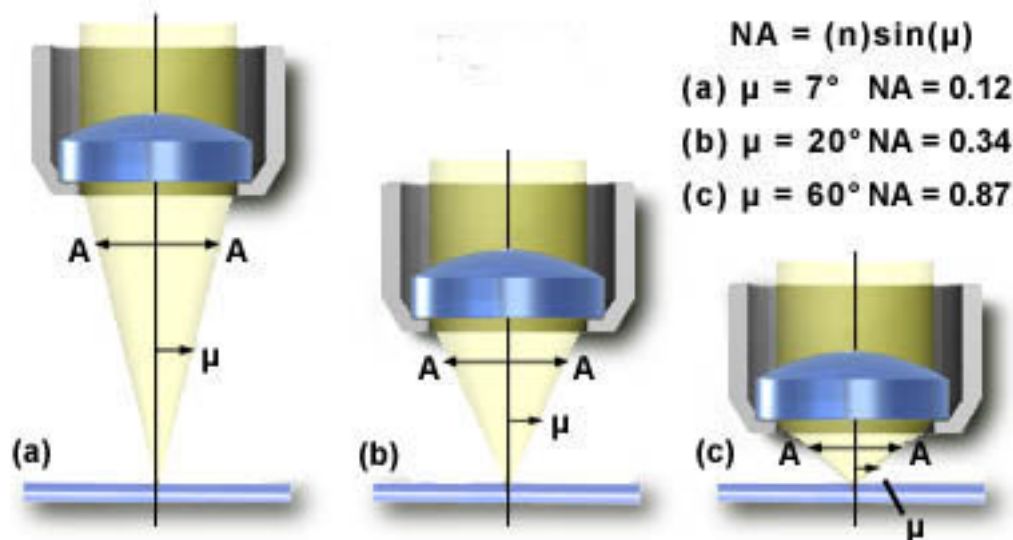


Figure 3: As the angle increases from 7° to 60° there is a 7 fold increase in N.A.
 Taken from <http://www.olympusmicro.com/primer/anatomy/numaperture.html>

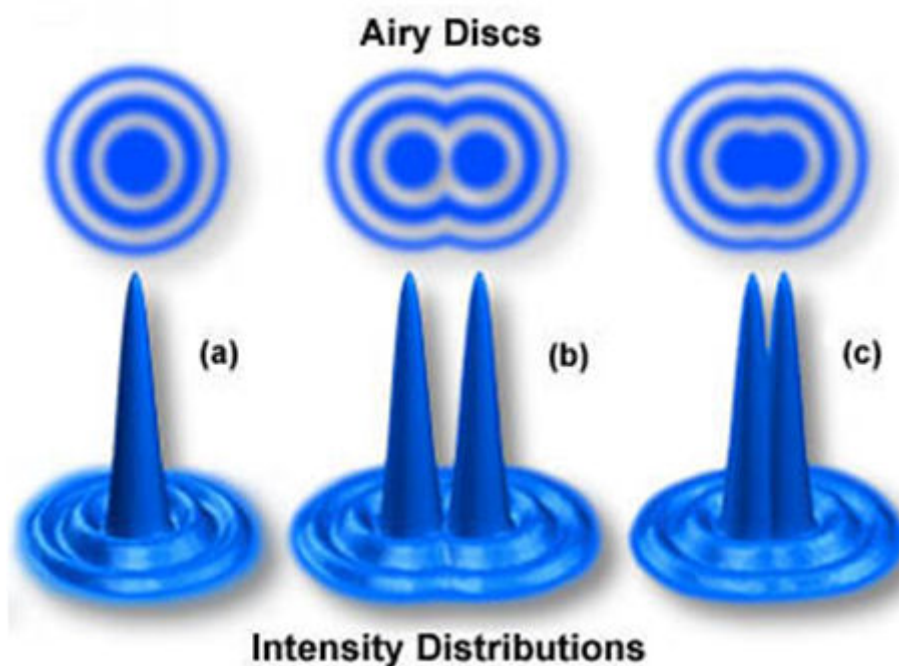


Figure 4: Schematic representation of Airy Disc intensity distributions. Adapted from <http://www.olympusmicro.com/primer/anatomy/numaperture.html>

2.1.8. Bleed Through

An important consideration for HCA is that the fluorescent dyes and proteins used in assays typically have broad excitation and emission spectra. As a result there can be significant bleed through from one fluorescent probe to another. At least four things can be done to minimize this:

1. The excitation wavelengths chosen should take into account the peak properties of the fluorescent targets to minimize cross excitation. With laser or LED light sources this is less of a burden. With other light sources, such as halogen, xenon or mercury lamps, careful selection of filters in the excitation path is

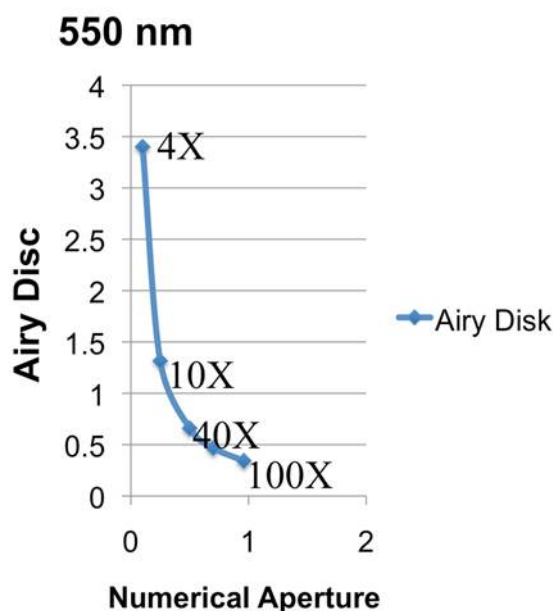


Figure 5: Airy disc versus numerical aperture (N.A.). As N.A. increases, the airy disc decreases.

required. In any case, absorption bands have tails towards shorter wavelengths. So a narrow band chosen to excite green fluorescent protein (GFP) will likely also excite a yellow fluorescent protein (YFP) (Figure 6).

2. The filters in the emission path must be optimized to minimize cross talk between the different fluorescence emitters and that is directly dependent on the dichroic filter in the optical path. Emission bands have tails towards the longer wavelengths. Fluorescence from GFP will bleed into the YFP channel. Keep in mind that imperfections in glass, optical materials and coatings used on filters in fluorescence detection commonly display multiple excitation or emission peaks; therefore it is recommended to review the specifications of the filters to understand how they perform.
3. Adopt a strategy to reduce problems with cross talk by having the brighter signal in the longer wavelength channels (Table 1).
4. Routinely assess cross talk between different channels. At a minimum, there should be control wells where the bleed through from the shorter wavelength channel into the longer wavelength channel is measured. In High Content Assays using primary and secondary antibodies, new lots of antibodies or just variations in handling on different days can lead to significant changes in fluorescent signals, with consequences for relative signal strengths in different channels. So controls need to be done on a routine basis.

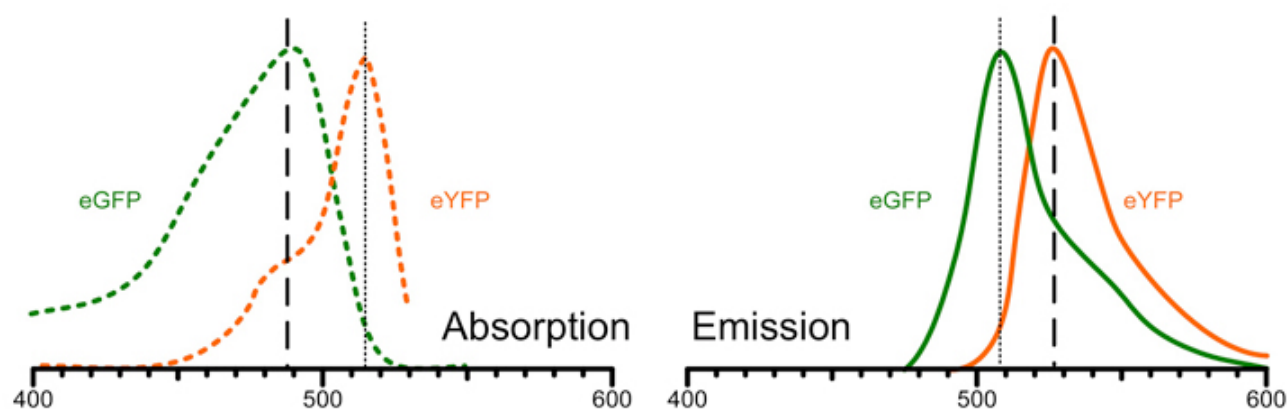


Figure 6: Fluorescent proteins often have overlapping excitation and emission spectra. eGFP and eYFP have substantial overlap, making clear separation of the signals difficult, even with the best filter choices.

Table 1: Making sure the signal from the emitter in the longer wavelength is brighter than the signal from the emitter in the short wavelength can minimize cross-talk between fluorescence emitters. If the brighter signal is from the shorter wavelength emitter, much of the signal detected in the long wavelength channel will be from the “wrong” emitter.

Fluorescence		Detection			
Green Label	Yellow Label	Green Channel		Yellow Channel	
		From Green (~ 100%)	From Yellow (~0%)	From Green (~50%)	From Yellow (~100%)
++	++	++	~	+	++
++	++++	++	~	+	++++
++++	++	++++	~	++	++
++++	++++	++++	~	++	++++

2.1.9. Detectors

HCI use two major types of detectors; digital cameras and photomultiplier tubes (PMTs). Digital cameras for HCS benefit from the large market for personal cameras that lead to decreased costs and increased chip size and defense needs for high sensitivity. HCI often use CCDs, EMCCDs and sCMOS cameras with high frame rates (100 FPS), large dynamic ranges (>20,000:1), broad spectral sensitivity (400-900 nm and higher), and high resolution (>2000 × 2000 pixels). While these cameras can provide high quality images, the files are large with consequences for image storage systems. The cameras are usually monochrome cameras. Color images are produced by acquiring images of the same field serially, using different optical filters. PMTs are based on a very mature technology, have extreme sensitivity to measure very low light intensity and fast responses with wide spectral sensitivity and are almost always used in conjugation with a laser source. To produce images, they are used in conjunction with a scanning technology that moves a light beam, typically a laser beam, across a sample. In HCS, the scanning is relatively slow but several PMTs can be used simultaneously to acquire data in different fluorescent channels.

2.1.10. Autofocus

HCI use two different approaches to focusing on the specimens. These are 1) laser-based systems that detect the bottom of the plate and 2) image analysis-based systems that step through the specimen and use algorithms to determine the optimal focus plane. Since focusing takes time it is usually not done every time an image is acquired in HCS. Testing is needed to determine the minimum number of times focusing needs to be done to

provide reliable data, which is related to the plate material used. The laser-based systems are fast but can perform poorly if the plates are not extremely flat or if the specimens are thick. The image analysis-based approaches are comparatively slow and have the caveat if fluorescent debris, artifacts, material or even clumps of cells which are not within the Z focus plane of uniformed cells in the well, the focusing typically fails. For these reasons it may be prudent to focus on fluorescence outside of typical lint debris that fluorescence in blue to violet wavelength.

2.1.11. Environmental Controls

HCA can be done on live cells to study cell movement, cell proliferation, cell death, and also to use various reporters to monitor protein interactions, membrane potentials or intracellular Ca^{2+} levels. This will require that the instruments control temperature, humidity and CO_2 levels. The larger instruments from most vendors have environmental controls as standard features or optional packages. If long-term time lapse imaging is planned then testing different instruments prior to purchase is recommended. While temperature and CO_2 are relatively easy to control, humidity is not and evaporation from multiwell plates can affect cell behavior. In addition, intense illumination of cells with lasers or lamps can damage or kill the cells. Therefore it is important to verify that the imager can detect critical features over time without causing cell damage.

2.1.12. Liquid Handling

Some instruments offer liquid handling to permit the addition of compounds, drugs, etc. to cells in individual wells. This is almost always done in live cell imaging situations to measure cell responses using fluorescence reporters to monitor membrane potentials or intracellular Ca^{2+} . Typically the liquid handling is done with a pipette like device, some with disposable tips.

2.1.13. High Content Imagers

There are many HCI on the market, with vendors releasing new models regularly. Therefore, it is impossible to have a resource that is truly comprehensive and current. Individuals interested in acquiring a new instrument are encouraged to survey the current market after first developing a detailed user requirements specification. International meetings, such as the Society for Laboratory Automation and Screening, PittCon, or CHI High Content Analysis are excellent venues to view demonstrations from many HCS vendors. There are a number of social media websites such as LinkedIn and Facebook focused on HCS/HCA that have members providing feedback about these instruments as well as dedicated user group websites. The Cold Spring Harbor Meeting on High Throughput Phenotyping is an exciting place to learn about cutting edge approaches.

2.1.14. Image and Data Analysis

Often the major factors that differentiate the High Content platforms from different vendors are in the software that acquire, analyze, and manage HC images. Most software packages from major vendors now offer advanced data analysis systems that allow tracking of entire screening campaigns. Perhaps more important, the software needs to have a comprehensive and user friendly system for developing a High Content Assay. While scientists often have a basic idea of the type of assay that will run, the optimal image analysis algorithms and features or parameters that need to be measured in a screen have to be determined using positive and negative referenced controls. The ease and speed at which this can be done is highly dependent on image analysis tools provided by the vendor. Newer software packages have improved GUIs based on real world workflows. Anyone acquiring a new HCA system should include image analysis tools in the user requirement specification.

2.2. Wide Field Imagers

These instruments are similar to and, indeed are often built around inverted research microscopes from major vendors such as Olympus, Nikon, Zeiss, etc. The hardware solutions offered by different vendors have evolved rapidly over the past 10-15 years and are now robust and provide excellent images quickly.

BD Biosciences distributes the BD Pathway 435tm, a unit with metal halide and transmitted light sources. This is also equipped with a Nipkow spinning disk for confocality (Note: BD is discontinuing their HCS instruments but there are many in academic labs and core facilities)

GE Healthcare markets the IN Cell Analyzer 1000 and 2000. The InCell 2000 is the new generation instrument; it uses a metal halide lamp. Camera options are 1392×1040 or 2048×2048 pixels. Transmitted light modes include bright field, phase and differential interference contrast.

IDEA Bio-Medical has a large instrument, the WiSCAN that uses a mercury light source for fluorescence and LEDs for transmitted light. The instrument uses a 512×512 water-cooled EMCCD camera for fast, sensitive imaging in a HCS environment. The Hermes 100 is a small bench-top instrument for individual labs. It uses LED light sources to allow two-color and transmitted light image acquisition.

MAIA Scientific markets the MIAS-2tm. This instrument can acquire 5 different transmitted light channels with a halogen light source and up to 8 fluorescent channels using a xenon light source. Imaging is done with a color camera and an intensified B&W camera.

Molecular Devices has one wide field imager, the ImageXpress Micro HCS system, which has an integrated fluidics system for delivering reagents in live cell imaging applications. It uses a xenon lamp and can use air or oil objectives.

Perkin Elmer sells the Operetta, a bench top widefield unit with a xenon lamp and an LED for transmitted light. It has a spinning disk confocal option

ThermoFisher (Cellomics) developed the first commercial HCS imager and now sells three wide field instruments. The ArrayScan VTI HCS Reader is an instrument suitable for a core facility or large laboratory. It uses a metal halide or LED light source and can be enhanced with a spinning disc confocal option. The Cellinsight is designed as a “personal” imager. It also uses an LED light source and is designed for use with four common dyes; Hoechst, FITX, TRITC and Cy5. The ToxInsight IVT Platform is designed to focus on identifying potential toxic liabilities in newly identified compounds. It is also a small footprint instrument using LEDs and a four-color approach to HCA.

Vala Sciences manufactures a Kinetic Image Cytometer (KIC) designed for kinetic analysis of calcium dynamics in an HCS system. It uses LEDs and large format cameras to acquire data.

2.3. Confocal HCA Imagers

Confocal microscopes use a light barrier with a fixed or adjustable pinhole to eliminate light that is in front or behind the focus plane of an objective. This gives much better depth resolution and improved contrast by rejecting light from out of focus sources. But it causes reductions in the light signal. It also only works for a single point in the specimen at any given moment. To overcome this problem the sample must be moved across the sampling point or the light beam and pinhole need to be scanned across the sample. One approach is the Laser Scanning Confocal Microscope (LSCM). The other is the Nipkow spinning disk that has multiple small pinholes or curved slits to increase illumination and the number of points in the specimen that can be imaged simultaneously. To obtain an optimal image with regards to light transmission and Z-axis resolution, the pinhole size must be matched precisely to the objectives Airy Disc. LSCMs have adjustable pinholes that can be varied depending on the objective and other factors, such as the wavelength of the illuminating light source. But the intense laser beam can bleach the specimen and it often takes a few seconds to scan a region of interest (ROI). Spinning discs sacrifice most of the illuminating light used to excite fluorescence in the specimen but can scan a ROI in a few hundred milliseconds and result in less bleaching and increases throughput. They are preferred for live cell imaging. Yokogawa has devised a dual spinning disc technology with lenses in the first disk that focus

light on the pinholes in the second disc. This increases the illumination of the specimen and, importantly for HCS, decreases image acquisition time.

Confocal imaging is usually more expensive in terms of capital investment and screening time. It is best used for imaging small intra-cellular structures, small cells, complex 3-D structures and samples with strong background fluorescence. HCS campaigns have been run using confocal imagers to eliminate the need to wash stains from cells, a big advantage if the cells are loosely adherent. Furthermore, the sharper images obtained via confocal methods could make image analysis process easier.

BD Biosciences distributes the BD Pathway 855tm, a Nipkow spinning disk system with mercury halide and transmitted light sources. It has an integrated liquid handler and integrated environmental control. It can also be used in wide field mode. This imager is often used for kinetic studies of signals relevant to physiologists, such as membrane potential or calcium (**Note:** BD is discontinuing their HCS instruments but there are many in academic labs and core facilities).

GE Healthcare markets the IN Cell Analyzer 6000. This is a line scanning LSCM with a variable aperture. It has 4 laser lines (405, 488, 561, 642) and an LED for transmitted light and a large format sCMOS camera. It has an integrated liquid handler and environmental control.

Perkin Elmer sells the Opera, a HTS system designed with water emersion lenses to give higher N.A. It uses laser based excitation combined with a Yokogawa dual spinning disc system to give confocality.

Molecular Devices has a point scanning LSCM, the ImageXpress ULTRA. This machine has four lasers and 4 PMTS that can be operated simultaneously or sequentially. It has options for air or oil objectives.

Yokogawa has two confocal imagers that exploit their dual spinning disc technologies. They have discs with different size pinholes, depending on objectives in use. The CellVoyger CV1000 is designed for long term live cell imaging with the option for oil immersion lenses. The CellVoyager CV7000 is an instrument designed for HTS, taking advantage of three large chip (2560 x 2160) cameras and a choice of lasers as well as halogen lamp and a LED for UV imaging. Live cell imaging is provided as well as liquid handling and water immersion lenses.

2.4. Laser Scanning Cytometers

These imagers are conceptually similar to a flatbed scanner with laser beams scanned across the entire surface of the plate and fluorescence detected with PMTs. They produce images equivalent to at maximum a low NA 20X objective and are good at detecting cells, including DNA content and colonies and even model organisms such as zebrafish, but not subcellular features or processes. LSCs have a very large depth of focus. They are often used to identify fluorescent intensities above a threshold. An example is nuclear translocation assays, where a diffusely localized protein in the cytoplasm gives a low signal but when concentrated in the nucleus gives a high signal. Other applications include cell proliferation, cell toxicity, protein kinase activation, and cell cycle analysis. This approach might be considered a medium content, high throughput technology.

The Acumen ^eX3 has 3 lasers (405, 488, 633nm) and 4 PMTS and has been used in many HTS projects.

Molecular Devices ImageXpress Velos Laser Scanning Cytometer (formerly IsoCyte) uses 2 lasers (selected from 405, 440, 488, 532, 633nm), 4 PMTs. It also uses light scattering as a method to detect non-fluorescent objects such as colonies.

The Compucyte iCyte is a hybrid instrument that uses laser scanning on an inverted microscope with objectives (10, 20, 40, 60, 100x) and up to four lasers (selected from 405, 488, 532, 561, 594, 633nm). It also uses 4 PMTs.

Slide based scanners: Some instruments offer measurement of transmitted light in different wavelengths using line scanners. When used with conventional histological stains, this can provide very useful images and

information from tissues that could be of interest in disease models. A major advantage of line-based scanners is the elimination or minimization of tiling to produce very large, high magnification images.

Aperio sells three slide scanners aimed at the pathology market. The ScanScope FL uses a mercury lamp, a 20x objective and a TDI line-scan camera to acquire images in up to 4 color channels. The ScanScope CS has 2 objectives (20x, 40x). The ScanScope AT has a 20x objective but is designed for automation with a slide loader that can hold up to 400 slides.

Hamamatsu markets the NanoZoomer, which uses TDI line-scans to acquire both transmitted light images of tissues stained, for example with H&E, PAS, or NBT stains and also fluorescence. It uses a 20x objective, a mercury lamp for fluorescence.

The Leica SCN400 and SCN400F uses a linear CCD device to acquire brightfield images, The SCN400F also can acquire fluorescence channels.

2.5. FACS like instruments

As mentioned previously FACS provides multidimensional data that can be considered in a high content approach. There are some instruments that cross the border from FACS to HCA by acquiring images and not just intensity data.

The Amnis ImageStream X uses lasers and LEDs to give darkfield, side scatter (785 nm) and fluorescence images of cells using 5 lasers (405, 488, 561, 592, 658) of cells passing through a flow cuvette.

3. Assay Concept and Design

Living cells, the basic building blocks of life, are an integrated and collaborative network of genes, proteins and innumerable metabolic reactions that give rise to functions that are essential for life. Conversely, dysfunctions in these same vital networks give rise to a host of diverse diseases and disorders. Although much less complex than *in vivo* models or complete organisms, cells possess the systemic complexity needed to study the interactions between different elements of the network and the responses of the network to external stimulations. Therefore more and more physiologically relevant cellular models are being used to validate targets or to evaluate drug efficacy and to predict potential adverse side-effects. Furthermore, advancements in cell isolation, cell line generation and cell differentiation technologies have led to more scalable and affordable cellular models, which in turn facilitate screening using more physiologically relevant cellular models. Due to its information-rich nature, high-content screening (HCS) has become the choice for many scientists to examine the complex effects of compounds or other reagents in physiologically relevant cellular models, not only against their intended targets, but also against other cellular targets and pathways (25-29).

Like standalone high-resolution microscopes, automated HCS systems can be utilized to study many cellular processes. Some of these processes, such as protein phosphorylation, cell surface ligand binding, molecular uptake, protein expression, cell cycle regulation, enzyme activation, and cell proliferation, can be analyzed by conventional methods, though image-based methods can often deliver comparably high quality results with multiple parameters. The strength of HCS is based on its ability to enable both target-based and phenotype-based assays for otherwise intractable cellular processes. These processes often play pivotal roles in cell survival and division, and can be visualized as intracellular protein translocation, organelle structure changes, overall morphology changes, cell subpopulation redistribution, and three dimensional (3-D) structure modifications. These assays not only have been used to study fundamental biological processes and disease mechanisms, but also have been applied to new drug discoveries and toxicity investigations.

3.1. Intracellular protein translocation:

Examples of HCS assays monitoring intracellular protein redistribution include translocation of a transcription factor from the cytoplasm to the nucleus to initiate or modulate gene transcription, internalization of G-protein coupled receptors (GPCR) to initiate a signaling cascade (30, 31), translocation of glucose transporter from the cytoplasm to the cell surface to facilitate glucose uptake (32), and recruitment of LC3B, an autophagy-related protein, to the autophagosome under conditions of stress (33, 34). In order to follow the translocation event, the protein must be labeled with a fluorescent probe, often by tagging/expressing the protein directly with a conjugated fluorescent protein marker (such as green or red fluorescent proteins (GFP or RFP)). This system then can be used to study the spatial and temporal effects of external stimulants in both kinetic and end-point fashions.

Different protein tagging technologies have been developed as potential substitutions of fluorescent proteins. For example, the SNAP-tag, which is a 20 kDa mutant of the DNA repair protein O6-alkylguanine-DNA alkyltransferase that reacts specifically and rapidly with benzylguanine (BG) derivatives (www.neb.com) The BG moiety can then be used to irreversibly label the SNAP-tag with a fluorophore. This technology allows one to label the protein in question using chemical fluorophores with different wavelengths and cell permeability, thus facilitating multiplex readout from the same cells. Halo-tag (www.promega.com) and fluorogen activating protein (FAP) (www.spectragenetics.com) are based on similar concepts, but using different proteins and probes. These proteins and their associated probes have no endogenous eukaryotic equivalent and are not toxic to cells when expressed at low levels. The covalent nature of these technologies makes them versatile for both live and fixed cell images.

The protein tagging technologies described above require overexpression of the proteins of interest. Sometimes stable cell lines are not feasible, and inducible expression systems could be used to circumvent the situation. Not surprisingly, this approach will require more intensive assay validations due to potential variations associated with the inducible expression systems. Overexpression of some proteins may disturb the delicate balance of the cellular network or the tags may disrupt the function or trafficking of the proteins in unknowable ways, and lead to results that are not physiologically relevant. Because of this, most scientists prefer antibody staining methods to track the intracellular locations of such proteins. However, antibody staining methods generally require chemical fixation of the cells, and so are limited to end-point reads (with the exception of cell surface proteins). There are many commercially available kits for specific protein translocations. These kits are validated by vendors but their uses are narrowly defined, and one has very limited options to change the compositions of the reagents as needed. Alternatively, there are many well-characterized antibodies available via different sources. Assay developers usually need to screen multiple antibodies to find one that works in the bioimaging-based assay, and to validate the assay using known stimulators and inhibitors. If assay developers decide to use proprietary antibodies raised in house, the selectivity of the antibodies must be critically examined, and the assay must be fully validated using known stimulators and inhibitors in related and unrelated pathways to ensure the observed translocation of proteins is specific to the biological event(s) of interest.

Lastly but not least important, it is imperative to develop image analysis algorithms and phenotype clustering statistical methods (if applicable) concurrently with the development of biological assays to make sure that the assay has optimal sensitivity towards the desirable phenotypes. These algorithms and methods must be validated using known stimulators, inhibitors and/or tool compounds. For compound screening, the same compound at different concentrations could lead to different phenotypes, due to the compound's different potencies on different pathways or due to toxic effects. Therefore, it is essential to test tool compounds in a broad dose response concentration range to find all potential phenotypes associated with the assay. This information can be used to define POSITIVE calling criteria for primary screening to minimize false positives and false negatives. These principles are applicable to all HCS assay formats.

3.2. Organelle structure changes

Examples of organelle structure change assays include the evaluation of mitochondrial membrane potential as a marker of cell health, cytoskeletal remodeling, quantification of lipid droplet formation in metabolic disease, formation of micronuclei during genotoxicity, and quantification of endocytosis or internalization for intracellular drug delivery (35, 36). Over the years, Molecular Probes® (Life Technologies) has developed many organelle-specific chemical dyes and fluorescently labeled antibodies against specific organelle markers. Recently, they also adapted the BacMam technology to express GFP-fusion constructs of different organelle markers. These dyes, antibodies and organelle markers cover a broad spectrum of wavelengths and can be used to examine the location and structure of multiple organelles simultaneously.

Development of HCS-amenable assays for structural changes can be a challenge, due to the heterogeneous morphologies of cells in dissociated cell cultures. Cell behavior is strongly influenced by local environment. There is evidence that some cell types at the edge of a colony will behave very differently than cells in the center of the colony (37-39). Recent developments in micro-patterned plate technology could be used to address this issue. These micro-patterned plates could provide niches that mimic the extra cellular matrix (ECM) of cells in tissues, and the organized patterns facilitate more uniform cell placement and adhesion to plates, thus making assay development and image analysis straightforward (40-43).

3.3. Morphology changes

Morphology change is a hallmark assay for high-content based screening. Many assays monitor cell process extension or tube formation as markers of disease. These include the measurement of angiogenesis for anti-cancer indications, oligodendrocyte differentiation for multiple sclerosis and other neurodegenerative diseases, and neurite outgrowth for different CNS indications (44). Another important area is related to cell differentiation associated morphology changes. Epithelial-mesenchymal transition (EMT) for oncology or fibrosis indications and stem cell differentiation are two notable examples (45-47).

Morphology changes can be directly monitored using bright-field image technology, or using fluorescent images with dyes or other markers that define the boundary of cells. However, assays reliant solely on morphological changes must be tightly controlled to avoid misinterpretation of results. For example, neurons, oligodendrocytes and astrocytes have very similar branched morphology, and differentiate from neural progenitor cells, though their functions are very different. Therefore, it is important to include cell-type specific markers in assays for phenotyping, preferably including both up regulated and down regulated markers, before making final conclusions.

3.4. Cell subpopulation redistribution

Most automated cellular imaging systems allow one to view a large population of cells at a time, often at the individual cell and organelle level. This allows one to run subpopulation analysis, including co-culture of multiple cell types to mimic tissue environment, cell-cell communication for signal transduction between cells (9 Li 2003), and determination of stem cell differentiation efficiency. Together with the multi-variant analysis ability, HCS technology empowers one to learn more about the interactions between the elements of the cellular network, such as the influence of cell cycle regulation and microenvironment on different signaling pathways, and gain in depth knowledge of the basic building blocks of our body.

The assay categories described above cover many bioassays under different biology events in different pathways and/or different cellular systems (Table 2, and Figure 7). Furthermore, a complex biological event could include multiple steps in different pathways. Frequently, there are specific imaging based assays for different steps involved in the complex biological event. Figure 8 illustrates key steps involved in apoptosis and available imaging methods (www.lifetechnologies.com). Apoptosis is a very highly regulated process leading to cell death.

The biochemical and morphological changes that characterize apoptosis include the activation of caspases, the loss of mitochondrial membrane potential, the loss of plasma membrane asymmetry, the condensing and fragmentation of the cellular DNA, cytoplasmic membrane blebbing, and apoptotic body formation. Finally, apoptotic cells will be destroyed by phagocytes. Inappropriate regulation of apoptosis could lead to diseases like neurodegeneration, autoimmune, AIDS, ischemia-associated injury, and cancers. Since there is no single parameter that defines apoptosis, a combination of imaging methods is recommended for reliable detection of apoptosis when conducting HCA. However, for high throughput compound screening, one may pick one or two imaging methods due to cost and screening logistic concerns. Choosing which imaging method(s) to be used is very dependent on the goal of the screening. To detect early stage apoptosis, caspase 8 activation and/or mitochondrial membrane potential assays could be used. To detect middle stage apoptosis, phosphatidylserine exposure or membrane permeability could be considered. Finally, to detect late phase apoptosis, DNA fragmentation assays could be used. However, in follow up assays, the hits from primary screening should be examined by a combination of multiple imaging methods in order to better understand the mechanism of actions.

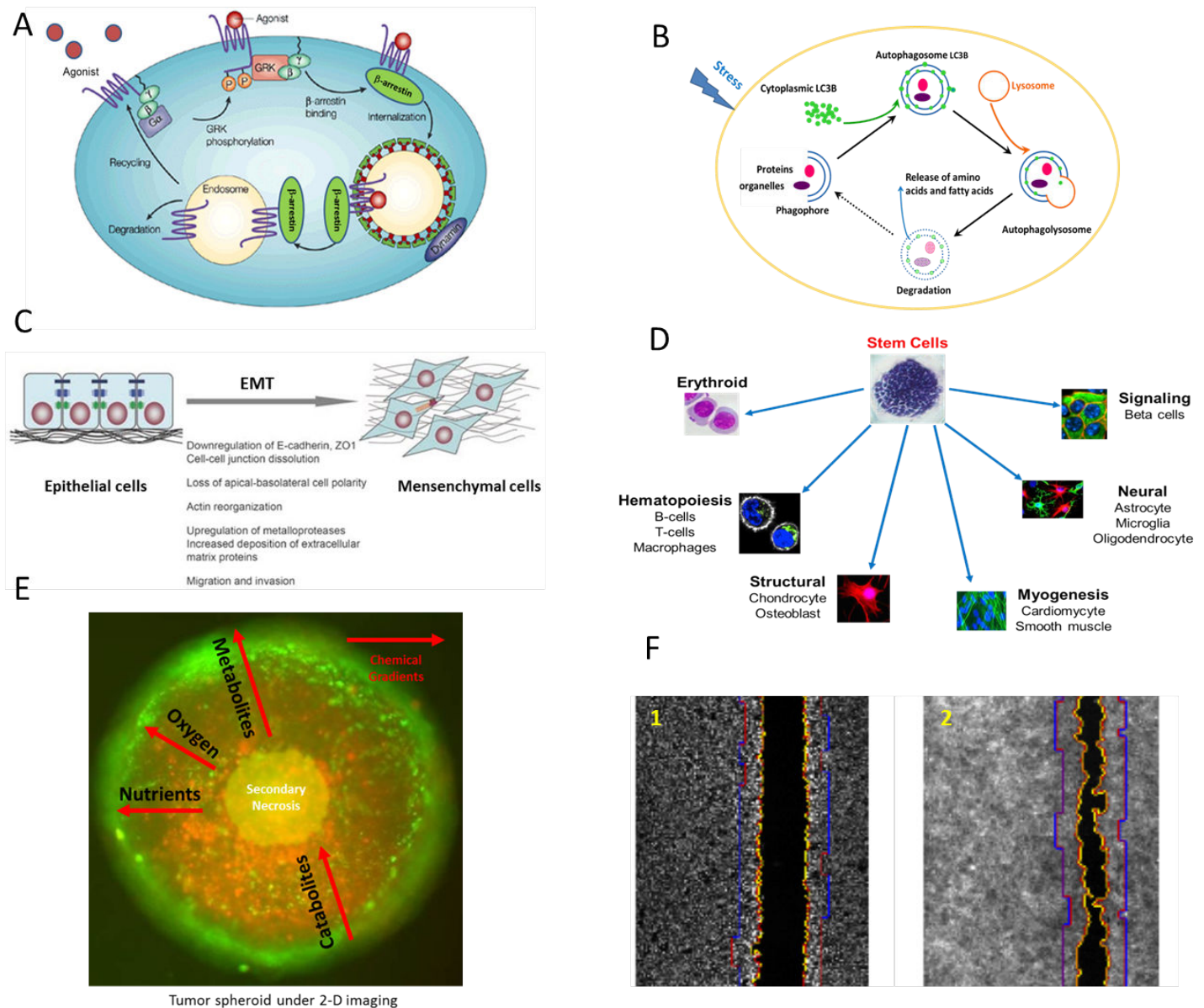


Figure 7: A) Beta-arrestin mediated GPCR internalization (Figure adapted from Nature.com with modifications). First, agonist-activated GPCRs are phosphorylated by GRKs (G-protein coupled receptor kinases) on their carboxyl-terminal tails. Second, arrestins translocate to and bind to the agonist-occupied, GRK-phosphorylated receptors at the plasma membrane. Third, arrestins target the desensitized receptors to clathrin-coated pits for endocytosis. Finally, receptors and arrestins are recycled or degraded. HCS can detect the internalization of GPCR by following GFP-tagged beta-arrestin (30, 31, 48). **B) Autophagy:** it is hypothesized that autophagy is up-regulated in cancer cells to promote survival in response to chemotherapy or other stresses. Autophagy includes multiple steps. The first step involves the formation and elongation of isolated membranes, or phagophores; in the second step, which involves the LC3B protein, the cytoplasmic cargo is sequestered, and the double-membrane autophagosome is formed. Fusion of a lysosome with the autophagosome to generate the autolysosome is the penultimate step. In the fourth and final phase, the cargo is degraded, and amino acids and fatty acids are released as nutrient for the cell. HCS can be used to monitor the aggregation of LC3B protein, thus following autophagy events (33,34). **C) Epithelial-mesenchymal transition (EMT)** (Figure adapted from Nature.com with modifications): it is hypothesized that EMT is a key step toward cancer metastasis or toward tissue fibrosis. During EMT, cell morphology is changed from cobblestone shaped epithelial cells to elongated mesenchymal cells. Meanwhile, epithelial cell markers, such as ZO-1 and E-cadherin, are down regulated, and expression of extracellular matrix proteins, such as collagens, is increased. Using cell-mask dyes for morphology changes and specific antibodies for different cell markers, HCS can be used to detect EMT (45). **D) Stem cell differentiation:** under certain physiologic or experimental conditions, stem cells can be induced to become tissue- or organ-specific cells with special functions. These specialized cell types have distinguished shapes and biomarkers and can be picked up by HCS using cell-mask dyes and specific antibodies for different cell lineage markers (46,47). **E) 3-D multiple cell type tumor spheroids** show many differences in biological functions compared to 2-D cultures (e.g. the chemical gradients within the 3-D tumor spheroids are much

similar to *in vivo* while 2-D models lack such gradients) , and resemble *in vivo* tumor tissue structure (Figure adapted from Dr. Michael A. Henson Group website with modifications. Green: live cells; red: dead cells). Therefore, the spheroids have gained momentum for applications in drug discovery. HCS technology with confocality provides ways to study the 3-D structure of the spheroids (49,50). F) HCS technology can be used to quantify cell migration, invasion and chemotaxis in 2-D or 3-D cellular models for wound healing, cancer metastasis and inflammation studies. Mechanic scratch or micro-patterned plate technologies could be used to create a cell-free area prior to assay start. Migration of cells into the cell-free area could be measured. The key for this assay is to distinguish migrated cells from proliferated cells in the scratched area. Micro-pattern technology also is used to create micro-conduit array plates with steady chemical gradients for chemotaxis assays (51, 52).

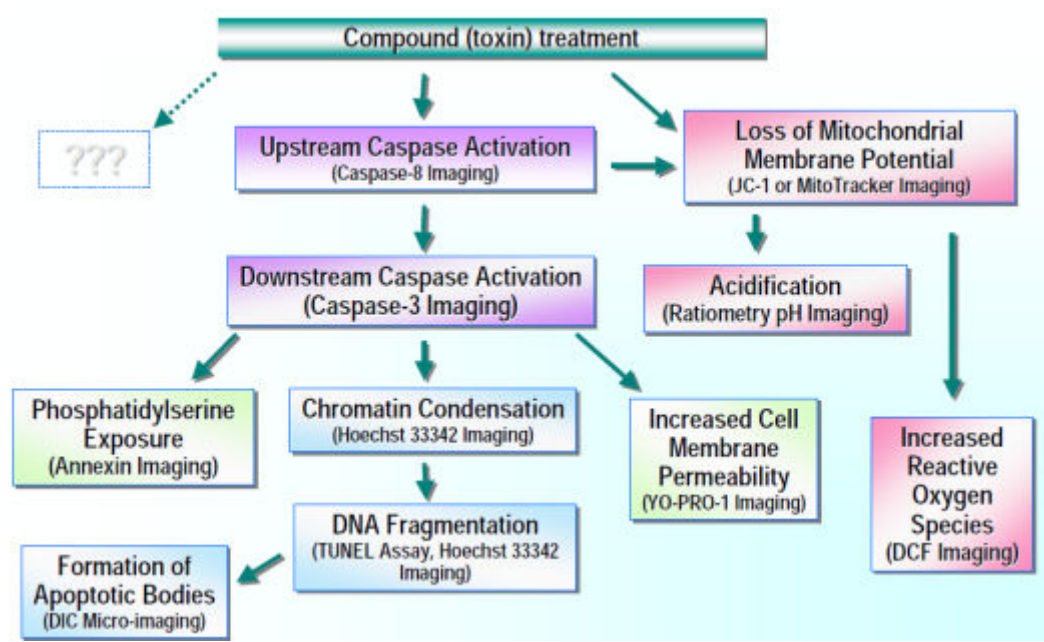


Figure 8: Key apoptotic steps and available imaging methods.

Table 2: Examples of bioassays

Assay Categories	Examples of Biology Events	Examples of Assays
Intracellular Protein Redistributions	Apoptosis	Caspases, cathepsins, calpains, Cyclins and PARP protein levels
	Autophagy	Autophagy protein LC3B aggregations
	Cytoplasm-nucleus translocation for nuclear receptors or transcriptional factors	AR, ER, GR, 5-LOX, ATF-2, ATM, beta-catenin, c-Jun, CREB ERK2, NF-κB, p53, SMAD, STATs
	Trafficking for cell surface receptors, ion channels and transporters	Beta-arrestin for GPCR internalization; ligand or receptor internalization for CB1, CB2, CRTH2, CXCR4, EGFR; Cytoplasm-cell surface membrane translocation for ion channels or transporters such as Glut1, Glut4

Table 2 continued from previous page.

Assay Categories	Examples of Biology Events	Examples of Assays
Organelle Structure and/or Function Changes	Apoptosis	Annexin V assay to detect externalization of phosphatidylserine, DNA fragmentation, mitochondria membrane potential, membrane permeability, nuclear condensation
	Autophagy	Autolysosome formation, mitochondria degradation
	Cell Division	Mitotic spindle structure by alpha-tubulin stain
	Cell polarization	Cytoskeletal re-arrangement by actin stain
	Drug delivery	Internalization of drugs via endocytosis
	Genotoxicity	Micronucleus assay to quantitate micronuclei in multinucleate cells; DNA damage indicated by phosphorylation of H2AX
	Lipid uptake and storage	Lipid droplet size and number
Morphology changes	Cell differentiation	Stem cell differentiation, epithelial-mesenchymal transition (EMT), oligodendrocyte differentiation
	Process extension	Angiogenesis, neurite outgrowth
Cell Subpopulation Redistributions	Anti-infectious	Percentage of cells infected
	Cell differentiation	Stem cell differentiation, epithelial-mesenchymal transition (EMT), oligodendrocyte differentiation
	Cell migration	Chemotaxi, wound healing, and cancer cell metastasis.

4. Cellular Models for High Content Experiments

High Content experiments depend on cell systems that serve as models for *in vivo*, typically human, biology. All models are measured by the extent to which they perform well in an assay and to the extent that they respond to stimuli in an authentic manner. Controversy exists concerning how well of *in vitro* cell systems accurately portray *in vivo* biology. Plating a single cell line on a two-dimensional surface in media with high levels of both oxygen and serum/growth factors may not model the *in vivo* situation sufficiently well for all investigations. Such systems give very robust signals in proliferation and apoptosis assays, but such responses are frequently muted *in vivo*, due to the target cells growing in an environment with multiple additional cell types. This section will explore how to insure that HCS assays best provide biologically or clinically meaningful results.

A discussion of cellular models must follow one on what needs to be modeled. In general, cell growth and the regulation of canonical signaling pathways have been modeled most frequently, particularly in the contexts of common cancers, glucose dysregulation in diabetes, neurodegeneration, pathological inflammation and toxicology. In these contexts, standard cell lines and culture conditions may be inadequate, but in other cases, such conditions may be fine. We will begin with experiments where the models are easier to establish and can be considered standard, and work towards models for more complex biological questions.

4.1. Cellular models for signal transduction pathways and other cell-autonomous responses

Much of pharmaceutical and biotechnology research is focused on finding modulators (typically inhibitors, but increasingly also to find agonists, potentiators and inverse agonists) of specific cellular target proteins. Studies on signaling pathways are also important to academic research. Such target-based research can make the search for a suitable model fairly straightforward. The easiest cellular models are immortalized and cancer cell lines. Although transformed, there are many examples of cell lines that retain the characteristics of the cell types they originated from. This includes important signaling pathways, such as estrogen receptor signaling in breast and

ovarian lines, insulin signaling in hepatic lines, and TNF- α responsiveness in immune cell-derived lines. Not all derivatives of a given cell type retain such properties, for example some breast cell lines have lost estrogen signaling. In these cases, the cell lines are better models of specific forms of cancer than of the original cell type, but then again, it is necessary to study signaling dysregulation in such diseases. Therefore, if you have a signaling pathway in mind, options for cell models can be found with a quick search of the literature. It is important to verify these cell lines are functional using known reference compounds, proteins, or other stimuli during assay development to ensure the desirable pathways are performing well in these cell lines.

The advantage of working with cell lines that are well-represented in the literature is that many additional properties of the lines that are important to consider will already be characterized and be manageable. Properties such as growth and metabolic rates can affect many assay types because some lines need to be attended more frequently than others. Other properties impact imaging assays more than other types. Colony morphology is one example. If the cells grow as clumps or clusters, then many cells will grow away from the well surface, making the imaging process more difficult than for lines that grow uniformly spread. Cell adherence can be a problem for imaging assays that require fixation and staining, as these steps add additional treatments and washing cycles to the process. Loosely adherent cells will be lost at each step unless care is taken to avoid disturbing them. This can be accomplished through automating sample preparation, where some instruments can control the rate and the placement of the reagent additions and wash steps. Common properties that can vary significantly between cell lines are summarized in Table 3.

Cancer cell lines and immortalized lines (lines that are not derived from tumors, but have inactivated senescence barriers) are easier and cheaper than primary cells, as they can be passaged in theory indefinitely; however mutations and functional response typically diminish over time. This allows both a single line to be used in experiments for many months or even years, and to expand cells prior to a screen, so that all of treatments (compounds, peptides, siRNAs, etc.) are used on cells of the same passage. Although cell lines are capable of near infinite growth, their properties do change over time and these changes can be exacerbated by inconsistent management of their growth. Understanding proper handling of cell lines by managing growth rate characteristics and cell passage number limitation is essential for consistent and biologically relevant studies. Sometimes it is necessary to sort cells to enrich the desirable cell type population. There are a few additional steps that ought to be taken when working with cell lines. Misidentification of cell lines is not rare (more than 20% error rates have been reported!). Cell lines can be mislabeled, contaminated or mishandled, making them inappropriate for the intended study. Examination of the cellular properties is essential; genotyping is inexpensive, so it is worth considering a deliberate evaluation phase for any line that is acquired through a commercial source or a collaborator.

Some cell lines are engineered for screening specific pathways. The cell lines used are chosen on the basis of their properties in cell-based assays, and the monitoring activity of the pathway through fluorescent proteins such as GFP fusion can make sample preparation much easier. In fact, they can be imaged live to better understand biological function and kinetics. Transcription factors expressed as GFP fusion proteins are common. Examples include FOXO and STAT family members, beta-catenin and TCF4/TCF7L2, NF- κ B, CREB and many others. GFP fusions to other proteins are used in other robust assays, including GPCR signaling components such as PTH receptor internalization and beta-Arrestin (30, 31, 48), even protein kinases such as p38-MAPKAP2 (53), AKT kinases are activated through a transient localization to the plasma membrane and MAP/ERK family kinases can be localized to the nucleus.

In cases where the screen or assay is not specifically tied to a single pathway, but is in fact targeting a cellular response, multiple pathways may contribute to the response. The effect may be different across cell lines, even lines that are genetically and phenotypically very similar. This heterogeneity has made it difficult for many experimental results to be extended, particularly to clinically significant therapeutics. This is becoming a well-recognized issue, and some groups, both academic and industrial, have transitioned to using panels of cell lines

that are defined by both signaling characteristics and genetic background, including amplification of oncogenes and chromosomal imbalances (54) The goal is to generate data that reflects properties of cell lines grouped by common properties as reflected in the disease state in question (such as cancer subtype). The process of selecting lines is the same as outlined above, but many cell lines would need to be selected and screened. The logistical challenges are out of the scope for this chapter, but scientists looking for novel therapeutic strategies should be aware of this approach. Phenotypic questions addressed by such panels include demonstrating that blocking autophagy, ER stress response or other survival mechanisms will lead to cell death (55). Image-based approaches to phenotypic assays present unique and very valuable additions to biology and drug discovery, but the value in a discovery made in a single cell line is potentially limited unless it can be generalized or placed in a tractable signaling context.

Table 3: Summary of common properties that can vary between cell lines

Property	Impact
Growth rate	Increase in cell number; affects confluence (some properties are affected by confluence and may need to be split more frequently)
Metabolic rate	Consumption of energy and nutrients; can change health of the cell, as well as assay conditions (pH in particular)
Colony morphology	Pattern of growth as cells divide; some lines will spread evenly, others will clump
Adherence	How strongly the cells bind to the plate; influenced by the materials used to coat the plate, some lines adhere better to a collagen or fibronectin coating on the plate surface
Heterogeneity	Cell lines that appear as mixed populations morphologically
Proportion of cytoplasm	Some lines have very little cytoplasm, making many imaging assays very difficult

4.2. Primary cell models.

4.2.1. Models using differentiated primary cells

Primary cells are intrinsically more difficult to acquire and handle than most cell lines. Primary cells have limited capacities for expansion (the Hayflick Limit), or may even be post-mitotic and cannot be expanded through normal cell passaging. It can be difficult to obtain many primary cell types. Most human cell types, including pancreatic β -cells, adipose, primary tumor and tumor-associated fibroblasts, kidney and liver hepatocytes or macrophages frequently require research collaboration or material transfer agreements with hospitals or specialized procurement facilities. Commercial sources are available, but are expensive. Primary cells from animal models, particularly rodent, are much more common. On-site animal facilities can make procurement simpler to plan for, but may require a researcher to isolate the cells themselves. The two biggest logistical challenges to using differentiated primary cells as experimental models are (a) donor consistency and (b) delivery schedule. These can be more manageable for animal sources but both can be major problems for human samples, particularly irregular delivery schedules. Differentiated human cells are collected during surgical or post-mortem procedures. Although disease tissue is frequently sought as a bona-fide model of the disease itself, sample heterogeneity is typically much greater and some samples cannot be used. This is true for non-cancerous samples as well, including hepatocytes or β -cells from diabetics and synovium samples from patients with Rheumatoid Arthritis. Even when samples can be used, there is frequent variability. Some primary cell models require media changes during the first few hours in culture, and the timing can vary from sample to sample. Samples are typically collected, isolated (purified) and shipped fresh within hours of collection, so advance warning is limited and a lab that depends on these will need to be prepared. In some cases, cells can be cryopreserved, greatly simplifying the experimental process.

4.2.2. Models using primary cells produced from differentiated stem cells

An alternative to using differentiated primary cells is to differentiate stem cells in the lab for the assays. Various adult stem cell types are available, and each can be used to generate different types of cells. Mesenchymal stem cells can be used to generate hepatocytes, skeletal muscle, adipose cells, and others. The extent to which they differentiate can be variable, creating a *de facto* co-culture system with cells that did not fully differentiate. In some cases, splitting and purifying the cells is not practical. Hepatocytes form very tight junctions, making separating them difficult and adipocytes have the unexpected property of being buoyant when they have accumulated significant lipid stores. In addition to the use of partially differentiated stem cells, protocols exist for differentiating pluripotent stem cells and iPS cells. These approaches are still under development and rather specialized.

4.2.3. Establishing primary cell models

Primary cells are valuable because they retain properties beyond what cell lines can provide. All cell lines have significant genetic and regulatory alterations. The price for a steady supply of cells is that many cell type-specific properties are greatly diminished or lost entirely. Loss of CDK inhibitors and telomerase, frequent activation of p53 and at least some chromosomal changes result in the degradation of cell type-specific functions; indeed many cell types are terminally differentiated, and this post-mitotic state is essential for physiology and morphology of the cell. Primary cells have a greater capacity to retain these properties, but they are affected by culture conditions, and therefore establishing proper culture conditions is essential to leveraging the benefits of using primary cells. For most cell models there is a strong primary literature history that describes the critical properties of the cell type in question and the culture conditions necessary to maintain them, although exceptions exist and some scholarship researching the models under consideration is important. Typically, conditions that need to be specified include media and supplements or the need for supporting feeder cells to produce native growth factors. Supplements in media for primary cells are typically titrated carefully to support growth but to avoid being higher than necessary. As such, the media may expire more rapidly than standard media preparations. In general, it is better to avoid proprietary media or supplement formulations because it is not possible to specify the experimental conditions and inter-lot variability can lead to failed assays. Therefore, some reverse engineering may be required to adapt the cell culture system to one that is appropriate for the assay being developed. As a quick example, primary human hepatocyte culture has been optimized for toxicological studies using commercial ITS (insulin-transferrin-selenium) formulations, but the level of insulin is far higher than normal, and precludes any insulin sensitivity of the hepatocytes. To adapt the hepatocyte culture system to one that can be used for the study of glucose regulation, the commercial ITS solution needs to be replaced with individual component stock solutions that can be independently varied. For proprietary formulations, manufacturers are typically reluctant to fully describe their composition, although they will often confirm whether specific materials or growth factors are present when asked as a specific question. Nevertheless, a lack of complete understanding of the culture conditions may lead to surprises later on.

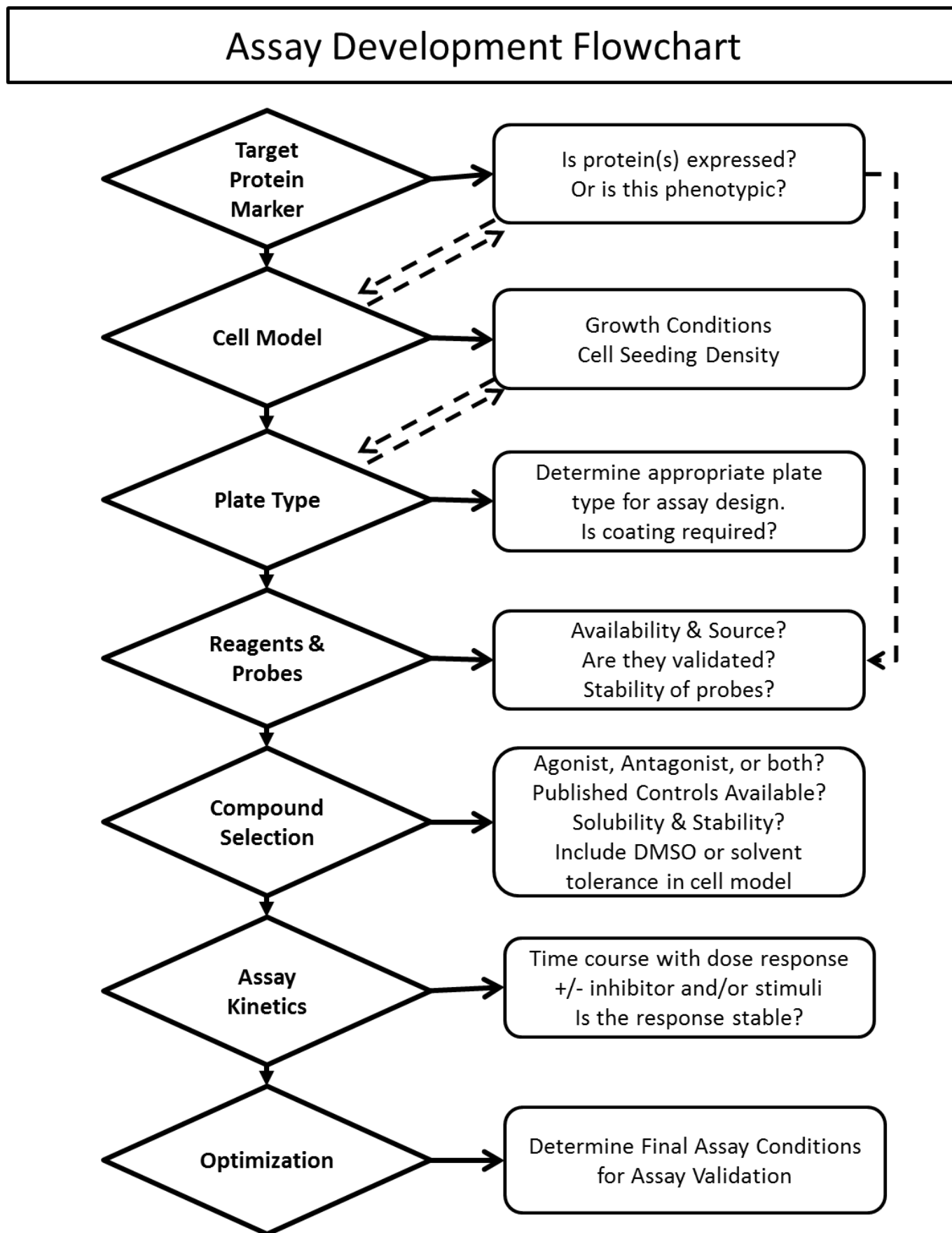
Beyond the media requirements, there are frequently additional specifications regarding seeding conditions. Cell density requirements are typically fairly rigid, particularly if they are high. Many post-mitotic cell types are plated at confluence, and deviating from this will cause the cells to dedifferentiate. This can be a very difficult step to optimize, as the fraction of surviving cells capable of adhering to the culture matrix will vary from sample to sample; for new samples, it may be necessary to plate cells across a range of seeding densities. The ability to work with a single batch of cryopreserved cells helps tremendously with this step more than any other. Addition of support extra-cellular matrices, such as collagen, laminin or fibronectin coated plates or basement membrane gels (e.g. Matrigel™) may be required. This is especially important for studies on certain cancer cells, such as breast cancer lines, primary neurons and hepatocytes. Often, it is not possible to omit these and maintain the cells for any length of time. Neurons, in particular are very sensitive to changes in substrate conditions. Different concentrations or types of poly-lysine, laminin or proteoglycans produce dramatic changes in neuronal

phenotypes. Even under optimal conditions in *ex-vivo situations*, these post mitotic cells will have a defined life span.

Standardizing primary cell culture conditions is essential for robust assay performance. For experiments where cells are used from a new source (patient or animal) for each experiment, responsiveness will vary, and separate normalizations will be required for each experiment. Endothelial cells, a proliferating primary cell used in angiogenesis experiments, form tube-like channels when plated on a basement membrane matrix. The dynamics of this process is affected by modest changes in source or lot, seeding density, passage number, basement membrane matrix composition and media. The first three factors mean that the assay responsiveness will change during repeated runs of an experiment, so historical performance comparisons are difficult. There is a significant literature from the HTS field on the number of controls that are needed per plate to give reliable normalization across plates and experiments (56). This is relevant to HCS as well.

5. Assay Development Considerations and Troubleshooting

5.1. Assay Development Flowchart



5.2. Target, Protein, Marker

It is important to consider the goals of the assay and whether the desired target protein/s is/are expressed in the cell model of interest, if the protein expression is constitutively active or requires activation or stimulation. It is suggested to examine relevant reference literature or the Gene Expression Omnibus for microarray data, then measure activity with a validated assay (e.g. Western Blot, ELISA, flow cytometry, etc). Another consideration is location, location, location; verify the location of the protein or marker probe expression within the cell model to determine if it is amenable for HCS.

5.3. Cell Model

Which Cell Line Should I Use?

It is highly recommended to review the literature and references to determine if the appropriate cell model or cell line is documented for the specific assay of interest. If the cell model or cell line of interest is not referenced in the literature then it is recommended to cross validate a known cell model or cell line with a known biological endpoint before proceeding with any unknown or orphan cell models. The source of the cell line must have documentation; if using a cell line from a collaborator or colleague then obtain as much background information as possible about the history and growth characteristics. Phenotype and genotype the cell line as required. If starting with a known documented cell line, it is best to purchase from established cell bank, e.g., ATCC with history of lot details and cell growth profile specifications. If using primary cells, stem cells, *ex-vivo* tissue then establish and document as much about the growth behavior in culture before transitioning to multiwell plates for further reference, reproducibility and evaluation of likelihood of success. For transient, stable, or inducible transfection or infection of reporter proteins, such as fluorescent proteins (i.e. GFP), then steps must be taken to further validate the cell line to determine the percentage of cells expressing the reporter after cell seeding, stimulation, and other treatment.

5.3.2. Growth Conditions

Define the media, serum, and other growth factors for optimal biological response. Please note, while the optimization of health of the cells and biological conditions are needed, high levels of serum can lead to compound absorption in the assay affecting the results. When and if possible reduce the amount of serum used to a minimal level without sacrificing the overall health conditions of the cells. When miniaturizing the assay to multi-well plates, it is required to verify and/or validate if it is able to reproduce the correct biological response. It is important to know how long cells can survive and respond “normally” in culture when designing these types of assays. Determine the sensitivity of the cells outside of normal optimal environmental conditions, i.e., outside of the incubator at room temperature to mimic plate handling timing, and if the cells can tolerate changes in temperature, pH or osmolarity fluctuations. Also consider whether the addition of HEPES buffer will minimize pH changes without altering the biological model response.

5.3.3. Cell Seeding Density

Determine the cell seeding density by initially plating cells to achieve at or near confluence of the monolayer or at desired density for biological outcome. For cell types that tend to form clumps, a cell strainer could be used to de-clumping. As a general rule, cells approximately 10 microns in diameter and proliferation doubling time less than 24 hours, seed ~5,000 cells per well for 96-well plates or ~1,500 cells per well for 384-well plate. From this point forward, dilute cells by increments of 500 to 1000 cells per 96-well in replicates and incubate overnight. Label cells with an indicator to identify cells, i.e., nucleic acid dye such as Hoechst 33342, DAPI, DRAQ5, or others; this can be counterstained with a cytoplasmic indicator such as Cell MaskTM or other live cell indicator such as CM-FDA (5-Chloromethylfluorescein Diacetate) or Calcein-AM. These fluorescent indicators will be used to determine if the image analysis algorithm can properly identify, segment (separate) individual cellular

objects. Perform statistical analysis of number of cellular objects per field or well to determine minimal number that can be used to provide a robust assay (see Section 6).

5.4. Plate Type

Often overlooked, the plate type chosen is critical to a successful screening campaign; keep in the mind the following when choosing a plate type.

5.4.1. Plate Material

Plates are generally made of glass, quartz, polystyrene or other composite materials. Each plate material has its advantages and disadvantages, so it is important to carefully consider what type is chosen to provide the best result in the assay.

1. **Glass and quartz** are one of the flattest and best optical materials made but are also expensive to purchase and therefore they are typically only used in specialized cases where the need for enhanced optics and flatness of the plate is required to resolve detection of subcellular structures or if capturing an entire well with a high numerical aperture, high magnification objective, i.e. 40X, 60X, 63X, 100X. Keep in mind glass and quartz material will likely require substrate coating for proper cell attachment.
2. **Polystyrene** based materials are the most common in HTS and have been adopted for HCS. The advantage of these plates is the cost is relatively low and most cell types can attach without basement substrate materials or coating.

5.4.2. Substrate requirements

The use of poly-D-lysine (PDL) coating can enhance attachment and spreading in many cells. This is commonly used in to improve attachment during compound treatment and subsequent processing and labeling steps, such as cytotoxicity assays. Extracellular matrix (ECM) proteins are used to coat plates to establish or mimic appropriate biological conditions of the assay. The most common ECM base substrates include Collagen-I, Collagen-IV, Fibronectin, Laminin, and Matrigel. If using glass bottom plates, then it is absolutely necessary to coat plates with ECM or PDL coating to promote cell adhesion and attachment. For cell spreading and migration assays it is important to test individual ECMs or a combination of these substrates as the outcome is highly dependent on the cell adhesion molecules expressed by the cells and the matrix molecules they interact with. In primary cells it is almost always necessary to use a biological substrate material to achieve appropriate conditions for an assay if feeder cells are not used. For example, appropriate substrates are required for optimal axon and dendrite outgrowth from neurons. Commercially available plates with pre-coated substrate materials are offered by many manufactures. It is recommended testing more than one lot of these plates to verify assay performance and robustness as variability in manufacturer lots are not uncommon.

5.4.3. Physical dimensions of the plate

Most plate manufactures follows SLAS standard format. Table 4 shows the approximate surface area and maximum volume for a single well, based on plate manufacturer. The flatness and bottom thickness of a plate are also important parameters. When matching a plate with numerical aperture (NA) microscope objective lenses, it is important to carefully determine the plate thickness. For example, higher NA objectives such as a 40X/0.95NA objective lens likely has a coverslip thickness of 0.17 mm. Be sure the plate bottom thickness is at or near the coverslip thickness of the objective lens and is appropriate for the working distance of the objective. In this case, do not use plate thickness near 1 mm as the microscope objective lens with high NA will fail to focus on the cells with clarity.

It is also important to determine if there is a need for evaporation wells or barriers to prevent loss of liquid in wells over time for longer term incubations, depending on your assay. Test plate types to ensure cell morphology and biological outcome are not altered. Additionally it is important that the plate does not leak over time before

scanning; if the wells dry out from leaks or from wicking, the autofocus (image based and laser based) will likely fail. Be cautious of this as potential damage from salt based storage buffer solution including sodium azide (NaN_3) on optics and electrical components inside the imager is possible.

Table 4: Approximate surface area and maximum volume for a single well

Plate Type	Surface Area / well	Volume
96-well	0.32 cm ²	< 300 uL
384-well	0.06 cm ²	< 110uL
1536 well	0.0023 cm ²	< 10uL

5.5. Reagents, Buffers, and Probes.

When beginning a new assay, if and when possible use a validated assay kit, commercial if available, to become familiar with the steps involved in performing the assay. Then decide if the assay will continue with the “kit” or if a “home-brew” assay will be developed. A home-brew kit requires further optimization, validation, and time, but often offers cost savings in larger screening campaigns. Buffers used for imaging assays include the following:

1. **Salt based solutions:** most common solutions are Hank’s Balanced Salt Solution (HBSS), Phosphate Buffered Saline (PBS), and Tris Buffered Saline (TBS).
2. **Permeabilization buffers:** salt solutions or water containing detergents such as Triton X-100, Tween-20, SDS, NP-40, or other detergents.
3. **Blocking buffers:** for antibody labeling, these are salt solution buffers containing protein such as BSA, fractionated antibody chains, or whole or fractionated serum from animal species that correspond to any secondary antibodies that will be used in the assay.
4. **Fixation buffers:** for cell preservation include formaldehyde, paraformaldehyde, glutaraldehyde, ethanol, methanol, acetone, and commercial customized propriety formulas. Typically the alcohol based fixatives serve as both a fixative and a permeabilization agent and useful for phospho-protein labeling. Combinations of multiple fixatives or even double fixation methods can improve preservation and fluorescent signal. Glutaraldehyde can provide stabilization of protein labeling but it auto-fluoresces so it is best avoided or used a low concentration, i.e. 0.01%. The presence of auto-fluorescence can be reduced with specialized treatments or quenchers but these may bring about other problems.
5. **Post staining buffer solutions:** prevent microbial growth includes salt solution (HBSS, PBS, TBS) with 0.01% NaN_3 . Be cautious as NaN_3 is toxic and can be dangerous when combined with metals or acids, so precautions are needed.

5.5.1. Optimization and development of an un-validated assay

Antibody and organelle probe selection requires researching the literature and other resources to determine a starting point of antibody or probe choice. Choosing an antibody typically involves choosing one or more antibody sources for differences in epitope recognition site or phosphorylation recognition. When choosing a probe, it is important to understand the different chemistry for binding to organelles or proteins, spectral properties of the probe, how the kinetics are altered over time, and stability of probes in live or fix end point assays.

5.5.1.1. Antibody optimization

As with other antibody based staining methods, blocking with serum from the same animal species as the primary antibody is best; an alternative is to use at least 3% w/w bovine serum albumin (BSA). As a general rule, use the recommended dilution by the supplier of the antibody or if not stated start at 1:50 dilution and dilute by 2-fold. If no signal is observed, increase the antibody concentration. Use 50 μL per well for cells seeded in 96-

well plate and dilute as necessary for other plate types; confirm the cell monolayers are completely covered with antibody solution. For example, if the supplier of the antibody suggests 1:100, use a titration scheme outlined in Table 5.

Incubate primary antibody for a minimum of 60 minutes at RT. Longer incubation times may be required to improve antibody binding or to optimize work flow process such as overnight incubation at 4°C. Use a secondary antibody reporter that is well established and be sure to measure secondary antibody staining alone without primary antibody to determine non-specific binding. Include no antibody staining to measure and establish background fluorescence of the cell type used, as some cell types are notorious for autofluorescence such as liver derived cells. By lowering the concentration of the fluorescent secondary reporter, the signal to noise ratio may improve. If two or more primary antibodies are used it is important to prove the secondary antibodies do not cross-react with an inappropriate primary antibody. If the signal in the antibody labeling is weak or undetectable, the use of other enhancement techniques may be required to boost the signal such as streptavidin binding complex or tyramide signal amplification.

Table 5: Example titration scheme for primary antibody

Content	Concentration of primary antibody
Negative Control	Unstained – No primary Ab and No secondary Ab
Negative Control	Non-specific Binding - Secondary Antibody Only, no primary
Experimental Conditions	1:50
	1:100
	1:200
	1:400
	1:800
	1:1600

Probe optimization

Probes include functional dyes such as calcium indicators, liposomes, lysosomes, mitochondria indicators, cytoplasmic, and nucleic acid probes. Dilute desired probe starting at the recommend manufacturer's concentration by 2-fold; additional increase concentration by 2-fold for at least one or more concentrations for a total of not less than 5 data points. Repeat concentration curve if signal is either too weak or if saturation is reached and reduce the concentration curve less than 2-fold to “dial-in” on the optimal concentration.

Not all probes are fixable and must be analyzed using live cell imaging techniques; proper design of live cell experiments with time dependent kinetics is absolutely critical to successful outcome. When planning a live cell experiment with untested bioprobes it is important to account for the time required for an HCS imaging device to acquire cells, fields or wells on a plate. For example, if a mitochondria probe requires 30 minutes to properly load in cells and fluorescence begins to decay or results in toxicity in 2 hours, then it is absolutely necessary the image acquisition is completed within this time period.

For fixable probes, determine the stability of the fluorescent signal following fixation and analyze plates appropriately. For example, measure probe fluorescent signal at time 0 post-fixation, then measure signal at day 1, 2, 3... and so on to determine the overall stability of the signal. It is also critical to establish the stability of the light source (lamp-based, laser, LED) in the high content imager to ensure it is functioning properly during the testing period. Use a known standard fluorescent dye, cells with label or other inert material to reference daily fluorescence during the study. As a general rule, most nucleic acid probes bind tightly and are very stable but organelle probes tend to be leaky and less stable over time.

5.6. Reference Compounds and Stimuli

If there are no known published reference compounds for the assay, then use untreated control or untreated control plus vehicle (i.e., DMSO) as a baseline to normalize the data. This is an acceptable approach and may be used for orphan targets or in phenotypic screenings assays when “references” are not established.

For known reference compounds, consider the commercial availability, the stability of the reference compound, and its solubility in solvent, media, or buffer. Also determine if a specific or specialized solvent is required, and its specificity for activation or inhibition.

Refer to literature to determine potential reagents to use as stimuli for signaling pathways. A few things to consider when selecting stimuli for assay are:

- What is the signal to noise ratio and window for the assay?
- Is the stimulus biologically relevant (e.g. cell types, pathways...)?
- Is it constitutively active
- Is the stimulus specific, or does it activate multiple pathways?
- Would different stimuli for the same receptor lead to activation of different signaling pathways?
 - If so, did you select the correct stimulus for the assay of interest?
- Is more than one stimulus required for the assay or to obtain an improved S.N? And is it biologically relevant?
- Does media, sera, or growth factor used to maintain the cells activate the signaling pathway in question or alter morphology or migration?
 - If so, one may need to search for suppliers for “conditional media” or find ways to remove the stimuli.
 - If the pathway is activated by serum, it may be necessary to incubate the cells in serum-free media for a few hours prior to starting the assay.

Determine the tolerance of the cells in the assay to chemical compound solvents, such as acetone, acetonitrile, chloroform, dimethyl sulfoxide (DMSO), ethanol, ethyl acetate, hexane, methanol, tetrahydrofuran, toluene, or water used to solubilize chemical compounds. Determine the maximum concentration of solvent a cell model or cells can withstand before assay performance is altered and/or results in detachment of cells or cytotoxicity.

DMSO is the most common solvent used in biological drug discovery and many compound libraries are delivered in DMSO. In most cases, a working concentration of DMSO for *in vitro* assays between 0.1 and 1% is acceptable; however, this needs to be confirmed for every assay model. Perform DMSO tolerance by beginning at either 8 or 4%, dilute 2-fold in media used in the assay to include 8, 4, 2, 1, 0.5% DMSO, then include 0.2, 0.1, 0.05, 0.01% DMSO. Also include an untreated (no DMSO) control. DMSO tolerance is used in the assay model design by mimicking compound addition for the assay. If a stimulus is applied, include DMSO concentration curve for both un-stimulated and stimulated to determine if the stimuli has an effect on DMSO tolerance. Based on these results chemical compounds stocks for screening can be made at the appropriate concentration for maximum solubility and delivery to cell plates for assay validation process. If dose response curves will be done in the assay, all wells need to have the same final concentration of vehicle (such as DMSO) to reduce variability and eliminate artifacts caused by synergies between the vehicle and compounds at some concentrations but not others, and to prevent compounds from crashing out in the solution. Other solvents mentioned above require a similar concentration curve as DMSO and may require a larger concentration range to determine both tolerance of cells to solvent and solubility of the chemical compound.

5.7. Kinetics of the Assay

5.7.1. Assay response stability

Determine if the response for the assay is stable or prone to degradation by performing a time course experiment. This is critical in fast response assays such as using an agonist to trigger calcium mobilization or in using a stimulus to activate signal transduction pathways. The time course will be dependent on the assay type chosen. For example, calcium flux assays must be performed in live cells and measured within seconds.

Alternatively, for signal transduction pathways, you must determine the half-life activation time following addition of stimuli in increments not less than 5 minutes for the first 30 minutes and increments not less than 10 minutes between 30 and 60 minutes.

Determine if inhibitor compounds can be added simultaneously or if a pre-incubation of inhibitor compound is required before adding stimulus. Be sure to determine this timing with the adaptation of automation and liquid handling devices in the laboratory. If simultaneously delivery of inhibitor compound and stimuli is not possible, determine the time course required to pre-incubate with the inhibitor compound starting at 5 minutes and increasing to 30 minutes or more as necessary to optimize work flow logistics for screening and determine if it improves signal to noise ratio. It is important in fast signaling pathways that the pH and temperature are stable during compound and stimuli additions as these can affect the biological outcome. Be sure to pre-warm stimuli plates, media and compounds to room temperature if necessary. Once a method is adopted, it is critical to maintain it throughout the validation process.

For assays with long incubation time, samples in the wells at the outskirts of the plate are frequently problematic due to evaporation or inconsistent temperature controls. To minimize impacts of evaporation, for small sample numbers, one could fill the outskirts wells with buffer or media only, and use other wells for your samples; for large scale screening, one could put a tray of water with antibiotics or NaN₃ in an incubator to provide sufficient humidity; for assays that are very sensitive to evaporation, one could put plates in small boxes padded with wet paper towels. To minimize effects of temperature fluctuations across the plate, one should make sure all plates and reagents used for the assay are warmed up to room temperature prior to the addition of cells, pre-plate the cells in the tissue culture hood, allow the cells to attach before moving the plates to the incubator; for assays that are extremely sensitive to temperature changes, one should not stack plates in the incubator.

5.7.2. Cell growth characteristics

Determine if serum withdraw or serum free conditions, or addition of supplementary cell growth components or chemicals affect cell morphology, migration, or assay endpoint in cells over time. These considerations are important in several assay endpoints including

- **Cell cycle analysis:** if cells reach confluence or if they are starved of serum or growth components, cell cycle arrest can occur, which can affect the endpoint measurement.
- **Dendrite, axon or neurite extension:** typically require growth factors from supplements or from feeder cells.
- **Cell motility and migration:** typically affected by serum withdrawal and addition of serum or growth factor supplements.
- **Signal Transduction Pathway:** serum withdrawal or low serum can increase the signal in assays such as NF- κ B (see NF- κ B Translocation Assay Development and Validation for HCS), MAPK kinase pathways such as ERK phosphorylation or p38 (53).

5.7.3. Live cell imaging

Not all live cell imaging assays need to be performed with an environmental controlled chamber; however, in screening operations it is important to know the challenges and difficulty to control the work flow if disrupted by

automation mishaps or other failures. If the sequence of processing plates is interrupted, this typically will result in variable in the assay data. To determine if your assay is amenable to live cell imaging conditions with or without environmental control in screening operations, you should perform a time-course study on the HCS instrument following assay treatment or in environmental-controlled conditions such as in an incubator. For example, once a bioprobe indicator completes recommended incubation time for detection, acquire images on the HCS device at time zero ($t=0$), then in subsequent time points over a 60 minute period, capturing images at 5 or 10 minutes intervals. There are several conditions that need to be considered when performing this operation including the image acquisition time per well or per plate and the exposure time per fluorescent probe. Photobleaching and phototoxicity are possible and may affect the results. When appropriate, use more than one plate and analyze multiple wells to measure the overall variability. If the time required to capture every well or selected wells on a plate does not exceed the degradation of the fluorescent probe measured then the assay, it can be used in screening operations as long as each plate has appropriate control wells for normalization.

5.8. Optimization

Verify methodology through documentation of all previous established assay conditions along with the DMSO or other solvent used for optimal assay performance for preparation of assay validation. Standardize and create an SOP protocol that will be referenced for assay validation. To reiterate, image analysis method should be developed alongside with the biological assay development to ensure optimal sensitivity is obtained for the desirable phenotypes.

6. Image Acquisition, Analysis and Data Interpretation

6.1. Introduction

Computer assisted image analysis is the key component to most high-content screening endeavors, since a microscope generated image can contain an immense amount of information. The goals of image analysis are simple – identify objects (usually cells), accurately measure features within, about or between these objects and extract knowledge from the features. But before an image can be analyzed a few things should be quickly reviewed.

6.1.1. Capturing a Good Image

Use your imaging platform to capture *good* representative images in an unbiased manner (see Section 2 – Image Technologies and Instruments). Verify that your workflow allows for automated capture of information about the identity of the plate being scanned (user information, time, plate ID, barcode, etc). Double check the location of well A1 to ensure the plate is not loaded in reverse. Having a designated well lacking cells or containing fluorescent beads can allow unambiguous identification of plate position. Ensure that your workflow will annotate the data with assay conditions and compound treatments that will be needed for data analysis.

Every image captured regardless of the quality generates data, good or bad, so it is imperative a *good* image is captured for subsequent analysis. Some aspects of acquiring a good set of images are predetermined by the experimental design. Images should include all the fluorescent and/or brightfield channels needed for the analysis. If the analysis will require 3-D analysis or different sample or time points in a series that are required for analysis, these images must be appropriately captured. Image fields should be taken in appropriately located, predefined positions within the sample wells (cells near the edge of the well can behave differently or the images may be distorted). Pixel resolution and magnification must also be selected to balance the level of detail vs. the number of objects (eg. cells) available for analysis and will depend on the type of objects you are setting out to analyze. Sometimes multiple fields of images from the same well must be taken to obtain enough objects with appropriate resolutions.

