Image-Based High-Throughput Screening, High-Content Analysis and High-Throughput Profiling

Part 2: Basic Concepts for 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.*

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.

High Content Screening (HCS) vs. High Throughput Profiling (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

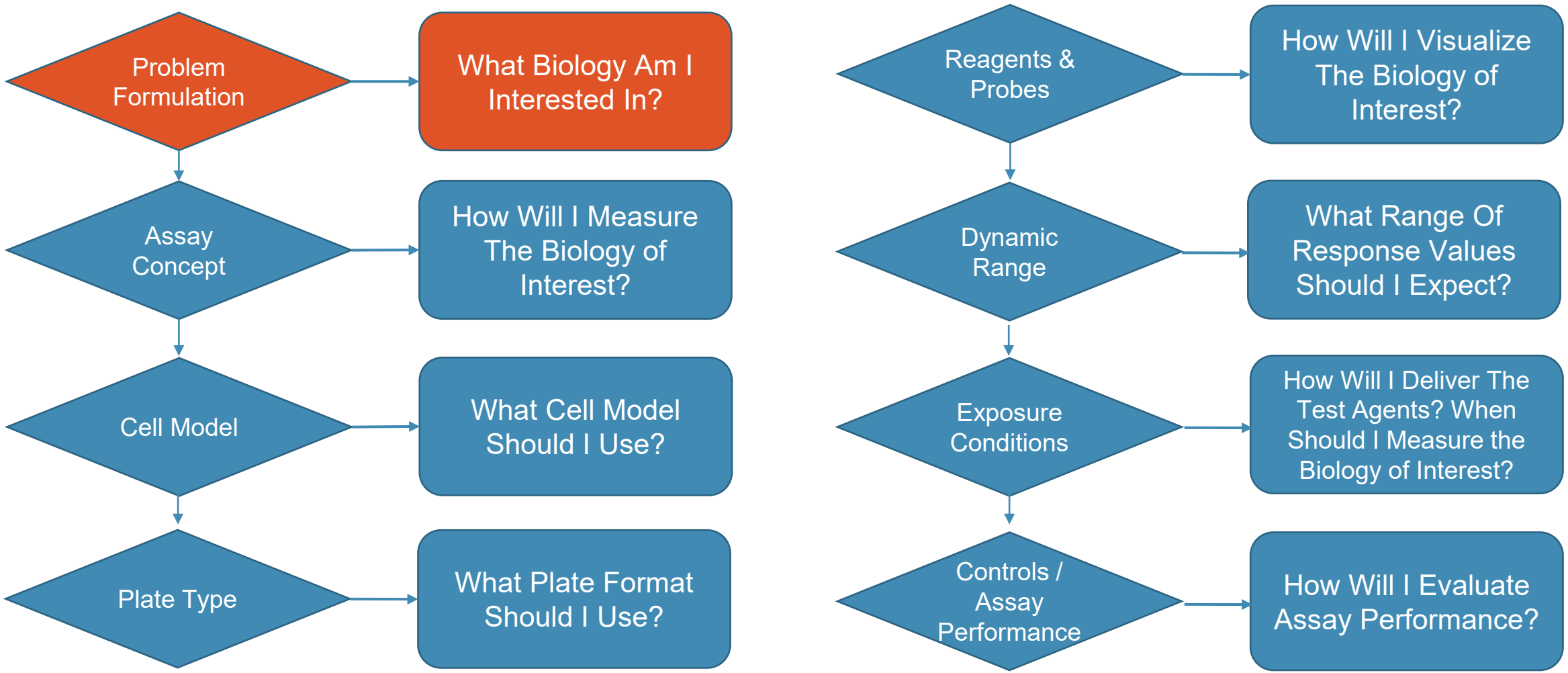
- Screening is a distinct strategy from profiling.
- Although both involve large-scale (high-throughput) imaging experiments, the goals differ:
    - **Screening:** The researcher aims to measure one or more phenotypes that are visually discernable, and choose a subset of hits for further investigation.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

