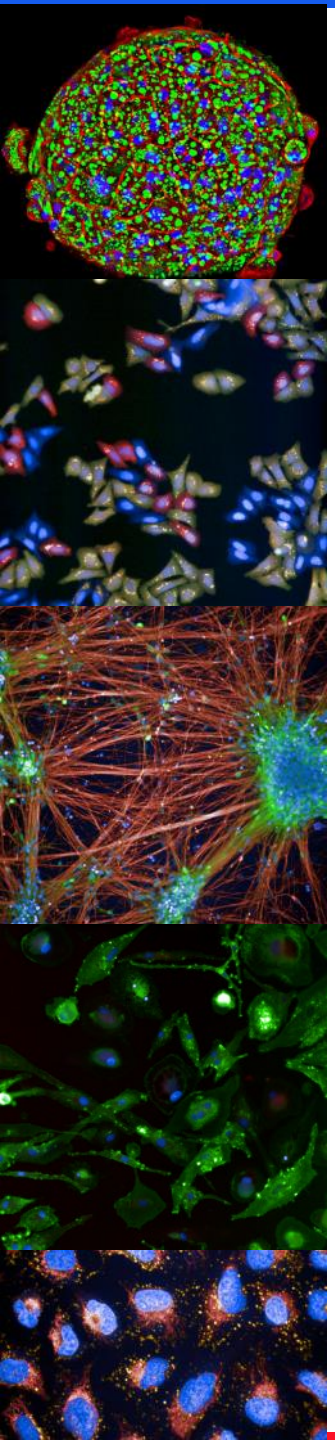

High Content 2019
6th Annual Conference
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Joseph B. Martin Conference Center

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

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Disclaimer

- *The views expressed in this presentation are those of the author(s) and do not necessarily represent the views of the U.S. Environmental Protection Agency, nor does mention of trade names or product represent endorsement for use.*

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

- **Buchser et al. (2012).** Assay Development Guidelines for Image-Based High Content Screening, High Content Analysis and High Content Imaging. In G.S. Sittampalam, A. Grossman & K. Brimacombe (Eds.), *Assay Guidance Manual*. Bethesda, MD: Eli Lilly & Company and the National Center for Advancing Translational Sciences.
- **Bray et al. (2017).** Advanced Assay Development Guidelines for Image-Based High Content Screening and Analysis. In G.S. Sittampalam, A. Grossman & K. Brimacombe (Eds.), *Assay Guidance Manual*. Bethesda, MD: Eli Lilly & Company and the National Center for Advancing Translational Sciences.
- **Taylor, D.L., Haskins, J.R., Giuliano, K.A. (Eds.)** *High Content Screening, A Powerful Approach to Systems Cell Biology and Phenotypic Drug Discover, 1st Edition*. New York, NY. Humana Press.
- **Johnston, P.A. & Trask, O.J., (Eds.) (2018)**. *High Content Screening, A Powerful Approach to Systems Cell Biology and Phenotypic Drug Discovery, 2nd Edition*. New York, NY. Humana Press.

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.

HCS vs. HTP

Applications in image-based profiling of perturbations

Juan C Caicedo[1,2], Shantanu Singh[1] and Anne E Carpenter[1]

Current Opinion in Biotechnology

Text directly quoted from Caicedo et al (2016). Curr Op Biotech 39:134-142

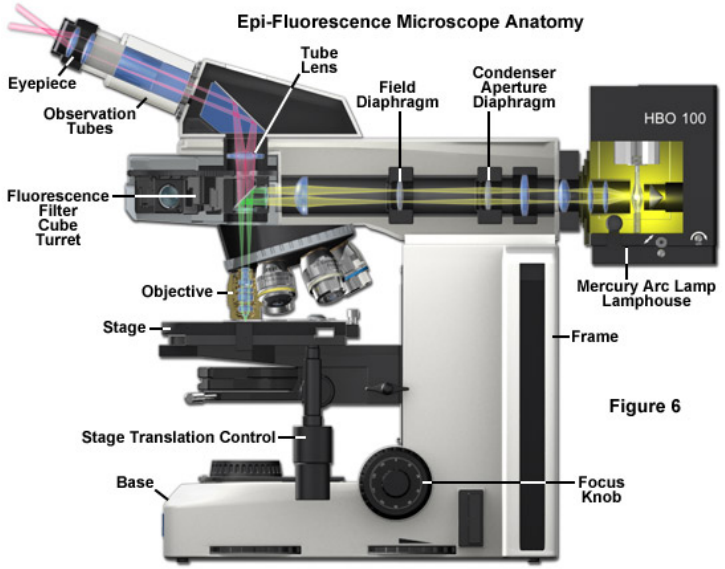
- Screening is a distinct strategy from profiling.
- Although both involve large-scale (high-throughput) imaging experiments, the goals differ:
    - **Screening:** The researcher aims to measure one or more phenotypes that are visually discernable, and choose a subset of hits for further investigation. 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?

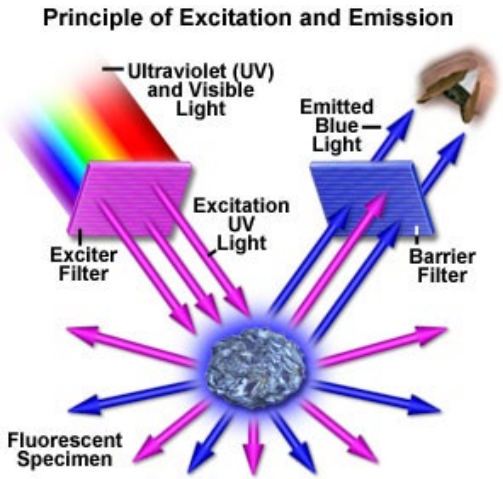
- HTS assay can provide information on the biological activity of test agents, however, they provide single (i.e. “low content”) readouts.
- HCS can be used evaluate many of the same biological processes as HTS assays, but provide more detailed information at the level of the individual cell or organism.
- 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**.

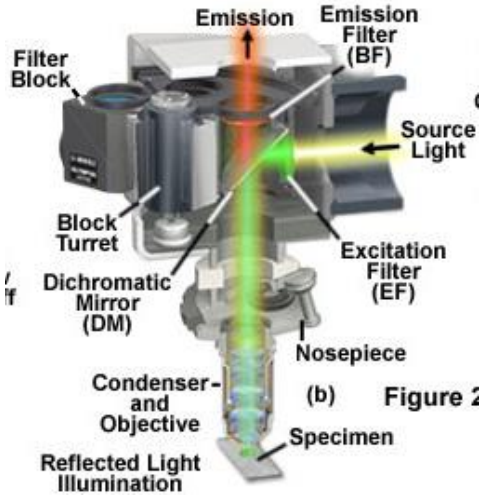
- Protein Expression
- Protein Translocation
- Protein Modifications
- Enzyme Activation
- Cell Surface Receptor Activation
- Molecular Uptake
- Cell Proliferation
- Cell Cycle Regulation
- Cell Viability / Apoptosis
- Cell Migration
- Cell-Cell Interactions
- Differentiation
- Cell Morphology
- Organelle 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!





https://www.olympus-lifescience.com/ http://zeiss-campus.magnet.fsu.edu/

Components of an HCS System (1)

Component	Type	Notes
Excitation Source	Lamps	• 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
Filter Sets	Excitation	• 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 fluoroprobe and optimized to minimize crosstalk between different probes when multiplexing.

Adapted from NIH Assay Guidance Manual

Components of an HCS System (2)

Component	Type	Notes
Objectives	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)

Component	Type	Notes
High Precision Automated Stage	--	• 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 Mechanism	Image-based	• 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.
	Laser-based	• Detect the bottom of the plate. • Comparatively faster than image-based methods. • Performs best with extremely flat imaging plates and this specimens.
Environmental Chamber	--	• Controls temperature, humidity and CO_2 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

- **Trask O.J. (2018)** *Guidelines for Microplate Selection in High Content Imaging.* In: Johnston P., Trask O. (eds) High Content Screening. Methods in Molecular Biology, vol 1683. Humana Press, New York, NY

> *"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."*

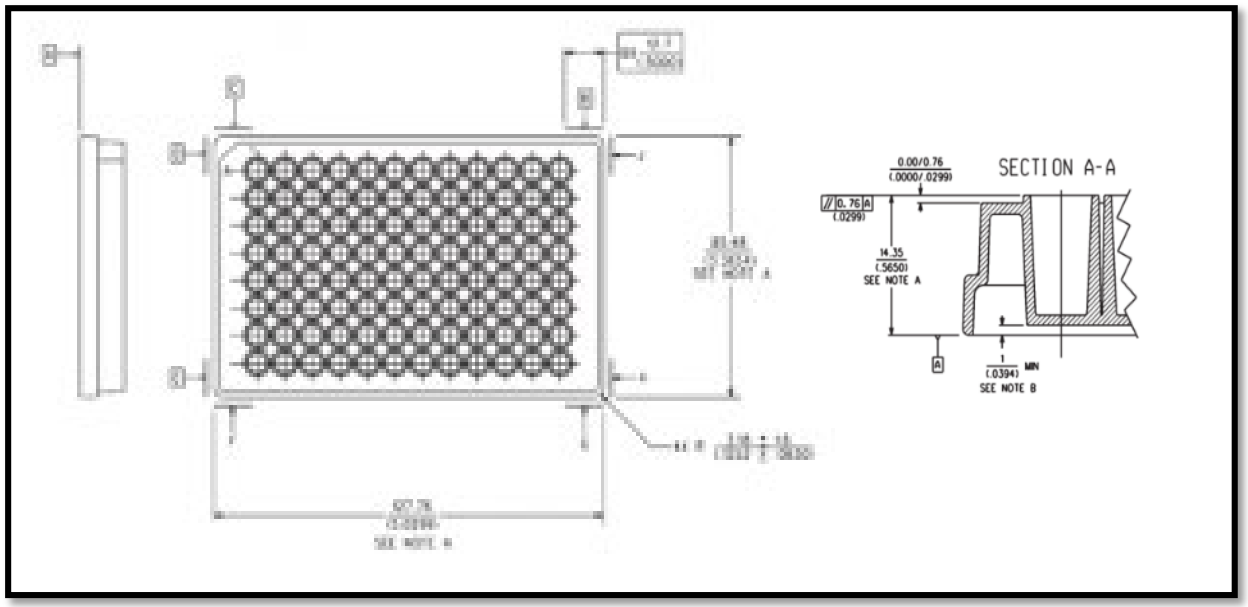
Variety:

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

Uniformity:

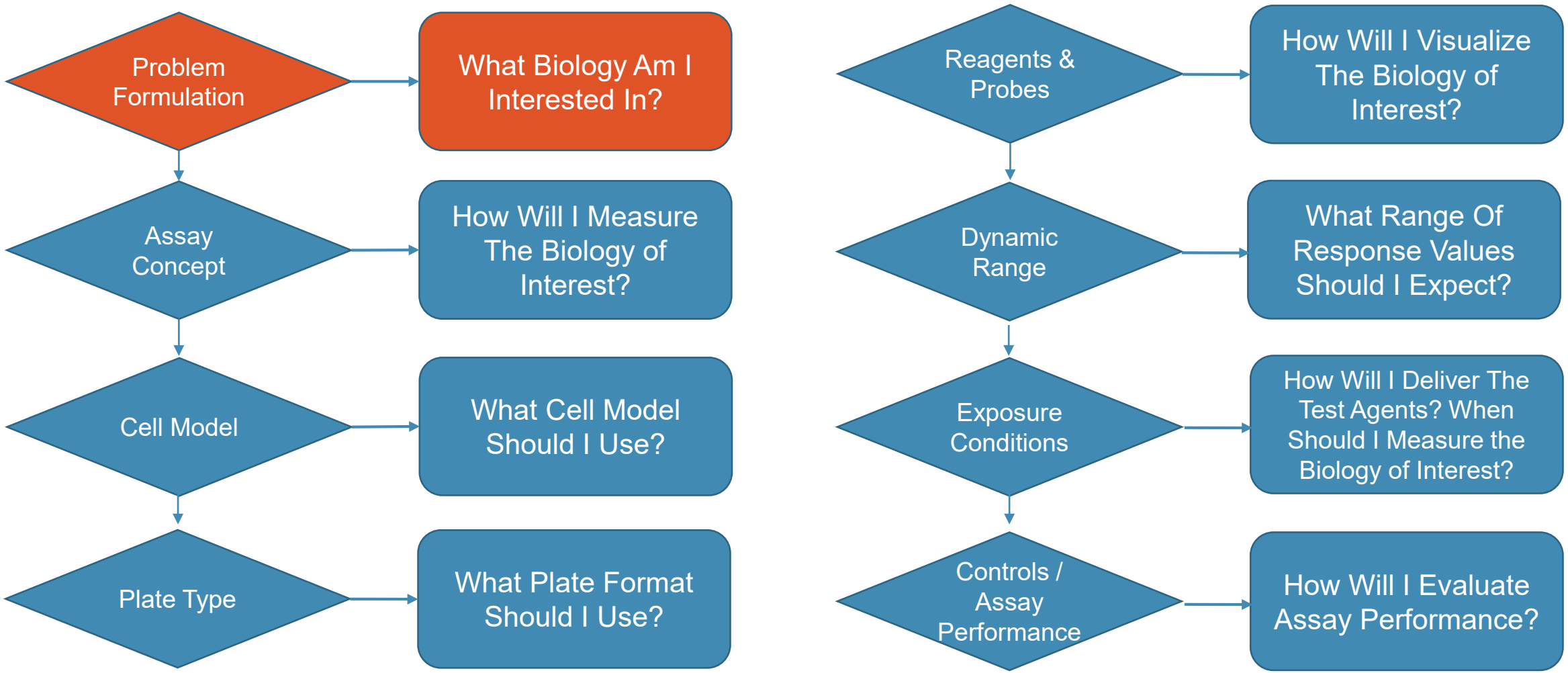
- SLAS / ANSI standards

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



https://www.wellplate.com/the-origin-of-microplate-standards/

Steps in HCS Assay Development



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

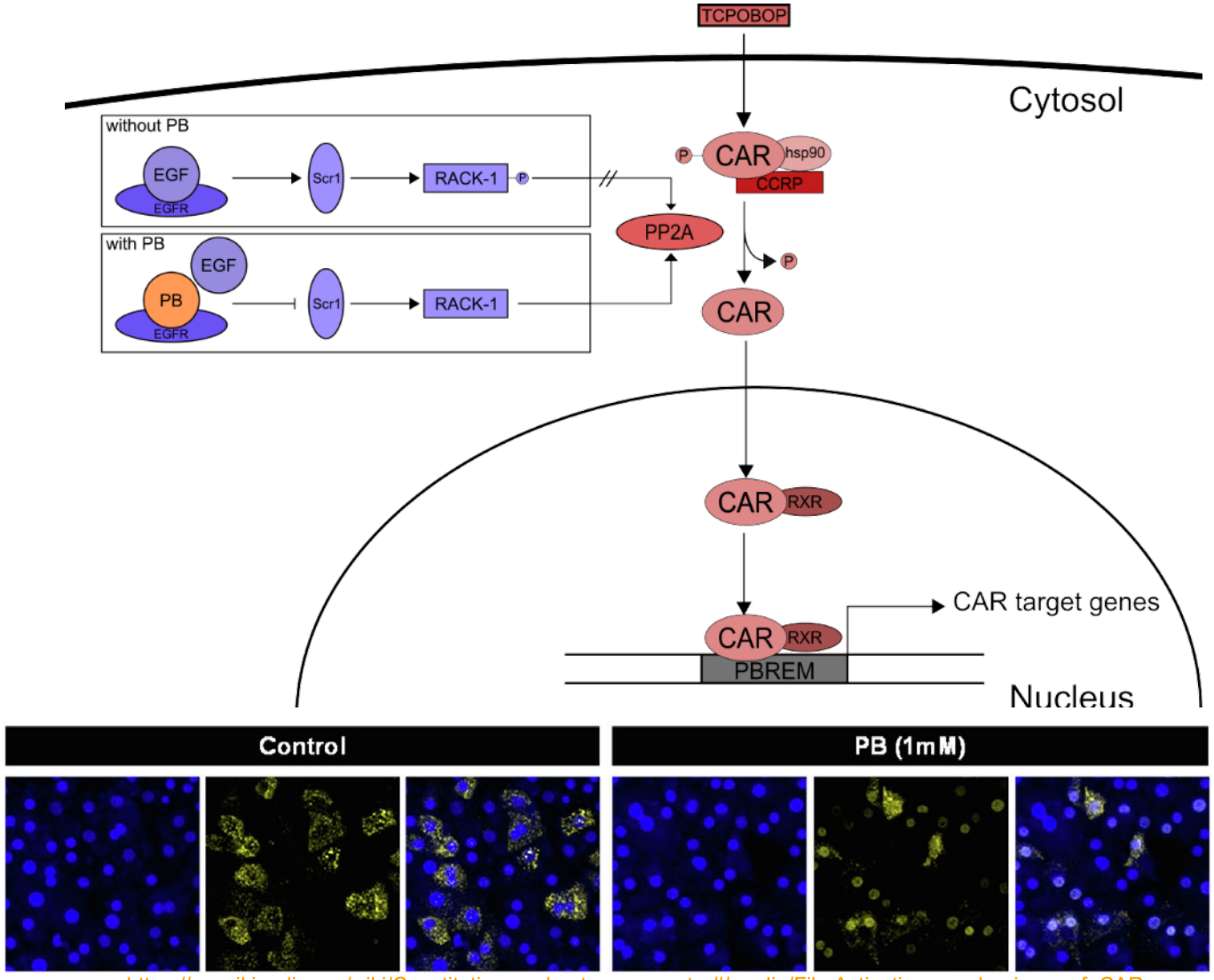
Goal(s): Identify nuclear receptor **activators**
Identify agents that **inhibit** nuclear receptor activation

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



https://en.wikipedia.org/wiki/Constitutive_androstane_receptor#/media/File:Activation_mechanisms_of_CAR.png

Mackowiak et al. (2019) PMID: 31306645

Example 2, Oxidative Stress & Apoptosis

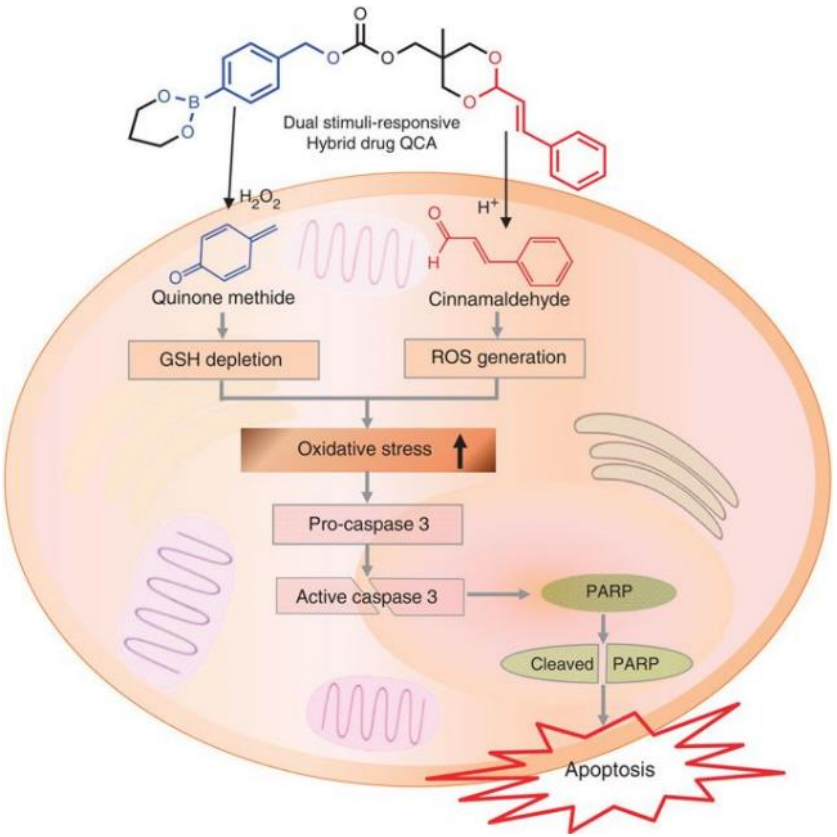
Goal(s): Identify chemicals that **produce** oxidative stress & **cause** apoptosis
Identify chemicals that **reduce** oxidative stress & **prevent** apoptosis

Characteristics:

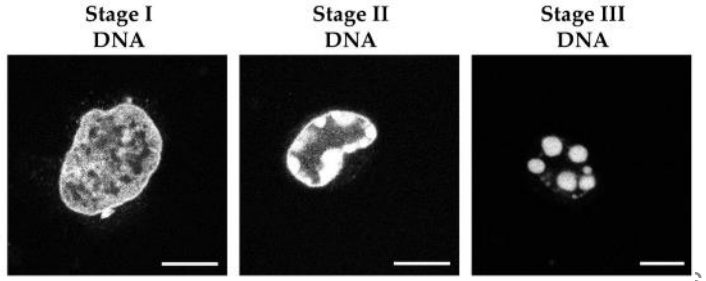
Oxidative stress can result from:
- Increased intracellular production of reactive oxygen species (ROS)
- Decreased levels of endogenous antioxidant molecules

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.

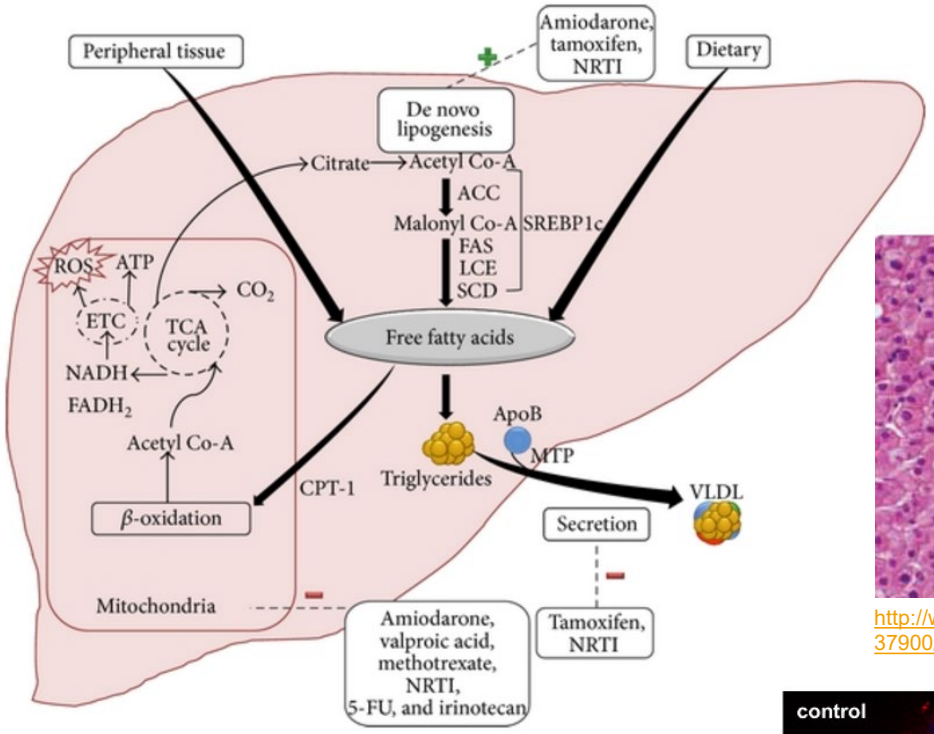
Characteristics:

Abnormal retention of lipids within a cell or organ.

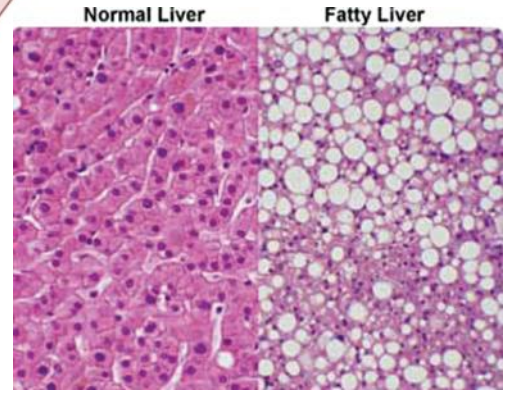
Occurs in the liver in response to:
- Dietary factors
- Chemical exposures
- Signals from peripheral tissues.

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

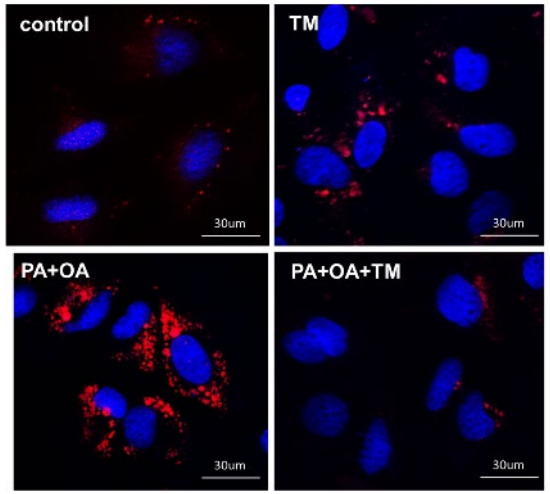
Hallmarks of steatosis includes:
- Accumulation of lipid droplets
- Production of ROS



https://www.researchgate.net/figure/Mechanism-of-drug-induced-hepatic-steatosis-steatohepatitis-ATP-citrate-lyase-ACL_fig1_281056564



http://www.theindependentbd.com/arcprint/details/137900/2018-02-19



https://journals.plos.org/plosone/article/figure?id=10.1371/journal.pone.0170591.g002

10.1016/j.taap.2011.02.013

Example 4, Neurite Outgrowth

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

Characteristics:

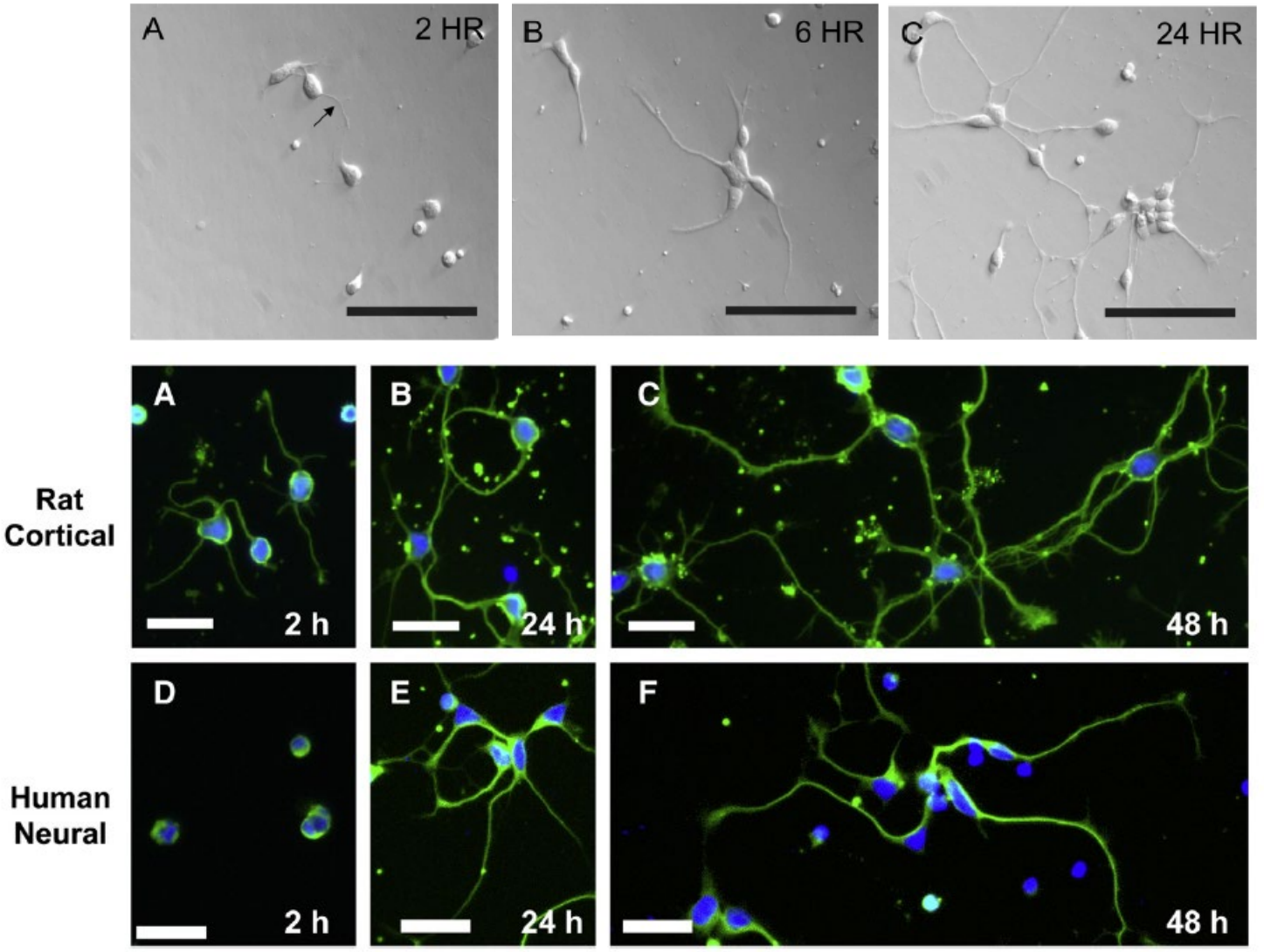
Neurite outgrowth involves extension of long, thin processes from the cell body of neurons.