Two aspects that may require fine adjustment on a day-by-day basis for a given screening campaign are the focus and exposure. An out-of-focus image, even slightly, will impact the apparent size and intensity of the objects and sub-objects within the image, and can quickly increase the noise within the analysis. Most platforms provide multiple ways to auto-focus fields before taking the image, and they should be tested for their accuracy, speed and robustness. A good exposure is also very important, and should aim to optimize the *dynamic range* of the detector such as a monochrome CCD camera or PMT (photo multiplier tube). The result is a gradient scale with dark intensity in an image as black pixels and bright intensity as white pixels representing low and high numbers respectively.

Finally, do the settings used to capture an image of a neutral control field also allow other images in the experiment to be acquired accurately and with high quality? Depending on the dynamic range of the image acquisition system and the intensities in different fluorescent or brightfield channels, it may be necessary to adjust the image acquisition settings using positive or negative controls. Compromises may need to be made because it is usually preferable to have all the images within one experiment taken with exactly the same parameters rather than have to spend time normalizing after the acquisition.

In the process of taking a good image you may want try to limit the time it takes to actually acquire the images; for example, ensure that exposure times are not needlessly long or that resolution is unnecessarily high. Long exposures generally mean more time needs to be spent in image acquisition, High Throughput and High Content Screening often need to balance the amount of time taken to achieve the desired assay quality with the throughput needed for the screening.

6.1.2. Overview of Image Analysis

You have the images, so now what?

Analyzing an image with the goal of measuring features within the image or objects requires several steps (Figure 9). Once the image is acquired, it often needs to be adjusted to get the best quality by use of flat-field or post acquisition background correction. Next a threshold is applied to identify objects from background followed by segmentation to separate individual objects. These objects are often further selected based on a variety of criteria and finally the features are extracted. While the primary object identification is usually done using a nuclear or cytoplasm specific stain in one channel to identify the cell, additional objects are often identified with other stains and acquisition channels to generate additional feature data from the image. All these steps will be discussed in detail in the following sections.

6.2. Segmentation (Image Processing)

Computer based image processing has been an important part of most industries since the late sixties when the first graphics programs were developed (57). Now, image analysis and processing is a normal end point for many biological assays. Most images processing in biology has a simple goal—to separate the *signal* from the *background*. This is accomplished in a few steps that involve optimizing the image, reducing background artifacts, and then applying a threshold.

6.2.1. Notes about Images

Images come in many forms, including different file types, resolutions, color depth, pages, stacks, montages, and usually have associated metadata. Image formats vary, and different platforms provide options for how to acquire and store images. These same considerations will also be important when processing the image.

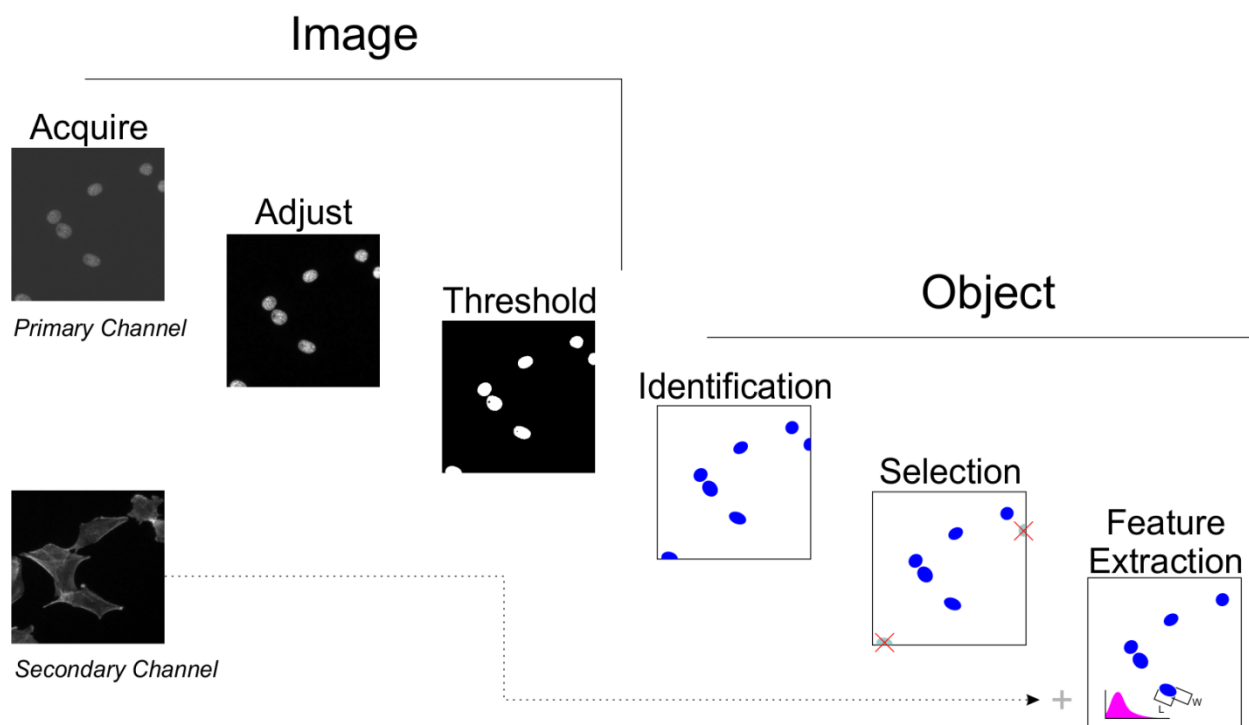


Figure 9: Typical Steps of Image Analysis. After acquisition, an image is adjusted to reduce noise and optimize the signal, then a threshold is applied and the information is converted into an object-based format. The objects are selected based on criteria and features of each object are extracted.

6.2.1.1. File Type

There currently is no universal image standard for HCS, although OME offers one such solution. Use a lossless format to work with images, such as TIF or BMP raster formats. Copies can be made in JPEG or PNG, but these formats will lose information so they should not be used as primary storage.

6.2.1.2. Resolution

Generally the image should be analyzed at the same resolution it was captured. In some situations, where the signal to noise is low, an image can be “binned” so that a group of pixels (2×2 for example) will be averaged into one pixel. This process decreases the resolution, noise and the image size for storage. It is generally more beneficial to do this during image acquisition, because it can reduce scan time, but could also be beneficial during analysis in some cases.

6.2.1.3. Color depth

Color or bit depth is a very important parameter of the image. It is the number of bits (1 and 0s) used to represent the staining intensity. The more bits associated with an image, the more shades of gray or color that can be represented. Larger bit depths expand the intensity range, allowing for the inclusion of pixels with fewer photons (previously black) or many more photons (previously white). Most professional cameras (including those used in HCS) operate in the 12 bit range, giving 4096 shades of intensity to work with, and this is generally preferred for image analysis due to practicality considerations. Larger dynamic range cameras may be used including 14-bit ($2^{14}=16,384$ shades) or 16-bit ($2^{16}=65,536$ shades). Unfortunately, the majority of computer displays (PC, Mac, Linux) are only capable of displaying 8 bits (256 shades) of information per color channel, causing some information in the acquired image to be lost upon viewing. 12-bit images will generally show up as black if you use standard software to display them. Programs like ImageJ are useful as they provide a solution to

automatically stretch the color depth to fit into the 8-bit range so it can be properly displayed, but remember that the actual image is actually more nuanced.

Users will commonly see images that are labeled 16 bit and 24 bit as well. 16 bit TIF images are a common “container” that can hold 12, 14, and 16 bit images, so if your camera is 12 bit, then these are actually 12 bit images in a 16 bit container. Similarly, 24 bit images are often combinations of the three color channels (R, G, B) each of which are 8 bit. Most image based software can read and translate these image variations.

Proprietary formats often group images together within one file. For example, TIF images support groupings of images, but this can make the TIF images complicated to work with. Often, multiple color channels will be saved in one TIF image. It is also possible to bundle multiple pages, frames of a time lapse, Z-stacks of images, or even montages of images within one image format. These types of groupings may not be natively read by image processing software and will therefore need conversion to separate and store individual images. The diversity of storage solutions adapted by instrument manufacturers makes reanalysis of images across software platforms challenging.

6.2.2. Image Optimization and Background Correction

Before an image can be processed for object identification, it often needs to be “adjusted” or optimized to achieve optimal contrast and reduce errors that would otherwise be confused as objects or alter the object tracing. There are two common “types of error” that are seen with microscopic images. These can be thought of as bias and imprecision or systematic and random. Bias or systematic errors are reproducible or predictable. Bias is an overall or local deviation in image intensity, which can be caused by variation in the output from the light source, uneven field illumination, optical aberrations, an artifact floating in the well, focus failures, or other reasons. On the other hand, all images contain some *imprecision* or random error, which we call noise. Random error arises from variations in the number of observed events (photons) stimulating a dye molecule, number of dye molecules, electrons emitted per stimulation, etc. These two classes of errors are present in any kind of measurement and imaging is no exception.

Bias correction is usually called “background correction” imaging platforms. Since background imperfections are generally low frequency (not in sharp focus), they are easily dealt with by two methods. The first group of methods takes the form of a moving average (a smoothing function) and can both reduce noise and reduce background. These functions are also called “rolling ball” methods. Background correction of this type is usually defined by a pixel radius to sample from and depends on both the size of the objects you are trying to identify and the size of the objects that tend to be causing artifacts. Slight changes in the sample size of the background function can have major effects on the image (Figure 10). For instance, if the background correction averages across 4 pixels and you are looking for features that are on average 4 pixels across, you will lose most of your sensitivity to that feature.

Filtering the image based on spatial frequency is also an effective way to eliminate out of focus background artifacts (a high pass filter will remove variations that change slowly, across many pixels, such as something that is out of focus, while retaining abrupt changes seen in objects in focus). In addition, microscope systems often provide a way of eliminating common artifacts produced in the optic pathway of the instrument. These are often termed “peripheral illumination” or “illumination” errors, and are produced as the light is channeled through the various lenses and apertures. The peripheral illumination artifacts can be easily corrected by sampling an empty plate, and using that image to compute a “flat field”. This operation may not eliminate the need for additional computational adjustments.

6.2.3. Object Identification

The primary goal of image processing is to distinguish the *signal* from the *background*. Once the background has been corrected, a threshold is typically set which cuts off the pixels which are too dim (in a fluorescent image) or

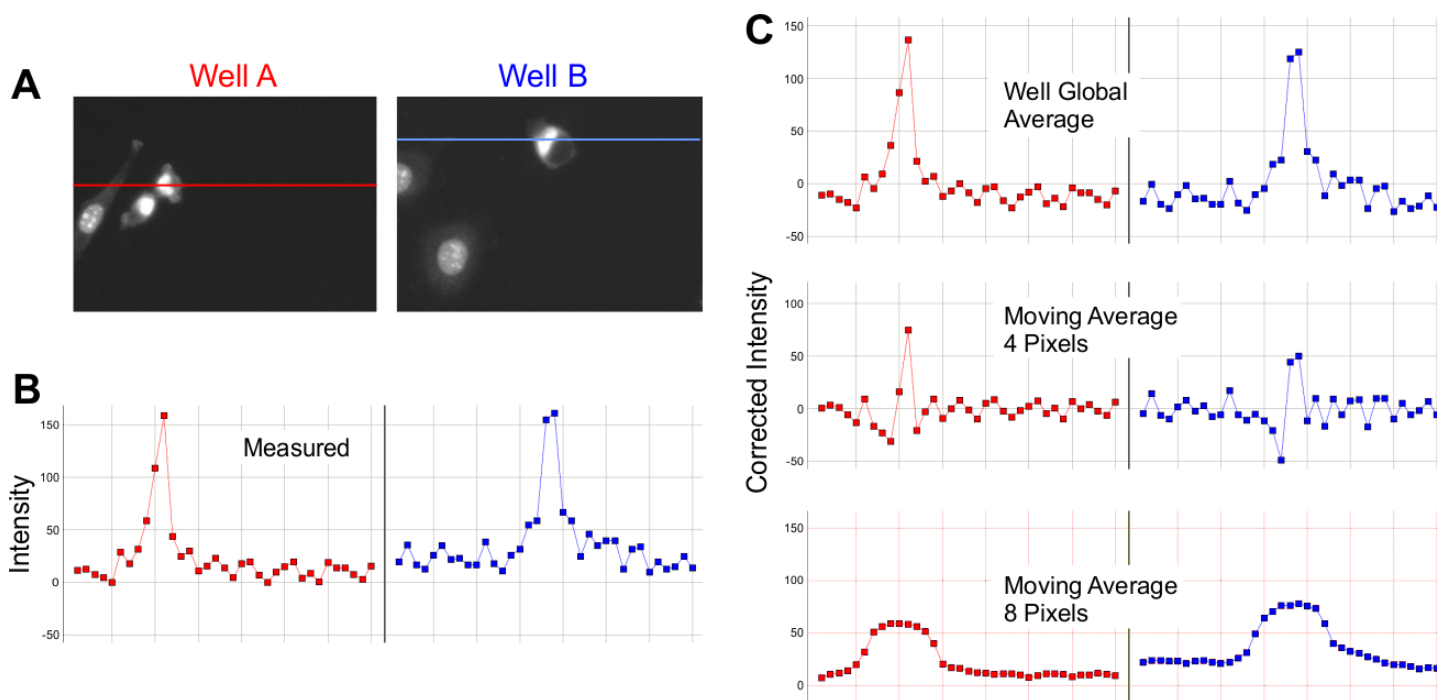


Figure 10: Background correction can have significant effects on image analysis. A) Two images from different “wells” are shown in raw grayscale, pre-corrected. A “line scan” is done across the image producing two scatter plots. B) Measuring the raw intensity for each well. C) Application of basic background correction on the two line scans. First, each point is simply subtracted from the global average of the scan. This preserves the full detail but shifts the baselines so they fall at zero. If a moving average (rolling ball) is applied with a radius of 4 pixels, the result is drastic, actually decreasing the signal-to-noise ratio and burying the peaks. An increase of the moving average radius to 8 pixels reduces the background noise and smoothes the scan.

too bright (in a brightfield image) and are thus ignored as background. Setting the threshold can be simple if images are taken with consistent exposures and with a very stable dye (Hoechst or DAPI for example). In these cases it is often possible to have a manual or fixed threshold which works across an entire plate. In other cases, more sophisticated approaches must be taken that account for changes in signal intensity with time or position.

Automated thresholding algorithms analyze the pixel intensities to determine which pixels are associated with background and which belong to the objects. If certain assumptions can be made, then these automated methods work well. The most common methods assume that the majority of the image pixels will be background, and uses an offset from the mode of the image histogram (Figure 11) to set the threshold.

The result of thresholding is a “binary” image or mask, which has only negative and positive pixels. After thresholding, image “segmentation” can divide positive pixels into separate entities or “objects”. This process can be a simple algorithm which scans through the image until a positive pixel is found, then scans all connected positive pixels which are added into the first object. This process is repeated until all the positive pixels are accounted for by objects.

Another type of segmentation is often preformed either before or after object identification with the goal of splitting apart two objects that are associated with one another. This is achieved, for example, by applying a watershed algorithm on the binary image (“fills” the image with water until boundaries are established) or searching for intensity peaks or computing shape features. Other segmentation algorithms may divide the entire image into a grid for subsequent object or pixel based segmentation. Improving and developing segmentation algorithms is an active area of research.

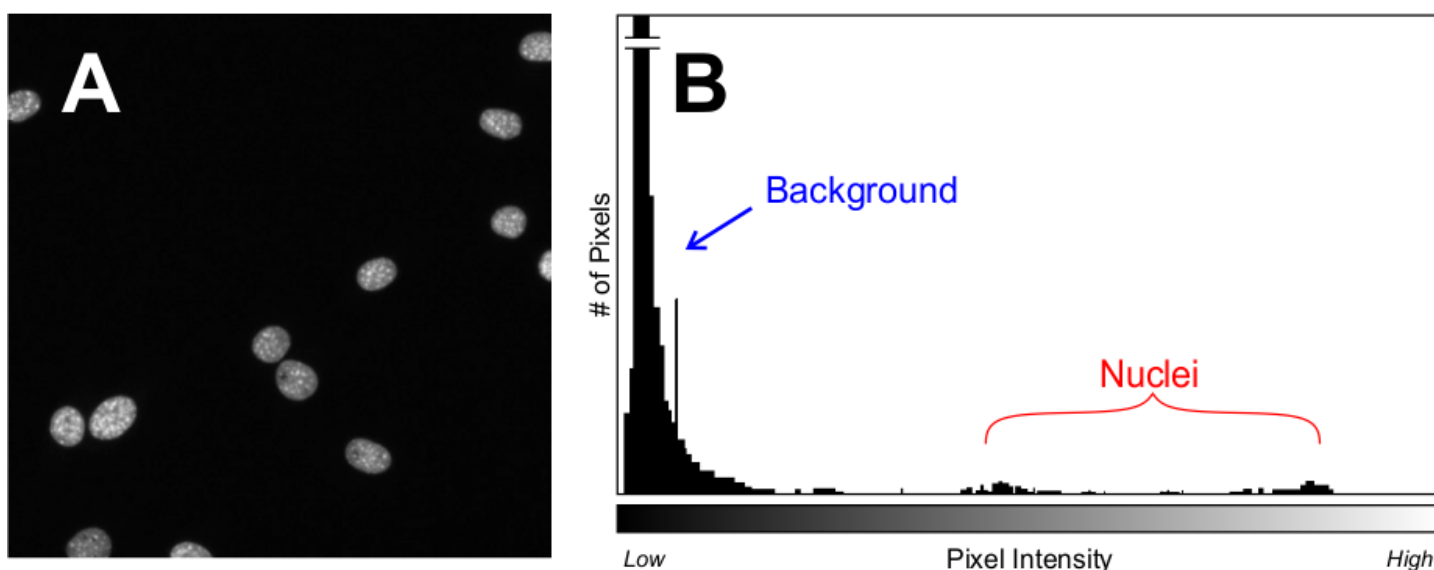


Figure 11: Image and Corresponding Histogram. A histogram plotting the number of pixels (Y-axis) which have a particular intensity (X-axis). Here, almost all the pixels are close to black, and only a small number occupy the lighter bins of the histogram.

6.2.3.1. Border or Edge Objects

Most image analysis software has options for inclusion or exclusion of objects which intersect the border of the image. These objects should only be included if complete sampling is most important (total counts for instance) and it is known that a small gap exists between image fields. If there is no gap between fields then these objects would be counted twice, which would overestimate the total count. Border objects should be excluded if information about particular object shape or structure is most important (i.e. size of cell or length of neurites).

The processing of objects provides a new set of feature data from the analytical software. Each object has many properties, i.e., shape, size, texture, and intensity that can be used in analysis. But usually objects are first “selected” to determine whether they are of interest in the analysis or just part of the noise. Object selection requires a training set or specific parameters to refine objects by their properties. For example, if objects are identified to represent cell nuclei in a homogenous culture of cells, then it is likely that the object area and object intensity criteria will be in a relatively small range, and all other objects are considered noise, debris or something else in the well. Processing can be done to identify objects similar to a known object or to identify objects that are different from a known object (i.e. training).

6.2.3.2. Secondary and Tertiary objects

To extract other features or to gain information about entities near the primary object, secondary and tertiary “sub-objects” are often identified. The simplest algorithm uses a mask or halo around the primary object at a predefined pixel width. Other algorithms use additional channels (from actin or cytoplasmic staining for example) to define the border of the secondary objects.

6.3. Feature Extraction (Object Processing)

The actual measurements generated from an image are called “features”. Usually these are cell or object-based measurements like number, size, shape, intensity, texture, or kinetic measurements. Most software analysis packages provide many more features per cell than is useful to ultimately report from a high content screen and therefore in it is important to understand what the features represent and how they are derived. This understanding will allow you to choose appropriate set to analyze.

There are no standards for output data features; therefore, there remains a wide range of interpretation of the generated output features for each manufacturer's image analysis algorithms. The specifics for each feature vary from platform to platform, but a few stand out for their broad use such as object counts, object size / shape and object intensity. It is important to read the manufacturer's description of each feature and to remember that features can be used in combinations.

For each of these measurements, there are two basic ways to determine their inclusion in downstream analysis. First, hypothesis driven: is a particular cellular feature which is being directly measured by a feature relevant to the dataset and worthy of inclusion. The advantage is that it will always be easy to interpret this data. The disadvantage is that these hypothesized features are often *not* the most robust features for measuring differences between samples and controls. The second method looks to best compare the sample phenotype to that of the controls. Both positive and negative controls could be used for comparison.

Before starting on analysis, it is very important to think about the organization of the various forms of data. This is primarily the images, the results of image analysis, and the metadata (experimental setup, etc). Other sections of the book will discuss this in detail (see Section 8 – Data Management for High Content Screening).

6.3.1. Intensity measures

Measuring intensity should be simple, it is after all the most basic measurement that comes from the image sensor and is related to the number of photons captured on the sensor during the exposure time. The numbers attained are usually just called intensity *units*, since the camera isn't scaled or calibrated. Raw intensities are processed over some unit of area to give a meaningful value. The unit of area is a single pixel, set of pixels, or an object area (a cell or nucleus). Usually, at least two measurements are given for a particular object. The "Sum" or "Total" measurement, and an "Average" or "Mean" measurement. The Sum or Total intensity represents an aggregation of all the pixel intensities combined to make up the unit area, so these are directly affected by object size. This is also sometimes called integrated intensity. The Average intensity feature is an average accumulation of pixel intensities across an area so that the number of pixels doesn't affect the measurement as compared to Sum intensity, and therefore is a feature that is orthogonal to area measurements. There can also be intensity features related to the variation of the intensity compared to surrounding pixel intensities.

6.3.2. Nuclear features

With the appropriate fluorescent probes, nuclear stain is one of the best markers for cell identification because of its distinct edge detection and relatively uniform staining. Common features include the "Nuclear Area" and the "Nuclear Intensity", which are simple and useful calculations. Most screening paradigms should include these measures at least in quality control assessment to identify abnormal nuclei. Cell death and proliferation can dramatically impact other features, and should be considered assessed for removal from primary analysis or used in gating strategies for measuring subpopulations. In cell death, apoptotic nuclei are often smaller and more intense, while necrotic nuclei can be larger. In addition, dividing cells may have smaller or brighter nuclei. For cell cycle assays, the Total or Sum nuclear intensity is the most useful measurement for distinguishing G0/G1 and G2/M phases but not S-phase due to variability of intensity measurements, since it most closely reflects DNA content.

6.3.3. Position measurement

Each object can be tracked for its relative XYZ position within the coordinate system of the platform or relative to other features of the cell or neighboring cells. As before, careful reading of the manufacturer's description of these measures is necessary to avoid confusion. Position measurements are very important for any analysis that involves populations of cells (clusters, colonies, stem cells, population analysis). Proximity to neighboring cells has also been shown to be an important factor in predicting viral infection, neurite outgrowth, autophagy and a host of other phenomenon (40). The user should be concerned with two things – from which point is the

reference for the position measurement (the field or image, well, entire plate), and what position within the cell *itself* (upper left point, centroid, bounding box).

6.3.4. Regional analysis

Measuring specific regions or compartments within or around a cell is important for many assays. As with NF- κ B translocation, for example, the cytoplasm must be distinguished from the nucleus to assess which compartment the protein is occupying. The fastest algorithms to process this information use a mask or halo, which is dilated out or contracted in from the primary object. If a nuclear stain was used a Nuclear Mask is constructed either by directly copying the primary object, or often constricting by 1 or 2 pixels. The Cytosolic Mask is then constructed by dilating a few pixels away from the nucleus and then is active over an additional width of pixels (creating a ring overlaying the cytoplasm, Figure 12). There are two important considerations when defining the width and distances of these masks. First, should the perinuclear area be part of the nuclear mask, the cytoplasmic ring, or excluded? Many biological processes (autophagy, nuclear import/export, protein synthesis, etc) take place in this perinuclear area, so its placement is often relevant. Second, how far out should the cytoplasmic ring or halo extend? Most cells have projections which extend some distances, but if this mask is being used to identify cytosol then extending the mask too far will include too much background, decreasing the value of the measurement. On the other hand, if a spot or strand (see below) is expected out in these processes, then the mask should be extended. Sometimes this outer ring is used to detect nearby objects that aren't even part of the cell, so it could be dilated quite far depending on the assay.

Cell area

Although the cytoplasmic ring discussed above produces a boundary whose area can be measured, the shape of this simple object doesn't reflect the true cellular boundary (also known as the cellular *extent*). Secondary object algorithms that use an additional channel which demark the cytoplasm, cytoskeleton, or membrane can return an accurate measurement of the cellular area. These other channels can build up objects from the pixels, where groups of similar pixels are combined into groups that are eventually defined as a particular kind of object (Figure 13).

6.3.6. Puncta identification

Spots, strands and sub-regions are important measures in many cell biological assays. Most image analysis software includes feature extraction to look for "sub-objects" or "spots" within a primary object. These are powerful measurements, but rely on consistent primary object identification and cell extents (at least approximated) to be useful. Sub-object identification usually works by setting another threshold and operates within a boundary defined by a mask (Cytoplasmic Mask or Cell Area for example). Puncta (spots or small regions) can be identified in these regions (Figure 12). Typical examples of small punctate regions that can be identified and measured are lipid rafts, ribosomes, micronuclei, mitochondria and autophagosome to name a few. Strands like actin or tubulin filaments can also be identified in a similar manner.

6.3.7. Edges

Tracing and analyzing neurites often uses different types of algorithms due to the semi one dimensional nature of these extensions. Algorithms rely on a basic method sometimes called "skeletonize". This involves converting pixels into line segments, which branch out and behave just like axons or dendrites. These lines can then be pruned to remove small extensions (noise) and are analyzed for length and branching. Gap junction or tight junction formation between neighboring cells could be analyzed by the similar way.

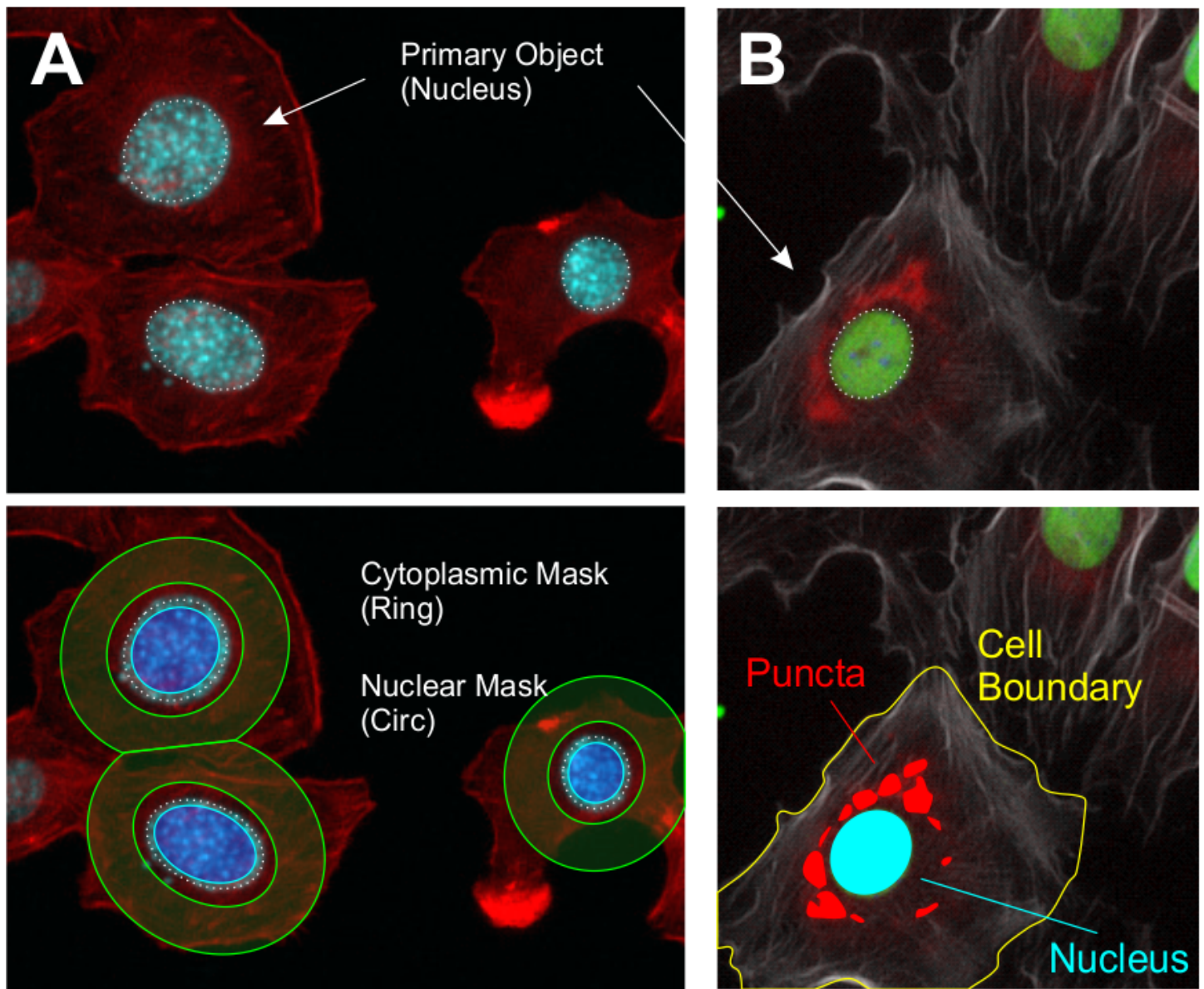


Figure 12: Regional Analysis, Masks, and Common Features. Images of cells in culture demonstrate primary object identification (dotted outline) and secondary analyses in the lower panels. A) Renal carcinoma cells stained with phalloidin (red) and Hoechst (blue) above. Below a 1 unit contracted nuclear mask (light blue outline) and a 2 unit dilated cytoplasmic ring (green). The relevance of the width of the ring is evident, since if the actin adhesion in the cell on right was an important feature, it would have been excluded from the mask. On the other hand, the masks on the two cells at the left do a good job of including cytoplasm and excluding background. B) mouse fibroblasts stained with phalloidin (gray), a nuclear protein (HMGB1, green), and an autophagic protein (LC3, red). Analysis of the cell boundary as well as cytoplasmic puncta in the red channel are displayed.

6.4. Informatics Analysis

High content screening seems simple: take good images, get good data. But once it is time to look at the data, it gets complicated. Once the images are analyzed, the results are a number of different features that quantitatively describe each and every cell present in the imaged samples. This means not only a lot of data, but a lot of multi-dimensional (multiple parameters) and hierarchical (embedded groups, cells are all related inside a well, wells are related by experimental treatments) data. This section will act as a quick description of points to think about when starting informatics analysis, and will be followed by advanced chapter(s) in areas such as machine-learning, image analysis for whole-organism, phenotypic clustering etc..

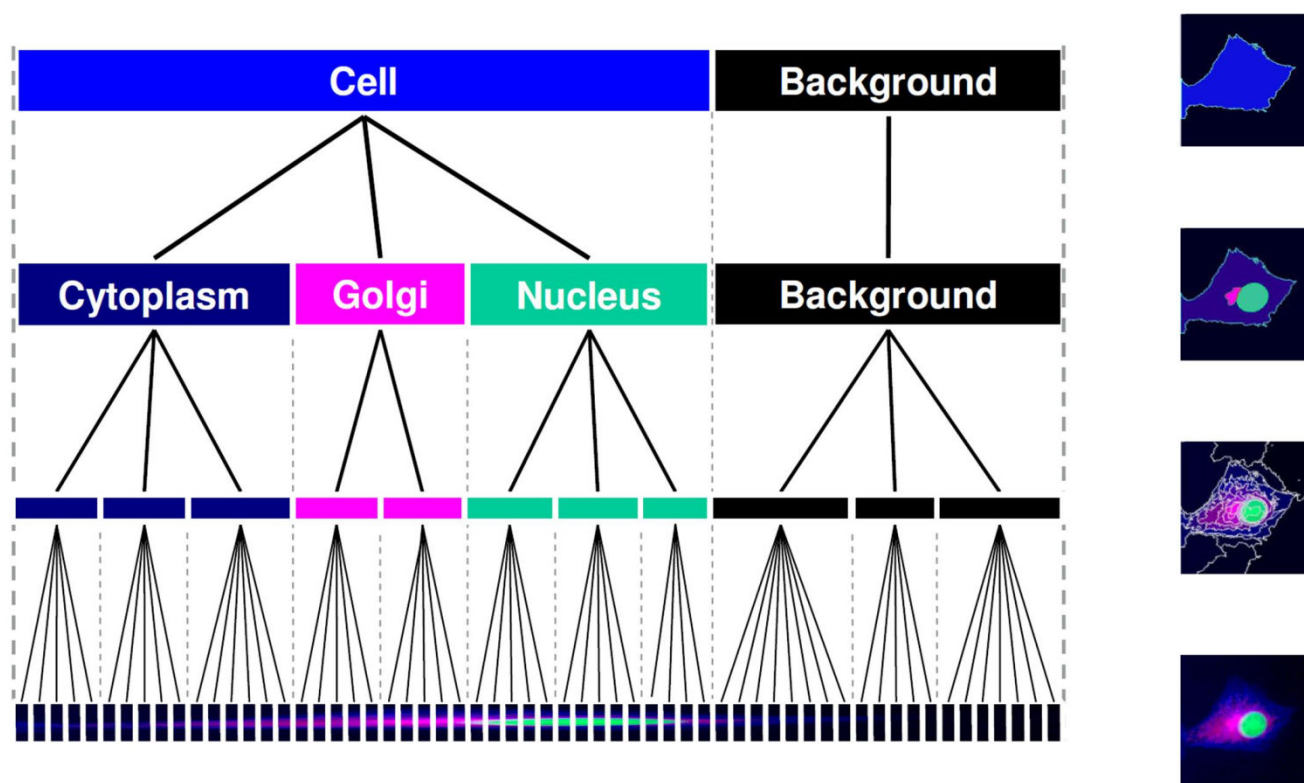


Figure 13: Pixels to objects. Using microscope channels that are derived from different cell strains, groups of pixels can be combined into a variety of objects, as in this example from Definiens: the cytosol, the golgi, and the nucleus.

6.4.1. A Brief Informatics Pipeline

An analytical pipeline (Figure 14) starts out with the raw cell-based image data. Immediately after, the metadata that describes the experimental conditions should be connected to raw data. A good next step is for a few standardized reports to be automatically generated. These reports should show well layout and plate overviews. The purpose of these reports is to get an overview of the current experiment and briefly check for large errors that can be easily spotted (such as edge effects).

A quality control / quality assurance step should be placed early in the pipeline. Many kinds of errors can occur during image acquisition or downstream analysis and common ones for the platform should be checked for in an automated or semi-automated way. Focus imperfections, incorrect exposures, background problems, artifacts, and tracing errors need to be identified so records which are affected by them can be excluded. At some point in a HCS campaign (at least in the beginning and the end, if not more frequently) images with mask overlays visible should be directly reviewed by a human observer to vet the images and ensure that tracing is correct. This process of manual image vetting can be assisted by software which let the user directly annotate the image or an attached data table with their findings (www.fastpictureviewer.com for example). Vetting the image analysis early in the screen can help to hone the algorithms and produce better data.

The data may be normalized at this point if normalization controls are built in. Some form of normalization is likely to be necessary to compare screening runs from separate days, etc.

The final steps of the analysis involve aggregating cell information together until the data can be analyzed at the treatment level (i.e. each record or row of the data table represents a particular compound or gene or condition tested in the assay). The simplest form of aggregation is to take the average (mean) or median, but one additional measure should often be included. While analyzing the cell-based data, thresholds should be set for

measurements of interest. Due to the fact that most measurements are not normally distributed, averages may produce inaccurate results. Therefore setting a threshold and aggregating the % of records above or below the threshold is sometimes preferred. Flow cytometry assays commonly call for this type of analysis called “gating”. Some form of a histogram (bar, cumulative, or 2 dimensional) is often utilized to aid in setting the threshold or gate.

Cell-level data may be aggregated up to treatment level directly, or taken to an intermediate “replicate-level” first. If replicate wells were used in the experiment, then the average nuclear intensity (for example) may be average for all cells within a well to produce replicate-level data. These replicates can then be further aggregated to treatment level, allowing for a different set of statistics to be used. The reasons to aggregate to different levels are usually statistical or based on weighting.

Finally, at the treatment level, the data should be in the simplest form, but still retain deviation information so statistics can be performed. All the data, but especially the treatment level data should be stored in a database and/or exported as charts. In addition, it should be possible to easily export the primary treatment data to software (such as Excel or Spotfire DecisionSite) so that personnel have easy access to it (also see Section 8, Data Management for High Content Screening)

6.4.2. Software for Data Analysis

To choose the best software to analyze the high content data, remember that ultimately, the purpose of the analysis is to make a decision or a figure. Decisions will need to be made about whether to proceed with a particular gene or compound, or whether an assay is working. Usually a figure with statistics will be needed to convince someone else of that decision. The ideal software would allow all the different forms of data to be present (completeness), and be able to operate on them quickly (speed). Being able to change the representation or the form of the data non-destructively (dynamically) is also an ideal characteristic. Below: a list of a few solutions.

Tibco Spotfire <http://spotfire.tibco.com/>

Databases (Microsoft Access for example) <http://office.microsoft.com/>

MATLAB <http://www.mathworks.com/products/matlab/>

R <http://cran.r-project.org/>

CellProfiler Analyst <http://www.cellprofiler.org/>

6.5. Image Analysis Solutions

HCS platforms are discussed elsewhere in this book (see Section 2 – Image Technologies and Instruments). Most of these vendors also produced Image analysis software that runs in real-time or just after the acquisition. But several good image analysis solutions exist that are free and open source. Two of these are listed below with examples demonstrating their basic use.

6.5.1. Free Open Source Image Analysis Software

6.5.1.1. ImageJ

ImageJ is a freely available open source, multi-platform project from the NIH. A closely related package “FIJI” (FIJI Is Just ImageJ) is usually preferred since it includes many useful modules and keeps itself up-to-date. Fiji and ImageJ are toolbox based and work much more like classic graphics software where an image is loaded, and then commands are run on it in real time and are destructive (i.e. they will change the image that has been loaded, such that if you save by accident, it would destroy the original image).

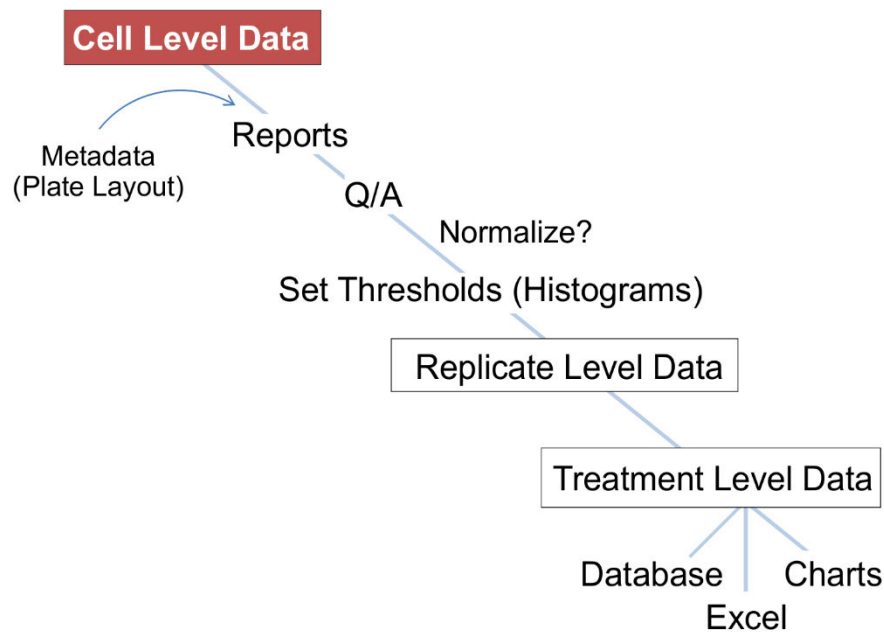


Figure 14: Informatics analysis pipeline

ImageJ can take advantage of multicore processors on most modern desktop and laptop machines. This means that programs can be written directly with multithread capabilities or that multiple scripts can be run simultaneously to greatly decrease the processing time (<https://www.ncbi.nlm.nih.gov/pubmed/17936939>).

Example:

Example image used are human renal cancer cells stained with Hoechst to mark the nucleus. The following case shows how to identify objects based on the nuclei.

1. File > Open Next Image (Figure 15A)
2. Background Subtraction (rolling ball)
 - a Or FFT and get rid of all the really low frequency stuff (but slower)
3. Image > Adjust > Window/Level > “Auto”
4. Image > Adjust > Threshold (Figure 15B)
5. Process > Binary > Make Binary (Figure 15C)
 - a Fill holes / Close (Figure 15D)
6. Process > Binary > Watershed (of touching)
7. Analyze > Analyze Particles (Figure 15E)
8. Apply Mask to original image, and other channels
9. Measure
10. Export results

6.5.1.2. CellProfiler

CellProfiler (<http://www.cellprofiler.org/>) is a free, open-source image analysis package that comes out of MIT’s Broad Institute from David Sabatini and Polina Golland’s lab by Anne Carpenter and Thouis Jones. It is a “pipeline” based tool which lets you add simple modules that work on a sequence of images {<http://genomebiology.com/2006/7/10/R100>}. Unlike more classic software, the modules don’t run until scheduled (by clicking analyze), and they are completely non-destructive. These tools allow for quick assay design since they are already tuned for the processing of cell biological images (for the most part). Below we load an example image (Figure 16).

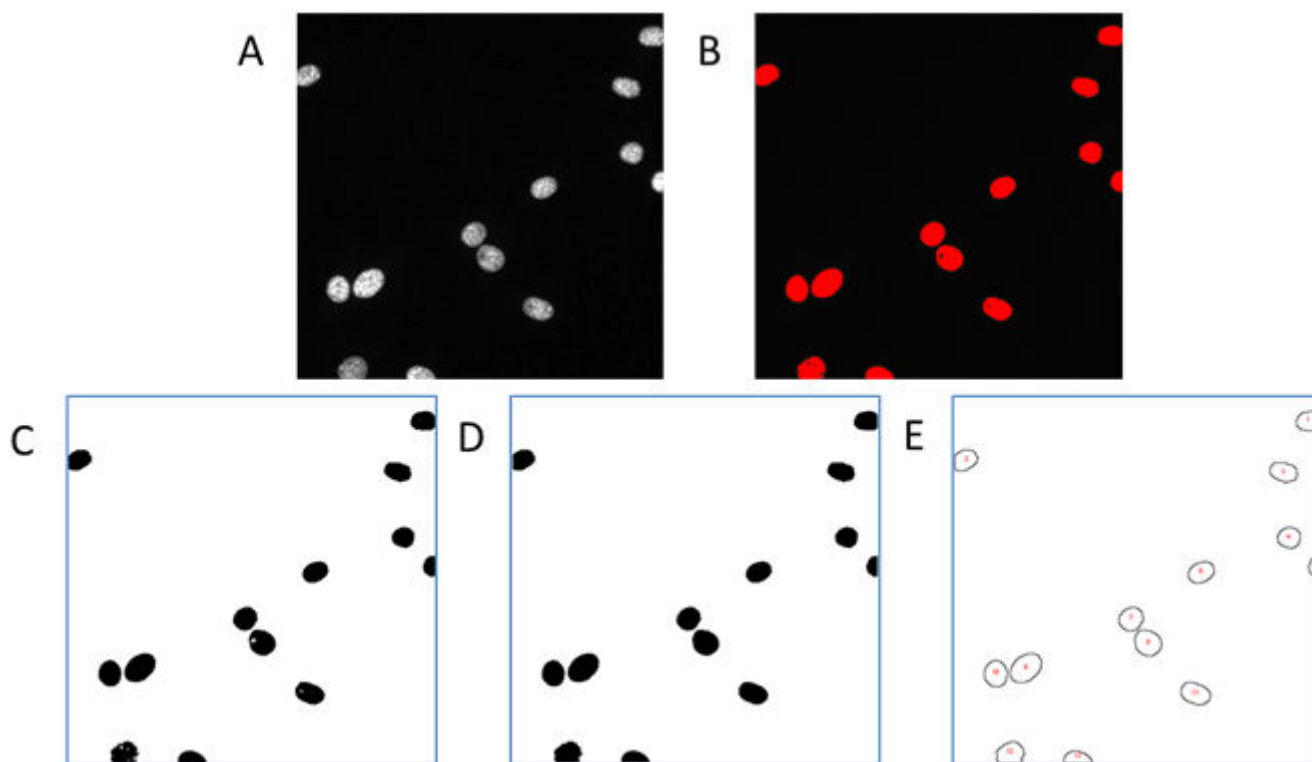


Figure 15: Example images from ImageJ analysis software. A) Representative image from Step 1. B) Representative image from Step 4 C and D) Representative images from step5 and 5a. E) Representative image from step 7.

Step 1: **LoadImages** – use “elsewhere” and specify a directory, enter in a part of the filename or TIF or BMP etc

Step 2: **RescaleIntensity** and background correction (Figure 17).

Step 3: Check “Allow Overwrite”.

See Figure 17 for representative images using CellProfiler for analysis.

Step 4: **ApplyThreshold**

Threshold (as above) can be done in an automated way or manually. Although CellProfiler has thresholding built into its PrimaryObjectIdentification module, it is nice to do it separately so that the results of the threshold are clear. Aside from manual, Otsu, MoG (Mixture of Gaussian) and Background methods are provided (global is usually the best sub-option). Otsu is the most automated, while MoG and Background assume that the amount of background vs. foreground is known. If it is constant among the images (for example because cultures had a very constant confluence) then these will give slightly better results. The background method is similar to many classic methods which assume the background predominates in the image and uses the mode of the histogram to set the threshold. In this example (Figure 18a), Otsu global is used (with all the other defaults – Figure 18b).

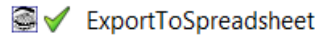
Then just have to take the measurements that you are interested in and export to spreadsheet!

MeasureObjectIntensity
MeasureObjectSizeShape

For Object Intensities – there is more than just the object mask information to consider, there is also which image should the masks be overlaid to make the intensity calculations. Here we selected the raw nuclear image,

but one could easily select a background-corrected image or even another channel. In CellProfiler, the MEAN Intensity measure is called “Intensity_MeanIntensity_” and the TOTAL Intensity measure is called “Intensity_IntegratedIntensity_”.

To measure the object size, area and shape, only the object mask is needed, so no image input is necessary. You will probably also want to uncheck the Zernicke features box, since these polynomials take a longer time.



After export, two files will appear in the default output director: “DefaultOUT_Image.csv” and “DefaultOUT_Nuclei.csv”. The first, “DefaultOUT_Images.csv” is important because it gives the list of images that were analyzed and the corresponding ID (an index) which can be used to match up additional information from the other spreadsheets.

CellProfiler has many powerful functions that are completely focused on Life Sciences research, including time lapse, worms, neuronal tracing, texture/granularity and neighbors. It is also helpful to free up some space by letting CellProfiler dispose its internal images using “Other > Conserve Memory”. CellProfiler can run very effectively on an enterprise multi-processor architecture (cluster computing), but is not currently configured to be able to run with parallel processes on a standard consumer machine.

6.5.2. Proprietary Image Analysis Software

In addition to the open source software described above, there are many proprietary image analysis software programs available. Table 6 provides a listing of some of these software programs.

Unlike the top four products, neither the Adobe nor Corel products are designed for image analysis. Since they are pervasive, many add-ons and custom scripts have been written for these to allow fairly sophisticated image analysis processes. But they are likely to require substantial more development.

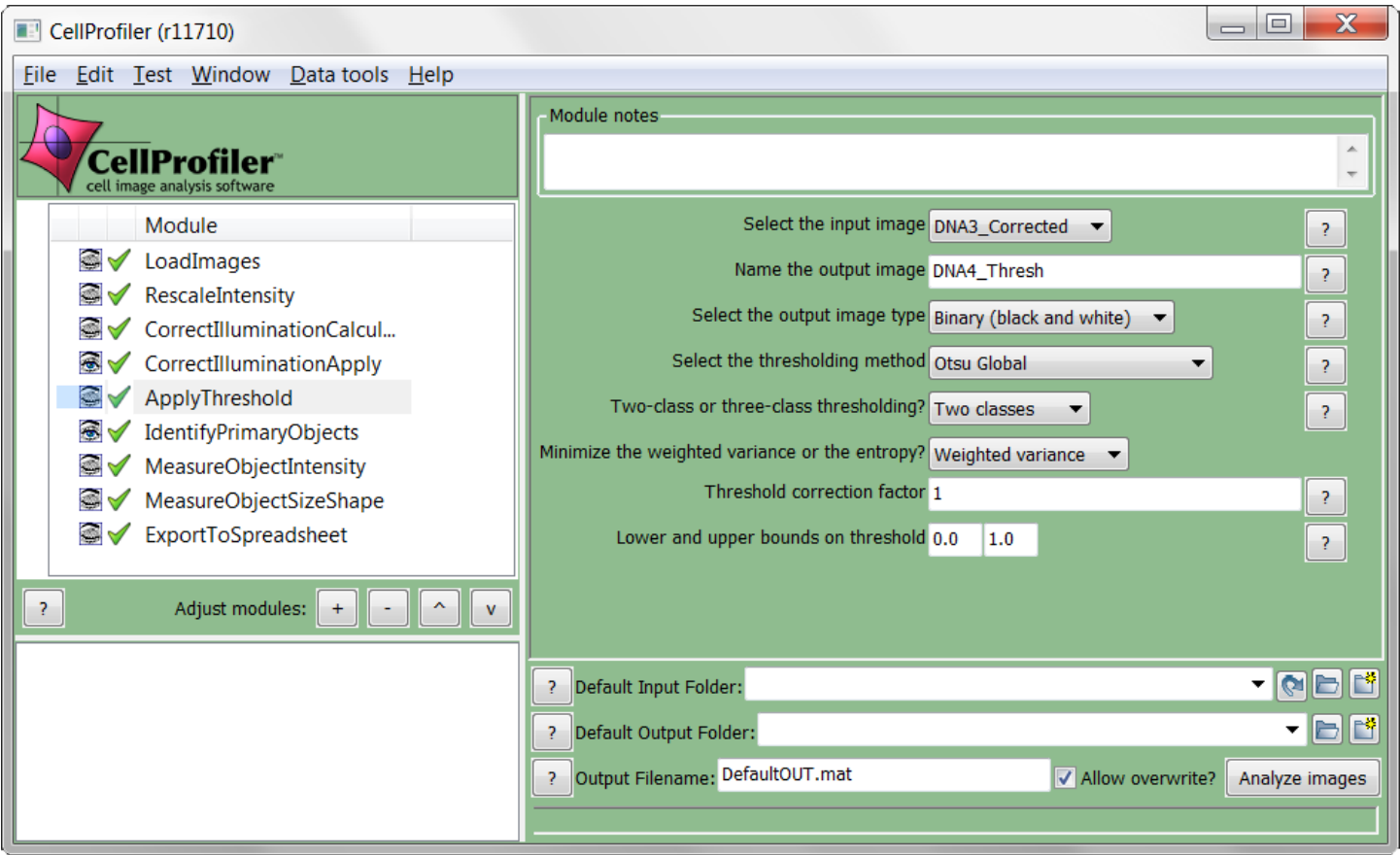


Figure 16: Example CellProfile image.

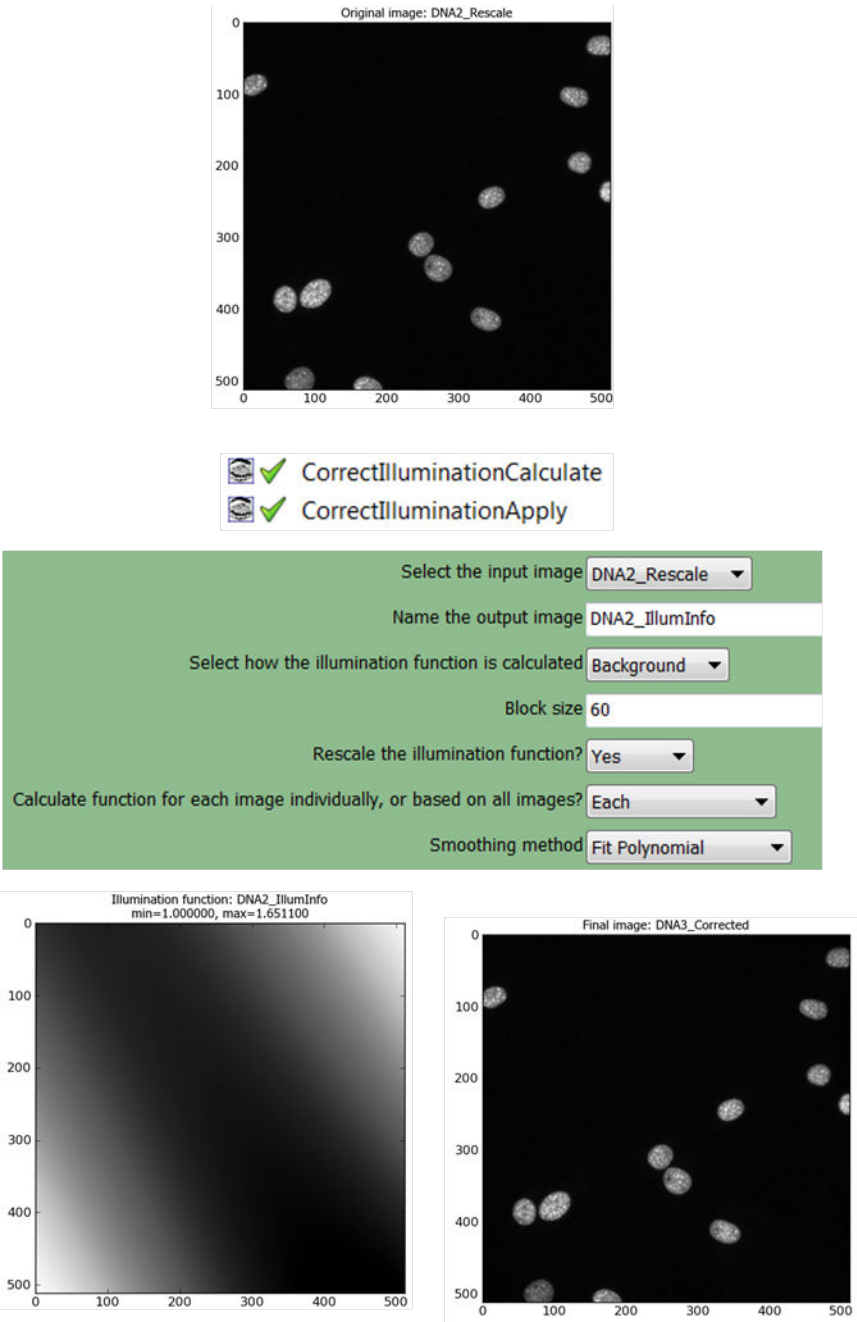


Figure 17: Representative screen captures using CellProfiler for image analysis.

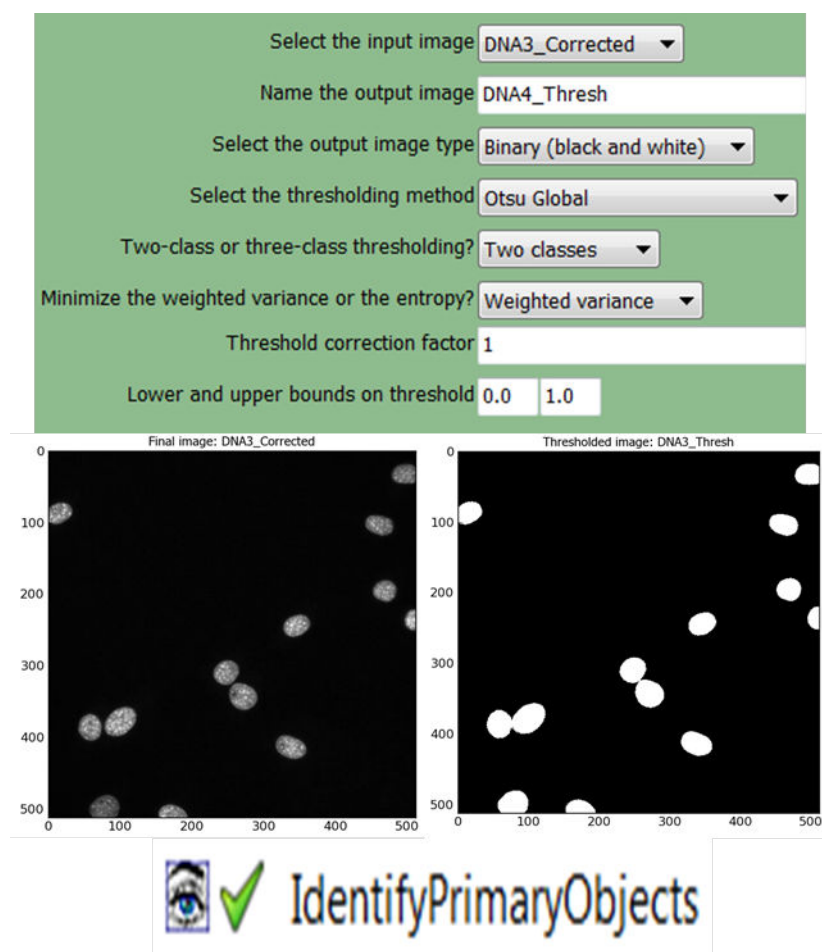


Figure 18a: Example using Otsu global

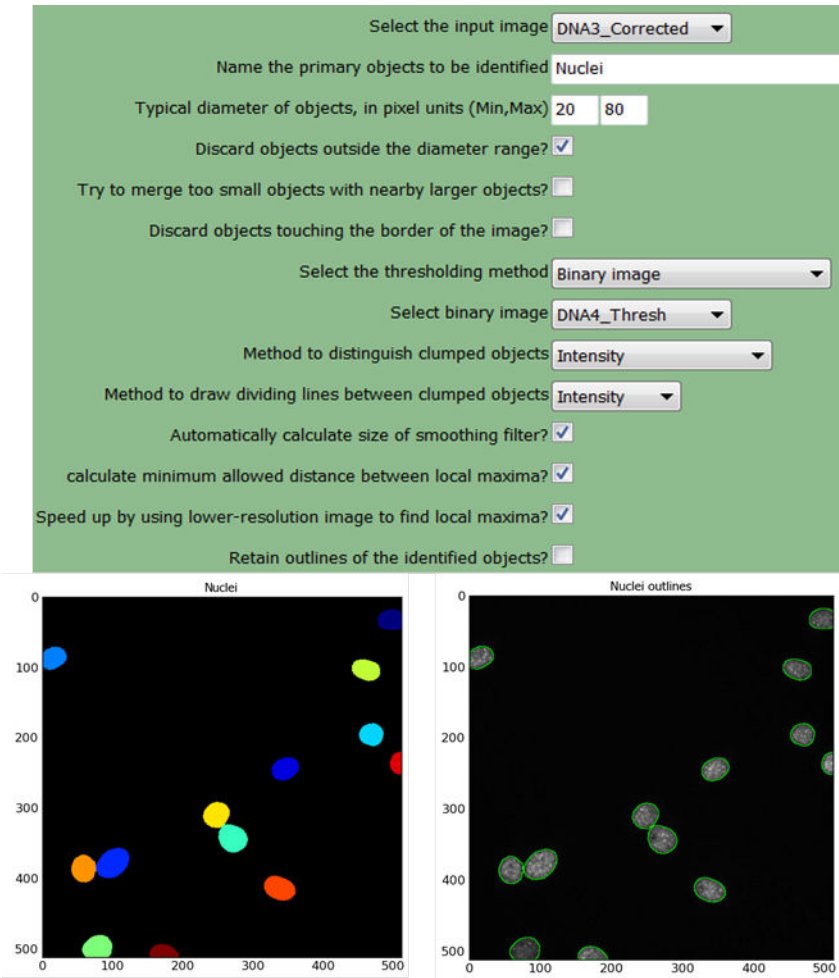


Figure 18b: Default Settings except for using the already-thresholded binary image and setting the typical diameter to match with the nuclear size of these cells.

Table 6: Examples of proprietary image analysis software

Company	Product	Website
Definiens	TissueStudio	http://www.definiens.com/
Media Cybernetics	ImagePro+	http://www.mediacy.com/
Mathworks	MATLAB	http://ww.mathworks.com/products/matlab/
Molecular Devices	Metamorph/MetaExpress	http://www.moleculardevices.com/
Adobe	Photoshop / Lightroom	http://www.adobe.com/products/photoshop-lightroom.html
Corel	Photopaint	http://www.corel.com/

7. Assay Validation for HCA Screens

This section on High Content Screen Assay Validation serves as an introduction to the topic but the reader is referred to the AGM HTS Assay Validation chapter.

HCA screens can be target defined but more often are phenotypic in nature and include measurements of dozens of features. Measured features include size, shape, intensity, texture, and dynamics. Simple examples include nuclear area and intensity, or derived measurements, such as nuclear or cytoplasmic translocation. The data is

usually based on analysis of the phenotype of single cells or objects within cells. The data can be reported for individual cells or the data from cells may be aggregated to produce data at the well or treatment level. HCA screens, unlike HTS cell based screens, often have large variability because of heterogeneous cellular populations in a given well. The data can also have large variability because the distributions of particular features are non-Gaussian when measured at the single cell level. There are many reasons for this, but the local cellular environment can have a very major effect on a given cell's response to a perturbagen (38-40, 58). There can be especially large inter-plate variability in HCA screens and this can be approached by each plate having appropriate positive and negative control wells that can be used to normalize data across plates and days (56, 59).

In HTS research plate variability studies commonly use a minimal of two signals: "Positive" and "Negative" signals. This makes the assumption that the study involves perturbagens, typically compounds that are known to be active in the assay. Many HCA phenotypic screens involve siRNAs or shRNAs to knockdown mRNA levels and subsequently protein levels or cDNA overexpression to express various proteins. In these cases it is not possible to construct dose response curves or even predict perturbagens that give "Negative" signals. Therefore standards used in HTS variability studies may not be useful in HCS assays using RNAi or over-expression approaches. During the assay development phase, it may be necessary to determine the mean response to a large number of treatments (100-1000) and then identify two or three treatments that reproducibly give a response at the level of the mean of the total set of treatments. This prescreen phase may also uncover treatments that can serve as robust positive controls. These can then be used in each plate to allow normalization across plates. The number of wells per plate in the negative reference group has a large impact on reliability of genome wide screens (56) and varies with the number of wells per plate. In 384 well plates, 16 negative control wells are acceptable and 20 or more is preferable to have acceptable false non-discovery rates (Figure 19).

There are different measurements of assay performance. Classic ones include signal to noise (S/N) and signal to background (S/B). The most widely used measurement to assess an assay is the so-called Z' -factor (60). This measurement gives insight into how much negative and positive controls are separated. The formula of Z' factor depends on the means of the positive and negative controls (μ_+ , μ_-) and their standard deviations (σ_+ , σ_-).

$$Z' \text{ factor} = 1 - \frac{(3\sigma_+ + 3\sigma_-)}{|\mu_+ - \mu_-|}$$

The constant factor "3" assumes the data has a Gaussian distribution and that 3 standard deviations would encompass 99% of the values. Assays with a Z' -factor between 0.5 and 1 are considered excellent. However, often HCA data is not Gaussian and can have long tails in one or both directions. Neurite length distributions are well known for having distributions with a very long tail in one direction. An experimental approach to dealing with non-Gaussian data is to use the one-tailed Z' factor which only uses samples between the positive and negative population means (CellProfiler Statistics Module). A recently introduced measure, which is an alternative to Z' -factor, is the strictly standardized mean difference (SSMD) (61).

Once an assessment measurement, such as the Z' -factor, is selected, then a series of validation experiments should be performed. These include:

1. A full plate with minimum and maximum signal conditions to determine Z' -factor
2. Full plates (5-10 plates at a minimum) with minimum and maximum signal conditions to assess edge effects and other patterns of variability (pipetting, etc.) between plates
3. Full plates with a range of DMSO concentrations to assess solvent tolerance
4. Full plates with minimum and maximum signal conditions in a compound dilution scheme to ensure expected EC_{50}/IC_{50} determinations are accurate
5. Three days of EC_{50}/IC_{50} runs with reference compounds to demonstrate reproducible Z' -factor and EC_{50}/IC_{50} values. Without reproducible EC_{50}/IC_{50} values, it is not possible to do reliable SAR studies.

Edge effects are readily observed in HCA screens, so much so that one should assume they exist and explicitly test for them at the beginning of assay development. There are several strategies to deal with edge effects, the most common being media-only wells around the outside of the plate and specialized plates which have water “motes” built in (from Thermo Fisher, Nunc, and Aurora Bio, for example), or simply use a water tray in the incubator or surround assay plates with wet paper towels. Another common practice is to pre-plate the cells in the tissue culture hood, allowing the cells to attach before moving the plates to the incubator, which normalizes seeding densities on the plate (62). Position effects are important, so having controls scattered through the plate and avoiding having hits always in the same part of the plate is advantageous (also see Section 5.7.1 Assay Response Stability).

Involving statisticians during the planning process of HTS and HCA campaigns is wise. Power analyses are generally better suited for non-discovery studies; therefore, HTS approaches which seek to control the false discovery rate (FDR) (63) and balance it with the false non-discovery rate (64) are generally used (65). Finding appropriate and realistic negative and positive controls is important in this effort, and negative controls often end up being especially difficult with HCA since the treatment procedures (transfection, for example) often manipulates some of the many parameters being measured. Because screening campaigns now can involve very large numbers of perturbagens, especially compounds, special statistical methods may be called for to eliminate systemic biases introduced into an entire screen or into some plates of a screen (66)

It is also very important to understand the signal derived from a particular analytical algorithm. A common issue with HCA is to misinterpret a particular parameter due to very strong results comparing the positive and negative control. For example, many small molecule inhibitors that produce a strong effect on the parameter of interest can also diminish or enhance cell viability. Changes in overall cell health tend to have a direct impact on other measured parameters. Cell morphology changes can be due to reduced viability or increased proliferation, with many cells “rounding” up, appearing as a decreased in size. With everything else being the same, the average intensity measurements of a cytosolic marker will increase since the cell occupies less horizontal space and more vertical space. The problem can be made worse by inappropriate use of background correction or other normalization schemes. The net result is that a small effect in the parameter of interest is made to look large and significant when compounded with other variables that the investigators may not actually be interested in studying. It is a multi-parameter analysis after all, so make sure to take into account all the parameters measured.

A review of recent HCA assay development papers shows that most published HCA assays, such as nuclear translocation assays and beta-arrestin internalization, involving compound screens have Z' factors greater than 0.7. However, most HCA screens of complex biological processes, such as neurite growth, angiogenesis and tube formation, do not report Z' factors. Those studies that do provide Z' factors report values of around 0.5 or less. This is considered within the range of screenable HTS biochemical assays. However, even lower Z' factor screens can contain considerable information and a Z' factor for a single parameter around 0 may still allow hits to be identified reliably (Figure 20). Importantly, the Z' factor and most other calculations only inform the user of the strength of the positive and negative controls, and may not necessarily inform about the assay if these controls are not realistic or appropriate.

For many large scale, truly multiple-parameter HCS based compound screens, it may be difficult to validate an assay using Z' factor calculated based on only one parameter, even on a derived parameter. An advantage of HCA screens is that by combining data from multiple output parameters including ratiometric scoring it is possible to improve Z' factors from 0.3 to 0.7 (67). Figure 21 illustrates one such alternative. In this assay, each derived measurement (or classifier) alone is unable to distinguish phenotype 1 and 2 (if one projects both red and blue does onto a single axis, there will be significant overlap between the two populations). But when all 3 derived measurements are used in a three-dimensional plot, one can make a clear decision surface to separate these two populations. These advanced statistical analysis and computation need support from experienced

statisticians or informatists, and must have comparable high-speed computational infrastructure in the research facility. Future HCS chapters will discuss alternatives to the Z' factor in greater detail.

8. Data Management for High Content Screening

High throughput screening technologies by definition generate large amounts of data. Within this field, however, high content screening methods stand out as they are capable of generating massive amounts of data – even when run in a non-high throughput mode. This is largely driven by the fact that a given well is characterized not by a single experimental readout (say, fluorescence) but first by a set of images with associated metadata and second, multiple numerical readouts derived from the well images. Given (uncompressed) image sizes on the order of 3 MB, a single 384 well plate imaged using a single field of view leads to 1.1 GB of image data alone. Assuming a small pilot screen of 10 plates run in single dose format, this generates 10 GB of data for that single run. While this is not particularly large, given today's storage systems, a medium sized lab can easily generate tens of such screens a month, and if dose response is considered, the image storage requirements increase by an order of magnitude. This implies corresponding increases in storage requirements including investment in software (for data management and analysis). Software costs are likely one-time investments (or will only increase slowly). It is useful to note that Open Source solutions can be employed on the software side, reducing initial costs, though of course, such solutions invariably require customization and maintenance and dedicated manpower.

8.1. Not Just Images

However, image capture and storage is just the first step in a high content screening experiment. Following imaging, the images must be registered in a repository, suitable for long-term storage and supporting efficient access by screening campaign, plate barcode and well location. Images will be processed to generate descriptors, numerical features that characterize aspects such as the number of cells, their shape, size, intensity, and texture and more complex features such as translocation of proteins, number of neurites and so on. Such analysis protocols can easily generate tens to hundreds of such features for *each* cell in a well – leading to millions of data points for a single plate. This numerical dataset must be stored and linked to the images (via plate barcode, well location and cell identifier). Finally, imaging and analysis metadata must also be recorded. This includes information such as focus settings, wavelength details, object masks, operator information and so on. These pieces of information are associated with different “levels” of the screening analysis– some are associated with the screen itself, others are relevant to individual plates or images and so on. Importantly, users may generate some metadata after the screen. Examples of this type of metadata are annotations, where a user might highlight a set of wells or even a selection of cells within a well for follow-up and include some free-text comment indicating their interest in the selection. Thus, this metadata must also be stored and linked back to images and numerical results. Any useful data management solution must be capable of supporting all these data types as well provide the flexibility to query this information in a variety of ways (68).

8.2. Image repositories

The key component of a HCS data management solution is a centralized location to collect images. In absence of a formalized management solution, the simplest approach is to simply acquire images in a file folder and inform users of folder location. This is clearly a crude and brittle approach that does not scale beyond one or two users. Invariably, folder locations may be forgotten, there is no explicit link between images and downstream data and images may not be accessible over a network easily. In addition, one must always work with the raw images, even though for many purposes (e.g., thumb nailing) they may not be required.

Most modern image repositories will employ a file system-based approach – where images are organized using a hierarchy of folders, usually located on a network-accessible storage device. But more importantly, the repository will also usually include a relational database system that records the file system path to the individual images

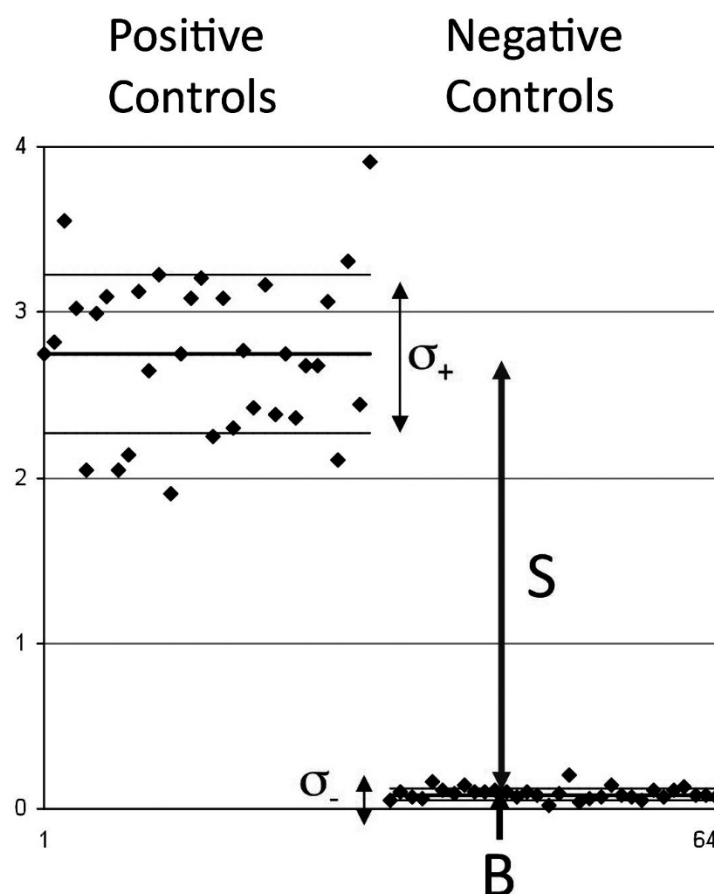


Figure 19: Standard measures of HTS assay performance:

Signal = mean of (C+) – mean (C-). Background = median (C-).

Signal to Background = S/B. σ_+ = Std Dev (C+), σ_- = Std Dev (C-).

$N = \text{SqRt}(\sigma_+^2 + \sigma_-^2)$ Signal to Noise = S/N

along with metadata such as plate barcodes, well location, imaging details (focus setting, wavelength, etc.). Import of images into a repository will usually convert them to some standardized image format specified by the system, generate thumbnail views, record meta-data and so on.

A user versed in SQL, MySQL, or Oracle can query the database to locate individual images via the database. But obviously such an approach does not lend itself to daily usage by bench scientists! To address this most vendors of image repositories will provide a graphical user interface allowing users to easily browse the image collection, searching for individual plates or wells, retrieve or archive, record notes and so on. In addition, some vendors will also include an application programming interface (API) that allows users to develop their own applications that interact with the image repository, without having to directly work with the internals (which may be subject to arbitrary changes).

Most image repositories are designed to work in an integrated fashion with a vendors imaging platform. All such repositories allow one to export and import images, though this task may not be easy. Usually, when loading images into a repository, they are converted into a common image format, such as TIFF. Some repositories may use specialized versions (OME employs the OME-TIFF format, which is a superset of the standard TIFF format).

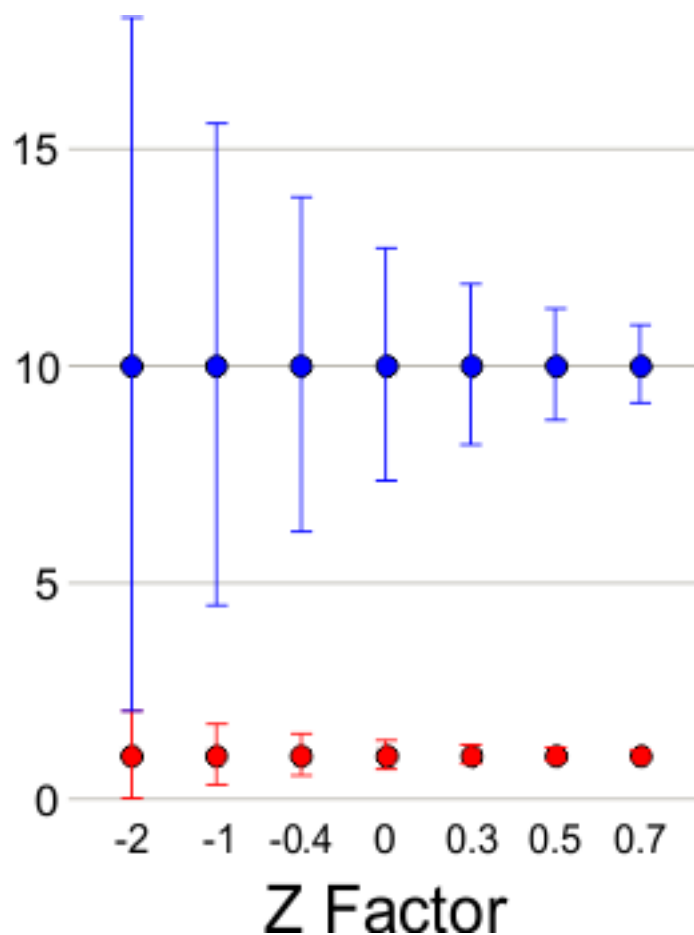


Figure 20: Z Factors comparing negative (red) and positive (blue) controls. The averages are fixed at 1 and 10, but the standard deviation is varied from 1 to 0.1 for the negative and 8 to 1 for the positive. A Z factor of -1 might still give significant results in a HCS assay.

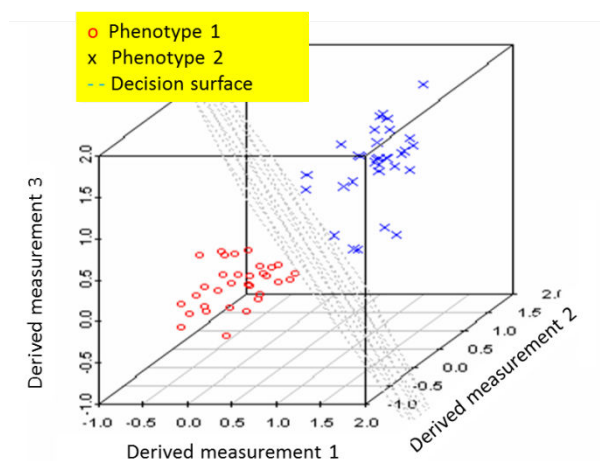


Figure 21: Validation of bioimaging based assay for primary screening using multiple parameter analysis.

The fact that the image repository is usually tightly integrated with a HC instrument's platform usually means that one is constrained to using the repository that is provided by the vendor. In other words, mixing and matching components of a HCS platform is not easy and in many cases impossible. Thus one cannot (usually) employ an image repository from vendor X and expect that the analysis or viewing applications from vendor Y

will work seamlessly. More often than not, such a mix will not work without significant investments in time and effort from the vendor (or custom development on the part of the user, requiring manual export of images from one repository and possibly manual import into another repository). For smaller laboratories, such restrictions on interoperability may not be a problem, given that they may only work with a single platform. For larger facilities, however, the lack of interoperability can become a major hindrance to the effective use of multiple imaging platforms. Recent software upgrades from several of the vendors are addressing this issue but it is an ongoing concern of the HCA community.

