High-Throughput Approaches to Measuring Cell Death

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Topic Introduction

High-Throughput Approaches to Measuring Cell Death

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Cell death is integral to developmental and disease processes, and high-throughput screening (HTS) has been instrumental both for understanding biological mechanisms underlying cell death and for discovering novel therapeutic agents targeting these pathways. The various cell death modalities and their distinctive morphological and biochemical features have led to the development of a staggering variety of assays to measure these features, many of which have been adapted to HTS format. Although not all cell death assays are readily amenable to a high-throughput format, the potential power of HTS assays and increasing accessibility to associated technology make it likely that new approaches will continue to emerge. In particular, many recent studies in this field have used multiplex assays and high-content imaging to measure several features concurrently. Here, we discuss a broad array of considerations for designing HTS cell death assays, including some common challenges and pitfalls. We aim to provide a framework for deciding the most appropriate biological readouts, assay strategy and mode, workflow, controls, validation, and bioinformatics.

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INTRODUCTION

Our understanding of the molecular pathways controlling cell death is rapidly improving, and with it our ability to modulate these pathways for therapeutic benefit. High-throughput screening (HTS) has been instrumental both for understanding the biological mechanisms underlying cell death and for discovering novel cytoprotective and cytotoxic agents targeting these pathways. In particular, HTS approaches have been used for drug discovery and pharmacogenomics, as well as for identifying genetic regulation of cell death (Dompe et al. 2011) and understanding mechanisms of drug resistance (Potratz et al. 2010) (Table 1).

Cell death can occur via multiple pathways, each showing distinct morphological and biochemical features. Accordingly, a staggering variety of assays have been developed to measure these features, many of which have been adapted to HTS format and are available commercially (Table 1). HTS assays for cell death can take many forms, depending on the specific question(s) being asked, the parameters being measured, and the nature of the library being used to modulate cell behavior (i.e., chemical or genetic). They are generally linked to a few common reporter systems that are easily adapted for use in the various high-throughput instruments available, including morphology, fluorescence, bioluminescence, and phenotypic selection (e.g., survival/resistance or synthetic lethality). Fluorescent protein reporters used in HTS to measure cell death or cell death signaling include Bax-GFP (Galluzzi et al.

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TABLE 1. Cell death assays amenable to HTS

Biological readout	Format	Commercial assay	Notes	Suppliers	Reference
Caspase activity	HC or PR	Caspase-Glo, ApoLive Glo (multiplex with cell titer Fluor) Caspase-3 activation kit	Various reagents available for specific caspases; multiplex capable; potential for nonspecific activation of substrates; end point	Promega Cellomics (Thermo)	Stec et al. 2012
Cell viability—ATP	PR	Cell Titer-Glo	End point	Promega	Boutros et al. 2004; Nguyen et al. 2006
Cell viability—redox status	PR	Rezazurin (alamar blue) Cell Titer-Blue	Nontoxic, can be multiplexed with HC	Life Technologies Promega	Bauer et al. 2010
Cell viability—live cell protease	PR	Cell titer Fluor	Nontoxic, can be multiplexed with HC	Promega	
Cell viability—esterase	HC or PR	Calcein-AM	Live cell only	Life Technologies, Sigma, BD	Gilbert et al. 2011
Protease activity	PR	CytoTox-glo	Dead cell protease detection; reflects membrane integrity; end point	Promega	Niles et al. 2009
DNA cleavage	HC	TUNEL	Measures DNA strand nicks associated with apoptotic nuclease activity; end point	Life Technologies	Parrish and Xue 2003
Nuclear morphology	HC	DAPI, DRAQ-5, BOBO-3, etc.	DNA binding dyes; end point and live cell; often multiplexed	Various (numerous)	De Pasquale et al. 1990
Mitochondrial function (membrane potential)	PR	MitoProbe JC-1CMXRos (mitotracker red)	Measures mitochondrial membrane integrity; end point and live cell	Life Technologies	Qiu et al. 2010
DNA synthesis	HC	EdU/BrdU	Usually coupled with measurement of DNA content	Life Technologies	Poon et al. 2008
Plasma membrane integrity (LDH release, protease release, DNA binding)	HC or PR	CytoTox-glo, CytoTox-ONE, CellTox Green Image-iT DEAD, Ethidium	Often coupled with other markers in multiplex assays particularly suited to kinetic assays, nontoxic	Promega Roche Life Technologies	Buenz et al. 2007

HC, high content; PR, plate reader

2010), GFP-H2B (Neumann et al. 2010), and protein fragment complementation (PCA/BiFC) (MacDonald et al. 2006).

