Basic Concepts in Imaging-Based High-Throughput Screening and High-Throughput Profiling Assay Development

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Disclaimer

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Objectives for This Session

• Explore basic concepts in imaging-based high-throughput screening (HCS) and high-throughput profiling (HTP) assay development to those who are new to this area of science.

• Provide examples from a variety of HCS assays to contextualize the assay development process.

• Discuss considerations for experimental design and methods for evaluating HCS and HTP assay performance.
Resources


Key Terms

• **High Throughput Screening (HTS):** Refers to large-scale experiments where combinations of robotic automation, liquid-handling devices, instruments for detecting assay-specific outputs and data processing and analysis pipelines are used to evaluate the biological effects of hundreds to thousands of agents (i.e. chemicals, siRNA, other) in parallel.

• **High Content Screening (HCS):** A HTS approach that combines automated fluorescence microscopy and quantitative image analysis to assess the biological activity of test agents on a specific process or cell function at the single cell level or single organism level.
  - Synonymous with High Content Analysis (HCA), High Content Imaging (HCI), Image Cytometry (IC), but higher throughput

• **High Content Profiling (HTP):** A HTS approach that combines automated fluorescence microscopy and quantitative image analysis to assess the biological activity of test agents by measuring a large variety of cellular features.

• **Feature:** A property of a cell or organism determined using quantitative microscopy.
• Screening is a distinct strategy from profiling.

• Although both involve large-scale (high-throughput) imaging experiments, the goals differ:

  • **Screening**: The researcher aims to measure one or more phenotypes that are visually discernable, and choose a subset of hits for further investigation. Assay design is based on *a priori* knowledge of a biological process of interest (ex. receptor translocation, ROS production, etc.).

  • **Profiling**: A broad spectrum of measurements is captured from each sample (unguided by prior knowledge) in order to reveal important differences and similarities with other samples.

• **Screening** depends on a biologist’s expertise to interrogate a particular phenomenon whereas profiling takes an unbiased approach to grouping samples, with a higher potential to capture unknown mechanisms.
Why Consider HCS or HTP?

• HTS assay can provide information on the biological activity of test agents, however, they provide single (i.e. “low content”) readouts.

• HCS can be used to evaluate many of the same biological processes as HTS assays, but provide more detailed information at the level of the individual cell or organism.

• HCS can be used to evaluate cellular responses that are not amenable to traditional HTS assays, such as changes in cell morphology or movement of proteins within a cell.

• The potential applications of HCS for interrogating biology are broad.

- Protein Expression
- Protein Translocation
- Protein Modifications
- Enzyme Activation
- Cell Surface Receptor Activation
- Molecular Uptake
- Cell Proliferation
- Cell Cycle Regulation
- Cell Viability / Apoptosis
- Cell Migration
- Cell-Cell Interactions
- Differentiation
- Cell Morphology
- Organelle Structural Changes
- Neurite Outgrowth
- Membrane Potentials
- Cell subpopulation redistribution
- Redox state
What is an HCS System?

• “Microscope-in-a-Box”
• HCS systems include:
  1. Many of the same physical components as traditional microscopes
  2. Specialized components to increase imaging throughput
  3. Image analysis software
  4. Data storage and management solutions
• Major Varieties:
  1. Wide-field imagers
  2. Confocal imagers
  3. Laser scanning cytometers
• Know the components of your HCS system so you can use it effectively!

## Components of an HCS System (1)

<table>
<thead>
<tr>
<th>Component</th>
<th>Type</th>
<th>Notes</th>
</tr>
</thead>
</table>
| **Lamps** | Excitation | • Broad wavelength emission from UV to IR.  
• Can excite many different fluorescent dyes, but power is low.  
• Can require frequent realignment for optimal performance.  
• Comparatively shorter lifespan than other source types.  
• Require careful selection of filter sets for fluorescence microscopy. |
| **Lasers** |            | • Fixed monochromatic wavelengths.  
• Substantial power, but wavelength may not be optimal for certain probes.  
• Long lifetimes, but relatively expensive to replace. |
| **LEDs**  |            | • Wavelength emission spectrum is narrow (not monochromatic) and varies by type.  
• Stable light output (compared to lamps)  
• Long lifetimes |
| **Excitation** | Filter Sets | • Bandpass filter that passes wavelengths that are absorbed by the fluorophore. |
| **Dichroic** |            | • Reflects light in the emission band (towards the sample) and transmits light in the excitation band (towards the detector). |
| **Emission** |            | • Bandpass filter that passes wavelengths emitted by the fluorophore. |
|            |            | • Excitation / Emission filter selection should take into account the peak properties of the fluorophore and optimized to minimize crosstalk between different probes when multiplexing. |
## Components of an HCS System (2)