There are certain software platforms that have been designed to work with multiple imaging vendor platforms. An example would be GeneData (<http://www.genedata.com/>), a commercial software package, which can access images and data stored by PerkinElmer, Thermo Fisher, Molecular Devices and OpenBIS.

8.3. Data retention policies

It is important to realize that while image repositories can be very large (greater than 50-100 TB), they are finite in size. As a result, it is infeasible to continuously add images to a repository, without some policy in place to delete or archive images. Such data retention policies are obviously local to an organization. In some cases, with low enough throughput, one can retain raw images for many years. But invariably this is not practical. A more realistic goal would be to provide sufficient space to store raw images for say two years on high-speed disk (exactly how much space would be required would depend on estimates of screening throughput) after which images would be converted to a lossy compressed form (such as JPEG). These images could be retained on the high-speed disk or else moved to a slower device such as tape. This set of data would be retained for longer periods – say 5 to 10 years. This of course will depend on the study design and format. In cases of GLP, longer-term data retention policies are mandated.

A primary role of retaining the raw images is to go back to them, say for reanalysis or visual examination. In many scenarios one can get away by conversion to a format that supports different levels of compression – allowing one to quickly access a high-resolution version or a low-resolution version from the same image. A primary example is the JPEG2000 format. One could argue that a reasonable level of image compression might not affect image analysis (though we are not aware of any benchmarks that have quantitatively measured this), and thus one could directly store images in a compressed format such as JPEG instead of the raw data (even compressed TIFFs).

8.4. Linking images and data

Handling images is obviously the core responsibility of a HCS data management system. However, images are just the first step and a standard task is to process the images to evaluate numerical features (cell counts, shape, size, intensity, etc.). As noted above, a 384-well plate can easily lead to millions of data points being generated. All this data must be stored and efficiently retrieved. In addition to numerical features, other forms of image related data such as overlays and masks must also be stored for rapid access. Most HCS management systems will make use of a backend relational database, and these are usually suitable to support the large storage requirements of high content image analyses. The use of such databases allows users to easily write back new numerical results or updated pre-existing data, say based on a new or updated calculation procedure. Obviously, it is vital that this numerical data be linked to the actual images (and even cells within an image) that they are associated with. For management systems provided by vendors, this link is always present. However, when one analyzes an image using software different from the vendor provided software, the link between numerical data, overlays, etc. and images is not present – unless somehow explicitly made. This is a bottleneck for many larger organizations that operate multiple imaging platforms, and solutions to this involve using an external data management system such as GeneData, Pipeline Pilot, or OpenBIS, or developing custom software to capture links between images and numerical analyses, overlays, compound IDs and other related information.

8.5. Commercial and Open Source solutions

All imaging hardware vendors provide a HCS data management system. In some cases, the default system may contain a small amount of functionality, sufficient for handling and viewing images of a single instrument used by a few operators. But in most cases, a more comprehensive management system will also be available. As noted above, such commercial management systems are invariably proprietary.

Depending on the scale of the HCS operation, one solution may be favored over another. Thus, a small lab, using a single instrument, is probably well served by employing the vendor provided data management system. For many such groups, the tools provided by the vendor to browse, analyze and annotate are sufficient. While we do not comment on a preferred system, most of the commercial vendors provide a capable image management solution. All of these platforms are able to export images in a variety of formats and also export numerical data obtained from image processing in standard formats (tab delimited, comma separated, etc.). In addition, many of these can be integrated with advanced visualization and reporting tools such as GeneData, SAS JMP, and Spotfire (<http://spotfire.tibco.com/>). However, given the high costs associated with these tools, they may be out of reach for smaller groups and in such cases it is paramount that image and feature data be exportable to tools such as Matlab, Excel, R and so on.

For larger operations that have multiple imaging platforms, the lack of interoperability between vendors is a significant bottleneck in the development of a unified interface to all the imaging data generated across platforms. While some progress has been made by commercial tools external to the imaging platform (such as Spotfire and GeneData), an integrated solution invariably requires custom development by the organization (69).

Such custom development can be impossible, when vendors employ completely closed systems and are unwilling to provide access to the internals via a public API. While such cases are becoming fewer, lack of a public API to all aspects of a vendors data management system should be considered a significant shortcoming and hindrance to integration with an organizations pre-existing informatics infrastructure.

On the Open Source side, there are relatively few comprehensive HCS data management systems. The two primary systems that are currently undergoing active development are the Open Microscopy Environment (OME <http://www.openmicroscopy.org/site>) and OpenBIS (<http://www.cisd.ethz.ch/software/openBIS>). The former system provides a comprehensive file system-based image repository, coupled with the use of PostgreSQL as the relational database that stores image locations, metadata and so on. The infrastructure provides a browser-based interface to the system and allows users to access images and perform some simple operations on them. Importantly, the OME infrastructure supports varying levels of security, allowing one to restrict images and their data to certain individuals or groups, with varying degrees of accessibility (read only, read write, etc.). A key feature of this system is that it provides a completely open, well defined API, allowing users to develop applications that interact with all aspects of the repository. This makes it much easier to integrate an OME repository with an organizations pre-existing infrastructure. It is important to note that the OME platform focuses only on image data management and not other aspects of a HCS workflow such as image analysis. However, a number of open source software packages such as ImageJ and CellAnalyst (associated with CellProfiler) can interact with an OME installation, thus enabling a fully Open Source HCS data workflow.

The OpenBIS platform is a more general biological data management platform that supports a variety of technologies including high content screening, sequencing and proteomics. In terms of functionality the system supports image export and import, metadata and annotations and also links to the KNIME workflow tool to allow integrated analyses. In addition, the system comes with a number of analysis modules built in. As with the OME platform, it exposes an API allowing users to develop novel applications on top of the OpenBIS platform.

8.6. Visualization and reporting

Efficient and robust management of imaging data is a key to ensuring reproducible and rigorous scientific studies. But equally important is the ability to interact with the data to enable mining and visualization of phenotypic data. There is an abundance of tools and techniques for the visualization of data, though visualizing datasets characteristic of high content screens requires certain capabilities. Primary among them is the ability to handle millions of data points on a plot, yet retain interactivity. Importantly, plots with millions of data points are not usually informative, so visualization platforms should have the ability to generate on the fly summaries of the entire datasets (e.g., density and contour plots, binned plots, etc.). A common visualization platform is Spotfire, which has extensive capabilities and is able to connect to a number of HCS data management systems. This allows it to integrate images with various plots – click on a data point in a scatter plot displays the image(s) associated with that data point. Such integration is vital to the analysis and exploration of high content data; in the absence of image viewing capabilities, one is faced with a mass of (usually uninterpretable) numbers. Another commercial platform for such visualization tasks is GeneData. However, Spotfire and GeneData are commercial tools and can be very expensive for smaller groups. Alternatives include Miner3D (<http://www.miner3d.com/>) and Tableau (<http://www.tableausoftware.com/>). While there are a number of cheaper or free visualization tools, they are not always intuitive to use and some (such as R) while providing very sophisticated visualization capabilities, lack easy interactivity.

It is important to note that most visualization platforms will also be tightly coupled to data mining capabilities, as one usually wishes to perform some analytical operation (clustering, predictive modeling, etc.) on the phenotypic data. While data mining of phenotypic data is out of the scope of this section we note that the platforms such as SAS JMP, Spotfire and GeneData provide extensive modeling capabilities.

Another class of application that is commonly used to interact with HCS data are workflow tools such as Pipeline Pilot and KNIME. Both tools allow non-experts to easily construct analysis pipelines, in which individual components perform specific tasks such as retrieve images from repository, perform thresholding and then calculate summary statistics over a plate. Note that such tools do not play a direct role in terms of managing HCS data, but serve to hide the data management system from the users, enabling them to interact with the data in a sophisticated manner. Some significant progressions have been made in this area (69).

8.7. Towards a Unified HCS Management System

Given the variety of vendors in the HCS field, it is not surprising that there are a many choices of HCS data management system solutions. However, given that the fundamental goal of such a system is to keep track of images, their meta-data and downstream analytical results, it is not unreasonable to desire a unified management system that allows interoperability between different vendor solutions. Unfortunately, this is currently not the case.

Given the current state of HCS management systems, any unified approach must recognize that some imaging systems will be black boxes and cannot be replaced with a common repository and associated components. One unification strategy is to implement a software interface that hides the details of individual HCS data management systems, shown schematically in Figure 22. The interface would provide access to raw images, thumbnails (if present) and associated meta-data. Depending on the scope of the repository, it may also provide read/write access to numerical data calculated from images. However, the latter is very specific to individual installations and in general the interface proposed here focuses on image repositories.

Then, new applications that are developed communicate with each system via the intermediate software interface. While this certainly allows one to have a uniform interface to multiple vendor platforms this is not an ideal situation. It is completely dependent on individual vendor platforms to provide a public, documented API, which is not always the case. Furthermore while such an interface allows an organization to develop custom

applications across all their imaging platforms, it does not necessarily allow specific vendor platforms to interact with other platforms (say, vendor platform X imports image from platform Y, performs an analysis and stores results in platform Z). In the end, this approach does not solve the fundamental interoperability problem.

What are the requirements for a unified HCS data management infrastructure? In fact very little! It is perfectly fine for each individual vendor to have a proprietary database with their own schema and formats. However, the key to supporting interoperability is the provision of a uniform API to all the data. If such an API were available, one vendor would be able to develop their applications independent of where the images and associated data are stored. One could argue that certain platforms provide certain advantages that are not available on other platforms. While this is certainly true on the hardware side, it is not clear how much vendors can (and do) differentiate themselves in terms of the actual data types that are managed by their systems. From this point of view, a set of industry standards for data management is not unthinkable.

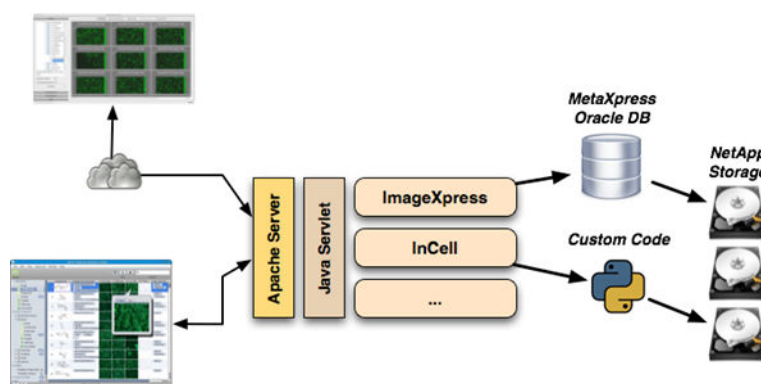


Figure 22: An overview of a software interface approach to providing a uniform interface to multiple imaging vendor platforms

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Advanced Assay Development Guidelines for Image-Based High Content Screening and Analysis

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Abstract

Automated microscopes are now widespread in biological research. They enable the unprecedented collection of images at a rate which outpaces researchers' ability to visually inspect them. Whether interrogating hundreds of thousands of individual fixed samples or fewer samples collected over time, automated image analysis has become necessary to identify interesting samples and extract quantitative information by microscopy. This chapter builds on the material presented in the introductory HCS section.

1. Experimental design for HCS

1.1. Controls

Whenever possible, positive and negative controls should be included in an assay. Using controls is required to calculate a performance envelope for measuring the assay quality and phenotype feature space (see "Assay Quality and Acceptance Criteria for HCS" section below).

However, positive controls may not be readily available. In these situations, an assay measuring a real biological process may still show a phenotype of interest under some conditions that can be observed and measured even if positive controls that induce high levels of cells with the phenotype do not exist. Once a condition is identified and demonstrates such a measurable change, then it can serve as a positive control going forward.

For profiling assays, in which a large variety of cellular features are measured to identify similarities among samples, and hence designed to have multiple readouts, several different positive controls for each desired class of outcomes may be necessary. However, these may not be known in advance. Long running assays will typically accumulate positive controls over time and may even change the perceived limits or dynamic range of the assay.

Ideally, a positive control is of the same type as the reagents to be screened (e.g. a small molecule control for a small molecule screen, and an RNAi-based control for an RNAi screen). However, any reagent that induces the phenotypic change of interest can serve as a positive control if necessary, even if artificial. For example, expression of a constitutively active form of a tagged protein or knockdown of a target by RNAi can simulate the effects of a sought-after small molecule in a screen. Although differences in modality may complicate direct quantitative analysis of such controls, such 'artificial' controls are often helpful during assay development and optimization, and provide a sense of the dynamic range to be expected in the screen.

In selecting controls, there is a temptation to select reagents with very strong effects as positive controls. This is often the result of undue emphasis on minimum criteria for acceptance for screening, such as a Z'-factor cutoff. Good judgment should instead prevail, and positive controls should be selected based on the intensity of the hits hoped to find. For example, it is not helpful to select a very strong positive control that yields a high-quality Z'-factor if it is not comparable to the strength of the expected hits sought in an actual screen. Instead, inclusion of moderate to mild positive controls, or decreasing doses of a strong positive control, is better in gaining a sense of the sensitivity of the assay to realistic hits.

The authors have observed several successful screens with sub-par Z' -factors or absent a positive control that nonetheless yielded high-value, reproducible, biologically relevant hits. As such, common sense should prevail by factoring in the complexity and value of hits in the screen and the degree of tolerance for false positives that can be filtered out in confirmation screens.

For plates of reagents from vendors, typically only the first and the last columns of a multi-well plate are available for controls, with the treated samples contained in the middle wells.

Unfortunately, this practice renders the assay susceptible to the well-known problem of plate-based edge effects, which lead to over- or under-estimation of cellular responses when normalizing by the control wells.

One strategy to minimize edge effects is to spatially alternate the positive and negative controls in the available wells, such that they appear in equal numbers on each of the available rows and on each of the available columns (1)(2). (Figure 1)

If the screener is creating a custom plate for an HCS run, ideally the control wells should be randomly placed across the plate in order to avoid spatial bias. However, this approach is rarely practical in large screens as it must be performed manually. Therefore, the chance of introducing a spatial bias effect by using a non-random control placement is an accepted practice due to the difficulty in creating truly random plate arrangements.

For screens run in multiple batches where the controls need to be prepared such as lentiviral shRNA screens which necessitate the creation of viral vectors, variation in viral preparation may be confounded with assay variation. One helpful strategy in this situation is to make a plate containing controls in one batch, freeze them and then thaw and use them a few at a time as the screen progresses. This method can help identify assay drift or batch specific problems.

For analytical approaches to correcting inter- and intra-plate bias, see the section "Normalization of HCS data" below.

1.2. Replicates

Like all HTS assays, the cost of replicates should be weighed against the cost of cherry-picking hits and performing a confirmation screen. HCS assays with complex phenotypes are often more difficult to score so more replicates are often needed. Experiments are normally performed in duplicate or higher replicate numbers in order to decrease both false positive and false negative rates. Performing replicate treatments offers the following advantages (2):

1. Taking the mean or median of the replicate measurements yields lower variability than with single measurements only.
2. Replicate measurements provide direct estimates of variability and are useful for evaluating the probability of detecting true hits. When combined with control measurements, a statistically significant change can be determined by comparing (a) the ability to distinguish treated wells from the controls, and (b) the ability to distinguish replicates of the same treatment.
3. Replicates reduce the number of false negatives without increasing the number of false positives.

Despite the multitude of good reasons for high replicate numbers, almost all large screens are performed in duplicate. Increasing the replicate number from 2 to 3 is a 50% increase in reagent cost which in the case of an HCS screen involving the tens or hundreds of thousands of samples can determine whether the screen is performed at all. HCS screens are normally performed by first screening all the samples, usually at a single concentration in duplicate, and then retesting all the hits in confirmation assays. The confirmation assays serve to filter out the false positives and are performed on a much smaller scale where it is easier to increase the replicate number and perform dose response studies if needed. In an HCS screen, preference is given to reducing

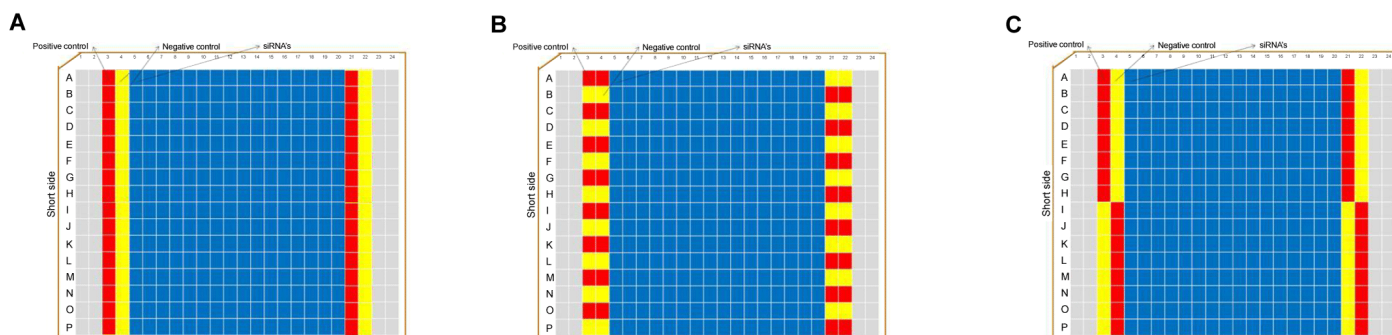


Figure 1: Location of sixteen positive controls (red) and sixteen negative controls (yellow) on a 384-well plate. In layout (A), both sets of controls are located on the plate edges in a regular pattern, and are susceptible to edge-based bias. In contrast, layouts (B) and (C) attempt to systematically decrease the edge bias by alternating the spatial position of the controls so that they appear in equal quantity on each of the rows and available columns. Adapted from (1).

false negatives because if a hit is missed during the first round of screening, it is irretrievable unless the screen is performed.

The number of treatment replicates needed is empirical and largely dictated by the subtlety of the observed cellular behavior: If a given treatment produces a strong biological response, fewer replicates will be required by virtue of a high signal-to-noise ratio. In certain cases, up to 7 replicates may be needed (3) but 2 - 4 is more typical.

Placement of replicate wells is subject to the same considerations in placement of control wells (see "Controls" above). Although randomization of the sample placement from one replicate to another is ideal, this is rarely done and for practical reasons, plates follow the same layout for all replicates. Where possible, both inter- and intra-plate replicate wells should be used for the purposes of ensuring robust normalization (see "Normalization of HCS data" below).

2. Assay Quality AND Acceptance Criteria for HCS

Z'-factor (not to be confused with z-score): While there are a number of different measurements of assay performance, the most widely used measurement to assess an assay is the so-called Z'-factor.

- Definition:* This criteria for primary screens has gained wide acceptance in the HTS community and is defined as (4):

$$1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

where μ_p and σ_p are the mean and standard deviation values of the positive control (or

alternately, the treated samples) and μ_n and σ_n are those of the negative control.

- Range and interpretation:* The Z'-factor has the range of $-\infty$ to 1, and is traditionally interpreted as follows: (Table 1).

It should be noted that the "ideal" case of $Z' = 1$ implies that the dynamic range $\rightarrow \infty$ or $\sigma_p = \sigma_n = 0$ (no variation). Neither of these scenarios represent realistic assays.

For moderate assays, the screener should consider the utility of mining hits that fall into the $Z' = 0 - 0.5$ range. Given the screening cost of eliminating a false positive (which is hopefully low) as compared to that of eliminating a false negative (potentially very costly, as mentioned above), a decision will need to be made whether to follow up on such hits or instead cherry-pick and repeat treatments, or re-screen entire plates.

- **Advantages:** While $Z' > 0.5$ has become a de facto cutoff for most HTS assays, $0 < Z' \leq 0.5$ is often acceptable for complex HCS phenotype assays, because those hits may be more subtle but still valuable. In comparison to other assay robustness metrics (5), advantages of the Z' -factor include:
 - Ease of calculation.
 - Accounts for the variability in the compared groups while properly ignoring the absolute background signal.
 - Often found in both commercial and open-source software packages.
- **Disadvantages:**
 - Does not scale linearly with signal strength. That is, an increased target reagent or a very strong positive control may achieve a higher Z' -factor that is disproportionate to the phenotype strength. The above ranges may not realistically represent more moderate screening positives which may still be biologically meaningful, e.g., RNAi screens where the signal-to-background ratio is lower than that of small-molecule screens (6).
 - The use of sample means and standard deviation. Statistically, this condition assumes that the negative and positive control values follow a normal (i.e., Gaussian) distribution. The presence of outliers or asymmetry in the distributions can violate this constraint. Such is often the case for cell-based assays but is rarely verified, and can yield a misleading Z' -factor. Conversely, attempting to correct for this by transforming the response values to yield a normal distribution (e.g., log scaling) may yield an artificially high Z' -factor (7).
 - The sample mean and sample standard deviation are often not robust estimators of the distribution mean and standard deviation. In the presence of outliers, these statistics can easily lead to an inaccurate measure of control distribution separation.

One-tailed Z' factor: This measure is a variant of the Z' -factor formulation which is more robust against skewed population distributions. In such cases, long tails opposite to the mid-range point lead to a high standard deviation for either population, which results in a low Z' factor even though the population means and samples between the means may be well-separated (unpublished work).

- **Definition:** This statistic has the same formulation as the Z' -factor, with the difference that only those samples that lie between the positive/negative control population medians are used to calculate the standard deviations.
- **Range and interpretation:** Same as that for the Z' -factor.
- **Advantages**
 - Attempts to overcome the Gaussian limitation of the original Z' -factor formulation.
 - Informative for populations with moderate or high amounts of skewness.
- **Disadvantages**
 - Still subject to the scaling issues described above for the original Z' -factor formulation.
 - Not available as part of most analysis software packages.

V-factor: The V-factor is a generalization of the Z' -factor to a dose-response curve (8).

Definition: Calculated as either:

$$1 - 6 \frac{\sigma_{fit}}{|\sigma_p - \sigma_n|} \text{ where } \sigma_{fit} = \sqrt{\frac{1}{N} \sum_{i=0}^n (f_{exp} - f_{mod})^2}, \text{ i.e., the root-mean-square deviation of a logistic}$$

model to the response data, and σ_p and σ_n are defined as above; or

$$1 - 6 \frac{\text{mean}(\sigma)}{|\sigma_p - \sigma_n|} \text{ if no model is used, i.e., the average of several replicates where } \sigma \text{ are the standard deviations of the data.}$$

- *Range and interpretation:* Same as that for the Z'-factor.
- *Advantages*
 - Decreased susceptibility to saturation artifacts, which reduce the variability of the controls.
 - Taking the entire response curve into account makes the V-factor robust against dispensing errors (which typically occur towards the middle of the dose curve, rather than the extremes as for the positive/negative controls).
 - The V-factor formula has the same value as the Z'-factor if only two doses are considered.
- *Disadvantages:*
 - Requires dose response data, which requires many more samples than statistics relying solely on positive and negative controls.
 - Not available as part of most analysis software packages (CellProfiler is an exception).

Strictly standardized mean difference (SSMD, denoted as β): This measure was developed to address limitations in the Z' factor in experiments with control of moderate strength.

- *Definition:* The SSMD measures the strength of the difference between two controls, using the formulation is (9)(10):

$$\beta = \frac{\mu_n - \mu_p}{\sqrt{\sigma_n^2 + \sigma_p^2}} \text{ where } \mu_n, \sigma_n^2, \mu_p \text{ and } \sigma_p^2 \text{ are defined as above for the Z' factor.}$$

- *Range and interpretation:* Acceptable screening values for SSMD depend on the strength of the positive controls used, as described in the following table (11) (these threshold values assume that the positive control response is larger than that of the negative control; if the converse is true, the threshold values are negative and the inequality signs are reversed): (Table 2).

Zhang *et al* (10) make the following recommendations to choosing the appropriate criterion:

- In chemical compound assays (which typically have positive controls with strong/extremely strong effects), use criterion (4) or (3).
- For RNAi assays in which cell viability is the measured response, use criterion (4) for the empty control wells (i.e, wells with no cells added).
- If the difference is not normally distributed or highly skewed, use criterion (4).
- If only one positive control is present in the experiment, use criterion (3).
- For two positive controls, use criterion (3) for the stronger control and criterion (2) for the weaker control.
- *Advantages* (6)
 - Ease of calculation.
 - Accounts for the variability in the compared groups.
 - Accommodates the effect size of the controls, through the use of different thresholds.
 - Lack of dependence on sample size.
 - Linked to a rigorous probability interpretation.
- *Disadvantages* (6)
 - Not available as part of most analysis software packages (CellProfiler is an exception).
 - Not intuitive for many biologists.
 - The thresholds are based on a subjective classification of control strength.

Area under the receiver operating characteristic curve (AUC) (12):

- *Definition:* The receiver characteristic curve (ROC) is a graph showing the proportion of true and false positives given a range of possible thresholds between the positive and negative control distributions (see

figure). This pictorial information can be summarized as a single value by taking the area under the ROC curve (also known as the AUC) (Figure 2).

- *Range and interpretation:* The AUC can assume a value between 0 and 1. An assay which generates both true and false positives at random would result in a diagonal line between (0, 0) and (1, 1) on the ROC. For such a case, the AUC would equal 0.5. Therefore, a usable assay must therefore have an $AUC > 0.5$ (and ideally much higher) although no cutoff criteria has been agreed upon by the community (Table 3).

Given that most screens will require a false positive rate of less than 1%, the right side of the ROC is typically less relevant than the left-most region. An alternate metric is to calculate the AUC from only the left-most region (12).

- *Advantages (6)*
 - Allows for the viewing the dynamic range of the data given positive and negative controls.
 - Does not assume that control distributions are normal (i.e, Gaussian)
 - Multiple thresholds for defining positives and the resulting trade-offs between true positive and true negative detection can be evaluated simultaneously.
- *Disadvantages (6)*
 - Requires a large sample size for calculation. Ideally, many replicates of positive and negative controls are needed.
 - Some information is lost when the AUC is used exclusively rather than visual inspection of the complete ROC. For example, two classifiers under consideration may have the same AUC but one may do better than the other at different parts of the ROC graph (that is, their curves intersect at some point). In this case, the relative accuracy no longer becomes a measure of the global performance, and restricting the AUC calculation to only a portion of the ROC graph is recommended.
 - Not available as part of most analysis software packages (though GraphPad's [Prism](#) is a commonly-used exception).

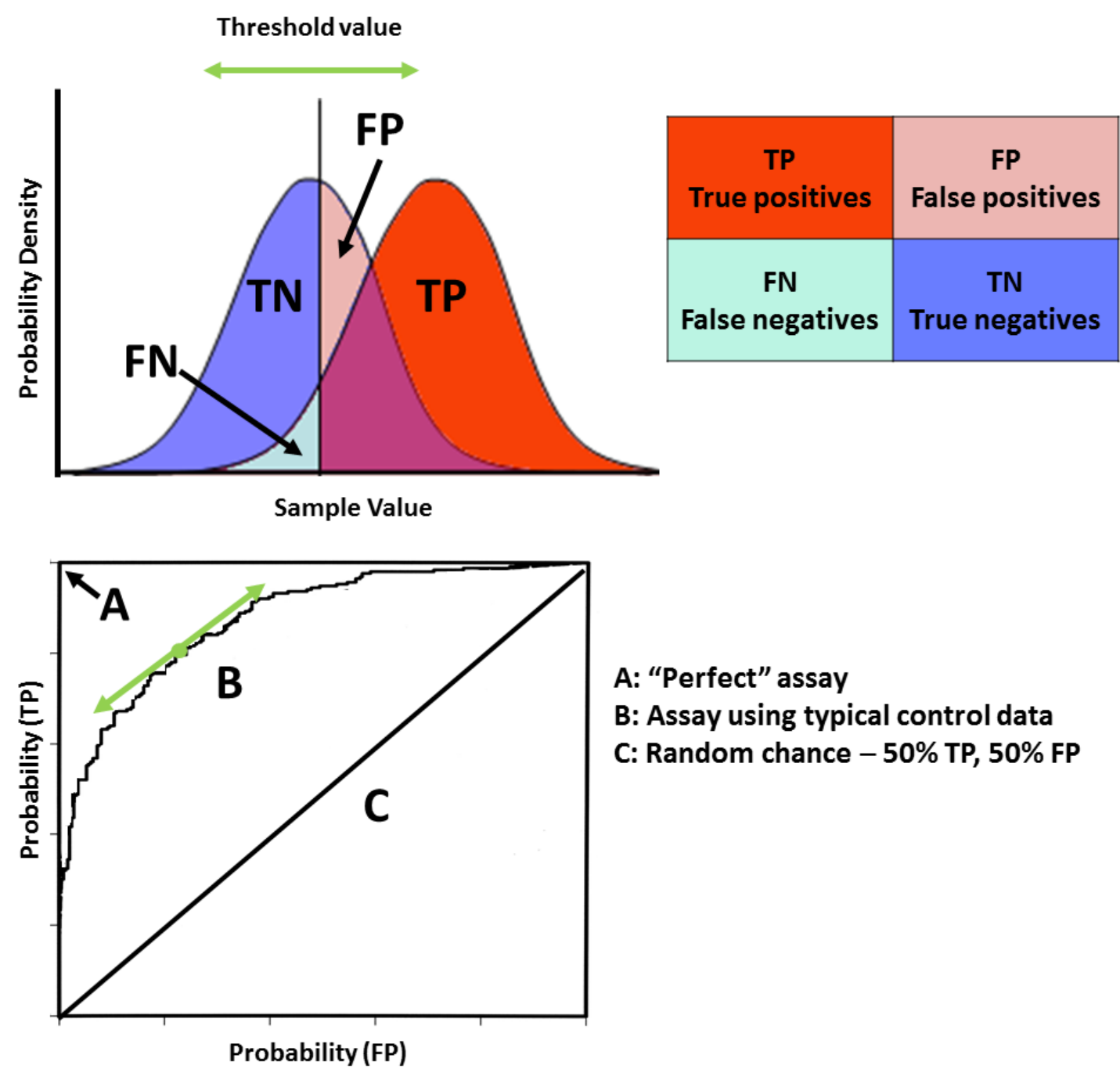


Figure 2: Details of a receiver characteristic curve. "Top:" Probability distributions of a hypothetical pair of control data. As a threshold value is varied, the proportions of actual and predicted positives and negatives drawn from the two distributions will also vary. "Bottom:" Three hypothetical ROC curves based on the table below. Adapted from (10).

Table 1: Interpretation of Z' values

Value	Interpretation
Z' = 1	An ideal assay
1 > Z' ≥ 0.5	Excellent: Good separation between the populations
0.5 > Z' ≥ 0	Marginal: Moderate to no separation of the distributions

Table 1 continued from previous page.

Value	Interpretation
$Z' = 0$	Nominal: Good only for a yes/no response
$Z' < 0$	Unacceptable: Screening is essentially impossible

Table 2: Interpretation of SSMD values (positive control response > negative control response)

Quality Type	1 Moderate Control	2 Strong Control	3 Very Strong Control	4 Extremely Strong Control
Excellent	$\beta \geq 2$	$\beta \geq 3$	$\beta \geq 5$	$\beta \geq 7$
Good	$2 > \beta \geq 1$	$3 > \beta \geq 2$	$5 > \beta \geq 3$	$7 > \beta \geq 5$
Inferior	$1 > \beta \geq 0.5$	$2 > \beta \geq 1$	$3 > \beta \geq 2$	$5 > \beta \geq 3$
Poor	$\beta < 0.5$	$\beta < 1$	$\beta < 2$	$\beta < 3$

Table 3: Interpretation of AUC values

Value	Interpretation
AUC = 1	A "perfect" assay.
AUC = 0.5	Poor: Performance is only as good as random chance.
AUC < 0.5	Worse than random chance.

3. Quality Control for HCS

Unlike most HTS assays, HCS data can be visually and sometimes automatically checked to identify and remove artifacts. In HCS assays, several fluorescent probes are often used simultaneously to stain cells, each labeling distinct cellular components in each sample. For screens in which the goal is to identify a small number of hits for a particular, known phenotype of interest, the candidate hits can often be screened by eye to eliminate false-positive artifacts. However, large screens and profiling experiments probing a broad spectrum of subtle morphological responses require more automated methods to detect and remove artifacts and systematic aberrations.

In general, the best approach to avoid imaging artifacts is to adjust the image acquisition settings to optimize the image quality (see the section "Capturing a Good Image" in the introductory [HCS chapter](#) of the AGM for more details). However, despite the screener's best efforts at acquisition and sample preparation, anomalies will still appear and end up polluting otherwise high-quality microscopy data.

Common artifacts that can confound image analysis algorithms are out-of-focus images, debris, image overexposure, and fluorophore saturation, among others. Because these anomalies affect a wide variety of intensity, morphological, and textural measurements in different ways, a single quality control (QC) metric that captures all types of artifacts, without also triggering on the unusual appearance of hits of interest, is not realistic. Instead, it is recommended to use either multiple metrics targeting the various artifacts that arise, or a supervised machine-learning approach.

3.1. Using targeted features for QC

3.1.1. Cell count as a quality control measure

Depending on the experimental context, a simple measure of quality is the calculated cell count. This metric can help identify problems with the following situations:

- **Per-image object segmentation:** If the screener has an idea of the typical number of cells in a given image, deviations from this range at the per-image level can be indicative of improper object segmentation. An unusually low apparent number may mean that neighboring cells are getting merged together or are absent due to cell death or incorrect cell plating, whereas a high apparent count may mean that cellular objects are being split apart incorrectly or an artifact such as compound precipitation is present.
- **Per-well heterogeneities:** Uneven distribution of cells have effects on cellular adhesion and morphology, and in multiwell plates, low cell counts may characterize the wells of the edge of the plate. Computation and display of per-well cell counts in a plate layout heatmap format can reveal the presence of such systemic artifacts, which can be corrected at the sample preparation stage (14).

3.1.2. Features for detecting out-of-focus images

Despite the use of autofocusing routines on automated microscopes, out-of-focus images are a common and confounding artifact in HCS. The rate of occurrence can depend on the cell type being examined and how adherent the cells are to the bottom of the well. Two measures are particularly useful in out-of-focus image detection (15):

- **Power log-log slope (PLLS):** This measure evaluates the slope of the power spectral density of the pixel intensities on a log-log scale; the power spectrum density shows the strength of the spatial frequency variations as a function of frequency. It is always negative, and decreases in value as blur increases and high-frequency image components are lost.
Typical in-focus values are in the range of $-1.5 \sim -2$; very negative values indicate a steep slope which means that the image is composed mostly of low spatial frequencies. It is recommended to plot the PLLS for a given channel as a histogram and examine outliers that are substantially less than -2 .
 - **Advantages:** Because the PLLS of natural images is relatively invariant, this metric is useful as an unbiased estimator of focus.
 - **Disadvantages:** The presence of bright artifacts (e.g, fluorescent debris) in an otherwise in-focus image can produce artificially low PLLS values (Figure 3).
- **Textural correlation:** This measure evaluates the dependency of each pixel on the grayscale value of its neighbors, for a given spatial scale. Given the proper spatial scale, this measure shows the separation between blurry/in-focus images: as the correlation of an image increases, the blurriness of the image also increases.
Of particular importance is the choice of spatial scale: a smaller spatial scale will pick up the blurring of smaller image features first, increasing the sensitivity. In general, it should be no larger than the diameter for a typical object (e.g, nucleus, speckle) in the channel of interest.
 - **Advantages:** Performance is generally insensitive to the amount of cell coverage.
 - **Disadvantages:** Dependence upon proper *a priori* selection of spatial scale. If the scale is too small, this metric starts reflecting the smaller in-focus image features rather than the amount of blur; too large a value, and it reflects the spatial proximity of similar cellular features.

The situation is more complicated if only a portion of the image is out-of-focus rather than the entire image (e.g., a section of a confluent cell cultures lifting off and "rolling up", causing a local change in the depth of field). In this case, the difference between such images and in-focus images will not be as distinct, but a more moderate shift in the metric values may still aid in establishing a reasonable cutoff.

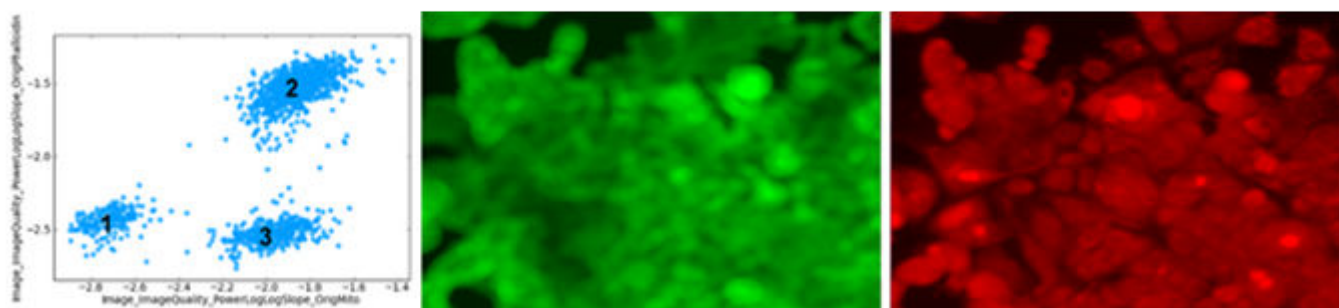


Figure 3: Illustration of PLLS performance for in-focus/out-of-focus MCF7 images. *Left panel:* Scatter plot of PLLS from the mitochondrial channel (x-axis) vs the phalloidin channel (y-axis) on a whole-image basis. If both channels were simultaneously blurred, we would expect that the measurements would cluster along a line. In this case, though, there are three clusters that are apparent: clusters 1 and 2 represent images in which both channels are blurred/in-focus, and cluster 3 where one channel is in focus but the other is not. An example image from cluster 3 is shown, both the blurry mitochondrial (center panel) and in-focus phalloidin channels (right panel).

3.1.3. Features for detecting images containing saturated fluorescent artifacts

Saturation artifacts are another common aberration encountered in HCS. Unusually bright regions in an image can be caused by debris contamination, aggregation of fluorescent dye, and/or inappropriately high exposure time or detector gain settings. Such regions can produce inaccurate intensity measurements and may impair cell identification even when such a region is small or not terribly bright.

The following metrics can be measured and examined to detect saturation artifacts:

- A useful measure is the percentage of the image that is occupied by saturated pixels. (Here, we define *saturation* as the maximum value in the image as opposed to the maximum bit-depth allowed by the image format.) In normal cases, only a small percentage of the image is at this value. Images with a high percentage value typically indicate either bright artifacts or saturated objects (e.g., non-artifactual dead cells). Further examination is required to determine which is the case, and whether the image is salvageable.
- The standard deviation of the pixel intensity is also useful for detection of images where a bright artifact is present but is not bright enough to cause saturation (Figure 4).

3.2. Using machine learning for QC

Machine learning provides a more intuitive way to train a computer to differentiate unusable from acceptable images on the basis of a sample set of each (the training set) and is particularly useful for detection of unforeseen aberrations. PhenoRipper is an open-source unsupervised machine-learning tool that can detect major classes of similar images and can be useful for artifact detection (16). The advantage of taking the machine learning approach is that it does not require a priori knowledge of the important features which identify the artifact(s). Disadvantages include the time required to create the training set and the expertise to run the analysis (17)

For more on machine learning applications, see the section "Machine learning for HCS" below.

4. Normalization of HCS data

In the process of performing an HCS screen, invariably small differences between samples, including replicates, appear despite the screener's best efforts at standardization of the experimental protocol. These include both systematic (i.e., stemming from test conditions and procedure) and random errors (i.e., stemming from noise). Sources of systematic error include (18):

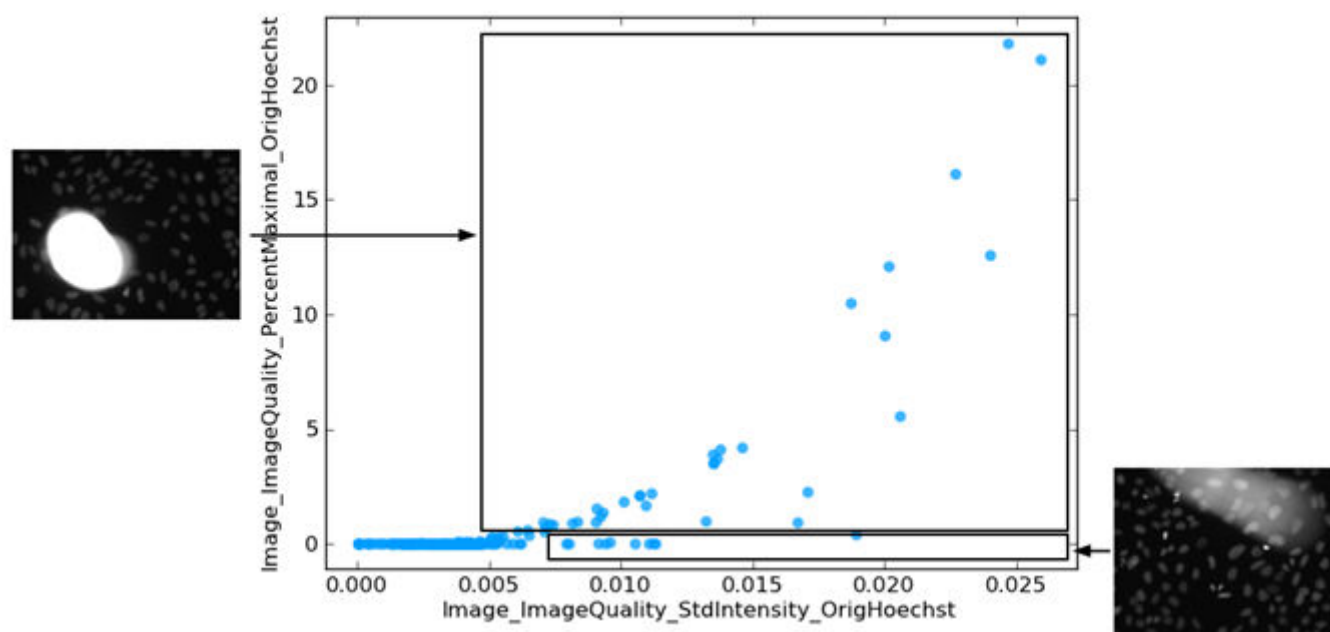


Figure 4: Illustration of performance of the percentage of pixels at maximum intensity and the standard deviation of the pixel intensities. The inset to the left shows an example image from the box at top where the percentage measure is high. The inset to the right shows an example image from the box at bottom where the image standard deviation is high but the percentage measure is low.

1. Errors caused by reagent aging, or changes to compound concentrations due to reagent evaporation or cell decay.
2. Anomalies in liquid handling, malfunction of pipettes, robotic failures and reader effects
3. Variation in incubation time and temperature differences, temporal drift while measuring multiple wells and/or multiple plates and reader effects, and lighting or air flow present over the course of the entire screen

The combination of these effects may generate repeatable local artifacts (e.g., border, row or column effects) and global drifts recognizable as consistent trends in the per-plate measurement means/medians which result in row and/or column measurements that systematically over- or underestimate expected results (19).

In addition, the cellular population context (e.g. cell density) has a profound influence of cell behavior and may account significantly to cell-to-cell variability in response to treatment (20); correcting for these effects involve sophisticated methods of modeling the cellular population response (21).

The overall impact of these variations depends upon the subtlety of the cellular response in question. For example, even if a positioned plate layout is used (rather than a random layout), a strong biological response may be sufficient to overcome any plate effects that may occur by virtue of a high signal-to-noise ratio.

If this is not the case, normalization is necessary to remove these systematic variations and allow the detection of variation originating from experimental treatments. The following results are ideal:

1. The feature ranges observed across different wells with the same treatment should be similar.
2. The feature distributions of the controls (whether positive or negative) should be similar.

Examples of software packages that include a variety of plate normalization techniques include GeneData Screener (<http://www.genedata.com>) and Bioconductor (22).

4.1. Inter-plate normalization

For screening purposes, ideally the control wells should be present on all plates. Negative controls should be present at minimum, but preferably both positive and negative, and subject to the criteria described in the "Controls" section above, with the expectation that the cellular response in the control wells is consistently similar between all samples. However, this is rarely the case; it is not uncommon for the means and standard deviations of the collected measurements to vary from plate to plate. Hence, *inter-plate normalization* is needed to reduce variability between samples across plates.

The choice of normalization method will depend on the particular assay. Methods of inter-plate normalization include the following (6)(2):

- **Fraction or percent of controls:** The most straightforward approach is division of each sample value by the mean of the (negative or positive) control measurement of interest. This approach requires a large number of controls for sufficient estimation of the mean (see "Replicates" above), and is appropriate in instances where the data is tightly distributed and close to normal. Because information about the sample variation is not included, this measure is sensitive to outliers in the controls. A more robust version of this calculation is to substitute the median for the mean (although the sample variation is still not taken into account).
- **Normalized percent inhibition:** When a reliable positive control is available, this approach is calculated

as $x'_i = \frac{H - x_i}{L - x_i} \times 100\%$ where x_i is the measured value, x'_i is the evaluated percentage, H is the

mean of the high controls, L is the mean of low controls. However, see the caveats with the use of positive controls in "Controls" section above.

- **Fraction or percent of samples:** When a high proportion of wells are expected to produce little to no response (e.g., many RNAi studies), the mean of all samples on a plate can be used for the percent of controls formula in lieu of a negative control. A more robust version of this calculation is to substitute the median for the mean, subject to the caveats above.
 - This approach is recommended if the assay lacks good negative controls that work effectively across all plates, and can provide more accurate measures due to the larger number of samples as compared to controls while reducing the need for large numbers of controls (6).
 - A variation on this approach that is unique to HCS is normalization of nuclei intensity measures by the DNA content using the mode of the DNA intensities, in cases where the large majority of the cells are in interphase in the cell cycle.
 - However, the assumption that the reagents have no biological effect should be confirmed for the particular assay. For example, it is not recommended where most of the wells have been chosen precisely because the response is differentially expressed or the overall response level between samples is changed. The following screens would violate this assumption:
 1. Confirmation screens in which phenotype-positive reagents are evaluated on the same assay plate.
 2. Primary screens targeting structurally or functionally related genes.
- **Z-score (or standard score) and robust z-score:** The z-score transforms the measurement population distribution on each plate to a common distribution with zero mean and unit variance. The formula is

$x'_i = \frac{x_i - \mu}{\sigma}$ where x_i is the measured value, x'_i is the normalized output value and μ and σ are the

mean and standard deviations, respectively. An advantage of this approach is the incorporation of the sample variation into the calculation. However, the method assumes a normal Gaussian distribution to the underlying data (which is often not the case in HCS) and is sensitive to outliers or otherwise non-

symmetric distributions. For an approach which is more robust against outliers, the robust z-score uses the median for the mean and the median absolute deviation (MAD) for the standard deviation.

- **Robust linear scaling:** In this method, distributions are normalized by mapping the 1st percentile to 0 and the 99th percentile to 1 (21). This approach does not make assumptions about similarity of the distribution shape. However, this approach does not guarantee that the distributions of the controls will be identical between plates.

4.2. Intra-plate normalization

On a given plate, systematic errors generate repeatable local artifacts and smooth global drifts. These artifacts often become more noticeable upon visualization of the measurements and it is helpful to display well values graphically in their spatial layout, such as through the use of positional boxplots, heat maps of assay measurements on a plate layout and trellis map overviews of heat maps across multiple plates (23) (Figure 5).

In general, it is highly recommended to prospectively avoid such artifacts through sample preparation optimization. For example, plate edge effects can be substantially mitigated by simply allowing newly cultured plates to incubate at room temperature for a period of time (14). Another approach is to simply avoid the edge effect by leaving the edge wells filled with liquid but unused for samples; special plates exist for this purpose (e.g., Aurora plates from Nexus Biosystems). However, in experiments that require a large number of samples to be processed, leaving the edge wells empty may not be practical in terms of cost.

4.2.1. Correction of systematic spatial effects

The following analytical approaches may be used if there is an observed positional effect:

1. **Global parametric fitting:** This approach fits a smooth function to the data based on the physical plate layout. The corresponding per-cell measurements at each position are then divided by the smoothed function. Care must be taken in selecting the function parameters. If the function is too smooth, it is then unable to accurately model the spatial variation due to systemic error; if the function is too rigid, it will over-fit the measurements and will not generalize to new data. Using splines to create the parametric surface is common.
2. **Local filtering:** Similar to the global parametric fitting approach, this method takes the physical plate layout into account. Assuming that the aberration is highly spatially localized, the measurements for each well are "denoised" using measures from adjacent wells, often the median calculated from a square neighborhood centered on the well to be normalized.
3. **B-Score:** This method locally corrects for systematic positional effects by iterative application of the Tukey median polish algorithm (19)(24). This approach is robust to outliers. However, it assumes that most samples in a plate have no biological effect (essentially using the entire plate as a negative control) and can produce artifacts if this assumption is violated.
4. **Model-based:** In either of the above cases, consideration must be given to whether all the samples on a plate can be used or just a subset. In a primary screen where the majority of the reagents can be assumed to have negligible or minor effect, the full set of samples can be used. In a confirmation screen, the spatial variation may be caused by samples that exhibit a moderate to large effect; hence, the representative samples should be drawn from the control wells. In this case, correction may be achieved using a diffusion model based on the control wells even though the location of the controls is often spatially constrained (25).

4.2.2. Illumination correction

The quantification of cellular fluorescence intensities and accurate segmentation of images is often hampered by variations in the illumination produced by the microscope optics and light sources. It is not uncommon for illumination to vary 1.5- to 2-fold across a single image when using standard microscope hardware.

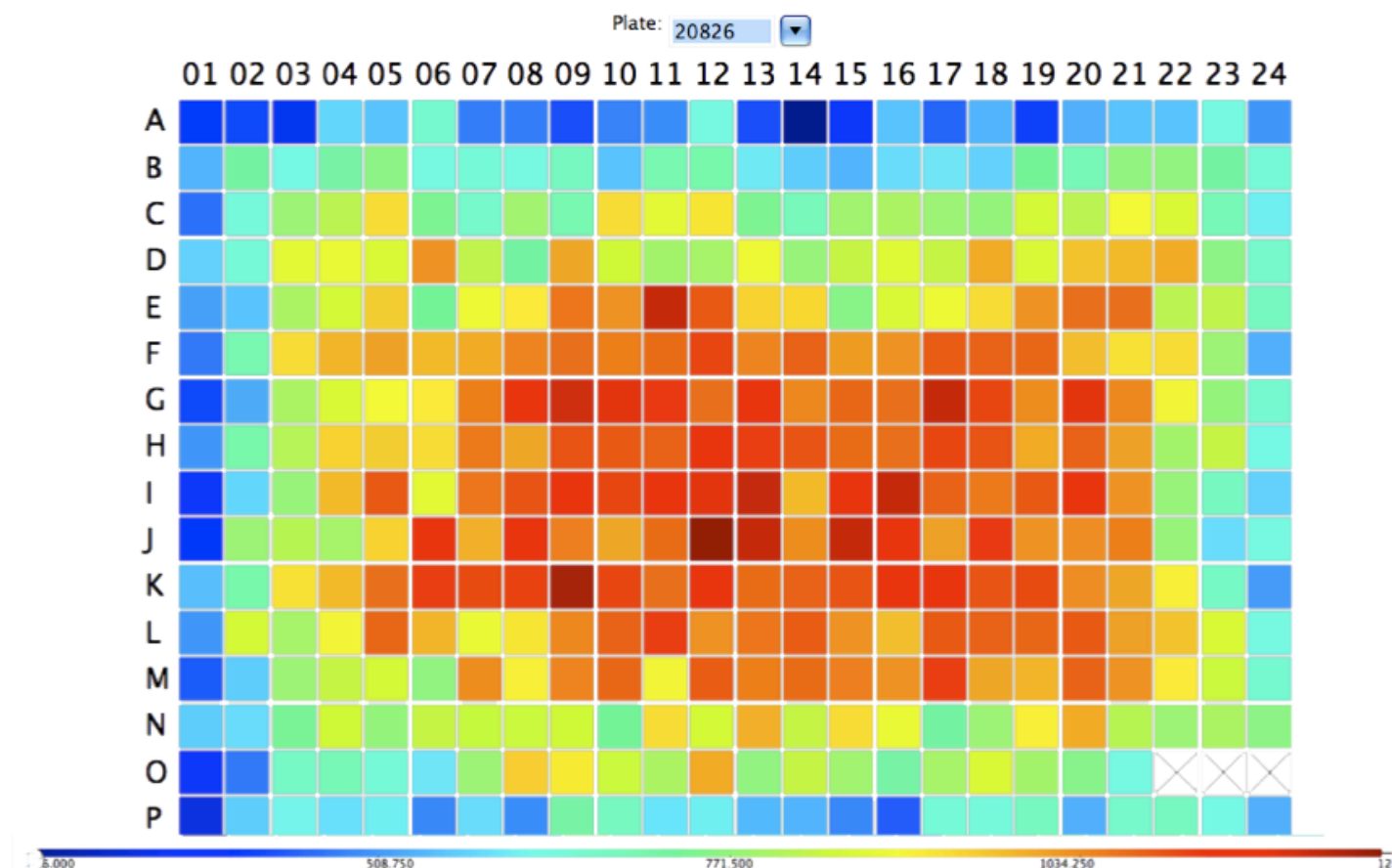


Figure 5: Heat map of values from a hypothetical 384-well plate containing edge effects.

Image acquisition software may mitigate these artifacts through the use of a reference image of a uniformly fluorescent sample (e.g. free fluorescent dye), which is then divided or subtracted from each collected image. This approach is described in "Image Optimization and Background Correction" section in the introductory [HCS chapter](#) and has the advantage of convenience and utility in cases where the illumination might change over time, e.g., light source aging. Disadvantages of this approach include:

- The underlying assumption is that the screener properly creates the appropriate referencing images and that conditions do not change between the acquisition of the reference images and the collection of the experimental images.
- Some software only allows one white reference image to be used to correct all wavelengths, which ignores differences in their respective optical paths and spectral characteristics.
- The fact that a uniformly fluorescent control is in a different chemical environment than a real sample is not taken into account.
- Typical methods do not account for different exposure times between the standard image and each collected image, although more sophisticated software allows for using a linear fit based on a series of images using a variety of exposure times for each wavelength.

A retrospective (i.e., post image acquisition) approach is an alternative (26). It bases the correction on all (or a sufficiently large subset of) the images from a plate for a given wavelength. This approach assumes that the actual cellular intensity distribution is distorted by a multiplicative non-uniform illumination function (additional sources of bias may also be considered if needed). This approach is applied to each wavelength condition because the spectral characteristics of the filters differ in addition to non-uniformities introduced by the

excitation lamp. Furthermore, it should be applied to each plate for a given image acquisition run unless it can be shown that observed patterns are consistent across plates. The methodology is as follows:

1. For all images of a given wavelength, smooth the image by applying a median filter. Because we do not want the small-scale cellular features to obscure the underlying large-scale illumination heterogeneity, the size of the filter should be large enough that any cells in the image are heavily blurred.
2. Estimate the illumination function by calculating the per-pixel average of all the smoothed images.
3. Rescale the illumination function so that the range is $(1, \infty)$ by dividing by the minimum pixel value, or more robustly, by the 2nd percentile pixel value (to avoid division-by-zero problems)
4. Obtain the corrected image by dividing the original image by the illumination correction function (Figure 6).

5. Measurement of image features

The quantitative extraction and measurement of features is performed by biological image analysis tools (e.g., CellProfiler (27), Fiji (28) and commercial software sold with HCS instruments). In addition to features that are straightforwardly related to the intended biological question, the extraction of additional features lends itself to serendipitous discoveries if mined correctly. A couple of examples illustrate this point:

- A phenotypic screen of 15 diverse morphologies in *Drosophila* cells revealed that cells with actin blebs and actin located in the periphery also tended to contain a 4N DNA content, a cell cycle relationship that would most likely not have been uncovered outside of an HCS context (29).
- Another study used a diffuse GFP reporter to look for clathrin-coated pit (plasma membrane) and intracellular vesicle formation. The researchers also took the opportunity to measure GFP signal representing translocation to the nucleus. Unexpectedly, such an effect was uncovered which was compound-specific and not previously described in literature (30).

Measured image features fall into the following types (31) (some of which are described under "Feature Extraction" in the introductory [HCS chapter](#)):

- **Counts:** The number of objects or sub-cellular objects per compartment (e.g., foci per cell in a DNA damage experiment). The number of objects per image may also be useful as a quality control statistic.
- **Size and shape:** Size is a descriptor of the pixel area occupied by a specific labelled compartment and includes such measures as area, perimeter and major axis length. Shape measures describe specific spatial features. Some examples include the aspect ratio (ratio of the height vs width of the smallest enclosing rectangle) as a measure of elongation, or compactness (ratio of the squared perimeter and the area). Zernike features (coefficients of a Zernike polynomial fit to a binary image of an object) are also useful as descriptors of shape.
- **Intensity:** The amount of a particular marker at a pixel position, assuming that the total intensity is proportional to the amount of substance labeled. The idea is that the presence or absence of the marker reflects a specific cellular state. For example, the total intensity of a DNA label in the nucleus is related to DNA content, which is useful for cell-cycle phase identification. Intensity measurements include the minimum, maximum, various aggregate statistics (sum, mean, median) as well as correlation coefficients of intensity between channels with different markers (useful for co-localization). If the target marker changes position, e.g. translocation from the nucleus to the cytoplasm, then the correlation coefficient of the stain between the two sub-compartments can provide a larger signal dynamic range than only measuring intensity in either the sub-compartment independently.
- **Texture:** A description of the spatial regularity or smoothness/coarseness of an object, which is useful for characterizing the finer patterns of localization. Textural features can be statistical (statistical properties of the grey levels of the points comprising a surface), structural (arrangements of regular patterns), or spectral (periodicity in the frequency domain).

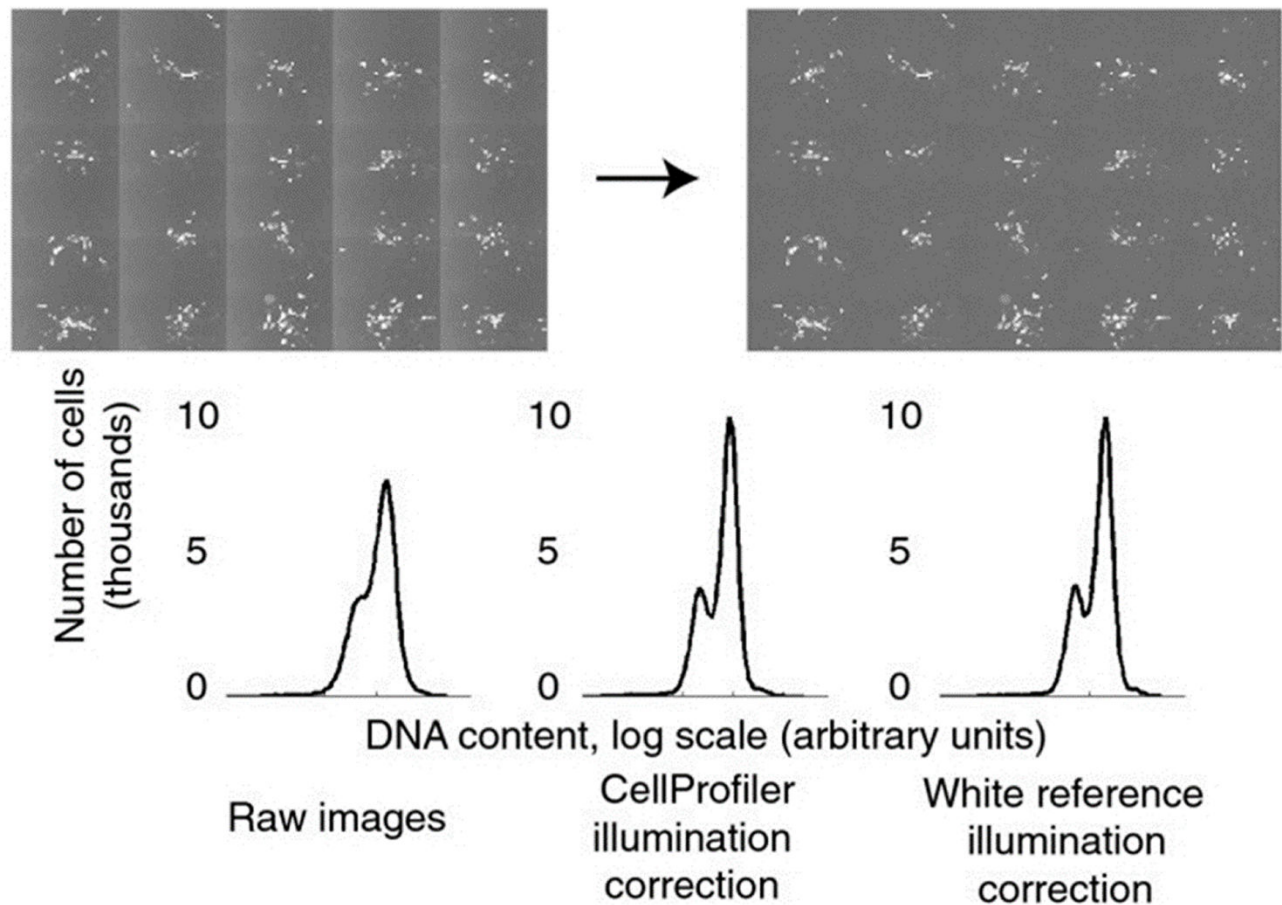


Figure 6: Top left: Example of uneven illumination from the left to the right within each field of view in a tiled grid of 5 x 4 images from a cell microarray. Top right: Correction of anomalies by CellProfiler. Bottom: Impact of anomalies and correction on *Drosophila* Kc167 DNA content data. Adapted from (27), copyright Carpenter et al.

- **Location:** The position of an object with respect to some other feature. Typically, the (x,y) location of an object within the image is not itself biologically relevant. However, relative positional features (e.g., absolute distance of foci from the border of an enclosing organelle) may be indicative of some physiological change.
- **Clustering:** A description of the spatial relationship of an object with respect to other objects. Examples of measures include the percentage of the perimeter touching a neighboring object and the number of neighbors per object.

It should be noted that while some of the suggested features are often difficult to use as direct readouts and are not biologically intuitive to interpret (e.g., texture and some shape features), they are often beneficial for machine learning approaches (see the section "Machine learning for HCS" below for more details). While spreadsheets are commonly used for storing cellular measurements, in order to contain the vast amount of per-cell information, across millions of cells, a database is a more feasible option for data storage and interrogation.

6. Machine learning for HCS

Generically, *machine learning* is defined as the use of algorithms capable of building a model from existing data as input and generalizing the model to make predictions about unknown data as output (32). The measurement of a large number of features lends itself to the use of machine-learning approaches for automated scoring of

samples, especially in cases where visual inspection or hand-annotation of images is time- and cost-prohibitive. In the context of HCS, machine-learning algorithms make predictions about images (or regions of images) based on prior training. We describe below two domains in HCS where machine-learning has proven useful: identifying regions of interest in images and scoring phenotypes (a brief discussion of machine-learning for quality control application is above).

6.1. Machine learning for image segmentation

Typically, the first step to an HCS workflow is the identification of the image foreground from the background, i.e., finding which pixels belong to each object of interest. For fluorescent images, often one of a number of thresholding algorithms is suitable for this purpose (33).

However, in cases where the pixel intensity of the foreground is not markedly different from that of the background (e.g., brightfield images), machine-learning approaches can be useful in classifying pixels as foreground and background based on other features, such as local intensity variation or texture. Because it is not trivial to choose *a priori* features that identify the foreground, a common approach is to extract a large number of image features, hand-select example foreground and background regions and then use machine learning to find combinations of features that identify the foreground class (or classes) of pixels from the background.

One open-source pixel classification tool is Ilastik (34). It classifies each pixel in an image by calculating sets of features that are linear combinations of the intensities of neighborhood pixels in order to identify textures and edges in the neighborhood of the pixel (35). The software then calculates a membership probability for each class based on these features using supervised machine learning based on hand-annotated pixels.

6.2. Machine learning for scoring phenotypes

Machine-learning can in theory be used to identify samples of interest based on features calculated from entire images. However, the actual application of this approach is rare in HCS, so we focus more on machine-learning based on per-cell features.

6.2.1. Feature extraction and normalization

Using one or two features for scoring phenotypes is a common approach, especially when the biological relevance of the features of interest are well-defined (17). However, hand-selecting the features necessary to distinguish phenotypes of interest versus negative controls is often intractable, especially if the phenotype is subtle, or simple linear combinations are insufficient. A machine learning approach lends itself well to this task, provided a sufficient variety of features are provided as "raw material." For more details on the types of features that can be extracted see the section "Measurement of image features" above. For complex phenotypes, the features that will contribute to the discriminating power of the classifiers will probably not be known in advance. Thus, in general, it is advisable to extract as many features as is practical and to use a machine-learning algorithm capable of choosing among them.

In many HCS experiments, the phenotype is dramatic enough that plate-to-plate variation and batch effects are the only confounding effects of concern and they can be removed adequately using the techniques mentioned in the prior section.

However, for more subtle phenotypes, the screener will need to model and remove the confounding effects more precisely in order to avoid obscuring the distinctions between phenotypes of interest.

For further details related to normalization, see the "Normalization" section above.

6.2.2. Supervised machine-learning for identification of particular phenotype sub-populations

For rare phenotypes that are nonetheless recognizable by eye, a researcher can generate a classifier to recognize cells with the phenotype of interest. Software packages that perform this task are Definiens Cellenger (36) and CellProfiler Analyst (29). Here we describe the workflow for CellProfiler Analyst, which is open-source (available at <http://www.cellprofiler.org>).

6.2.2.1. Creating a classifier

In CellProfiler Analyst, an interactive training session with iterative feedback is used to create a classifier for supervised machine learning as follows:

1. The software presents cells to the researcher for sorting, either selected randomly from the assay or taken from a specific plate with positive or negative controls. The screener manually sorts these into phenotypic "bins" to create a *training set*. Preferably, the screener will sort clear examples of the phenotype(s) in question; cells with an uncertain phenotype can be ignored, while keeping in mind that all cells will eventually be scored by the computer. Here, we refer to cells showing a phenotype of interest as "positives" (Figure 7).

Additional bins can be added, but as few bins as necessary should be used for the relevant downstream analysis as adding too many bins can decrease the overall accuracy.

If uncertain about the classification of a particular object, it can be ignored or removed from the list of objects under consideration. However, keep in mind that the final scoring will ultimately assign **all** objects to a class.

A note of caution: Sampling of a phenotype from only the control samples can lead to "overfitting," a scenario in which the machine-learning algorithm preferentially learns features which are irrelevant to the phenotype itself (e.g., spatial plate effects), leading to a classifier which does not generalize well and has poor predictive performance. It is thus preferred to select individual cells from a variety of images in the experiment.

2. After enough initial examples are acquired for the training set (typically, a few dozen or so), the screener then requests the computer to generate a tentative classifier based on the sorted cells. The screener sets the number of rules for distinguishing the cells in each of the classification bins. Here, we define a *rule* as a feature and cutoff representing a decision about the cell. It is recommended to use a smaller number of rules (e.g., five) at the early stages of defining a training set in order to accumulate cells spanning the full range of the phenotype and avoid training for a too-narrow definition of the phenotype (Figure 8).
3. At this point, the goal is to refine the rules by adding more cells to the training set. The screener then requests cells that the classifier deems as belonging to a particular phenotype.
4. The screener refines the training set, correcting errors by moving misclassified cells to the correct bin and re-training the classifier by generating a new set of rules (Figure 9).
5. By repeating the two steps above, the classifier becomes more accurate. If needed, the number of rules should be increased to improve accuracy (see below). At this point, the screener should save the training set for future refinement, to re-generate scores and for experimental records. It is advisable to do so before proceeding to scoring the entire experiment because scoring may take a long time for large screens.
6. When the accuracy of the classifier is sufficient, the screener can then scores all cells in the experiment so that the number of positive cells in each sample can be calculated (Figure 10).

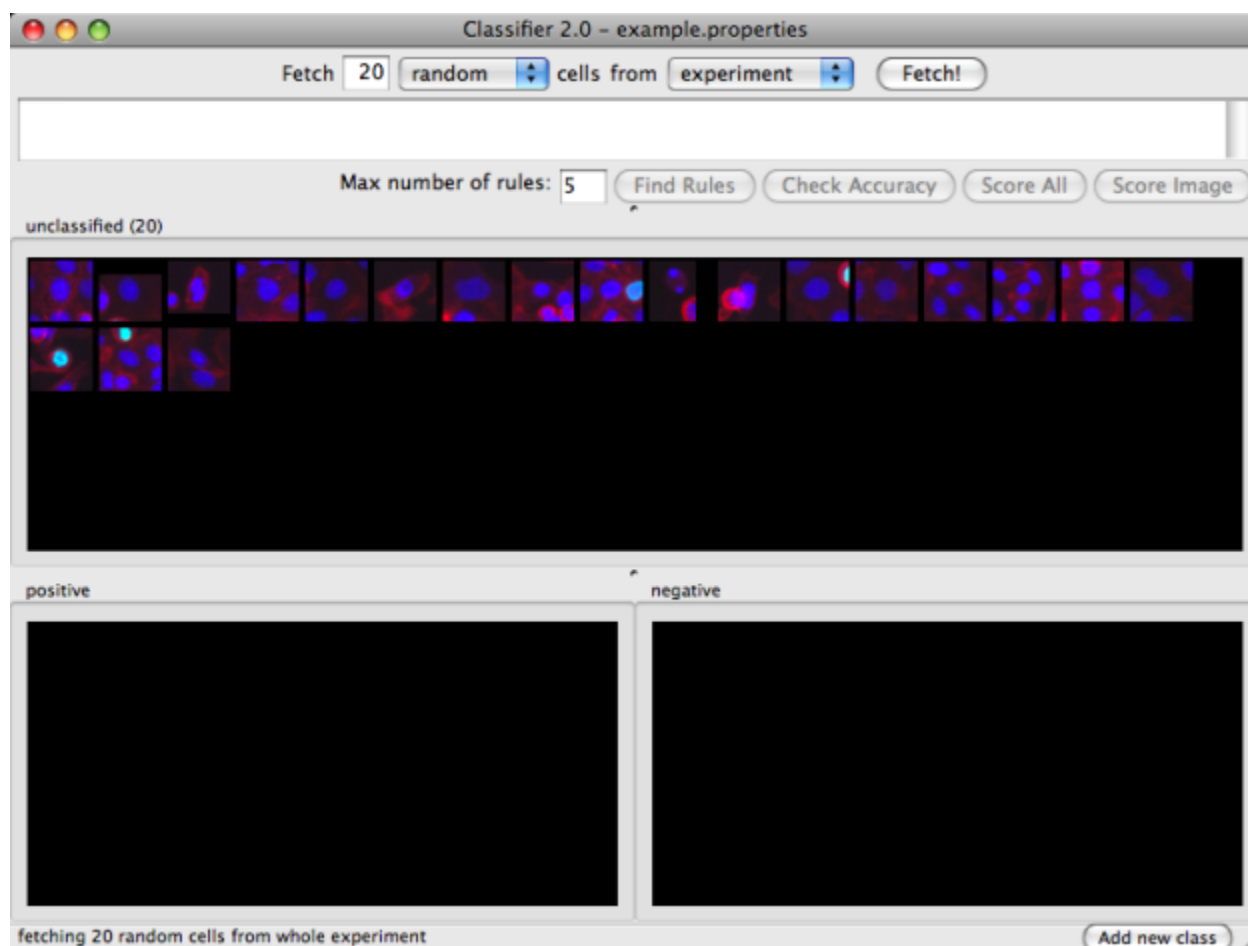


Figure 7: Twenty unclassified cells are presented to the screener for initial sorting using the CellProfiler Analyst phenotype classification tool.

6.2.2.2. Obtaining rules during the training phase and assessing classifier accuracy

When the first pass at sorting sample cells is finished, the maximum number of rules allowed needs to be specified prior to generating the initial set of rules.

During the initial training step, it is best to use a small number of rules (typically 5 to 10) in order to avoid defining the phenotype too narrowly. Doing so will help insure identification of the minimal set of features covering the wide range of object characteristics represented in the training set.

As training proceeds, if the number of misclassifications does not improve, the number of rules may be increased to allow the machine-learning algorithm to capture more subtle distinctions between phenotypes. However, using more rules does not always result in greater accuracy. In particular, increasing the number of rules above 100 is unlikely to improve classification accuracy and is computationally expensive to calculate.

Based on prior experience with 14 phenotypes in human cells, an upper limit of 50 rules is recommended for complex object classes (that is, to the human eye, one that involves the assessment of many features of the objects simultaneously) (29).

The most accurate way to gauge the performance of a classifier is to fetch a large number of objects of a given phenotype from the whole experiment. The fraction of the retrieved objects correctly matching the requested phenotype indicates the classifier's general performance. For example, if a screener fetches 100 positive objects but find upon inspection that 5 of the retrieved objects are not positives, then the classifier is estimated to have a

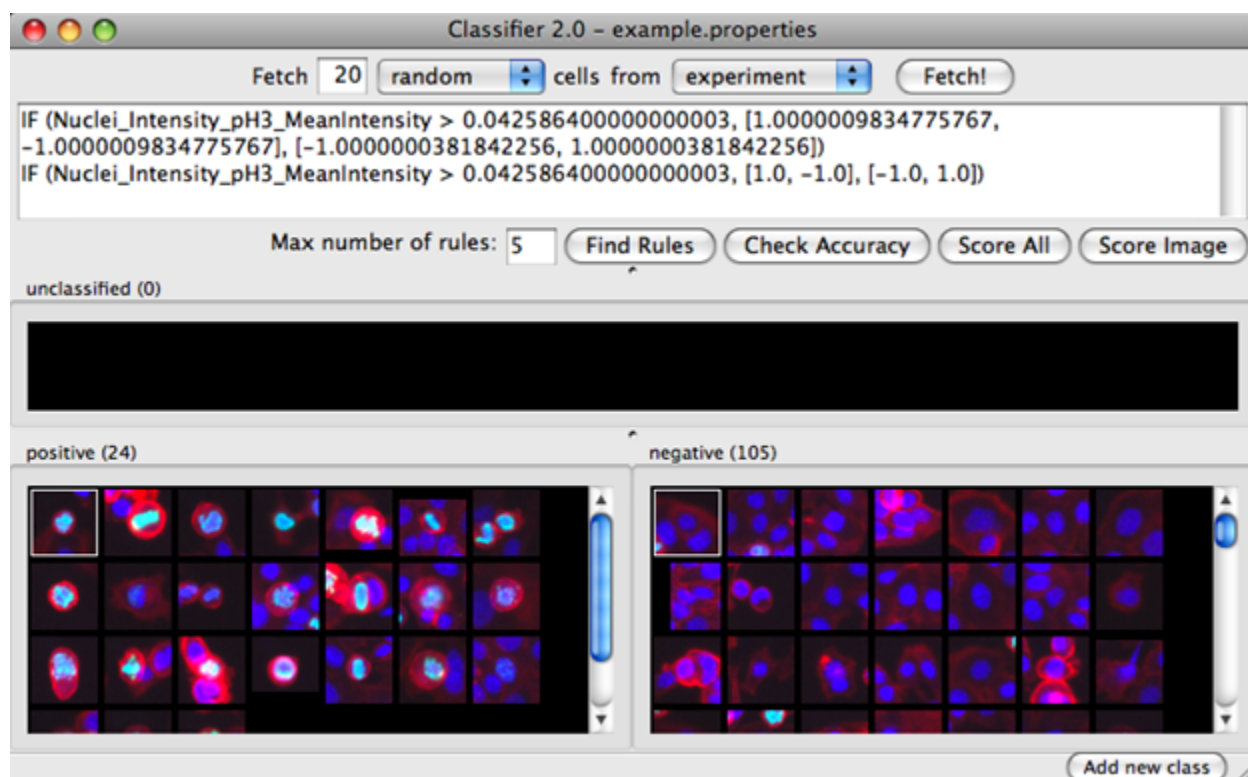


Figure 8: The set of rules after initial sorting using the CellProfiler Analyst classifier tool. In this example, only 2 rules were found out of the specified maximum of 5, both pertaining to the mean pH3 intensity of the nuclei channel, indicating that this feature was sufficient to achieve perfect classification on the training set.

positive predictive value of 95% on individual cells. Note that the classifiers' ability to detect positives and negatives must be interpreted in the context of the actual prevalence of individual phenotypes, which may be difficult to assess a priori. For studies or screens where the data is gathered over time, re-training the classifiers on the larger data set can increase their robustness.

Cross-validation is a standard method for estimating classifier accuracy, with important caveats discussed below. One version of this approach is to use a sub-sample of the training set for training a classifier and then use the remainder of the training set for testing. The optimal number of rules may be assessed by plotting the cross-validation accuracy for the training set as an increasing number of rules are used, where values closer to 1 indicate better performance. Two features of the plot are useful for guiding further classification:

1. If the accuracy increases (that is, slopes upward) at larger numbers of rules, adding more rules is likely to help improve the classifier (if the line slopes downward, this may indicate more training examples are needed).
2. If the accuracy is displayed for two sub-sampling percentages (say, 50% and 95% of the examples are used for training), and the two curves are essentially the same, adding more cells to the training set is unlikely to improve performance.

A note of caution: The accuracy in these plots should not be interpreted as the actual accuracy for the overall experiment. These plots tend to be pessimistic, as the training set usually includes a disproportionate number of difficult-to-classify examples (Figure 11).