Common challenges when measuring cell death and apoptosis by HTS include the highly dynamic and multifaceted nature of cell death signaling pathways, a diversity of responses to specific stimuli, the heterogeneity of cell lines, and defining the most appropriate timing for the assay readout. Avoiding reliance on a single morphological or biochemical feature by deploying two or more complementary biological readouts can help avoid many of these potential pitfalls. Recent advances in single-cell analysis may also be a powerful means to overcome some of these issues (Snijder et al. 2012).

Although not all cell death assays are readily amenable to a high-throughput format, increasing interest in HTS assays and their potential power as well as increasing accessibility to technology make it likely that new approaches will emerge. We discuss here a broad array of considerations when designing HTS cell death assays. Our intention is to provide a framework for deciding the most appropriate biological readouts, assay strategy and mode, workflow, controls, validation, and bioinformatics. We also provide a protocol that encompasses high-throughput imaging and plate reader-based assays for cell viability and caspase 3/7 activity (see Protocol: **A High-Throughput, Multiplex Cell Death Assay Using an RNAi Screening Approach** [Falkenberg et al. 2014]).

INITIAL CONSIDERATIONS

In designing an HTS assay for cell death, it is critical to first determine the measurement most relevant to the particular signaling pathway or phenotype of interest. For a particular assay to be adaptable to high-throughout format, it should satisfy the normal criteria related to reproducibility, sensitivity, and

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specificity. However, there are three key additional considerations: (1) compatibility with multiwell format readouts (e.g., a plate reader or high-content imager), (2) compatibility with a high-throughput workflow (i.e., the assay should not be too complex or time consuming), and (3) expense of substrates and specialized reagents, particularly when being used in large quantities.

From a biological perspective, it is important to consider the various cell death modalities when deciding which assay(s) may be most appropriate. For example, apoptosis and necrosis are distinguishable by very specific biochemical and morphological features. Many assays that measure cell viability as a surrogate of cell death are unable to distinguish between these modalities or discern the effects of cytostasis.

A selection of cell death assays with demonstrated utility in the HTS format is shown in Table 1. These assays represent a reasonably broad spectrum of the biological mechanisms relevant to the various cell death modalities, including proliferation (cell number), protease activity (e.g., caspases), cell cycle (GFP-cyclins, DNA content and synthesis), cell viability (e.g., membrane permeability, metabolic substrates), cellular energetic or reductive potential (e.g., ATP or NADH levels), mitochondrial function (cytochrome c release, membrane potential), and DNA cleavage (e.g., TUNEL). More recently, multiplex assays combining multiple readouts in a single workflow have been used, as described in the accompanying protocol (Protocol: **A High-Throughput, Multiplex Cell Death Assay Using an RNAi Screening Approach** [Falkenberg et al. 2014]). Further, in vitro and in vivo selection-based screens are also possible in high-throughput format (e.g., using arrayed or pooled shRNA/ORFeome screens or survival of mixed populations).

ASSAY MODE

As noted in Table 1, there are many cell death assays to choose from. However, there are limited tools for performing these assays. Principally, high-throughput cell death assays can be measured using high-content imaging (via a dedicated microscope that is fully automated with computer-learned algorithms for analysis), an intermediate low-resolution imager for simple cell counting, or a microplate reader that quantifies fluorescence or luminescence. The distinction to note is that high-content imaging generally reflects a specific number of cells in a well, whereas plate reader quantitation represents the entire contents of the well.