<table>
<thead>
<tr>
<th>Component</th>
<th>Type</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Objectives</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Dry       |      | • Different magnifications.  
|           |      | • Objectives with a higher numerical aperture will collect more light, thereby reducing exposure times and increasing throughput. |
| Wet       |      | • Different magnifications.  
|           |      | • Used with water or oil interface to the bottom of the imaging plate.  
|           |      | • Collect more light than dry objectives.  
|           |      | • More difficult to use than dry objectives. |
| **Detectors** |      |       |
| Digital cameras (CCDs, EMCCDs, cCMOS) |      | • High frame rate (i.e. the frequency which consecutive images can be acquired).  
|           |      | • Large dynamic range.  
|           |      | • Broad spectral sensitivity (400 – 900 nm & higher).  
|           |      | • High resolution / large format.  
|           |      | • Monochromatic.  
|           |      | • Produce large files that must be managed with an image storage solution. |
| PMTs      |      | • Extreme sensitivity (can measure very low intensities of light).  
|           |      | • Fast response.  
|           |      | • Broad spectral sensitivity.  
|           |      | • Almost always used with a laser source.  
|           |      | • Produce an image by being used in conjunction with scanning technology (i.e. mobile source). |

Adapted from NIH Assay Guidance Manual
# Components of an HCS System (3)

<table>
<thead>
<tr>
<th>Component</th>
<th>Type</th>
<th>Notes</th>
</tr>
</thead>
</table>
| High Precision             | Automated       | • Submicron resolution and repeatability.  
• Required for precise image acquisition in multi-well format.  
• Facilitates acquisition of multiple unique fields-of-view within a well.  
• Specialized applications (image stitching, object detection with low mag. pre-scan). |
| Hi-Speed                   | Autofocus       | • Step through the specimen and use algorithms to determine optical focal plane.  
• Comparatively slower than laser-based methods.  
• Can be used with non-uniform or thick specimens (to a point).  
• Debris, dust and other imaging artifacts can cause failures. |
| Environmental Chamber      | --              | • Controls temperature, humidity and CO₂ levels within imaging device  
• Allows for real-time and kinetic measurements in live cultures |
| Liquid Dispensers          | --              | • Allows for automated addition of reagents or test substances during imaging runs  
• Peripheral or integrated. |
| Plate Handling Automation  | --              | • Automated loading and unloading of plates from the imaging instruments  
• Used for high-throughput, high-volume applications |

Adapted from NIH Assay Guidance Manual
HCS Microplates


"While the highest priority in the experimental design is selection of a biological model, the choice of microplate can alter the biological response and ultimately may change the experimental outcome."

Variety:
- Formats (96-, 384-, 1536, etc.,)
- Well geometries (round, square, u-, etc.,)
- Materials (glass, polystyrene, olefin)

Uniformity:
- SLAS / ANSI standards

**Key consideration:** Does the plate thickness and the skirt height match the working distances of the imaging objective?

Steps in HCS Assay Development

- **Problem Formulation**
- **Assay Concept**
- **Cell Model**
- **Plate Type**
- **What Biology Am I Interested In?**
- **How Will I Measure The Biology of Interest?**
- **What Cell Model Should I Use?**
- **What Plate Format Should I Use?**
- **Reagents & Probes**
- **Dynamic Range**
- **Exposure Conditions**
- **Controls / Assay Performance**
- **How Will I Visualize The Biology of Interest?**
- **What Range Of Response Values Should I Expect?**
- **How Will I Deliver The Test Agents? When Should I Measure the Biology of Interest?**
- **How Will I Evaluate Assay Performance?**

*Even though these are depicted as a linear sequence, that is not always the case.*

Adapted from NIH Assay Guidance Manual
Problem Formulation

• When developing an HCS assay, begin by asking:

  • What is the biological process of interest?
  