The relationship between accuracy on individual cells versus accuracy for scoring wells for follow-up is complicated, because false positives and false negatives are often not evenly distributed across wells in an

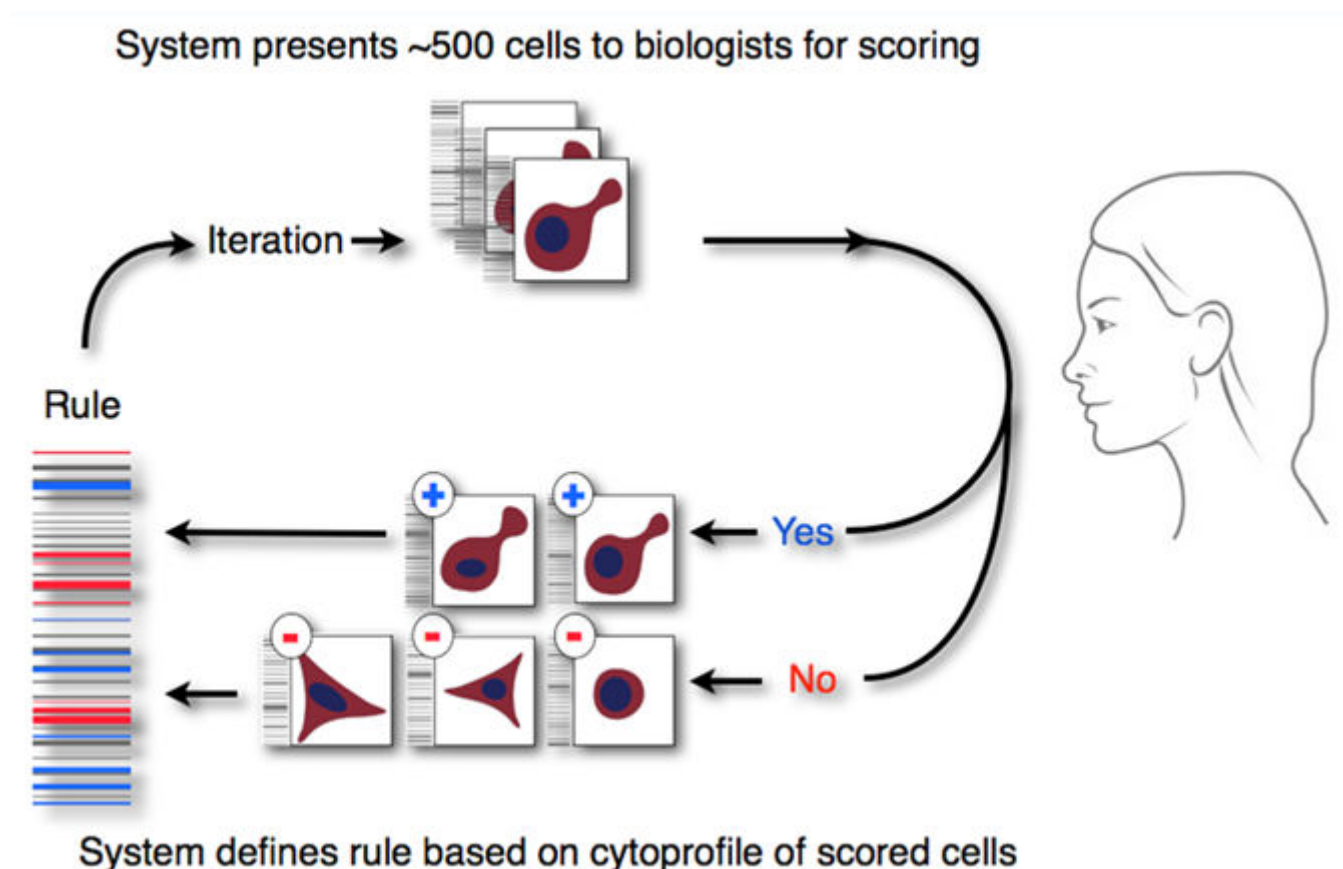


Figure 9: Illustration of the iterative machine learning workflow. Adapted from (29), copyright The National Academy of Sciences of the USA.

experiment. In practice, improving accuracy on individual cells leads to better accuracy on wells, and in general, the actual goal is per-well accuracy more than per-cell accuracy.

7. Whole-organism HCS

For those experiments in which the molecular mechanisms in question cannot yet be reduced to biochemical or cell-based assays, screeners can search for chemical or genetic regulators of biological processes in whole model organisms rather than isolated cells or proteins. The advantages of performing HCS in an intact, physiological system include the increase in likelihood that the findings from such experiments accurately translate into the context of the human body (e.g., in terms of toxicity and bioavailability), simplification of the path to clinical trials, and reduction of the failure of potential therapeutics at later stages of testing.

While a number of small animals are amenable to whole-organism HCS (e.g., zebrafish embryos, *Drosophila* fruit fly larvae, etc), this section of the chapter will focus on novel HCS techniques developed for the *Caenorhabditis elegans* roundworm.

7.1. *C. elegans* HCS

The nematode *C. elegans* is an increasingly popular choice for enabling HCS in whole organisms due to the following advantages:

- Manually-analyzed RNAi and chemical screens are well-proven in this organism, with dozens completed (37).

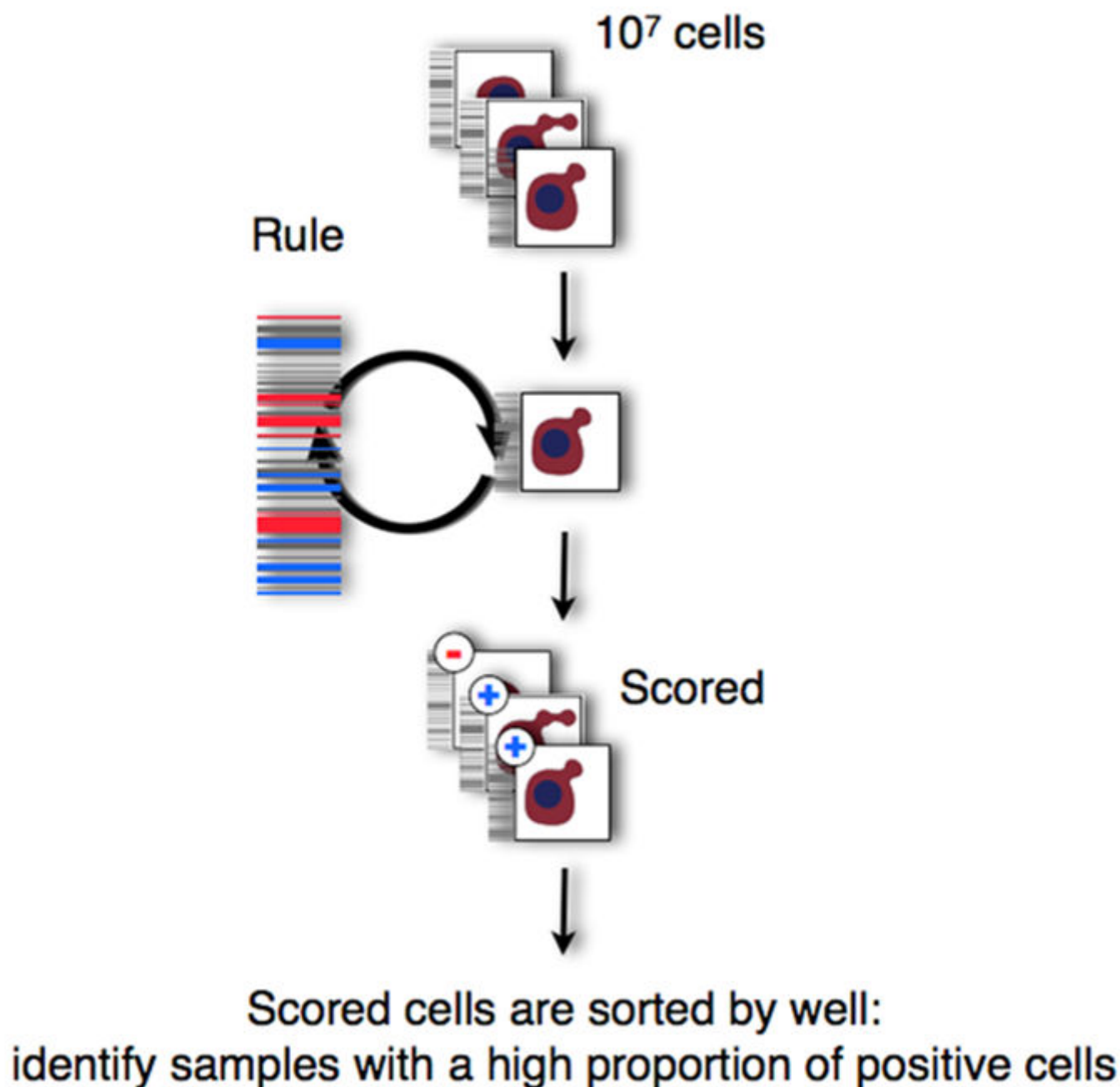


Figure 10: Illustration of the final cell scoring workflow. Adapted from (29), copyright The National Academy of Sciences of the USA.

- Many existing assays can be adapted to HCS; instrumentation exists to handle and culture *C. elegans* in HTS-compatible multi-well plates.
- Its organ systems have high physiologic similarity and genetic conservation with humans.
- *C. elegans* is particularly suited to assays involving visual phenotypes: physiologic abnormalities and fluorescent markers are easily observed because the worm is mostly transparent.
- The worms follow a stereotypic development pattern that yields identically-appearing adults, such that deviations from wild-type are more readily apparent.

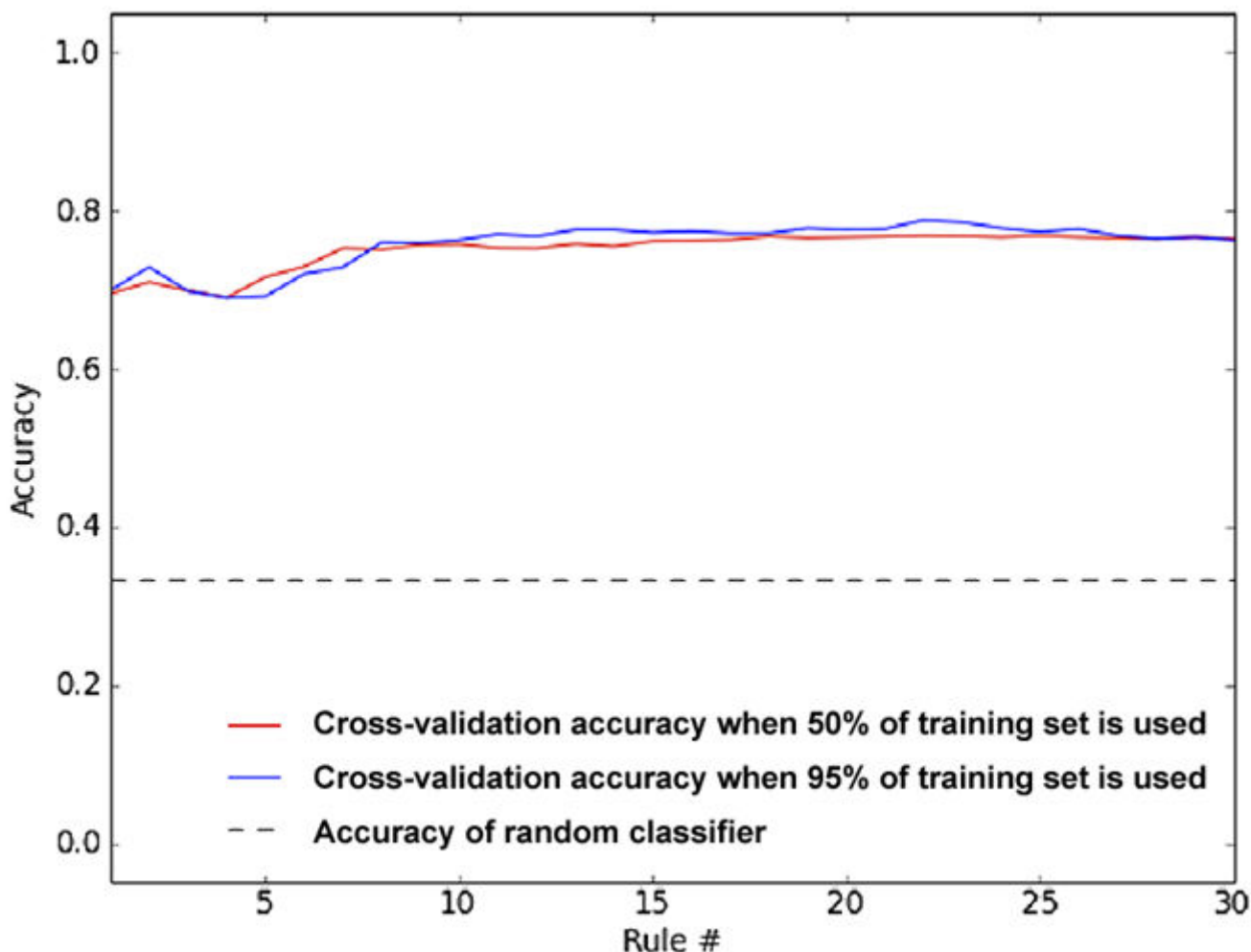


Figure 11: Plot displaying the cross-validation accuracy of a 3-class classifier with 30 rules. Note that the accuracy does not increase for more than 10 rules.

Microscopy imaging and flow cytometry are the primary HCS methods for *C. elegans*, as plate readers do not offer per-worm or morphological readouts and often cannot measure bulk fluorescence from worm samples due to their spatial heterogeneity within the well.

- **Flow cytometers:** Systems such as the COPAS Biosort (Union Biometrica) can be used for the automatic sorting of various "large" objects, including *C. elegans*, using the object size and intensity of fluorescent markers. Such a system is capable of differentiating some phenotypes using fluorescent intensity changes as the readout (e.g., isolating of mutants with reduced RFP-to-GFP intensity ratios as compared to wild-type worms (38) or signature extraction based on GFP intensity profiles created along the length of the worm (39)). One limitation of this approach is low spatial resolution. Another disadvantage is that retrieval of worms from the multi-well plates typically used in screening becomes a rate-limiting step, reducing the throughput.
- **Automated microscopy:** Defining worm phenotypes in HCS has also been enabled through the use of image data. Standard 6- or 12-well assays can be miniaturized to 96- or 384 well plates by dispensing a precise number of worms within a specified size/age range into the desired number of multiwell plates (using a COPAS sorter, for example). The worms are typically transferred from agar to liquid media to minimize imaging artifacts. A paralytic drug may be added to slow worm movement, minimizing

misalignment between subsequently imaged channels. Alternately, microfluidics may also be used to stabilize worm position (40).

Below is an HCS image analysis workflow tailored for worms dispensed into multi-well plates (41), using brightfield images of each well, along with the corresponding images from additional fluorescence wavelengths:

1. **Well identification:** In order to restrict worm detection to the region of interest, delineate the well boundary within the brightfield image; the well interior is typically brighter than the well exterior, lending itself to simple thresholding. In order to avoid artifacts at the well edge, use morphological erosion to contract the well border by a few pixels.
2. **Illumination correction and masking of pixel intensities:** Often the brightfield image will exhibit illumination heterogeneities which must be corrected prior to worm detection (see the "Illumination_correction" section above). Calculate an illumination correction function (ICF) from the brightfield image and then correct the image by dividing by the ICF. It is recommended to mask the image using the eroded well image and use the result for creating the ICF, otherwise the ICF will be distorted by the sharp features at the well edge. At this point, the illumination-corrected image is then masked with the eroded well image in order to restrict worm identification to the well area.
3. **Worm foreground identification:** Identify the worms as the image foreground using image thresholding on the brightfield image. Because the brightfield images are usually high-contrast, an automatic thresholding method such as Otsu is typically effective here. It is helpful to impose size criteria in order to remove objects that are likely to be spurious, e.g., debris, embryos, and other artifacts.
4. **Make population-averaged measurements if desired:** At this point, quantification of the additional fluorescent markers within the worm regions can be performed. For example, if a viability stain (e.g., SYTOX Orange) was used as part of a live/dead assay, image segmentation (i.e. partitioning the foreground pixels into individual worms) is not necessary, and the workflow would continue by identifying the SYTOX-positive pixels from the fluorescent image using automatic thresholding, measuring the total pixel area occupied by the worm foreground and the SYTOX foreground, and calculating the ratio of the SYTOX foreground total area and the worm foreground area to yield the final per-well readout of worm death.
5. **Make per-worm measurements if desired:** For some assays, it is preferable to identify individual animals rather than a whole-well readout (e.g., pathogen screens). While non-touching worms can usually be delineated in brightfield images based on the differences in intensities between foreground and background, image intensity alone is not sufficient for touching and overlapping worms. For these assays, algorithms are required that separate touching and overlapping worms. Moreover, edges and intensity variations within the worms often mislead conventional segmentation algorithms. Here, we describe a recent algorithm that employs a probabilistic shape model using intrinsic geometrical properties of the worms (such as length, width profile, and limited variability in posture) as part of an automated segmentation method (distributed as a toolbox in CellProfiler) (42) (Figure 12)
 - a. **Identify worms as described in the above workflow:** in this case, however, only the brightfield images are acquired from each well
 - b. **Construct a worm "model":** Once the worm foreground is obtained, construct a model of the variations in worm morphology by creating a training set of non-touching representative worms. This is done by saving binary images of a number of non-touching worms and using the "Training" mode of the UntangleWorms module in the CellProfiler worm toolbox.
 - c. **Apply the worm model to worm clusters:** Once the model is created, apply the model on the images using the "Untangle" mode of the UntangleWorms module. The result of this operation will be identify the individual worms from the worm clusters as well as exclude artifacts such as debris, embryos, etc.
 - d. **Quantify fluorescent markers:** Measure the various features available for each worm, such as morphology, intensity, texture, etc. The delineated worms can also be mapped to a common atlas

(using StraightenWorms) so that spatial distribution of staining patterns may be quantified (Figure 12).

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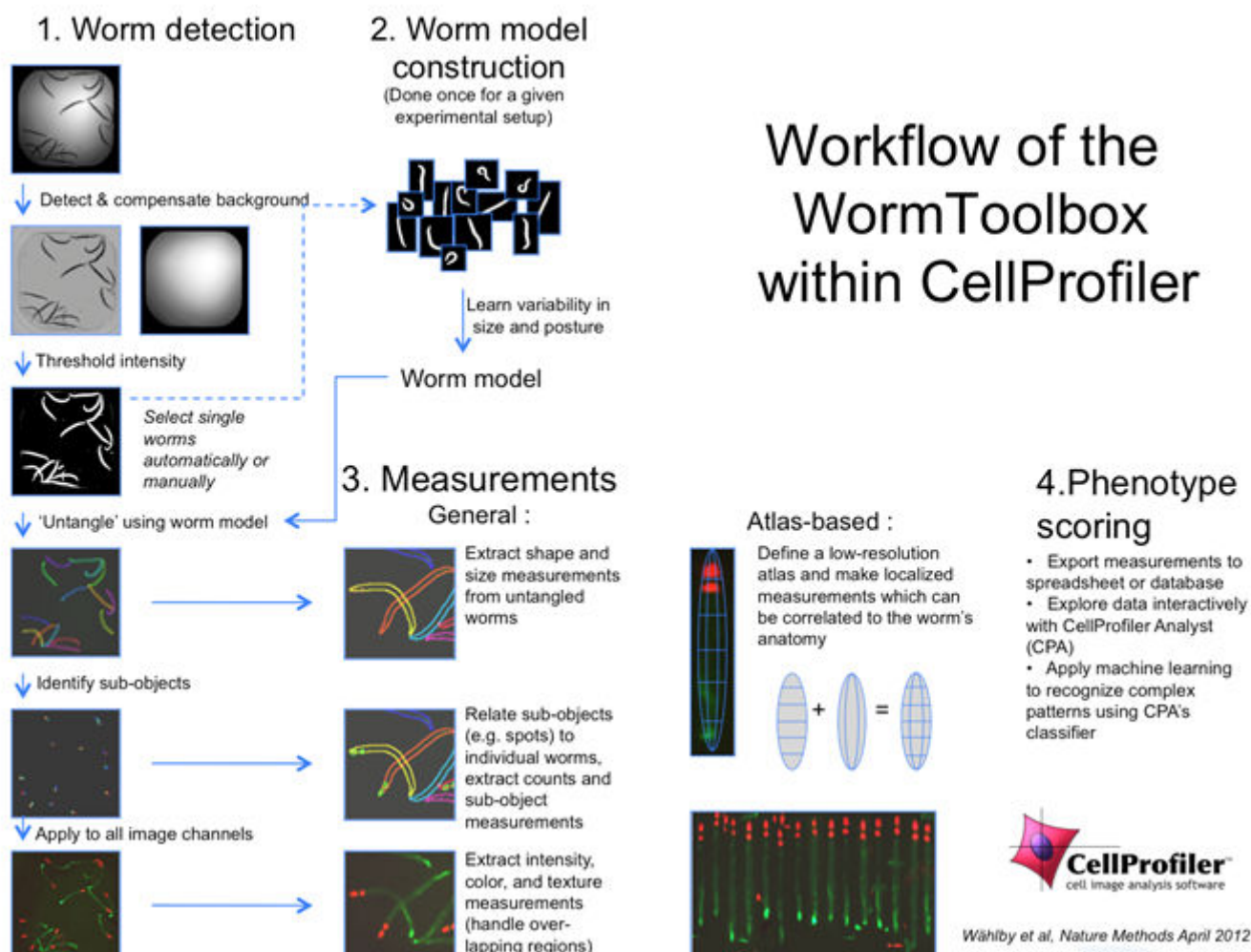


Figure 12: Workflow of the WormToolbox in CellProfiler. Adapted from (42), contributed by Carolina Wählby.

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Nuclear Factor Kappa B (NF- κ B) Translocation Assay Development and Validation for High Content Screening

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Abstract

In this assay study design, activation through stimulation or inhibition of the Nuclear Factor κ B (NF- κ B) pathway demonstrates translocation of NF- κ B protein from the cytoplasm to nucleus as measured using automated fluorescent microscopy computer-assisted image analysis technology better known as high content screening (HCS), High Content Analysis (HCA), High Content Imaging (HCI), or Image Cytometry (IC). This approach offers a better understanding of novel drug targets by examining the sub-cellular spatial distribution of the target proteins and the effects of target perturbation on cellular processes. Cell imaging provides multi-probe detection and is advantageous over other methods in quantifying spatial measurements such as protein translocation or redistribution, receptor internalization, cell morphology, cell motility, cell cycle, cell signaling, and others. The limitation of traditional microscopy and image analysis has been a log jam of sample throughput, day-to-day variability, poor standardization and of the need for specialized personnel to operate and oversee instrumentation. Automated fluorescent microscopy systems offer one solution for studying cell function in a “high content screening” (HCS) mode that allows the capability of measuring multiple cellular characteristics in a non-biased fashion. This chapter details the assay development, optimization, and validation for running a compound screening campaign and includes descriptions of the cell model, stimuli to activate NF- κ B pathway, time course kinetics, effects of serum on assay window, validation of proinflammatory cytokines, reference control inhibitor compound optimization, and examples of nomenclatures used by many manufacturers of HCS instruments or software algorithms.

Introduction and background

There is still much that remains unknown within the biology of NF- κ B despite the fact that in the year 2011 NF- κ B celebrated its 25 year anniversary (1). NF- κ B, also referred to as the Rel family of proteins, was first described in 1986 as a protein which binds to specific decameric DNA sequences (ggg ACT TTC C) in mature B-cells nuclear fraction and the immunoglobulin kappa light chain enhancer of plasma cells (2). The subunits of NF- κ B or Rel family proteins are part of a structurally related family; currently five different proteins have been identified: p50, p52, p65, Rel-B and c-Rel. The structure of NF- κ B resembles a “butterfly” in appearance with DNA binding in a pocket between two domains (Figure 1) (3). All identifiable Rel proteins contain a conserved N-terminal region, called the Rel Homology Domain (RHD). The RHD contains the DNA-binding and dimerization domains and the nuclear localization signal of the Rel proteins. p50 (also called NF- κ B B1) and p65 (RelA) were the first NF- κ B proteins to be identified. In 1988, I κ B was described as a specific inhibitor of the NF- κ B ability to translocate and turn on transcription factors (4). Seven known I κ B's have been identified: I κ B- α , I κ B- β , I κ B- γ , I κ B- ϵ , Bcl-3, p100 and p105. All known I κ B's contain ankyrin repeats (30-33aa) that mediate non-covalent interaction between I κ B and NF- κ B. NF- κ B p50 subunit crystal structure was first described by two groups (5, 6). NF- κ B has similarities with NF-AT family of proteins with both having interaction with members of FOS and Jun family of proteins (7). Activation of NF- κ B signaling is triggered in a series of steps through either the classical canonical pathway, the alternative non-canonical pathway, or the atypical I κ B independent pathway (8-11) (Figure 2). NF- κ B was initially discovered in B-cells, although B-cells do not express NF- κ B until

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stimulated by LPS or cytokines (12). NF- κ B is generally thought to be constitutively active and located in the cytoplasm in most cell types, until induced by a stimulus to migrate to the nucleus. Exceptions are cornel keratinocytes and vascular smooth muscle cells, which have been shown to have nuclear NF- κ B in the absence of activation (13). Following activation, translocation, and transcription of NF- κ B in the nucleus, re-accumulation of I κ B α in the cytoplasm is thought to occur within 60 minutes (14, 15). Furthermore, if I κ B α is re-synthesized it may enter the nucleus and then interacts with NF- κ B complexes and inhibit DNA binding (16). Dimerization of NF- κ B subunits results in the association of NF- κ B proteins with DNA. The C-terminal region of the Rel homology domain is where dimerization takes place. The nuclear localization sequence (NLS) that regulates transport of activated NF- κ B complexes into the nucleus is located near the end of the C-terminal end of the Rel homology domain. With the exception of Rel B, all Rel protein family members contain a phosphorylation site for PKA approximately 25aa to the N-terminal of the NLS (17).

Translocation of NF- κ B is a critical step in the coupling of extracellular stimuli to the transcriptional activation of specific target genes. NF- κ B is activated by pro-inflammatory cytokines (IL-1 α , IL-1 β , TNF- α); bacterial toxins (LPS, exotoxin B); viral products (HIV-1, HTLV-1, HBV, EBV, Herpes Simplex); and cell death stimuli (O₂-free radicals, UV light, γ -radiation) (17, 18). I κ B proteins are rapidly phosphorylated following induction by triggering cytokine receptors. I κ K- α and I κ K- β are both responsible for modification of I κ B complex. NF- κ B in response to activation of these cytokines is associated with triggering cellular defense genes (17). Upon cell stimulation, the nuclear localization signal on NF- κ B becomes exposed and the protein translocates to the nucleus, where it turns on transcription factors and induces specific gene expression.

The NF- κ B activation has direct screening applications for drug discovery for many pharmaceutical companies and in medicine for several therapeutic indications, most notably, inflammatory tissue injury, where NF- κ B controls the gene expression of a variety of pro-inflammatory mediators (11, 19, 20). In addition, NF- κ B regulates a number of genes that are involved in mediating tumorigenesis, metastasis, proliferation (21, 22) and apoptosis (23-26). Vascular cell adhesion molecule-1 (Vcam-1) found on endothelial cells following exposure to cytokines or LPS is involved in regulation of NF- κ B (2, 21), however, it requires an IRF-1 site that is located 3' to the TATA box (27). Other genes that regulate NF- κ B and are involved in the immune response and inflammation include peptide transporter (TAP-1), proteasome subunit LMP2 (28), MHC class II variant chain genes (29), and other unknown genes. Inducible nitric oxide synthase (iNOS) is regulated by NF- κ B activation, which results in an increase in nitric oxide production (30) and the inducible cyclooxygenase (COX-2), which generates prostanoids (31). Transcriptional regulation of p53, c-myc, and ras gene can be controlled by NF- κ B activation (32).

Principle of the Assay

NF- κ B is typically present and resides in the cytoplasm of most cells as a complex with members of I κ B inhibitor family of proteins. Both the size of this complex and I κ B masking of the nuclear localization sequence of NF- κ B prevents NF- κ B entry through the nuclear membrane. Once I κ B is phosphorylated and degrades, the nuclear localization sequences become assessable and NF- κ B is free to translocate to the nucleus (Figure 3). Microscopy images reveal strong evidence of the redistribution of NF- κ B from the cytoplasm to the nucleus following cytokine stimulation (Figure 4).

Assay Development Considerations

Image Analysis Algorithm to Detect NF- κ B Translocation

The basic principle to detect NF- κ B translocation is dependent on identifying the nucleus of a cell using one of many nucleic acid probe reporters, typically Hoechst, DAPI, or DRAQ5.

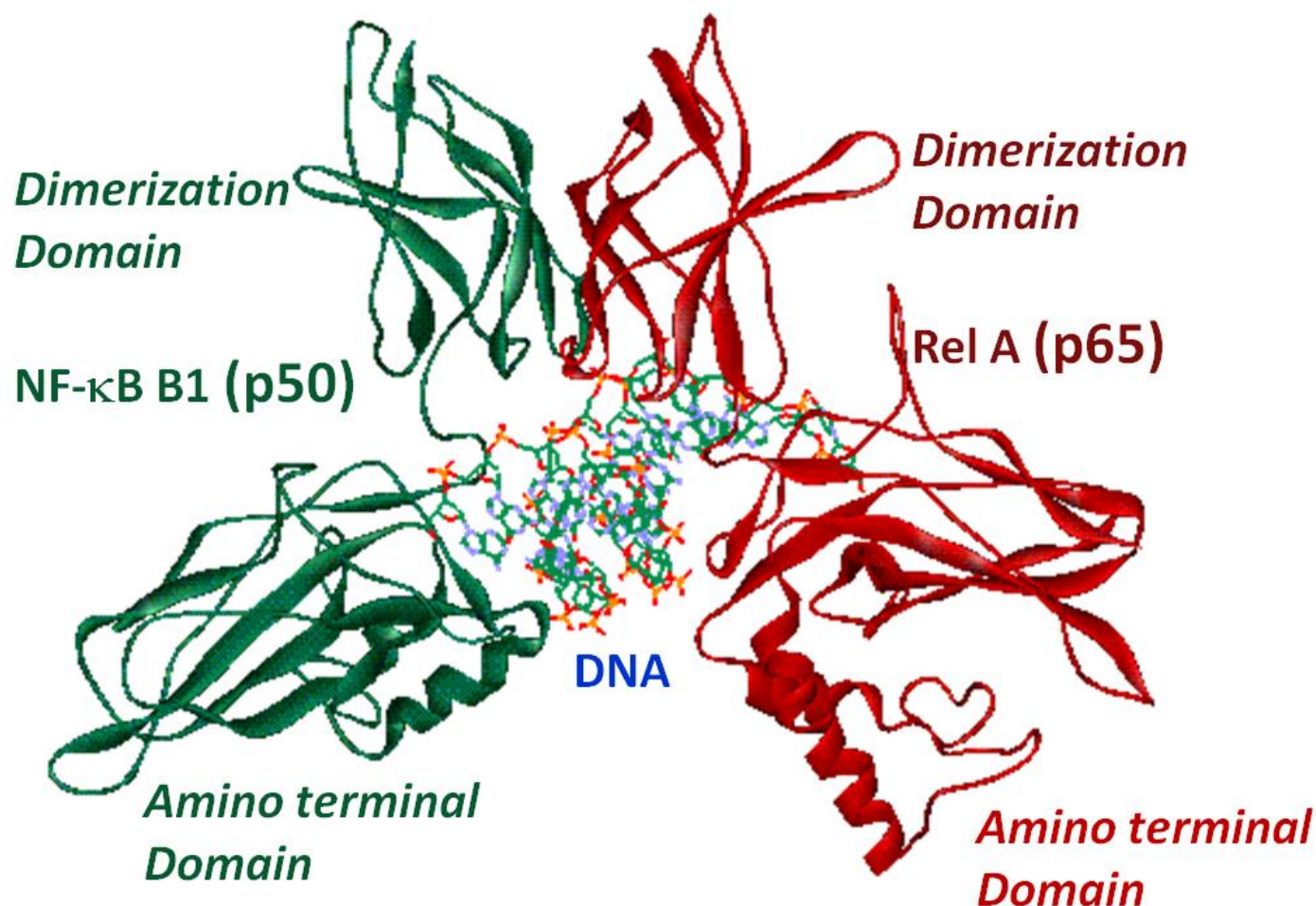


Figure 1: Structure of the NF- κ B/DNA complex. There are two subunits of NF- κ B, p50 (green) and p65 (red). The representation of the ribbon structure is viewed down the DNA helix axis with hydrophobic core Adapted from Chen F, et al (33).

Once the nucleus is identified using image analysis process it is masked, and then a secondary mask overlay is created to either encompass the entire cell boundaries or a subset area of the cytoplasmic area. Within this secondary masked area (nucleus and/or cytoplasm) labeled NF- κ B can then be quantified. Most available algorithms report two critical features; (A) NF- κ B intensity in the nucleus and (B) NF- κ B intensity in the cytoplasm. Once determined, a translocation value is calculated by measuring the average intensity of “difference” of the NF- κ B protein between the identified cytoplasmic region and nuclear region (Cyto-Nuc Difference). Additionally, a “ratio” of the average intensity of the nuclear region to average intensity of the cytoplasmic region can be calculated to represent this translocation value; Nuc/Cyt Ratio. Both the “difference” and “ratio” features reflect a biological phenotype of NF- κ B translocation. The data feature output from images is commonly reported as a well summary value but the calculation begins at the single cell level as shown in Figure 5. An example of well level data nomenclature describing these key features from different imaging platforms is showcased in Table 1. Representative images and algorithm overlays of HeLa cells in a dose response with IL-1 α are shown in Figure 6. Differences at the plate level (96 wells) in cells treated with stimuli (Max) or with DMSO or inhibitor compound (Min) are used to calculate Z-factor values (34) using CytoNuc Difference and Nuc/Cyt Ratio, highlighted in Table 2.

Additional, there are other valuable HCS output features that are useful including identifiable objects (cells), variability of intensity across area within the nucleus or cytoplasm, morphological measurements including the size, shape, length, aspects of individual cells or objects within a cell. For compound screening, the use of

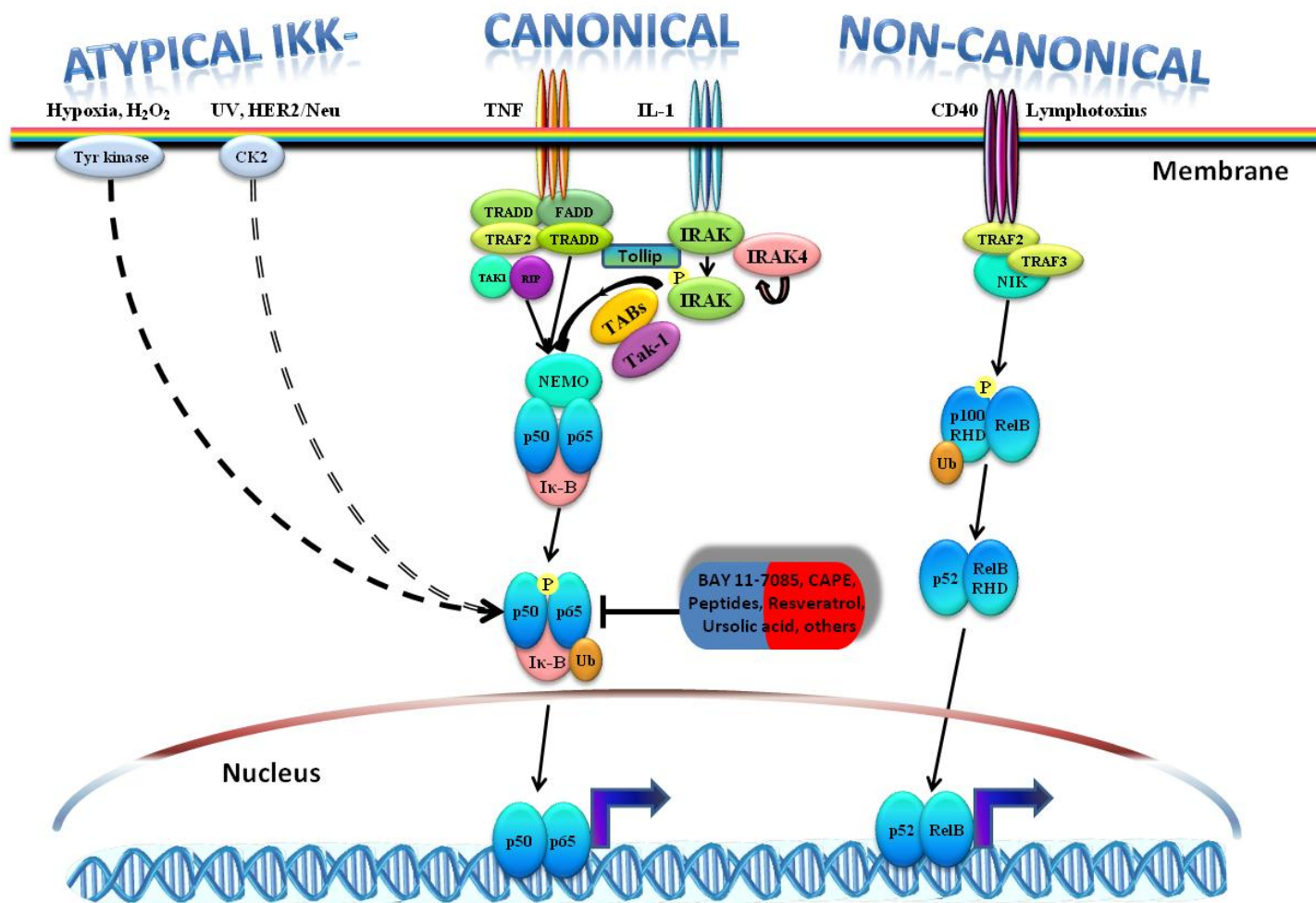


Figure 2: Activation of NF-κB Signaling Pathway. There are currently three recognizable pathways to activate NF-κB, the canonical, the non-canonical, and the atypical IκK independent pathways. NF-κB is naturally inhibited by IκB. Upstream activating signal (e.g., binding of TNF-α, IL-1α, LPS, CD40, Lymphotoxin, UV, HER2/Neu, H₂O₂, or other unknown ligands to its receptor) causes phosphorylation of IκB by IκK (IκB kinase). This triggers the degradation of IκB through the ubiquitin system (Ub), where the target molecule is masked by a chain of ubiquitins for degradation by the 26S proteasome. The free unbound NF-κB can then translocate to the nucleus and activates transcription (22). NF-κB translocation is reduced by inhibitor compounds such as BAY 11-7085, other IκB kinase inhibitor compounds, and other means.

nucleus or cytoplasm area intensity may also be helpful to identify nuisance fluorescent compounds that display the same or similar spectral properties as the probes used in the assay design and development. However, keep in mind that these compounds could also be classified as “false positive” activate compounds from the screen. In this case, careful follow ups needs to be employed b measuring basal autofluorescence levels in cells without fluorescent reporters and with compound(s) in question.

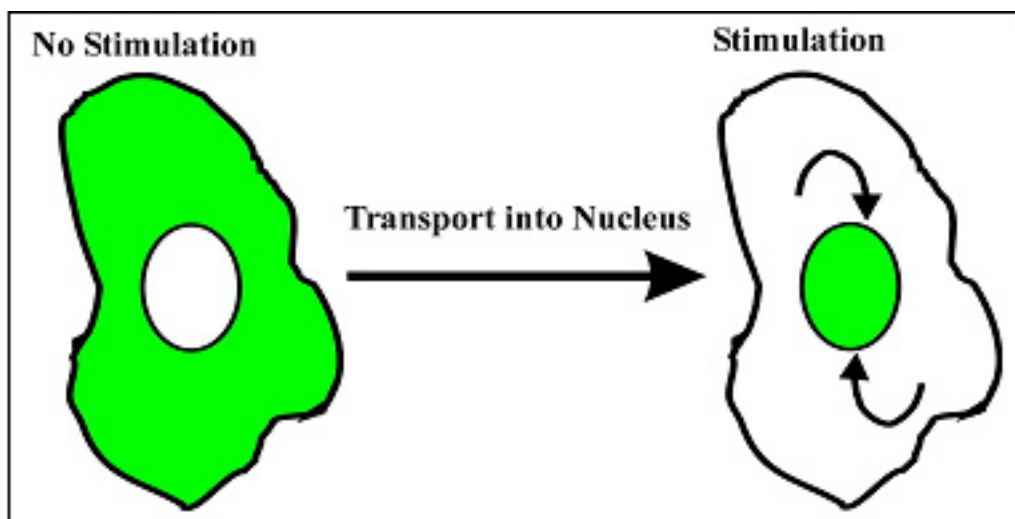


Figure 3: Diagrammatic view of the principle of NF- κ B activation. NF- κ B (green), normally resides in the cytoplasm, once activated by stimuli (IL-1 α , TNF- α , etc) it translocates to the nucleus.

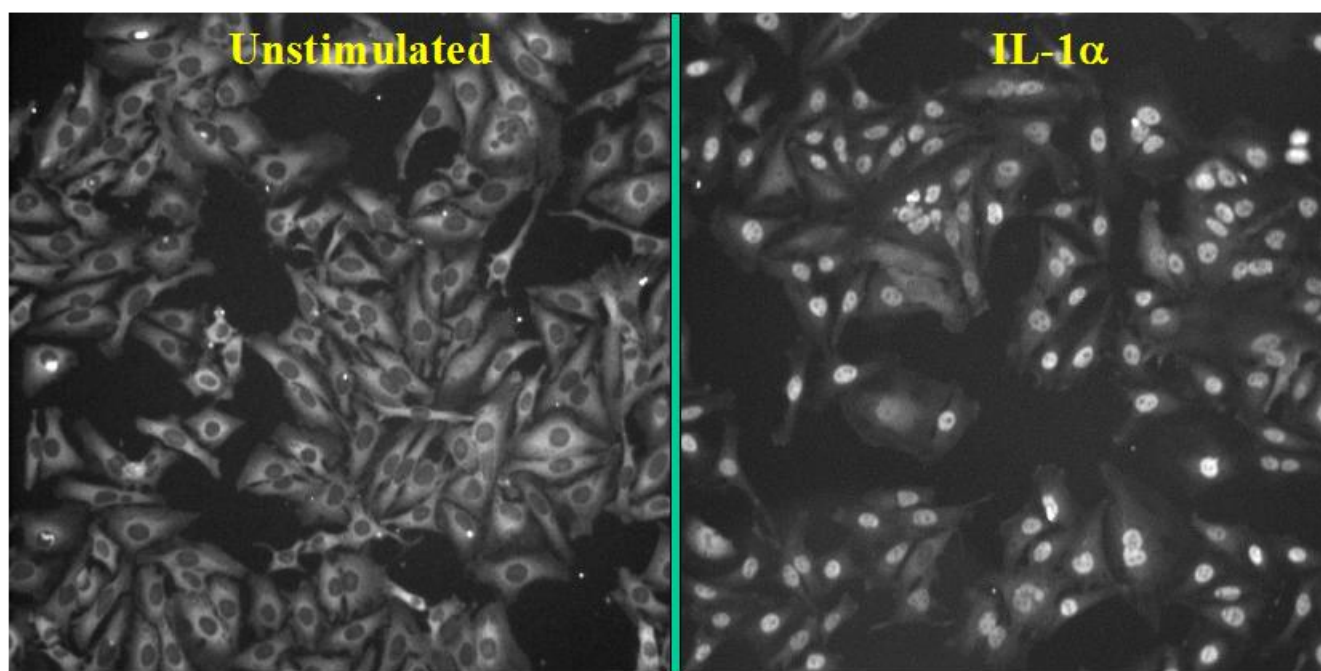


Figure 4: HeLa cells unstimulated or stimulated with 25 ng/ml IL-1 α for ~40 minutes. Cells were then fixed and labeled with NF- κ B-p65 polyclonal antibody and secondary AlexaFluor488 with counterstain of Hoechst 33342. (Left). Unstimulated cells showing a shaded cytoplasm containing NF- κ B-p65 with a dark nucleus “donut hole” counter stain. (Right). Following activation of NF- κ B, the nucleus appears bright as represented in white and the cytoplasm is no longer shaded indicating the protein translocated from the cytoplasm to the nucleus.

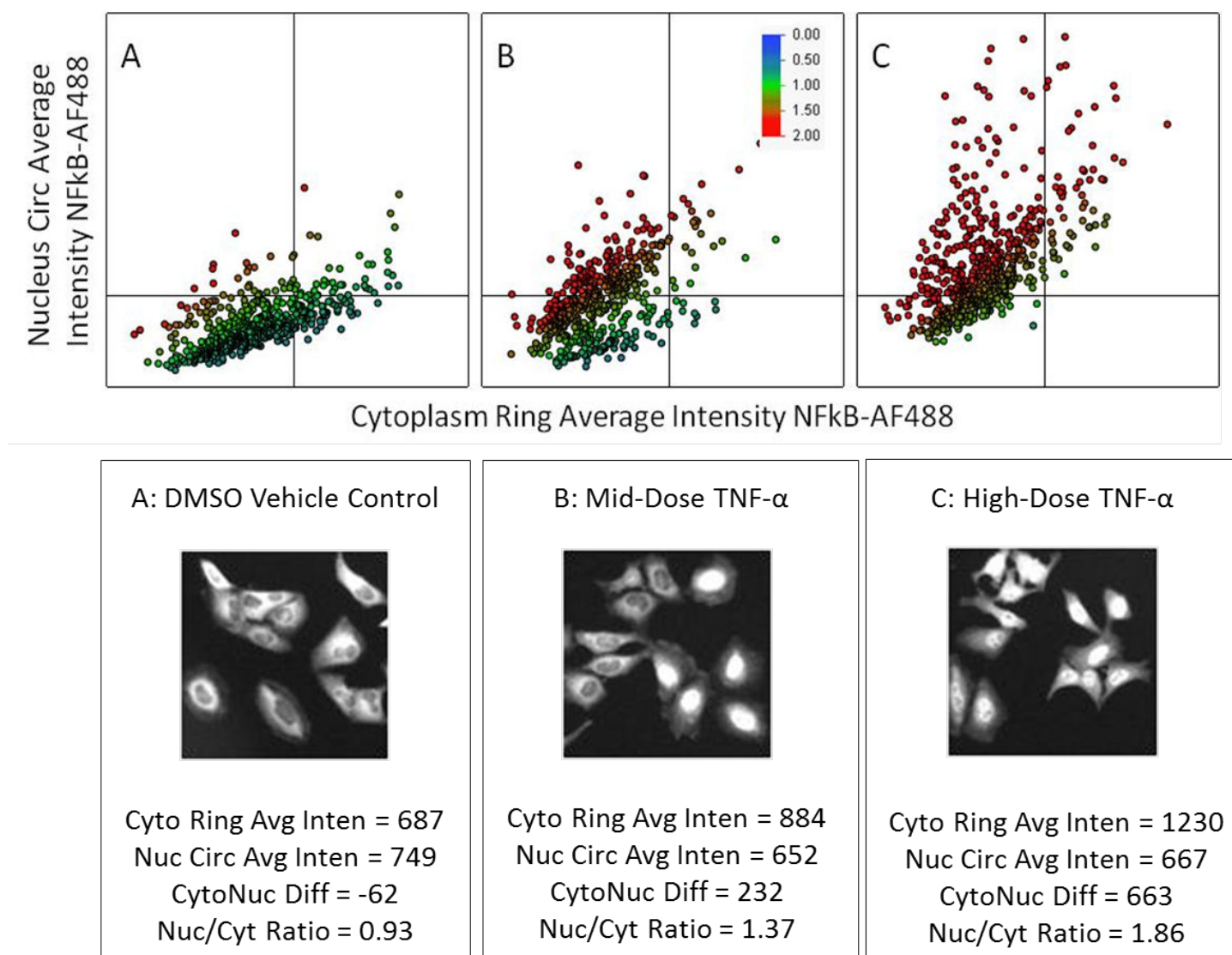


Figure 5: Multiparametric plot (Top) and corresponding images (Bottom) of NF-κB-AF488 average cytoplasm ring and nucleus circ intensities at the single cell level in HeLa cells following TNF-α dosing for 35 minutes. Each “dot” or “bubble” represents a single cell with a total of approximately 550 cells per graph; xy axis crossbar set at grayscale intensity value of 900. The color difference from blue to red is dependent on ratiometric calculation of NF-κB-AF488 Nucleus Circ Average Intensity divided by the Cytoplasm Ring Average Intensity (Nuc/Cyt Ratio); scale is display in graph B for all. (A) Vehicle control (B) Mid dose of TNF-α (C) High dose of TNF-α. Note the cell objects shift from cytoplasm ring average intensity (x-axis) to the nuclear circ average intensity (y-axis) and increase in Nuc/Cyt Ratio (red bubbles) with increasing TNF-α concentration.

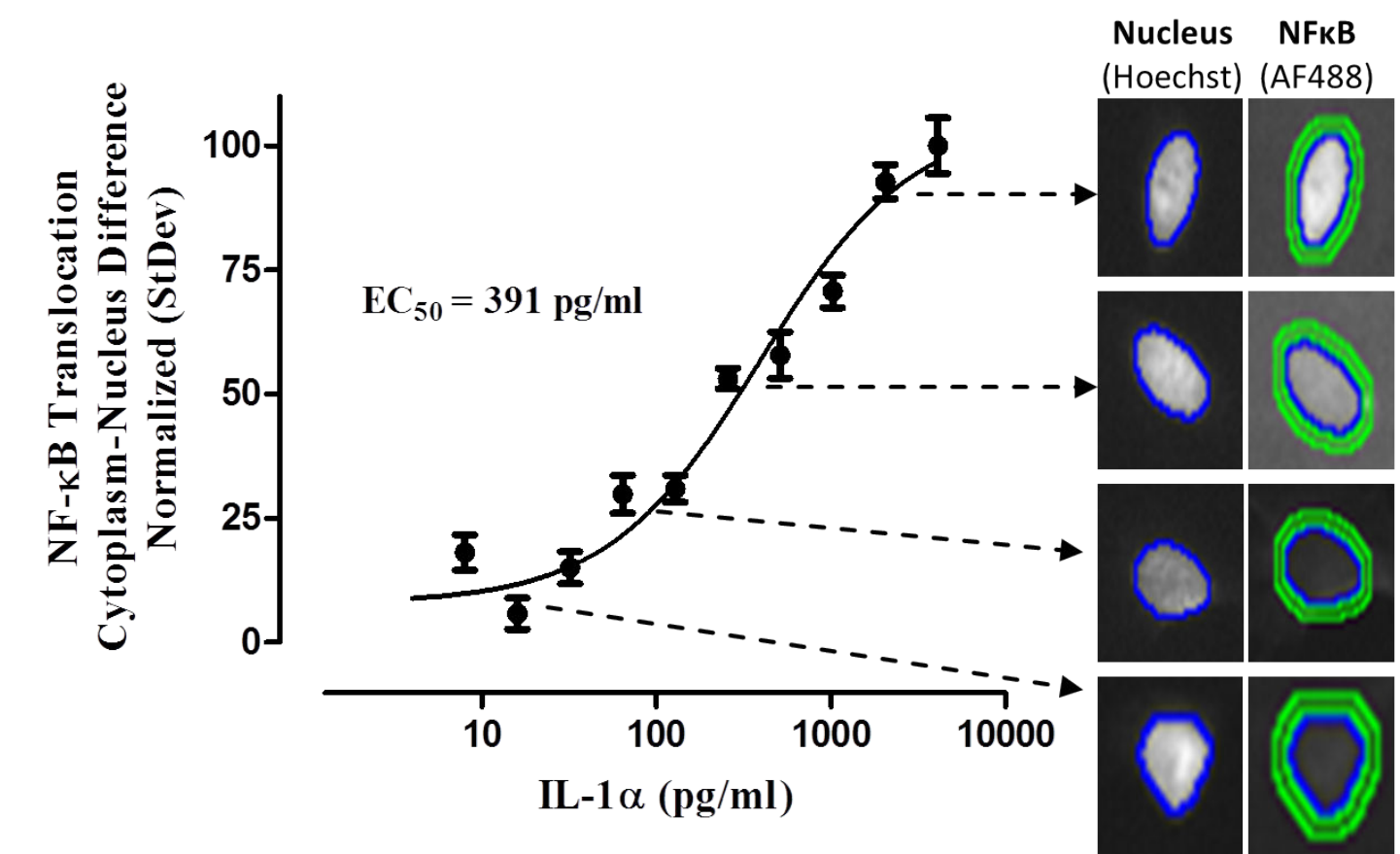


Figure 6: Nuclear Factor κB Translocation in HeLa cells following 30 minute incubation with IL-1α. Cells labeled with Hoechst 33342 are masked with a blue ring outline to identify the nuclear area. Dilation of this mask to identify cytoplasm area is masked and highlighted with two green rings to identify fluorescence of NF-κB-p65 antibody labeling. Note, the first green ring (inner) is dilated away from the outer nuclear boundary; it is then copied and expanded to represent the outer ring. The area between these two rings represents the cytoplasm area measured. The algorithm can output either the difference between the average intensities in the green and blue mask area or output a ratio of between the blue and green mask areas. Although difficult to see in these images NF-κB is expressed in the cytoplasm at low doses of IL-1α.

Table 1: Algorithm nomenclature output comparison of HCS feature data to measure NF-κB autofluorescence only.

Feature	BD AttoVision	Cell Profiler	Definiens Developer	GE HealthCare	MDC MetaXpress	Perkin Elmer	Thermo Scientific
Intensity inside the cytoplasm area	Cyt (Variable)	Variable	Variable	Cell Intensity	Outer Area	Intensity cytoplasm Alexa nnn mean (Variable)	RingAvgInten
Intensity inside the nucleus area	Nuc (Variable)	Variable	Variable	Nuc Intensity	Inner Area (AF488)	Intensity nucleus Alexa nnn mean (Variable)	CircAvgInten
Difference, between intensity in cytoplasm area minus the intensity in nucleus area	Nuc-Cyt (Variable)	Variable	Variable	N/A	N/A	Variable	CytoNuc Diff

Table 1 continued from previous page.

Feature	BD AttoVision	Cell Profiler	Definiens Developer	GE HealthCare	MDC MetaXpress	Perkin Elmer	Thermo Scientific
Ratio, intensity in nucleus area divided by the intensity in the cytoplasm area	Nuc/Cyt (Variable)	Variable	Variable	Nuc/Cell Intensity	Inner/Outer Intensity Ratio	Variable	NucCyt Ratio

Table 2: NF- κ B-p65-AF488 HCS feature data output comparison of Nuc/Cyt Ratio and CytoNuc Difference measurements in HeLa cells. The minimum (min) signal is the baseline constitutive expression of NF- κ B and the maximum (max) is NF- κ B response following ~35 minute incubation with either 25ng/ml IL-1 α or 25ng/ml TNF- α ; data includes the percent coefficient of variation (% CV) from 96-wells. The Z factor is calculated as described in literature (34)

IL-1 α	Ratio: Nuc/Cyt	Difference: Cyto-Nuc Diff	TNF- α	Ratio: Nuc/Cyt	Difference: Cyto-Nuc Diff
Signal (Min / Max)	1.0 1.84	-75.2 523	Signal (Min / Max)	0.957 1.86	-38.3 495
% CV (Min / Max)	4% 8.1%	3.5% 4.5%	% CV (Min / Max)	2.4% 6.7%	3.7% 5.4%
Z factor	0.64	0.76	Z factor	0.73	0.73

Assay Detection and Limitation

The assay described below will quantify the redistribution of NF- κ B from the cytoplasm to the nucleus upon activation using antibodies to NF- κ B p65 subunit in intact cells following fixation. Stimulating cells with proinflammatory cytokines such as IL-1 α and TNF- α activates the canonical NF- κ B pathway and therefore the noncanonical pathway (CD40, LT), or the atypical I κ B independent pathway (UV, Hypoxia) will likely be non-responsive in this assay format. In addition, this assay can indirectly measure I κ B degradation since phosphorylation of I κ B is required before degradation of I κ B α and subsequent translocation of NF- κ B subunit to the nucleus. This assay does not directly measure phosphorylation of NF- κ B protein.

This assay can be multiplexed with other biofluorescent probes. For example, other kinase related bioprobes to measure MAPK kinase pathways such as phospho-JNK-1/2 or viability indicators may provide important information about the biology or selectivity of the compound. The critical consideration for developing this assay is the dose and time using more than one stimulus to triggering multiple signaling pathways.

Assay Development

Cell Model

HeLa cells are endothelial cells from the American Tissue Culture Corporation (ATCC), catalogue number CCL-2, original isolated from the cervix of a 31yo black female with adenocarcinoma, were selected as the cell line as choice for this assay for several reasons. (A) HeLa cells are well documented in the literature to illustrate NF- κ B redistribution; (B) HeLa cells are relatively flat once seeded in plates and therefore image well using widefield 2-D bright-field and fluorescent microscopy; (C) there are commercial available validated kits and antibodies to detect redistribution of NF- κ B.

Note: Other cells such as Swiss 3T3, BHK, HepG2-C3A, Rat hepatocytes, LLC PK, HMVEC, CHO-K1, HT1080, HCT-116, U 2-OS, and others are also known to express NF- κ B but before adopting one of these cell lines or other unlisted cell models, it is recommended to validate assay performance with a known cell line such as HeLa.

Cell culturing and harvesting

Maintenance of the HeLa cell line or cell of choice should be carried out following recommendations from the ATCC or the source of the cell line. Typically splitting cells 2-3 times per week is required for optimal growth and performance in the assay. Do not allow cells to become over confluent in tissue culture flasks. If this occurs, it will be necessary to thaw a new vial of cells before proceeding with assay development, validation, or screening. Grow cells in complete media, and then adjust serum concentration prior to cell seeding as necessary (see [Effects of Serum](#) for additional information). Upon harvest of cells with trypsin or other cell detachment methodology it is recommended to filter the cells through a cell strainer (40-70 microns) to remove large clumps before cell counting and seeding. If aggregates are not removed this will negatively impact cell object identification and segmentation with image analysis algorithms.

Cell Seeding Number Determination

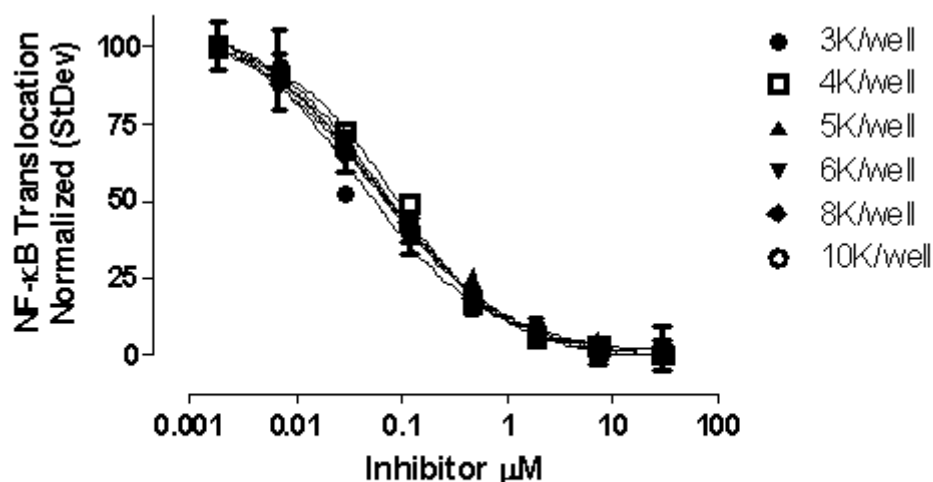
Determine the optimal cell seeding density as described in the assay development HCS chapter by seeding cells at approximately 5,000 cells/well for a 96-well plate as the median starting point; then dilute or increase the cell density by approximately 40-50%. (*Note: In 384-well plates, approximately 2,500 cells/well is recommended*). Incubate cells overnight, and then label as described in subsequent sections with reporter probes. There is some variability but no significant difference in cell number (3,000 to 10,000 cells/well) in 96-well plate format from the NF- κ B inhibitor compound IC₅₀ curves (Figure 7). However, since identification and segmentation of individual cells in the well is optimal for accurate image analysis measurement of NF- κ B, 5,000 cells/well was chosen. An NF- κ B reference compound inhibitor, IK202, was used to determine cell number. The number of cells per well was optimized based on reviewing images to observe cell-to-cell contact. Higher concentrations of cells per well resulted in rejection of more cells due to piling of cells, massive cell contact clumps, and poor image analysis segmentation. However, the number of fields required to collect 500 cells, the assay defined threshold, was decreased (Figure 8). The optimal cell seeding density for HeLa cells is 5,000 cells/well for 96-well plate. *{data not shown}*.

Cell Passage Limitations

It is critical to gauge the number of cell passages in the assay before a noticeable decline is observed. Cells with many passages may not survive, become contaminated, or fail to respond in the assay over time. If possible, continue passaging cells at the onset of development with a fresh thaw of cells and maintain a weekly stock over time; then measure NF- κ B response in comparison with lower passage cells. HeLa cells with different cell splitting passage numbers up to 40 from ATCC and the same lot were tested to compare sensitivity to TNF- α cytokine to activate translocation of NF- κ B and to determine if the response was altered. Even though the R² of the curve fitting was similar; there was a significant shift in the NF- κ B translocation EC₅₀ response to cytokines in cells with high passage number. A significant loss in responsiveness of NF- κ B translocation was observed with an increase in calculated EC₅₀ values directly correlated with increasing passage number (Figure 9). Based on these findings it is recommended to use cells with as low passage number as possible and as a general rule never exceed passage of 20.

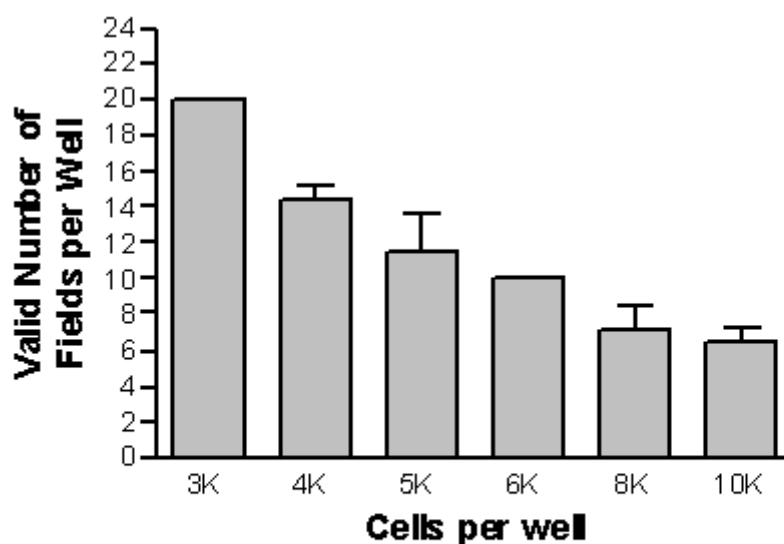
Activators of the NF- κ B Signaling Pathway

Activating NF- κ B pathway using cellular cytokines as described in the introduction is critical to establish an assay window. Although several stimuli have been identified in activating NF- κ B pathway, three cytokines, IL-1 α , IL-1 β , and TNF- α gave the most robust and best EC₅₀ values of all stimuli tested (Figure 10). IL-1 α , IL-1 β family members and TNF- α cytokines activate NF- κ B through different up-stream mechanisms. Platelet-derived growth factor (PDGF) also activates the translocation events of the NF- κ B pathway to a lesser extent and is described to work through Ras and PI3 kinase signaling. (33). Lymphotoxin (LT) α 1/ β 2, LT α 2/ β 1, and IFN- γ showed little or no activation of NF- κ B (the latter stimuli data is not shown). Additionally, anisomycin and



	3K/well	4K/well	5K/well	6K/well	8K/well	10K/well
EC50	0.03777	0.09116	0.06331	0.06138	0.06414	0.05735

Figure 7: Cell Number Determination. HeLa cells seeded overnight, treated with reference compound inhibitor for 15 minutes and subsequent 25ng/ml TNF- α for ~35 minutes, and then fixed and stained for NF- κ B-p65-AF488. Plates were analyzed on HCS imager to determine NF- κ B translocation using CytoNuc Difference; data was normalized to control and plotted in GrapPad Prism using non-linear regression 3-parameter fit.



	3K/well	4K/well	5K/well	6K/well	8K/well	10K/well
Mean	19.75	15.06	11.38	8.250	6.125	5.375

Figure 8: Field numbers required to collect 500 objects (cells) using a 10X/0.3NA objective, no magnifier and a 1392x1024 camera pixel array sensor.

phorbol-12-myristate-13-acetate (PMA) were also tested but did not show NF- κ B-p65 activation as robustly as IL-1 α , IL-1 β , or TNF- α stimulus. This knowledge is important if considering multiplexing NF- κ B with other signaling pathways. In this assay design, development, and validation, HeLa cells will be stimulated with both

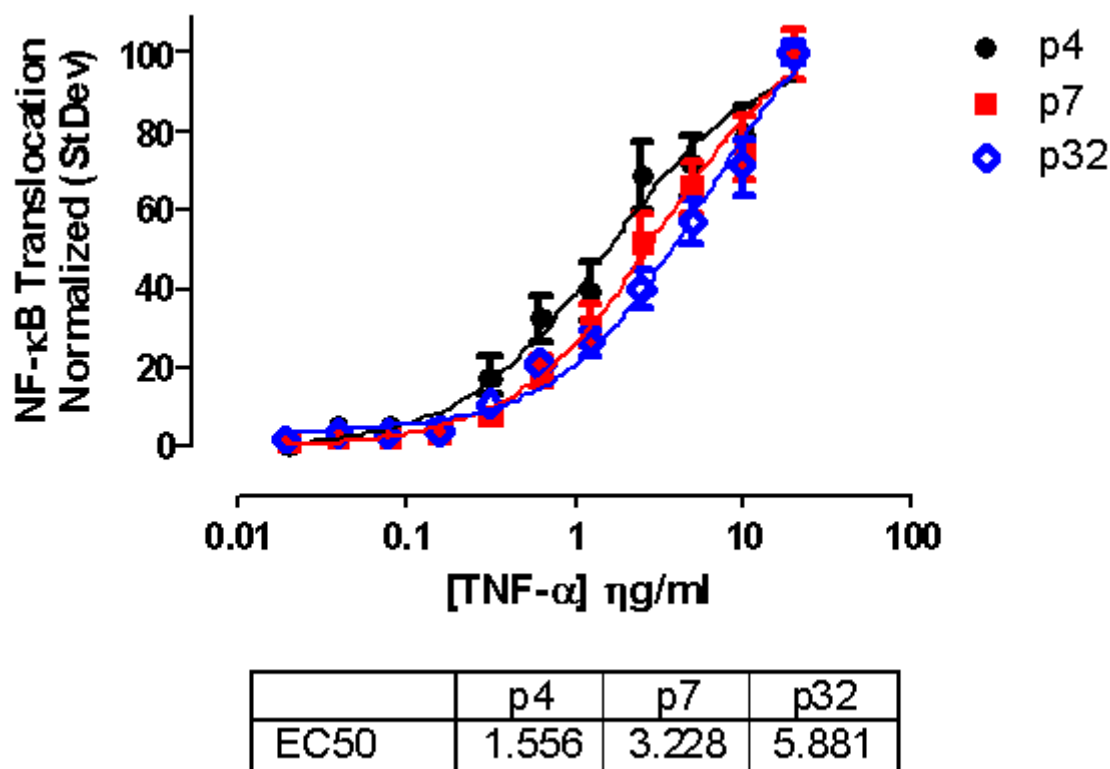


Figure 9: Cell Passage Number Comparison. Different cell splitting passages of HeLa cells seeded at 5,000 cells/well overnight and treated with dose response of TNF- α for ~35 minutes, then fixed and stained to measure NF- κ B translocation. Plates were analyzed on HCS imager to determine NF- κ B translocation using CytoNuc Difference calculation; data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit.

IL-1 α and TNF- α following compound treatment, however, for compound profiling and screening, either stimuli alone will work and choice of cytokine is depended on the upstream or downstream target and biology.

Plate Type

The NF- κ B assay is considered a very robust HCS assay using HeLa cells and likely can be adapted to many different SLAS standard plate types including 384-well or even 1536-well formats, however it is recommended to first perform the proof of concept in a validated format such as the 96-well, as discussed within, then move to another well plate format. In this assay sterile 96-well tissue cultured treated Perkin Elmer (Packard) View plates were chosen with no extracellular matrix proteins or PDL substrate coating.

Reagent & Probes

There are a few validated assay kits and antibodies in market that can be used for this assay, or alternatively development of an in-house or “homebrew” assay protocol kit can be developed. In the next section, details of developing and comparing with a commercially available kit are described. The primary advantage of using a commercial kit is it is “ready to use” with “cookbook” instructions included. If just measuring a few plates, then this is a preferable option. The benefit of developing an in-house “homebrew” kit is to know what antibodies, buffers, and reagents are being used in the assay as this may not be disclosed by commercial sources of kits. In addition, the cost for running many plates is reduced once an in-house kit is developed. This chapter should reduce development time and costs. An example comparing a commercial kit with an in-house kit using alternative antibody sources and the process to cross validate their relative performance is described in next section.

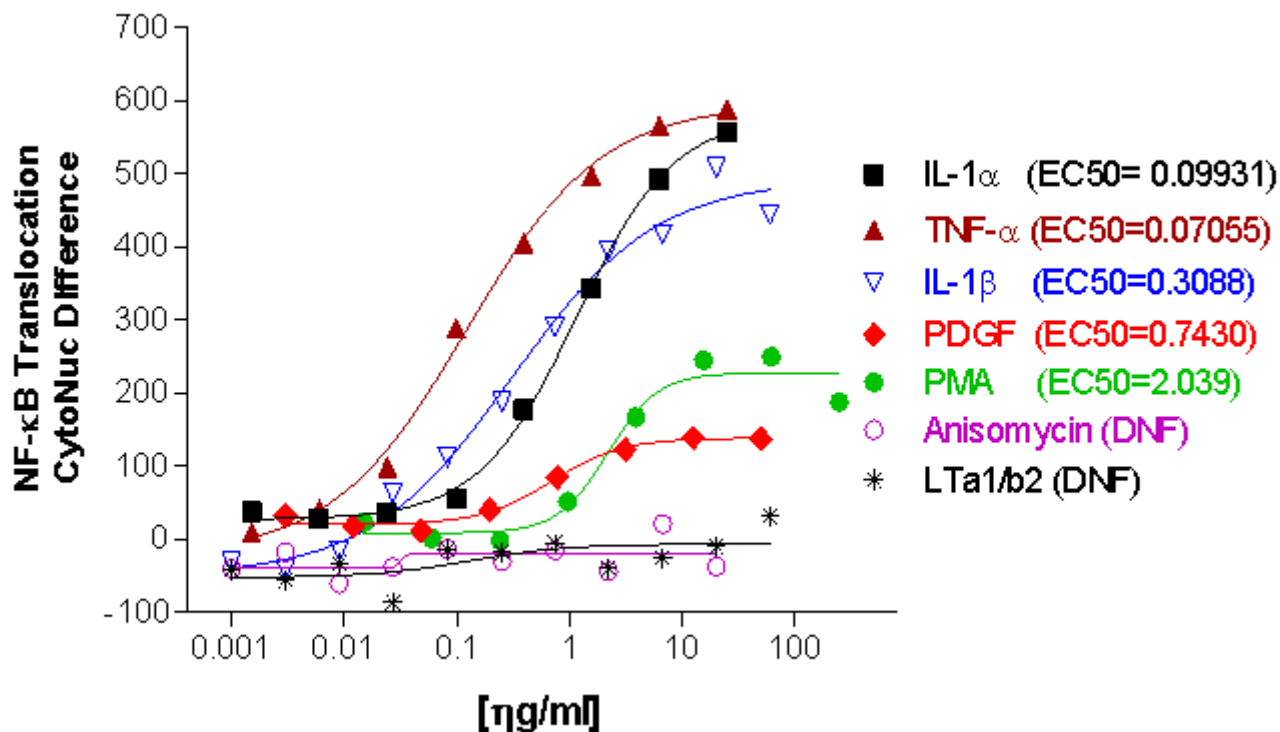


Figure 10: Activation of NF- κ B-p65 with Different Stimuli. HeLa cells seeded at 5,000 cells/well overnight and treated with stimuli for 30 minutes, then fixed and labeled with NF- κ B-p65-AF488 and Hoechst33342 to measure NF- κ B translocation. Plates were analyzed on HCS imager to determine NF- κ B translocation using CytoNuc Difference calculation; data expressed as raw unit values (y-axis) from algorithm using non-linear regression 3-parameter fit was done in GraphPad Prism; standard deviation error bars (n=3) was removed for visualization.

Note: Before beginning the assay development process determine if a fixed endpoint assay is appropriate for the biological question. Alternatives to the classic antibody recognition and binding approach is the use of a fusion protein of NF- κ B with a fluorescent protein reporter such as GFP, mCherry, or HaloTag® (Promega, Madison, WI) are other options to consider for measuring protein function.

Commercial Kit Validation & Development of In-house “Home-Brew” Kit

The initial assay development was performed using commercial available validated kits for NF- κ B. Additional, several different commercial available antibodies were screened for signal-to-noise ratio performance that targeted the p65 subunit of NF- κ B (Appendix-1). Although other antibodies worked well in the evaluation, based on performance and comparable EC₅₀ calculated values, the rabbit polyclonal IgG NF- κ B-p65 antibody from Santa Cruz (SC-372) was used for assay development and validation procedures (see Figure 11).

Since the commercial kit contains other unknown and perhaps proprietary reagents, i.e., antibody, buffers, and reagents including PBS; PBS containing 0.5% Triton X-100; PBS containing 0.01% Tween-20 for testing and comparison with “in-house” buffers and commercial kit; it is always important to verify the signal-to-noise ratio window and insure that assay variability is not sacrificed.

Comparisons of different lots of NF- κ B antibody from Santa Cruz were done at three different concentrations of primary antibody following stimulation with IL-1 α . There were no significant differences observed (Figure 12). Lot-to-lot variability was evaluated several times with new antibody lot shipments and no significant differences in the calculated EC₅₀ values were observed.

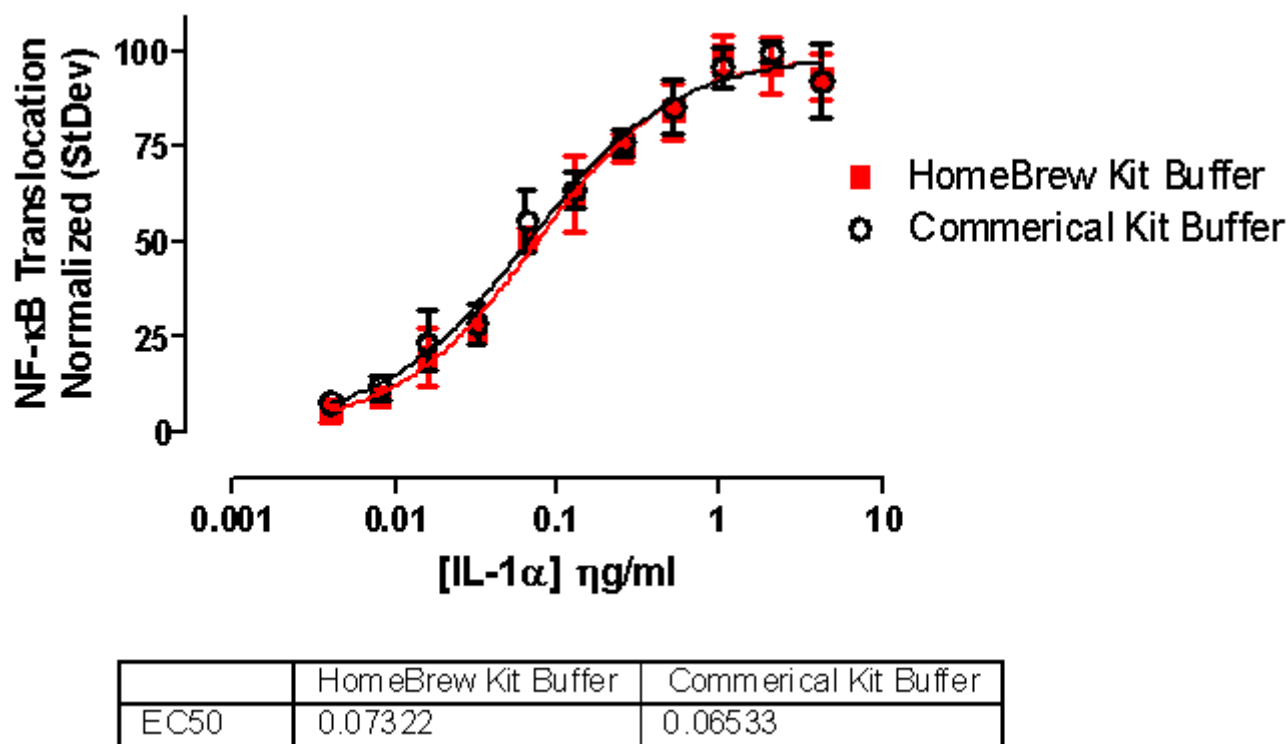


Figure 11: Comparison of Commercial and HomeBrew Buffers. HeLa cells seeded at 5,000 cells/well overnight and treated with dose response of IL-1 α for ~35 minutes, then fixed and stained to measure NF- κ B translocation using SC-372 antibody. Plates were analyzed on HCS imager to determine NF- κ B translocation using CytoNuc Difference calculation; data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit.

Fixation comparison of two different forms of formaldehyde solution

Formaldehyde, 37% stock solution (Sigma) and Ultra-pure Formaldehyde, methanol-free 10% stock solution (Polysciences) were compared. HeLa cells were treated with IL-1 α for about 30 minutes, followed by 1:10 dilution of fixation buffer in PBS for 10 minutes. An approximately 3-fold shift in the EC₅₀ values was observed (Figure 13). For this assay, 3.7% formaldehyde from Sigma containing methanol was used. It is possible higher concentrations of Ultrapure formaldehyde may be used but this needs confirmation by testing.

Note: At the time of testing 16-20% paraformaldehyde solution was not readily available from commercial sources and should be considered as alternative source.

Please refer to institutional safety guidelines before working with formaldehyde; suggestions are in Safety Consideration Guidelines Precautions. Formaldehyde is specifically regulated by OSHA, so be sure you are in compliance with the OSHA standard (http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_id=10075&p_table=STANDARDS).

Stability and Lot Variability of Cytokines

Variability of cytokines lots were measured to determine variability of the assay if new reagents were introduced to the assay during the screening campaign or used in subsequent screens. Please note, it is highly recommended to order all reagents before the start of the experiment and/or screen. From this experiment and several other experiments, both IL-1 α (data not shown) and TNF- α EC₅₀ responses and calculated EC₅₀ values indicated very high reproducibility (Figure 14). In addition, the activity and stability of cytokine performance was measured after one or more freeze-thaw cycles (Figure 15).