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:

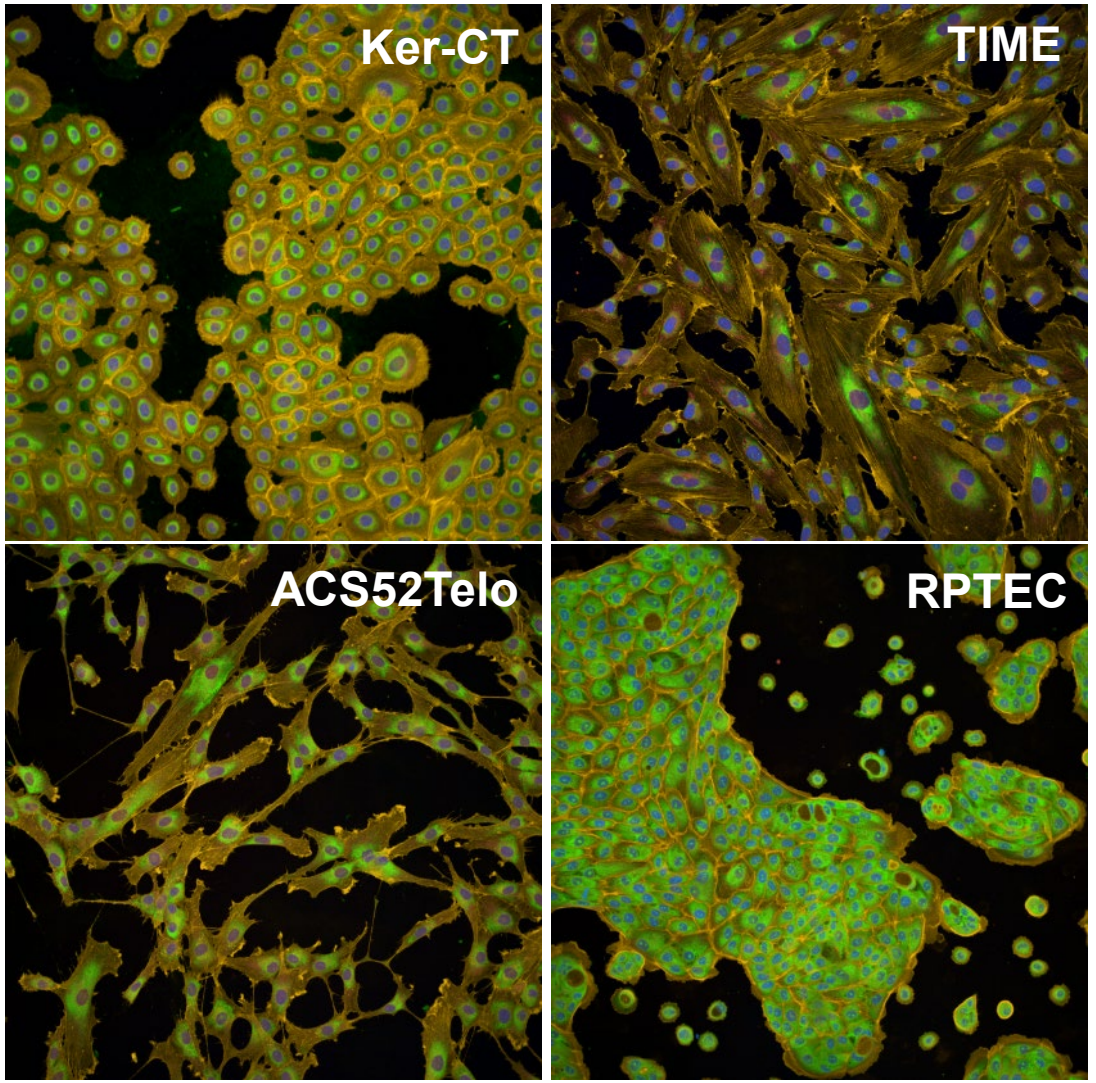
- Growth substrates
- Soluble growth factors
- Support from feeder cells



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



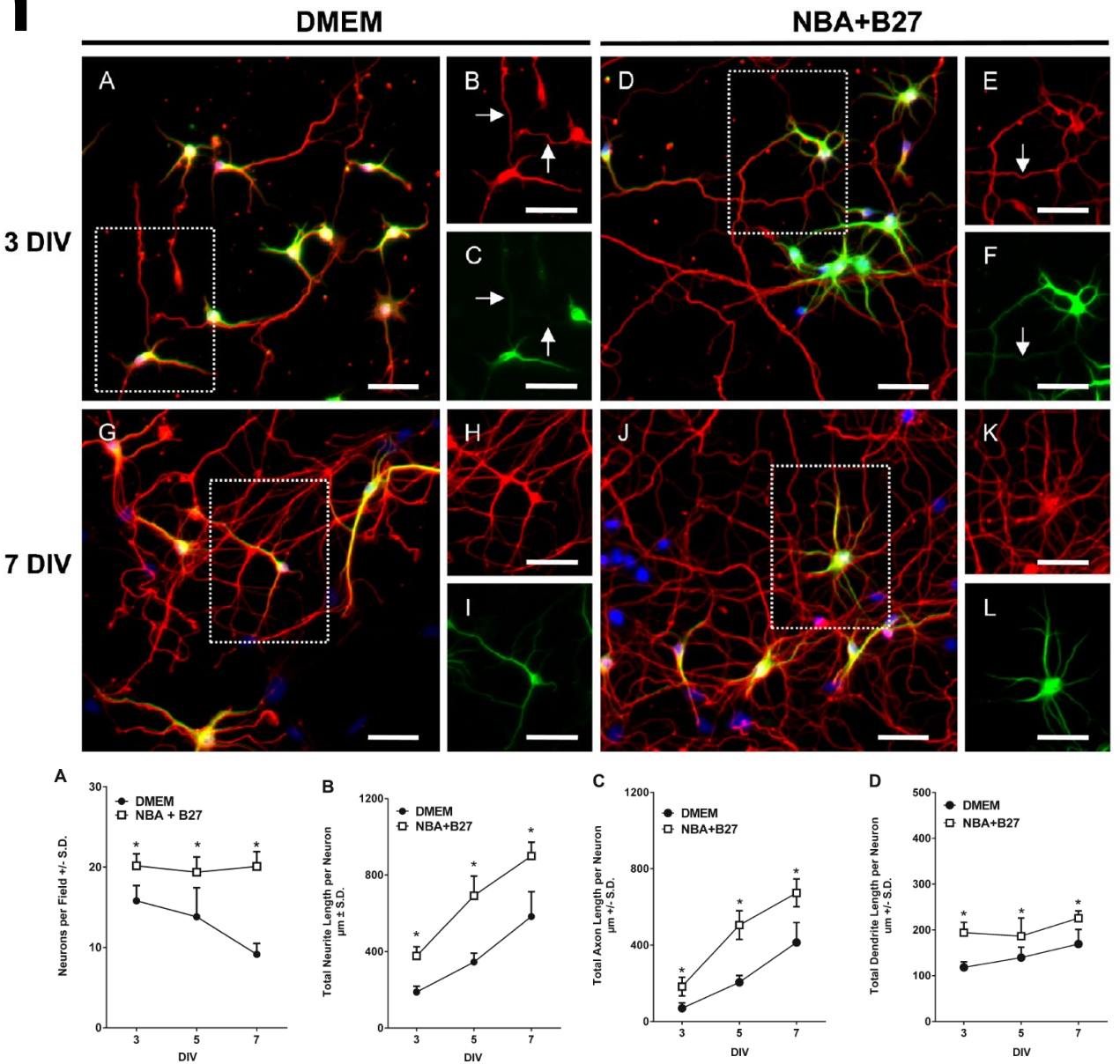
Willis, Nyffeler & Harrill. Unpublished Results

10.1007/s11626-015-9873-3

Cell Model Optimization

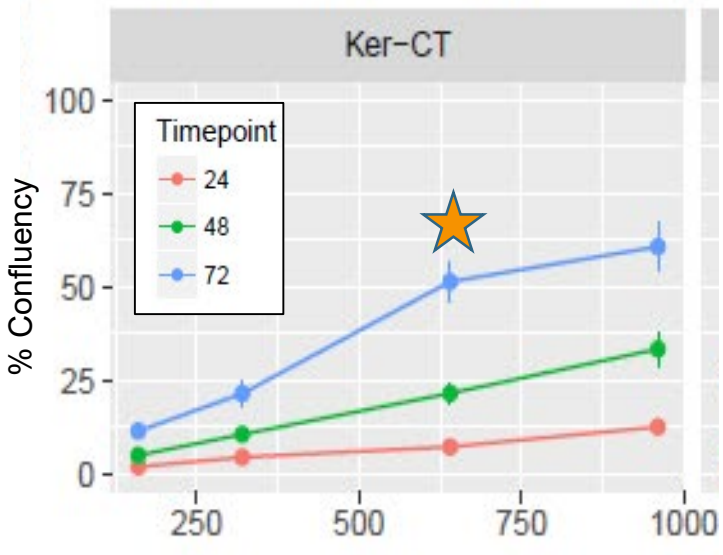
- **Cell models can require optimization of many parameters:**
  - Media formulation
  - Growth atmosphere (i.e. CO_2 / O_2)
  - Plate coating / growth substrate
  - Seeding density
  - Passaging
  - Maintenance in culture
  - Stimuli / Stressors
  - Labeling strategy

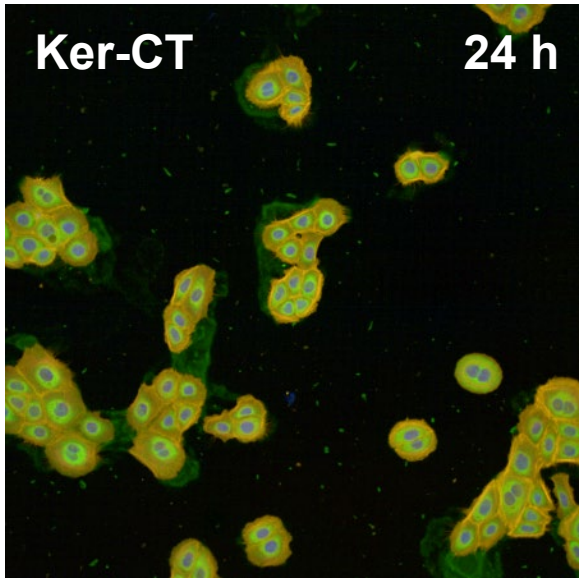
- Variability, sensitivity and dynamic range of HCS measurements can vary for the same cell type under different growth conditions.
- A key to reproducible HCS results is optimization and consistent preparation of cell cultures.

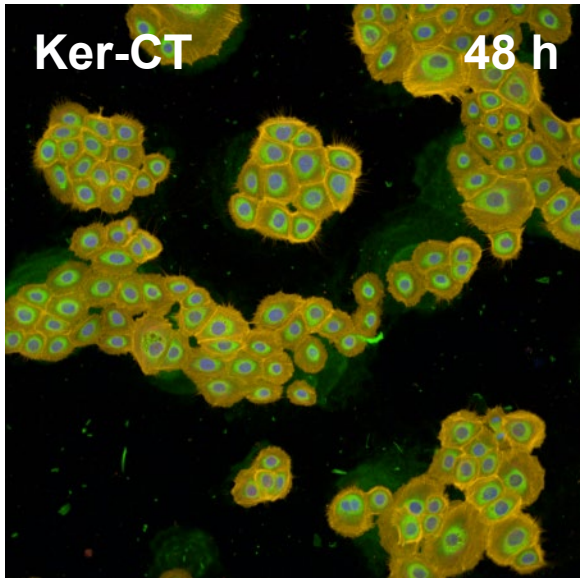


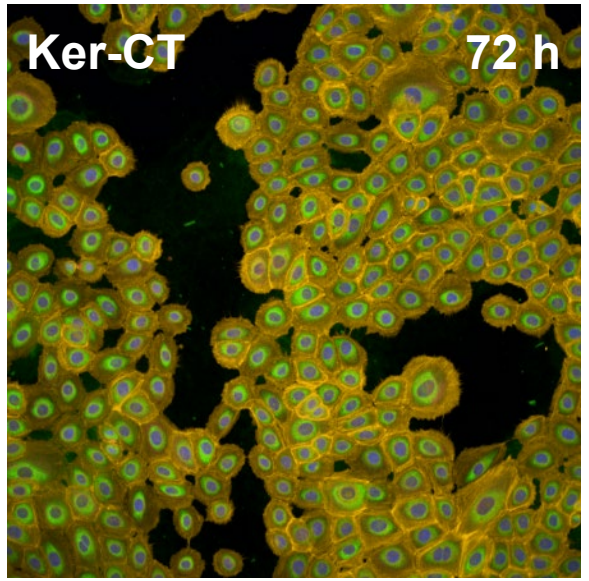
Harrill et al. (2015). PMID: 23142577

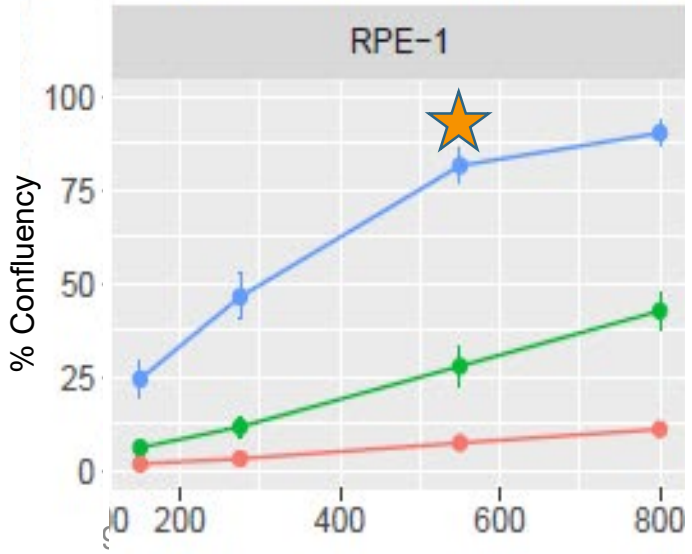
Cell Model Optimization (2)