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

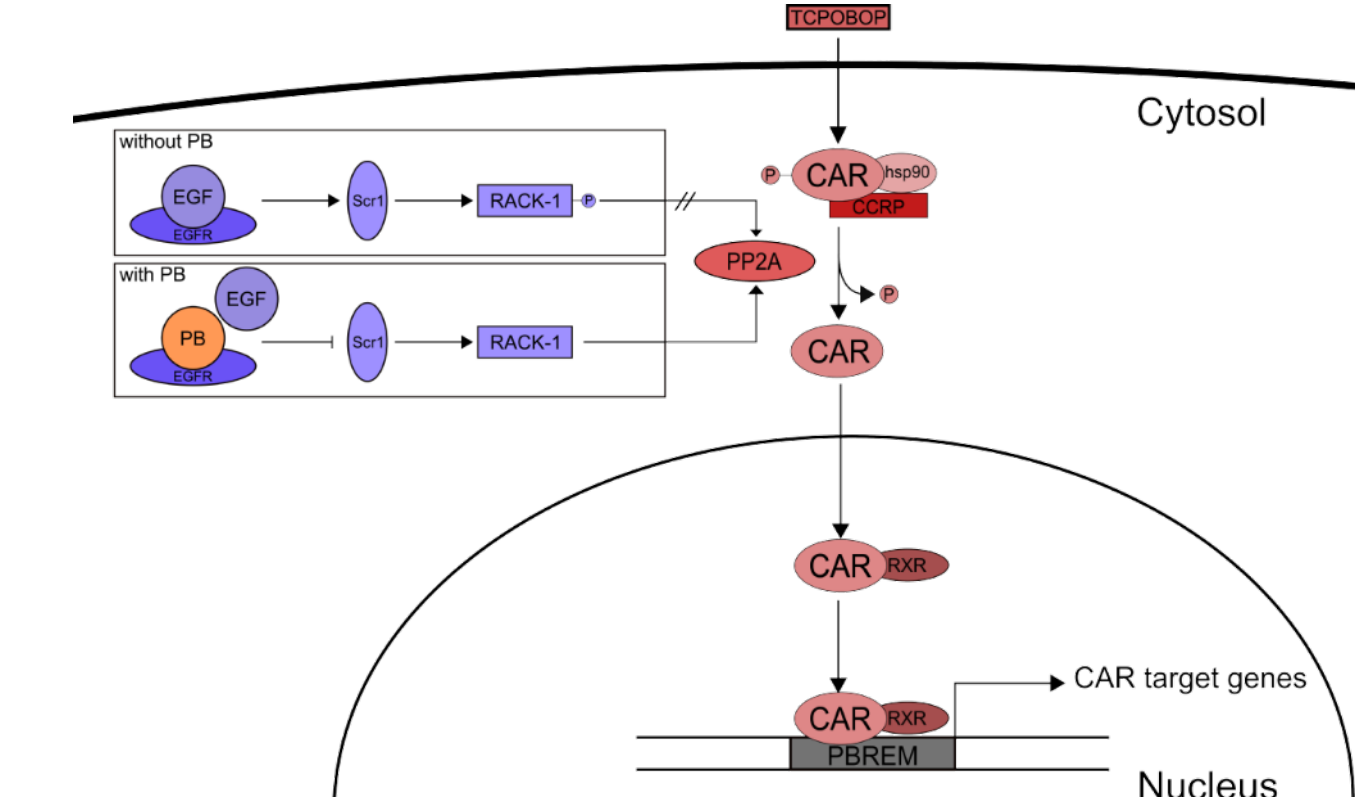
Goal(s): Identify nuclear receptor **activators**
Identify agents that **inhibit** nuclear receptor activation

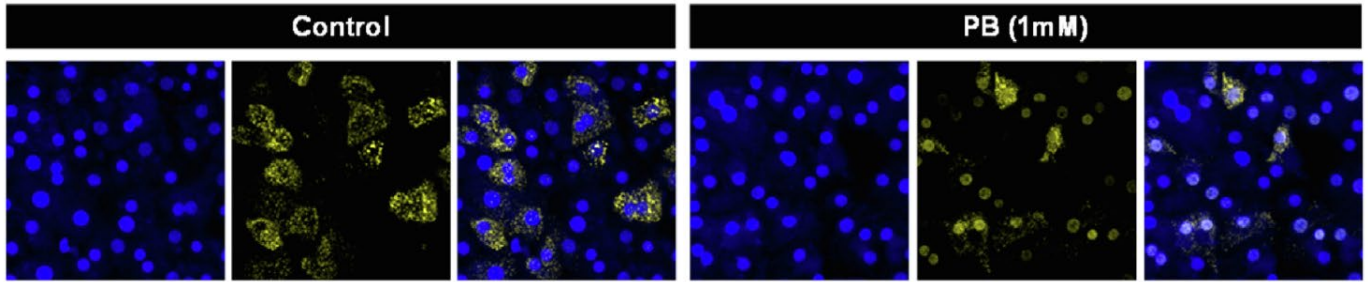
Characteristics:

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

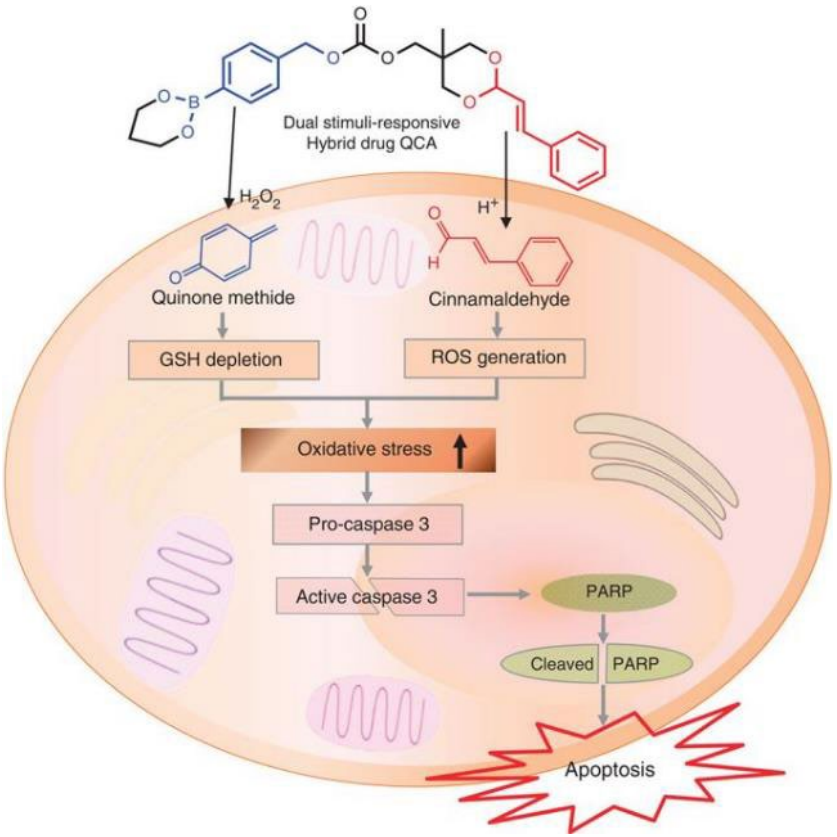
Goal(s): Identify chemicals that **produce** oxidative stress & **cause** apoptosis
Identify chemicals that **reduce** oxidative stress & **prevent** apoptosis

Characteristics:

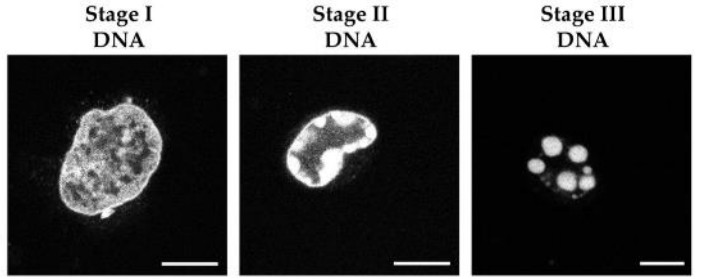
Oxidative stress can result from:
- Increased intracellular production of reactive oxygen species (ROS)
- Decreased levels of endogenous antioxidant molecules

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.

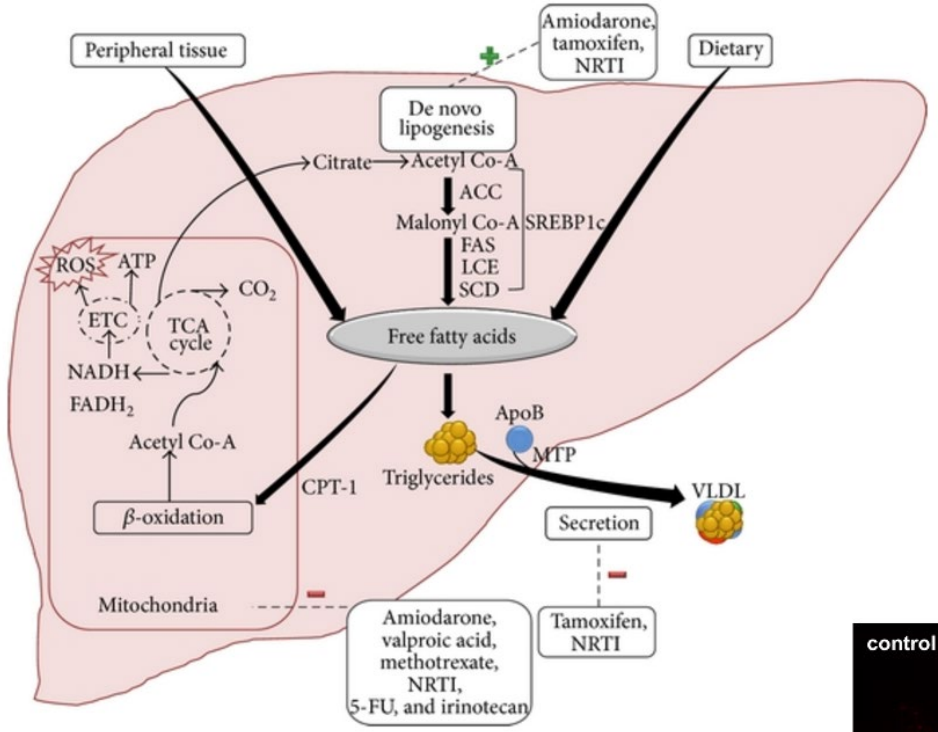
Characteristics:

Abnormal retention of lipids within a cell or organ.