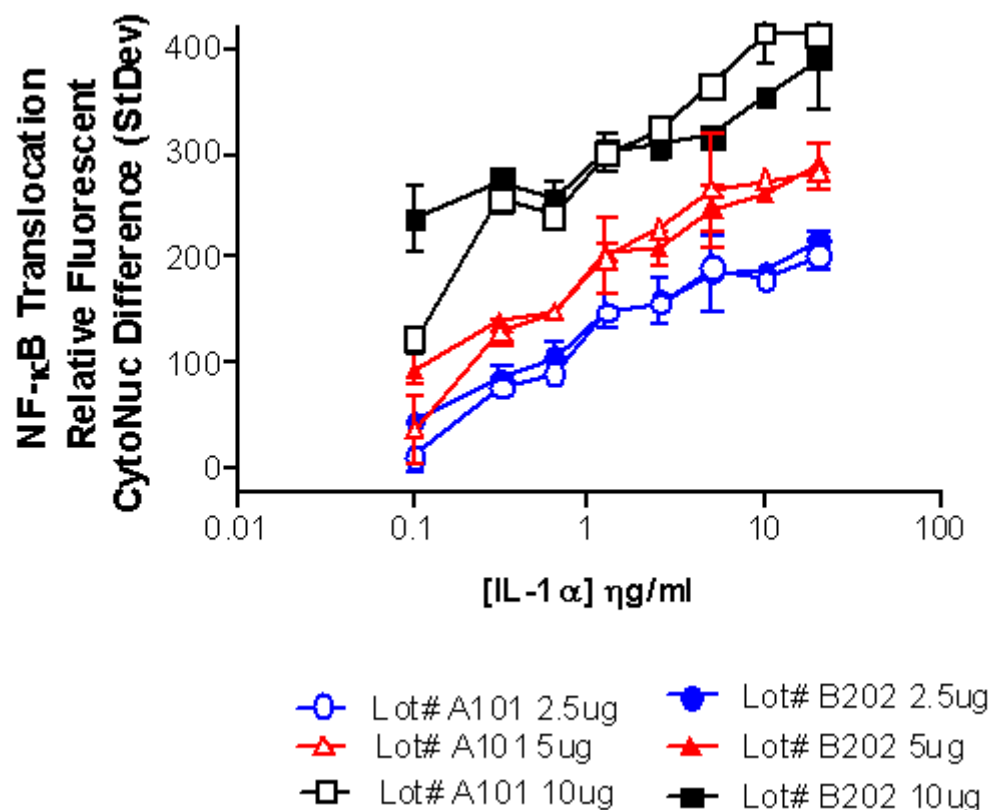


Figure 12: Lot-to-Lot Variability Comparison of Santa Cruz SC-372 NF-κB-p65 Antibody. HeLa cells seeded at 5,000 cells/well overnight and treated with dose response of IL-1α for ~35 minutes, then fixed and stained to measure NF-κB translocation using two different lots of SC-372 antibody and at different concentration dilutions. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was not normalized and plotted with “raw” numbers in GraphPad Prism.

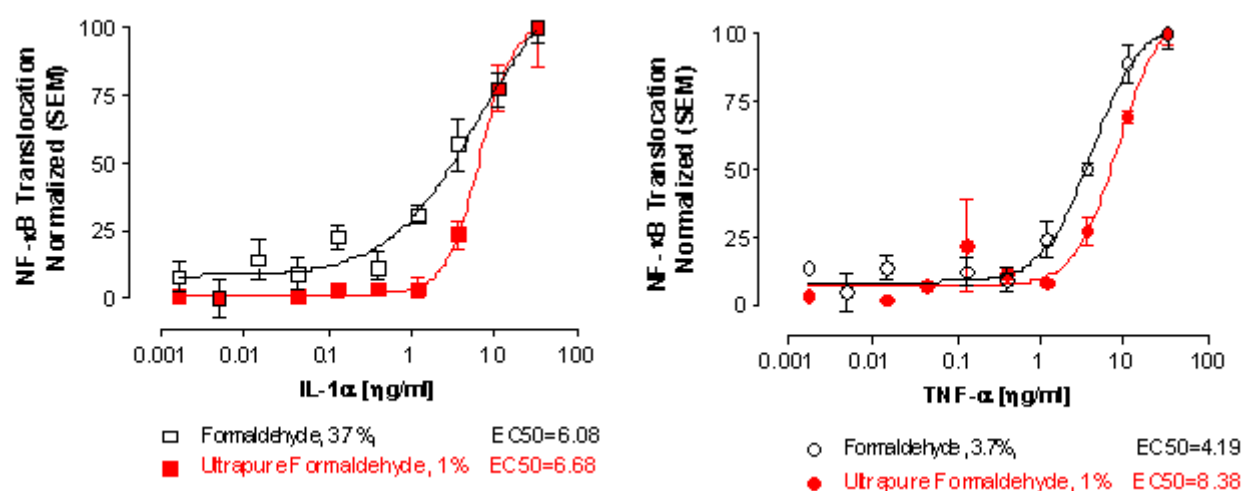


Figure 13: Comparison of Formaldehyde Fixatives. HeLa cells seeded at 5,000 cells/well overnight and treated with either dose response of IL-1α (left) or TNF-α (right) for ~35 minutes, then fixed with either 3.7% formaldehyde or 1% Ultrapure Formaldehyde and stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit.

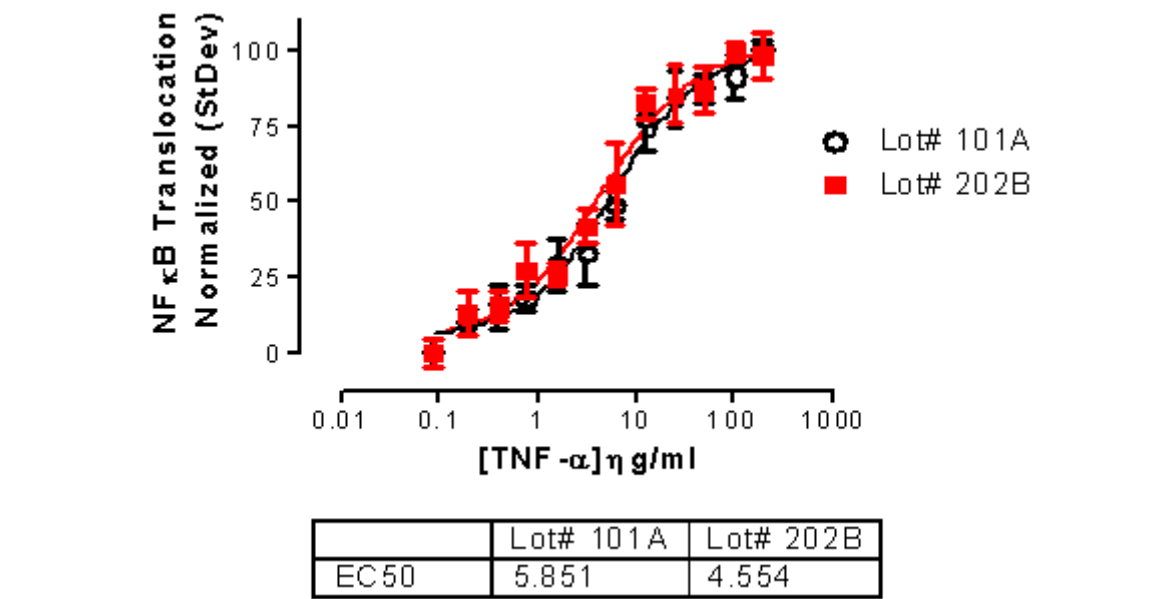


Figure 14: Lot variability of tumor necrosis factor alpha. HeLa cells seeded at 5,000 cells/well overnight and treated with dose response of TNF-α for ~35 minutes, then fixed and stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit.

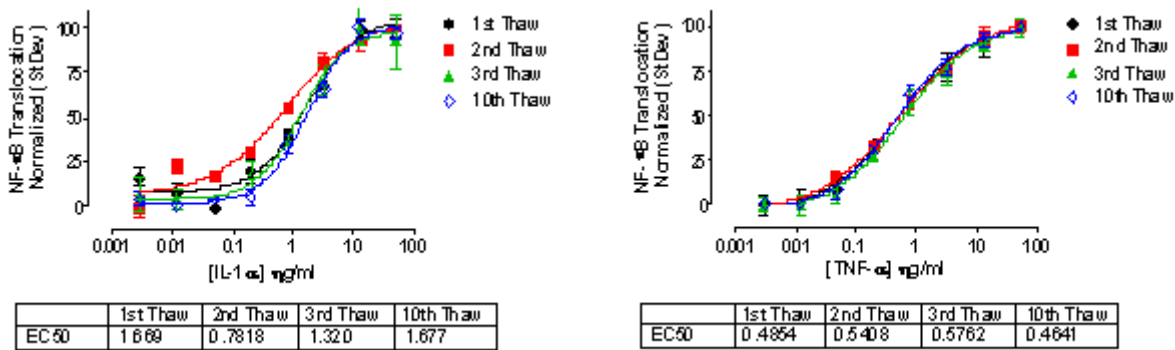


Figure 15: Stability of cytokines following multiple freeze-thaw cycles. Following the reconstitution of the cytokine per manufacture suggestion, cytokine reagents were store at -80°C, and then allowed to thaw at room temperature before use. Samples were then re-frozen at -80°C multiple times. Translocation of NF-κB was performed on HeLa cells following treatment with IL-1α (left) or TNF-α (right) as previously described and data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit.

Stability of NF-κB detection in cells following fixation

The NF-κB protein in HeLa cells is stable for several days at 4°C prior to staining with NF-κB antibody following fixation with 3.7% formaldehyde for 10 minutes, washed and stored in PBS. This provides flexibility in screening operations if a “Stop Point” is required in the workflow. It is recommended that cells stained with NF-κB antibody be imaged as soon as possible, and not to exceed 14-days (Figure 16). For logistics in screening operations including robotics, liquid handling, and cell plate handling to reduce flutations in pH and temperature, 35 minutes was chosen.

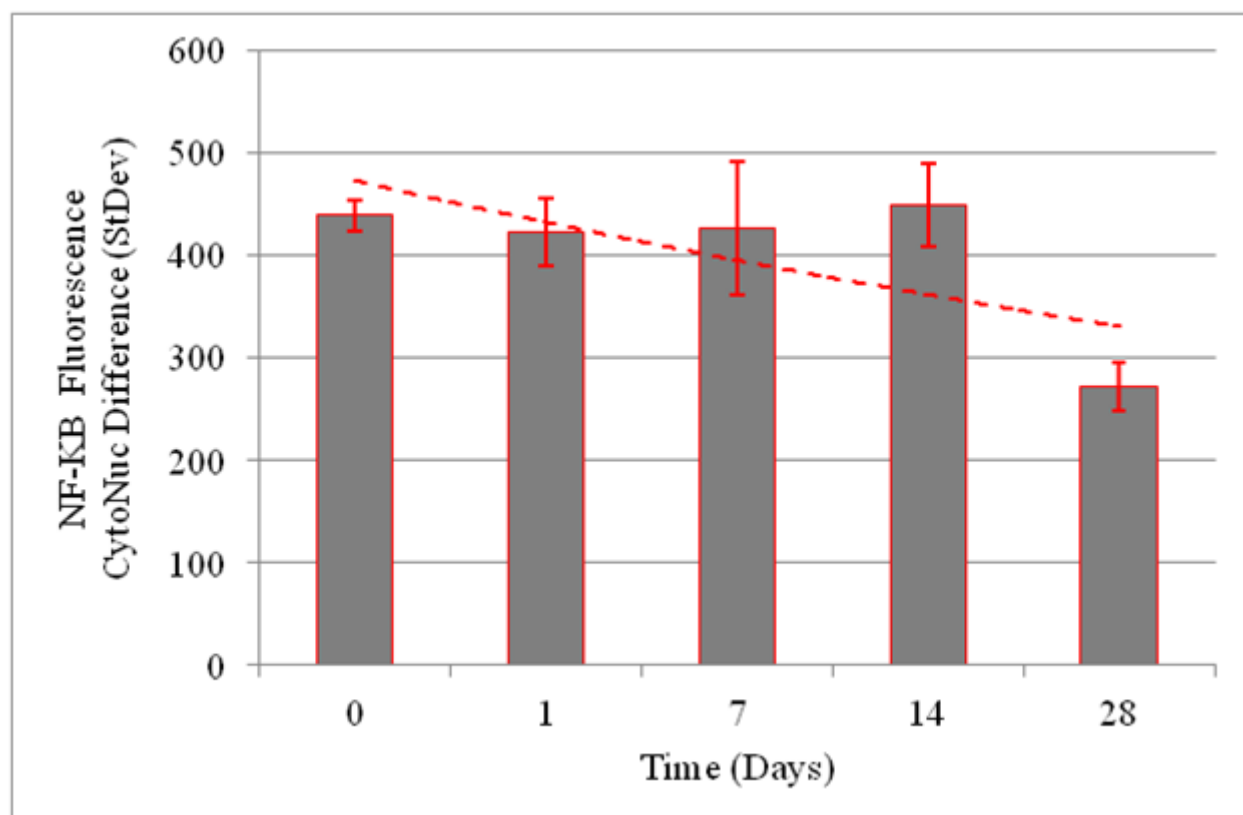


Figure 16: Stability of NF-κB-p65-AF488 complex post staining and fixation. Using several plates, HeLa cells seeded at 5,000 cells/well overnight and treated with 25 ng/ml of TNF-α for ~35 minutes, then fixed and stained to measure NF-κB translocation at the maximum signal. At different time points (days), plates were analyzed on HCS imager to determine NF-κB translocation fluorescent intensity measurements using CytoNuc Difference calculation; raw data was used and plotted for comparison.

Time Course of Cytokine Stimulation

Determine the optimal time window for NF-κB translocation by performing a time dependent stimulation with known stimuli. HeLa cells seeded at 5,000 cells/well in a 96-well plate overnight were then treated with 50 ng/ml of IL-1α to establish a time course for activation and redistribution of NF-κB. Following incubation with cytokine, cells were fixed at different times and then labeled with NF-κB antibody using an indirect staining method with secondary fluorescent antibody. Images of cells expressing fluorescent antibody are captured using HCS imager and analyzed for NF-κB expression. Time ½ = 24 minutes (Figure 17). Based on these results 30-60 minutes proved to be an excellent window to capture NF-κB translocation.

Effects of Serum

HeLa cells treated with cytokines IL-1α and TNF-α were tested with different concentrations of fetal bovine serum (FBS) to determine if serum affects the assay performance and window. While the assay window did not diminish with concentration of serum, the sensitivity of cytokine stimulation as calculated by EC₅₀ values of NF-κB translocation was significantly different; IL-1α sensitivity with 0.5% FBS increased ~3-fold as compared to 10%FBS, while TNF-α sensitivity increased by ~ 2.5-fold using 0.5%FBS as compared to 10%FBS (see Figure 18). Based on this data and subsequent data 0.5% FBS concentration was chosen for validation experiments.

DMSO Tolerance

HeLa cells seeded at 5,000 cells/well in 100μL media containing 0.5%FBS were allowed to attach overnight. Cells were then incubated with DMSO concentrations up to 10% for 30 minutes (2X the dosing time for compound

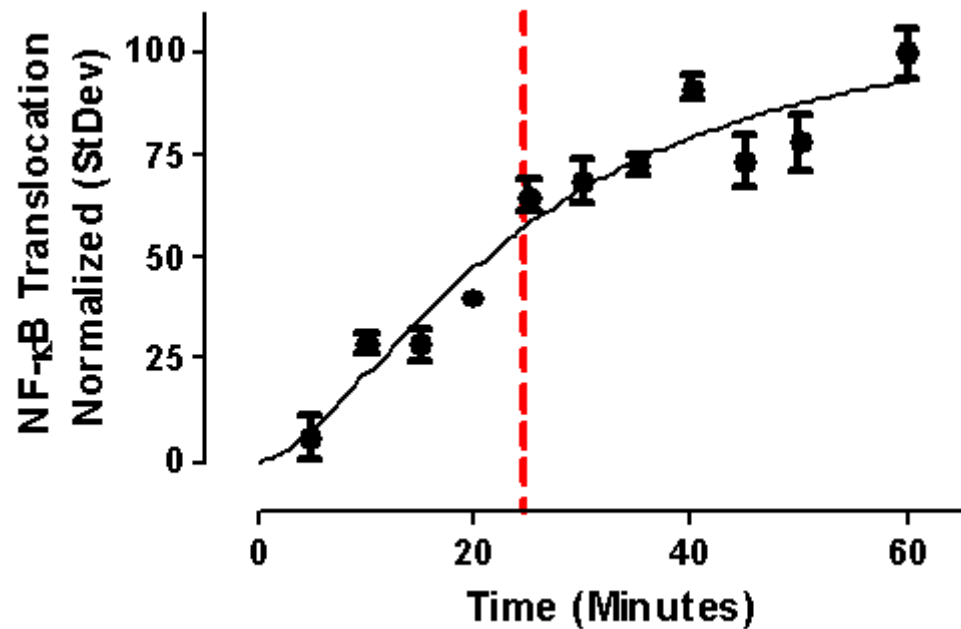


Figure 17: NF-κB Translocation time course kinetics. HeLa cells seeded at 5,000 cells/well overnight and treated with 25ng/ml of IL-1α over time, at 5 or 10 time minute intervals, cells were fixed and then stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized and plotted in GraphPad Prism using non-linear regression one-site binding to calculate the ½ time response, 24 minutes.

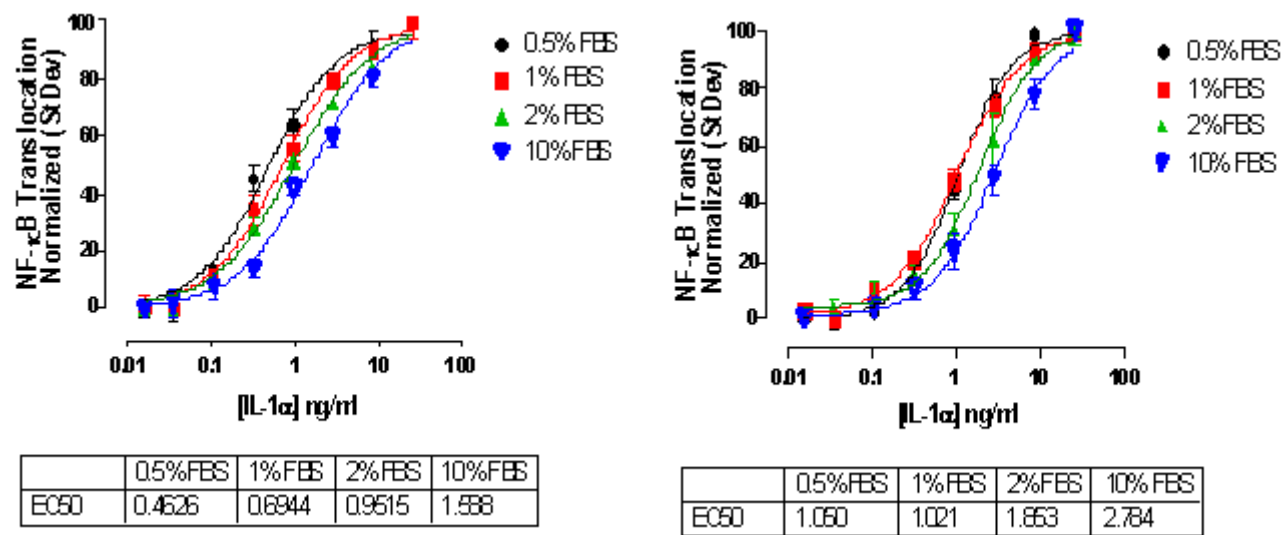


Figure 18: NF-κB translocation effects from serum concentrations. HeLa cells seeded at 5,000 cells/well overnight in 10% Fetal Bovine Serum (FBS), were then removed from complete serum by washing with serum-free media and replaced with 0.5%, 1%, 2%, or 10% FBS and treated with dose response of either IL-1α (left) or TNF-α (right) for ~35 minutes. Cells were then fixed and stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized and plotted in GraphPad Prism using non-linear regression 3-parameter fit.

treatment) followed by 30-minute incubation of cytokine. The increase and subsequent decrease in signal at DMSO concentrations greater than 1% on the graph is a result in change in cell morphology and cytotoxicity respectively (Figure 19). At 5% DMSO, the cell's cytoplasm area shrunk as a result of increase in nuclear size,

thus the image analysis algorithm measurement is reflective in the data showing an increase in CytoNuc translocation (see figure 19). At 10% DMSO, the cells are fragmented, dissociated, or detached from the plate and therefore not possible to quantify cytoplasm or nuclear fluorescent expression.

Reference Compounds

Dosing Time of compounds on Cells

It is highly recommended to pre-determine the duration of exposure dose of an inhibitor compound prior to stimulation with secondary stimuli such as cytokines or growth factors. Please keep in mind and consider the following:

- Is it possible to co-add the inhibitor compound and stimuli simultaneously?
- How does this affect the S:N window or assay variability?
- Is a pre-incubation required to maximize window and assay variability?
- Is there an advantage for the workflow in screening operations?

For this assay a 15 minute pre-incubation of inhibitor compound was chosen to make certain the compound exposure to the cells was saturated prior to stimulating with cytokines. Comparative data suggests simultaneous co-addition of both the inhibitor compound and stimuli does not affect the performance of the assay window (data not shown). But keep in mind these are reference compounds and unknown chemicals in a compound library may not readily penetrate cells before NF- κ B is activated.

Inhibitors of NF- κ B Pathway

There are several known inhibitors of NF- κ B pathway that have been published such as Bayer's compound, BAY 11-7085, which was initially selected as the control inhibitor reference compound for assay development and validation because it was commercially available and showed IC₅₀ activity of about 10 μ M (Figure 20).

Additionally there were two compounds identified in-house named IK101 and IK202 with improved potency. These compounds were chosen as reference compounds in the assay validation, although BAY 11-7085 and BAY 11-7082 compounds both could serve as reference compounds. The IK101 and IK202 compounds, with IC₅₀ values of less than 2 μ M and 0.3 μ M, respectively, were used for demonstration and performance for assay validation purposes (Figure 21). Inhibitor compounds were pre-incubated with cells for 15 minutes followed by cytokine addition for about 30 minutes.

Reference Compound Stability of Freeze-Thaw Cycles

HeLa cells treated with reference compounds (IK101 or IK202) that underwent multiple freeze-thaw cycles over a course of several weeks were tested for stability and/or loss of activity. Compounds were dissolved in 100% DMSO and aliquoted in cryovials and stored at -80°C for later use. After up to 10 freeze-thaw cycles there is no significant evidence of loss of compound activity (Figure 22).

Performance and Validation Pre-Screen

Following the optimization in the prior steps it is critical to validate the process procedure for screening operations before testing unknown chemicals. In Figure 23 and Figure 24, the reproducibility of cytokine stimuli is determined over a course of three independent assays and at least 3 different days to mimic the time it may take to screen an entire library. This requires independent harvesting of cells from flasks, cell seeding, and so on over 3 or more days. In Figure 25, the reference compound inhibitor IK202 was tested for reproducibility over 3 independent experiments, again to mimic screening operations. The results were acceptable for proceeding with Z-factor determination.

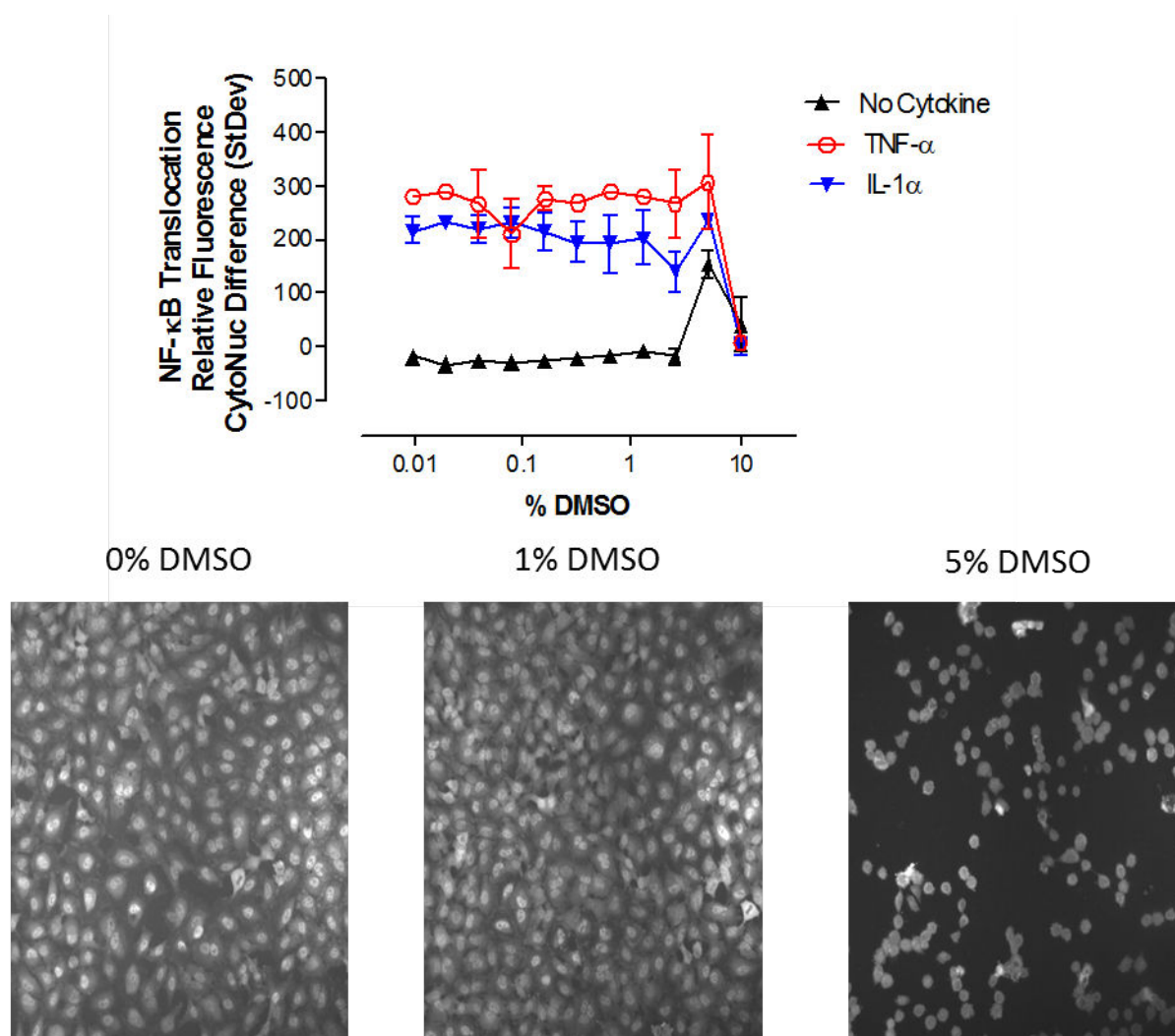
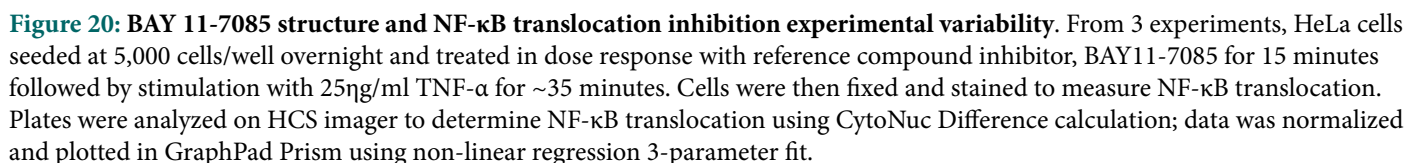
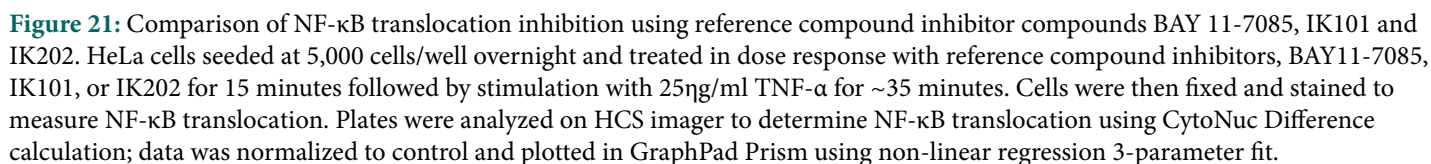


Figure 19: DMSO tolerance. HeLa cells seeded at 5,000 cells/well overnight and treated DMSO for 15 minutes followed by stimulation with either media only containing 0.5% FBS, 25ng/ml IL-1 α , or 25ng/ml TNF- α for ~35 minutes. Cells were then fixed and stained to measure NF- κ B translocation. Plates were analyzed on HCS imager to determine NF- κ B translocation using CytoNuc Difference calculation; raw data was used and plotted for comparison (top) and images captured using 10X/0.3NA objective (bottom) show differences in concentration.

To determine the robustness and variability of single dose addition across the entire plate using liquid handling and robotics, at least 2 full plates with minimum and maximum responses are required to calculate a Z-factor. Z-factor is calculate using the formula $1 - [3 \times (\text{standard deviation of positive control} + \text{standard deviation of negative control}) / (\text{mean of positive control} - \text{mean of negative control})]$ (33). The values from the Z-factor are indicative of the variability of the data, values of less than 0 is considered too much overlap in the positive and negative signal; 0 – 0.5 is considered a marginal assay and 0.5 – 1 is considered an excellent assay.

For the NF- κ B assay, the maximum response (Max or positive) is cytokine addition and the minimum (Min or negative) response is the reference compound inhibitor, i.e., IK202 + cytokine. Additional a Mid response can be added to verify ~50% reduction in the assay window. As mentioned earlier and throughout this chapter measuring translocation of NF- κ B with HCS can use one or more HCS data features, namely intensities of the Cytoplasm-Nucleus Difference (CytoNuc Diff) or the Nucleus to Cytoplasm Ratio (Nuc/Cyt Ratio). In Figure 26 (IL-1 α) and Figure 27 (TNF- α), both the CytoNuc Diff and Nuc/Cyt Ratio is reported. Interestingly, the TNF- α stimuli showed equivalent Z-factor values, however, the IL-1 α stimuli Z-factor was significantly different; the CytoNuc Diff was 0.76 and the Nuc/Cyt Ratio was 0.64. Both are considered excellent screenable assays.



Reagents for assay are listed in Table 3.

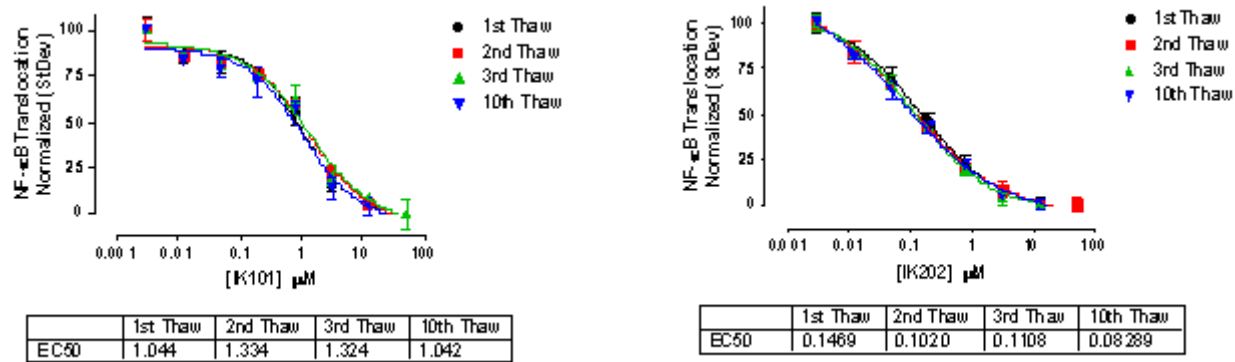


Figure 22: Freeze-thaw cycle stability of reference compound inhibitors IK101 and IK202. Reference inhibitor compounds were made at 10mM in DMSO and stored at -80°C, and then allowed to thaw at room temperature before use. Compound vial samples were then re-frozen at -80°C multiple times following thaw. HeLa cells seeded at 5,000 cells/well overnight and treated in dose response with reference compound inhibitors IK101 (left) or IK202 (right) for 15 minutes followed by stimulation with 25ng/ml TNF-α for ~35 minutes. Cells were then fixed and stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit.

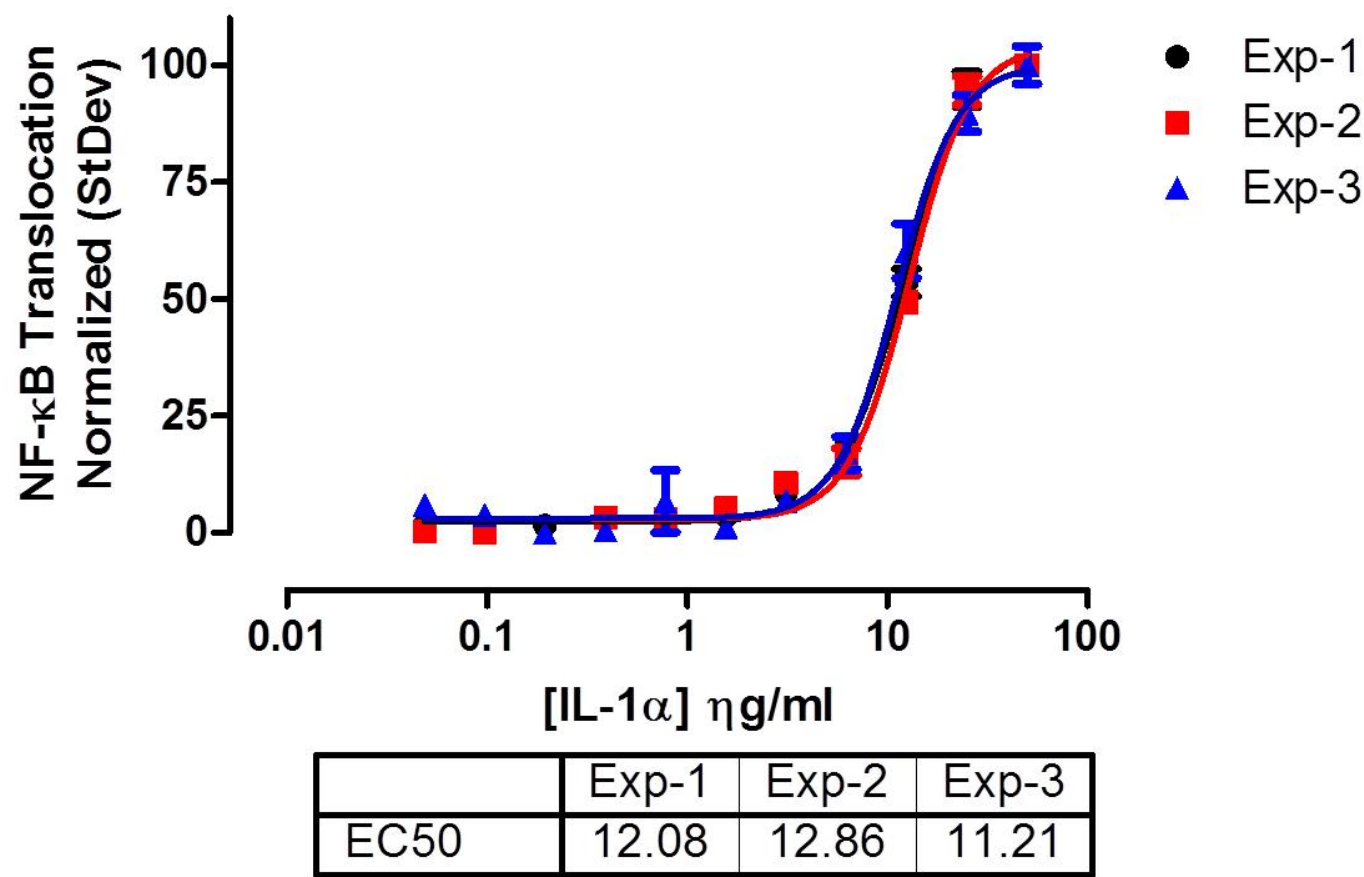


Figure 23: IL-1α dose response experimental variability. HeLa cells seeded at 5,000 cells/well overnight and treated with IL-1α in dose response for ~35 minutes were fixed and then stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized and plotted in GraphPad Prism using non-linear regression 3-parameter fit.

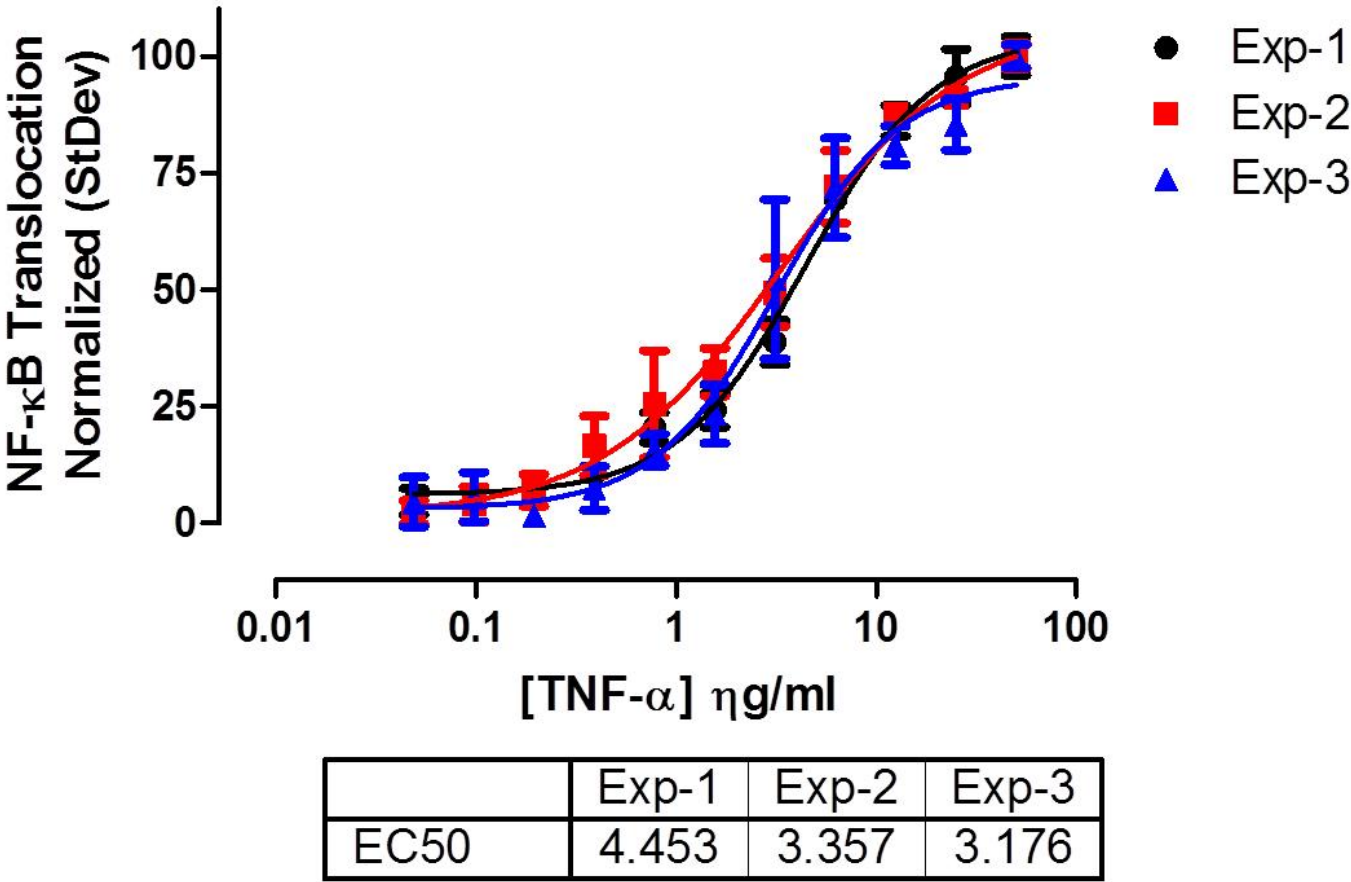
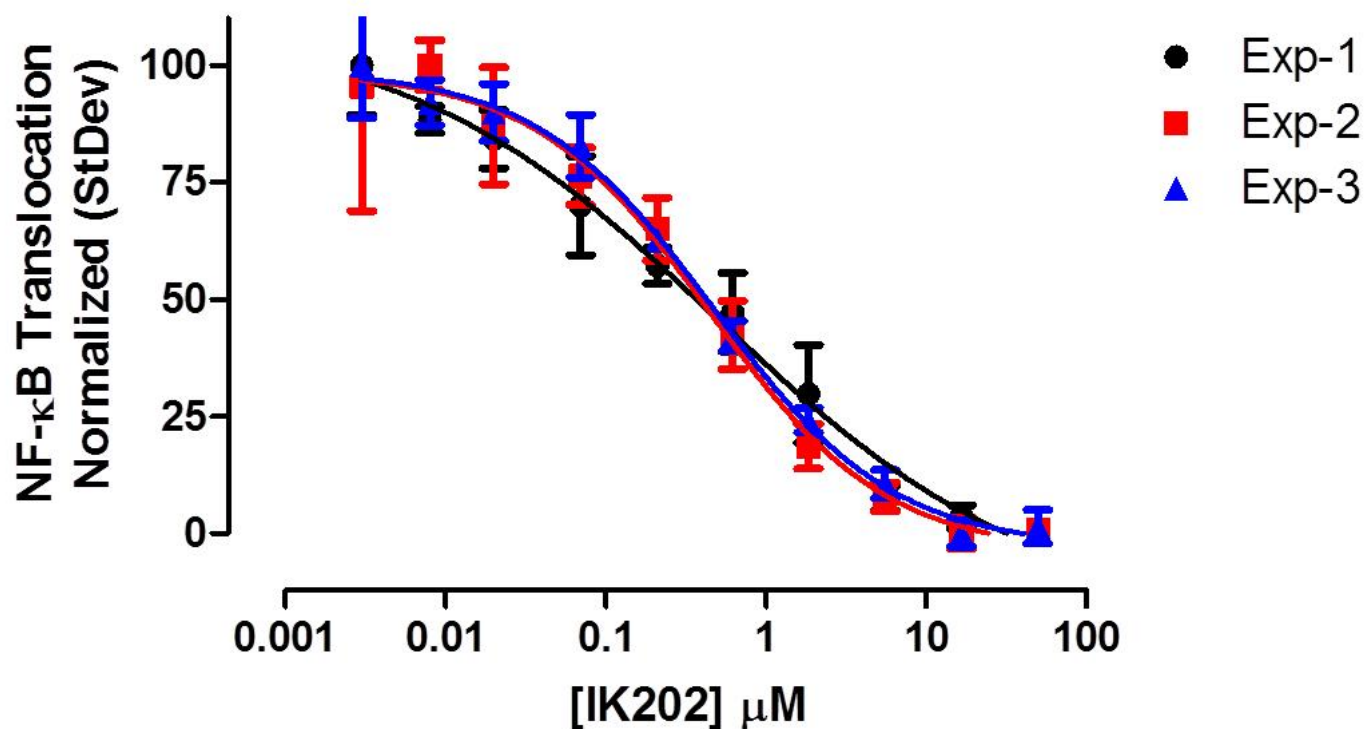


Figure 24: TNF-α dose response experimental variability. HeLa cells seeded at 5,000 cells/well overnight and treated with TNF-α in dose response for ~35 minutes were fixed and then stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized and plotted in GraphPad Prism using non-linear regression 3-parameter fit.



	Exp-1	Exp-2	Exp-3
EC50	0.4985	0.4474	0.4717

Figure 25: Reference compound inhibitor IK22 dose response 3 day experimental variability. HeLa cells seeded at 5,000 cells/well overnight and treated in dose response with reference compound inhibitors IK22 for 15 minutes followed by stimulation with 25ng/ml TNF- α for ~35 minutes. Cells were then fixed and stained to measure NF- κ B translocation. Plates were analyzed on HCS imager to determine NF- κ B translocation using CytoNuc Difference calculation; data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit.

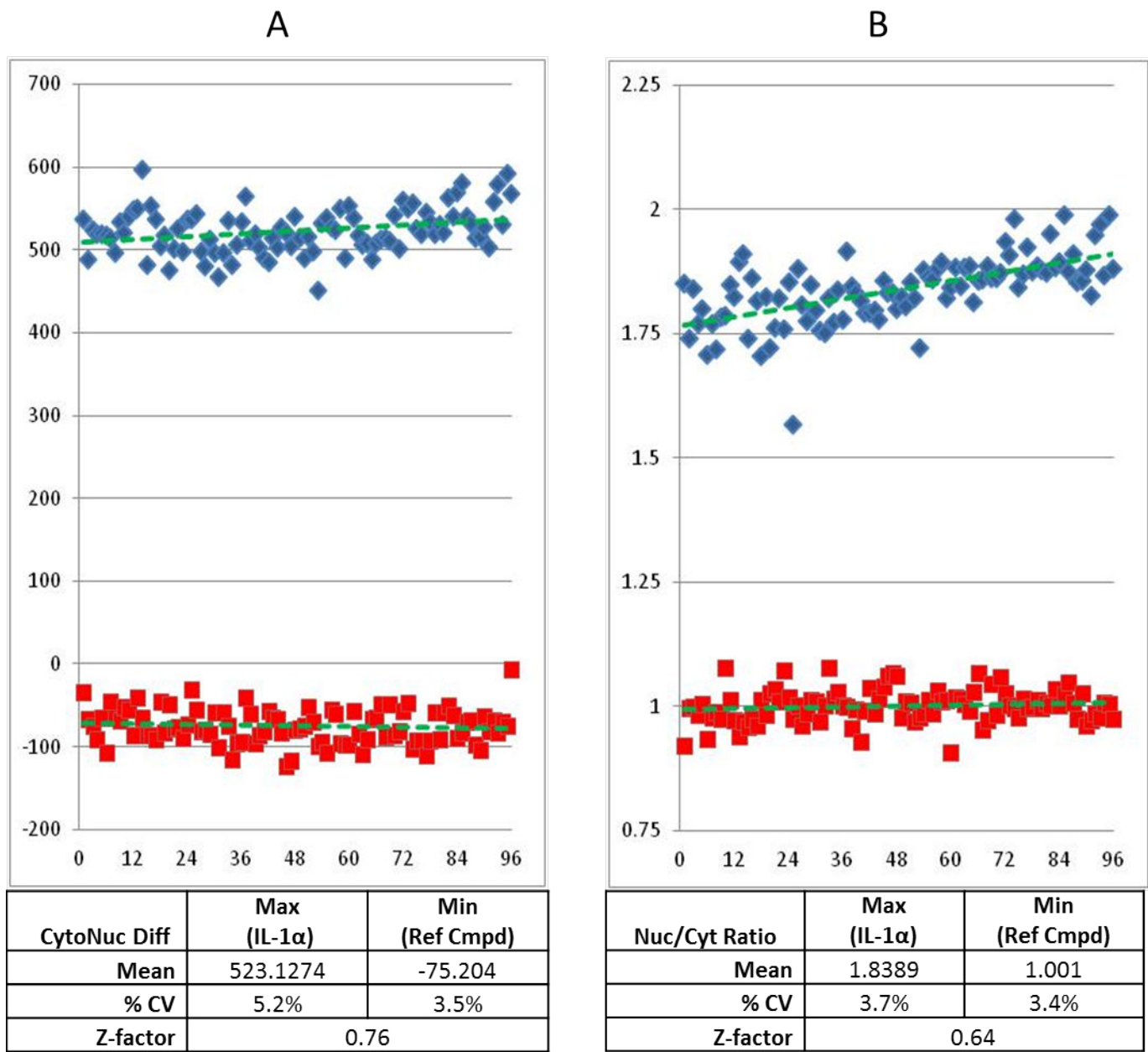


Figure 26: NF-κB translocation IL-1α stimuli Z-factor calculation & graph display. HeLa cells seeded at 5,000 cells/well overnight into 3 different 96-well plates were treated with 0.5% DMSO for maximum response (Red), media for unstimulated (green), and reference compound inhibitors IK202, 50μM for minimum response (blue) for 15 minutes followed by stimulation with 25ng/ml IL-1α for ~35 minutes. Cells were then fixed and labeled to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference (Panel A) or Nuc/Cyt Ratio (Panel B) calculation. Raw data values from HCS instrument were used to calculate the mean response (left axis) and standard deviation (right axis). Z-factor was calculate using the formula $1 - [3 \times (\text{standard deviation of positive control} + \text{standard deviation of negative control}) / (\text{mean of positive control} - \text{mean of negative control})]$ (33).

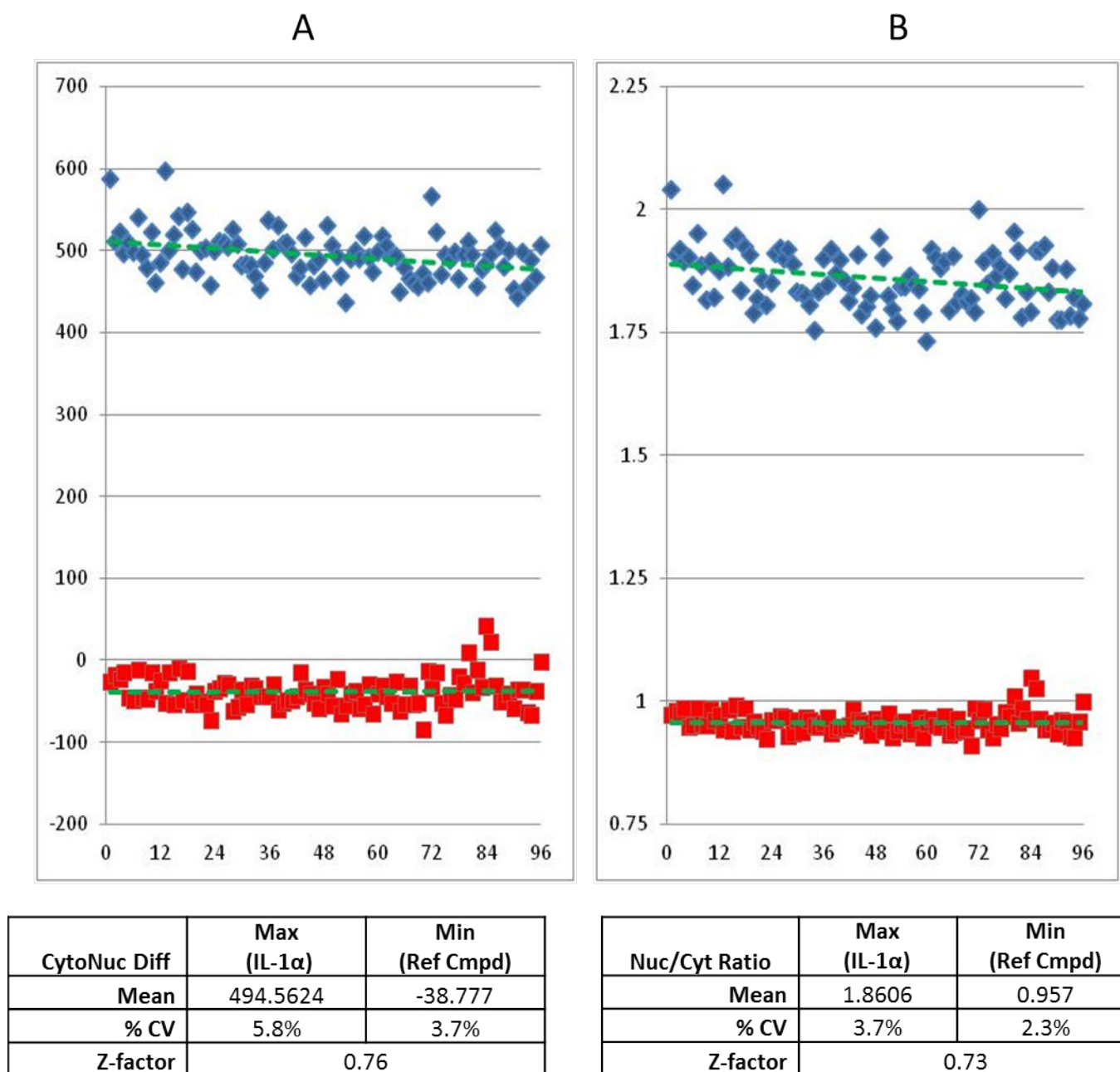


Figure 27: NF- κ B translocation TNF- α stimuli Z-factor calculation & graph display. HeLa cells seeded at 5,000 cells/well overnight into 3 different 96-well plates were treated with 0.5% DMSO for maximum response (Red), media for unstimulated (green), and reference compound inhibitors IK202, 50 μ M for minimum response (blue) for 15 minutes followed by stimulation with 25 η g/ml TNF- α for ~35 minutes. Cells were then fixed and labeled to measure NF- κ B translocation. Plates were analyzed on HCS imager to determine NF- κ B translocation using CytoNuc Difference (Panel A) or Nuc/Cyt Ratio (Panel B) calculation. Raw data values from HCS instrument were used to calculate the mean response (left axis) and standard deviation (right axis). Z-factor was calculated as previously described.

Table 3: List of materials and reagents

Reagents	Vendor	Cat #
Alexa 488 goat anti-rabbit IgG (H+L, 2 mg/ml)	Invitrogen	A-11008
Albumin from Bovine Serum (BSA)	Sigma	A-2153
Dimethylsulfoxide (DMSO), 78.13 g/mole	Thermo	20684

Table 3 continued from previous page.

Reagents	Vendor	Cat #
DPBS (PBS), Mg ²⁺ and Ca ²⁺ free	Lonza	17-512Q
Fetal Bovine Serum, Defined	Thermo	Sh30070
Formaldehyde, 37%	Sigma	F-1268
*Formaldehyde, Ultrapure, MeOH free, 10%	Polysciences	04018
*Paraformaldehyde, Ultrapure, MeOH free, 16%	Polysciences	18814-20
HeLa cells	ATCC	CCL-2
Hoechst 33342	Invitrogen	H-21492
L-Glutamine, 200 mM	Lonza	17-605E
Minimum Essential Medium Eagle (EMEM)	Lonza	12-662F
NF-κB p65 (C-20) Rabbit Polyclonal IgG, 200 µg/ml	Santa Cruz	SC-372
Penicillin/Streptomycin, 10000 Units	Lonza	17-602E
Polyoxyethylensorbitan monolaurate (Tween-20) 10%	Roche	1332465
Recombinant Interleukin -1α (rh IL-1α)	R&D Systems	200-LA
Recombinant Tumor Necrosis Factor-α (rh TNF-α)	R&D Systems	210-TA
t-Octylphenoxypoly-ethoxyethanol (Triton X-100), 10%	Roche	1332481
Trypsin-EDTA, 1X (0.05%)	Invitrogen/Gibco	25300-054
Water, Reverse Osmosis (RO-H ₂ O)	House	
Reference Compound(s)		
BAY 11-7082	Enzo	BML-EI278
BAY 11-7085	Enzo	BML-EI279
Consumables		
12-Place Dilution Reservoir	USA Scientific	1301-1212
150 cm ² Cell Culture Flask (T-150)	Corning	430825
96-well Packard View Plates	Perkin Elmer	6005182
96-well V-bottom Plates	Nunc	245128
Backing Tape, Black	Perkin Elmer	6005189
Cell Strainer, 70 µM	BD Falcon	352350
Plate Seals	Excel Scientific	100-SEAL-PLT
Reagent Reservoir, 50 ml	Corning (Costar)	4870
Storage Mats, 96-well plate	Corning (Costar)	3092

* Alternative fixative solution; recommend a final solution of 4% paraformaldehyde but test in assay model to verify performance.

Preparation of Stock Reagents:

1. Reference compound (IK202), 10 mM: Make a 10mM stock solution by dissolving 10mg IK202 compound (MW=202.3) into 4.95 ml DMSO. Store at -80°C.
2. Hoechst 33342, 2 mg/ml: Make a 2 mg/ml stock solution by dissolving in 100% DMSO. Protect from light. Store aliquots at -80°C ~indefinitely.

3. IL-1 α , 10 $\mu\text{g}/\text{ml}$: Make a 10 $\mu\text{g}/\text{ml}$ stock solution by dissolving in PBS containing 0.1% BSA. Store aliquots at -80°C for up to 6 months.
4. TNF- α , 10 $\mu\text{g}/\text{ml}$: Make a 10 $\mu\text{g}/\text{ml}$ stock solution by dissolving in PBS containing 0.1% BSA. Store aliquots at -80°C for up to 6 months.

Working Solutions per 96-well Plate (Prepared daily)

1. Inhibitor compound: Dilute in EMEM media without serum at 5X final concentration (50 μM final at 250 μM). For IK202 compound, dilute 25 μl of 10mM stock into 1ml of EMEM media. Note final DMSO concentration should not exceed 1%.
2. IL-1 α : Make working solution at 6X (150 ng/ml) the final assay concentration, which is 25 ng/ml . Dilute 15 μl of 10 $\mu\text{g}/\text{ml}$ cytokine into every 1ml of EMEM media needed to complete the assay. For one 96-well plate dilute 45 μl of 10 $\mu\text{g}/\text{ml}$ cytokine stock in 3ml of EMEM. Note, for EC_{50} , start at 40 ng/ml final and dilute 1:3 in PBS or medium. For time course or kinetics study, use 25 ng/ml .
3. TNF- α : Make working solution at 6X (150 ng/ml) the final assay concentration, which is 25 ng/ml . Dilute 15 μl of 10 $\mu\text{g}/\text{ml}$ cytokine into every 1ml of EMEM media needed to complete the assay. For one 96-well plate dilute 45 μl of 10 $\mu\text{g}/\text{ml}$ cytokine stock in 3ml of EMEM. Note, for EC_{50} , start at 40 ng/ml final and dilute 1:3 in PBS or medium. For time course or kinetics study, use 25 ng/ml .
4. Formaldehyde: Dilute 37% formaldehyde 1:10 with PBS. Warm to 37°C before use, this must be prepared fresh with each assay run.
5. 0.1% Triton X-100: Dilute 250 μl of 10% Triton X-100 in 24.75 ml of PBS. Prepare fresh daily.
6. 0.01% Tween-20: Dilute 50 μl of 10% Tween-20 in 50ml of PBS. Prepare fresh daily.
7. Primary Rabbit anti-NF- κB -p65 polyclonal antibody: Make 5 $\mu\text{g}/\text{ml}$ 1 $^{\circ}$ antibody solution by diluting antibody 1:40 in PBS (137.5 μl of antibody into 5.5ml of PBS). Prepare fresh daily.
8. Goat anti-Rabbit Alexa-488 Secondary Antibody / Hoechst Stain: Make a 10 $\mu\text{g}/\text{ml}$ 2 $^{\circ}$ antibody solution by diluting antibody 1:400 in PBS (27.5 μl of 2 $^{\circ}$ antibody into 5.5ml of PBS). Add 5.5 μl of 2 mg/ml Hoechst 33342 stock solution for a 2 $\mu\text{g}/\text{ml}$ solution (1:1000 dilution). Prepare fresh daily and protect from light.

Plate Layout

Plate controls used as experimental references can be placed in any wells throughout the plate as long as the “data analysis calculator” is programmed to interrupt the plate layout conditions and concentrations of compound. For simplicity and a guideline use the example plate layout illustrated in Box 1 with controls on the outside columns and all test compounds in the center of the plate. Edge effects for the assay are minimal because of the short incubation period.

Box 1: Example of a Plate Layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO											DMSO
B	DMSO											DMSO
C	MIN											MAX
D	MIN											MAX
E	MIN											MAX
F	MIN											MAX
G	DMSO											DMSO
H	DMSO											DMSO

Protocol for finding Inhibitors (Antagonist) in the NF- κ B Pathway

Note: All washes and buffers are at 100 μ l unless indicated otherwise. Assays can be performed with automated hand-held pipettes for less than 10-plates. For more than 10-plates it is recommended to use automated plate washers and liquid handling devices.

Day-1

1. Seed approximately 5,000 cells/well in 100 μ l volume of EMEM media containing 0.5% FBS, 2 mM L-Glutamine in 96-well plate (recommend using automated cell dispensing device for uniformity)
2. Allow cells to attach overnight in complete medium at 37°C, 5% CO₂, 95% relative humidity.

Day-2

3. Transfer 25 μ l 37°C pre-warmed compound from v-bottom compound plates to cell plate using automated liquid handling device.
4. Incubate for 15 minutes at 37°C, 5% CO₂, 95% relative humidity.
5. Transfer 25 μ l 37°C pre-warmed cytokine (IL-1 α or TNF- α) from v-bottom compound plates to cell plate using automated liquid handling device.
6. Incubate for 35 minutes at 37°C, 5% CO₂, 95% relative humidity.
7. Remove all media using hand-held aspirator or with automated liquid handling device.

Note: option to add high concentration of fixative directly to the cells or remove media and add final concentration of fixative buffer.

8. Immediately fix cells by adding 100 μ l of “pre-warmed” 3.7% formaldehyde solution in a vented hood or with an automated liquid handling device.
9. Incubate at room temperature for 10 minutes with plate lid.
10. Remove formaldehyde and replace with 100 μ l of PBS using an automated liquid handling device. Please read safety precautions before working with formaldehyde.

Note: At this step, you can stop the experiment and store plate at 4°C for several days by filling wells with salt solution and sealing to prevent evaporation.

11. Add 100 µl of 0.1% Triton X-100/PBS working solution and incubate 5 minutes at room temperature (RT). Use automated liquid handling device as needed.
12. Wash plate 2X with 100 µl PBS at RT, leaving PBS on cells.
13. Use 96-head aspirator to remove buffer or use an automated liquid handling device. DO NOT ALLOW WELLS TO DRY.
14. Immediately add 50 µl of 1^o antibody using hand-held pipettor or automated liquid handling device. Incubate for 60 min at RT.
15. Remove antibody and discard. Wash plate 1X with 100 µl of 0.01% Tween 20/PBS.
16. Incubate for 15 minutes at RT.
17. Wash 2X with 100 µl PBS at RT, leaving PBS on cells.
18. Use 96-head aspirator or automated liquid handling device to remove buffer. DO NOT ALLOW WELLS TO DRY.
19. Immediately add 50 µl of 2^o antibody containing Hoechst dye per well.
20. Incubate 1-hour in the dark at RT. Protect from light by using black tape plate lids or foil.
21. Wash with 100 µl of 0.01% Tween20/PBS. Incubate for 10 minutes at RT.
22. Wash plate with 100 µl PBS at RT
23. Add 200 µl PBS and seal plates.
24. Analyze plates on High Content Imaging device. If required, plates can be store at 4°C in dark for future analysis. Do not freeze plates.

Protocol for Activators (Agonist) of NFκB Pathway

Note: All washes and buffers are at 100 µl unless indicated otherwise. Assays can be performed with automated hand-held pipettes such as Matrix for less than 10-plates. For more than 10-plates, automated plate washers, Multidrop, or MultiMek should be used.

Day-1

1. Plate approximately 5,000 cells/well in 100 µl volume of EMEM media containing 0.5% FBS, 2mM L-Glutamine in 96-well plate (Multidrop)
2. Allow cells to attach overnight in complete medium at 37°C, 5% CO₂, 95% relative humidity.

Day-2

1. Transfer 25 µl of 37°C pre-warmed unknown agonist or known agonist such as cytokines (IL-1α or TNF-α) from v-bottom compound plates to cell plate using automated liquid handling device.
2. Incubate for 35 minutes at 37°C, 5% CO₂, 95% relative humidity.
3. Remove all media using hand-held aspirator or with automated liquid handling device.

Note: option to add high concentration of fixative directly to the cells or remove media and add final concentration of fixative buffer.

4. Immediately fix cells by adding 100 μ l of “pre-warmed” 3.7% formaldehyde solution in vented hood or with an automated liquid handling device.

5. Incubate at room temperature for 10 minutes with plate lid.

6. Remove formaldehyde and replace with 100 μ l of PBS using an automated liquid handling device. Please read safety precautions before working with formaldehyde.

Note: At this step, you can stop the experiment and store plate at 4°C for several days by filling wells with salt solution and sealing to prevent evaporation.

7. Add 100 μ l of 0.1% Triton X-100/PBS working solution and incubate 5 minutes at room temperature (RT). Use automated liquid handling device as needed.

8. Wash plate 2X with 100 μ l PBS at RT, leaving PBS on cells.

9. Use 96-head aspirator to remove buffer or use an automated liquid handling device. DO NOT ALLOW WELLS TO DRY.

10. Immediately add 50 μ l of 1^o antibody using hand-held pipettor or automated liquid handling device. Incubate for 60 minutes at RT.

11. Remove antibody and discard. Wash plate 1X with 100 μ l of 0.01% Tween20/PBS.

12. Incubate for 15 minutes at RT.

13. Wash 2X with 100 μ l PBS at RT, leaving PBS on cells.

14. Use 96-head aspirator or automated liquid handling device to remove buffer. DO NOT ALLOW WELLS TO DRY.

15. Immediately add 50 μ l of 2^o antibody containing Hoechst dye per well.

16. Incubate for 1-hour in the dark at RT. Protect from light by using black tape plate lids or foil.

17. Wash with 100 μ l of 0.01% Tween20/PBS. Incubate for 10 minutes at RT.

18. Wash plate with 100 μ l PBS at RT

19. Add 200 μ l PBS and seal plates.

20. Analyze plates on High Content Imaging device. If required plates can be store at 4°C in dark for future analysis. Do not freeze plates.

Note: Cells will respond to cytokines in the presence of serum and NF- κ B will translocate to nucleus. For step-3, be sure to adjust either the volume of medium or the amount of cytokine.

Logistics Analysis of Protocol

1. Projected # plates/day: state number of plates that can be processed per day
2. Suggested equipment: automated cell dispensing devices, automated liquid handling devices, 96-well aspirator, HCS imager
3. Timing issues, stop points: After fixation step
4. Stability and process studies: Compound and cytokine freeze-thaw stability acceptable
5. Dealing with multiple lots of reagents: None
6. Reagent and supplies availability: None
7. Flow Chart: None

8. Safety considerations: Example, formaldehyde use, see appendix-6
9. Data Collection Issues: state electronic location of data storage
10. Estimated throughput: state number of plates that can be processed per day
11. Batching Plates/Stop Points:
12. 3.7% formaldehyde fixation – STOP POINT - plates can be stored for several days at 4°C in PBS.
13. Permeabilization with 0.1% Triton X-100 – BATCHING – handle as many plates that can be processed under 15-minute incubation period.
14. Wash steps – BATCHING – do not allow wells to dry and do not introduce air bubbles during wash and aspiration steps. Handle one plate at a time unless automation devices are used.
15. Post staining, after plate is sealed – STOP POINT – store plates at 4°C in the dark and read on HCS Imager within 14-days.

Cell Based Screen

1. Cell Handling for HeLa cells
 - a. Cell Bank – *location of cells in the cell bank*
 - b. Continuous Passage – Yes, maximum passage is not known.
 - c. Transfection – No
 - d. Proposed Schedule – continuous passage
 - e. Cell Culturing Guidelines:
 - i. Subculturing: Remove medium, and rinse with PBS. Remove the solution and rinse cell monolayer with Trypsin-EDTA solution. Remove and allow flask to sit at room temperature for 3-5 minutes or until the cells round up. Lightly tap flask to detach cells. Add fresh culture medium containing at least 0.5%FBS, aspirate and dispense into new culture flasks.
 - ii. Split Ratio: A subcultivation ratio from 1:2 to 1:10 is recommended
 - iii. Fluid Renewal: 2 to 3 times per week
2. Propagation: Suggested medium: Minimum essential medium Eagle with 2 mM L-glutamine, fetal bovine serum, 10%; 1% (100Units/ml) Penicillin/Streptomycin.
Temperature: 37°C , 5%CO₂
3. Freeze Medium: culture medium 90%; DMSO, 10%

Compound Libraries

It is recommended to use a library of compounds to test the sensitivity and robustness of the assay for both negative and positive responses. There is a unique NF-κB inhibitor set of 14 -compounds available from Calbiochem (Cat#481487 InhibitorSelect™ NF-κB Signaling Pathway Inhibitor Panel). There are now 4 versions of this library with the latter ones containing IκK inhibitors. Additional there are other larger compound library collections commercially available for purchase including LOPAC. For this assay the Calbiochem kinase Library, otherwise known as the Millipore EMD 539744 InhibitorSelect™ 96-Well Protein Kinase Inhibitor Library-I was the only commercial library available. Interestingly, this 80 chemical compound library contains no NF-κB or IκK inhibitor compounds and this is reflected in the results. For single point determination at 50 μM, two compounds showed an increase in NF-κB CytoNuc difference was a result in changes in the morphological characteristics of the cell in assay both with IL-1α and TNF-α stimulation (Figures 28 and 29). No activates were confirmed with IL-1α or TNF-α stimulation IC₅₀ curves follow-ups from 50μM, 3-fold in duplicates.

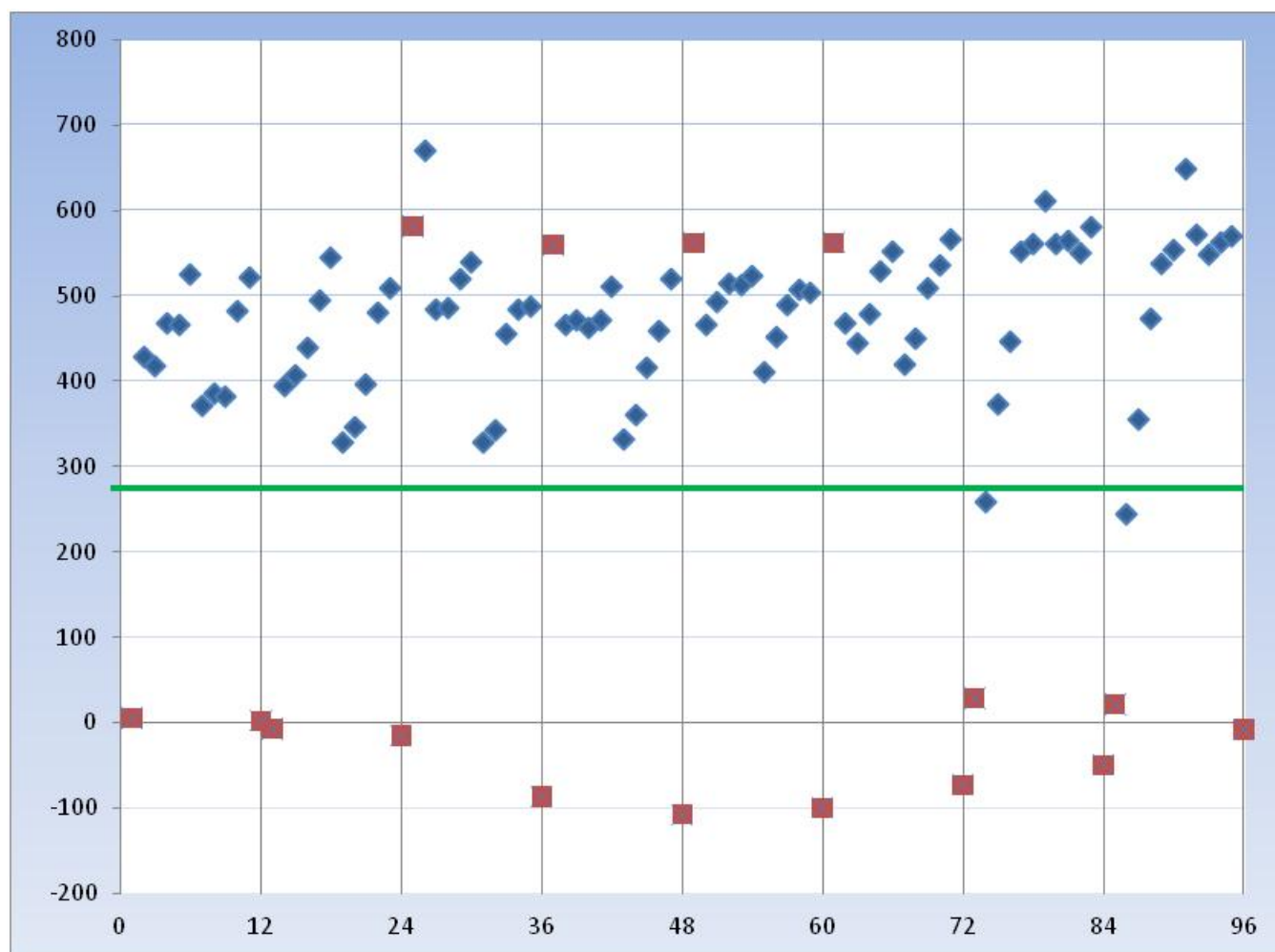


Figure 28: Results from Calbiochem kinase library (EMD 539744 InhibitorSelect™) screen. HeLa cells seeded at 5,000 cells/well overnight and dosed with a single dose of 50 μ M compound for 15 minutes followed by stimulation with 25ng/ml TNF- α for ~35 minutes. Cells were then fixed and labeled to measure NF- κ B translocation. Plates were analyzed on HCS imager to determine NF- κ B translocation using CytoNuc Difference calculation. Raw data values from HCS instrument were used to calculate the mean response. Controls represented as “red squares” and compound tested shown in “blue triangles”. Green line represents ~50% activity; points below this line are considered “hits”. No inhibitors were confirmed; one compound showed an increase in NF- κ B Nuc-Cyt difference that was a result in changes in the morphological characteristics of the cell in assay both with IL-1 α and TNF- α stimulation. Images not shown.

Other Considerations

Detection of Cytotoxicity and Fluorescent Compounds in NF- κ B Translocation Assay

One of the powers of HCS is providing the capability to measure morphological and intensities of bioprobe markers at the single cell level. The combination of these capabilities allows the ability to measure cell populations and subpopulations captured in the image. If a compound dosing concentration is the direct or indirect cause of cytotoxicity it is often measureable with good precision by using population statistics of the number of cell objects detected in the well in comparison to a vehicle control well. Optional, cytotoxic and viable dyes can be used in combination with the biomarker indicator as an reporter. HeLa cells seeded overnight, then pre-incubated with 10-point compound dose response starting at 50 μ M, $\frac{1}{2}$ log for 15 minutes and subsequently treated with TNF- α for 35 minutes, were stained with NF κ B antibody as previously described. The inhibition of NF- κ B in the presence of the known reference compound recorded an IC₅₀ of 0.2095 μ M using 3-parametric fit

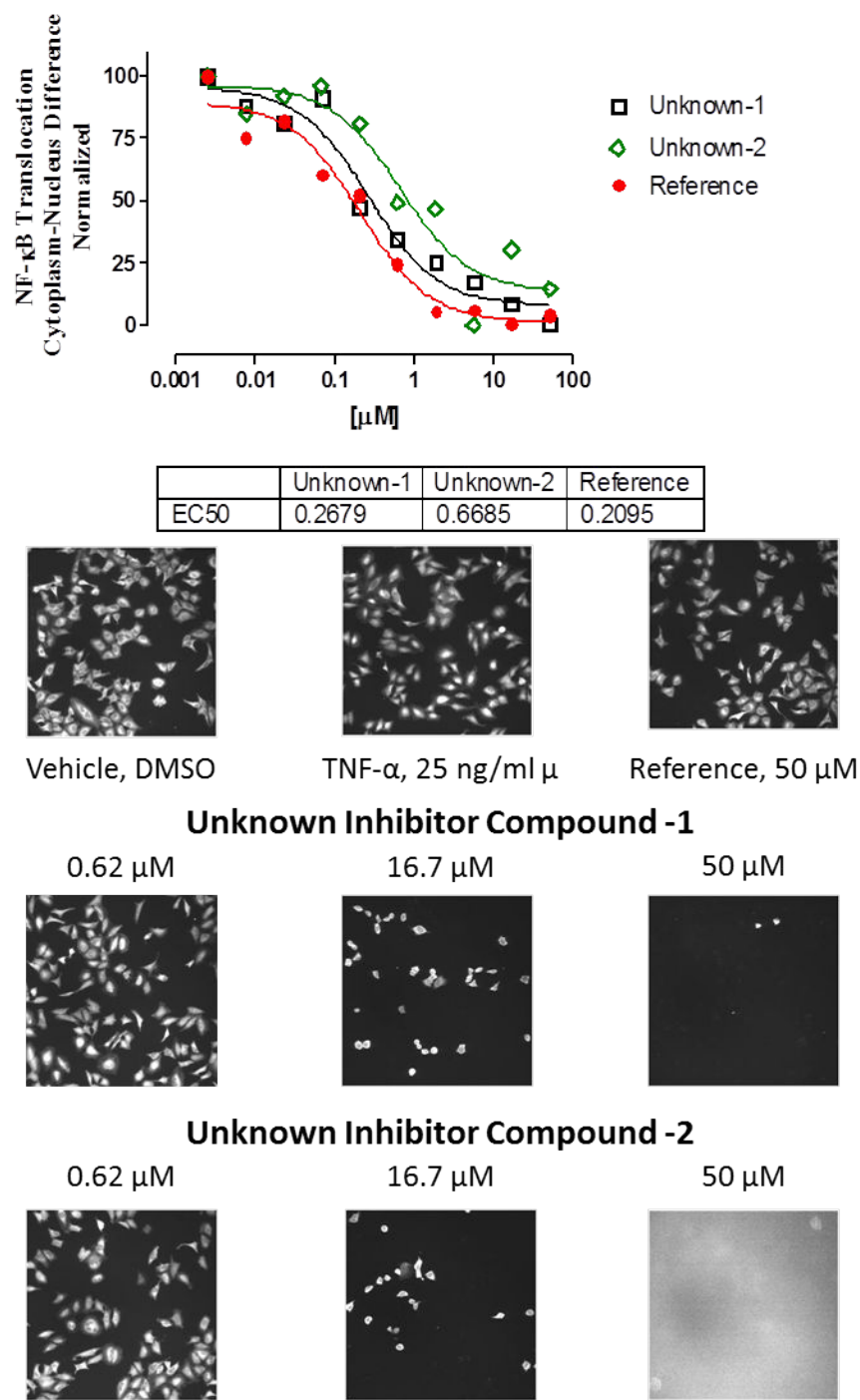


Figure 29: Reference compound and unknown inhibitor dose response with corresponding images. Top panel is a comparison of reference compound inhibitor and two unknown inhibitors identified as actives in screen. HeLa cells seeded at 5,000 cells/well overnight and treated in dose response with reference compound inhibitors IK202 or two unknown inhibitor compounds for 15 minutes followed by stimulation with 25ng/ml TNF-α for ~35 minutes. Cells were then fixed and stained to measure NF-κB translocation. Plates were analyzed on HCS imager to determine NF-κB translocation using CytoNuc Difference calculation; data was normalized to control and plotted in GraphPad Prism using non-linear regression 3-parameter fit. Bottom panel of images: (A) vehicleI DMSO) control; (B) TNF-α, 25ng/ml control; (C) IK202, 50μM reference compound inhibitor; (D) Unknown inhibitor compound-1, 0.62μM; (E) Unknown inhibitor compound-1, 16.7μM; (F) Unknown inhibitor compound-1, 50μM; (G) Unknown inhibitor compound-2, 0.62μM; (H) Unknown inhibitor compound-2, 16.7μM; (I) Unknown inhibitor compound-2, 50μM.

in GraphPad Prism, whereas the unknown inhibitor compounds recored and IC₅₀ of 0.2679 μM and 0.6685 μM. The differences in these values is somewhat subtle in the curve fitting; however, the key differences is observed in

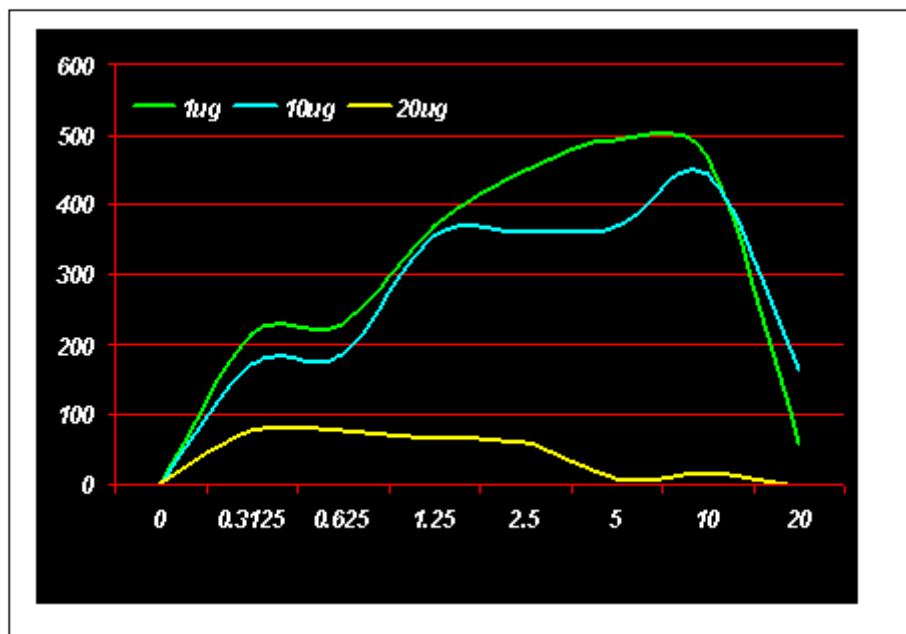
the loss of cells with increasing concentration of the compound above the recorded IC₅₀ value. It is important to always review and record the number of cellular objects, fields, or other HCI feature parameters that provide information about an alteration in the cell seeding density in the well in addition to other key HCS feature values including reviewing the images to validate the results (Figure 30).