High-Content Imaging Mode

High-content imaging enables quantitation of specific cellular features on a per cell basis (Snijder et al. 2012). It requires robust assays and protocols and can be highly specific (e.g., measuring a particular molecular process in a cell death pathway) or more general (e.g., counting cells and distinguishing live from dead). Imaging assays typically require a lot of dedicated time and effort to develop because of the inherent nature of "teaching" the computational algorithm the specific intricacies of the phenotype, followed by statistical validation. Image analysis relies on quantifying a statistically representative number of cells and fields per well. Some of the more straightforward assays include quantitation of either healthy or dying cells using DAPI or Hoechst staining and defining the size of a healthy versus condensed nucleus. More detailed analysis includes detection of caspase activity and/or cytochrome C release from mitochondria. One particularly challenging screen used GFP:H2B reporter cell lines to follow cytokinesis via live imaging (Tsui et al. 2009), whereas another quantified an exhaustive array of basic cell features such as cell division, proliferation, survival, and migration (Neumann et al. 2010). The cell death features that can be measured by high-content imaging are extensive (see Table 1 for selected examples) and can often be modified from low-throughput approaches. Other considerations for assay development and design are outlined below in "Assay Design and Strategy."

The following hardware is needed for high-throughput imaging:

- A specialized microscope with multiple objectives and multiple fluorophore capabilities that is capable of reading at least 96- to 384-well format plates.

- Automation to deliver plates to the instrument (depending on the number of plates, automation improves throughput for plates that take many hours to read and allows images to be acquired overnight and through the weekend).
- Barcode read-enabled instrumentation for plate label incorporation into file names of all data acquisition.
- Software that is intuitive, allows visualization of all well data, and can export cell features in a manageable data format (e.g., .xls, .csv, or .txt).
- Brightfield imaging. It is not often used in high-content screening, but it can be useful for visualizing cells and can be used in conjunction with, for example, β-galactosidase senescence assays.
- Sufficient data processing and storage capacity, and IT support. Image size depends on many parameters such as the image resolution (2×2 or 1×1 binning), the number of fluorophores, the number of fields imaged, the number of cells counted, and the number of parameters measured. Together, the amount of data from a single 384-well plate can range from several Gb to ~25 Gb. Factor those figures into a large compound screen or genome-scale RNAi screen, in the order of 60 or more library plates, screened in duplicate and multiple terabytes are easily required. The amount of data collected and ultimately stored for the purpose of probity should not be underestimated; a large server and storage capacity is absolutely required, particularly for screening facilities.

Because experience and technical knowledge can be a significant barrier to accessing this technology, a local field application specialist or dedicated microscopy specialist can be invaluable.

Consider the following when you plan for high-content imaging:

- The appropriate cell density must be empirically determined; for example, overconfluent cells will not be easy to segment and identify.
- The complexity of the assay should be considered in terms of minimizing wash steps and the feasibility of performing all steps in a relatively defined period.
- The number of channels to be imaged will vastly alter the imaging time.
- The number of cell features collected must also be considered. Capturing information that you do not intend to use increases data storage dramatically, so think carefully about the information required. It is always possible to reanalyze images after they have been captured.

Plate Reader Mode

Most plate reader assays measure the response of the entire cell population within the well, although some instruments are capable of multiple sampling across individual wells. Assay development is generally much faster using a plate reader-based assay compared with a microscopy-based assay but will be limited to only one or two key parameters. It is important to note that in comparison with image analysis, in which information can be obtained on a single-cell level, detail can be lost when taking an aggregate measurement. Often, a plate reader-based viability assay will be multiplexed into an imaging-based screen in which cell health is not easily measured (e.g., wound healing [Simpson et al. 2008]). Because there is no permanent record of the well contents as there is for imaging, it is imperative to confirm during assay development that the specific assay readout accurately depicts what is visible. For example, when measuring cell health using an ATP-based readout, a parallel plate must be evaluated at least visually but if possible, by accurately counting nuclei-stained cells on a high-throughput microscope. This step is essential both to validate the assay and to ensure there is a significant correlation between cell number and the relative viability reading. Once confirmed, a screen can continue with only the plate reader quantitation, but we recommend a quick visual inspection of randomly selected plates before addition of fluorescent or luminescent reagents. If quantifying a phenotype that is not easily interpreted visually (e.g., caspase activity), then benchmark-

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ing the readout against a more traditional low-throughput approach (FACS-based annexinV/PI or active caspase-stained cells) is essential. Regardless of the assay, a comparable dynamic range and signal-to-noise ratio should be detected between the positive and negative controls in high-throughput versus traditional low-throughput methods.