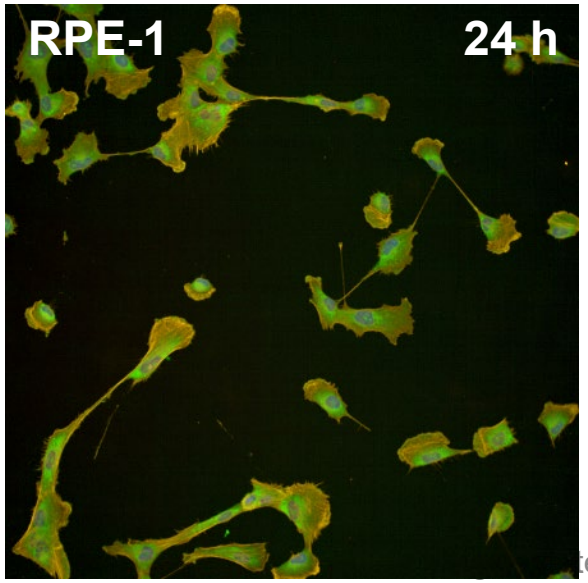


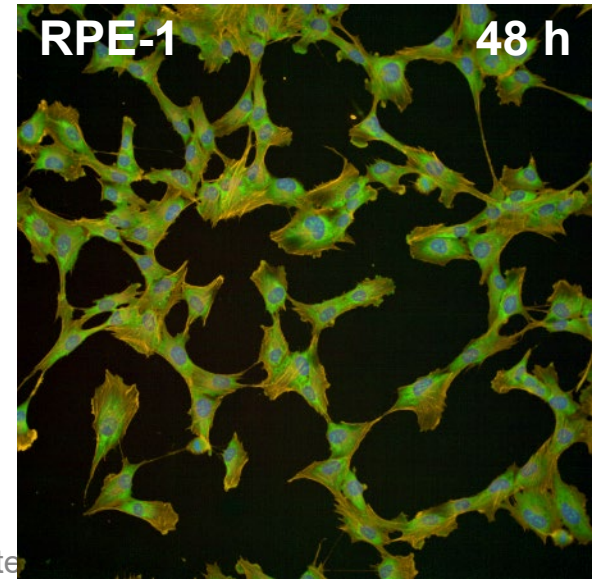


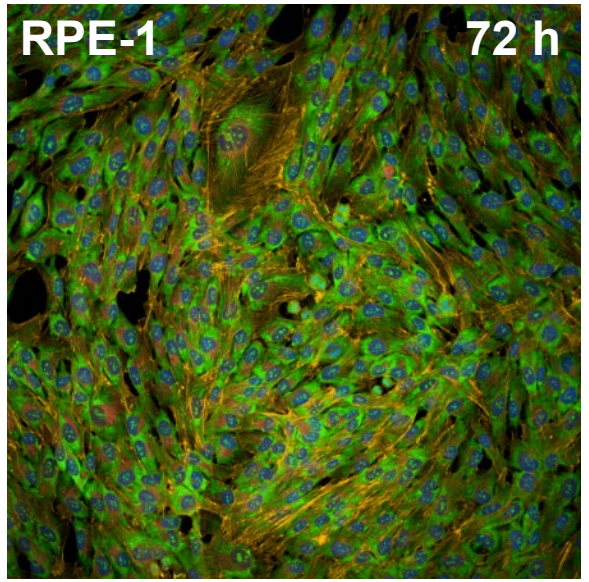












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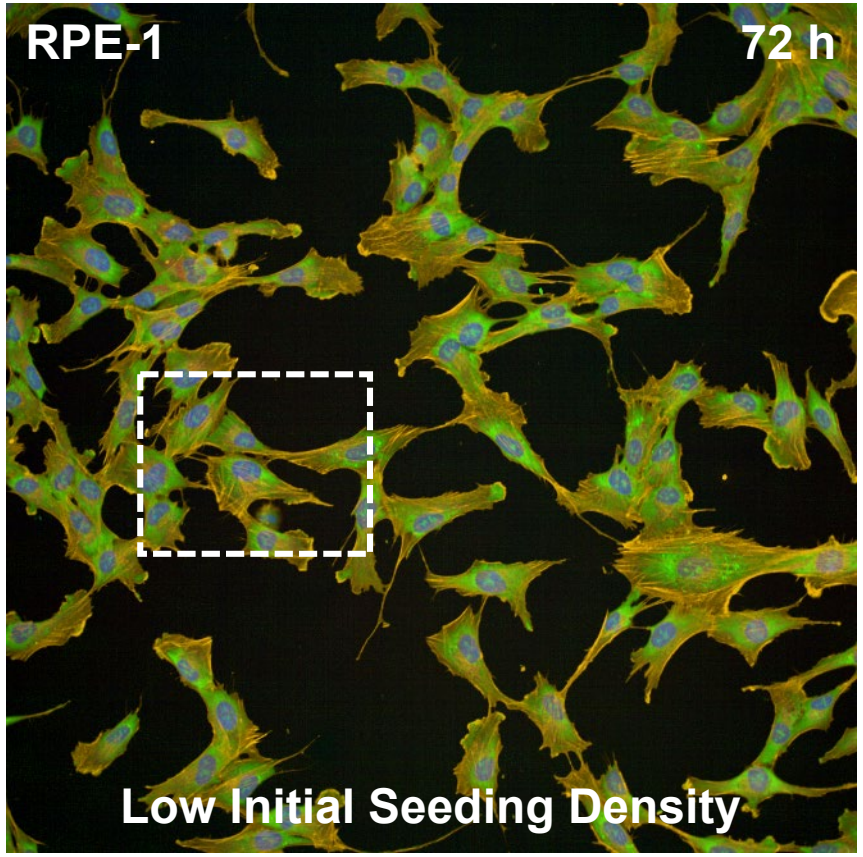
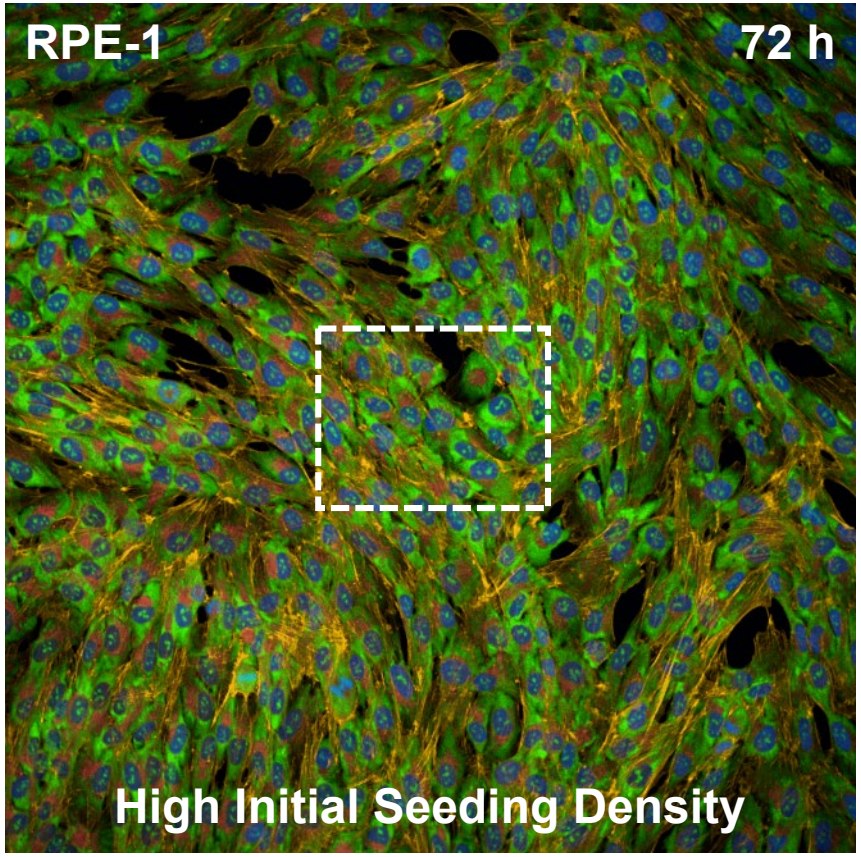
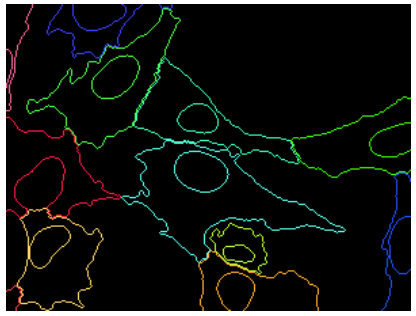
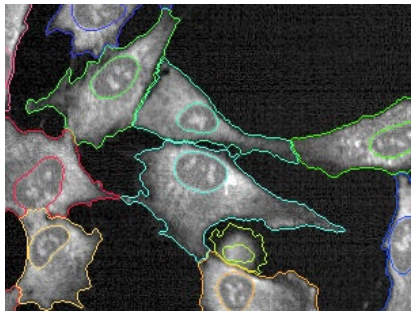
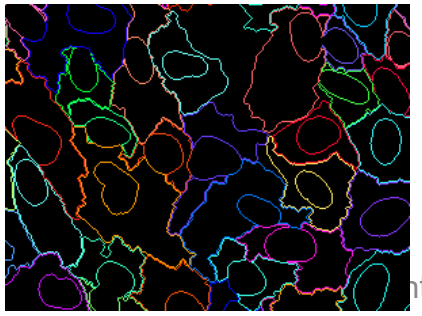
Cell Model Optimization (3)

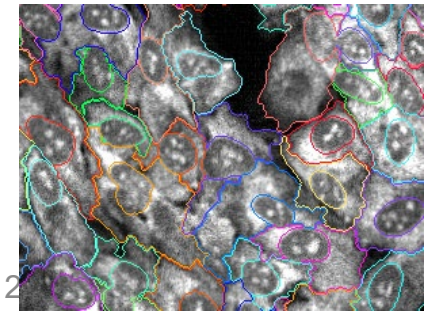












Overly confluent cultures:

- 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.

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

- 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

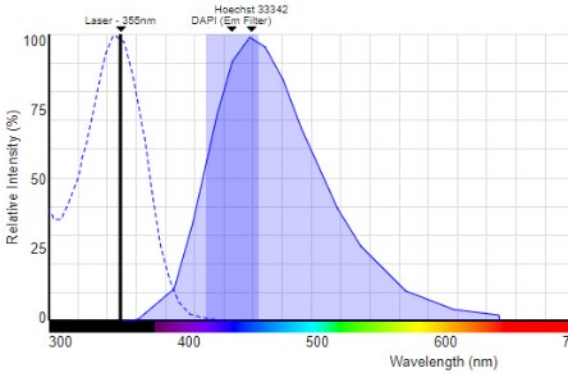
Heterologous protein expression constructs that may be incorporated into genomic DNA or encoded in extranuclear expression vectors.

- **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.

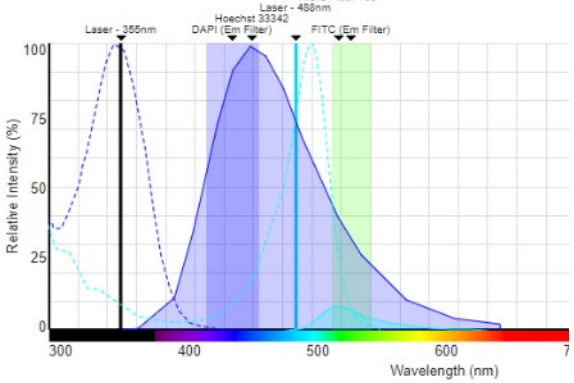
Multiplexing of Fluorescent Probes

- 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.

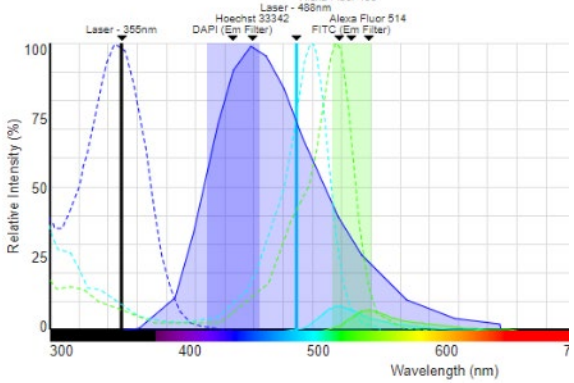
DAPI



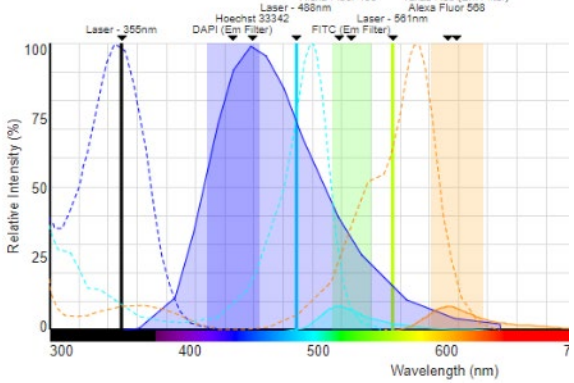
DAPI + A488



DAPI + A488 + A541



DAPI + A488 + A568



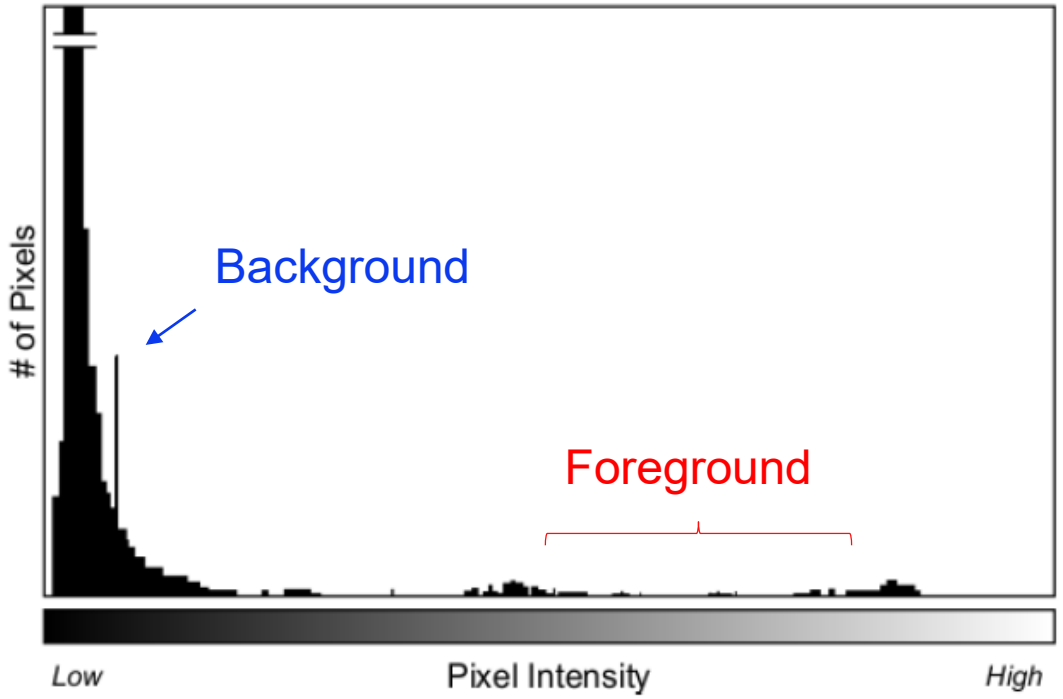
https://www.thermofisher.com/order/spectra-viewer