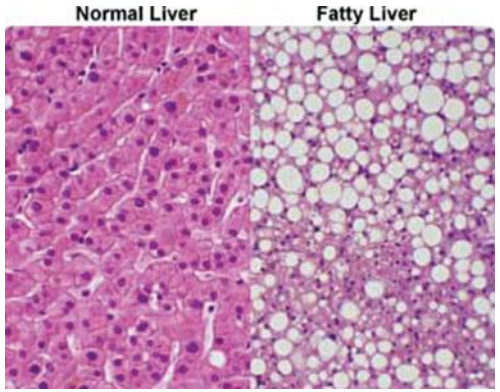
Occurs in the liver in response to:
- Dietary factors
- Chemical exposures
- Signals from peripheral tissues.

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



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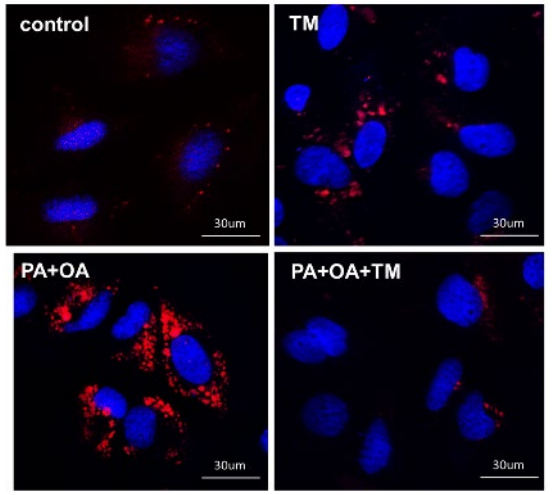


https://journals.plos.org/plosone/article/figure?id=10.1371/journal.pone.0170591.g002

10.1016/j.taap.2011.02.013

Example 4, Neurite Outgrowth

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

Characteristics:

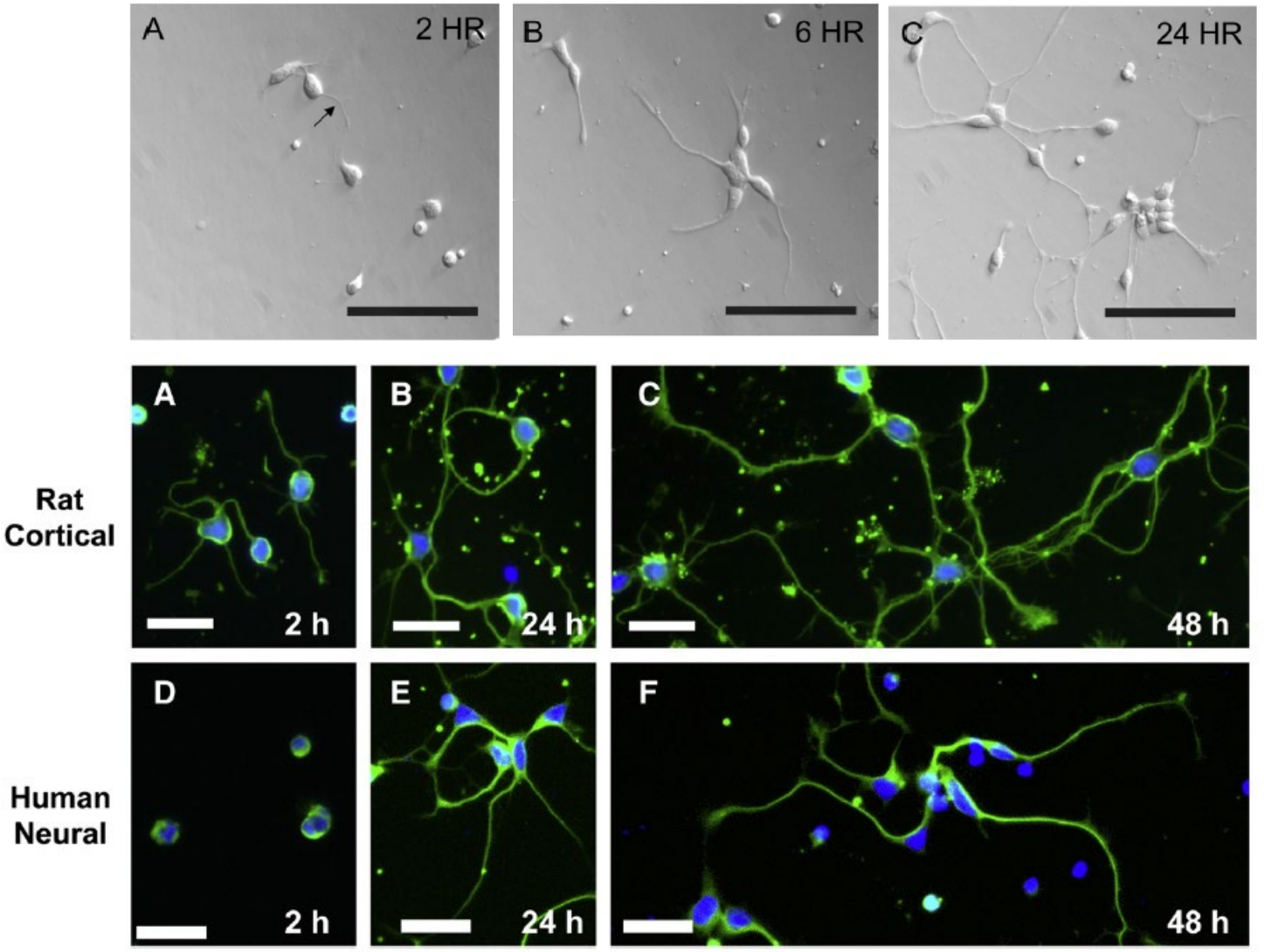
Neurite outgrowth involves extension of long, thin processes from the cell body of neurons.