Plate reader assays involve the following instrumentation and software:

- Plate management automation. Automation provides timed accuracy and allows the researcher to continue working while plates are reading.
- Software to batch export files, so all the plates read at a single time point can be incorporated in one output file (individual files work fine, but it is better to keep all data in one file).
- Barcode read-enabled instrumentation to incorporate the barcode into the output file to ensure correct plate ID, thus eliminating any concerns if plates are accidentally stacked out of sequence.
- A plate reader that permits multi-sampling injection. These readers can be very useful for kinetic studies and luminescence assays.

Optimize gain and exposure times to ensure high sensitivity and dynamic range without "overflowing" the sample (when the gain has reached maximum capacity for readout value).

Adherent Versus Suspension Cells

One of the most critical considerations in defining the assay type and mode of measurement is the choice of cell type, which ideally reflects the biological question you are asking. The most suitable cells from a biological standpoint, however, may not always be the best choice for the assay. For example, suspension cells in particular are not easily amenable to high-throughput assays from the perspective of handling and assay readouts, but they can perform sufficiently well in plate reader assays if media changes are possible (or can be avoided) during the workflow and if staining is optimal. If suspension cells can settle or be centrifuged to the bottom of the plate, imaging might be possible but will still be challenging.

ASSAY DESIGN AND STRATEGY

Many different elements must be considered collectively when planning and executing an HTS approach.

The Model System

Understanding the limitations and advantages of your model system, and in particular the cell line(s) being used, is critical to a successful high-throughput screen. Are you investigating cell death in a global fashion with no mechanistic insight? Do you need to distinguish growth inhibition versus cell death? Cytostasis is a common drug response that is difficult to detect with assays that measure ATP content, in part because of the rate of proliferation during the course of the assay (especially if only 2–3 d). However, to understand the mechanism driving the death response, the mode of action of the drug must be considered (see "Drug Action"). Answers to these questions will help establish the assays most suitable for evaluating a particular emergent phenotype (Table 1). Importantly, the cell line(s) chosen should reflect the biological question being asked. For example, if you are performing an siRNA screen to identify novel ovarian cancer oncogenes, an appropriate ovarian cell line should be chosen (such as a tumorigenic line) in which loss of a gene target will result in death or a less aggressive phenotype. Powerful insights have been gained using synthetic lethal genetic approaches with isogenic cell lines (with genetically altered lines created, for example, by gene ablation or overexpression in a parental line) or matched sensitive-versus-resistant cell lines derived from the same genetic background (Luo et al. 2009). We highly recommended that cell lines be routinely assessed for mycoplasma contamination. You must be "in tune" with the cells being used and understand their morphology,

passaging and seeding densities, transfection conditions, and response to any treatment. This is critical in being assured that the cells are responding as expected throughout the course of an assay. Importantly, pilot assays should be performed to statistically evaluate cell responses, because differences that appear satisfactory on first glance may often not be statistically robust.

Drug Action

Many chemotherapeutics kill by inducing apoptosis via a caspase activation cascade, but their mechanisms for doing so vary. Targets include intrinsic and extrinsic pathways, mitochondria, and death/growth factor receptor pathways (Brunelle and Zhang 2010). Understanding the mechanism of action for the drug you are studying will help you devise sophisticated analyses, which may not be amenable for a primary screen but will often be suitable for triaging hits in a lower-throughput, secondary screen format (i.e., one plate versus 20 plates). The mechanism of action also informs the type of screening approach to be used, particularly with respect to the timeframe and duration of the screen (e.g., compound versus siRNA screen; synthetic lethal or drug resistance).

An understanding of drug action also directly affects the choice of controls to include on each plate. Positive and negative controls become vital for establishing the dynamic range of the death response. For example, the extent of cell survival in control wells determines the concentration of drug required to induce cell death or sensitize cells to death. If screening for drug resistance, the drug concentration used should be no greater than that needed to produce 90% cell death in control wells. This will avoid drug concentrations so toxic that even resistant cells struggle to survive and assay sensitivity is compromised.