  • What are the characteristics of the biological process of interest?
  
  • What could you measure to evaluate effects on the biological process of interest?
  
  • What is the goal of the study?
Example 1, Nuclear Receptor Activation

Goal(s): Identify nuclear receptor **activators**
Identify agents that **inhibit** nuclear receptor activation

Characteristics:

Requires the receptor to be expressed and functional.

Appropriate stimulus required to activate receptor:
- Ligand binding
- Post-translational modification
- Dissociation from chaperone

Activation results in:
- Translocation within the cell
- Association or dissociation with a binding partner
- Post-translational modification
- Transcription / translation of regulated gene products


Mackowiak et al. (2019) PMID: 31306645
Example 2, Oxidative Stress & Apoptosis

Goal(s): Identify chemicals that **produce** oxidative stress & **cause** apoptosis
Identify chemicals that **reduce** oxidative stress & **prevent** apoptosis

Characteristics:

Oxidative stress can result from:
- Increased intracellular production of reactive oxygen species (ROS)
- Decreased levels of endogenous antioxidant molecules

Oxidative stress leads to apoptosis via:
- Cleavage of pro-caspase 3 to activated caspase-3
- Cleavage of PARP by activated caspase-3

Hallmarks of apoptosis include:
- Reduction in nucleus size
- Nucleus fragmentation
- Loss of plasma membrane integrity

Noh et al. (2015). PMID: 25892552
Kihlmark et al. (2001). PMID: 11707516
Example 3, Steatosis

Goal(s):
- Identify chemicals that cause steatosis.
- Identify chemicals that are protective against steatosis.

Characteristics:

Abnormal retention of lipids within a cell or organ.

Occurs in the liver in response to:
- Dietary factors
- Chemical exposures
- Signals from peripheral tissues.

Reflects an impairment of the normal processes of synthesis and elimination of triglyceride fat.

Hallmarks of steatosis includes:
- Accumulation of lipid droplets
- Production of ROS
**Example 4, Neurite Outgrowth**

**Goal(s):** Identify chemicals that **inhibit** or **enhance** neurite outgrowth.

**Characteristics:**

Neurite outgrowth involves extension of long, thin processes from the cell body of neurons.

Neurites contain a variety of cytoskeletal proteins that vary according to neurite type (i.e. axons vs. dendrites).

Neurite networks become more complex over time.

NOG May require:
- Growth substrates
- Soluble growth factors
- Support from feeder cells

Harrill et al. (2011). PMID: 21354195
Choice of Cell Model

• The choice of cell models is guided by many factors:
  - Representative Biology
    - Availability
    - Scalability
    - Reproducibility
    - Ease of Use
    - Growth Characteristics
    - Morphology
    - Cost
    - Complexity
    - Compatibility with Assay Concept

• Types of cell models:
  - Cancer cell lines
  - Immortalized cell lines
  - Stem cell-derived or iPSCs
  - Primary cultures
  - 2-D versus 3-D
  - Uniform versus mixed
Cell Model Optimization

- Cell models can require optimization of many parameters:
  - Media formulation
  - Growth atmosphere (i.e. CO₂ / O₂)
  - Plate coating / growth substrate
  - Seeding density
  - Passaging
  - Maintenance in culture
  - Stimuli / Stressors
  - Labeling strategy

- Variability, sensitivity and dynamic range of HCS measurements can vary for the same cell type under different growth conditions.

- A key to reproducible HCS results is optimization and consistent preparation of cell cultures.
Cell Model Optimization (2)

Ker-CT

RPE-1

% Confluency

Timepoint

- 24
- 48
- 72

24 h

48 h

72 h

Ker-CT

RPE-1

24 h

48 h

72 h

Willis, Nyffeler & Harrill. Unpublished Results
Cell Model Optimization (3)

Overly confluent cultures:

- Inaccurate segmentation
- Obscure the biology of interest

Willis, Nyffeler & Harrill. Unpublished Results
Assay Concepts, Overview

• HCS assays are based on:
  1. The use of fluorescent reagents and probes.
  2. Identification of labeled objects.
  3. Measuring fluorescent intensity, shapes and spatial relationships between objects and/or pixels (i.e. texture).