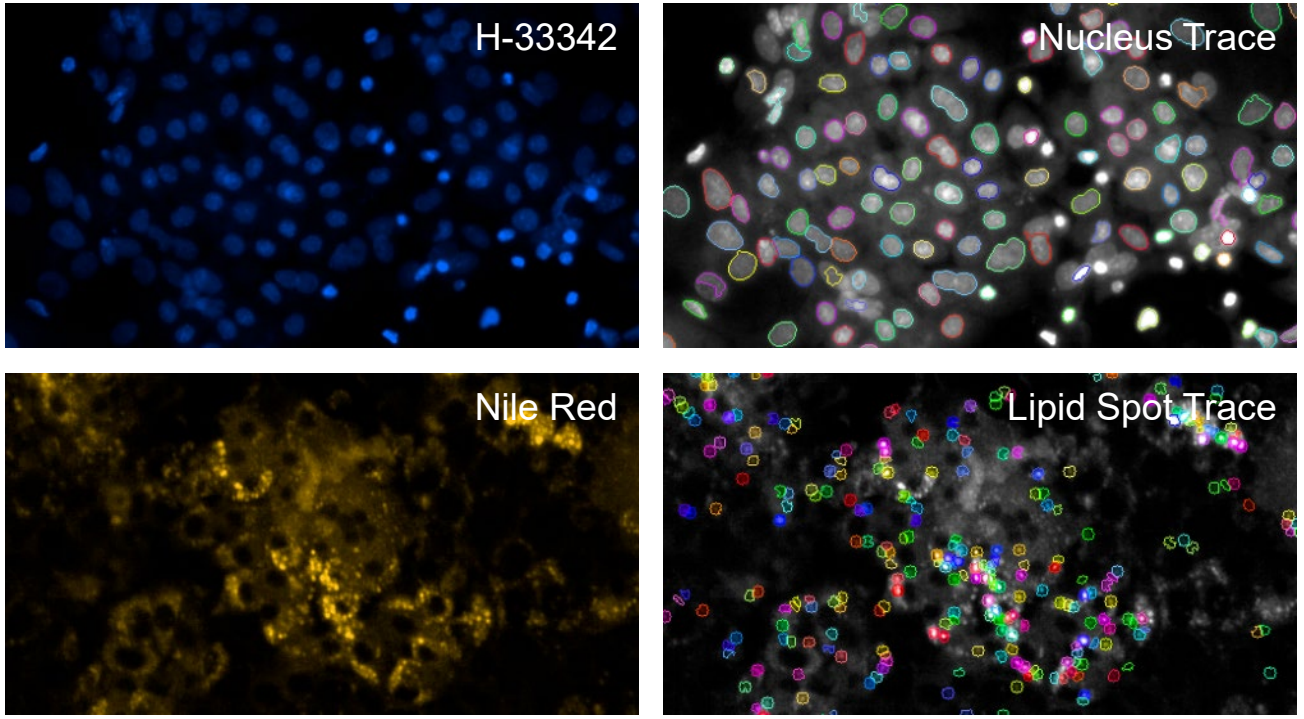
Rules-of-Thumb:

- 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

Identification of Labeled Objects

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





Tucker, Nelson, Harrill, Chorley: Unpublished Results

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

Assay Concepts – Apoptosis (1)

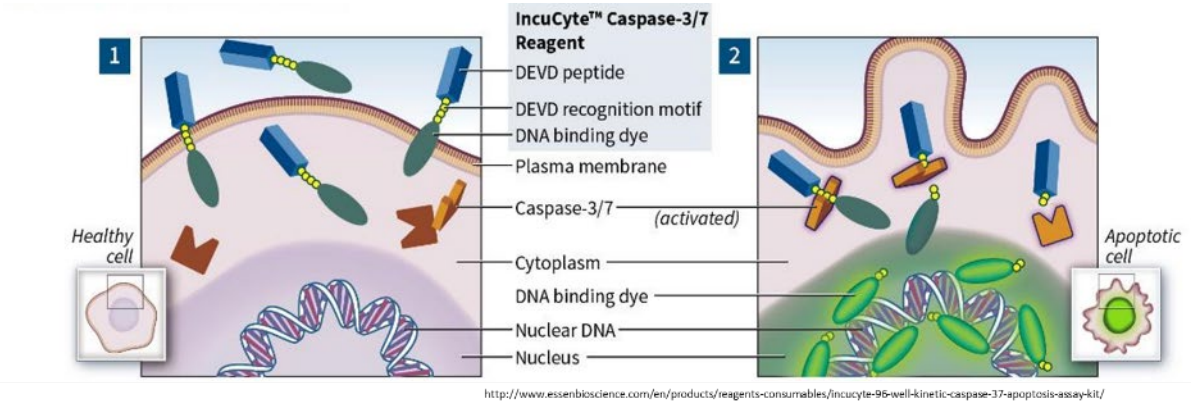
Cell Model:
- MCF-7 Adenocarcinoma cells

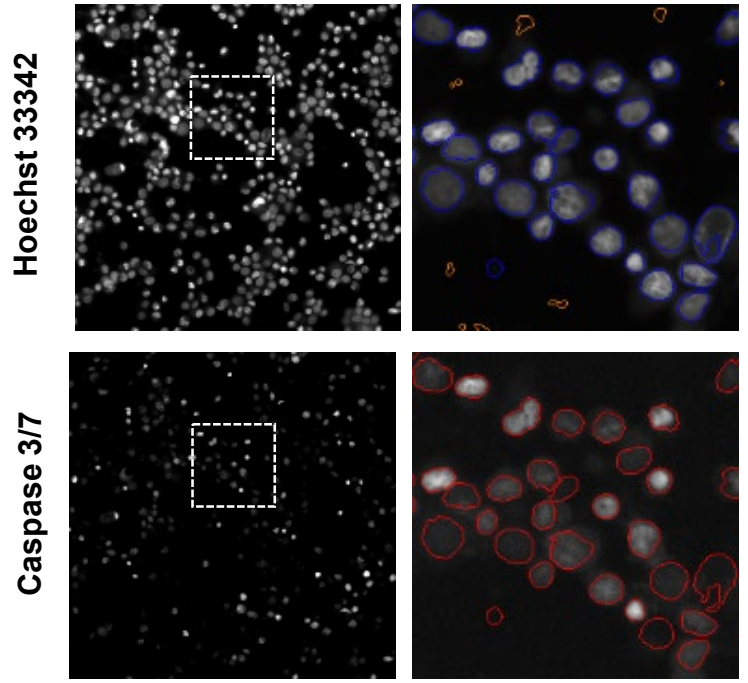
Biology of Interest:
- Apoptosis

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.

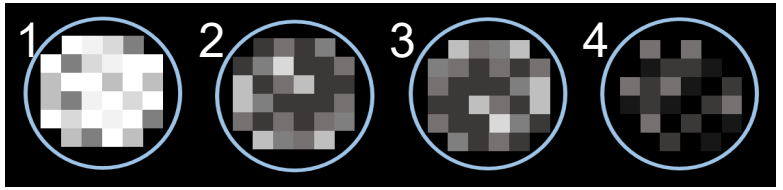


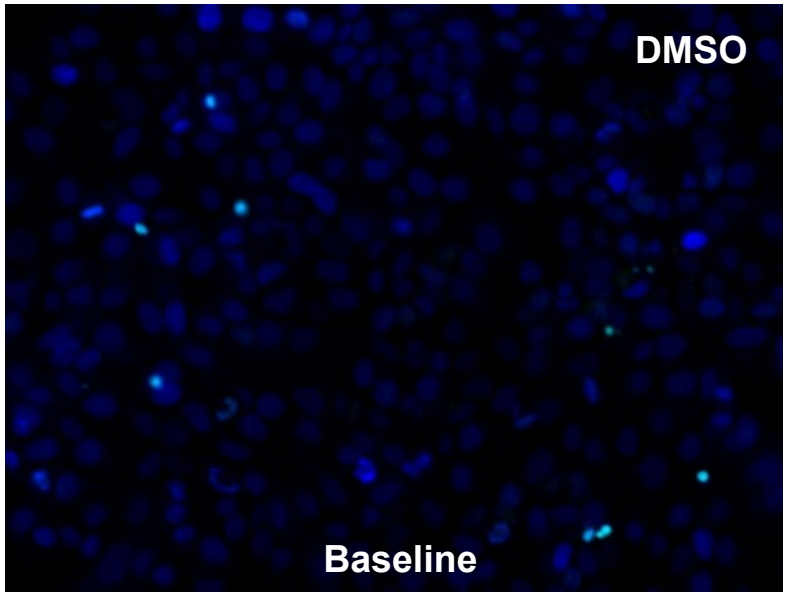


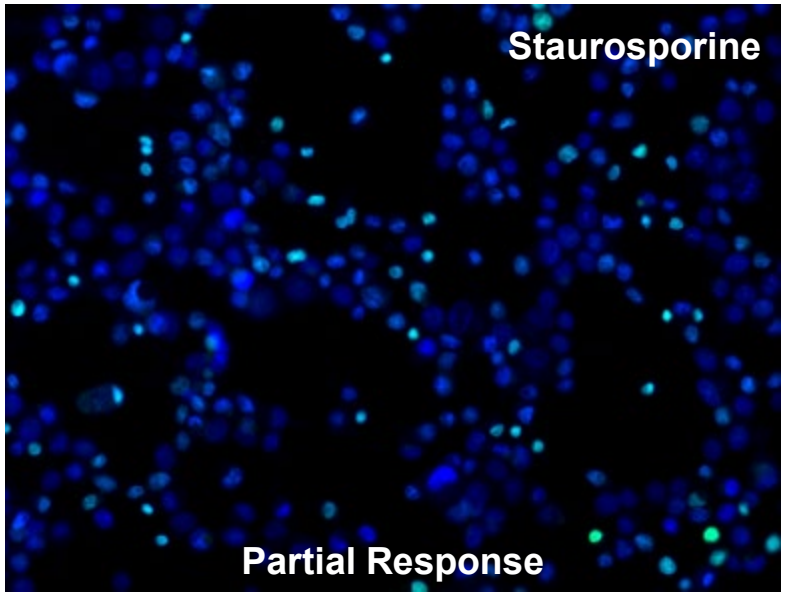
Willis, Nyffeler & Harrill, Unpublished Results

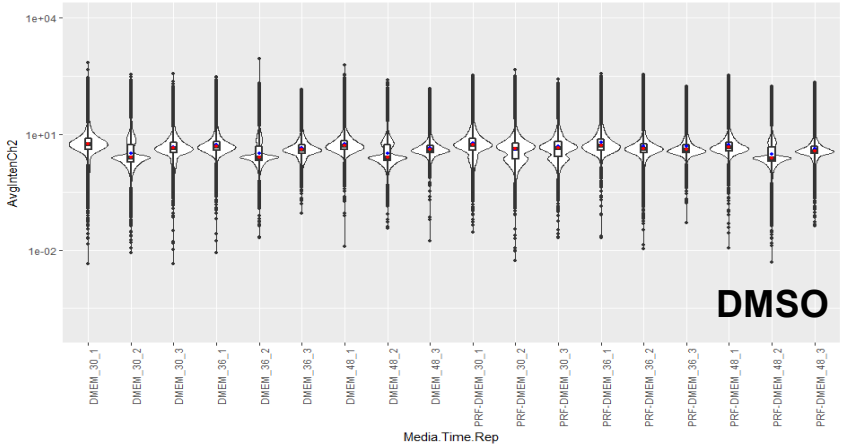
Assay Concepts – Apoptosis (2)

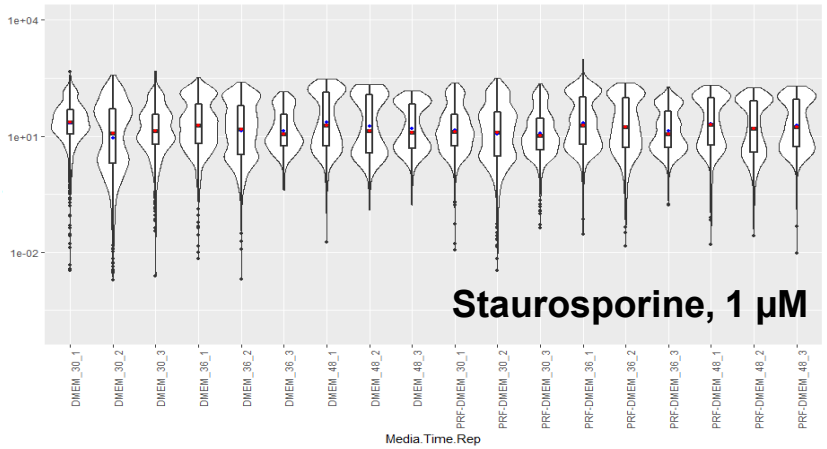
- Average fluorescent pixel intensity within each selected object
  - 1 > 2 ≈ 3 > 4

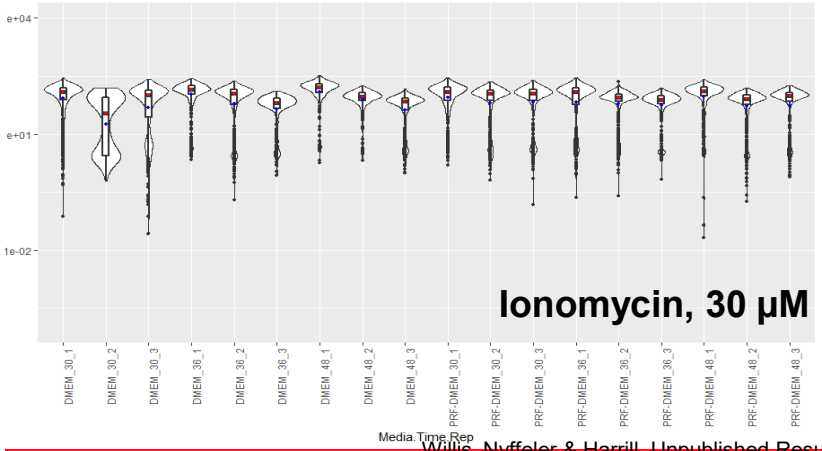












Willis, Nyffeler & Harrill, Unpublished Results

Assay Concepts – Apoptosis (3)