Maximizing Data Density

In general, plate reader-based assays produce relatively limited amounts of data, as only one or two parameters can be measured in an assay. In contrast, high-content imaging is capable of producing seemingly endless amounts of multidimensional cell feature data. This can be a distinct advantage, but identifying the most meaningful data and the best way to extract it is critical. Recent outstanding publications that capture exhaustive numbers of cell features are clearly the result of many years of dedicated effort, from acquisition of data to advanced computational analysis (Neumann et al. 2010; Snijder et al. 2012). These huge, multidimensional approaches can be particularly daunting and in reality are not currently achievable for most researchers outside specialist facilities. Keeping such publications in mind, however, it is important to be aware of the main questions being asked, the key data to be collected (specific cell features, timing of response, live versus fixed cells), and what additional information can be collected that could add value—not necessarily in the present but possibly in the future. If cell-based imaging is set up thoughtfully, encompassing key elements like nuclei staining, a cell mask, and possibly a mitochondrial marker, and a significant number of cells and fields are imaged, archived images can always be reanalyzed with new algorithms to answer different or related questions.

Live Versus Fixed Analysis—Temporal Context

There are clear advantages to quantifying cell death over a time course to establish the rate and mechanism of cell death, particularly when looking for the most effective drug or gene target. Live imaging, however, is not significantly high throughput if one is trying to keep the timing between treatment and imaging consistent, given the time it takes to capture multiple sites in a 384-well plate. There are several dyes that can be used to track live cells, but the production of a cell line harboring a fluorescent reporter system (Tsui et al. 2009) is a very efficient and consistent tool for tracking cells. Another consideration is that live imaging can generate an enormous amount of data, depending on the time course. For example, compare imaging five fields every 30 sec for 3 h with imaging five fields once every hour for 24 h. An exciting new product, the CellTox Green Express Cytotoxicity Assay (Promega), involves a nontoxic dye that binds to DNA in cells with compromised membrane integ-

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rity; the dye can be presented in culture media over the time course of an experiment, emitting fluorescence quantified by a plate reader or microscope.

Fixing cells and staining with fluorescently labeled antibodies remains the most common method for image-based analysis, and the range of different fluorophores matching the read capabilities of a high-content microscope allows multiplexing. A stable fluorescent reporter cell line also performs very well for fixed end point readouts and can significantly reduce screening costs by not requiring any antibodies. With fixed cell staining, it is possible to accurately conclude an experiment within a defined window of time; the method is generally robust enough that imaging can occur over days, if necessary, or the samples can be stored at 4°C and imaged later.

Cell Density

Cell density is critical for assay success and must be considered at multiple points during assay development. The appropriate density at which to start and end the assay is affected by the number of population doublings the cells must undergo and the timeframe of efficacy of the drug or other treatment used (e.g., siRNA knockdown). Optimal transfection density, for example, is tied to cell number and lipid concentration, and the final cell density is generally based on controls.

In the context of a high-throughput chemical screen, compounds are generally serially diluted over a broad dose–response range and applied to cells plated at a density that results in controls reaching ~90% confluence at the assay end point (cells can be seeded before or at the same time as drug addition). When performing a synthetic lethal functional genomics screen, cell density at transfection is optimized based on the desired end point density and transfection/infection efficiency. The end point density is a combination of the level of death in the positive and negative controls, the length of drug treatment, and the response time after treatment. Drug activity and response can be acute (hours), or cells can be cultured in drug for days. Overconfluent cells at an end point can cause misleading viability data and affect the measurement of other variables, because such cells can effectively alter their physiological state and responsiveness. For siRNA screens, the knockdown window is normally 72–96 h. This timeframe can be extended effectively as far as 120–144 h, but you must verify these times in your cell line. Starting density can then be extrapolated by knowing the end point cell number and estimating the doubling time over the course of the assay. The optimal transfectable density must also be considered, and is usually ~30% confluence. If the density is too sparse, cells would not proliferate well and the lipid may be toxic, and if the density is too high, the transfection efficiency will be dramatically reduced and the effective length of time in culture (especially in a 384-well format) will be reduced.

Instrument-specific aspects of imaging also are affected by cell density. Microscopes can have difficulty defining and counting confluent cells, and imaging very sparse wells can take extraordinary lengths of time as the microscope struggles to find cells and subsequently a focal plane (i.e., increasing sampling time). In general, if sparse wells is not the phenotype you are studying, we recommend defining a minimum threshold cell number to be obtained in three to five fields, which if not met, will cause the microscope to move to the next well; there is clearly no need to waste time imaging nothing.