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:

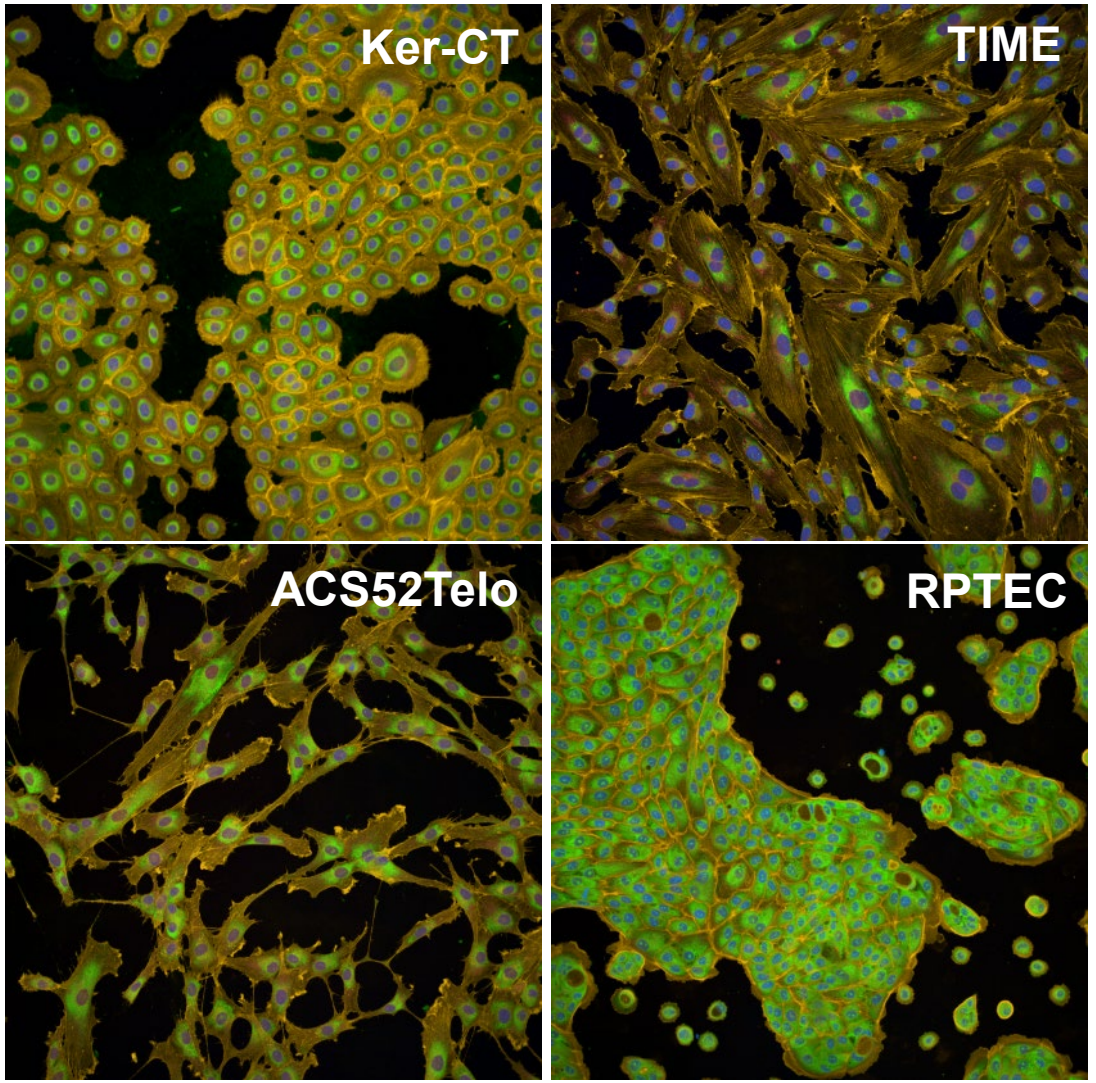
- Growth substrates
- Soluble growth factors
- Support from feeder cells