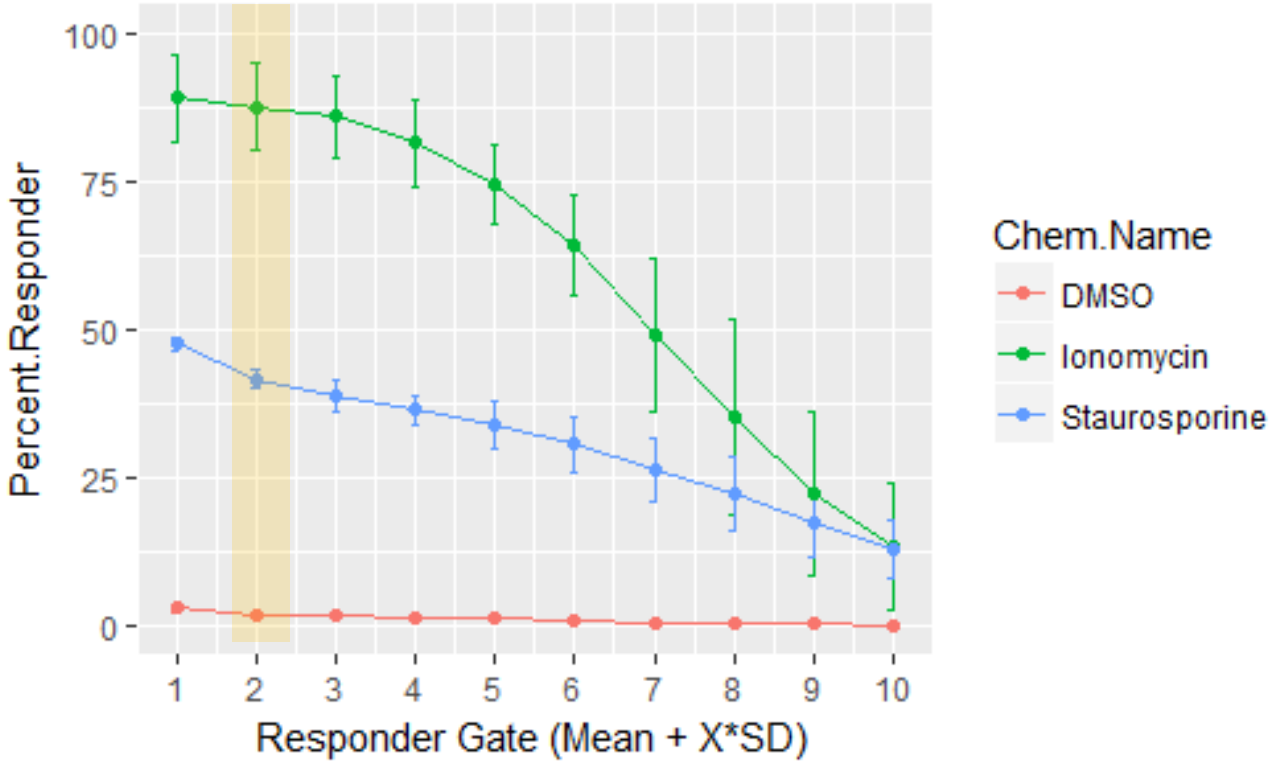
Two Approaches →	Mean Intensity	Percent Responder
Description	Well level mean of AvgIntenCh2 values.	Percent of cells with AvgIntenCh2 values above range of control.
Example	Breier et al. (2008), Culbreth et al. (2012)	Vogt et al. (2005), Foldes et al. (2011)
Detect Rare Events ?	NO	YES

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).



Assay Concepts – Steatosis

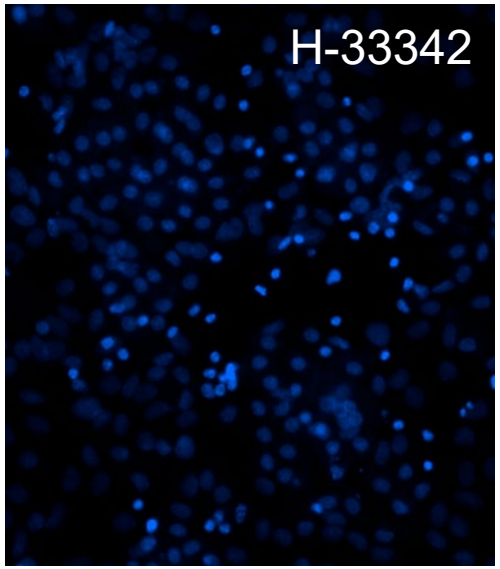
Cell Model:
- Differentiated (2-D) HepaRG.
- Mixed culture:
  - “Hepatocyte-like” cells
  - “Cholangiocyte-like” cells.

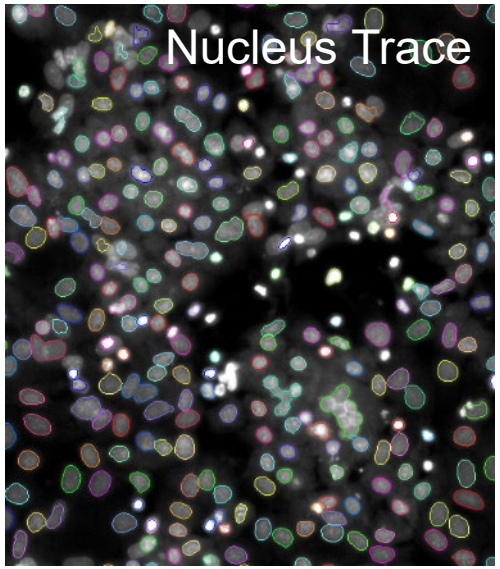
Biology of Interest:
- Lipid accumulation in hepatocyte-like cells is biology of interest.

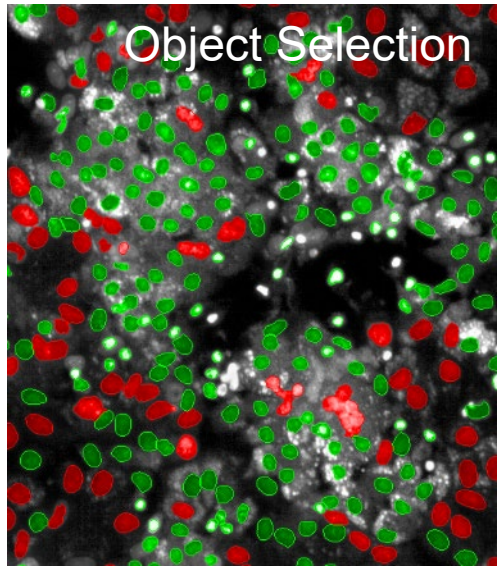
Visualization Approach:
- Nile Red labeling of lipids.
- Nucleus counterstain

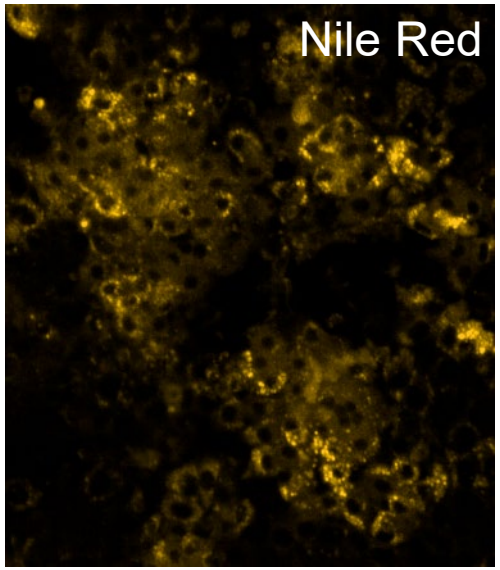
Image Analysis Approach:
- Identify labeled nuclei
- Select nuclei of interest based on morphology
- Define a Region-of-Interest (ROI) around each cell
- Identify lipid droplets within each ROI.

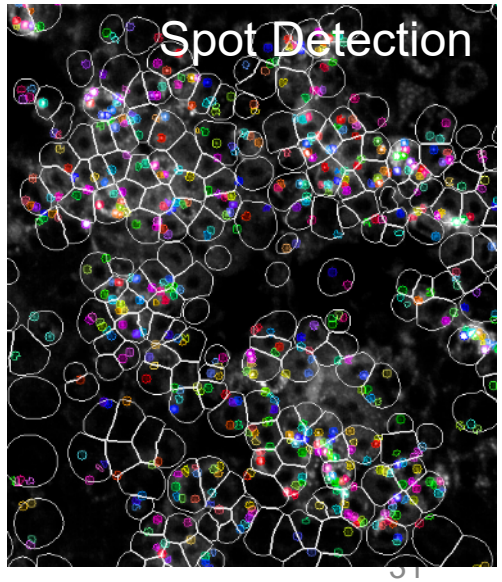
Intuitive Output:
- Average number of lipid droplets per hepatocyte-like cell











Tucker, Nelson, Harrill, Chorley: Unpublished Results

Assay Concepts – Neurite Outgrowth

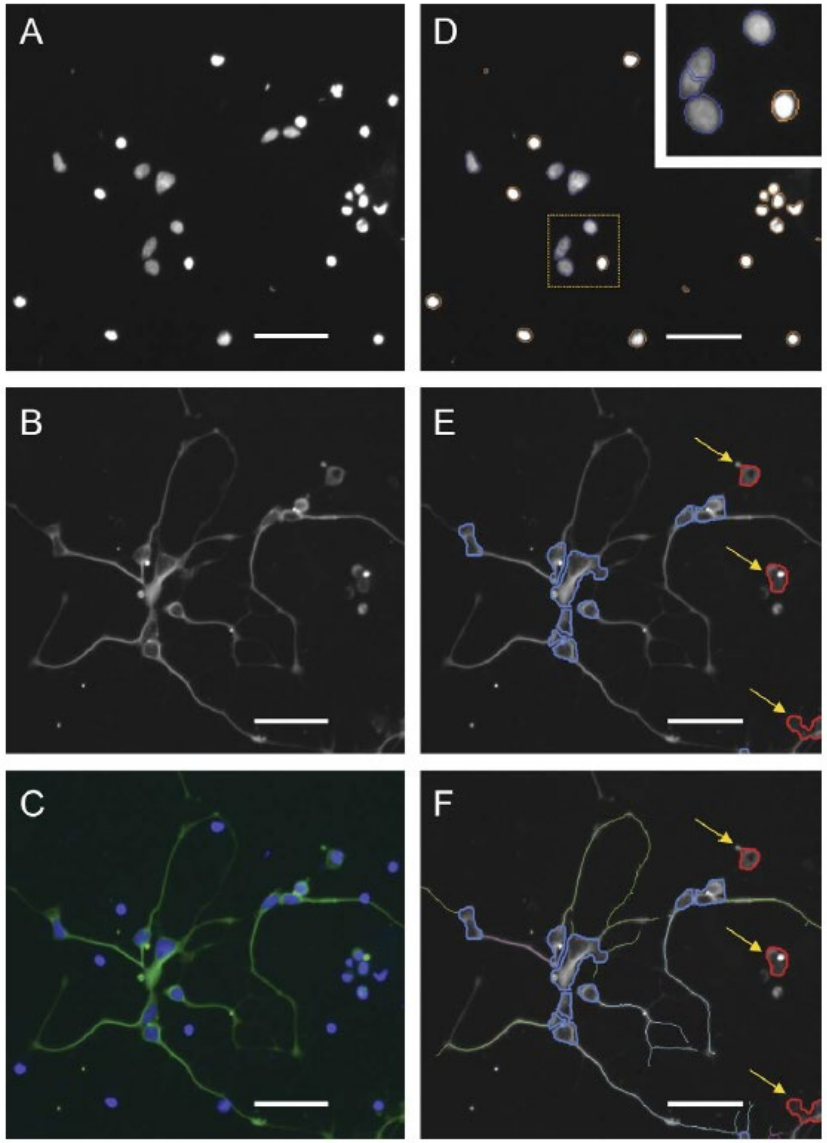
Cell Model:
- hESC-derived neural cultures

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



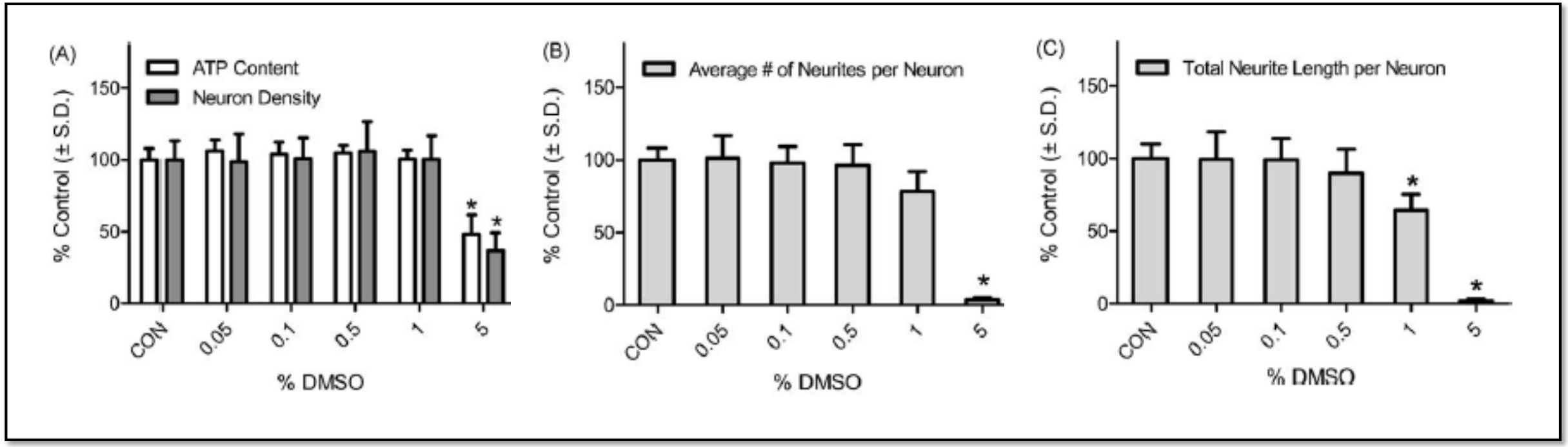
Harrill et al. (2010). PMID: 20188755

Assay Controls for HCS (1)

Control Type	Description
Untreated	An assay well that did not receive any test agent.
Vehicle	An assay well that received the test agent delivery vehicle (i.e. DMSO), but did not receive a test agent.
Positive	An assay well that received a test agent known to produce an expected effect in an assay.
Negative	An assay well that received a treatment that is not expected to have an effect in an assay.
No Label	An assay well containing cells (treated or untreated), but were not labeled with detection reagents.