Controls

Appropriate and robust controls, including negative (or no effect), positive (assay-specific biological controls), and technical controls, are essential to any high-throughput approach, especially for screens that might take weeks to complete. These controls must be defined by your biological question and the technical considerations inherent in the specific platform used. Assay-specific controls must be both informative and statistically robust. It is not unusual that a suitable positive control cannot be found for a specific RNAi screen, in some ways reflecting the discovery-based nature of this approach. In practice, however, it is often possible to set up viability screens using known potent death genes identified from numerous screening efforts (such as PLK1, COPB2, WEE1, and AURKA). Controls not only report the technical elements of a screen on a per-plate basis, but are also essential for normalization during data analysis, not just on a per-plate basis but also for comparison of all plates.

The same controls must be included in every plate, with sufficient replicates to ensure statistical significance, and their location on the microplate depends on the format of the screen's library. Where possible, edge columns and rows should be avoided to minimize potential edge effects. Statistical analysis of controls is outlined briefly below and in detail in the accompanying protocol (see Protocol: **A High-Throughput, Multiplex Cell Death Assay Using an RNAi Screening Approach** [Falkenberg et al. 2014]).

Dynamic Range and Magnitude of Effect

The dynamic range of an assay represents the breadth between positive and negative control values, such that they are statistically significant from each other, allowing "hits" to be identified from all the samples. The signal-to-noise ratio reflects the level of signal above background, which should also be significantly higher. Luminescence assays tend to give very low background (i.e., in media-only conditions) and very robust, highly sensitive readings that result in large numbers. Signal-to-noise issues can be more pervasive in imaging assays, depending on how clean the background staining is and how strong the signal is. However, postprocessing correction can significantly improve signal-to-noise ratio and segmentation performance (Poon et al. 2008).

Timing of Effect

Drug mechanism of action and concentration can directly affect the rate of cell death. During assay development, it is important to establish the window of activity for treating cells such that the subsequent effects can be measured. If possible, we recommend evaluating multiple time points (if working with an end point assay, set up as many replicate plates as required). For example, in a synthetic lethal RNAi screen, stimulation with drug for 4 h results in control cells that are largely still viable but primed and sensitized for death. The time it takes for the maximum number of control cells to die must then be evaluated and the window for the assay based on that. The best option for some screens may be a series of kinetic measurements rather than a single end point. If that approach is too daunting on a genome scale, then consider an end point screen followed by a kinetic screen with a smaller high-confidence gene/compound list.

Workflow Considerations

When working in high-throughput mode, the sheer number of plates necessitates a trade-off between complexity and throughput, whereby workflows are often simplified to increase throughput. This does not mean compromising on experimental rigor. Thus, all assays must be reviewed in light of the question, "Can I make all of these manipulations within a defined window of time so each plate is treated equally?" For example, traditional low-throughput immunohistochemistry might require fixing, blocking, and staining, with three washes between each step. This process is very difficult to automate, because of the time the steps require and the forceful effect of the dispensing and media change apparatus on cells (which, although determined to be acceptable at the outset of a screen, can dislodge the cells after days of culture and high repetition). The use of commercial assays must be reviewed in the same manner; incubations for very short time frames (e.g., 10 sec followed by 1 min) are not possible with large numbers of plates. To avoid compromising the outcome of the experiment, the design of the assay must take into account the trade-offs between quick and relatively easy procedures versus those that require extensive processing.

Understanding Assay Limitations

There is no "one size fits all" assay that is suitable for every high-throughput cell death approach. It is, therefore, critical to be aware of exactly what is being measured and what, if any, limitations there might be on those measurements. For example, the stability of reagents after they are added to cells determines when assay outputs must be measured, and data quality can deteriorate quickly outside of that time frame. Many reagents can have diminished activity when freeze–thawed, and plate readers

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and microscopes can take many minutes or hours to read a plate—conditions that might not be compatible with the assay or throughput requirements.