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



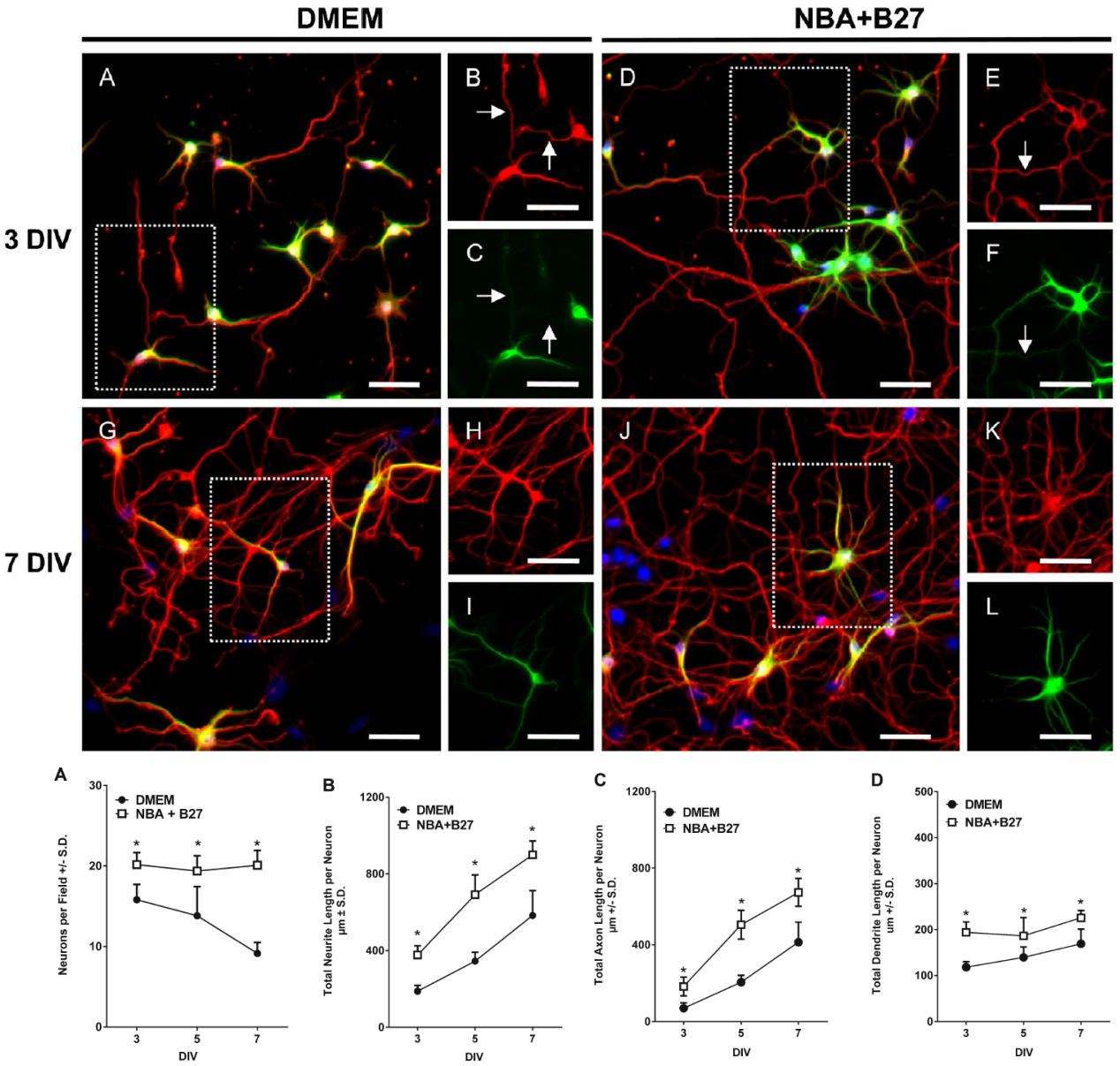
Willis, Nyffeler & Harrill. Unpublished Results

10.1007/s11626-015-9873-3

Cell Model Optimization (1)