Assay Controls for HCS (2)

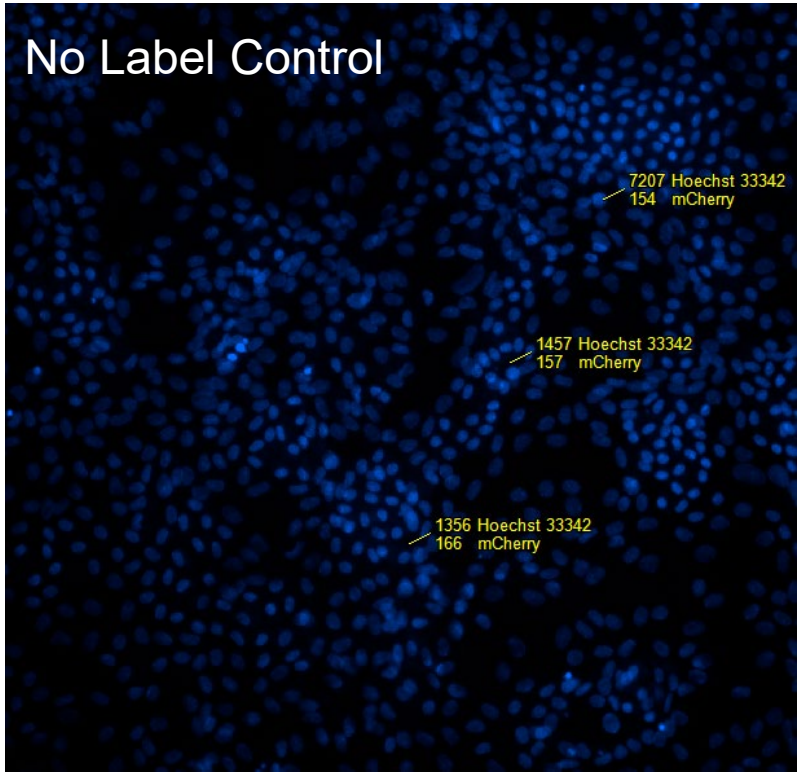
- **Untreated vs. Vehicle:** Determine “vehicle tolerance” → At what concentration does the vehicle affect the assay

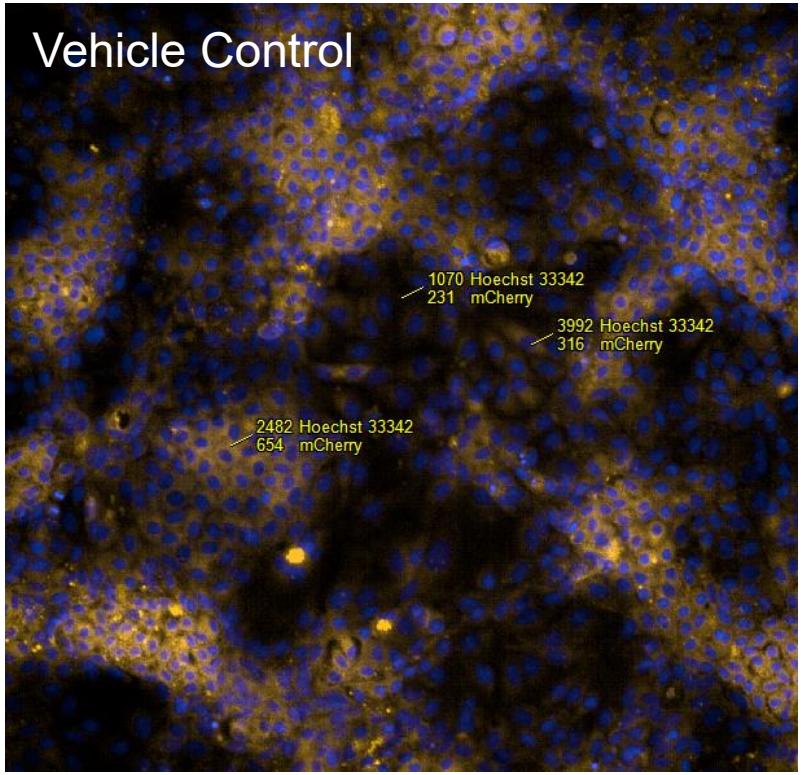


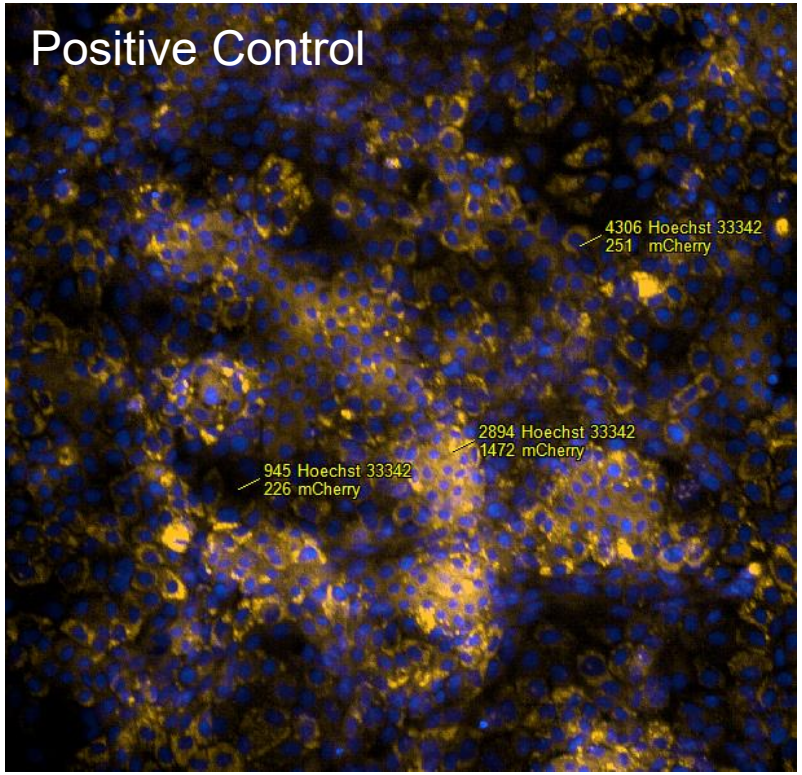
Harrill et al. (2010). PMID: 20188755

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	20	No L	20	20	20	20	20	20	20	20	20	20	20
B	6	6	6	6	6	6	6	6	6	6	6	6	No L	6	6	6	6	6	6	6	6	6	6	6
C	2	2	2	2	2	2	2	2	2	2	2	2	No L	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	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
E	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	U	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	0.06	U	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	0.02	U	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	0.006	U	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	20	DMSO	20	20	20	20	20	20	20	20	20	20	20
J	6	6	6	6	6	6	6	6	6	6	6	6	DMSO	6	6	6	6	6	6	6	6	6	6	6
K	2	2	2	2	2	2	2	2	2	2	2	2	DMSO	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	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
M	0.2	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
N	0.06	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
O	0.02	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
P	0.006	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

= No Label Control

= Untreated Control

= Vehicle Control

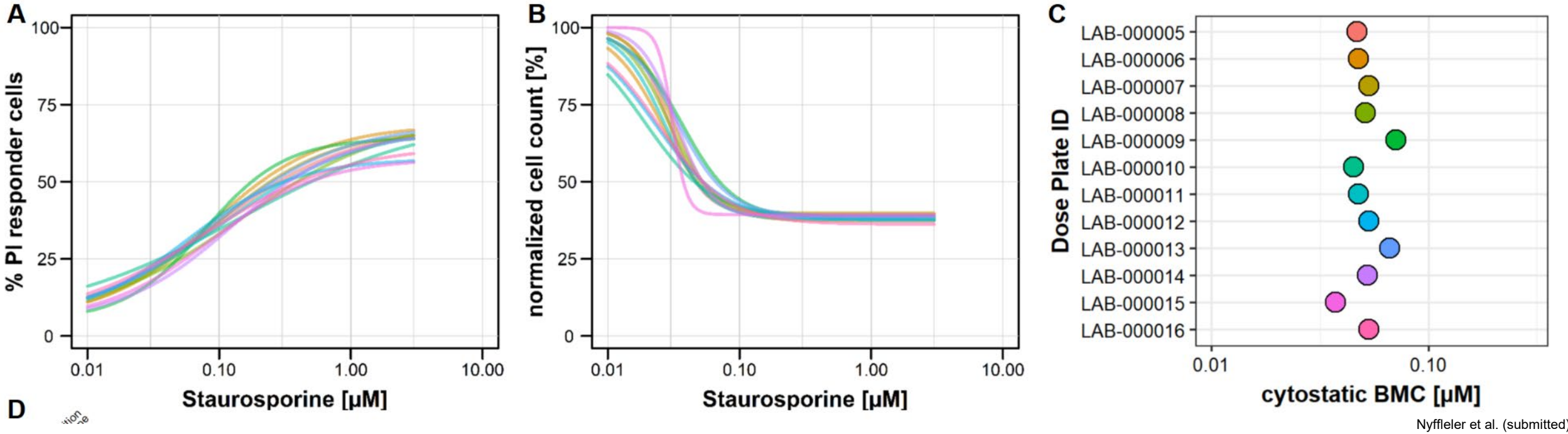
= Positive Control

= Negative Control

= Test Chemicals

Assay Controls for HCS (5)

- **Reproducibility of potency values in HCS screen of cell viability.**

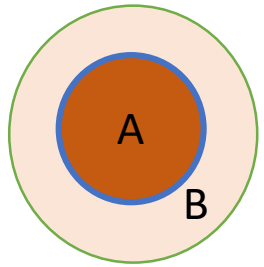


Nyffleler et al. (submitted)

Dynamic Range

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

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

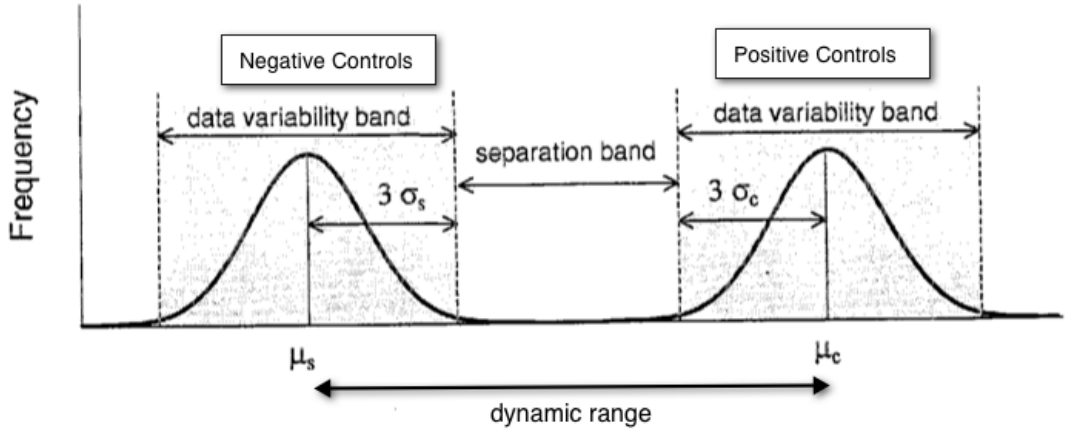


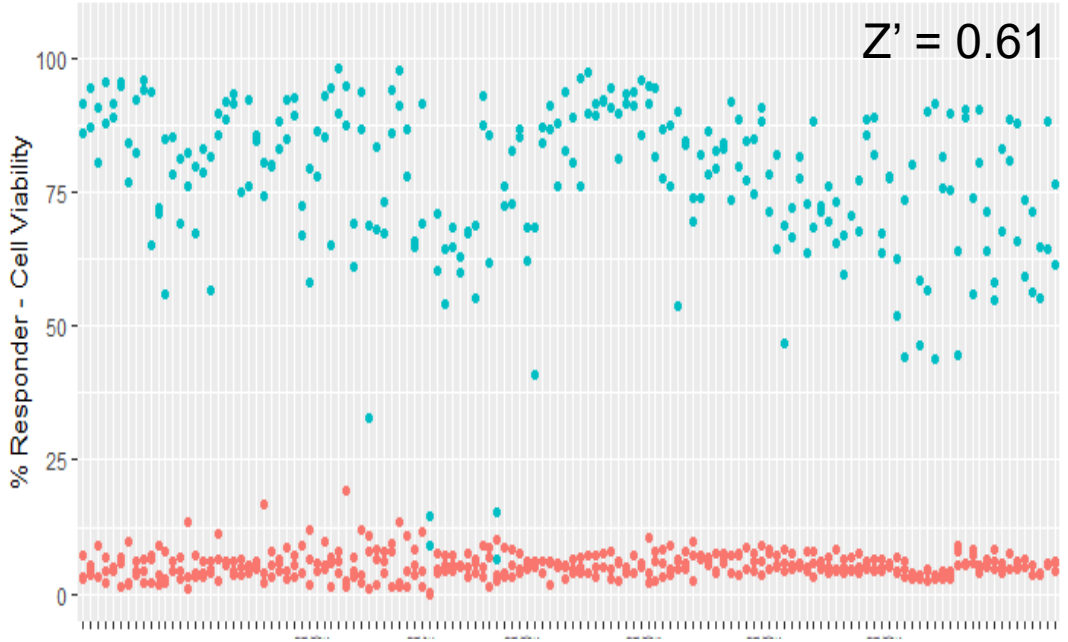
- **Empirical Dynamic Range:** The difference in values between control conditions and the most efficacious positive control condition.