• In HCS, the assay concept can be designed to provide intuitive measurements of the biology of interest.

<table>
<thead>
<tr>
<th>Concept</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear Receptor Activation</td>
<td>• Ratio of fluorescent intensity inside vs. outside nucleus</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>• Nucleus area, Nucleus shape, % of caspase positive cells</td>
</tr>
<tr>
<td>Steatosis</td>
<td>• Number of lipid droplets per cell</td>
</tr>
<tr>
<td>Neurite Outgrowth</td>
<td>• Total neurite length per neuron</td>
</tr>
</tbody>
</table>

• Multiplexing of reagents and/or measurement of different features can provide information on different aspects of the biology of interest.
Synthetic & Biological Reagents

• Fluorescent Probes:
  • Molecules that absorb light of a specific wavelength and emit light of a different (typically longer) wavelength.
  • Some probes change their fluorescence emission properties in response to a binding event, chemical reaction or change in their immediate environment.

• Small Molecules:
  • Low molecular weight chemical that binds to a specific protein or molecule within a cell.
  • Examples: Endogenous fluorescence or conjugated to a fluorescent probe.

• Sensors:
  • Probes that change fluorescent properties after interaction with a reactive or charged molecule within a cell.
  • Examples: ROS / RNS sensors; voltage sensors

• Antibodies:
  • Immunoglobulins that recognize and binds to specific antigens.
  • Examples: Fluorescent probe-conjugated primary or secondary antibodies
Genetic Reagents

Heterologous protein expression constructs that may be incorporated into genomic DNA or encoded in extranuclear expression vectors.

- **Fusion Proteins:**
  - Created through transcription and translation of a genetic sequence that encodes two or more proteins, resulting in formation of a single polypeptide.
  - For imaging applications, designed as a protein of interest with a fluorescent protein (i.e. GFP, RFP, etc.) attached to the N- or C-terminal end.
  - Expression driven by the presence of a constitutively-active promoter into the genetic sequence.

- **Fluoro-tagging:**
  - Heterologous expression of a fusion protein consisting of the protein of interest and a enzyme that reacts with a particular class of fluorescent probes.
  - Results in a fluorescent-labeled fusion protein, but provides flexibility regarding the wavelength of the fluorescent probe.

- **Fluorescent reporters:**
  - Heterologous expression of a fluorescent protein driven by activation of a promoter sequence.
  - Used to detect activation of intracellular signaling pathways by endogenous proteins.
Multiplexing of Fluorescent Probes

- Fluorescent probes may be multiplexed to provide information on different aspects of the biology of interest.
- Care must be taken during multiplexing of probes to minimize cross-talk across fluorescent channels.

Rules-of-Thumb:
- Targets with higher signal should be imaged in lower wavelengths than targets with lower signal.
- Routinely evaluate cross-talk between channels using single-plex labeling and evaluation of “off-channel” images

https://www.thermofisher.com/order/spectra-viewer
Identification of Labeled Objects

- **Segmentation**: Separation of signal from background

- Once objects have been identified, their properties and associations with other objects can be measured as endpoints in an HCS assay.

Tucker, Nelson, Harrill, Chorley: Unpublished Results
Cell Model:
• MCF-7 Adenocarcinoma cells

Biology of Interest:
• Apoptosis

Visualization Approach:
• Caspase cleavage site peptide (DEVD) conjugated to fluorophore and quencher
  • ThermoFisher CellEvent™ Caspase 3/7 OR
  • IncuCyte™ Caspase-3/7 Reagent
• Nucleus counter-stain (Hoechst-33342)

Image Analysis Approach:
• Identify nuclei and select nuclei of interest
• Measure caspase 3/7 intensity within nucleus mask
• Score cells as “responder” or “non-responder”

Intuitive Output:
• Percentage of caspase-positive cells.
Assay Concepts – Apoptosis (2)