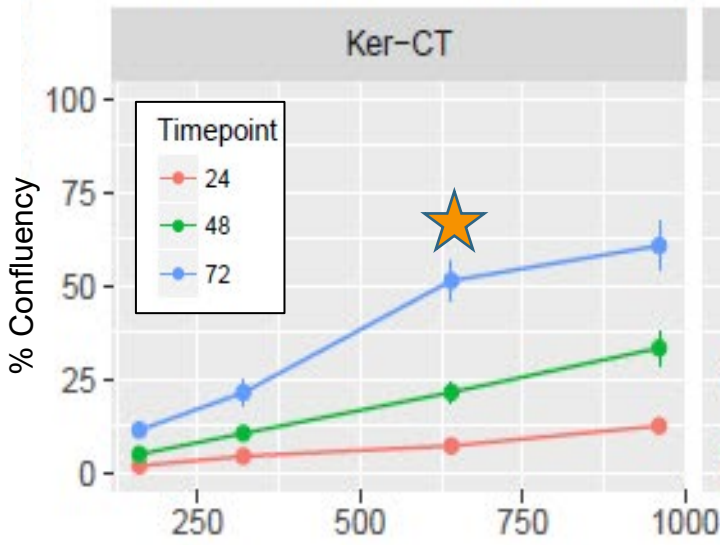
- **Cell models can require optimization of many parameters:**
  - Media formulation
  - Growth atmosphere (i.e. CO_2 / O_2)
  - Plate coating / growth substrate
  - Seeding density
  - Passaging
  - Maintenance in culture
  - Stimuli / Stressors
  - Labeling strategy

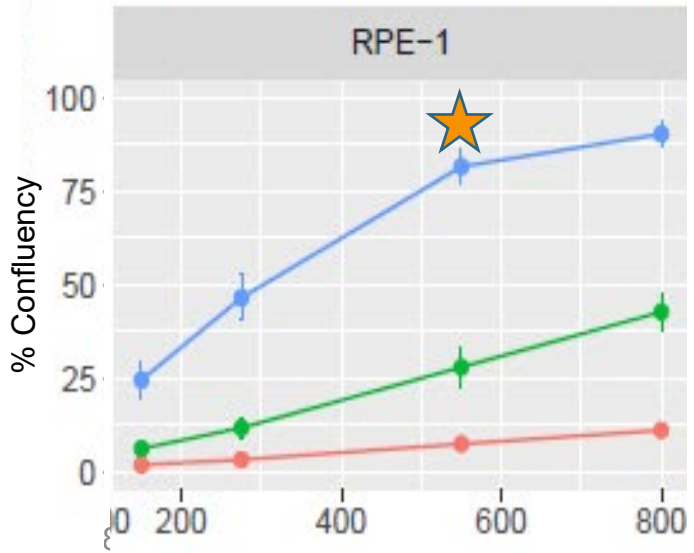
- Variability, sensitivity and dynamic range of HCS measurements can vary for the same cell type under different growth conditions.
- A key to reproducible HCS results is optimization and consistent preparation of cell cultures.