To determine if an unknown compound(s) is fluorescent in the visible spectra when screening a chemical compound library using HCS assays can be achieved by using intensity measurements in the nucleus or cytoplasm regions of interests (ROI). These compounds can create a false positive and are not commonly identified until follow ups are underway in protein redistribution assay such as NF κ B where fluorescence can occupy both the cytoplasm and nuclear ROI. If a suspect compound is thought to be fluorescent in the HCS assay, this can be verified using a high content imager by measuring the baseline autofluorescence of cells using vehicle control, then measure any increase in fluorescence following compound treatment. Evidence of compound fluorescence in the cells is typically observable using fluorescent microscopy images not shown.

Screening Commercially Available NF- κ B Antibodies (Appendix 1)

NF- κ B Antibody Staining (Santa Cruz, SC-372)

Secondary labeling concentration; 1, 10, 20 μ g/ml.



Graph of commercially available antibodies evaluated

X-axis represents 1°Ab concentration with 10 μ g/ml 2°Ab;

Y-axis represents average fluorescent intensity of NF- κ B difference between the cytoplasm and nucleus subtracted from the 2°Ab control. Values listed below x-axis are the raw data (CytoNuc Diff).

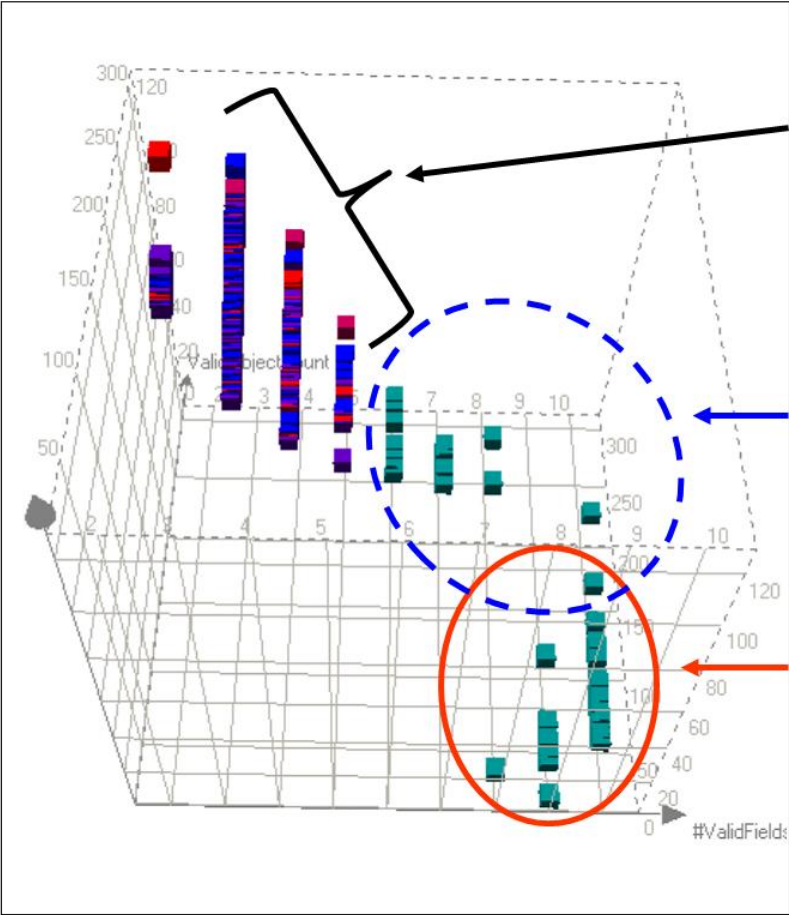
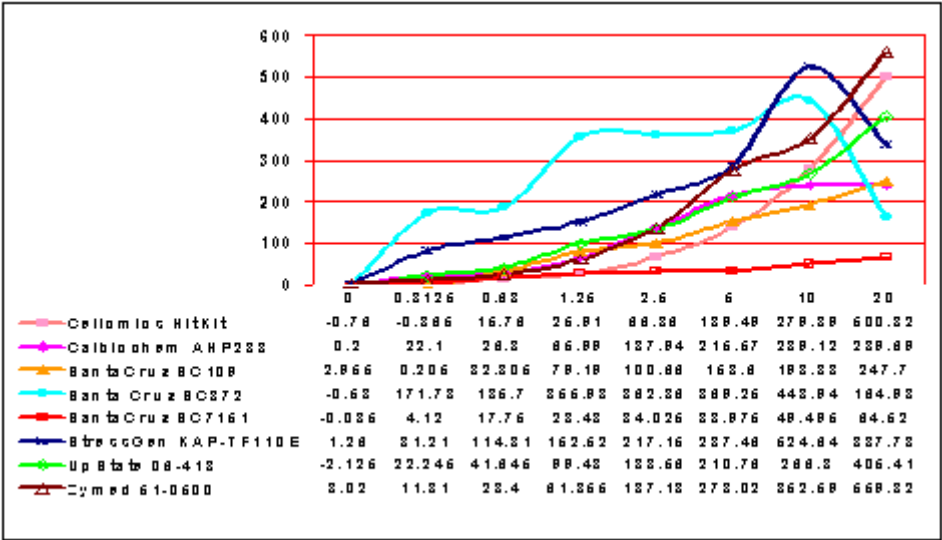


Figure 30: SpotFire 3-D graph visualaization: well level plate HCS features showing separation between normal cell distrubution, altered morphology, or cell loss and/or cytotoxic effects. X axis = number of valid fields; Y axis = valid object count; Z axis = average cell density/field



Safety Considerations: Guidelines & Precautions (Appendix 2)

Please follow institutional laboratory safety considerations and special precautions with regard to formaldehyde and paraformaldehyde use. Training is commonly required for all personnel using formaldehyde and paraformaldehyde

Formaldehyde and Paraformaldehyde Use Guidelines in the Laboratory:

1. All stock solutions greater than 4% must be maintained in a fume hood.
2. Working solution of less than 4% and less than 37°C can be used in the following areas:
 - Fume hood
 - Biosafety cabinet
 - Automation liquid handling robots with standard setup with <4% formaldehyde solution in appropriate reservoir positions. Following addition of formaldehyde, plate lids must cover open plates to minimize formaldehyde exposure during a 10-15 minute incubation period. Transfer of formaldehyde to and from the liquid handling robot reservoir requires a safety container that is larger than the stock reservoir container to collect any spill. It is recommended that the volume of working solution of formaldehyde does not exceed 100ml at any given time. If additional formaldehyde solution volume is required than add as needed.
3. Transport of formaldehyde working solution between laboratories and pre-warming to 37°C requires standard lid cover to prevent potential exposure.
4. Formaldehyde waste should be less than 0.1% before it is discarded; be sure to verify per institution or government ordinance.
5. Formaldehyde mixture with DMSO requires normal DMSO disposal procedures.
6. Stock solutions of 37% formaldehyde contains methanol and disposal of this stock solution should be treated as flammable if appropriate.
7. Report any excessive spill of formaldehyde to your safety department

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High Content Screening with Primary Neurons

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Abstract

Advances in microscopy, instrumentation, and image analysis software, combined with improved protocols for acquiring and maintaining primary neuronal cultures, have made high content analysis (HCA) with primary neurons a routine procedure in many labs. Additional improvements in robotics and automation allow the high-throughput application of HCA in high-content screening (HCS). Given its multiplexed nature, HCS provides an unprecedented amount of multiparametric kinetic and morphogenic information from cells. It holds promise for better understanding of molecular signaling networks, and for easing the bottleneck in the drug discovery process, particularly relating to neurological disorders. Here we describe protocols for preparing different neuronal types and discuss advantages, challenges, pitfalls, and detailed workflows for utilizing them in HCS with various perturbagens.

1. Introduction

High-content screening (HCS) has emerged as a new approach for drug target validation and lead optimization before costly animal testing (1,2), as well as a valuable tool for understanding complex molecular processes in a cellular context (3). Despite years of progress, in understanding the etiology and genetics of numerous disorders afflicting the nervous system, the number of disease modifying therapeutics remains modest. HCS offers a number of advantages over conventional high-throughput screening (HTS) approaches when studying neurons, which will be discussed below.

1.1. What is HCS?

HCS is a screening method based on multiparametric analyses of large scale image data extracted from multiple targets in thousands or millions of cells in multiwell plates (1,2,4). Thus, although high-content analysis (HCA) refers to the extraction of multiparametric information from image data, HCS is the automated medium-to-high-throughput application of HCA in screening campaigns. The concept of HCA dates back to the late 1990s, when the CellomicsTM initiative conceptualized it as a strategy for cataloging multiple molecular processes inside intact cells (5). The initiative was bolstered by the development of fluorescent-protein biosensors that allowed time-dependent examination of processes ranging from membrane activity and cell signal transduction to energy metabolism and cell physiology (2). By utilizing the correct combination of probes, and by spatially (and sometimes temporally) resolving readouts of individual cells, HCA can provide quantitative measurements of nuclear size/shape, DNA content, organelle shape and function, protein modification and intracellular localization, cell movement, and cell shape. HCS can thus produce an enormous amount of morphometric and kinetic outputs.

1.2. Why HCS?

The majority of drug discovery campaigns have utilized HTS methods as originally introduced by the pharmaceutical industry (6). Most HTS assays screen for *in vitro* interactions with a single target, typically using a spectrophotometric or fluorescent readout (7,8). This approach has been extremely successful in identifying

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probes and drug-like molecules with a wide range of selectivities towards intended targets. However, the rate at which new drugs have been reaching the clinic has decreased (9,10). One possible explanation is the difficulty of capturing the complexity of interactions that occur within cells or whole tissues using target-based screening. Although target-based HTS offers a streamlined pipeline for sifting through millions of compounds and conditions, the added burden of performing screens in more complex systems with expanded assay readouts may well be worth the effort (1). However, cell-based screening is usually agnostic to molecular mechanisms of action, hence subsequent determination of drug targets and lead compound optimization can be quite difficult. Fortunately, advancements in chemical proteomics and comprehensive activity panels are quickly improving the outlook for target identification.

The informational gain from investigating signaling networks using unbiased genetic screens in cellular systems, by way of HCS, has also been highlighted (3). Most of our knowledge of signal transduction pathways has come from forward genetic screens in model organisms. These methods have been extremely successful in delineating many of the canonical pathways and their major transducers, they do not sufficiently address the spatial and temporal complexities of signal transduction within cells. For example, different neuronal cell types exhibit significantly distinct RNA expression profiles (11) that likely underlie distinct physiologies. Cell-based genetic screens using various genetic perturbagens, gain- or loss-of-function analyses, will be vital for elucidating the intricate signaling circuitry within specific neuronal types. This may prove essential for identifying appropriate drug targets for the notoriously cell-specific neurological diseases.

The technology for high content analysis (HCA) is becoming more affordable and readily available to both industry and academia, encouraging such endeavors as HCS in primary neurons (12). Technical obstacles have, until recently, hindered screening with primary neuronal systems, including the difficulty of obtaining, culturing, and transfecting primary neurons (13,14). However, new culturing and transfection methodologies now offer a more practical platform for HCA, and by extension HCS, in these cells.

2. Preliminary Assay considerations

Therapeutic agent discovery in the nervous system has thus far trailed behind other areas, implying the need for more relevant screening models (15,16). Choosing a cell type for HCS may be the most important element of the experimental design, while also being the most challenging. Many of the conditions and reagents of the experiment are tailored to the chosen cell type, upon which large investments of time, effort, and money are ultimately made. Thus, a well-reasoned choice of the most relevant cellular model is critical. Additional consideration must be given to the usually narrow range of conditions in which the assay yields the largest separation between positive and negative controls. Choosing the most relevant substrate, treatment conditions, incubation time, and immunostaining protocols can all pose additional challenges that must be overcome before an HCA assay is considered to be appropriate for screening. Detailed protocols appear at the end of this chapter.

2.1. Neuronal Type

Several options are currently available for HCS, each with its own advantages and drawbacks (17). Primary cells are far more physiologically relevant than immortalized cell lines (18), and neurons differentiated *in vivo* best recapitulate actual neuronal subpopulations (12). Primary neurons have gene and protein expression profiles that better resemble those of differentiated cells *in vivo*, and are thus more appropriate for drug target validation (12). Numerous considerations factor into the choice of age and type of primary neurons to be used.

2.1.1. Embryonic versus adult neurons

Historically, primary neurons isolated from embryonic brain have been more frequently utilized than neurons isolated from postnatal or mature brain. This is due to the relative ease of isolating embryonic neurons, in addition to increased viability, improved regenerative ability, and reduced glial growth in embryonic cultures

compared to their adult derived counterparts (19–23). Terminally differentiated CNS neurons are post-mitotic in culture, although neuronal cultures may contain precursors that divide in permissive culture conditions (24). Reduced glial numbers in embryonic brains combined with culture conditions that retard glial growth and proliferation (25) allow for the preparation of relatively homogenous low density neuronal cultures, which can be useful in simplifying immunostaining, HCA readout, and culture consistency. However, the improved regenerative and survival capacity of embryonic neurons combined with the lack of additional cell types that would more reliably recapitulate the biological environment can, for example, lead to false hits (or false negatives) in a screen for neuroprotective agents relevant to the adult CNS. Thus, one should be aware of the balance between choosing the most relevant cellular model and maintaining assay screening suitability. It may prove most efficient to perform HCS with a more convenient cellular model, and to use a secondary filtering assay, such as a slice culture that better recapitulates the cellular and molecular environment of the CNS, to reduce numbers of false positives prior to animal testing (26,27).

2.1.2. Choosing the source of primary neurons

Many types of primary neurons of mouse, rat, or human origin have been successfully used in cell-based screening, and in particular HCS. Regardless of species, neuronal cells of the same type and anatomical origin tend to behave similarly in culture. Cortical neurons have been used in large scale overexpression screens for neurite growth promoting genes (28). Hippocampal neurons have also been used in overexpression (29) and chemical (26) screens for agents that promote neurite growth. Cerebellar granule neurons (CGNs) have been used in overexpression studies, knockdown screening with siRNA (30), and neurotoxicity testing (31). Dorsal root ganglion (DRG) neurons have been used in chemical screens for compounds that mitigate neuronal injury in hyperglycemic conditions (32) as well as knockdown studies of axonal degeneration (33). Retinal ganglion cells (RGCs) have been used to screen, by overexpression, developmentally regulated genes (34). Given the differences in gene expression profiles, as well as morphological dissimilarities among different neuronal types, variability in their respective responses to certain perturbagens is expected (Figure 1). This highlights the importance not only of choosing primary neurons over cells lines, but also specifically selecting the neuronal cell type that best recapitulates the target cell population.

2.2. Choosing a substrate

In most cases, it is preferable to perform HCS with purified neurons attached directly to a culture substrate rather than on or around non-neuronal cells. Non-neuronal cells can rapidly outgrow neuronal counterparts in culture, or confound phenotypic analysis due to neuronal attachment and subsequent migration of non-neuronal cells. Additionally, studying cultures that contain mixed cell types requires the consideration of indirect effects mediated by non-neuronal cells, which can complicate mechanistic interpretations. Therefore, protocols have been developed for either purifying neuronal cells prior to culturing or optimizing conditions for neuronal growth at the expense of other proliferating cell types, such as glia. The elimination of a non-neuronal cell scaffold, however, creates the requirement for an alternative substrate to allow cell adhesion and neurite growth. Polylysine treated surfaces promote cell adhesion and viability of primary neurons (35). Polylysine also promotes reproducible neurite growth in low-density cultures. Both poly-D-lysine (PDL) and poly-L-lysine (PLL) have been used with success, and early studies showed that while the D isomer minimizes aggregation of neurons better than the L isomer, the two elicit little difference otherwise (35). More recently, polyethylenimine was reported to allow adhesion and growth of primary neurons while completely immobilizing the cells after adhesion (36). Polylysine can also constitute a basal adhesive substrate upon which proteins can be loaded. For example, laminin allows growth and spreading of cultured neurons (37) and promotes extension of longer neurites (38) than those grown on polylysine alone. This can be desirable when searching for agents that inhibit axon growth, as it can increase the dynamic range of the assay readout. Inversely, neurite growth inhibitory components, such as chondroitin sulfate proteoglycans (CSPG) and/or myelin derived proteins (39–45) can be applied to inhibit neurite growth and increase biological relevance of the assay when searching for agents that

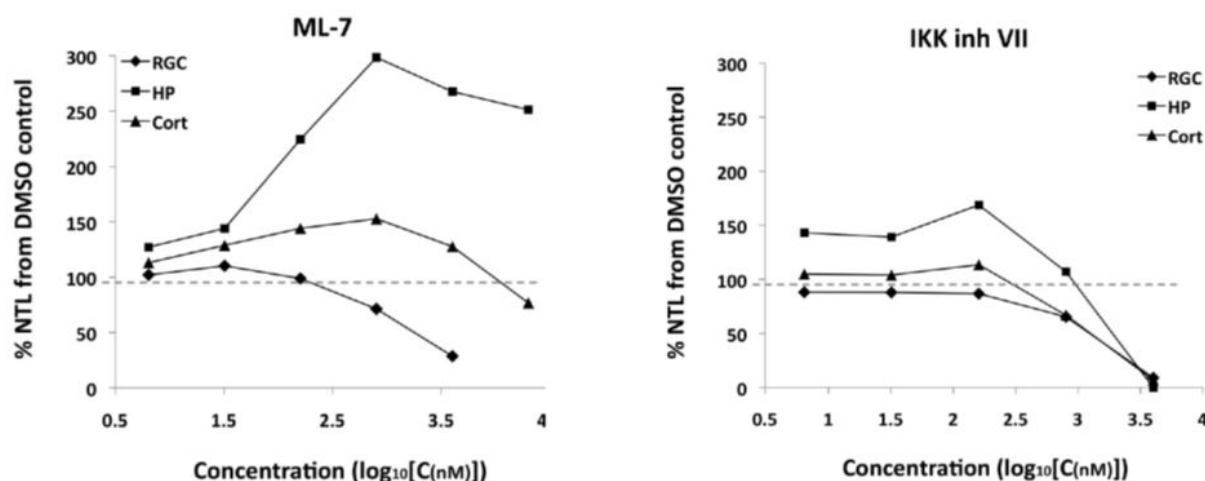


Figure 1: Neurite total length (NTL) in various neuronal cell types treated for 2DIV with protein kinase inhibitors ML-7 and IKK inh VII, relative to DMSO treated controls. HP: E18 hippocampal neurons, CGN: postnatal (P8) cerebral granules neurons, Cort: postnatal (P5) Cortical neurons, RGC: postnatal (P8) retinal ganglion neurons (*unpublished data*).

can promote neurite growth on inhibitory substrates (Figure 2). Naturally, any modifications to the substrate will add complexity to the assay and data interpretation.

3. Assay Development

3.1. Cell culture media

Synthetic media require serum supplementation to maintain survival of cultured primary neurons. In certain cases, supplementation with growth or trophic factors may be necessary. Low density cultures can be sustained directly by an astrocyte feeder layer (46), or indirectly using glial condition media (47,48). Serum supplements, however, introduce significant experimental variability as a result of differences in donor age, sex, nutrition, and physiological state (49). Similar variability can result from conditioned media. To circumvent this problem, defined media supplements were developed to replace serum (49–51). The widely used supplement, B27, was developed and optimized in conjunction with a DMEM derived formulation, Neurobasal, and the B27/Neurobasal combination has been shown to support growth of various neuronal types (25). The B27 supplement was later reformulated into SN21 to further minimize lot variability and enhance the quality of cultures (52). Other reformulations, such as the NeuroCult™ SM1 supplement, are optimized to give reproducibly high numbers of functional neurons and reduced glial cell contamination.

Despite attempts to fully define the culture media, lot and vendor variability can introduce significant noise into the experiment. It is thus preferable to test different lots of reagents and then obtain enough of well-qualified reagents for an entire screening campaign. Of course it is essential that appropriate positive and negative controls be run to ensure reproducibility.

3.2. Cell density and plating method

Desired plating density depends on the application and readout of the assay. High density cultures are required for HCS protocols utilizing neural networks or cellular monolayers, and may be more amenable for screens of neurotoxic or neuroprotective agents (53). Low density cultures, on the other hand, are required for HCS protocols focused on cellular (particularly neuritic) morphology. Unfortunately, however, neurons grow relatively poorly at low density, and small variations in local densities can cause significant differences in neurite morphology (Figure 3). Thus, a sufficient number of cells must be evaluated to accommodate the large variability

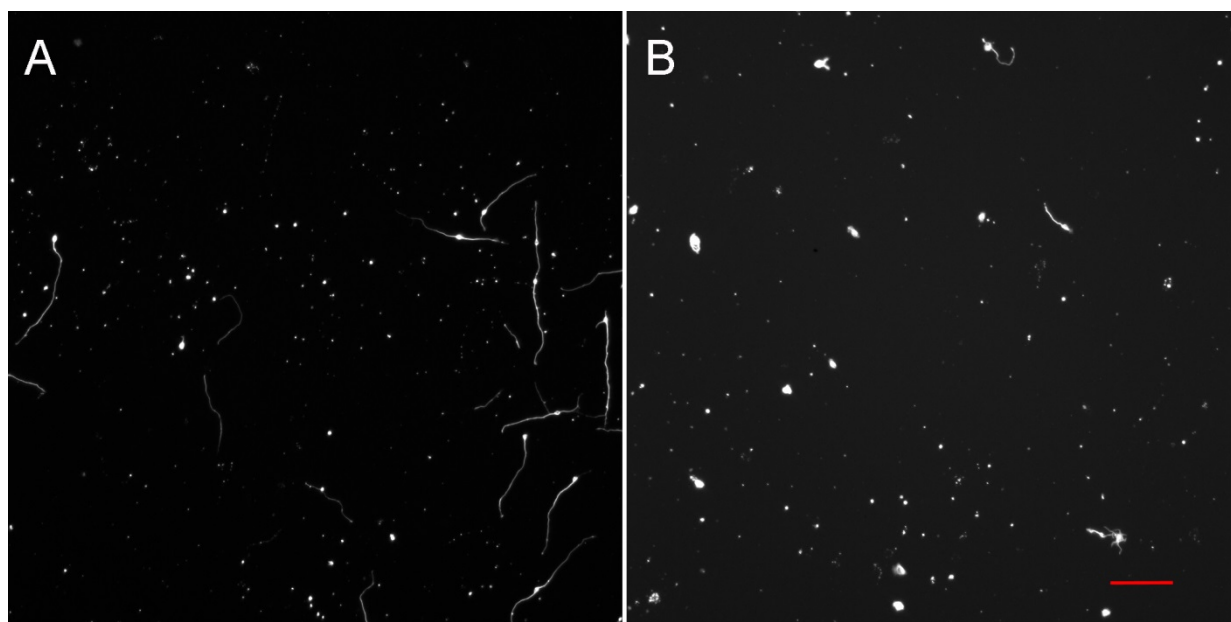


Figure 2: Postnatal (P8) CGN cells grown on (A) PDL/Laminin and (B) PDL/Laminin/CSPG. Scale bar 100 μm (*unpublished data* (29,71,74)).

and non-normal distribution of morphological properties within low density cultures. Usually, 96-well plates offer sufficient area for a low density culture screen with enough neurons per condition/well, although the duration of the experiment depends on the rate at which the neurons extend and overlap processes (overlapped processes render automated cell feature extraction [segmentation] difficult or impossible). For assays that allow or require higher cell densities, 384 well plates may be used. If cells become overlapped sooner than the effect of treatment can be observed, lowering the cell density may be required. This can be achieved by seeding fewer cells or switching to larger area wells. However, because low cell number can dramatically increase noise in the data, it may be more prudent to switch to a larger well size rather than decrease cell number. This is especially true for screens involving transfection, which is often inefficient (transfection rates of 10-50%), and also decreases neuronal viability.

Edge effects are widely recognized in screening departments and can lead to high well-to-well variability and deterioration of overall assay performance (54). Numerous factors can contribute to edge effects, including thermal gradients and differential evaporation rates. An easy work around is to load peripheral wells with water or buffer and avoid using them in the assay.

When seeding cultures of any density, it is best to keep the neurons as uniformly dispersed as possible within the wells. This minimizes perturbations due to variations in local conditions and maximizes the number of cells that can be included in the analysis. When loading cells pre-diluted in media, it helps to do so at low speed to avoid creating too much turbulence which can lead to patterning of cells as they sediment to the bottom. If, on the other hand, cells are being spread in a relatively small volume over a larger volume of media pre-equilibrated within the well, it helps to gently lift and release the entire volume once or twice to insure proper dispersion.

3.3. Endpoint assays and fixation

It has been suggested that the full potential of HCS will be realized with time-lapse imaging of live cells (12). Temporal analysis of cellular behaviors can lead to improved understanding of the phenomena underlying observations made in a phenotypic screen and allow for better differentiation between primary and secondary consequences of treatment (55). However, the magnitude and technical requirement for such an undertaking might be inconvenient for medium to high throughput screens, or perhaps unnecessary for endpoint assays.

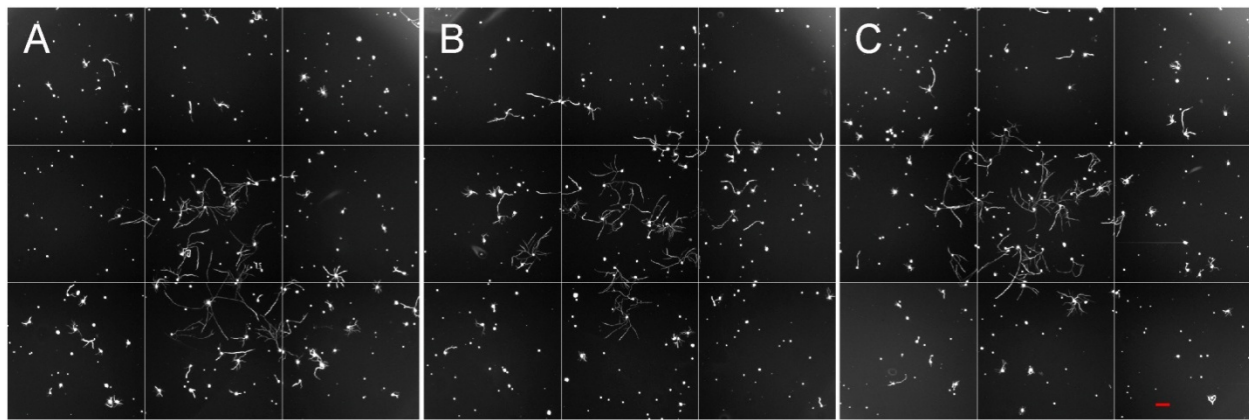


Figure 3: A, B, & C, 9 field image montages of RGC cells grown inside wells of 96-well plates, showing differences in neurite length correlated with cell location and cell density (*unpublished data*).

Additionally, longer-term time-lapse studies, especially those using fluorescence, can result in damage or cell death (though current imaging with sensitive cameras and low light levels can mitigate this problem). Live imaging can also limit the types of features that can be easily measured, such as phospho-epitopes on signaling proteins (which require antibody staining), making an endpoint assay more information-rich. This raises the question of when to terminate the experiment and how to fix the cultures for imaging. Ideally, incubation times should be calibrated to give maximum separation between positive and negative controls (see Section 3.8). This information is normally acquired empirically. The fixation method will depend on the subsequent steps for analyzing the cultures. Typically, cells are fixed in 4% paraformaldehyde in phosphate buffer. Sucrose can be added to the fixative to enhance preservation of membrane structure and processes during cross-linking. Sucrose also increases the density of the fixing solution, which causes it to drop to the bottom of the wells and ensure rapid fixation. In certain cases, paraformaldehyde can mask antigenic epitopes from recognition by specific antibodies. In those cases, other fixatives, such as cold methanol, can be used.

3.4. Washing

Post-fixation, cultures require multiple rounds of washing, to remove fixative and other reagents. It is important to adopt a washing procedure that is gentle enough to avoid washing away cells and preserve their delicate processes while also being rapid and efficient. A plate washer can be used if the proper settings can be deployed to avoid harsh suction or excessive flushing. It is prudent to avoid complete drying of cells during washing. If a plate washer is used, it is best to leave a few millimeters of wash buffer over the cells at times during the wash cycle. If plates are being washed manually, it is best to use a multichannel suction adapter with pins shorter than the full depth of the well. It helps to take out and dispense fluid gently and at low speed, preferably along the wall of the well rather than directly over the cells.

3.5. Permeabilization and blocking

Labeling with primary antibodies prior to permeabilization ensures that only surface proteins are identified. However, the fixation step itself can cause permeabilization, even if no detergent is present (56). Therefore, it is important to perform staining for the protein of interest along with an exclusively intracellular protein to identify instances of compromised plasma membrane integrity. For staining intracellular proteins, a permeabilization step is normally required, usually with Triton X-100 (0.2-0.3%, 5-10 mins). Milder detergents, such as digitonin and leucoperm, may be useful when Triton X-100 compromises antigenicity. Saponin (0.02%) can be used to permeabilize cell membranes while keeping intracellular organelle membranes intact. Preparation and storage of solutions of Digitonin and Saponin is not always straightforward so it is helpful to consult the literature (57). In all cases, non-specific binding sites should be blocked to decrease background staining. This

can be achieved with a 10% bovine serum albumin solution, fish gelatin, or goat serum. As an example, goat serum may help with background caused by nonspecific binding of secondary antibodies raised in goat. By adding detergent to the block, the permeabilization and blocking steps can be combined to minimize wash steps. In this case, a lower percentage of Triton X-100 (0.03%) can be used to slow down the permeabilization process, allowing sufficient time for blocking (up to one day).

3.6. Staining and visualization

Quantitative HCA measurements of neuronal morphology are commonly carried out using immunohistochemistry with fluorescent dyes. Hoechst and DAPI dyes are routinely used to stain DNA and identify nuclei. Immunostaining of cytoskeletal markers, such as tubulin, is commonly used for visualizing cell morphology. Tubulin β III-specific monoclonal antibodies that preferentially label neurons can be used for preparations that contain a substantial number of non-neuronal cells. Tubulin antibodies will also allow for the detection of neurites; however, other antibodies need to be used to distinguish dendrites from axons. Antibodies to dendrite-selective markers, such as MAP-2, or axon-specific markers, such as Tau1, can be used for that purpose. For quantification of synapse formation, co-localization of presynaptic markers, such as synapsin, with postsynaptic puncta can be measured (58,59). It is generally convenient to also stain for tubulin because automated HCA software will typically require a complete cell body mask in order to correctly assign structures to their corresponding cell body. Co-staining of other markers can be done simultaneously, provided antibody compatibility is ensured. Primary antibodies for different antigens, such as MAP2 or β III tubulin, must be raised in different species (for example mouse and rabbit) if they are to be used simultaneously in the assay. Secondary antibodies should preferably be from the same species (for example goat or donkey). It is important to pre-absorb secondary antibodies with potentially cross-reacting primary antibodies. Thus, a goat-anti-mouse secondary antibody should be preabsorbed on a rabbit IgG column to eliminate cross reactivity. Pre-absorbed antibodies are readily available from commercial sources. It is crucial to ensure that the optical filter sets used for imaging have appropriate excitation/emission filters so as to capture relevant signals from a given combination of fluorophores, while minimizing “bleed-through” from one channel to another. An alternative method to secondary immunostaining is to use primary antibodies that are directly conjugated to fluorescent tags. These are readily available from various suppliers. Conjugation kits are also available for labeling primary antibodies with a variety of fluorophores, infrared dyes, and quantum dots.

Plasma membrane specific dyes can be used for delineating cell bodies and neurites. CellMask™ Deep Red has been used with success in a neurite growth assay, where it was applied in the fixative solution along with a nuclear stain, dramatically decreasing the time and reagents required for the staining process (60). However, while these stains do survive fixation, they do not survive permeabilization, and so their use must be reserved for assays where external morphology and/or extracellular staining are the only endpoints.

3.6. Data analysis

In addition to HCS parameters that can be extracted from virtually all cell types, which include cellular/nuclear/organellar size and brightness, HCS with neurons can produce readout parameters related to neuron-specific morphologies, such as axon and dendrite count, branching, and length. All vendors of major HCS imaging systems provide image analysis software solutions for neuronal HCS (e.g., Figure 4).

Additionally, there are other commercial (61) and open source (62–64) feature extraction solutions. Given the sheer amount of data produced from HCS image analysis, it is helpful to develop batch processing scripts to facilitate data analysis and quality control.

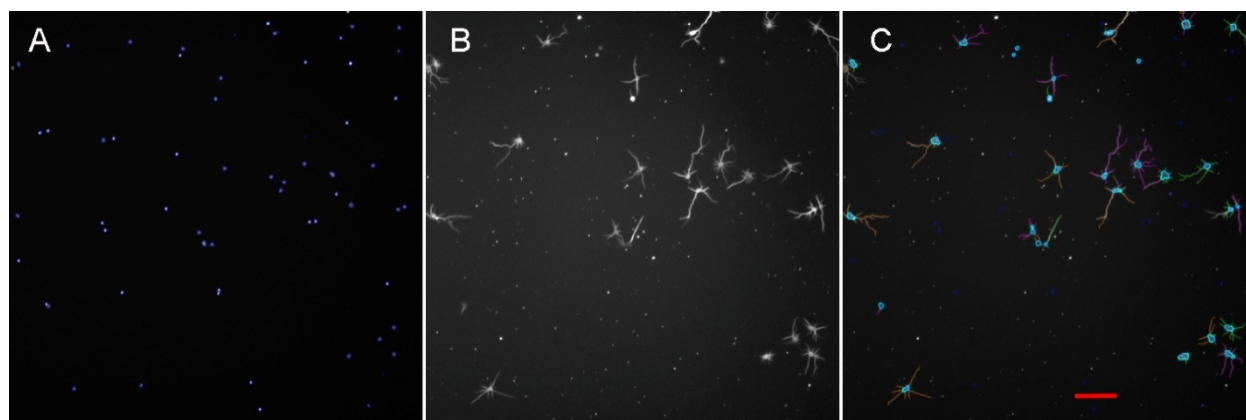


Figure 4: HCA of neurons in culture. Neurons in 96 well plates immunostained for nuclei (Hoechst – Panel A) and β III-tubulin (cell bodies and neurites – Panel B). The images were automatically traced using the Neuronal Profiling BioApplication (Cellomics) to yield dozens of morphological measurements for each neuron in the well (Panel C). Scale bar 100 μ m (*unpublished data*).

3.7. Assay suitability and quality control

The suitability of a screening assay is a measure of its ability to reproducibly distinguish positive from negative controls. Due to inter-prep variability and differences in the behaviors of dissociated primary neurons in culture, it is essential to run the proper controls in every screening experiment to evaluate the consistency of responses to treatment. Because of variability in absolute readout values, means, coefficients of variation, and signal to noise ratios, it is difficult to compare assay performance across multiple assay formats or even within repetitions of the same assay. To resolve this issue, a dimensionless statistical parameter, Z' -factor, was defined to assess the “screening window” of a bioassay (65). A Z' -factor value of ≥ 0.5 reflects a large separation band between the populations of positive and negative control readouts, and thus indicates good screening suitability (Figure 5).

The Z' -factor is, however, based on a single selected readout. HCS offers the option of collecting a large number of readouts per screen. Incorporating additional parameters in the definition of assay hits can distinguish between actives with distinct mechanisms of action. For that purpose, a multiparametric Z' -factor was proposed (66). This multiparametric value is calculated by scaling Z' values from multiple readouts, and thus requires post hoc calculation of a weight for each considered readout. A potentially more objective metric for distinguishing actives in a screen is the multidimensional perturbation value (mp-value) (67). The strength of this approach lies in evaluating exactly how similar or dissimilar treatments are from each other or from controls, and thus no *a priori* definition of hits is required. However, this can make the definition of hits less clear-cut. In most cases, HCS focuses on one select parameter, and the suitability of the assay is evaluated using a one dimensional Z' -factor.

4. Additional Considerations

4.1. Controls

Due to the complex morphology of neurons compared to most other cells types, these cells manifest a relatively large range of measurable phenotypes. Consequently, a number of factors can influence the phenotypes of cultured primary neurons, including preparation, reagents, and culture conditions. This introduces a significant amount of variability (noise) in the data that can mask effects from treatment. It is possible to minimize circumstantial variability and improve detection of treatment effects by including normalization controls within individual plates (29). A sufficient number of controls is important for reliable normalization (68). Negative controls in compound screens usually receive the equivalent amounts of solvent (e.g. DMSO)—it is important to determine beforehand whether the solvent at relevant concentrations has any discernible effects on the assay

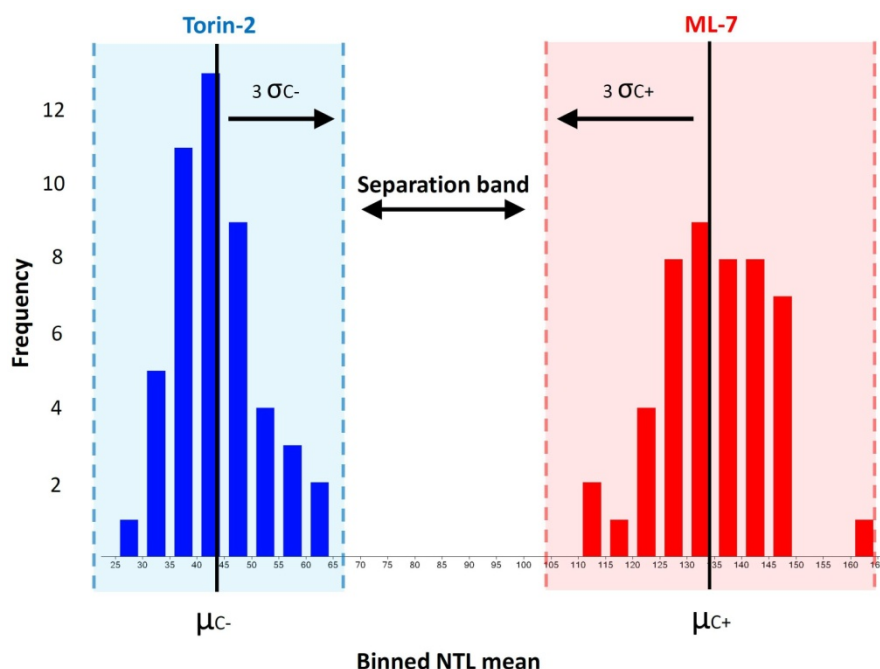


Figure 5: Bar plot representing binned values of neurite total length (NTL) averages from wells (96-well plates) containing cultured hippocampal neurons treated with ML-7 (neurite growth promoter) or Torin-2 (neurite growth inhibitor). Each block shows the mean of the population with three standard deviations above and below. This pair of controls yields a Z' -factor >0.7 , as evidenced by the complete separation of the two populations (*unpublished data*).

readout. Choosing controls for RNA/DNA screens is less straightforward. “Control” plasmids or oligonucleotides may unevenly influence phenotypic parameters. It may be necessary to screen a large number of different constructs to determine population behavior and select controls that yield population mean values.

4.2. Cell viability assessment

Accurate quantification of cell death is a critical component of HCS with primary neurons. Cell viability is the most important readout in neurotoxicity screens, but is important when analyzing data for any screen. As mentioned previously, certain phenotypes have non-normal distribution in low-density neuronal cultures, and a minimum number of viable (or valid) cells is required to obtain reliable and reproducible results. Thus, it is important for quality control purposes that individual data points on cellular phenotypes are obtained from healthy neurons in suitable numbers. Therefore an assessment of cell viability is required. Several methods exist that assess viability by interrogating different aspects of the cell - nuclear morphology, caspase activation, membrane integrity, and mitochondrial function. Each of these indicators answers the question of viability in a different way. For example, examining caspase activation using antibodies against activated caspase 3 or 9 is a measure of apoptotic death. Dying cells tend to have smaller nuclei that stain more brightly with DNA stains (due to DNA compacting and fragmentation), hence the nuclear morphology and staining intensity can be used as an indicator of cells death (Figure 6 unpublished data), provided the proper controls are considered (29).

This, however, may not work equally well for all neuronal types. In the case of live cell imaging, cell viability can be assessed by examining membrane integrity. Membrane impermeable fluorescent dyes can be used to identify dead cells by positive or negative selection. For example, a DNA-binding fluorescent impermeable dye like SYTOX® will fluoresce inside dead or dying cells with breached cellular and nuclear membranes. Inversely, a cell permeable dye like Calcein AM will enter live cells where it will be cleaved into a fluorescent membrane-impermeable derivative, thus fluorescing inside live cells. Another widely used marker for cell viability is mitochondrial function as indicated by the presence of a mitochondrial membrane potential, which can be

examined using commercially available dyes such as MitoTracker® or Tetramethylrhodamine, Ethyl Ester, Perchlorate (TMRE).

Considerations for small molecule screening

4.3.1. Concentration of small molecules

Choosing a single concentration for a cell-based small-molecule screen is complicated by a multitude of factors including, but not limited to, differences in solubility, membrane permeability, binding affinity, selectivity, and toxicity. A single concentration screen accepts the caveat of exaggerated false negatives. Ideally, a concentration of a compound that is close to IC_{50} or K_D of the target should be tested. However, even slight chemical modifications can dramatically alter the binding affinity within a group of chemically related compounds, making it unrealistic to find a single concentration that elicits effects in all actives. Of course, in cell-based screenings, most molecular targets are unknown. Therefore, it is highly desirable to obtain a dose response in HCS of primary neurons, typically in the low nanomolar to micromolar range.

4.3.2. Time and duration of treatment

Two important considerations for cell-based screening of small molecules include time of administration of the compounds and duration of incubation. Depending on the experimental model, the exact time for adding the compounds may be of biological relevance or purely technical relevance. Low density cultures are often allowed a recovery period before perturbation (typically a few hours to a day). Assays that examine effects on networks of neuronal processes, for example, will require the necessary time for such networks to form following plating. Thus, pre-treatment time should be considered in the overall time of the assay and cultures must be verified to remain viable and healthy over the course of the entire experiment. It is most helpful if the duration of incubation is calibrated to maximize the dynamic range of the readout. To achieve this, the Z' -factor can be calculated for control positives (promoters) and negatives (inhibitors) at multiple time points, and the window with the largest Z' -factor can be adopted for the screen.

4.3.3. DMSO tolerance

DMSO is by far the most commonly used solvent for small-molecule and drug-like compound libraries. DMSO can induce toxicity in neurons at concentrations greater than 0.5% v/v. At high enough concentrations, DMSO can also affect neuronal morphology and function (Figure 7). It is thus advisable to keep final DMSO concentrations as low as possible, preferably below 0.5% v/v. It is also useful to keep DMSO concentrations constant in serial dilutions of stock compounds in media, to eliminate DMSO concentration effects as a confounding variable.

4.4. Considerations for genetic screening

4.4.1. Overexpression vs knockdown

Overexpression screens with primary neurons have identified developmentally regulated transcription factors (34), kinases, and phosphatases (29) involved in axon regeneration. Knockdown screens have also been used to identify regeneration associated kinases (30) as well as kinases involved in neurodegeneration (53). The choice between overexpression and knockdown depends on the exact question that the assay is attempting to answer; each method brings its own advantages and drawbacks. Overexpression can produce false negatives due to issues arising from auto-regulation, inappropriate posttranslational modification, novel activities at high expression levels, and lack of activation by stoichiometrically insufficient trans-activators. Knockdown with RNAi can suffer from severe off-target effects (69), and may be less efficient in neurons compared to other cell types (70). Ultimately, a validation with one approach of hits identified using the other approach will increase the likelihood of biological relevance.

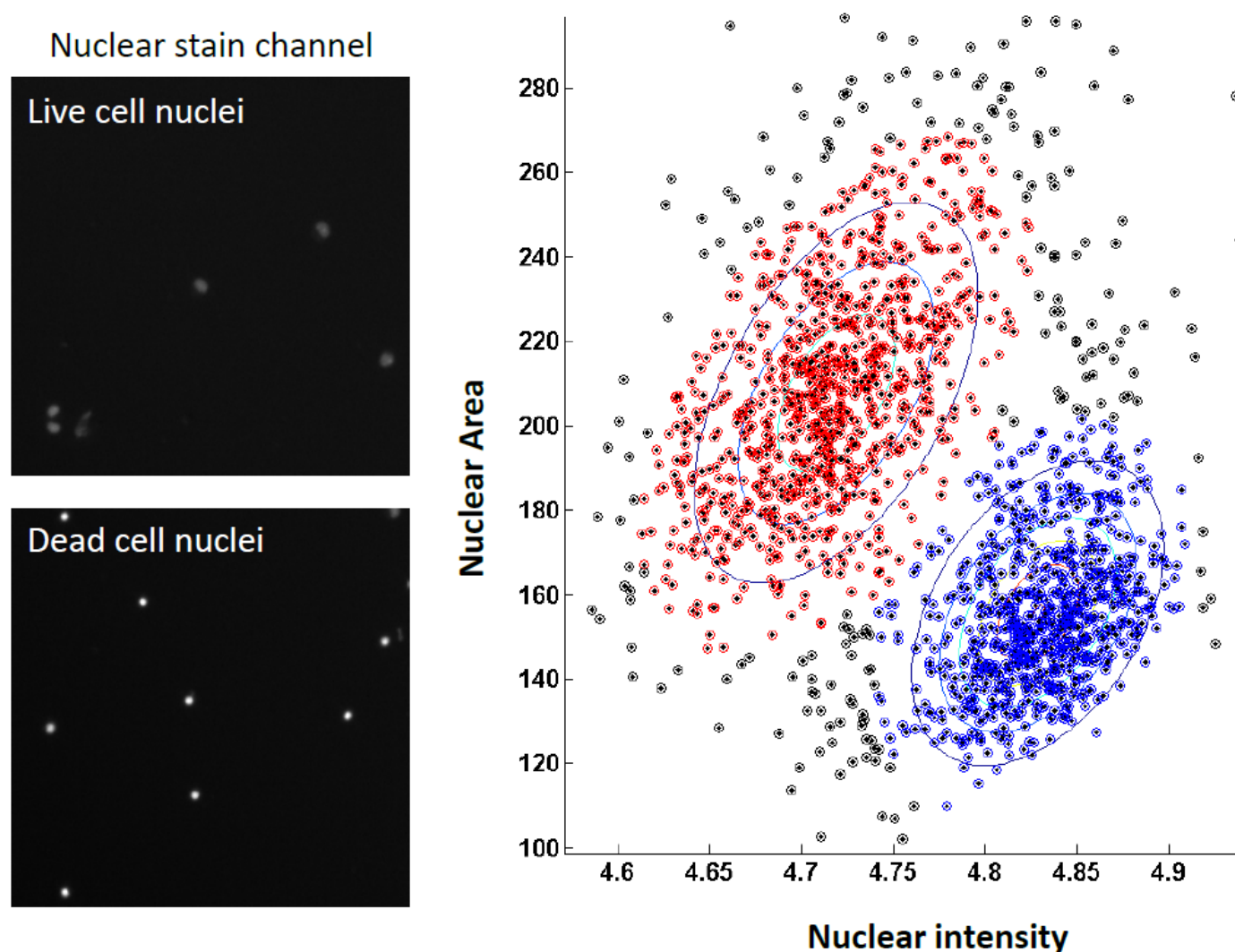


Figure 6: Small and bright nuclei are the result of heterochromatin condensation, while big dim nuclei correspond to cells that are alive. This distinction can be used to measure cell survival in nuclei-stained cultures. A Gaussian fit searches for two clusters within the population as live [large nucleus, low intensity] or dead [small nucleus, high intensity] (*unpublished data and 29*).

4.4.2. Transfection

Transfecting primary neurons can be challenging for multiple reasons, including difficulties in obtaining sufficient quantities of purified neurons and problems encountered in automated liquid handling. Traditional lipid-based methods yield low transfection rates in neurons and induce cell death. Electroporation can yield more efficient transfection for HCS, and is likely preferable (29,71). RNAi is reported to be less efficient in neurons than in other cell types (70), and longer turnover rates of proteins can lead to false negatives. Magnetic transfection may enhance siRNA introduction into neurons (53). Viral transduction, which overcomes many of these problems, has been successfully used in HCS with primary neurons (33).

4.4.3. Identification of transfected or transduced cells

A common issue with overexpression of cDNA libraries is the lack of a tag or a co-expressed marker. Thus, for transfection or transduction efficiencies below 100%, a marker is required for identifying modified cells. New libraries are more commonly being made with tags, such as Myc or FLAG. If tagged constructs are used, a

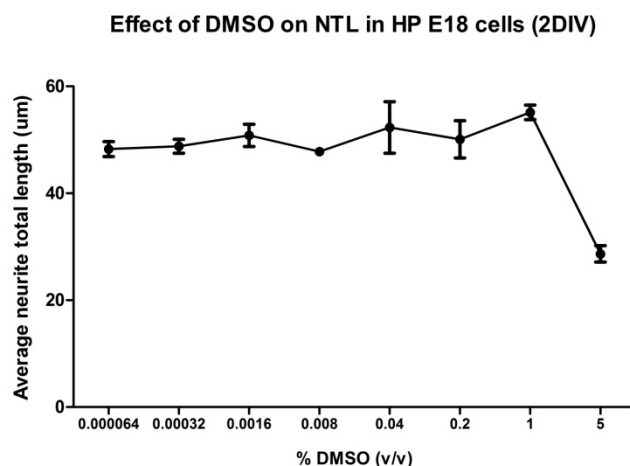


Figure 7: Effect of DMSO concentration on neurite length in E18 hippocampal neurons. Each point represents an average of 6 data points $\pm$ SEM. These data suggest that it would be best to keep the final DMSO concentration below 1% to avoid complicating any analysis related to neurite length (*unpublished data*).

follow-up validation experiment with the untagged proteins may be required to eliminate possible false positives, though false negatives would persist (as discussed before).

Alternatively, a coexpressed fluorescent protein can be introduced via an Internal Ribosomal Entry Site (IRES) (72). Another strategy is to use plasmids expressing the self-cleaving 2A peptide (73), though such plasmids may exhibit variability in expression of the second (reporter) gene. Finally, a co-transfection strategy can be used in which the gene of interest is administered at a 4-6X molar ratio relative to a reporter plasmid. This generally results in 80-90% of fluorescently marked cells also expressing the gene of interest (29) (Figure 8).

5. Protocols for HCS with Primary Neurons

5.1. Small molecule screening with hippocampal neurons - Overview

This bioassay utilizes primary hippocampal neurons isolated from embryonic (E18) rat brain and is optimized for identifying small molecules that can promote neurite growth in those neurons. The cells are cultured on a semi-permissive substrate, poly-D-lysine, which produces moderate neurite growth and allows for a large dynamic range in neurite length readout (growth promoters can reach 300-400% of control neurite growth). The assay typically produces a very good Z' factor and has low false discovery rate. In its current format, it is suited for low throughput screening (with a practical average of 100-200 compounds per experiment), though the high Z'-factor makes it suitable for higher throughput screening.

5.1.1. Reagents

1. NbActiv4 Cell-culture media (BrainBits cat. no. Nb4-500)
2. NeuroCultTM SM1 Supplement (Stemcell cat. no. 05711)
3. Hibernate E (Brainbits-HE-Ca-500mL)
4. 2.5% (wt/vol) trypsin; aliquot for single use (0.5 ml) and store at -20°C
5. DNase 30 mg/mL in ddH₂O (Sigma-Aldrich cat. no. D-5025)
6. Polylysine solution, 0.5 mg mL^{-1} in HBSS (prepared from poly-L-lysine, molecular weight 30,000–70,000 kDa; Sigma-Aldrich, cat. no. P2636)
7. HBSS (LifeTechnologies cat. no. 14175-103)
8. 4% PFA 4% Sucrose in PBS

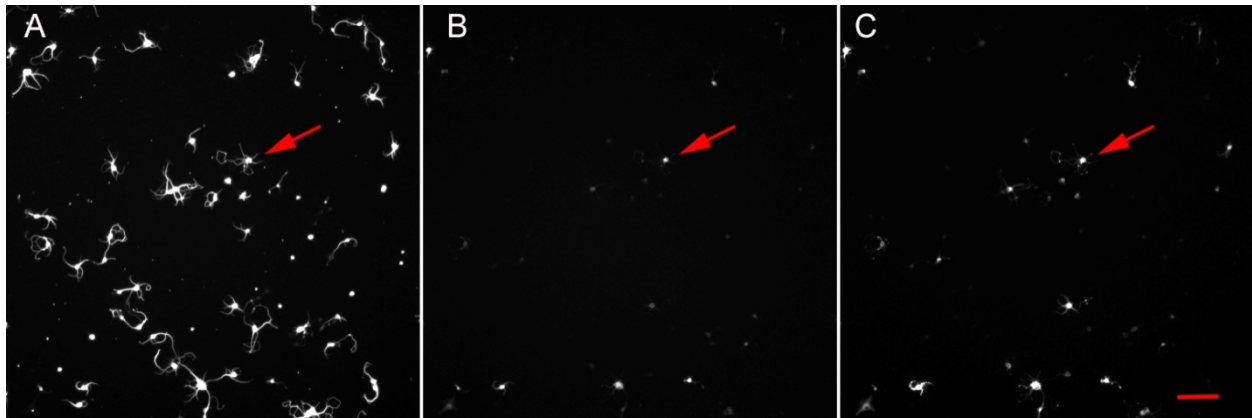


Figure 8: Postnatal (P5) cortical neurons (1) simultaneously transfected with GFP (1) and mCherry (1). Arrows indicate a cell exhibiting fluorescence in both green (1) and red (1) channels, indicating double transfection. The high co-transfection rate suggests that a fluorescent marker can be used as a marker for transfected cells in this experiment. Scale bar 100 μ m (*unpublished data*)

9. PBS
10. DMSO
11. Blocking and permeabilization buffer (0.2% fish gelatin, 0.03% Triton X-100, in PBS + 0.02% azide)
12. Mouse anti-tubulin β III antibody
13. Hoechst solution (10 mg/mL)
14. Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) (Lifetechnologies cat. no. A-11029)

5.1.2. Equipment

1. Laminar flow hood, able to accommodate a dissecting microscope
2. Tissue culture incubator at 35.5 °C with humidified 5% CO₂ atmosphere
3. Water bath at 37 °C
4. Dissecting microscope
5. Dissecting tools (sterilized): fine-tipped forceps, microdissecting scissors
6. Hemacytometer or automated cell counter
7. Sterile plasticware: 5-, 10- and 25 mL serological pipettes, tissue culture dishes and bacteriological dishes, 15 mL and 50 mL conical centrifuge tubes
8. Sterile glass Pasteur pipettes
9. 96-well plates
10. Filter-plugged pipette tips (1200, 200, and 20 μ L)

5.1.3. Pre-coating culture plates

1. On the day before the experiment, load PDL solution in 96-well plates to coat (50 μ L/well)
2. Wash plates next morning with HBSS or PBS (150 μ L $\times$ 4 washes) then leave in buffer until plating

Comment: For larger well size, the volume of PDL coating solution should be increased roughly proportional to the well area.

5.1.4. Preparing the Cells

1. Euthanize timed pregnant rat carrying E18 embryos using an IACUC approved method
2. In a laminar flow hood, remove the embryos and place in a petri dish containing Hibernate E
3. Dissect pup brains and collect hippocampi in 15 ml conical tube containing Hibernate E with SM1 (2% v/v)

4. Prepare dissociation media by combining 4.5 ml of Hibernate E (without SM1) with 0.5 mL of Trypsin and 100 μ L of DNase solution
5. Carefully decant hippocampi then add dissociation solution. Incubate at 37 °C for 15-20 min, occasionally swirling the tube
6. Using cotton plugged Pasteur pipettes (flame polished), remove dissociation media then add 5 mL of Hibernate E containing SM1. Swirl the tube to thoroughly wash the tissue. Allow the tissue to settle to the bottom of the tube then carefully remove the rinse solution. Repeat this step 5 times to dilute out trypsin and DNase and remove debris from lysed cells
7. Remove final rinse media from the tube then add 1 pipette-full of rinse media
8. Using the narrow-bore flame-polished Pasteur pipette (pre-wetted with rinse media), triturate until all cells are dissociated and no chunks of tissue remain. It's best to perform less than ten triturations as it adversely affects cell viability
9. Bring volume up to 8-12 mL using Hibernate E containing SM1, then mix well and determine cell concentration

Comment: Plating cells immediately after trituration will prevent clumping and improve overall quality of the culture.

5.1.5. Plating

1. Dilute cells in culture media to a final concentration of 10,000 cells/ml
2. Aspirate HBSS from PDL coated plates
3. Load 150 μ L of cell solution in the middle 48 wells (See Figure 9)
4. Allow cells to adhere for 2 hours in tissue culture incubator prior to treatment

Comment: Use a multichannel manual pipette and load cells gently. Alternatively, use the slowest speed setting on an automated pipette. This helps avoid patterning in the plated cells and promote even spreading.

5.1.6. Preparing Compound Dilution Plates

1. Place culture media in a 96-well plate (new – uncoated) as shown in Figure 10 (solution A = culture media, solution B = culture media + 0.8% DMSO)
2. Add 1 μ L of compound stock solution to each of the top wells containing 124 μ L. This will produce a final DMSO concentration of 0.8% and final compound concentration of 80 μ M (starting with a 10 mM stock solution in DMSO). Control wells receive 1 μ L of DMSO
3. Using a multichannel manual p200 pipette, mix the solutions in the top row then move 25 μ L to the below row. This creates a 1:5 dilution of compound while DMSO is held constant at 0.8%. Repeat serial dilutions until the last row. This format allows for screening 3 compounds per plate at 6 concentrations with duplicate wells per condition.
4. Place compound dilution plates in tissue culture incubator for 1-2 hours to equilibrate

For calculating the Z'-factor of the assay, include an additional plate (or more) with the corresponding treatment format as shown in the schematic in Figure 11 (C_+ is a compound that produces the desired bioassay readout – in this case neurite growth promotion, and C_- is a compound that produces the opposite effect – or neurite growth inhibition. Both compounds must be prepared at 4X the corresponding concentration where their maximal effects are observed. See Figure 11)

5.1.7. Treatment

1. Using a multichannel manual p200 pipette, aspirate 50 μ L from the final dilution (row G) and slowly add to corresponding row of recipient cell plate (4X dilution, 50 μ L of compound solution into 150 μ L of cell

A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
B01	B02	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	B11	B12
C01	C02	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	C11	C12
D01	D02	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	D11	D12
E01	E02	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	E11	E12
F01	F02	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	F11	F12
G01	G02	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	150 μ l	G11	G12
H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12

Figure 9: Plating schematic

A01	A02	Compound 1		Compound 2		Compound 3		DMSO		A11	A12
B01	B02	124 (A)	124 (A)	124 (A)	124 (A)	124 (A)	124 (A)	124 (A)	124 (A)	B11	B12
C01	C02	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	C11	C12
D01	D02	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	D11	D12
E01	E02	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	E11	E12
F01	F02	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	F11	F12
G01	G02	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	100 (B)	G11	G12
H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12

Figure 10: Schematic of compound dilution plate

A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
B01	B02	4X C _H	4X C _H	4X C _H	4X C _H	4X C _L	4X C	4X C	4X C _L	B11	B12
C01	C02	4X C _H	4X C _H	4X C _H	4X C _H	4X C _L	4X C _L	4X C _L	4X C _L	C11	C12
D01	D02	4X C _H	4X C _H	4X C _H	4X C _H	4X C _L	4X C _L	4X C _L	4X C _L	D11	D12
E01	E02	4X C _H	4X C _H	4X C _H	4X C _H	4X C _L	4X C _L	4X C _L	4X C _L	E11	E12
F01	F02	4X C _H	4X C _H	4X C _H	4X C _H	4X C _L	4X C _L	4X C _L	4X C _L	F11	F12
G01	G02	4X C _H	4X C _H	4X C _H	4X C _H	4X C _L	4X C _L	4X C _L	4X C _L	G11	G12
H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12

Figure 11: Schematic for additional assay plate containing corresponding treatment

culture). Repeat going up the rows (increasing concentration). Final compound concentration in screen: 0.0064, 0.032, 0.16, 0.8, 4, and 20 μ M (in 0.2% DMSO)

2. Be sure to avoid creating too much turbulence in the wells to avoid detaching recently adhered cells.
3. Return plates to incubator and culture for 48 hours

Comment: Do this for 5 or less plates at a time to avoid leaving the cells too long out of the incubator, which could lead to changes in the pH of culture media and affect cell viability.

5.1.8. Fixing the cultures

1. Remove media from plates and immediately replace with 100 μ l of warm (37 °C) PFA/sucrose solution. Gently inverting plates will remove media without damaging delicate neurites
2. Fix for 15-20 min
3. Rinse with PBS (200 μ L /well $\times$ 3)

Comment: Exercise sufficient care when handling PFA and perform all steps inside a fume hood with sufficient protection. Be sure to properly dispose of waste PFA solution

5.1.9. Staining and imaging

1. Remove PBS and add 100 μL of blocking/permeabilization buffer (PBS, 0.2% fish gelatin, 0.03% Triton X-100, 0.02% azide) and incubate overnight at 4 $^{\circ}\text{C}$ (can be stored over the weekend). If faster permeabilization and blocking are required, cells can be permeabilized in a higher Triton X-100 concentration (0.3% in PBS) for 1 hour and then blocked for 1 hour.
2. Add 100 μL of primary antibody solution (mouse anti-Beta III tubulin in blocking buffer) and incubate overnight at 4 $^{\circ}\text{C}$.
3. Rinse wells with PBS (200 $\mu\text{L} \times 3$)
4. Remove PBS and add 100 μL of secondary antibody solution (Goat anti-mouse Alexa 488, 10 $\mu\text{g}/\text{mL}$ Hoechst, 0.2% fish gelatin, 0.02% azide, in PBS). Shake gently on a rotating shaker for 2 hours.
5. Rinse wells with PBS (200 $\mu\text{L} \times 5$)
6. Image plates using a Cellomics ArrayScan VTI in 2 different channels for nuclear staining (Hoechst) and cell body/neurite staining (βIII -tubulin). Typically, nine fields per well are imaged with a 5X objective and automatically traced by the Neuronal Profiling Bioapplication. To get reliably reproducible results, at least 200-300 cells should be measured per condition.

5.1.10. Data analysis

1. Export plate data in Excel sheet format.
2. Filter out artifacts, cells that died upon plating, debris, and so on. This can usually be achieved by applying filters on phenotypic parameters. A set that works well for this assay includes the following cut off (NeuriteTotalLength > 10 μm , NeuriteMaxLengthWithoutBranching < 500 μm , MinCellBodyArea > 100 μm^2 , MaxCellBodyArea < 3000 μm^2 , MaxNeuriteBranching < 50). Additional parameters can be derived empirically and added as required. This leaves only the valid neurons in each well.
3. For each drug condition, normalize the data to the controls in the corresponding row. For example, to calculate the effect of Compound 1 on neurite total length (NTL) at 20 μM , use the following formula

$$\text{Compound1 \%NTL (20 } \mu\text{M)} = \frac{\text{AVG}(\text{B3}_{\text{NTL}} + \text{B4}_{\text{NTL}})}{\text{AVG}(\text{B9}_{\text{NTL}} + \text{B10}_{\text{NTL}})} \times 100$$

Where B3NTL and B4NTL are the averages of neurite total length in wells B3 and B4, and B9NTL and B10NTL are the averages of neurite total length in wells B9 and B10.

4. Calculate the Z-score for each attribute using the following formula

$$\text{Compound1 Z-score NTL (20 } \mu\text{M)} = \frac{\text{AVG}(\text{B3}_{\text{NTL}} + \text{B4}_{\text{NTL}}) - \text{AVG}(\text{B9}_{\text{NTL}} + \text{B10}_{\text{NTL}})}{\sigma_{\text{controls}}}$$

Where σ_{controls} is the standard deviation in all DMSO wells within the plate (columns 9[B→G] and 10[B→G])

5. Calculate the Z'-factor for the assay using the positive and negative control (CH/CL) plate(s) according to the following formula

$$\text{Z'-factor} = 1 - \frac{(3\sigma_{\text{CH}} + 3\sigma_{\text{CL}})}{|\mu_{\text{CH}} - \mu_{\text{CL}}|}$$

Where μ_{CH} and μ_{CL} are the means for the positive and negative (neurite growth promoter and neurite growth inhibitor) control wells respectively, and σ_{CH} and σ_{CL} are the respective standard deviations.

Comment: Hits can be identified as compounds that fulfill the following criteria: %NTL > 130% & NTL Z-score > 1.5 & % valid neurons > 60% & valid neurons Z-score > -4. Hits must be confirmed in two independent screens. These criteria usually yield an average false discovery rate of ~ 10%.

Comment: Considering the rather large size of datasets generated from this kind of experiment, it is advisable to automate the data analysis process, provided that sufficient attention is given to quality control and validation of all data points.

5.2. Plasmid DNA screening with cortical neurons – Overview

This bioassay uses mixed cortical cells from early postnatal rat pups, aged P1 to P7, and is optimized for delivery of plasmid DNA via electroporation. The use of postnatal neurons, as opposed to embryonic, is beneficial when screening for genes or compounds that may be relevant to age-related reductions in neurite outgrowth. Such cultures, however, are subject to challenges associated with isolating and maintaining postnatal neurons. As animals age postnatally between P1 and P7 neurons become increasingly difficult to dissociate, and the mechanical force needed to remove neurons from the surrounding matrix increases, which leads to increased mortality during cell preparation. Sequential digestion with papain followed by trypsin, continual agitation during enzyme treatments, and brief triturations are critical to balance the relative difficulty in dissociating cells with the need for large numbers of viable cells to support screening efforts. It is also important that cells be provided with appropriate survival factors in the growth media. This protocol utilizes a relatively complex growth media, which is then conditioned by exposure to glial cultures. Glial conditioning increases cell viability and neurite outgrowth. This procedure will yield approximately 5 million cells from three rat pups, with transfection efficiencies of approximately 40%.

5.2.1. Preparing 1mg/mL poly d-lysine (PDL) stock solution

Reagents:

1. PDL: Poly-D Lysine (Hydrobromide) Mol. Wt. 30,000-70,000
PDL 100 mg Powder (Sigma [P7886](#)).
2. 10X HBSS: Hank's Balanced Salt Solution: KCl 53.7 mM, KH₂PO₄ 4.4 mM, NaCl 1.3 M, NaHCO₃ 41.66 mM, Na₂HPO₄ 3.38 mM, Hepes 99.87 mM.
3. This recipe makes 10x, should be pH 7.2, to 1Liter
 - a. 4 g KCl (MW: 74.5)
0.6 g KH₂PO₄ (MW: 136)
76.5 g NaCl (MW: 58.4)
3.5.g NaHCO₃ (MW: 84)
35.8 g Na₂HPO₄ (MW: 142)
23.8 g HEPES (MW: 238.3)
 - b. After adjusting pH, sterilize by filtration
 - c. Dilute to 1x with water before use

Tools:

1. Sterile hood (Bio-safety cabinet)
2. 0.22 µm Filter (Nalgene 595-4520)
3. Multichannel pipette trough
4. 96-Well Falcon Plate (Falcon 353072)
5. 15 mL Falcon tubes (Falcon 352097)

6. 100 mL Bottle
7. 10 mL and 50 mL Pipette (Falcon 357551 and 357550)

Protocol:

1. All preparation should be done in the HOOD
2. Spray inside of the Hood with ethanol to kill Bacteria before use
3. If PDL ships as 100 mg powder, make it into a 10x solution (stock PDL) as follows.
4. Using a 50 mL pipette, transfer 100 ml 1x HBSS into the PDL container.
5. Mix with the pipette in order to dissolve powder.
6. Filter the stock PDL solution through a 0.22 μ m filter into the 100 mL bottle attached to the vacuum.
7. Using a 10 mL pipette, make 10 mL aliquots of the PDL solution in 15 mL tubes.
8. Label the tubes with the date/ Name/ PDL concentration (1 mg/mL)/ Cat No. / Lot No.
9. Store @ -20°C for further use.

5.2.2. Pre-coating plates with PDL (100 μ g/mL) for further laminin coating

Purpose: Coat plates with PDL to augment cell adhesion, final concentration of 100 μ g/mL.

Reagents and Tools:

1. Multichannel pipette trough
2. Multichannel pipette (1200 μ L)
3. 1x HBSS (Gibco 14175)
4. Box of 1200 μ L Tips
5. 96-Well Falcon Plate (Falcon 353072)
6. 5 mL and 50 mL Pipette (Falcon 357543 and 357550)
7. 100 mL Bottle

Protocol:

20 x 96 Well Plates: 90 mL 1x HBSS, 10 mL PDL Stock Solution

1. Thaw one aliquot of 10 mL PDL Stock solution on the 37 °C water bath
2. Using a 50 mL pipette, transfer 90 mL of 1x HBSS into a 100 mL Bottle
3. Add the 10 mL PDL stock solution and mix
4. Transfer the total volume to a trough
5. Using multi-channel fill 100 μ L/well in the inner 48 wells of the 96-well plates
6. Store the plates inside a plastic box in the cold room over night or until use

10 x 24 Well Plates: 90 mL 1x HBSS, 10 mL PDL Stock Solution

Follow exactly the same procedure but filling 400 μ L/well.

5.2.2. Coating plates with laminin (10 μ g/mL) after PDL pre-coating

Reagents and Tools:

1. Falcon PDL-coated plates
2. 1x HBSS (Gibco 14175)
3. Mouse Laminin I, 1 mg/mL, 100 μ L aliquot (Cultrex 3500-010-01)
4. Multichannel pipette (1200 μ L)
5. Multichannel pipette trough
6. Pasteur glass pipette
7. 15 mL Falcon tubes

Protocol:**1 x 96 well Plates: 3 mL 1x HBSS, 30 µL Laminin**

1. Thaw laminin aliquot on ice
2. Using Pasteur glass pipette with vacuum remove the PDL from wells or invert the plate gently.
3. Wash 5 times with sterile distilled water. Leave the last wash while you do step 4.
4. In a 15 mL tube add 30 µL of laminin solution per 3 mL of HBSS per plate and transfer to a multichannel trough.
5. Remove the water from the wells and add 60 µL of laminin solution in the inner 48 wells of the 96-well plates.
6. Incubate plates overnight @ 37 °C, 5%CO₂ in the incubator.

1 x 24 well Plate: 7.5 mL 1x HBSS, 75 µL Laminin

Follow exactly the same procedure but adding 75 µL of laminin solution per 7.5 mL of HBSS per plate and filling the wells with 300 µL of Laminin solution final concentration 10 µg/mL.

5.2.3. Preparing the transfection reagents: intraneuronal buffer (INB) stock solutions and INB solution**Reagents:**

1. Potassium Chloride, Powder (KCL) FW: 74.55 (J.T.Baker-3052-01)
2. HEPES Sodium Salt FW: 260.29 (Omnipur-5380)
3. Calcium Chloride (CaCl₂) FW: 147.02 (Fisherbiotech-BP510-500)
4. Ethylene glycol-bis(2-aminoethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA) FW: 380.35 (sigma-E4378-25g)
5. Magnesium chloride hexahydrate (MgCl₂) FW: 203.31 (sigma-M2670-500g)

Protocol:

1. Prepare the following solutions:
 1. 1 M KCL
 2. 1 M HEPES
 3. 0.5 M EGTA (Needs to be dissolved @ pH8 with 10 M NaOH Solution)
 4. M CaCl₂
 5. M MgCl₂
2. Check the pH for the solutions

INB Preparation

1. Add in a 50 ml Falcon tube:
 - H₂O: 42 mL
 - 1 M KCL: 6.75 mL
 - 1 M HEPES: 0.5 mL
 - 0.5 M EGTA: 0.5 mL
 - M CaCl₂: 0.1 mL
 - M MgCl₂: 0.1 mL
2. Check the pH of the INB; it should be about 7-8.

3. In the white hood, filter the INB through a 0.22 μ m filter and leave 10 mL (labeled as filtered INB) in a Falcon tube for the transfection itself and pour the rest in a multichannel through to wash the transfection plate (BTX plate).

5.2.4. Preparing culture media: enhanced neuronal buffer (END) supplement recipe

1. 50 ml 100x Sato Stock:
 - Add the following to 50ml Neurobasal medium:
 - 0.5 g transferrin [Sigma #T1147]
 - 0.5 g BSA [Sigma #A4161]
 - 12.5 μ L progesterone [Sigma #P8783] (from stock: 2.5 mg/100 μ L EtOH)
 - 80 mg putrescine [Sigma #P7505]
 - 500 μ L sodium selenite [Sigma #S5261] (from stock: 4 mg/100 μ L 0.1N NaOH + 10 mL NB)
2. 50 mL 100X T3 [Sigma #T6397-100 mg]:
 - Dissolve 3.2 mg triido-thyronine [Sigma #T6397] in 400 μ L 0.1 N NaOH
 - Add 30 μ L to 60 mL DPBS [Invitrogen #14287072]
 - Filter 0.22 μ m filter, DISCARDING the first 8 mL
 - Aliquot and store at -20 °C
3. 10 ml 1000X NAC [Sigma #A8199-10G]:
 - Dissolve 50 mg N-acetyl cysteine in 10 mL NB (will be yellowish in color)
 - Make aliquots and store at -20 °C

To prepare an aliquot to add to 100 mL Neurobasal (calcium free) [Invitrogen 12348017] add:

- 1 mL 100X Pen/Strep [Invitrogen #15140122]
- 1 mL Sato stock
- 1 mL 100X T3
- 1 mL 100X L-glutamine [Invitrogen #25030149]
- 100 μ L 1000X NAC
- 2 mL 50X B27 [Invitrogen #17504044] (This was replaced by Neurocult supplement SM1 from Stemcell Technologies, item #:05711)

Mix:

- 50 mL of Pen/Strep
- 50 mL of Sato (1)
- 50 mL of T3 (2)
- 50 mL of L-Glutamine
- 5 mL of NAC (3)
- 100 mL of SM1 supplement

Make aliquots of 6.1 mL and freeze at -20 °C.