• Average fluorescent pixel intensity within each selected object
  • $1 > 2 \approx 3 > 4$

DMSO Staurosporine Ionomycin

Baseline Partial Response Full (i.e. Uniform) Response

Willis, Nyffeler & Harrill, Unpublished Results
Assay Concepts – Apoptosis (3)

<table>
<thead>
<tr>
<th>Two Approaches</th>
<th>Mean Intensity</th>
<th>Percent Responder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td>Well level mean of AvgIntenCh2 values.</td>
<td>Percent of cells with AvgIntenCh2 values above range of control.</td>
</tr>
<tr>
<td>Example</td>
<td>Breier et al. (2008), Culbreth et al. (2012)</td>
<td>Vogt et al. (2005), Foldes et al. (2011)</td>
</tr>
<tr>
<td>Detect Rare Events?</td>
<td>NO</td>
<td>YES</td>
</tr>
</tbody>
</table>

Q: How is the % Responder Gate Determined?

A: Based on central tendency of control data plus a multiplier of variability.

• Example: Mean + 2*SD.

The stringency of the responder gate affects the assay window (i.e. the ability to detect a response).
Cell Model:
- Differentiated (2-D) HepaRG.
- Mixed culture:
  - “Hepatocyte-like” cells
  - “Cholangiocyte-like” cells.

Biology of Interest:
- Lipid accumulation in hepatocyte-like cells is biology of interest.

Visualization Approach:
- Nile Red labeling of lipids.
- Nucleus counterstain

Image Analysis Approach:
- Identify labeled nuclei
- Select nuclei of interest based on morphology
- Define a Region-of-Interest (ROI) around each cell
- Identify lipid droplets within each ROI.

Intuitive Output:
- Average number of lipid droplets per hepatocyte-like cell
Assay Concepts – Neurite Outgrowth

Cell Model:
• hESC-derived neural cultures

Biology of Interest:
• Extension of neurites from the cell body

Visualization Approach:
• B-tubulin immunocytochemistry
• Nucleus counter-stain (Hoechst-33342)

Image Analysis Approach:
• Identify nuclei and select nuclei of interest
• Used positional information from nucleus channel and b-tubulin labeling (shape) to identify cell bodies & count cells.
• Trace and measure neurites.

Intuitive Output:
• Neurite length per neurons

Harrill et al. (2010). PMID: 20188755
## Assay Controls for HCS (1)

<table>
<thead>
<tr>
<th>Control Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>An assay well that did not receive any test agent.</td>
</tr>
<tr>
<td>Vehicle</td>
<td>An assay well that received the test agent delivery vehicle (i.e. DMSO), but did not receive a test agent.</td>
</tr>
<tr>
<td>Positive</td>
<td>An assay well that received a test agent known to produce an expected effect in an assay.</td>
</tr>
<tr>
<td>Negative</td>
<td>An assay well that received a treatment that is not expected to have an effect in an assay.</td>
</tr>
<tr>
<td>No Label</td>
<td>An assay well containing cells (treated or untreated), but were not labeled with detection reagents.</td>
</tr>
</tbody>
</table>
Assay Controls for HCS (2)

- **Untreated vs. Vehicle:** Determine “vehicle tolerance” → At what concentration does the vehicle affect the assay

Harrill et al. (2010). PMID: 20188755
Assay Controls for HCS (3)

- **No Label Control**: Compared to other control well types to evaluate potential imaging artifacts inherent to the cell model or introduced by the labeling reagents.

Tucker, Nelson, Harrill, Chorley: Unpublished Results
## Assay Controls for HCS (4)

|   | 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 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20  | 20  | No L | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| B | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6   | 6   | No L | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  |
| C | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2   | 2   | No L | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| D | 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6  | 0.6  | No L | 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6|
| E | 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2  | 0.2  | No L | 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2|
| F | 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06  | 0.06  | No L | 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06|
| G | 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02  | 0.02  | No L | 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02|
| H | 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006  | 0.006  | No L | 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006|
| I | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20  | 20  | DMSO | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| J | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6   | 6   | DMSO | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  | 6  |
| K | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2   | 2   | DMSO | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  | 2  |
| L | 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6  | 0.6  | DMSO | 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6| 0.6|
| M | 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2  | 0.2  | DMSO | 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2| 0.2|
| N | 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06  | 0.06  | DMSO | 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06| 0.06|
| O | 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02  | 0.02  | DMSO | 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02| 0.02|
| P | 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006  | 0.006  | DMSO | 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006| 0.006|