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

HCS Assay Performance

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

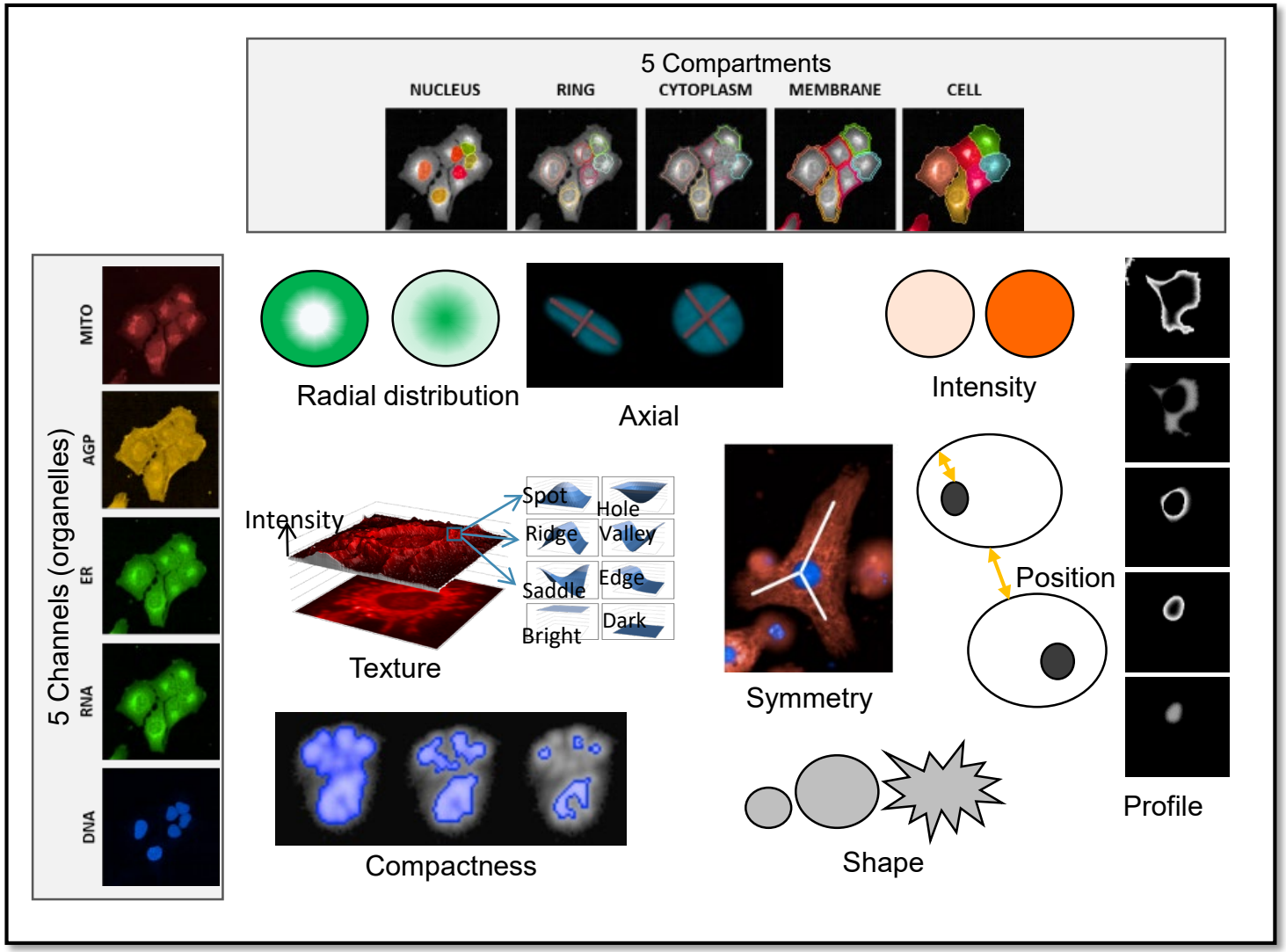




Z-factor	Interpretation
1.0	Ideal. Z-factors can never exceed 1.
between 0.5 and 1.0	An excellent assay.
between 0 and 0.5	A marginal assay.
less than 0	There is too much overlap between the positive and negative controls for the assay to be useful.

https://en.wikipedia.org/wiki/Z-factor

High Throughput Profiling (HTP)

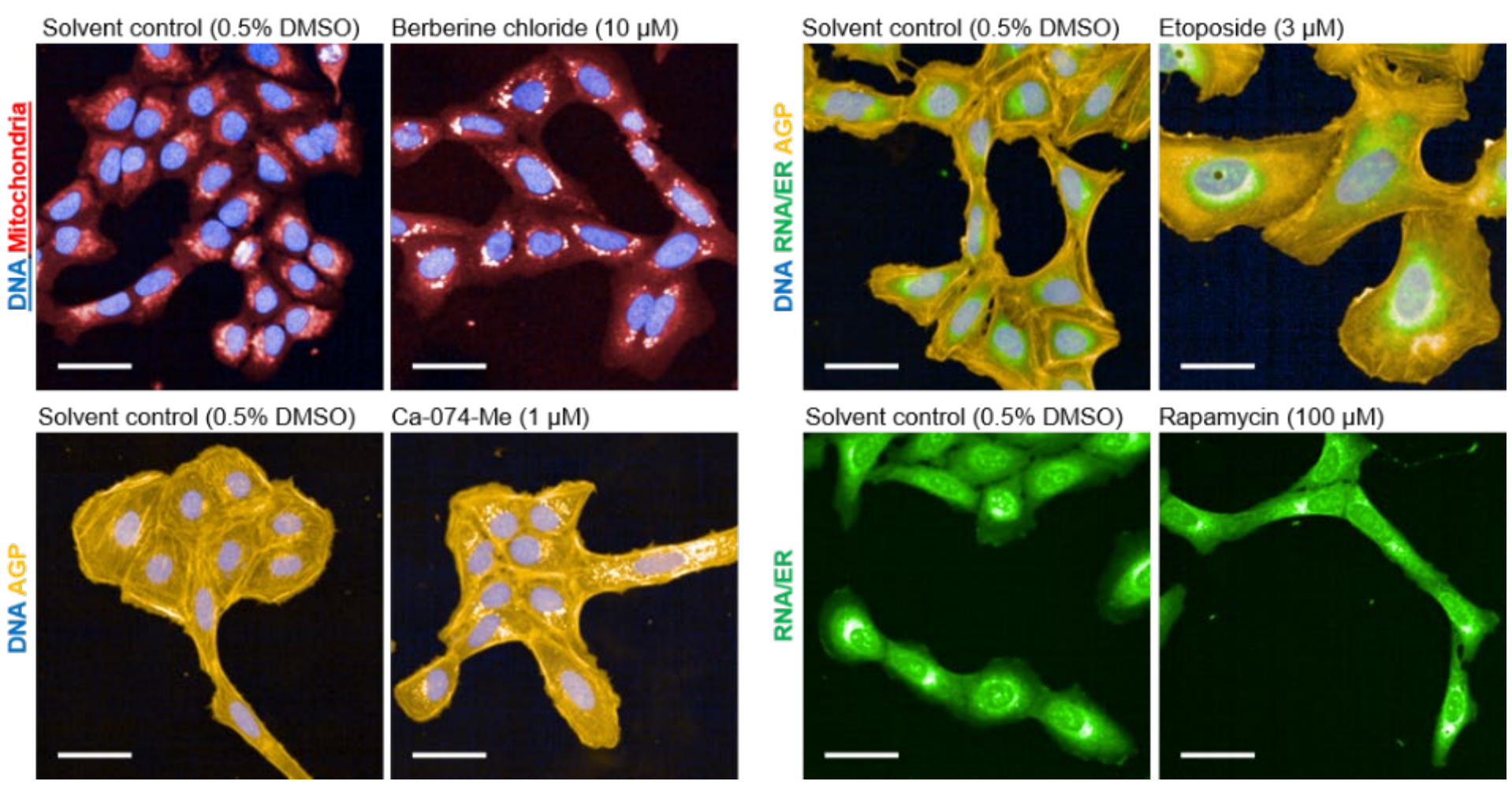


~ 1300 endpoints

- 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

Phenotypic Reference Chemicals (1)

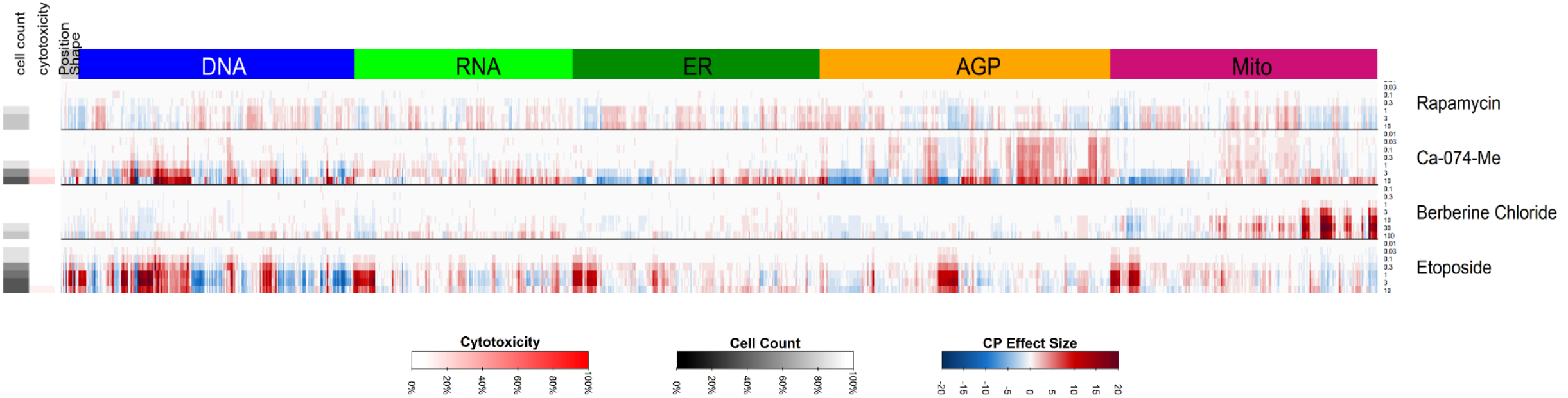
- A set of chemicals that elicit reproducible but distinct profiles of phenotypic effects.
- The profile may be specific for a particular channel / organelle or affect many components of the cell.



Nyffeler et al. (submitted)

Phenotypic Reference Chemicals (2)

- Individual features are measured, normalized to vehicle control and scaled to facilitate comparisons across features.



Nyffleler et al. (submitted)

HTP, Example Plate Design

	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	20	20	20	20	20	20	20	20	20	6	2	0.6	DMSO
B	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	2	0.6	0.2	DMSO
C	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0.6	0.2	DMSO
D	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	0.6	0.6	0.6	0.6	0.6	0.6	0.6	2	0.6	0.2	DMSO
E	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	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.6	0.2	0.06	DMSO
F	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.2	0.06	0.02	DMSO
G	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.06	0.02	0.006	DMSO
H	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.02	0.006	0.002	DMSO
I	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	2	2	DMSO	DMSO
J	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	0.6	0.2	DMSO	DMSO
K	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0.6	0.2	DMSO	DMSO
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	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.2	DMSO	DMSO
M	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	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.02	DMSO	DMSO
N	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.002	DMSO	DMSO
O	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.0002	DMSO	DMSO
P	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.00002	DMSO	DMSO

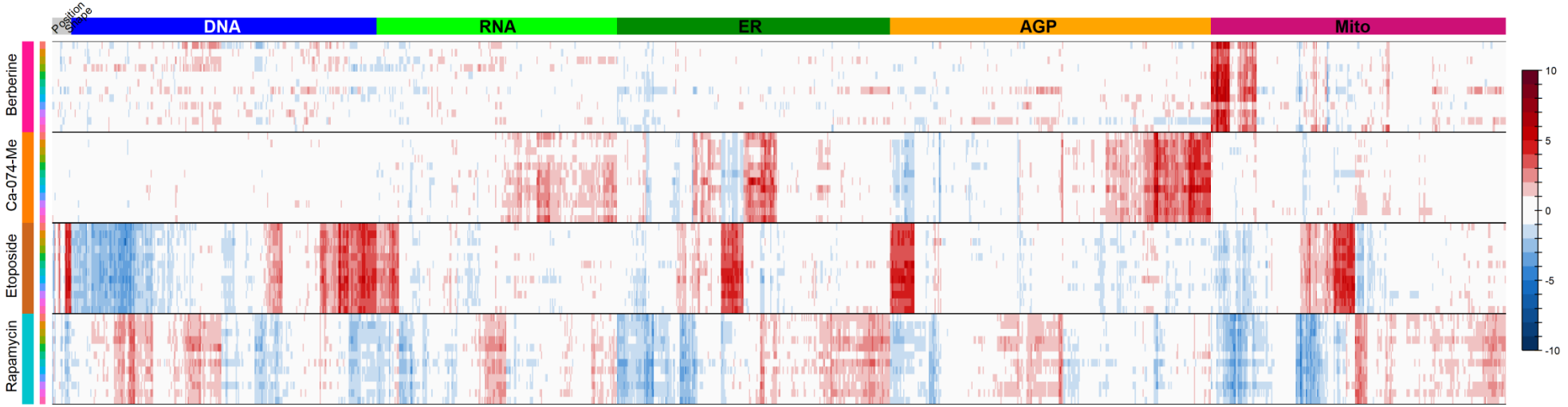
= Vehicle Control

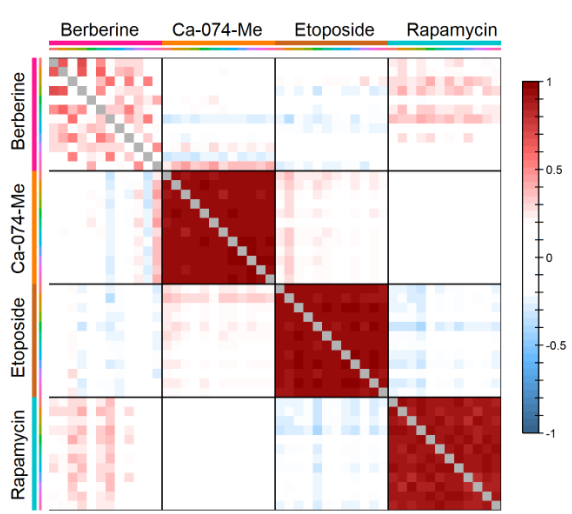
= Cytotoxicity Reference Chemical

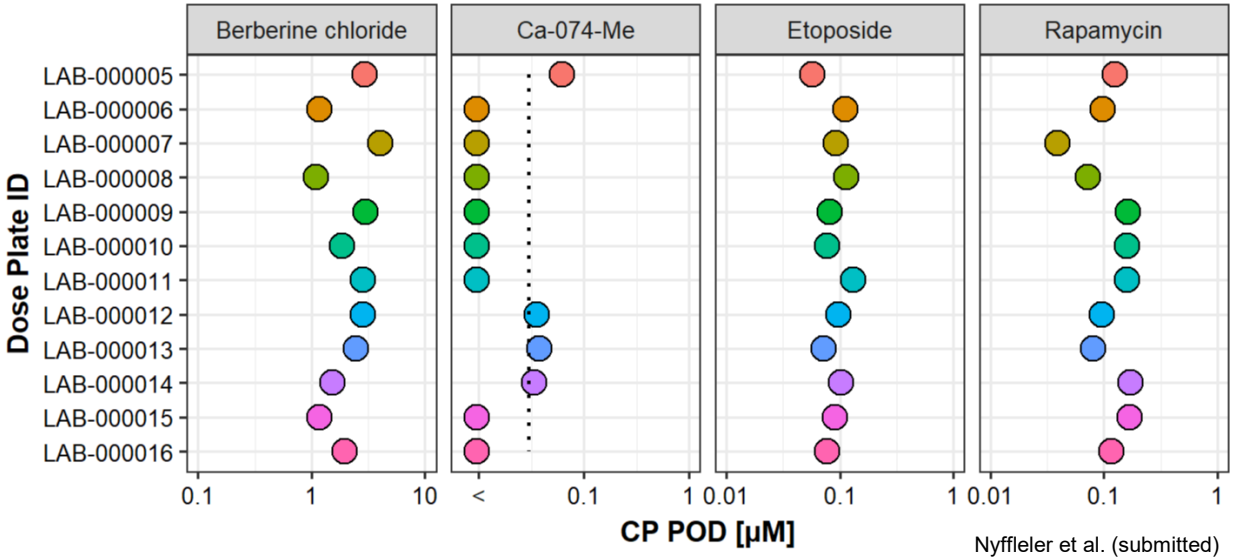
= Phenotypic Reference Chemical

= Test Chemicals

Evaluating HTP Assay Performance







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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!

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