5.2.5. Glial cell culture

Culture Media

1. 430 mL MEM with Earle's Salts (Gibco 11095-080)
2. 15 mL Glucose 20% in 1X PBS
3. Dissolve and filter through a 0.22 μ m filter to sterilize
4. 5 mL Anti-Anti (Antibiotic/Antimycotic) (Gibco 15240)
5. 50 mL Horse Serum (Gibco 26050-070)

Dissociation Media

1. 18 mL HibernateE (-CaCl) (Brainbits-HE-Ca-500 mL)
2. 2 mL Trypsin (2.5%, Gibco 15090)

Prepare for Culture

1. Prepare 500 mL Culture Media (see above)
2. Prepare 20 mL Dissociation Media
3. Thaw two 50 μ L aliquots of DNase (30 mg/mL, Sigma-D5025) on ice
4. Prepare Culture Dishes
 - a. Place 4 mL Culture Media in Falcon 3002 dishes
 - b. Place 25 mL Culture Media in T75 Flask (Thermoscientific-156499)
5. Thaw at least 6 mL of horse serum
6. Set up in hood
 - a. 2 forceps
 - b. 1 spatula (to transfer slices)
 - c. 1 scalpel
 - d. 1 razor blade
 - e. 1 Petri Dish
 - f. 20 mL Hibernate E on ice
 - g. Pasteur pipette with bulb

Protocol

1. Remove at least 4 P1 or P2 rat brains and place in ice-cold Hibernate E (-CaCl)
2. Under dissecting scope, remove meninges and make 2 transverse slices of brain using scalpel.
3. Using forceps as tiny clippers, remove cortex from brain slices.
4. Once all the cortex pieces are collected, transfer to lid of Petri dish. Mince with razor blade.
5. Using Pasteur pipette, transfer minced cortex to dissociation media (get rid of bubbles).
6. Add 1 aliquot of DNase to trypsin solution
7. Incubate in trypsin for 15 minutes with constant shaking.
8. Prepare a 50 mL tube with 3 mL of Horse serum in the bottom.
9. Place tube containing cortical cells and trypsin solution in hood and let the pieces settle to the bottom (takes a few minutes).
10. Remove supernatant and place in tube with horse serum (to stop trypsin reaction)
11. Add another 10 mL of dissociation media to the tube with the cortex pieces. Add 1 aliquot of DNase.
12. Incubate another 15 minutes, 37 °C, with constant shaking.
13. Add 3 ml of horse serum to second dissociation. Triturate 10 times with 10 mL pipette attached to electric pipette aid.
14. Filter the original supernatant and the new triturated cells supernatant through a 70 μ m filter, and into a fresh 50 mL tube.
15. Pellet cells at 180G for five minutes.
16. Remove supernatant and resuspend in 10 mL of culture media.
17. Plate 1 mL of resuspended cells per T75 flask or more if desired.

Comments:

1. Cell viability will be quite low the next day and large amounts of cellular debris will be visible. This is not a cause for concern. After consistent media changes over the next few weeks, the surviving cells will proliferate and make a nice monolayer in about 2-3 weeks.

2. To help prevent the overgrowth of microglial cells, when changing the media, strike the flask sharply to dislodge microglia, which tend to be loosely adherent, prior to replacing the media.

5.2.6. Passage of glial cultures

Comment: 1 confluent Glial flask can be split into 5 new flasks.

Culture Media

1. 430 mL MEM with Earle's Salts (Gibco 11095-080)
2. 15 mL Glucose 20% in 1X PBS pH-7.4 (Gibco-70011)
3. Dissolve and filter to sterilize
4. 5 mL Anti-Anti (Antibiotic/Antimycotic) (Gibco 15240)
5. 50 mL Horse Serum (Gibco 26050-070)

Protocol

1. Prepare Glial media and add 25 mL per flask (Thwermoscientific-256499).
2. Incubate at 37 °C until plate the cells.
3. Take the Glial flask to split and remove the media.
4. Add 10 mL of 1X HBSS (Gibco-14175) to wash the Glial culture. Move gently to the sides.
5. Remove the HBSS and add 5 mL of trypsin 0.05% (Invitrogen-25300062-500 mL).
6. Gently move the flask to spread the trypsin.
7. Incubate for 5 min, 37 °C.
8. Add 5 mL of Glial media to stop the trypsin (volume of trypsin=volume of media). Move the flask to spread the media evenly.
9. Transfer the suspended Glial culture to a 50 mL tube.
10. Centrifuge at 1000rcf for 5 min. Remove supernatant.
11. Resuspend using 1 mL of Glial media first and then add more media (1 mL of media per each new flask).
12. Put 1 mL of cells into each pre-warmed T75 flask. Gently move flask to spread cells evenly.

5.2.7. 24-well transfection of cortical neurons

Reagents

1. Hibernate E (Brainbits-HE-Ca-500 mL)
2. 24-well Falcon plates coated with PDL + laminin
3. Papain (Worthington, #3126)
4. ENB Media 50% Glia Conditioned
5. Intraneuronal Buffer solution (INB)
6. 50X SM1 Neuronal Supplement (Stemcell-05711)
7. DNase solution (Sigma-D-5025)
8. Trypsin 2.5% (Gibco-15090)

Tools

- Sharp forceps
- Spatula (for transferring brain slices)
- Razor blade
- Scalpel
- BTX Transfection plate (BTX-45-0450)

Protocol

- The day before the experiment: Prepare ENB media (see protocol)
- On the day of the experiment prepare INB transfection buffer (see protocol)

Fill culture plates with media

1. Filter the ENB Media.
2. Optional: add forskolin to the ENB Media (1 μ L of 5 mM stock per mL). Mix thoroughly.
Note: forskolin can be used to increase transcription from CMV promoters and in this way enhance expression from plasmids. Researchers should weigh this technical advantage against the potentially confounding effects of elevated cAMP signaling.
3. Add 400 μ L per well of the media to the 24-well plate. Be sure to avoid air bubbles at the bottom of the wells. If you get air bubbles, tap the plate to make the bubbles go up.
4. Put the plate on the incubator until use.

Prepare Hibernate E solutions

1. In a 50 mL Falcon tube place 20 mL of room temperature Hibernate E (for rinsing papain)
2. In another 50 mL Falcon Tube place 50 mL of Hibernate E + SM1 (add 1 mL 50X SM1)

Prepare 15 mL tube for Papain incubation

1. Put 10 mL of hibernate E in tube, put at room temp.
2. Add enough Papain to have 20 U/mL. usually 160-210 μ L.
 $200\text{U}/(\text{U}/\text{mg} \times \text{mg}/\text{mL}) = \text{mL} \rightarrow \times 10^{-3} = \mu\text{L Papain}$
3. Place tube in water bath.
4. Transfer brain to a Petri dish containing ice-cold hibernate E (-CaCl)

Prepare Transfection plate

1. Before using the INB buffer (Intraneuronal Buffer), filter it using a 10 mL syringe and 0.22 μ m filter.
2. Rinse BTX plate with 150 μ L/well of INB, 5 times.
3. Leave in the hood to dry. Put under the UV light for 15 min if you are reusing the transfection plate.

Set up Hood for Dissection

1. Tools (forceps, scalpel, razor blade, transfer spatula)
2. Pipette with bulb
3. Ethanol
4. 60 mm Petri dish
5. Start thawing DNase and Trypsin aliquots on ice.

Harvest Brains and dissecting cortex out

1. Let the pups sleep on ice for 10 min
2. Cut head off P1 rat with large scissors
3. Using small scissors make incision along midline of scalp. Clear the skin away.
4. Make cuts through skull, starting at the base of the skull and working around the later edges. This creates a large flap that can be peeled off.
5. Scoop whole brain into a 10 cm dish containing 10-12 mL ice-cold Hibernate E.

6. Remove meninges from brain (Back to Front)
7. Use scalpel to prepare a thick transverse section of middle 2/3 of brain.
8. Remove cortex from section (see Figure 12)
9. Repeat process for another brain, placing pieces of cortex from both brains together.
10. Get the dissociation media ready
11. Remove Papain solution from water bath and place in hood with cap open.
12. Add 50 μ L of DNase solution to papain.
13. Place a suction bulb on a glass pipette.
14. Using a flat spatula, transfer pieces of cortex to the lid of a Petri dish.
15. Using razor blade, mince the pieces of cortex.
16. Use pipette to transfer pieces of cortex into papain. This will take several repetitions. Be careful to avoid bubbles. Use the pipette to remove any bubbles.
17. Place conical tube with Papain + DNase solution and cortex in 37° C incubator on a shaker. Incubate for 30 minutes.

During incubation, prepare the following:

1. Clean up hood and organize pipettes and tip boxes
2. Be sure that Trituration Media (Hibernate E + SM1) is waiting at room temperature.
3. Lightly fire polish a glass pipette and attach it to suction bulb.
4. Attach a glass pipette to the vacuum.
5. Label 3 15 mL conical tubes as follows:
6. "Cell collection"
7. "Cell count"
8. "Transfection"
9. Set out 5 mL of buffer INB at room temperature.

Rinse Papain, triturate, add Trypsin

1. Spin cortex at 10G for 1 minute.
2. Remove supernatant; replace with 5 mL Hibernate E + SM1.
3. Spin cortex at 10G for 1 minute.
4. Remove supernatant, replace with 1.5 mL Hibernate E + SM1
5. Add 5 μ L DNase.
6. Triturate 3 times.
7. Spin cortex at 10G for 1 minute.
8. Remove supernatant; replace with 10 mL Hibernate E (NO SM1).
9. Spin cortex at 10G for 1 minute.
10. Remove supernatant; replace with 9 mL Hibernate E (NO SM1).
11. Add 1 mL trypsin + 50 μ L DNase.
12. Incubate 30 minutes, 37 °C, shaking.
13. During incubation start loading the plasmids in the transfection plate. First, the INB, then each plasmid. When each column is loaded, cover it with tape to avoid confusion and evaporation.

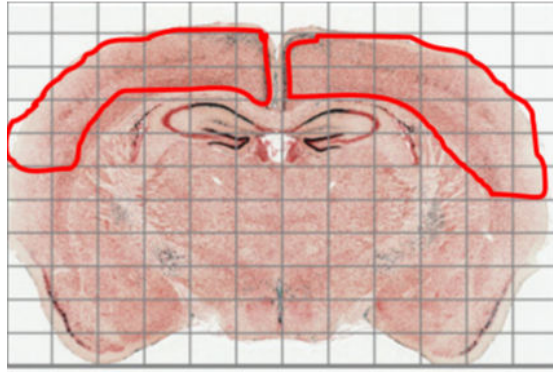


Figure 12: Diagram of rat cortex (outlined in red) in rat brain section.

Rinse, Triturate

The purpose of this trituration paradigm is to minimize the number of times a dissociated cell passes through the pipet tip. Tissue aggregates are trituated briefly, resulting in dissociation of cells from their surface. These cells are then removed to a separate container before further trituration of the aggregates. This approach is critical to maintain viability.

1. Spin cortex at 10G for 1 minute.
2. Remove supernatant; replace with 5 mL Hibernate E + B27.
3. Spin cortex at 10G for 1 minute.
4. Remove supernatant, replace with 1.5 mL Hibernate E + B27
5. Add 5 μ L DNase.
6. Triturate 3 times.
7. Let cortex settle for 2 minutes (set timer).
8. Remove the supernatant and place in “collection tube”.
9. Repeat steps 4-8 about five or six times, until about 10-12 mL of cell suspension is collected.

Determine Cell Concentration

1. Gently invert “Cell Collection” tube until the cells are evenly distributed (some probably settled to a pellet on the bottom during triturations).
2. Transfer 1ml of media to “Cell Count” tube.
3. Spin “Cell Count” tube at 80G, 3 minutes, room temperature.
4. Remove supernatant, and re-suspend pellet in 1 mL of Hibernate E + SM1.
5. Combine 90 μ L of cells with 10 μ L of trypan blue. Use hemacytometer to count number of HEALTHY cells.

Transfection and plating of cells

1. Calculate the volume needed for 1 million cells (We use 500,000 cells per column)
2. Vol. needed= amount of cell we want/ Cell count
3. Transfer that volume from “Cell Collection” tube to “Transfection” tube.
4. Spin down cells, 80G, 5 minutes, room temperature.
5. Resuspend cells in 500 μ L INB (250 μ L per column of BTX plate). INB IS TOXIC TO CELLS. FROM THIS POINT ON, WORK AS FAST AS YOU CAN WITHOUT MAKING MISTAKES.
6. Place 25 μ L of INB + cells in each well for transfection. Mix just one time, gently.
7. Cover transfection plate with tape. Use razor blade to make sure tape is not blocking the contact points.
8. Transfect cells with electroporator (350V, 300us, 1x)

9. Remove tape from transfection plate.
10. Add 100 ml of Hibernate E + SM1 to each well. Gently mix 2x.
11. Using multichannel pipette, transfer 25 μ l to wells in culture plate.
12. Let grow for two days, 37°C, and proceed with the Immunohistochemistry.

5.2.8. Fixative: paraformaldehyde 4%, sucrose 4%

Comment: Paraformaldehyde (PFA) fixative is very dangerous and much care should be taken while following this procedure! PFA in powdered form is very dangerous. Wear a mask and measure in a chemical fume hood! Do not allow yourself or anyone in the lab to be exposed to the powder or fumes coming from the hot water.

Materials

Set everything in the fume hood before starting:

1. Scale, spatula and weight plate.
2. PFA (Sigma P6148-500g)
3. Sucrose (FLUKA Biochemika_84097-1kg)
4. PBS 10x
5. 1 N NaOH
6. Glacial Acetic Acid
7. Distilled H₂O

For 250 mL:

- 10 g PFA
- 10 g Sucrose
- 200 mL H₂O
- 25 mL 10X PBS

Procedure:

For a 250 mL solution: 10 g of PFA and 10 g of Sucrose

1. Heat water (200 mL) to 60°C (don't allow it to go above 65 °C) and stir continuously
2. Weigh the PFA. Clean the scale of any remaining PFA.
3. Add the PFA and the sucrose to the water. Let stir for a few minutes.
4. Add 1 pellet of NaOH (or a few hundred μ L of NaOH 1N). Add only one at a time and wait a few minutes to see if more is necessary (until the solution turns clear).
5. Add 25 mL of 10X PBS.
6. Adjust pH near to 7.4 with pH strips using Glacial Acetic Acid.
7. Take to final volume with distilled water.
8. Filter and aliquot (12 mL/tube).

Note that the immunohistochemistry protocol calls for two distinct solutions: a BLOCKING BUFFER, which is used in the initial blocking step, and then an ANTIBODY BUFFER in which antibodies are applied.

Antibody Buffer

1. 200 mL ddH₂O
2. 1.75g NaCl (Sigma-Aldrich-S9625- 10 kg)
3. 1.2g Trizma Base (Sigma-T1503- 1 kg)
4. 2g BSA (Sigma-A9418- 100g)

5. 3.5g lysine (Sigma L2513- 25 g)

6. 0.02% Na Azide (2 mL of stock solution, which is 2 g in 100 mL H₂O)

Adjust to pH 7.4 and filter sterilize

Blocking Buffer:

To Antibody Buffer Add

1. 20% Normal Goat Serum (Gibco-16210): 2 mL/10 mL Antibody solution
2. 0.2% TritonX: 200 μ L from 10% Triton solution (prepared from 1 mL Triton X-100 (Omnipure-9410) in 9 mL 1XPBS).

Blocking is done for 30 minutes @ room Temperature

Antibody incubations are done in Antibody Buffer only (no serum or triton)

5.2.9. Immunohistochemistry for cortical neurons on 24-well falcon plates

Reagents & Instruments

1. Multichannel pipette P 1200.
2. Triton X 10% (1 mL Triton X-100 (Omnipure-9410) in 9 mL 1XPBS)
3. Goat Serum (Gibco-16210)
4. Blocking Buffer
5. 4% PFA/4% Sucrose solution
6. Hoechst (Sigma-2495-100 mg)

Procedure

1. In the fume hood: Remove the media from the plates (use a tray for easy handling).
2. Add 400 μ L per well of PFA4%/Sucrose4% and incubate for 30 min.
3. Discard the PFA and wash with 400 μ L/well of PBS 1X at least 5 times. Wait a few seconds between washes.
4. Prepare the blocking solution: 10 mL of Blocking Buffer (enough for 1 plate) 20% of Goat Serum: 2 mL/ 2% of Triton X: 200 μ L Triton 10%.
5. Remove the last wash and add 400 μ L/well of the Blocking Buffer.
6. Incubate for 30 min.
7. Prepare the Primary antibody in Antibody Buffer (for example Rb anti- β -tubulin III, 1:500) (Sigma-T2200-200 μ L)
8. Discard the Blocking Buffer and add 400 μ L/well of the primary antibody in Antibody Buffer. Incubate overnight at 4 °C.
9. Wash at least 5 times with PBS 1X, 400ul/well.
10. Prepare the Secondary Antibody in Antibody Buffer. Ex: Alexa Fluor GaRb 647 1:500 (invitrogen-A21244) and Hoechst 1:1000.
11. Add 400 μ L/well of the secondary antibodies and incubate for 1-2 hr in the dark.
12. Rinse the plate with PBS 1X at least 5 times.

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Phospho-ERK Assays

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Abstract

Extracellular signal-related kinase (ERK1/2 or p42/44) is a kinase in the mitogen-activated protein kinase (MAPK) family and phosphorylation of ERK (p-ERK) can be used as a common end point measurement for the activation of many classes of G protein coupled receptors (GPCR). This chapter addresses the use of PerkinElmer's AlphaScreen[®] SureFire[™] assay to measure ERK phosphorylation. This chapter is intended to assist in the development and optimization of p-ERK assays by providing sample protocols, factors to consider during assay optimization and data analysis details for the agonist and antagonist modes.

Introduction

Extracellular signal-related kinase (ERK1/2 or p42/44) is a serine/threonine kinase that acts as an essential component of the Mitogen-Activated Protein Kinase (MAPK) signal transduction pathway. There are two MAPKs which play an important role in the MAPK/ERK cascade, MAPK1/ERK2 and MAPK3/ERK1. Many biological functions are mediated by this pathway through the regulation of transcription, translation, and cytoskeletal rearrangements, including cell growth, adhesion, survival and differentiation. The MAPK/ERK cascade also plays a role in the initiation and regulation of meiosis, mitosis, and postmitotic functions in differentiated cells by phosphorylating a number of transcription factors. About 160 substrates have been identified for ERKs and many of these substrates are localized in the nucleus to participate in the regulation of transcription. However, there are other substrates located in the cytosol or in other cellular organelles that are responsible for processes. The MAPK/ERK cascade is also involved in the regulation of lysosome processing and endosome cycling through the perinuclear recycling compartment (PNRC); as well as in the fragmentation of the Golgi apparatus during mitosis.

Activation of ERK1/2 is commonly used to measure the functional outcomes for G protein coupled receptors (GPCRs). The α -subunit of G proteins can be categorized into different subclasses, $G_{ai/o}$, G_{aq} , and G_{as} , that trigger different signaling cascades. For GPCR targets, one utility of measuring p-ERK is its relevance across multiple receptor classes (G_{aq} , $G_{ai/o}$, and some G_{as}). G_{as} -coupled receptors increase cAMP and G_{ai} receptors decrease cAMP levels through the stimulation or inhibition of the adenylate cyclase pathway. G_{aq} -coupled receptors are known to work through the activation of the Phospholipase-C (PLC) pathway, causing increases in intracellular calcium. The advantage of measuring phosphorylated ERK (p-ERK) is that it is a common endpoint, despite initialization from different α -subunits and therefore different cascades. Now due to newer technology, there are options for developing cell-based screening assays with high throughput capability (Figure 1).

Measuring p-ERK can provide an alternative read-out for receptors. Detection of p-ERK can also potentially provide some advantages that supplement calcium and cAMP assay results when assessing GPCR drug candidates.

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Note: The content of the Assay Guidance Manual will be updated quarterly with contributions and new chapters to ensure the manual stays relevant to the current technologies and best practices used in the rapidly changing field of drug discovery and development. The chapter is currently in the process of being updated to reflect the current state of the field with respect to p-ERK assays and technologies. Therefore, it is possible that the most up-to-date information may not yet be included, but will be added in forthcoming chapter updates.

Overview of Technology

There are multiple methods currently used to measure p-ERK:

- AlphaScreen® SureFire™ ERK Assay – High throughput capability using bead proximity-based AlphaScreen technology.
- ELISA - Enzyme linked immunosorbent assays require wash steps and long incubations (often overnight). However, measurement with an Acumen reader can provide images of cells if this is desirable.
- Meso-Scale Discovery Assays – Electrochemiluminescence-based method providing medium to high throughput screening.
- LICOR - Infrared fluorescence-based method providing medium to high throughput screening.
- Western Blot Analysis – This method can be labor intensive and offers limited throughput.

AlphaScreen SureFire ERK Assay

General Background

Detection of activated ERK is enabled by immuno-sandwich capture of endogenous phosphorylated ERK in cell lysates. Antibody-coated AlphaScreen beads generate a highly amplified signal when in close proximity, due to binding of p-ERK (Figure 2; <http://www.PerkinElmer.com>, <http://www.TGR-Biosciences.com>).

Characteristics of the AlphaScreen SureFire ERK Assay

Measurement of p-ERK can be done in many ways, but the kit combining PerkinElmer's AlphaScreen technology and TGR BioSciences' SureFire cellular ERK assay is a popular method of measurement. Published work indicates that ERK1/2 activation is pharmacologically similar to previously established responses in other assay formats (1). Here are some characteristics of this assay format:

- Can be used with primary or cultured cells (adherent or non-adherent)
- Cloned or endogenous receptors (transient or stable transfection)
- Non-radioactive
- Detects agonists, antagonists, or orphans
- Homogeneous assay
- No wash steps
- One day assay
- Automatable for high throughput (384- or 1536-wells)
- Highly sensitive and low background
- Can detect broad affinities
- Specialized reader is required

Sample Protocol

See the PerkinElmer protocol for AlphaScreen SureFire p-ERK assay kits or the TGR Biosciences SureFire Cellular ERK assay protocol for more detailed information. Additional information is also available for one versus two plate protocols and non-adherent cells.

Figure 1: ERK1/2 can be activated by GPCRs which couple to different G protein subclasses and transduce the signal by different pathways. Functional separation of these signals is achieved by spatially distinct pools of ERK1/2 within the cell (1).

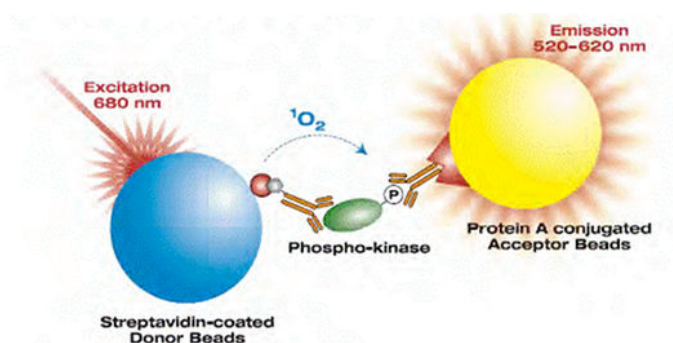


Figure 2: AlphaScreen SureFire ERK Assay Principle.

Suggested Assay plate: PerkinElmer, Proxiplate-384 well plate (half volume), #6006280

Schematic Outline of the SureFire Cellular ERK Assay

1. Plate cells into 96- or 384-well proxiplate for 24 hours at 37°C
2. Starve cells with low serum or serum-free medium. This optional step helps to keep the basal phosphorylation levels low (times vary based on the cell type).
3. Add inhibitors (time may vary)
4. Add agonist for 5 to 15 minutes
5. Lyse cells for 10 minutes at room temperature
6. Transfer 6 µl lysate to assay plate
7. Add 10 µl reaction mix containing beads for 2 hours at room temperature
8. Read plate

Assay Formats

Agonist Mode:

- a. Cells are stimulated with agonist and optimal time and temperature for stimulation are determined.

- b. Max response is maximum p-ERK produced by full agonist stimulation (Figure 3).
- c. Min response is p-ERK produced in the presence of stimulation buffer without agonist.
- d. Relative EC₅₀ is obtained from the concentration response curve.
- e. Detection of GPCR-induced ERK1/2 activation in transfected cell lines (Figure 4).

Antagonist Mode:

- a. Cells are pretreated with diluted test antagonist at 37°C for 1-2 hours.
- b. Agonist is added at the EC₈₀ concentration and incubated at room temperature for 15 minutes.
- c. Inhibition of the agonist response is quantified.
- d. Max response is maximum p-ERK produced by full agonist stimulation.
- e. Min response is p-ERK produced in the presence of stimulation buffer without agonist.
- f. Relative IC₅₀ is determined from the concentration response curve (Figure 5).

Assay Optimization

The following conditions should be optimized for the best assay performance:

- a. Cell titration to minimize the baseline ERK1/2 phosphorylation level and to maximize the signal window.
- b. Serum starvation parameters (if required). This may be necessary to reduce background.
- c. DMSO tolerance study.
- d. Time course for compound incubation.
- e. Time course for agonist stimulation.
- f. Optimization of instrument set-up.

Helpful Hints for Performing SureFire AlphaScreen ERK Assays

- a. Cells are grown to confluence in microplate wells prior to assaying for ERK1/2. This is important because contact inhibition significantly lowers the level of background ERK1/2 phosphorylation, synchronizes the responsiveness of the cells, and maximizes the signal window.
- b. Cells should be harvested from flasks for seeding into microplates at approximately 70-90% confluence. Cells should be detached from the flasks using conditions as mild as possible and allowed to adhere to plates for at least 24 hours prior to the assay.
- c. Monitor the cell passage number; determine empirically whether the cells will lose responsiveness at passages beyond a maximum limit.
- d. Assay incubation temperature should be at least 22°C.
- e. To eliminate “edge effects”, increase the reaction volumes from 11 µl (4 µl cell lysate and 7 µl reaction mix) to 16 µl (6 µl cell lysate and 10 µl reaction mix).
- f. Avoid bubbles in the assay wells.
- g. Because AlphaScreen beads are light sensitive, add beads and incubate assay plates in low light conditions.
- h. Read the plates using the Envision Alpha Turbo module to avoid an “edge effect” in data consistency.

Websites

www.TGR-Biosciences.com

www.PerkinElmer.com

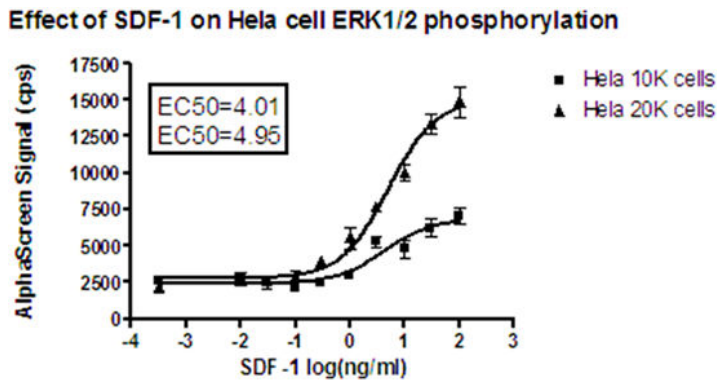


Figure 3: Agonist concentration response curves. ERK1/2 activation with cells expressing endogenous GPCRs.

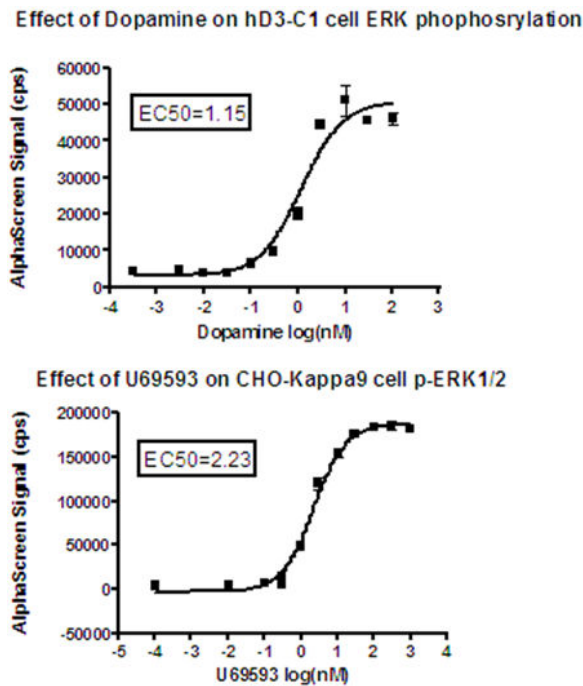


Figure 4: Detection of GPCR-induced ERK1/2 activation in transfected cell lines. A) Effect of dopamine on hD3-C1 cell ERK phosphorylation. B) Effect of U69593 on CHO-Kappa9 cell p-ERK1/2.

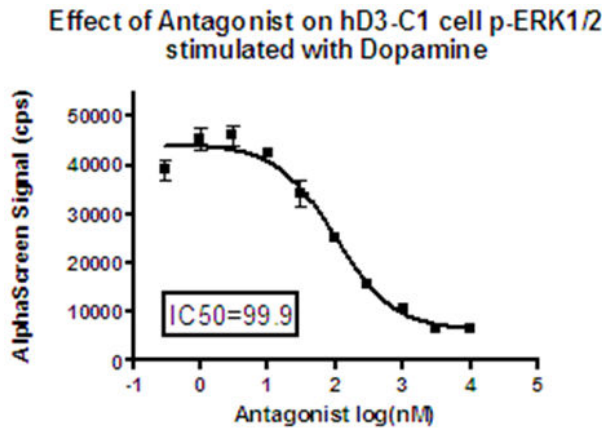


Figure 5: Antagonist concentration response curve.

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IP-3/IP-1 Assays

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Created: May 1, 2012.

Abstract

Activation of G-protein coupled receptors (GPCR) that couple to $G_{\alpha\theta}$ and $G_{\beta\gamma}$ and subsequent activation of phospholipase C β (PLC- β) can be detected through the measurement of D-myo-inositol 1,4,5-triphosphate (IP3) in cells. This chapter describes technologies that can be used to develop robust assays for screening compounds, more specifically the use of Homogeneous Time-Resolved Fluorescence Assay (HTRF) for IP3 measurement. A sample preparation protocol, parameters for assay optimization and examples of data analysis are provided.

Introduction

Note: The content of the Assay Guidance Manual will be updated quarterly with contributions and new chapters to ensure the manual stays relevant to the current technologies and best practices used in the rapidly changing field of drug discovery and development. The chapter is currently in the process of being updated to reflect the current state of the field. Therefore, it is possible that the most up-to-date information may not yet be included, but will be added in forthcoming chapter updates.

Agonist stimulation of G protein coupled receptors that are coupled to $G_{\alpha q}$ (or to $G_{\beta\gamma}$ subunits) leads to activation of phospholipase C β (PLC- β) followed by production of D-myo-inositol 1,4,5-triphosphate (IP3). IP3 initiates the release of Ca^{2+} from intracellular stores before it is rapidly degraded to IP2 then IP1 (Figure 1). Activation of this pathway is usually measured by detection of intracellular calcium using fluorescent calcium indicator dyes and fluorescence plate readers (FLIPR). Although calcium assays are robust and easily amenable to HTS, there are some important limitations: calcium flux is very rapid and transient, and does not allow detection of constitutive activity (or inverse agonism); interference by fluorescent and nuisance compounds is a problem; and sensitivity is often insufficient to allow the use of primary cells. Alternatively, it is possible to measure IP3 production directly or indirectly as a read-out of PLC- β activation.

Overview of Technology

Traditional assays for total inositol phosphate accumulation used radioactivity and were complicated and not amenable to HTS. In addition, IP3 production is very rapid and transient before it is metabolized to IP2 and IP1. There are a few alternative technologies available for measurement of IP-3 including AlphaScreen (Perkin Elmer) and HitHunter™ Fluorescence Polarization (DiscoverX). Recently a homogeneous time resolved fluorescence assay for IP1 (IP-One HTRF®), has been developed by Cisbio (see below). This assay format takes advantage of the fact that lithium chloride (LiCl) inhibits the degradation of IP1, the final step in the inositol phosphate cascade, allowing it to accumulate in the cell, where it can be measured as a substitute for IP3. Data from Cisbio show that the assay can be used with endogenously or heterologously expressed receptors in either adherent or suspension cells, to quantitate the activity of agonists, antagonists, and inverse agonists. Agonist EC_{50} 's and antagonist IC_{50} 's using the IP-One HTRF® assay correlate very well with those from calcium assays and traditional IP3 detection assays.

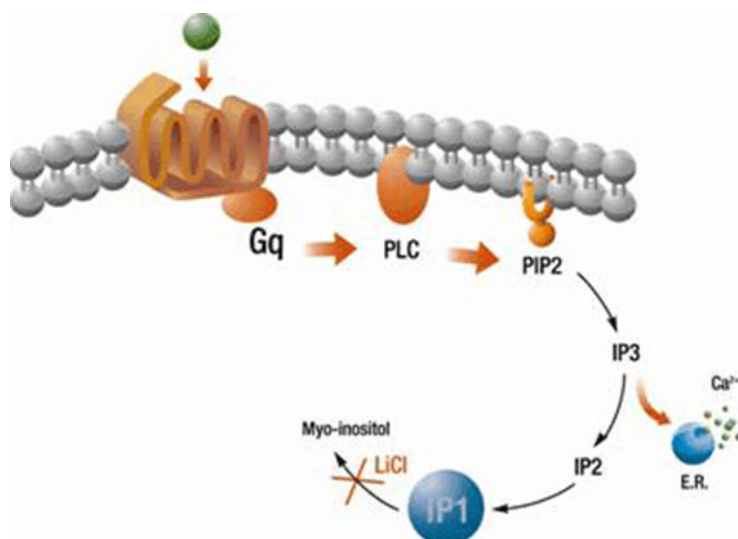


Figure 1: Activation of $G_{\alpha q}$ Pathway (Reprinted from Cisbio with permission).

IP-One HTRF® Technology (Cisbio)

General Background

The IP-One HTRF® assay kit allows direct quantification of myo-Inositol 1 phosphate (IP1) in cultured cells. The assay is a competitive immunoassay. IP1 produced by cells (in the presence of LiCl) after receptor activation competes with an IP1 analog coupled to a d2 fluorophore (acceptor) for binding to an anti-IP1 monoclonal antibody labeled with Eu Cryptate (donor). The resulting signal is inversely proportional to the concentration of IP1 in the sample. A standard curve is constructed to convert raw data to IP1 concentration (Figure 2).

See the following link for more information: <http://www.htrf.com/products/gpcr/ipone/>

Sample Preparation Protocol

Assay may be conducted in 96-well, 384-well or 1536-well formats. Only white plates should be used for IP-One HTRF. Suggested plate types:

- 96 half-well plate Costar cat # 3688 (white, opaque flat bottom, TC-treated). Total working volume = 100 μ l.
 - 96-well Costar cat # 3917 (white, opaque flat bottom, TC-treated). Total working volume = 200 μ l.
 - See the following link for additional plate recommendations: <http://www.htrf.com/technology/assaytips/microplate/>
1. Adherent cells may be seeded into tissue culture treated, white microplates 24 hours before assay. Just before the assay, media is removed from adherent cells and replaced with Stimulation Buffer (included in the kit). Note: buffers containing phosphate can not be used.
 2. Alternatively, cells may be prepared in suspension using the Stimulation Buffer provided in the kit, and plated immediately before the assay.
 3. Serial dilutions of the IP1 standard included in the kit are made using Stimulation Buffer, and pipetted into the assay plate for the standard curve.
 4. Cells are pre-treated with antagonist compounds prepared in Stimulation Buffer for 15-30 minutes at 37°C, 5% CO₂.

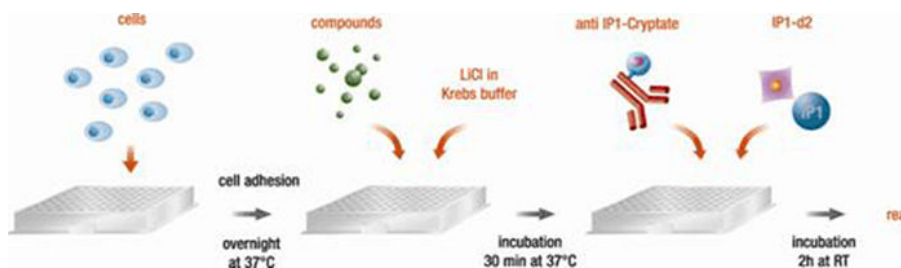


Figure 2: IP1 HTRF Assay Protocol (Reprinted from Cisbio with permission).

5. Agonist prepared in Stimulation Buffer is added and plates are incubated at 37°C, 5% CO₂ for required stimulation time (to be optimized).
6. Diluted IP1 d2 conjugate is added to wells.
7. Diluted anti-IP1 Eu Cryptate is added to wells.
8. Plates are incubated for 1 hour at room temperature.
9. Plates are read on an HTRF[®] compatible reader (eg: Envision, Tecan GENios, BMG Rubystar). See the following link for other readers: http://www.htrf.com/technology/htrfmeasurement/compatible_readers/
10. Excitation is at 320 nm. CisBio recommends using a ratiometric measurement for HTRF[®] emissions at both 620 nm and 665 nm. Emissions at 620 nm are used as an internal reference and emissions at 665 nm reflect the biological response. The ratio of 665/620 allows normalization for well-to-well variability and interference due to assay components.

Signal Stability: plates may be read repeatedly for determination of kinetics, and signal is stable for at least 24 hours at RT.

Results and Data Analysis

The ratio of absorbance 665/ absorbance 620 nm emissions is calculated. A standard curve is plotted of Ratio 665/620 vs IP-1 concentration using non-linear least squares fit (sigmoidal dose response variable slope, 4PL). Unknowns are read from the standard curve as nM concentration of IP1. Ratio 665/620 of unknowns should fall on the linear portion of the standard curve. Increased accumulation of IP-1 will result in a decrease in signal (Figure 3).

Assay Formats

Agonist Mode:

Cells are stimulated with agonist for optimum time and increase in IP1 produced by receptor activation is quantified. Max response is maximum IP1 produced by full agonist stimulation. Min response is IP1 produced in the presence of stimulation buffer and the absence of agonist. Relative EC₅₀ and Relative Efficacy (% maximum activity of a test compound relative to the reference agonist) may be obtained from concentration response curve (Figure 4).

Antagonist Mode:

Cells are treated with test antagonist compound for approximately 15 minutes. Agonist is then added at approximately EC₈₀ concentration and incubated for optimum time. Inhibition of the agonist response is quantified. Max response is IP1 produced by EC₈₀ concentration of agonist in the absence of compound. Min response is IP1 produced in the presence of stimulation buffer and the absence of agonist or test compound.

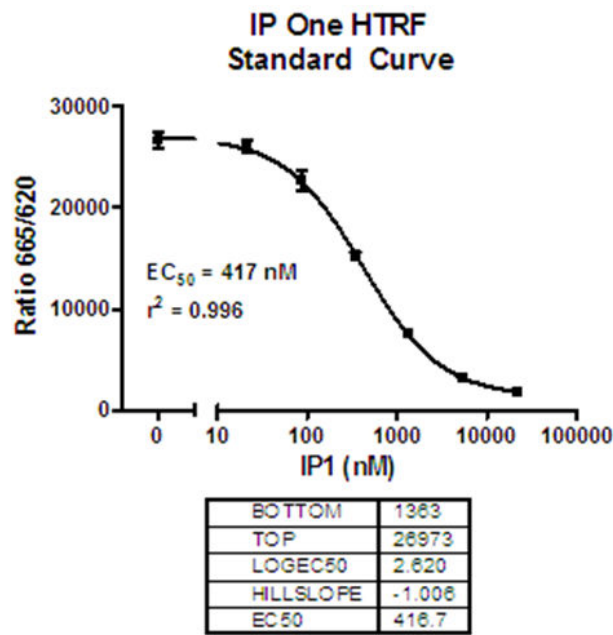


Figure 3: 1 Standard Curve.

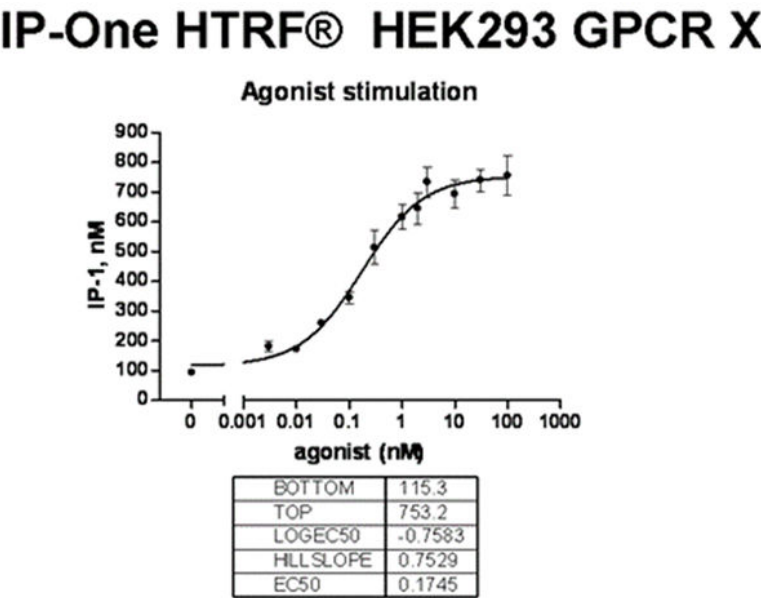


Figure 4: Agonist concentration response curve

Relative IC₅₀ may be obtained from concentration response curve and used to calculate antagonist K_b (Figure 5).

Inverse Agonist Mode:

Cells expressing a constitutively active receptor are treated with test compound for optimum time (in the absence of agonist). Inhibition of the basal response (IP1 produced in the presence of stimulation buffer alone) by test compound is quantified. Max response is basal level of IP1 produced in cells expressing the constitutively active receptor during the incubation time. Min response is basal level of IP1 produced in cells without the

IP-One HTRF® HEK293 GPCR X

Inhibition of agonist stimulated IP-1 accumulation

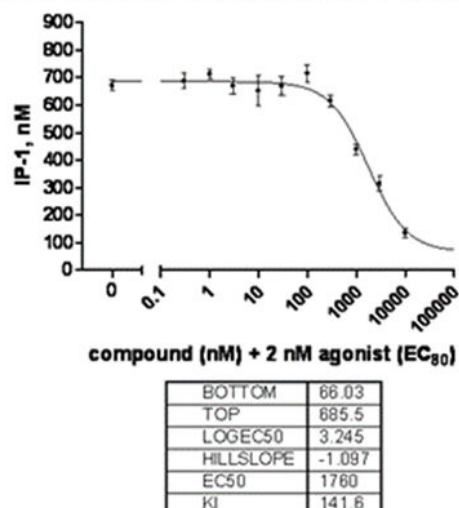


Figure 5: Antagonist concentration response curve

receptor. Relative EC₅₀ Inverse and Relative Efficacy Inverse (% maximum response of reference inverse agonist) may be obtained from concentration response curve (Figure 6).

Assay Optimization

The following parameters should be optimized to ensure that the level of IP-1 produced in the wells falls within the linear range of the standard curve, signal window is maximized and variability is acceptable:

- cell number
- preincubation of cells with stimulation buffer
- agonist stimulation time
- incubation time after addition of conjugates

See the following link for Cisbio recommendations for assay optimization: <http://www.htrf.com/files/resources/ip-one%20nature.pdf>

Web Sites

<http://www.htrf.com/resources/>

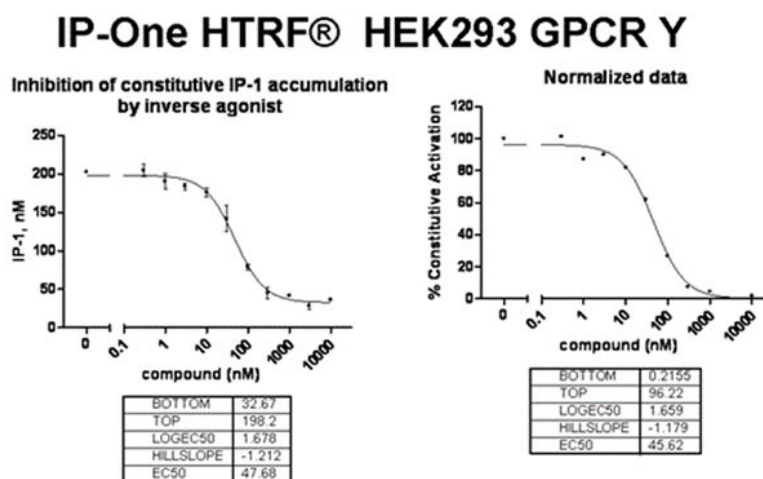


Figure 6: Inverse agonist concentration-response curve

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Cardiomyocyte Impedance Assays

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Created: February 25, 2015.

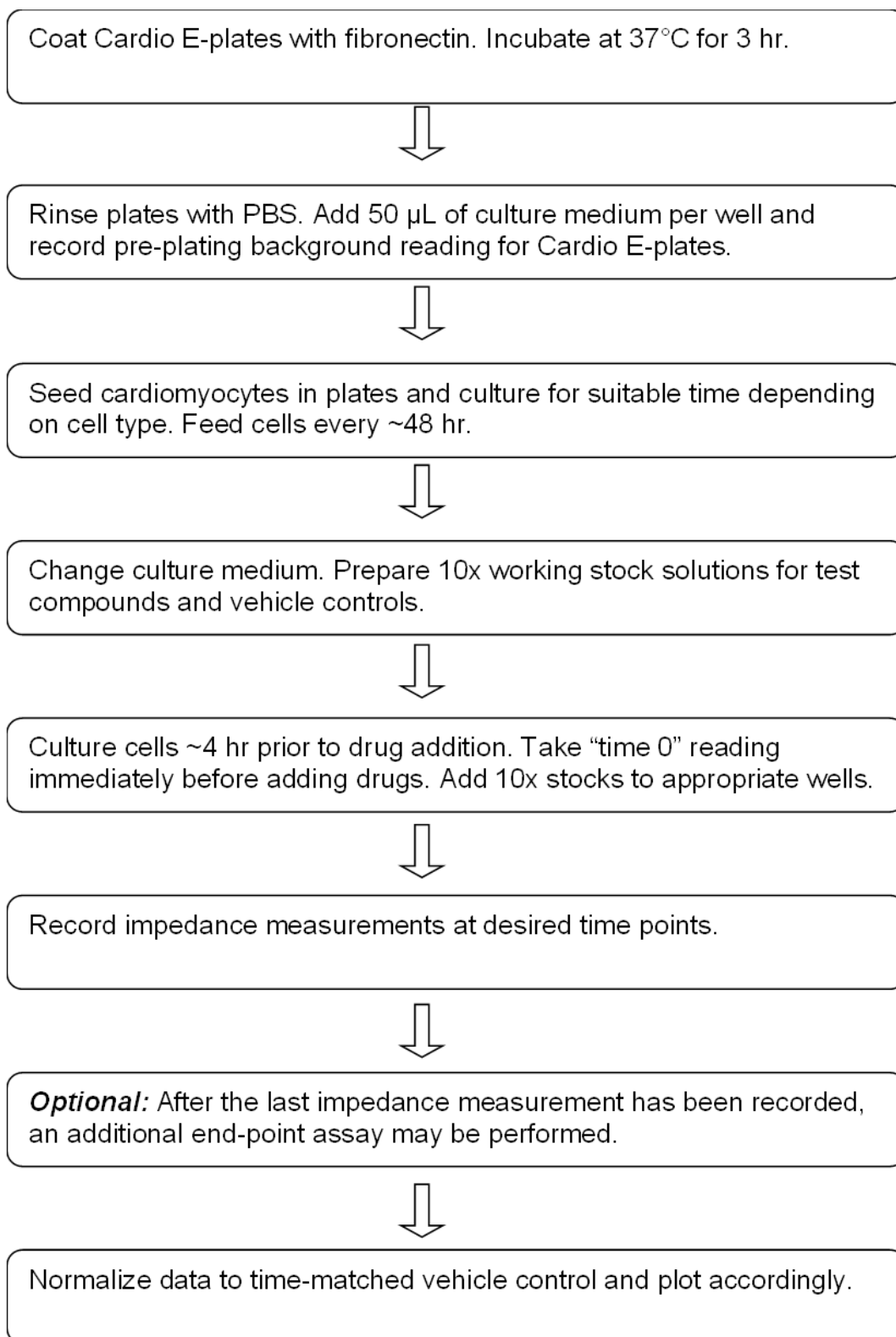
Abstract

Cellular impedance assays have been broadly utilized as a label-free approach for toxicity and drug discovery screening. The xCELLigence RTCA Cardio instrument sets itself apart from other impedance technologies by its rapid data acquisition rate (12.9 msec). This speed is fast enough to detect the contractile activity of beating cardiomyocytes, offering a relatively high throughput and robust strategy to noninvasively monitor the effects of test compounds on cardiomyocyte function. This chapter introduces the fundamentals and applications of cardiomyocyte impedance assays. A protocol detailing cardiomyocyte culture, data acquisition, and data analysis using the xCELLigence RTCA Cardio system is provided and considerations for assay design and data interpretation are discussed.

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Flowchart



Introduction

Cellular impedance assays have proved to be a robust and versatile label-free approach to study whole cellular behavior. The technology utilizes electrodes that are incorporated into the bottom of each well of tissue culture

plates. Weak alternating current (AC) is applied between the electrodes with tissue culture medium as the electrolyte. Impedance is calculated using the AC version of Ohm's law where impedance (Z) is the ratio of voltage (v) / current (I). Cells are seeded into the wells and attach to the bottom of the well. The cell layer covers the electrodes, thus impeding current flow between the electrodes to varying degrees, depending on cell number, morphology, adhesion, and cell-cell contacts. Cell morphology and adhesion changes are therefore quantified without exogenous detection labels or dyes, allowing noninvasive, continuous, and label-free monitoring of cellular events.

After cells adhere to the bottom of an impedance plate, current flows between the electrodes through a combination of extracellular and transcellular paths. The frequency of the electrical current influences the preferred path; lower frequency currents tend to take extracellular (paracellular) paths while higher frequency currents have a propensity to pass capacitively through cell membranes (transcellular) (1, 2). Because these paths are preferentially sensitive to different cellular functions and/or events, controlling current frequencies has allowed researchers to tailor cellular impedance platforms to suit diverse screening applications. Different instruments have been used to quantitatively measure a wide variety of cellular events, including cell viability and growth, migration, cell-cell and cell-matrix contact, and GPCR and kinase signaling (reviewed in 3).

Application of impedance to cardiomyocytes has recently been introduced by the xCELLigence RTCA Cardio instrument (ACEA Biosciences, San Diego, CA) that produces a single frequency (10^4 Hz) to enable accelerated data acquisition (12.9 msec) (4, 5). The number of data points collected per second (~ 78) easily allows for quantitative monitoring of impedance changes associated with the physical movement of spontaneous, synchronously beating cardiomyocytes. This relatively high throughput, label-free approach to detect cardiomyocyte beating has several advantages: 1) it allows for continuous, real-time monitoring without the need of exogenous detection reagents, 2) it produces robust, quantitative data, and 3) it offers versatile data analysis.

Concept and Overview of xCELLigence RTCA Cardio System

The xCELLigence Real-Time Cell Analysis (RTCA) Cardio instrument exploits impedance changes across the cardiac monolayer to evaluate both cardiomyocyte health status and contraction-induced morphology changes. Cardiomyocytes are cultured on 96-well Cardio E-Plates, which contain interdigitated gold microelectrodes incorporated into the bottom of each well. Spontaneously beating cardiomyocytes from several sources, including freshly isolated rat neonatal cardiomyocytes as well as human stem cell-derived cardiomyocytes have been used successfully with the RTCA Cardio system.

The system is composed of the RTCA Cardio Station, the RTCA Control Unit, and the RTCA Analyzer (Figure 1). To monitor impedance, the Cardio E-plate is placed in the RTCA Cardio Station, which is housed within a standard tissue culture incubator. The analyzer connects to the station through a ribbon cable that passes through the sealed door of the incubator. The control unit operates the software and acquires and displays the data in real time, while the analyzer sends and receives the electronic signals between the control unit and the station. Impedance is measured and Cell Index (CI) is calculated by subtracting base line impedance from impedance at any given time and dividing by a constant value (6). Because cell index is a relative measure of impedance, it is exquisitely sensitive to changes in cell number, attachment, and morphology. Change in any of these factors produces a measureable change in CI values and thus allows for determination of cell status over assay time (Figure 2).

The contractile activity of beating cardiomyocytes results in small transient changes in impedance that are a fraction (generally 0.1%) of the overall impedance (Figure 3). The hallmark feature of the xCELLigence RTCA Cardio System is the fast data acquisition rate (12.9 msec), allowing the detection of minute morphological changes of a spontaneously beating cardiomyocyte monolayer, thereby enabling a readout that is downstream of both mechanical and electrical elements of contraction. A demonstration that impedance measures the physical

movement of contraction rather than the electrophysiological properties of cardiomyocyte beating can be seen with blebbistatin, a small molecule myosin II inhibitor, which causes a reduced impedance beating pattern in the absence of altering the action potential as detected by multi-electrode array (MEA) (Figure 4). The current version of the analysis package of the RTCA Cardio Instrument Software is capable of quantifying several beat parameters including beat rate (BR), beat amplitude (amp), beat duration (IBD₅₀, IBD₉₀), rising time (T_r), falling time (T_f), beating pattern similarity (BS), and beating rhythm irregularity (BRI) (Table 1 and Figure 5). Therefore, in addition to monitoring changes in overall cellular status (morphology/attachment/viability), changes in beating characteristics can also be assessed.

A few publications have cross-validated the Cardio System with other detection methods using positive control compounds with well-established molecular mechanisms of action. For example, Guo et al (7) tested 28 compounds covering different known cardioactive mechanisms (inhibitors of various cardiac ion channels, GPCR agonists, etc) and showed comparable detection with both impedance and electrical field potential using microelectrode arrays (MEA). In a follow-up study, this group demonstrated concordance between impedance and patch clamp electrophysiological data using compounds with established clinical arrhythmic liability (8). Scott et al (9) demonstrated similar assay performance metrics between the impedance assay and a field stimulation IonOptix (optical-based) measure of cardiomyocyte contractility, using 30 inotropes and 19 non-inotropes. These compounds were recently tested for effects on Ca²⁺ transients using the FLIPR Tetra instrument, and gave results comparable to that seen with IonOptix and impedance (10). Thus, impedance data overlaps favorably with that derived from other detection methods, and in some cases can detect compounds that are not detected in methods that quantify “upstream” endpoints such as the action potential and calcium flux (e.g. blebbistatin, Figure 4). Finally, high-content imaging has been used to detect structural cardiotoxins (i.e. causing morphological damage and toxicity as opposed to affecting the mechanical properties of the cardiomyocyte) (11). Such compounds have not yet been systematically tested in the Cardio System. It will be interesting to determine the overlap between these two methods for detecting structural cardiotoxicity.

RTCA Cardio Control Unit

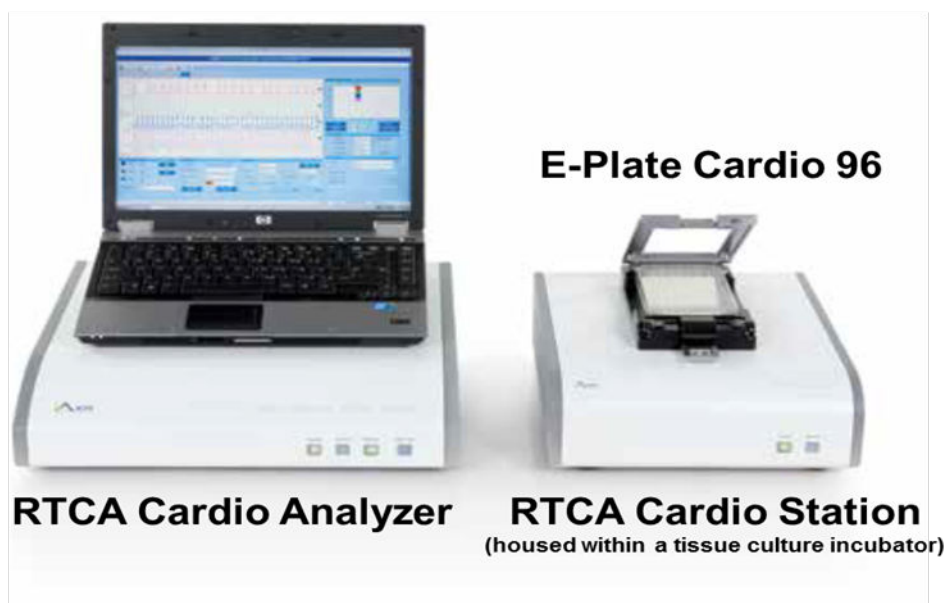


Figure 1: xCELLigence RTCA Cardio instrument (ACEA Biosciences). The RTCA Analyzer and Control Unit (left) connect to the RTCA Cardio Station (right), which is housed within a standard tissue culture incubator.

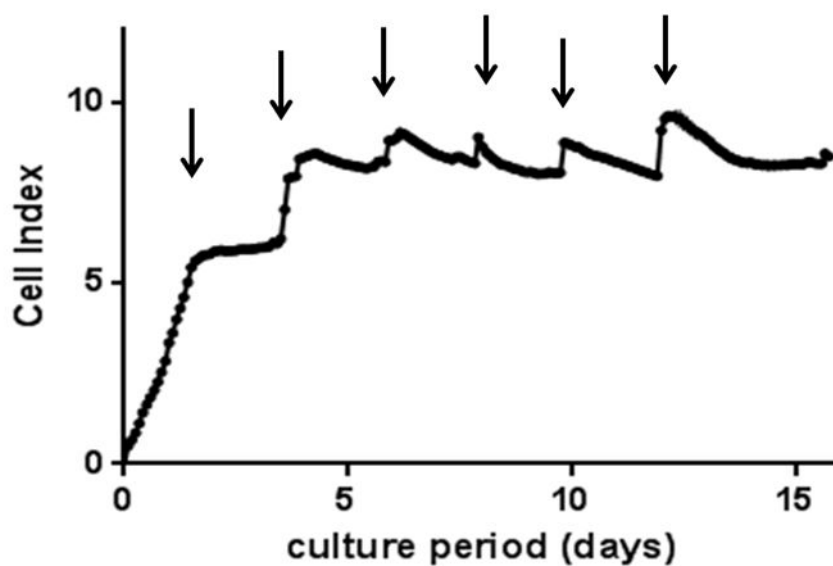


Figure 2: Changes in cell index over the course of cardiomyocyte culture. iPSC (30,000 plateable iCell® cardiomyocytes per well) were seeded on an E-plate 96 and overall impedance, displayed as cell index, was monitored for 16 days (mean $\pm$ SD of 3 wells). Medium changes indicated by black arrows.

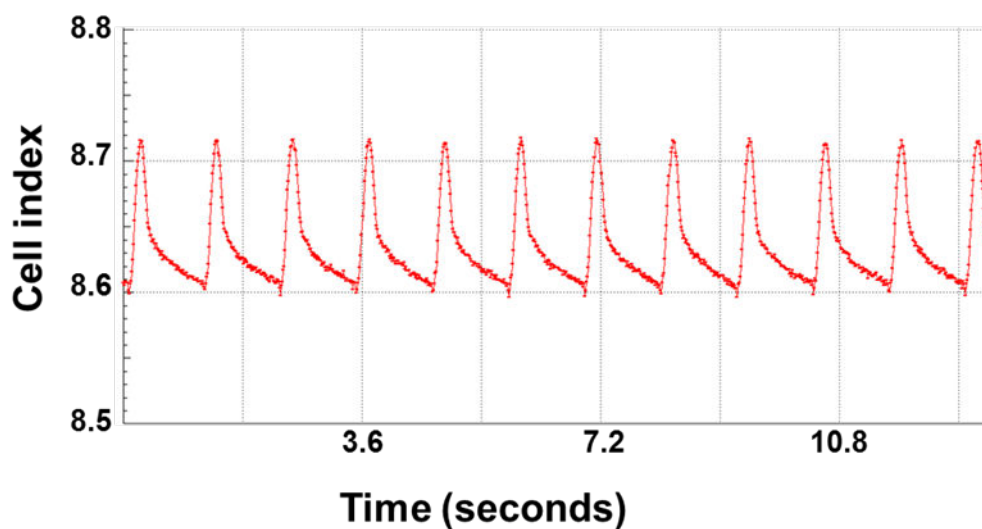


Figure 3: Change in cell index of beating cardiomyocytes. Note the relatively small fluctuation in cell index (8.6-8.7) compared to the overall cell index of ~ 8.6 , equaling an $\sim 0.09\%$ change in impedance.

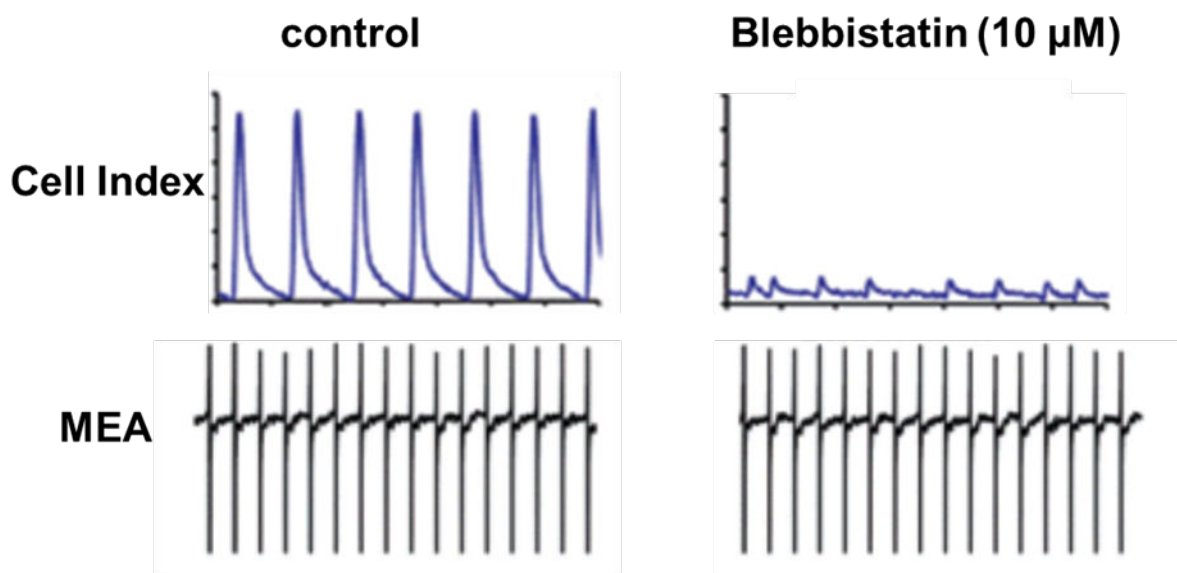


Figure 4: Effect of blebbistatin, a myosin II inhibitor, on mouse embryonic stem cell-derived cardiomyocyte impedance and MEA profiles (Adapted from 5). Blebbistatin treatment ($10\ \mu\text{M}$) results in inhibition of impedance signals but does not have an effect on field potential recordings measured by MEA.

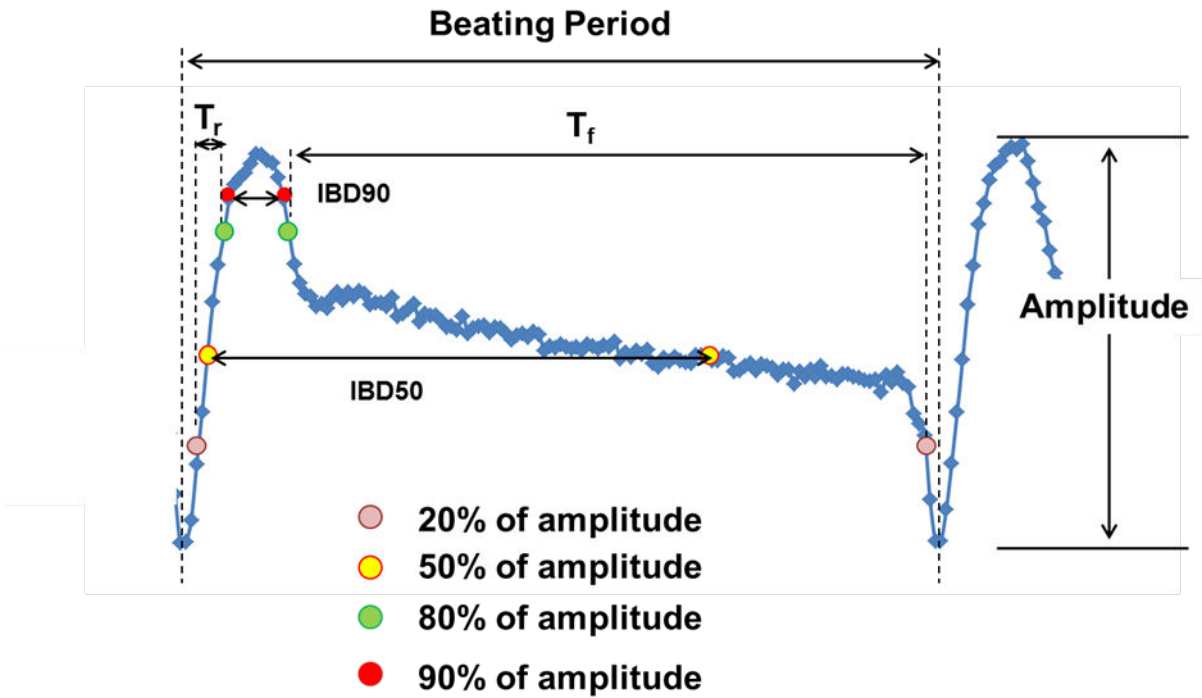


Figure 5: Definition of beat parameters. RTCA Cardio Software identifies and quantifies several beat parameters including amplitude, beating period, T_f , T_r , IBD₅₀, and IBD₉₀.

Table 1: Definition of beat parameters. RTCA Cardio Software identifies and quantifies several beat parameters.

Parameter	Abbreviation	Definition
Beat rate	BR	Number of beats per minute
Beat amplitude	amp	Cell Index difference between one negative peak to the following positive peak
Beat duration	IBD ₅₀ , IBD ₉₀	Duration between two adjacent points sitting at 50% or 90%, respectively, of maximal amplitude
Rising time	T_r	Time the signal rises from 20% peak height to 80% peak height
Falling time	T_f	Time the signal falls from 80% peak height to 20% peak height
Beating period		Time between each positive or negative peak
Beating rhythm irregularity	BRI	The CV (SD/avg) of all of the beating periods in one sweep
Beating pattern similarity	BS	A comparison of the beating compared to a selected base time (score1 for exactly the same -1 to exactly opposite)

Sample Protocol

This protocol provides basic instructions for cardiomyocyte culture, compound treatments, data acquisition, and analysis using the RTCA Cardio system. As an example, a description of how to culture commercially available induced pluripotent stem cell-derived cardiomyocytes (iPSC-CM) from CDI International (Madison, WI) for use with the RTCA Cardio system is offered. These cells can be maintained for extended durations (≥ 3 weeks), thereby enabling measurement of acute and sub-acute drug-induced effects.

Cell Plating and Culture

1. Dilute fibronectin (Sigma cat# F1141) to 10 µg/mL with sterile DPBS.
2. Coat wells of E-plate Cardio 96 (ACEA cat# 06417051001) with 50 µL of 10 µg/mL fibronectin. Incubate plates at 37°C for 3 hr. Alternatively plates can be incubated at 4°C overnight.
3. Aspirate fibronectin solution and rinse wells once with 200 µL DPBS.
4. Add 50 µL of iCell® plating medium (CDI cat# CMM-100-110) to each well.
5. Record pre-plating background impedance measurement for each plate.
6. Thaw iCell® cardiomyocytes (CDI International) for 4 min in a 37°C water bath. Transfer cells to a 50 mL conical tube and slowly add room-temperature plating medium drop-wise to a final volume of 5 mL plating medium/vial of cells. (Note: iCell® cardiomyocyte viability is highly dependent on the thawing process. Be sure to follow instructions provided by the manufacturer.)
7. Count cells and, taking into account the plating efficiency for the specific lot number of cells, dilute the cell suspension in plating medium to a final concentration of 6×10^5 plateable cells/mL.
8. Seed 30,000 plateable cells per well by pipeting 50 µL of the cell suspension/well. (Note: Significant edge effects can occur with the beating pattern. Therefore it is suggested that the outer wells are filled with buffer and not used for experimentation. This results in 60 wells of each plate being used per experiment.)
9. Allow cells to settle undisturbed for 30 min at room temperature to ensure even distribution of cardiomyocytes.
10. Culture cells undisturbed for 2 days at 37°C / 7% CO₂.
11. Aspirate medium and add 100 µL maintenance medium (CDI cat# CMM-100-120) per well. (Note: Take caution when replacing the maintenance medium. When aspirating spent medium, do not touch the bottom of the well as this might result in the removal of cells and cause an altered beating rhythm. Gently and slowly add fresh maintenance medium to the side of each well to reduce disturbance to the monolayer.)
12. Culture cells for an additional 10-12 days, carefully replacing maintenance medium every other day.

Compound Addition and Data Acquisition

1. Cells will have a consistent, stable, and synchronous beating pattern after approximately 10-14 days in culture; this window is the optimum time for compound addition and data acquisition.
2. On the day of compound addition, replace maintenance medium with 90 µL fresh medium ~4 hr before taking time zero reading and return cells to incubator.
 - a Temperature decreases reduce myocyte beating, and therefore several steps should be taken to reduce the time that cell cultures are out of the incubator (< 2 min). Maintenance medium should always be warmed to 37°C, for both cell culture and for preparing 10x stocks of test compounds. If possible, liquid handling steps should be automated.
3. Prepare 1000x stocks of test compounds in appropriate vehicle. Dilute stocks and vehicle controls 1:100 in maintenance medium for a 10x working stock in a 96-well cell culture plate. (Note: Vehicle should not exceed 0.1% final concentration and vehicle controls should always be included in the assay.)
4. Take time zero reading for each plate immediately before compound addition.
5. Add 10 µL of working stock to appropriate wells.
6. Collect data at desired time points (3 sweeps at 30-60 sec per time sweep). iCell® cardiomyocytes have a relatively long-term stable beating pattern and therefore cardiomyocyte beating function can be monitored for ≥ 72 hr.
7. If desired, an additional end-point assay can be performed AFTER the last reading is taken (e.g., cell viability can be assessed using commercial kits such as CellTiter-Glo® Luminescent Cell Viability Assay, Promega cat# G7570).

Data Analysis and Results

1. The total impedance and each of the beating parameters are recorded for every sweep performed by the RTCA software.
2. Under the “plot” tab, the beating pattern can be visualized by selecting the desired wells (right side of screen) and time (under “axis scale”). The RTCA software will identify the negative and positive peaks for each of the beats. The peaks can be visualized by checking the box next to “peaks” under “axis scale”. A “+” indicates a positive peak and a “-“ indicates a negative peak identified by the software. In most cases, the default settings correctly identify the positive and negative peaks. However, in some cases, the settings may need to be adjusted by tuning the waveform peak shape, threshold, and noise filter in order to correctly identify the peaks of the beating pattern.
 - a. After verifying correct peak identification, export the desired beat parameters into excel for data manipulation (Figure 6).
 - i. Select the desired beat parameters (left hand box).
 - ii. Select the desired time points (upper right hand box).
 - iii. Export parameters into excel in list and/or plate format (lower right box).
 - b. Export CI values into excel and calculate changes over the treatment period (Figure 7). CI changes provide valuable information on cell health, attachment, and morphology status.
 - i. Select the desired wells (upper right of screen).
 - ii. Select the desired time points (under “axis scale”).
 - iii. Right click on the cell index graph (upper left of screen).
 - iv. Click on “copy data in list format” or “copy data in matrix format”.
3. Total impedance and beating characteristics will vary slightly between wells, and therefore treating each well as an independent unit will improve the data precision. To compare the effect of tested compounds, data should be transformed first to percent of time zero reading for each well and subsequently to percent of time-matched vehicle control. Concentration-response curves can then be generated and EC₅₀ / IC₅₀ values can be calculated (Figure 8).
4. An emphasis on caution interpreting overall CI changes: several factors contribute to the impedance readout and a decrease in cell index is not always indicative of cell death. As an example, treatment of cardiomyocytes with blebbistatin causes a decrease in CI after a 20 hr exposure period. However, an end-point assay performed using CellTiter-Glo® Luminescent Cell viability Assay shows that cellular ATP levels remain comparable to that of control (Figure 9), indicating that while cells have detached from the surface of the well, they are still viable. This example illustrates the importance of a secondary assay to measure viability in conjunction with cell index information to judge cytotoxic effects.

Assay Optimization

- Cell number per well
- Time of cardiomyocyte culture prior to drug addition
- Time points for impedance measurements to encompass acute versus subacute effects
- Number of sweeps and sweep time for robust data
- Tolerability of cardiomyocytes to appropriate compound vehicles

Export Setup

Export D2.

Export Items

☒ Select All

- ☒ BR: Beating Rate
Method: PPC - Positive Peak Co
- ☐ NBR: Normalized Beating Rate
Method: PPC - Positive Peak Co
- ☒ Amp: Amplitude
Method: WP - Whole Peak
- ☐ NAmplitude: Normalized Amplitude
Method: WP - Whole Peak
- ☒ IBD50
- ☒ IBD90
- ☒ Tr: Rising Time
- ☒ Td: Falling Time
- ☒ BRI: Beating Rhythm Irregularity
Method: PPB - Positive Peak Pe
- ☒ BS: Beating Pattern Similarity
- ☒ BP: Beating Pattern

Export Time

From: 238:44:59

To: 261:05:38

Normalize Time: 238:43:58

Base Time: 238:20:05

Normalize Well:

Export Format

☒ Select All

- ☒ List Format
- ☒ Plate Format

Export Data

Cancel

Figure 6: Screen shot of beat parameter export setup page. Beat parameters can be selected (left hand box) for the desired time points (upper right hand box). Parameters can be exported into excel in list and/or plate format (lower right box).

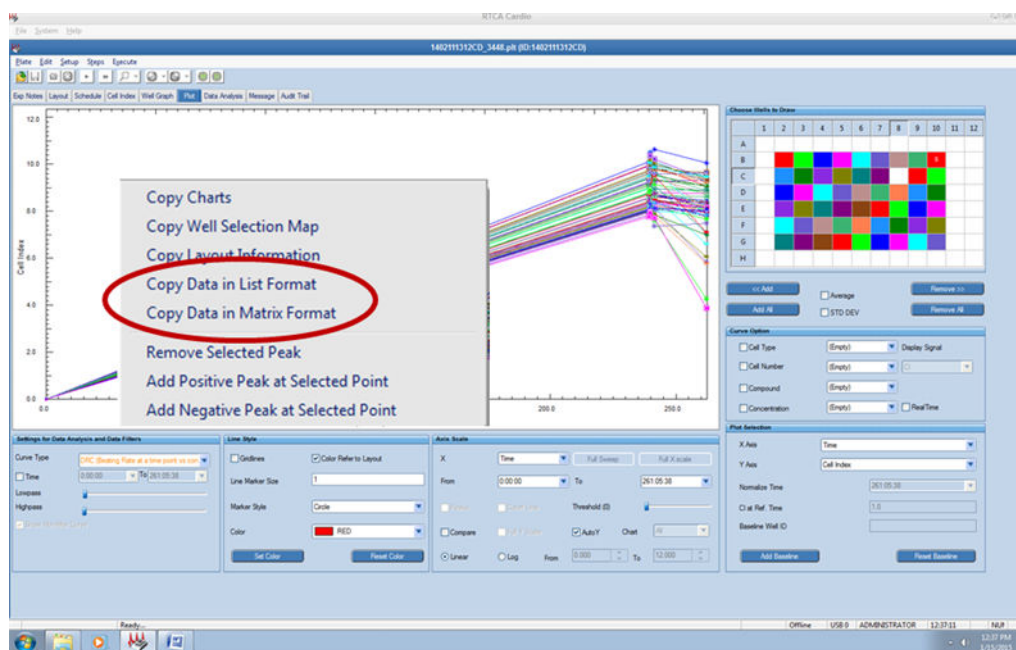


Figure 7: Screen shot of cell index export page. Cell index can be exported (left hand graph) for the desired time points (selected under “axis scale”). Parameters can be exported into excel in list and/or plate format.

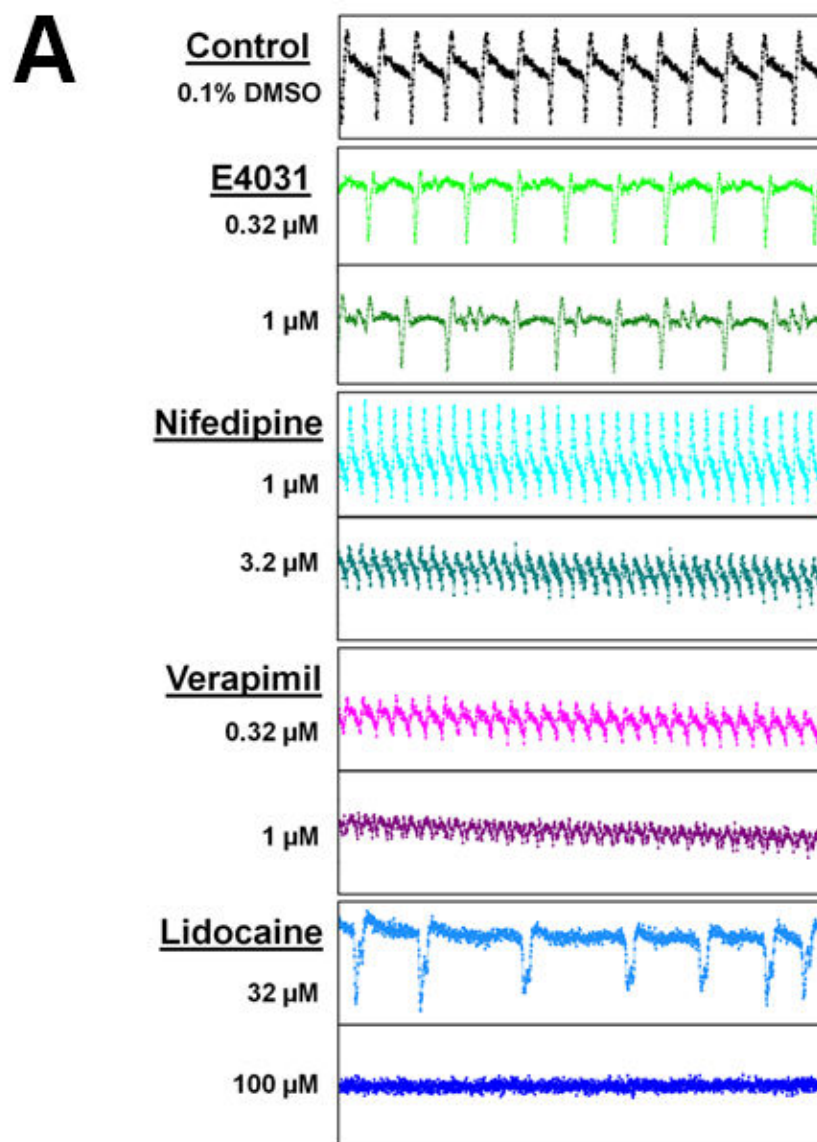
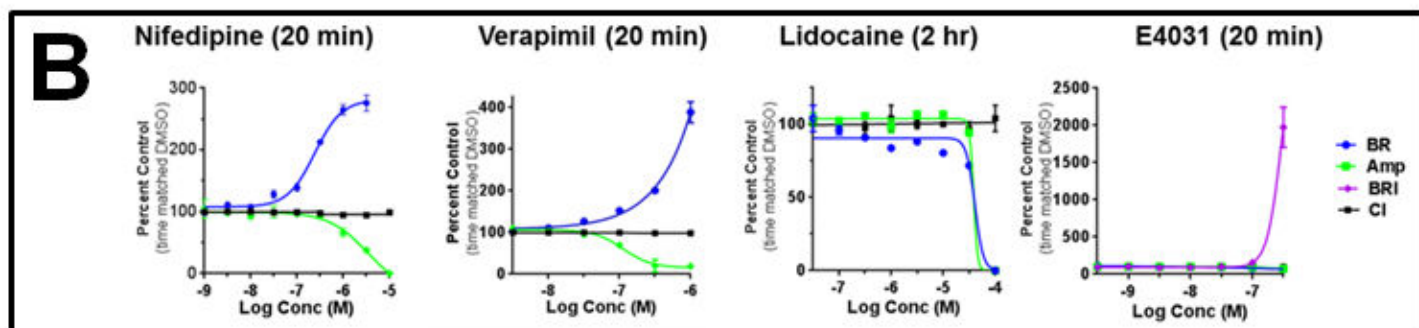


Figure 8: Examples of compound-induced changes in CM impedance profiles. Effects of a hERG-Ikr blocker (E4031, 20 min), sodium channel blocker (lidocaine, 2 hr), and L-type calcium channel blockers (nifedipine and verapamil, both 20 min). Shown as (A) raw traces and (B) concentration response curves (mean $\pm$ SD) for beat rate, beat amplitude, and BRI (E4031 only). Concentration-response curves were derived for each parameter by first calculating percent of time zero for each well and then percent of time-matched vehicle control.