- **No Label Control**
- **Untreated Control**
- **Vehicle Control**
- **Positive Control**
- **Negative Control**
- **Test Chemicals**

9/27/2019 SBI2 High Content 2019 36
Assay Controls for HCS (5)

- Reproducibility of potency values in HCS screen of cell viability.
Dynamic Range

- **Theoretical Dynamic Range**: The range of values that could be measured or calculated for an assay endpoint.

<table>
<thead>
<tr>
<th>Concept</th>
<th>Endpoint</th>
<th>Theoretical dynamic range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>% Responder</td>
<td>0 to 100 %</td>
</tr>
<tr>
<td>Nuclear Receptor</td>
<td>Intensity in Nucleus</td>
<td>Background to Upper Limit of Camera [16-bit: 0 to 65,536]</td>
</tr>
<tr>
<td>Activation</td>
<td>Intensity Ratio</td>
<td>(ROI_{A,bkgd} / ROI_{B,max.intensity}) &lt; 1 &lt; (ROI_{A,Max Intensity} / ROI_{B,bkgd})</td>
</tr>
<tr>
<td>Steatosis</td>
<td>Lipid Spots / Cell</td>
<td>0 to ????</td>
</tr>
<tr>
<td>Neurite Outgrowth</td>
<td>Neurite Length</td>
<td>1 uM to ????</td>
</tr>
</tbody>
</table>

- **Empirical Dynamic Range**: The difference in values between control conditions and the most efficacious positive control condition.

- Characterizing the empirical dynamic range is an important step in evaluating the performance of an HCS assay.
**HCS Assay Performance**

Estimated Z-factor = $1 - \frac{3(\hat{\sigma}_p + \hat{\sigma}_n)}{|\hat{\mu}_p - \hat{\mu}_n|}$

<table>
<thead>
<tr>
<th>Z-factor</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>Ideal. Z-factors can never exceed 1.</td>
</tr>
<tr>
<td>between 0.5 and 1.0</td>
<td>An excellent assay.</td>
</tr>
<tr>
<td>between 0 and 0.5</td>
<td>A marginal assay.</td>
</tr>
<tr>
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<td>There is too much overlap between the positive and negative controls for the assay to be useful.</td>
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Plates → [Z' = 0.61](https://en.wikipedia.org/wiki/Z-factor)
High Throughput Profiling (HTP)

• In contrast to HCS, HTP assays measure hundreds to thousand of phenotypic features and the endpoints reported are not always intuitive.

• The highly-multiplexed nature of HTP assays requires a modified approach for evaluating assay performance.
  • Reference chemicals
  • Profile concordance

~ 1300 endpoints
Phenotypic Reference Chemicals (1)

- A set of chemicals that elicit reproducible but distinct profiles of phenotypic effects.

- The profile may be specific for a particular channel / organelle or affect many components of the cell.

Nyffeler et al. (submitted)
Phenotypic Reference Chemicals (2)

- Individual features are measured, normalized to vehicle control and scaled to facilitate comparisons across features.
## HTP, Example Plate Design

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= Vehicle Control
= Cytotoxicity Reference Chemical
= Phenotypic Reference Chemical
= Test Chemicals
Evaluating HTP Assay Performance

Nyffeler et al. (submitted)
Summary

• HCS assays are powerful tools for interrogating biology.

• HCS assays can evaluate many aspects of cellular biology that are not amenable to evaluation non-imaging based methods.

• HCS assay concepts are customizable to a biology of interest and provide intuitive outputs.

• There are many interconnected steps in development of a successful HCS assay.

• Many resources and examples that can help you along the way!
Acknowledgements

• EPA NCCT (CCTE)
  • William Mundy (ret.)
  • Johanna Nyffeler
  • Clinton Willis
  • Daniel Hallinger
  • Brian Chorley
  • Gail Nelson
  • Nyssa Tucker