Assay Reproducibility

The use of liquid-handling automation for high-throughput approaches is essential for reducing variability. Reproducibility can be controlled by maintaining consistency on the timing of all aspects of the experiment, including cell dispensing, media changes, stimulation or drug treatment, and the assay itself, performed at the same time and the same way each time. In other words, a 2-min shake and 10-min incubation must be exactly that for each plate, each time it is done. The coefficient of variation (for the same sample treatment over multiple wells) is the key measurement to consider when assessing assay reproducibility (defined as the standard deviation divided by the mean, expressed as a percentage). Ideally, this number is ~10%, with values higher than 25% being unacceptable. The vast majority of high-throughput screens are performed with multiple technical replicates (at least duplicates), and depending on the assay, multiple biological replicates. Variability can also arise from the materials used in screens. We recommend banking a large number of vials of cells at the same passage before starting a screen and to using a fresh passage for each set of transfections or compound addition. Assay variability can be introduced by changes in lot number of essential reagents, including serum, compound or dilution batch, transfection lipid, media and additives, primary and secondary antibodies, and death assay reagents. We recommend that when possible, you purchase enough resources for the entire primary screen and any subsequent validation. All lots must be validated before embarking on a screen.

All liquid-handling instruments, including those that do nanoliter or microliter dispensing, cell dispensing, media changes, and siRNA transfection mixing (the actual mixing step is very important to ensure homogeneity in a very small volume), must be subject to routine quality control for volumetric dispensing, using a fluorescent reagent such as tartrazine. Microscopes should be routinely evaluated for consistent light intensity, even illumination field, and filter integrity to ensure that the exposure level is constant (these issues are of special concern if exposure times start to alter over experiments that last weeks). Bulbs should be changed well within their expected life span, although light source deterioration generally occurs long before the bulb goes out. The new LED light system microscopes have addressed these major limitations by having an extremely long life and more even illumination.

Validation Screens

The discussion points above are a broad overview centering on the concepts for a primary, first-pass screen that aims to identify and quantify the occurrence of cell death but generally not provide specific mechanistic insights. In general, once a hit list of primary candidates has been obtained, the researcher needs to be prepared to evaluate orthogonal assays to refine this list. These validation screens may come in the form of alternative high-throughput assays, or a shift back to a lower-throughput system that relies on more standard assays, which in this field often involve flow cytometry-based measurements.

INFORMATICS

No high-throughput approach is possible without strong computational and biostatistical support. Bioinformatics approaches range from determining assay robustness to collating a comprehensive compound library or genome-scale RNAi data. Robust statistical methods must be used to identify the most potent and consistent phenotypes from the complete screen data. The Z'-factor, a statistical measure of the dynamic range between positive and negative controls, encompasses the average and standard deviation of each control, which must be at least two standard deviations from each other. To ensure statistical significance, this method requires a large number of wells for each control reagent;

we recommend at least 10 of any control in a 384-well plate. For RNAi screening, a value of >0.3 is acceptable, whereas compound screening requires a value of >0.7. Great emphasis is placed on the Z'-factor during assay development and thereafter during the screen to ensure controls are consistent from week to week. The Z'-factor calculation is as follows:

$$Z'\text{-factor} = 1 - (3 * \text{sd}_{\text{hc}} + 3 * \text{sd}_{\text{lc}})/|\text{mean}_{\text{hc}} - \text{mean}_{\text{lc}}|,$$

where $*$ indicates "multiplied by," "mean" indicates the average, "sd" indicates the standard deviation, "hc" indicates the high-value control, and "lc" indicates the low-value control.

For an siRNA/miRNA screen, the most common approach for defining screen hits is to use a z-score or robust z-score (sample-based normalization) (Birmingham et al. 2009). This approach standardizes the strength of an siRNA phenotype relative to the rest of the sample distribution, reported as the number of standard deviations away from the mean. The z-score can be calculated as follows. A z-score approach can be applied to a plate of data or an entire genome's worth of data to identify hits:

$$z\text{-score} = (\text{sample value} - \text{sample mean})/\text{standard deviation}.$$

An alternative to the z-score is the robust z-score, which takes into account the plate median and median absolute deviation to eliminate the effects of outliers:

$$\text{robust } z\text{-score} = (\text{sample value} - \text{sample median})/\text{sample median absolute deviation}.$$

For a compound screen, an IC_{50} (half maximal inhibitory concentration; usually growth inhibition or cell death) measurement is made for the entire population assayed and used to compare the different treatments.

The number of "hits," or targets that are significantly identified in the screen, will vary for many reasons, including the cell line responsiveness to the assay, the actual assay itself, and the robustness of the experimental design and its controls. The number of hits selected will depend on the end user and his or her goals for the screen. Many researchers choose to integrate published interaction data with their hit list to triage the candidates to a manageable number.

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