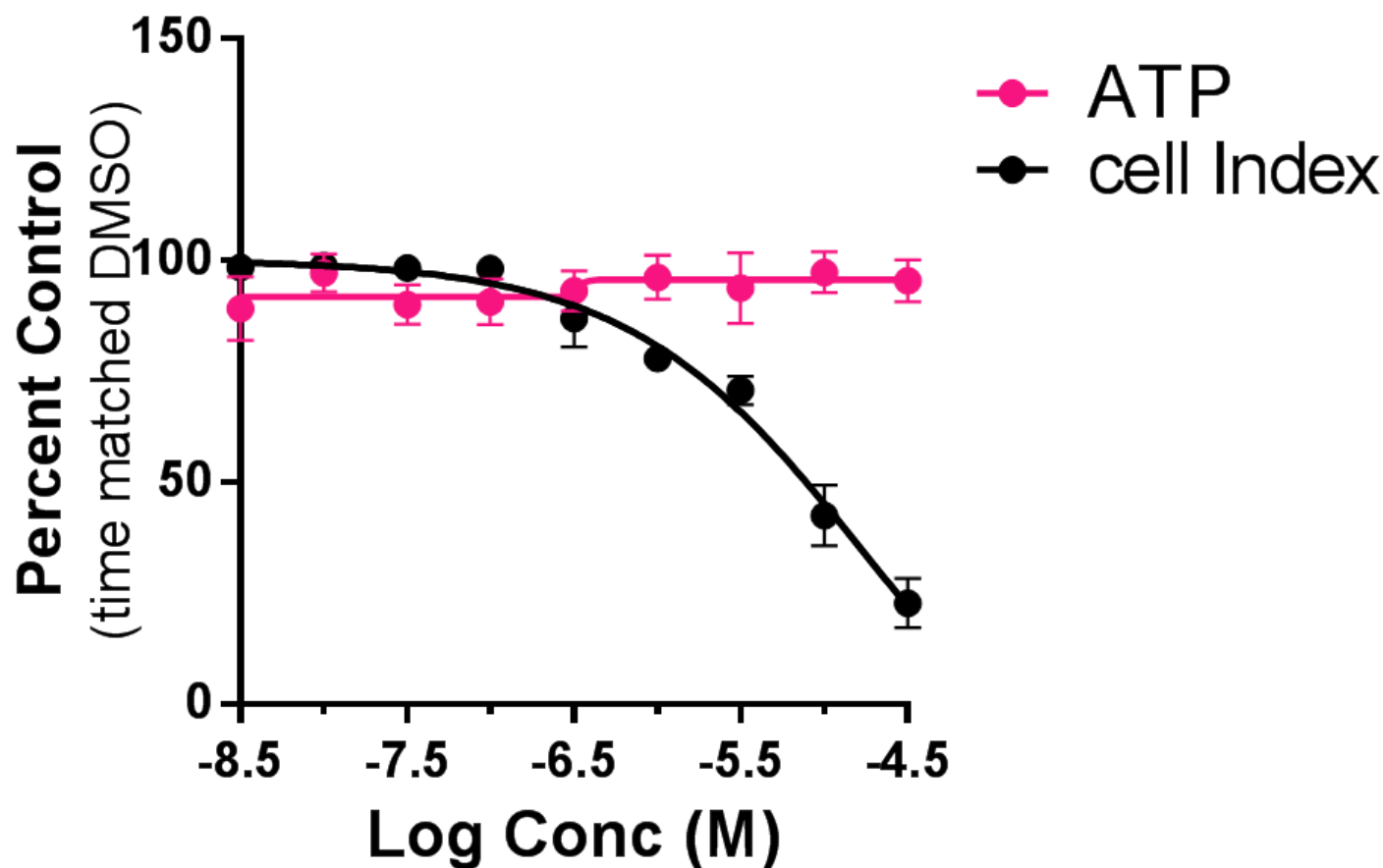


Figure 9: Effect of blebbistatin (20 hr) on CI and cellular ATP levels. Concentration-response of blebbistatin (20 hr) on cellular ATP levels and cell index (mean $\pm$ SD). Concentration-response curves were derived for each parameter by first calculating percent of time zero for each well and then percent of time-matched vehicle control.

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Screening for Target Engagement using the Cellular Thermal Shift Assay - CETSA

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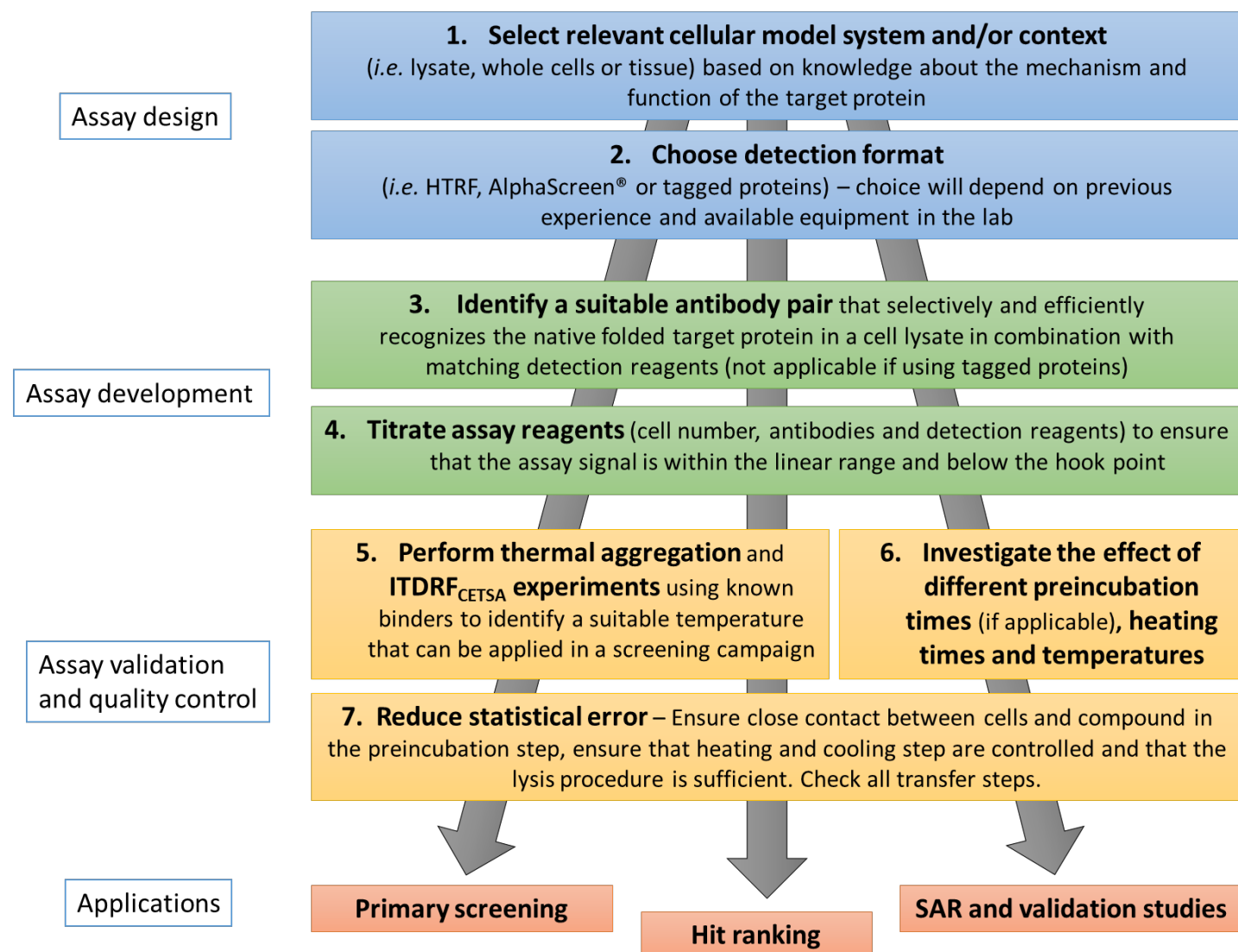
Abstract

The direct measurement of drug-protein interactions in living cells is a major challenge in drug discovery research. Using the cellular thermal shift assay or CETSA such measurements can be achieved, in principle, in any cell samples and in microtiter-plate format. This chapter starts with an overview of CETSA and then continues to provide thorough guidance in the development, optimization and application of a microplate-based protocol using AlphaScreen® as the detection format. Significant parts of the experimental descriptions are applicable also to other detection modalities. Each step in the assay development and validation process is exemplified by real case data from the development and validation of a fully screen-compatible live-cell assay for thymidylate synthase (TS; encoded by TYMS). When possible, the descriptions are kept general such that it allows for translation to other target proteins and efforts are made to point out crucial steps and considerations in the experimental design. At the end of the chapter there is a section devoted to insights from our screen adaptation experiences, troubleshooting and a discussion on tentative applications of microplate-based CETSA.

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Flow Chart



Introduction

Confirmation of direct binding to the intended target proteins in living systems, *i.e.* target engagement, is critical in the pharmacological validation of new chemical probes and drug candidates (1,2). Several exciting methodologies to achieve this are currently emerging, many of which are based on measurements of proximal biomarkers or the use of labelled molecules (tracers) or proteins to follow the binding process in microplate format (3–5). Here, we focus on the complementary use of thermal shift assays in live cells. These assays rely solely on ligand-induced thermodynamic stabilization of proteins, thus eliminating the requirements for tracer generation and protein engineering. As such this methodology provides a particularly attractive alternative for screening in primary cells, tissues, animal models and patient-derived material (6–8). Together with other developments, this technology opens up the possibility to track drug target engagement throughout the discovery process, in principle from primary screening to the treated patient. This information will help to ensure resources are invested on the best candidate compounds in target-based drug discovery programs.

CETSA Background

As with traditional melting temperature (T_m) shift assays (9–14), CETSA relies on protein stabilization as a result of ligand binding. Simply explained, when unbound proteins are exposed to a heat gradient, they begin to unfold or “melt” at a certain temperature. The mid-point of these transitions, *i.e.* where the concentrations of native and denatured proteins are the same, is generally referred to as the apparent melting temperature. Ligand-bound proteins, however, are stabilized by their interacting partner and will therefore melt at a higher temperature when exposed to the same heat gradient, resulting in a so called T_m shift. This term is however reserved for equilibrium processes, whereas CETSA is based on quantification of remaining levels of stabilized protein following irreversible aggregation of thermally unfolded proteins (see Figure 1 for a schematic outline). Thus to reflect the non-equilibrium nature of these experiments, the ligand-induced stabilization is more appropriately referred to as thermal aggregation temperature (T_{agg}) shifts.

In practice a typical CETSA experiment involves the following steps:

1. Drug treatment of the cellular system of choice (lysate, whole cells or tissue samples).
2. Transient heating of the cells to thermally denature and precipitate proteins that are not stabilized by ligand.
3. Controlled cooling and lysis of the cells.
4. Removal of precipitated proteins (if necessary).
5. Detection of remaining soluble protein in the supernatant/soluble fraction.

Based on the nature of the studied target protein and the cellular system chosen, experimental aspects of these steps will vary. Examples of possible variations include the choice of protein source (cell lysate, intact cells, biopsies or tissue homogenates), the length and means of sample treatment with ligand before heating, the heating time and temperature range applied and the procedure used for cell lysis (if applicable). The need for sample workup, such as the separation of the remaining stabilized protein from the denatured and precipitated material, as well as the ways to do so, is directly linked to the choice of detection method. This, in turn, depends on the demands for sample throughput, as well as prior knowledge and instrumentation available in the laboratory.

Experimental Formats

In general CETSA experiments assess drug target engagement in two different modes. The first setup serves the purpose of comparing the apparent T_{agg} curves for a target protein in the presence and absence of ligand when subjected to a temperature gradient. The aim is to assess the potential ligand-induced thermal stabilization (Figure 2a). The second alternative is to generate a so called isothermal dose-response fingerprint (ITDRF_{CETSA}). Here, the stabilization of the protein is studied as a function of increasing ligand concentration while applying a heat challenge at a single temperature (Figure 2b). It is common practice to first establish the T_{agg} curve for the unliganded protein, such that the isothermal challenge can be applied at a suitable temperature around or above the T_{agg} . Both formats allow for the ranking of compound affinities to a single protein target, but for structure activity relationship (SAR) studies ITDRF_{CETSA} experiments are often more suitable.

Assay Design

Before performing a CETSA experiment on a new target protein, some important considerations and choices must first be made to ensure that the ligand treatment and transient heating steps reflect the biology of interest in the best possible way. Naturally this means selecting an appropriate cellular model system in which the target protein is expressed, but considerations should also be made with regards to culture conditions and cellular status if these factors are suspected or known to affect the protein levels, regulation or ability to bind ligands. In

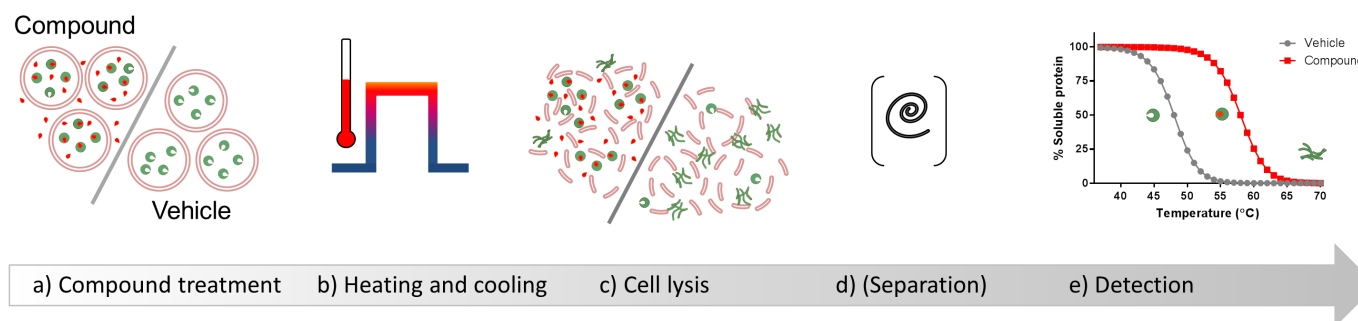


Figure 1: Illustration of the general assay procedure. The cells are incubated with compounds or controls followed by a transient heating step, cell lysis, an optional step in which the irreversible aggregates are separated from the soluble proteins and finally detection illustrated by the graph showing remaining levels of soluble target protein.

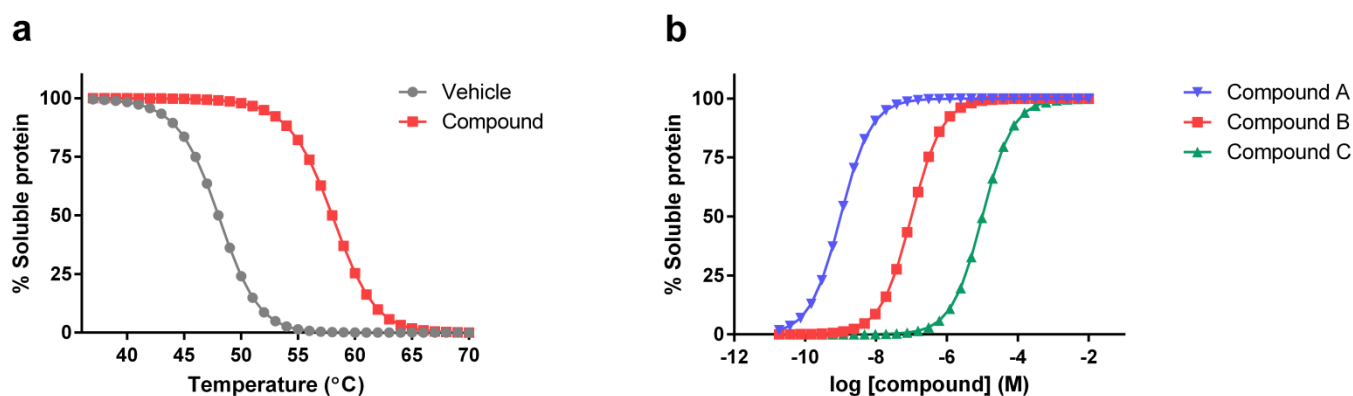


Figure 2: Anticipated results from CETSA experiments performed in two different ways. a) Example T_{agg} curve for a target protein in the presence (red squares) and absence (grey circles) of ligand when subjected to a heat gradient. b) Example ITDRF_{CETSA} curves for three compounds with different apparent potencies.

addition, depending on affinity reagent availability, throughput demands and equipment in the lab, a suitable detection technology that is amenable to microplate-based measurements must be decided upon. A general overview to these considerations is summarized in Figure 3 and the following sections intend to discuss the various aspects of assay design in more detail.

Model Systems

The CETSA method has been validated for a range of different cellular model systems of various complexity and relevance to clinical situations (6,15). An important prerequisite of the CETSA experiments is that they are performed under conditions where protein unfolding results in irreversible precipitation, such that ligand-induced stabilization can be assessed through remaining levels of soluble protein. Hence experiments can be performed in cell lysates if the endogenous protein is difficult to express and purify in a relevant form for more traditional target-based screening or if it is desirable to avoid the barriers of serum binding and cell permeability

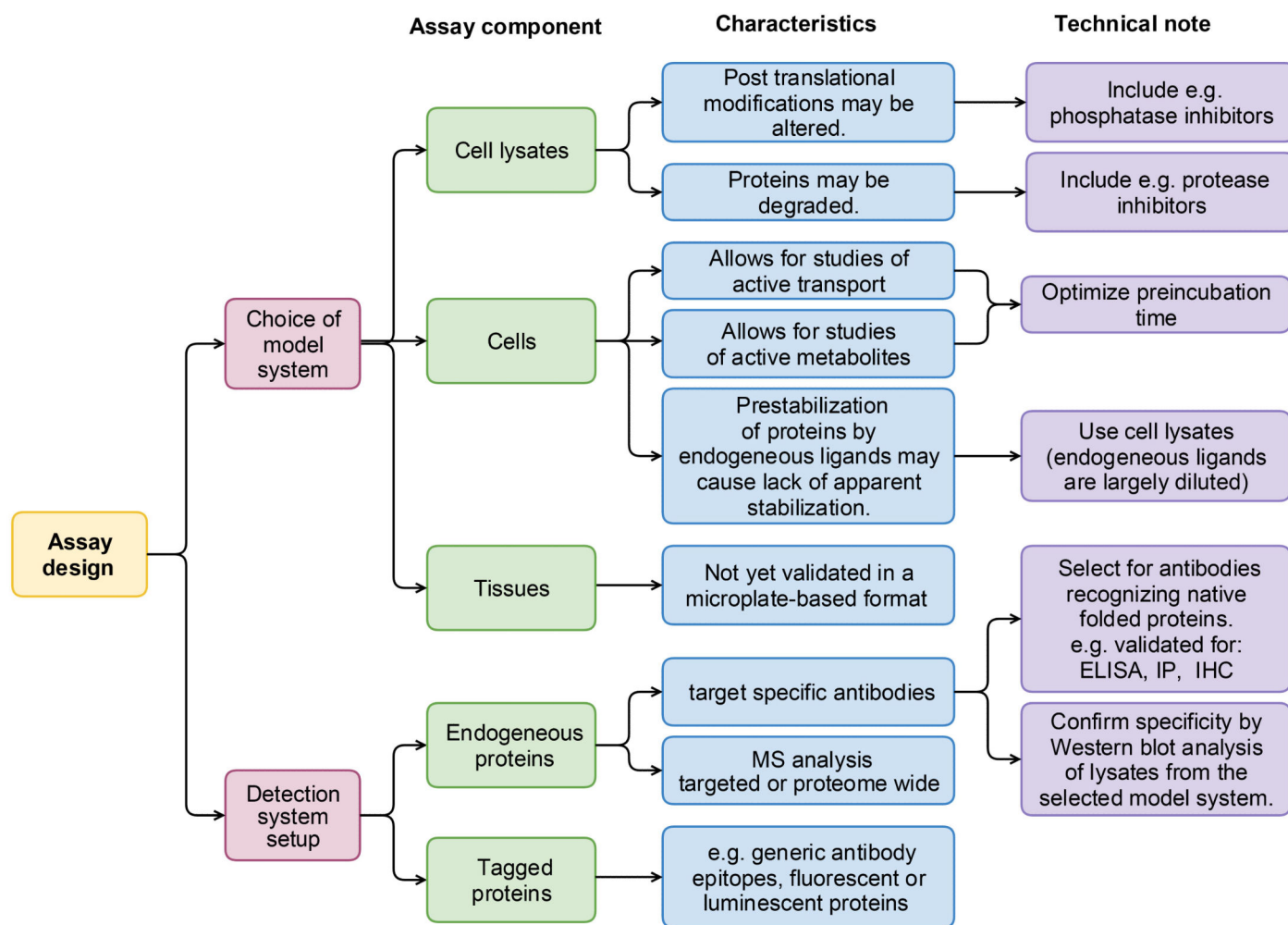


Figure 3: Illustration of general considerations and technical notes related to assay design. Figure created using Gliffy software.

associated with live cell assays. These aspects can, on the other hand, be addressed by the use of relevant cellular model systems as exemplified by drug efficacy, drug transport, drug activation, off-target effects and drug resistance studies in the original CETSA publication, which also included an example of monitoring drug distribution in animals (6).

It is likely that the level of complexity of the model system will increase with the maturity of a drug discovery program and potentially start with cell lysates or cells that overexpress a tagged protein to facilitate detection. This will likely be more prevalent for novel target proteins as these generally come with a less mature repertoire of affinity reagents to support detection of endogenous protein levels. As the project matures and enters late preclinical and clinical phases there will be a need to confirm response in primary cells, which is feasible if there are affinity tools available for achieving the protein quantification. Recent developments in the mass spectrometry (MS) field (16) may also come to support such efforts in case suitable affinity reagents are missing.

Detection Formats

As already mentioned, the basis of CETSA is that the vast majority of proteins denature and precipitate at elevated temperatures, whereas ligand-bound proteins are stabilized and thus remain folded in solution. In the original publication the stabilized protein in the soluble fraction of the samples was detected using western blotting (WB) (6). WB-based detection has since been adopted in the majority of reported applications of

CETSA in the literature (15), with the aim to study the effect of only one or a few compounds on individual target proteins. In general, WB-based CETSA experiments are relatively simple to establish because they only require one affinity reagent, namely a specific antibody directed towards the protein target of interest, and utilize equipment already available in most biochemistry labs. A detailed protocol for this procedure was recently published (7).

Many CETSA applications will, however, require a more systematic, parallel processing of samples. This includes, for example, hit qualification activities where thousands of hits from high-throughput screening campaigns are ranked based on their concentration-dependent responses in downstream follow-up assays. In such cases it is necessary to move CETSA to a microplate-based (or equivalent) format so that all addition and treatment steps can be performed using automated liquid handling and the transient heating and cooling can be achieved using plate-compatible equipment. Naturally such microplate formatting of the assay involves miniaturization and a minimization of the number of assay steps to improve on assay throughput. This reduction of steps also serves to diminish well-to-well variability and consequently improve on assay quality.

Detection of the amount of remaining soluble protein can be achieved using a broad range of available protein quantification assays. These include, but are not limited to enzyme-linked immunosorbent assay (ELISA) and variations thereof, proximity ligation assays and dot blots. Although these methods are compatible with a microtiter-plate format, the throughput is limited due to the requirement of wash steps and/or separation steps before detection. An optimal detection method should instead be homogenous and allow for the quantification of remaining soluble protein against a background of the same protein in denatured and aggregated forms, such that the separation step can be eliminated. The detection must also be achieved against a cell lysate background to avoid the sample workup and wash steps. Well-proven homogeneous detection methods in which antibodies or other affinity reagents recognize the folded protein structure in a cell lysate are, for example, AlphaScreen® (Amplified Luminescent Proximity Homogeneous Assay Screen) or time-resolved fluorescence resonance energy transfer (TR-FRET)-based assays. Applications are also emerging where the detection is greatly facilitated by using engineered proteins in which a signaling entity is incorporated (*e.g.* the Society for Laboratory Automation and Screening (SLAS) 5th Annual International Conference & Exhibition) (17,18).

Although not generally applicable to high-throughput microplate formats, even though this may be changing (16), it is worthwhile noting that detection can also be achieved using MS. This was recently adopted in an extension of the CETSA methodology in a technique referred to as thermal proteome profiling (TPP) or thermal-stability profiling, which allows for the simultaneous measurement of the entire melting proteome (19–21). Consequently this method allows for studies of the apparent selectivity of individual compounds or for unbiased target identification activities for compounds with unknown mechanisms of action in cell lysates and live cells. However, great care must be taken to ensure that these studies are undertaken with an understanding of the thermodynamic prerequisites for the magnitude of thermal shifts as these vary broadly among proteins and ligands (22). As a consequence, T_{agg} shift signatures must be considered highly apparent unless they are extended to include multiple ligand concentrations and temperatures such that the difference in shift sizes for different binding events can be accounted for (23).

Assay Development

The following section provides a detailed description of the various assay development and optimization steps to establish a homogeneous CETSA assay in live cells. Each step is illustrated by experimental data for human thymidylate synthase (TS) and specific consideration is given to point out potential challenges in the assay development and to provide guidelines for resolving such complications. A major aim in establishing this assay was to enable the detection of endogenous protein levels, with the intent to demonstrate potential utility for primary cells, thus requiring the use of a homogeneous immunoassay for detection. Although the following descriptions and illustrations will largely reflect this choice of assay readout, attempts will be made to separate

specific issues from more general considerations that are also applicable to other readouts. In the case of tagged proteins with a signaling entity, the reader is directed to the Assay Validation section.

Antibody Screening

AlphaScreen® is a proximity assay based on two bead types referred to as donor and acceptor beads, both of which possess a relatively large surface area for conjugation of biomolecules (24). Excitation of the donor beads results in the generation and release of singlet oxygen, which travels in solution and results in excitation and light emission from the acceptor beads when they are close in space, *e.g.* only when a complex is formed that involves both bead types. The system allows for the formation of complexes consisting of these beads functionalized with affinity reagents that recognize the target molecule of interest. An advantage of the AlphaScreen® technology, compared to *e.g.* TR-FRET assays, is the significantly longer distance tolerated for the proximity signal (24). This supports the use of multicomponent complexes in which the target-directed affinity reagents are recognized by capture antibodies or other affinity reagents on generic conjugated beads (25), prompting us to adopt this detection modality in the screening for suitable antigen-directed antibodies (7,8).

A starting point and prerequisite for the development of any homogenous, antibody-based assay is to identify a suitable antibody pair with sufficient affinity and selectivity for the target protein. Each pair consists of two antibodies that simultaneously recognize the native soluble target protein in a complex background of denatured and aggregated proteins. If the antibodies also recognize the denatured and aggregated protein it will be necessary to separate soluble from denatured protein prior to detection, *e.g.* by centrifugation or filtration. Depending on the number and quality of antibodies available, the strategies for selection of a good pair will vary. If there are numerous available antibodies it is recommended to test as many combinations as possible. A general guideline is to acquire and test antibodies that are validated to recognize the target protein in a fully folded form *i.e.* antibodies validated for ELISA, immunoprecipitation (IP) or immunohistochemistry (IHC). It is also good to include antibodies raised against different epitopes of the target protein (C-terminal, N-terminal and internal regions).

It should be pointed out that a key limiting factor in the selection of antibodies is the possible appearance of ligand-induced suppression of antibody recognition and thereby quenching of the anticipated signal (7,8). This is likely due to a conformational change of the target protein upon ligand binding. Failure to carefully validate the affinity reagent pair comes with an obvious risk for false negatives or unnecessarily large reagent investments if this is discovered late in the process. Known binders, ideally from a broad range of different structural classes, are therefore included in the very first experiments and throughout assay validation.

Herein the establishment of a microplate-based CETSA will be exemplified based on donor and acceptor beads functionalized with capturing antibodies directed towards mouse-derived and rabbit-derived IgG respectively (Figure 4a). Once a suitable antibody pair has been identified it is an option to conjugate the antibodies directly to the AlphaScreen® beads. This serves to improve the assay signal, as it shortens the distance between the beads, but most importantly it removes the need to carefully titrate multiple assay components. Alternatively the user has the option to move to alternative screening compatible assay formats such as TR-FRET assays. If the project aim does not require the use of primary non-engineered cells, assay development can be further simplified by the use of proteins with different reporter functionalities (Figure 4b).

Experimental Setup:

In this experiment all combinations of the selected antibodies are tested at a single concentration. The initial antibody screen is preferably done using recombinant protein; however, it can also be performed in a cell lysate if follow-up experiments ensure that the correct protein is selected for. A good starting point is to use antibody and protein concentrations at or slightly below the binding capacity of the AlphaScreen® beads. It is also worthwhile mentioning that screening scenarios in which the protein exists in multiple forms with regards to

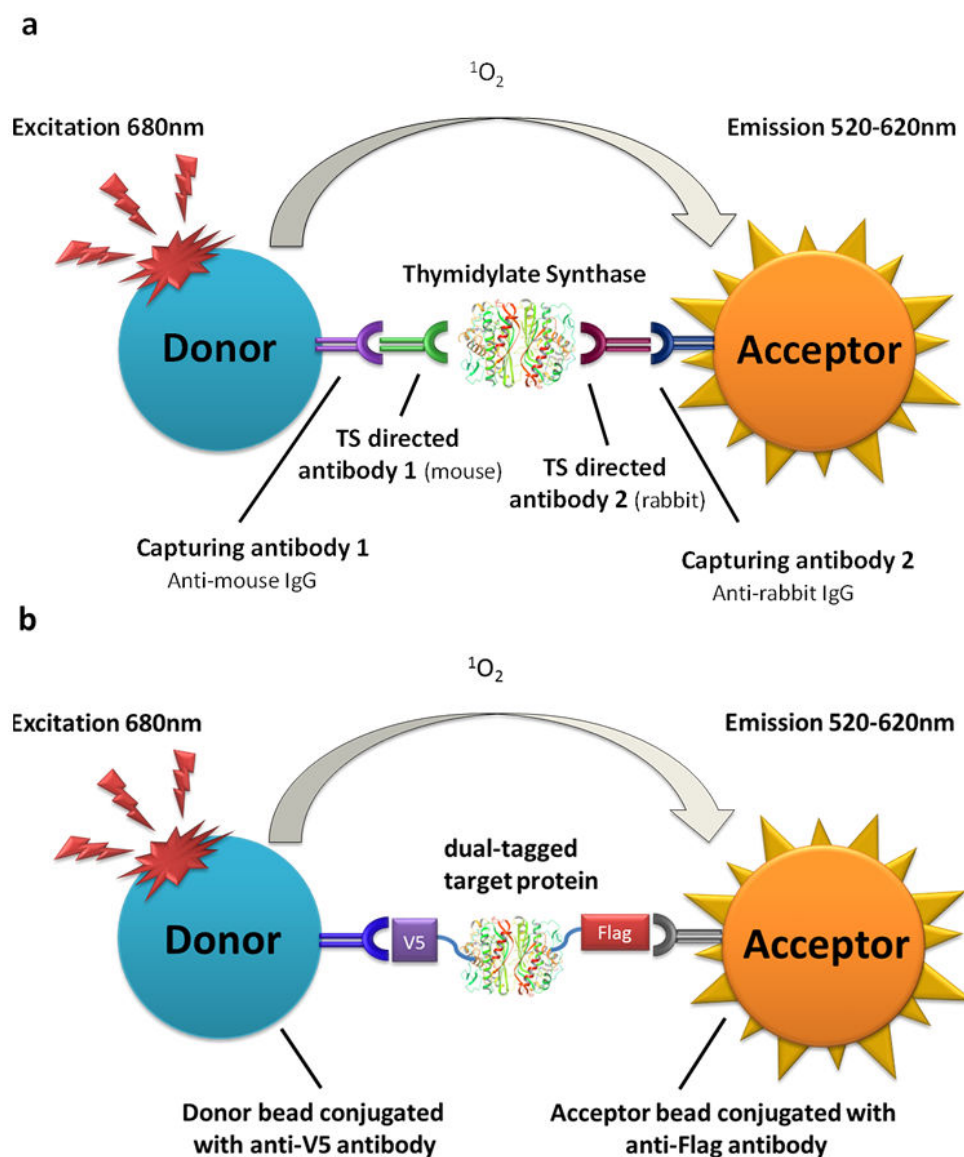


Figure 4: Illustration of two AlphaScreen® setups for detection of endogenous and tagged proteins, respectively. Both setups are based on the formation of a multicomponent complex consisting of the donor and acceptor beads functionalized with different affinity reagents that recognize the target protein of interest. a) Setup for detection of endogenous protein, in this case exemplified by TS, consisting of donor and acceptor beads functionalized with capturing antibodies (anti-mouse IgG and anti-rabbit IgG) recognizing anti-target antibodies (from mouse and rabbit) raised against different epitopes of the target protein. b) Example of a tentative setup for the detection of tagged proteins with dual epitopes, allowing the use of generic beads conjugated with antibodies towards these epitopes. Other options include for example GFP, luciferase and beta-galactosidase tagged proteins to circumvent the need for an immunoassay.

post-translational modifications may exist. If so this fact must be carefully considered in the antibody selection process. As a rule of thumb it is useful to always compare the observed data with those obtained using WB-based detection (or possibly mass spectrometry) as the size resolution helps to identify the presence of multiple forms.

As already mentioned, ligand binding can result in suppression of the anticipated signal; thus it is strongly recommended to include known binders in the very first experiments (if available), preferably from different structural classes (7,8). These controls are then included throughout the assay development and validation process to ensure that the selected antibody combinations produce a robust signal to background without any impact of the compound on the protein quantification.

It is important to also include a negative control lacking the target protein. If recombinant protein is not available, the negative control can consist of a lysate that has been heated well above the aggregating temperature of the target protein. Although this procedure also removes a significant portion of other proteins it can serve as a first estimate of the background signal. As described below, we also recommend that seeding experiments are used to validate the specificity of the signal, *i.e.* addition of recombinant protein to a non-modified cell lysate to see that the signal scales linearly with added protein concentration.

Assay Protocol:

All additions are made directly to an AlphaScreen® compatible detection plate.

- a. Add recombinant protein (or cell lysate) and preincubate with known binders or only buffer (or heated lysate) at room temperature. Depending on the sensitivity of the target protein it may be necessary to add specific supplements to the buffers such as protease or phosphatase inhibitors to avoid degradation or alterations of post-translational modifications.
- b. Prepare solutions of all different antibody combinations and add to the samples and incubate for a minimum of 30 minutes at room temperature.
- c. Add acceptor and donor beads, seal the plate, centrifuge briefly and incubate for 2 hours or overnight at room temperature under subdued light before reading the plate in an AlphaScreen® compatible plate reader.

The results of such an example antibody selection screen based on four mouse-derived and three rabbit-derived antibodies are shown below in Figure 5. Note that depending on the affinity reagents and bead types it can be possible to add a mixture of these reagents at the same time. In some cases better results are achieved when the acceptor and donor beads are added separately with an incubation time in between the additions (see generic AlphaScreen® guidelines if necessary).

Kinetics of the Antibody Recognition:

A factor that may be significantly altered when moving from an assay system using recombinant protein to a more complex system, such as a cell lysate, is the time required to establish equilibrium for all interactions involved in the detection system. The kinetics of antibody recognition of the target protein in a cell lysate may be impacted if the antibodies also bind to other proteins. A relatively fast establishment of the equilibrium and a signal that remains stable over time is desirable as this provides maximal flexibility in the assay procedure and allows accommodation of both smaller and larger screen batches. The experiment is also essential to establish that the target protein is stable and that the aggregates formed during the heat challenge do not resolubilize in the buffers and conditions applied for detection.

Experimental Setup:

This experiment serves to investigate the kinetics of antibody recognition by comparing the influence of different incubation times (for example 2h, 6h and overnight). Included in the incubations is the pair of antibodies and AlphaScreen® beads together with a cell lysate containing the target protein at endogenous levels. Because it is not recommended to read an AlphaScreen® sample multiple times (due to light induced signal deterioration) it is necessary to prepare one set of samples for each incubation time and antibody pair. A negative control consisting of a heated lysate to represent the background signal is also included for all conditions.

Assay Protocol:

All additions are made directly to an AlphaScreen® compatible detection plate.

- a. Add lysate samples.
- b. Add solutions of the selected antibody combinations.

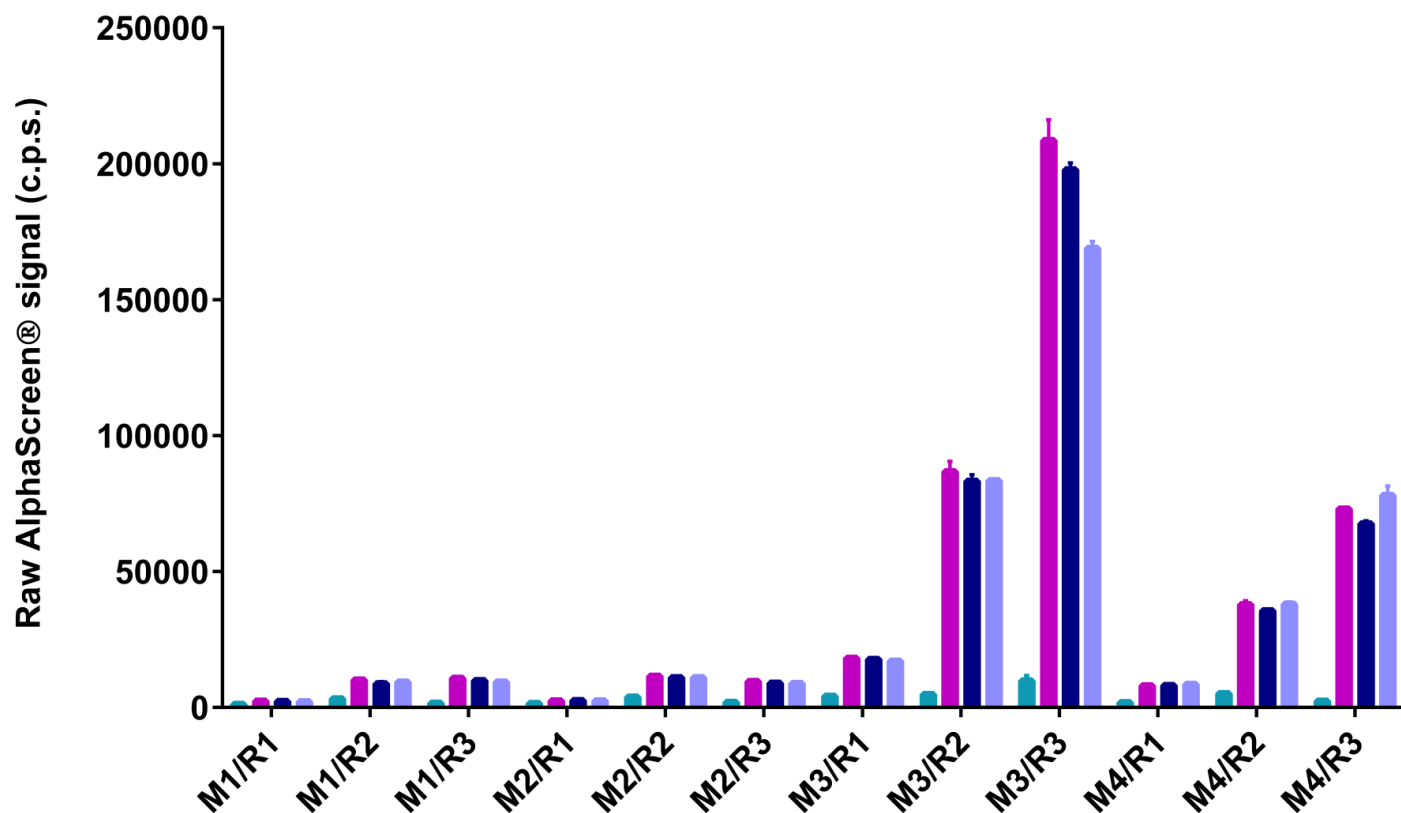


Figure 5: Illustration of the results from a screen of antibody pairs for recognition of recombinant TS. All combinations of four mouse-derived (M1-M4) and three rabbit-derived antibodies (R1-R3) were evaluated. Colors denote buffer only (teal), the presence of 2 nM of TS (magenta) or the addition of two known binders: 100 μ M deoxyuridine monophosphate (dUMP) (dark blue) and 100 μ M dUMP and 10 μ M raltitrexed (lavender) to the 2 nM TS condition. Known inhibitors were included to investigate the risk of compound induced loss of target recognition. In this case, four antibody pairs (M3/R2, M3/R3, M4/R2 and M4/R3) were selected for further testing. Data are provided as the average and range from experiments done in duplicate at one independent test occasion. Adopted with permission from Macmillan Publishers Ltd: Nat. Commun. 7 (2016), copyright (2016) <http://www.nature.com/ncomms/2016/160324/ncomms11040/abs/ncomms11040.html>

- c. Add acceptor and donor beads, seal the plate, centrifuge briefly and incubate for 2 hours, 6 hours or overnight at room temperature under subdued light before reading the plate in an AlphaScreen® compatible plate reader.

The results of such an example examination of antibody recognition kinetics are shown for four different antibody pairs recognizing human TS in Figure 6.

Titration of Assay Reagents

Given the nature of many immunoassays, *i.e.* the use of a multi-component complex involving several saturable components (binding capacity of antibodies and beads) to generate the assay signal, it is essential to carefully titrate both antibody and target protein concentrations. Titrations could also involve the AlphaScreen® beads, but in general we have stayed with the recommended concentrations. The aim of the experiment is to ensure that the assay is applied below the hook point and that an increase in target protein scales linearly with the assay signal (26). Titrations are still necessary, but much simplified, in assays using fewer components such as TR-FRET with the labels placed directly on the antibodies or the use of tagged proteins carrying a signaling entity.

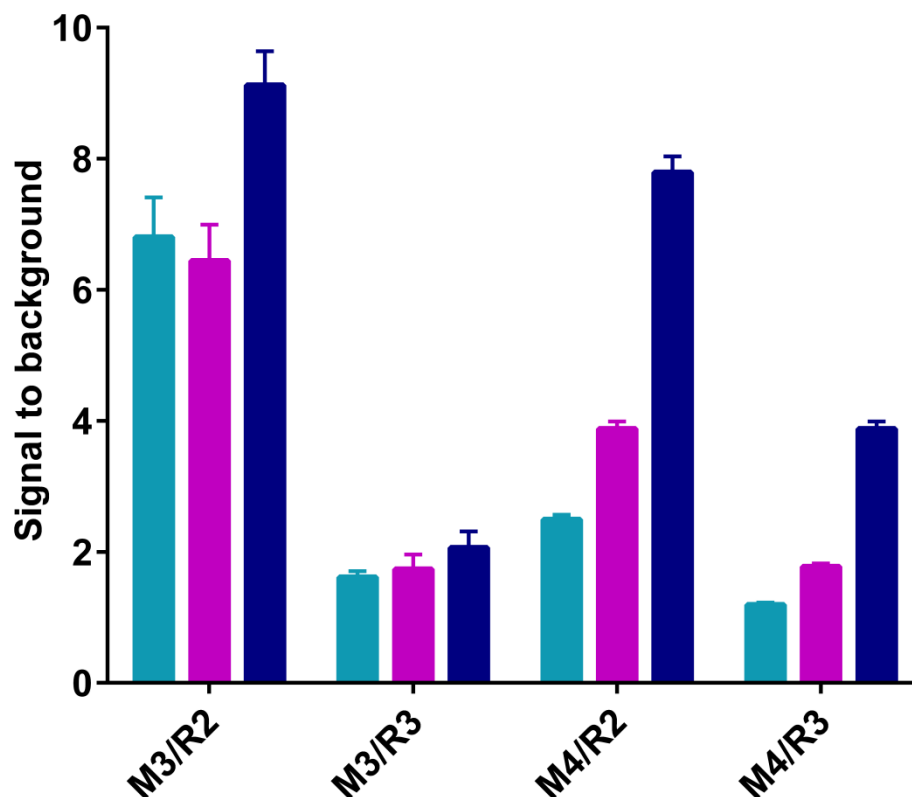


Figure 6: Illustration of the kinetics of antibody recognition of TS in K562 cell lysates. The signal to background of the different antibody pairs is given as a function of time after addition of antibodies and beads: 2h (teal), 6h (magenta) and 18h (dark blue). The combination of M3 and R2 was selected for further assay development based on a relatively fast and stable signal development and a high signal to background. Data are provided as the average and standard error of mean (SEM) from experiments done in triplicate at one test occasion. Adopted with permission from Macmillan Publishers Ltd: Nat. Commun. 7 (2016), copyright (2016) <http://www.nature.com/ncomms/2016/160324/ncomms11040/abs/ncomms11040.html>

Experimental Setup:

This experiment serves to perform a crosswise titration of both target-directed antibodies using cell lysate from unheated cells. The purpose is to identify the optimal concentration of each antibody and the experiment should include an appropriate control, such as lysates from cells heated sufficiently above the protein T_{agg} . Once optimal conditions have been established, a titration of the target protein must be performed by making a serial dilution of the lysate using the optimized antibody concentrations for detection. Depending on the outcome of these two experiments it may be necessary to make another iteration of the antibody titration, *e.g.* if the result from the lysate titration indicates that the first experiment was performed at target protein concentration at or above the hook point. Another option is to do a cross-titration of both antibodies against several dilutions of the lysate in order to accomplish both titrations at once.

Assay Protocol for the Initial Antibody Titration:

All additions are made directly to an AlphaScreen® compatible detection plate.

- Add the lysate samples.
- Prepare a crosswise titration of the two antibodies and add these to the lysate samples.

- c. Add acceptor and donor beads, seal the plate, centrifuge briefly and incubate for 2 hours or overnight at room temperature (depending on outcome of the above described experiments) under subdued light before reading the plate in an AlphaScreen® compatible plate reader.

The result of such a crosswise titration is shown in Figure 7 for the selected antibody pair recognizing human TS.

Assay Protocol for the Target Protein/Cell Number Titration:

All additions are made directly to an AlphaScreen® compatible detection plate.

- a. Prepare a dilution series of the cell lysate and transfer samples into the detection plate.
- b. Add antibodies at optimized concentrations to the lysate samples.
- c. Add acceptor and donor beads, seal the plate, centrifuge briefly and incubate for 2 hours or overnight at room temperature under subdued light before reading the plate in an AlphaScreen® compatible plate reader.

Example of the result of such a cell lysate titration is shown in Figure 8 for human TS.

Addressing the Specificity of the Antibodies

Even if the selected antibodies may show excellent specificity one by one in a WB-based detection setup, it is necessary that this holds true also in a dual recognition homogenous assay format.

Experimental Setup for Examining Antibody Specificity:

Such validation can be achieved by seeding a dilution series of recombinant protein in a cell lysate from both unheated and heated cells, as well as in cell medium. It is advised to use a fairly low lysate concentration for this experiment in order to ensure that the experiment is performed below the hook point of the detection system. A proportional increase in signal suggests that the signal is specific for the target protein and that recognition can be achieved in the context of a diluted cell lysate. Additional experiments that can be performed include competition experiments with antigen peptides for each of the antibodies and these can be performed at higher cell lysate concentration to further demonstrate sufficient selectivity.

Assay Protocol for Examining Antibody Specificity:

All additions are made directly to an AlphaScreen® compatible detection plate.

- a. Add lysate and medium samples.
- b. Prepare and add a dilution series of recombinant protein (including the appropriate post-translational modifications if this is the purpose of the assay).
- c. Add antibodies at optimized concentrations to the samples.
- d. Add acceptor and donor beads, seal the plate, centrifuge briefly and incubate for 2 hours or overnight at room temperature under subdued light before reading the plate in an AlphaScreen® compatible plate reader.

Results from an example of a seeding experiment based on recombinant human TS are illustrated in Figure 9.

Assay Validation

The following sections intend to give an overview of suggested procedures for the validation and optimization of a microplate-based CETSA assay. The different steps are illustrated by experimental data from the development of a live cell assay for TS including the final screening protocol. As for earlier sections the aim is to highlight generic considerations that are applicable also to other readouts.

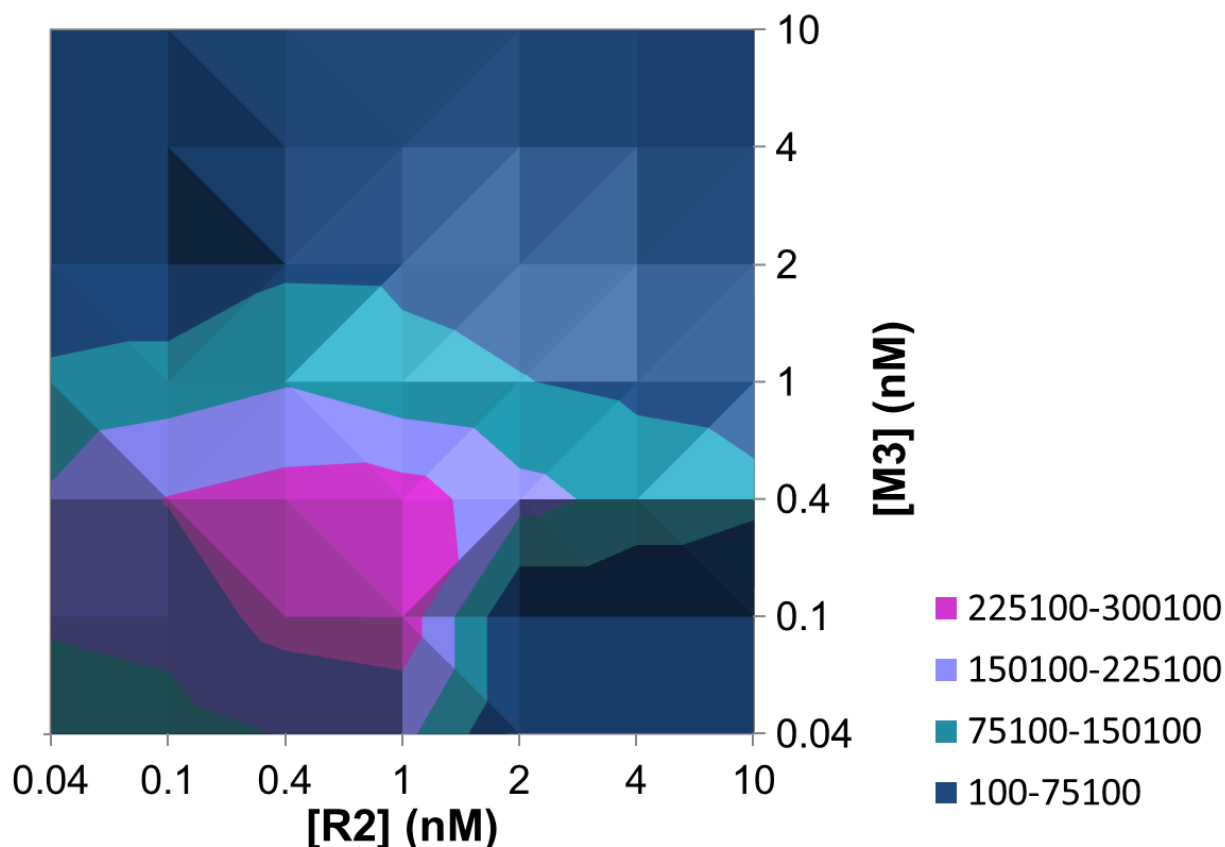


Figure 7: Illustration of the results from a crosswise titration of an antibody pair for the recognition of TS in a K562 cell lysate. Data are shown as a 3D contour graph where the concentrations of the antibodies directed towards TS are varied on the x-axis (R2) and y-axis (M3). The color scale is based on raw AlphaScreen® signal (c.p.s.). Based on these results 0.4 nM M3 and 1.0 nM R2 was selected for the final assay. Note the hook effect occurring at elevated concentrations of either antibody. Data are provided as the average from experiments done with $n=2-4$ for each condition obtained at one test occasion. Adopted with permission from Macmillan Publishers Ltd: Nat. Commun. 7 (2016), copyright (2016) <http://www.nature.com/ncomms/2016/160324/ncomms11040/abs/ncomms11040.html>

Tagg Curves and ITDRF_{CETSA} Experiments

As already mentioned CETSA experiments are principally designed in two different ways. T_{agg} curve experiments are carried out to assess any apparent ligand induced stabilization of the target protein, whereas ITDRF_{CETSA} experiments are used to study the apparent stabilization as a function of increasing ligand concentration. A pharmacological validation of the homogenous assay may be achieved by quantitatively comparing T_{agg} and ITDRF_{CETSA} data with those obtained using an alternative, validated readout. This confirmation could for example be achieved using the original WB approach. Such assay validation is optimally performed for a broad range of known binders. If ligands of different structural classes or compounds that bind to different binding sites are available, it is highly recommended to include examples of all of these.

Experimental Setup T_{agg} Curves:

For CETSA, as with other thermal shift assays, it is recommended to use saturating compound concentrations in order to achieve sufficiently sized apparent shifts in aggregating temperature (22). Hence it is often necessary to perform the T_{agg} experiments at higher compound concentrations than with other assays (see below for more quantitative qualifications of these recommendations). A suggested starting point is 5-20 times above the cellular EC_{50} value provided that solubility and toxicity is not a problem. If the cellular EC_{50} is not known one should consider also potential cell permeability and serum binding properties of the compounds when estimations are

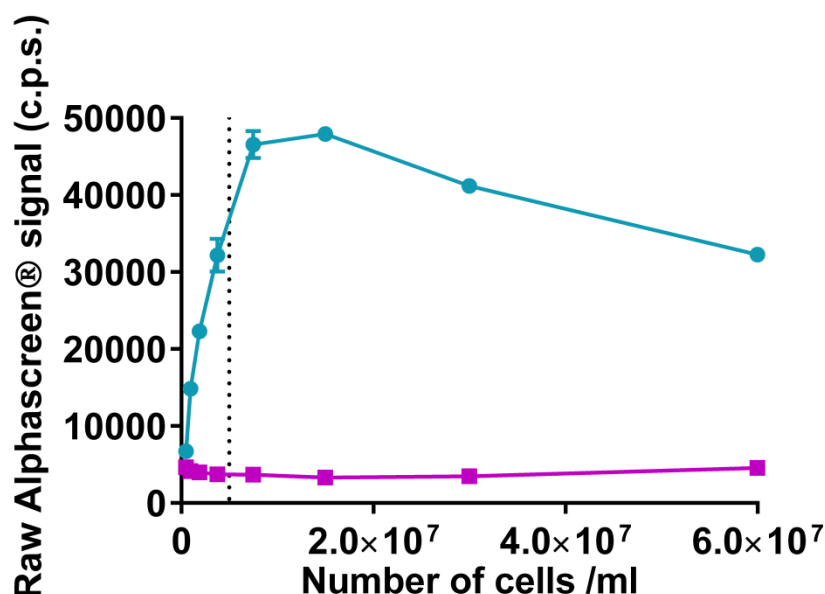


Figure 8: Illustration of a K562 cell lysate titration for recognition of TS. Cell lysate from unheated K562 cells are represented by teal circles and heated cells by magenta squares respectively. Based on these results it was decided to use 5 million cells per ml (dotted line) in the final assay to ensure that the assay is performed within the linear range of detection. Data are shown as the average and SEM from triplicate samples obtained at one test occasion. Adopted with permission from Macmillan Publishers Ltd: Nat. Commun. 7 (2016), copyright (2016) <http://www.nature.com/ncomms/2016/160324/ncomms11040/abs/ncomms11040.html>

done based on biochemical IC_{50} values. The experiment must include temperatures both above and below the expected aggregating temperature to cover the complete aggregation curve.

Assay Protocol T_{agg} Curves:

Compound treatment is performed in a PCR plate and the detection is done in an AlphaScreen® compatible detection plate.

- Prepare two cell aliquots. Treat one with compound and the other with the corresponding concentration of DMSO and preincubate both samples at 37°C (see the section “Variation of the preincubation time probes cellular uptake and metabolism” for more details).
- Transfer aliquots of samples from each treatment to a PCR plate.
- Subject the PCR plate to a transient heat gradient (see the section “Variations to the transient heating step” for details regarding the heating time) in a PCR machine with separate heat zones (PCR machines with a plate gradient option could alternatively be used). Make sure to always include a controlled cooling step back to room temperature as the aggregation that occurs downstream of target protein denaturation may occur also during this step. Failure to carefully control both the heating and cooling between all wells of the microtiter plate may result in plate edge effects.
- Add lysis buffer to the PCR plate and mix thoroughly to ensure complete lysis of the cells.
- Transfer aliquots of the samples to the detection plate and add antibodies.
- Add acceptor and donor beads, seal the plate, centrifuge briefly and incubate for 2 hours or overnight at room temperature under subdued light before reading the plate in an AlphaScreen® compatible plate reader.

An example of T_{agg} data and ligand-induced stabilization are illustrated for TS in Figure 10.

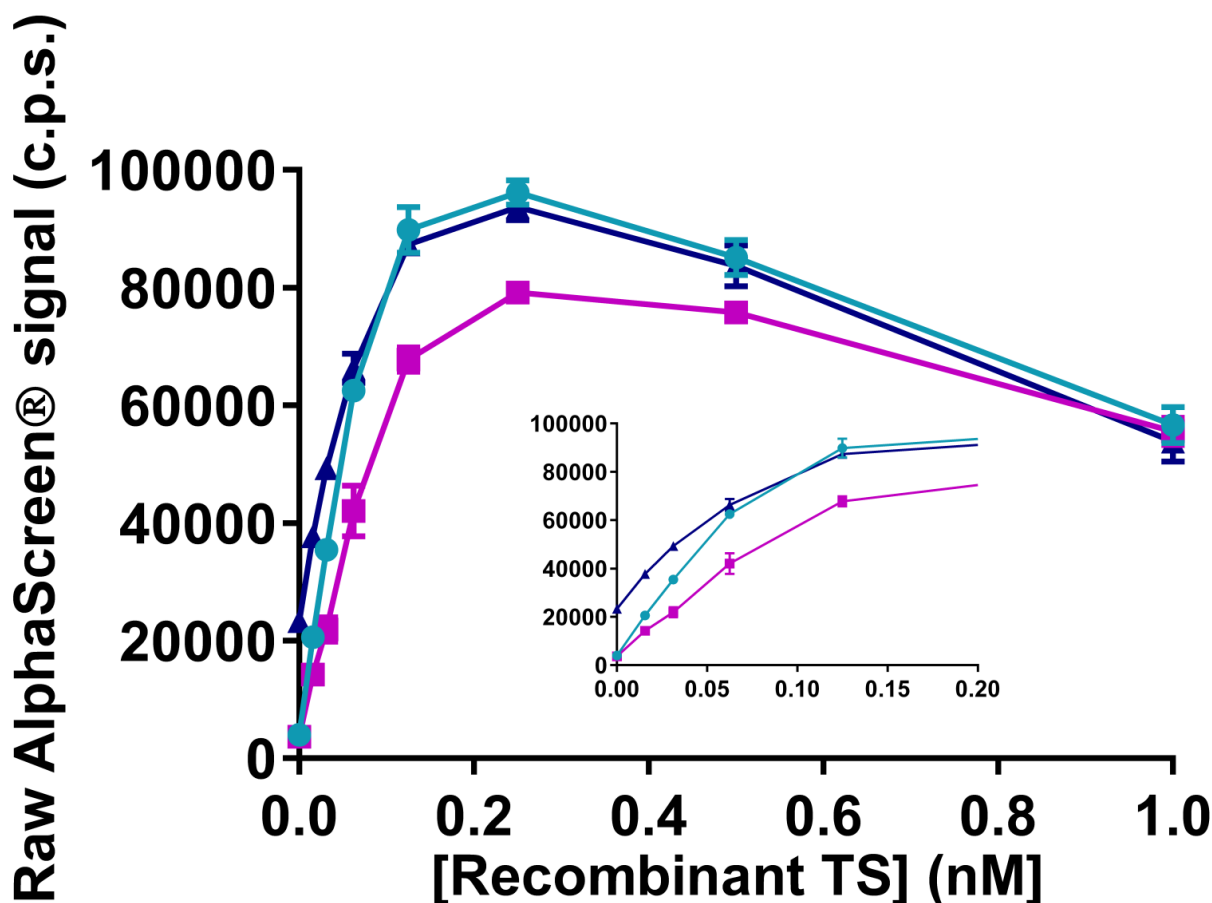


Figure 9: Illustration of the results from a seeding experiment addressing antibody specificity. Data are shown as a function of added recombinant human TS seeded in unheated cell lysates (blue triangle), lysate from heated cells (magenta square), or to cell medium without any cells (teal circle). The insert illustrates the same data, but restricted to the 0-0.2 nM concentration range. To ensure that at least the initial part of the titration is performed below the hook point it is necessary to use a rather diluted cell lysate for this experiment. In this case the cell lysate background corresponded to 1 million K562 cells per ml. A signal increase in line with the increasing concentration of seeded recombinant TS is observed in both unheated and heated lysates, although the background levels in the absence of TS are different which reflects the TS levels in K562 cells. In the absence of the cell lysate background the signal increase is somewhat steeper. Based on these data we conclude that the antibodies selectively recognize TS also in the cell lysates. Data are shown from one test occasion as the average and SEM from triplicate samples. Adopted with permission from Macmillan Publishers Ltd: Nat. Commun. 7 (2016), copyright (2016) <http://www.nature.com/ncomms/2016/160324/ncomms11040/abs/ncomms11040.html>

Experimental Setup ITDRF_{CETSA}:

The choice of temperature for the transient heat challenge in ITDRF_{CETSA} experiments is selected based on the results observed in the preceding T_{agg} experiments. A general rule of thumb is to select a temperature near or above the apparent T_{agg} such that a sufficiently large assay window is obtained. The best assay windows are obtained if a temperature is chosen such that the target protein levels of the unliganded T_{agg} curve is close to baseline, although the user should be aware that much elevated temperatures is associated with a loss of response to the ligand (see below for further details).

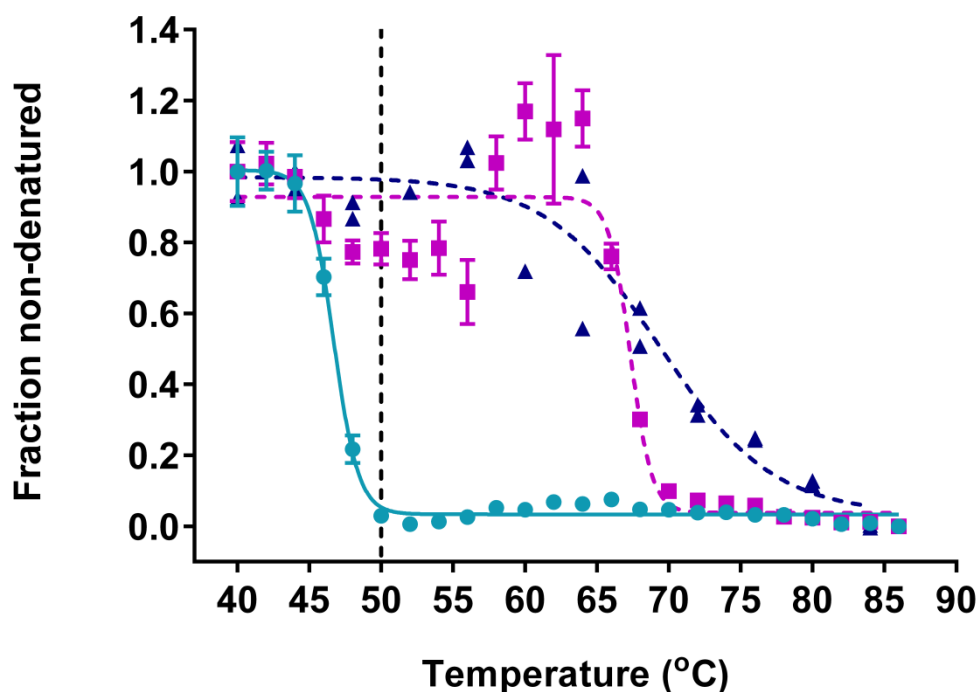


Figure 10: Illustration of T_{agg} curves for TS in K562 cells. Data are shown for unliganded TS in the presence of DMSO (green circle), floxuridine (blue triangle) and raltitrexed (magenta square). The vertical dotted line at 50°C represents the temperature selected for the isothermal experiment. Note that high T_{agg} values should not be considered as reliable measures of potency and permeability as these temperatures may influence cell membrane integrity (6). Data are provided as the average and SEM from two independent experiments performed in duplicate for raltitrexed and as individual data points from one experiment in duplicate for floxuridine. Adopted with permission from Macmillan Publishers Ltd: Nat. Commun. 7 (2016), copyright (2016) <http://www.nature.com/ncomms/2016/160324/ncomms11040/abs/ncomms11040.html>

Assay Protocol ITDRF_{CETSA}:

Compound treatment is performed in a PCR plate and the detection is done in an AlphaScreen® compatible detection plate.

- Serially dilute compounds and transfer the dilution series to a PCR plate.
- Add cells to the plate and preincubate at 37°C (see the section “Variation of the preincubation time probes cellular uptake and metabolism” for more details).
- Subject the PCR plate to a transient heat challenge (see the section “Variations to the transient heating step” for details) in a PCR machine. Include a controlled cooling step to room temperature.
- Add lysis buffer to the PCR plate and mix thoroughly to ensure complete lysis of the cells.
- Transfer aliquots of the samples to the detection plate and add antibodies.
- Add acceptor and donor beads, seal the plate, centrifuge briefly and incubate for 2 hours or overnight at room temperature under subdued light before reading the plate in an AlphaScreen® compatible plate reader.

Example data from an ITDRF_{CETSA} experiment for TS are illustrated in Figure 11.

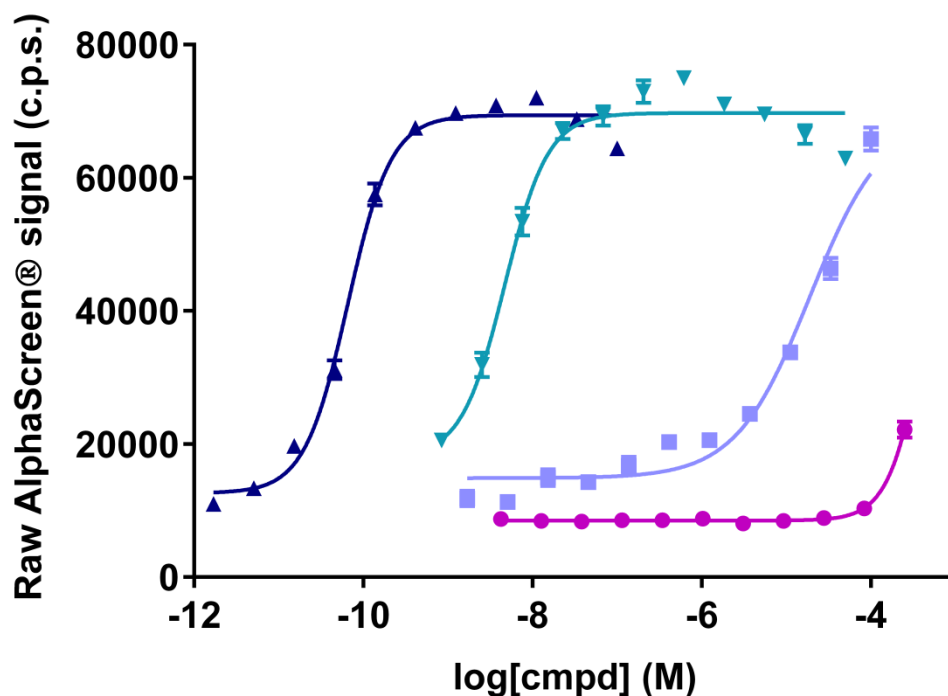


Figure 11: Results of ITDRFCETSA experiments in K562 cells with known and a novel TS ligands. ITDRFCETSA data illustrating the apparent stabilization of TS after 2 hour preincubation with floxuridine (blue upwards triangle), 5-fluorouridine (FUR) (teal downwards triangle), and 5-FU (lavender square) and for the novel TS ligand CBK115334 (magenta circle). Data are provided as the average and SEM from one independent experiment done in quadruplicate. Adopted with permission from Macmillan Publishers Ltd: Nat. Commun. 7 (2016), copyright (2016) <http://www.nature.com/ncomms/2016/160324/ncomms11040/abs/ncomms11040.html>

Variation of the Preincubation Time Probes Cellular Uptake and Metabolism

One factor that may influence the apparent stabilization of the target protein in a live cell-based system is the length of the preincubation time with compound. When working with live cells it is important to allow enough time for the compounds to cross the cellular membrane and where it applies allow for the subsequent intracellular metabolic activation of the compounds. In general 30-60 minutes is enough, but in the cases where metabolic activation is required prior to target engagement, longer preincubation times may be necessary (8). One should be aware that longer preincubation times may alter protein levels and post-translational modifications, thus impacting measured protein levels for reasons other than ligand-induced target protein stabilization.

Experimental Setup Preincubation Time with Compounds:

The appropriate choice of preincubation time varies with target protein and perhaps more importantly the nature of the ligands. In cases where there is reason to believe that cellular uptake is slow or that intracellular activation is required prior to target protein binding it is useful to conduct preliminary experiments in which the preincubation time is varied. This can be done by applying the same ligand concentration series to multiple plates, on which preincubation times prior to the transient heating step are varied.

Assay Protocol Preincubation Time with Compounds:

Compound treatment is performed in a PCR plate for variable times at 37°C. Detection is done in an AlphaScreen® compatible detection plate.

- a. Serially dilute compounds and transfer the dilution series to multiple PCR plates.
- b. Add cells to the plate and preincubate at 37°C for several different time periods, e.g. for 30 min, 1h, and several hours.
- c. Subject the PCR plates to a transient heat challenge in a PCR machine. Include a controlled cooling step to room temperature.
- d. Add lysis buffer to the PCR plates and mix thoroughly to ensure complete lysis of the cells.
- e. Transfer aliquots of the samples to the detection plate and add antibodies.
- f. Add acceptor and donor beads, seal the plates, centrifuge briefly and incubate for 2 hours or overnight at room temperature under subdued light before reading the plate in an AlphaScreen® compatible plate reader.

Data from this type of experiment is illustrated in Figure 12 for TS. As the known drugs targeting TS require intracellular enzymatic activation it serves as a clear example of the importance of the preincubation time. In most cases such a dramatic change in apparent potency over time will not occur, but it illustrates the potential to use this technology for measuring kinetics of ligand uptake and metabolism.

Variations to the Transient Heating Step

As already mentioned, CETSA technology is based on measurements of remaining levels of soluble protein following a transient heat challenge, which serves to thermally denature and irreversibly precipitate proteins that are not stabilized by ligand. The majority of studies reported in the literature have adopted the procedure described in the original study (15), which involved a three minute transient heating step with the temperature range adjusted to fit the thermal stability of the protein of interest. Importantly, it has been shown that transient heating up to 60-65°C for this time period does not have an acute impact on cell membrane integrity (6,7). It is, however, important to emphasize that the non-equilibrium nature of this procedure results in apparent measures of target protein stability and that both T_{agg} and $ITDRF_{CETSA}$ values are expected to change with both the temperature and the duration of the heat challenge (7,23). Consequently users must be aware that the choice of temperature and duration of the heat challenge can also affect the relative ranking of compounds and that this will be especially pronounced when studying ligands with long off-rates or ligands from different structural classes where the binding thermodynamics can vary greatly.

The careful generation of CETSA data in which the transient heating step is varied in a systematic way for several model systems will allow a better understanding of what models to apply for more quantitative interpretations. From the extensive literature on equilibrium systems, it is known that temperatures well above the T_{agg} of the unliganded protein and excessive heating times should be avoided as they drive the continuous unfolding and aggregation process (14). However, the possibility to use temperatures around or even below the apparent T_{agg} will be strongly dependent on the assay statistics obtained as the signal to background of the assay is dependent on the unfolding of a sufficiently large portion of the protein. There is an option to reproduce what is done in isothermal denaturation assays, *i.e.* to apply a temperature challenge well below the T_{agg} for prolonged periods of time, but this comes with an increased risk of heat stress responses, at least in live cells. Practically, however, a further reduction of assay volumes to facilitate rapid and homogeneous sample heating, as well as alternative means to achieve this more efficiently are potential future developments of the technology.

Screening Protocol for a 384-well based CETSA Assay

For screening purposes, the same conditions as in the $ITDRF_{CETSA}$ experiments are applied, although in most cases only one compound concentration will be tested. An example of a screening protocol applied in the case for human TS is outlined below, while a schematic illustration of the assay procedure is available in Figure 13.

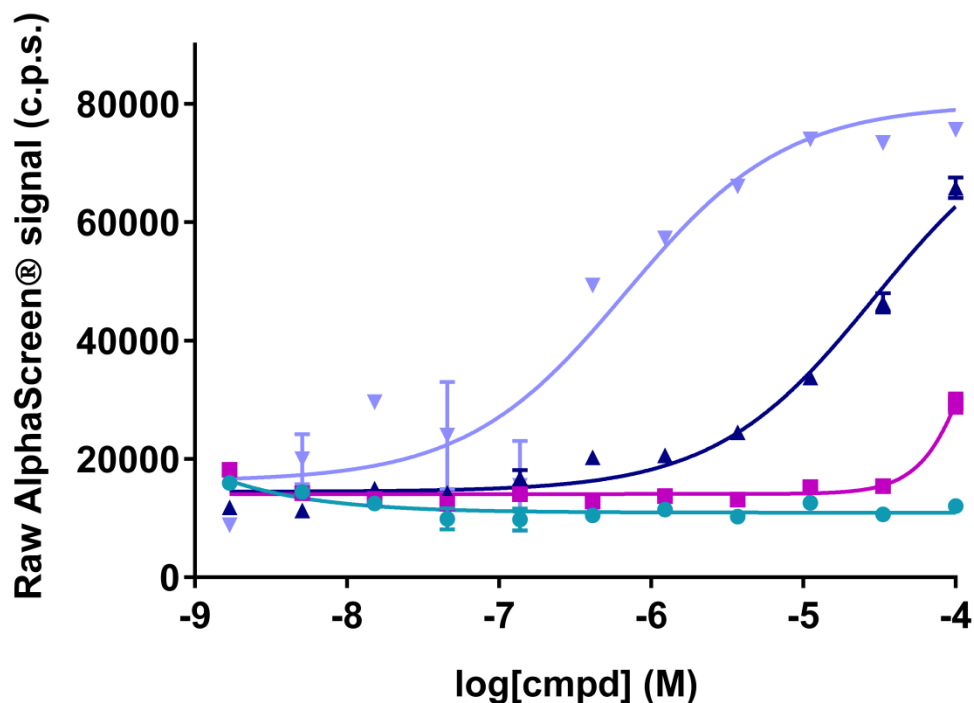


Figure 12: Illustration of a preincubation time experiment with 5-fluorouracil (5-FU) using K562 cells. ITDRF_{CETSA} data obtained after various lengths of preincubation time, as shown here for 10 min (teal circle), 30 min (magenta square), 2h (blue upwards triangle) and 6h (lavender blue downwards triangle). 5-FU is an example of a compound requiring metabolic activation prior to target engagement. Data are provided as the average and SEM from experiments done in quadruplicate at a single test occasion. Adopted with permission from Macmillan Publishers Ltd: Nat. Commun. 7 (2016), copyright (2016) <http://www.nature.com/ncomms/2016/160324/ncomms11040/abs/ncomms11040.html>

Assay Protocol Screen Format:

- Transfer of 5 μ l diluted compound solutions into individual wells of a 384-well PCR plate using a Bravo liquid handling instrument.
- Addition of 5 μ l K562 cell suspension to all wells.
- Preincubation of samples for 2h at 37°C.
- Heating to 50°C for 3 min in a PCR machine, followed by controlled cooling down to 20°C.
- Lysis by addition of 10 μ l lysis buffer using a Flexdrop IV.
- Thorough mixing to achieve complete cell lysis followed by transfer of 3 μ l of the diluted samples to a 384-well Proxiplate using a Bravo liquid handling instrument.
- Addition of a mix of antibodies, acceptor and donor beads under subdued light to the 384-well Proxiplate using a Multidrop™, centrifuge briefly and incubate for 2 hours or overnight at room temperature under subdued light before reading the plate in an EnVision plate reader.

In this protocol the majority of intracellular TS will denature and irreversibly aggregate unless stabilized by ligand. Inclusion of controls at relevant positions of the plate allows for standard normalization of the data to compare the potential stabilizing effects of the tested compounds with those of the positive controls. Further details on data analysis are available in the original study (8).