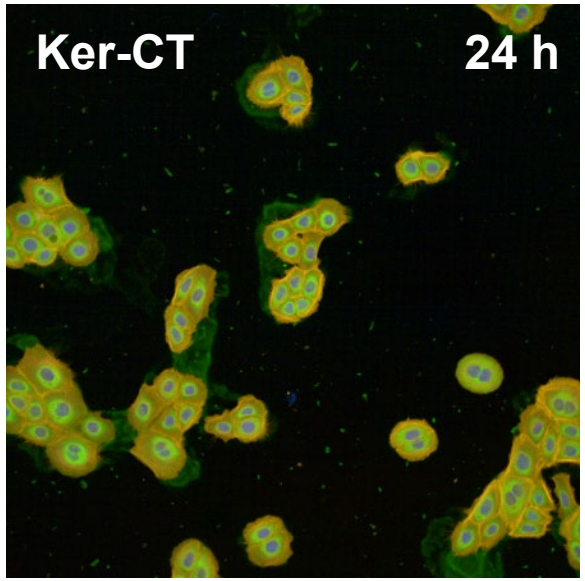


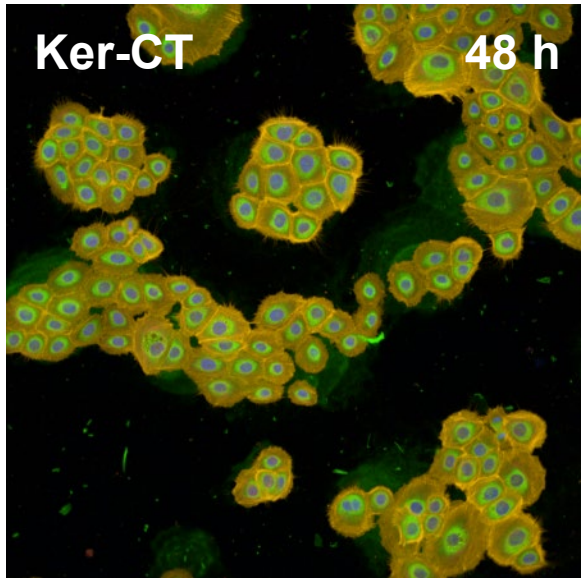
Harrill et al. (2015). PMID: 23142577

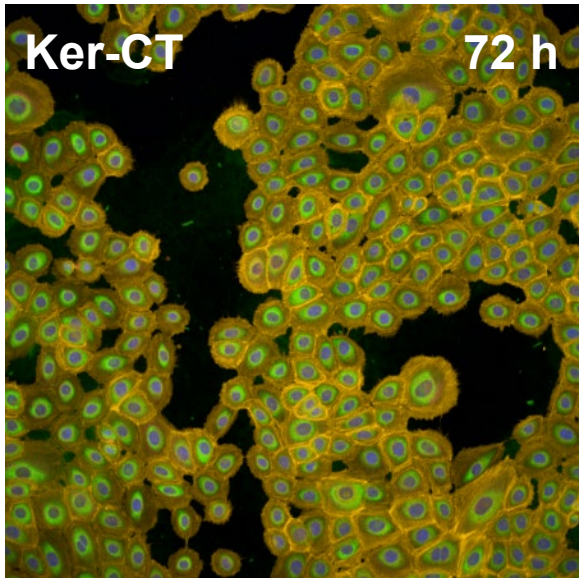
Cell Model Optimization (2)

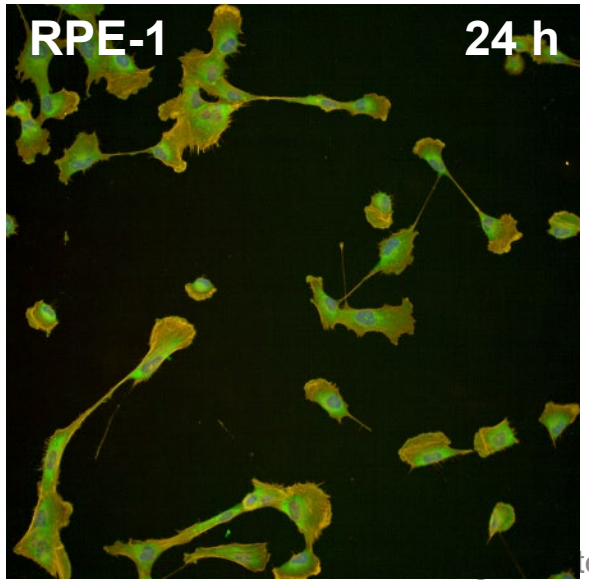


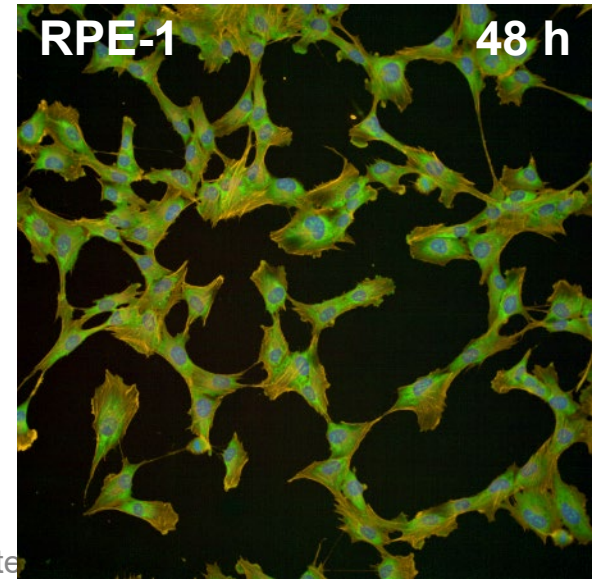


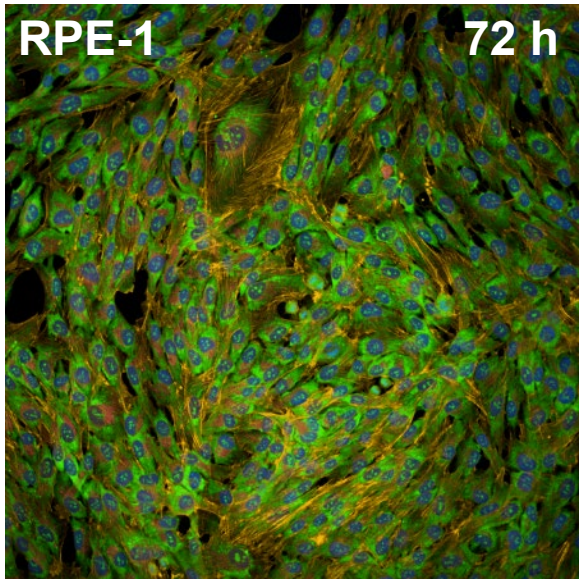












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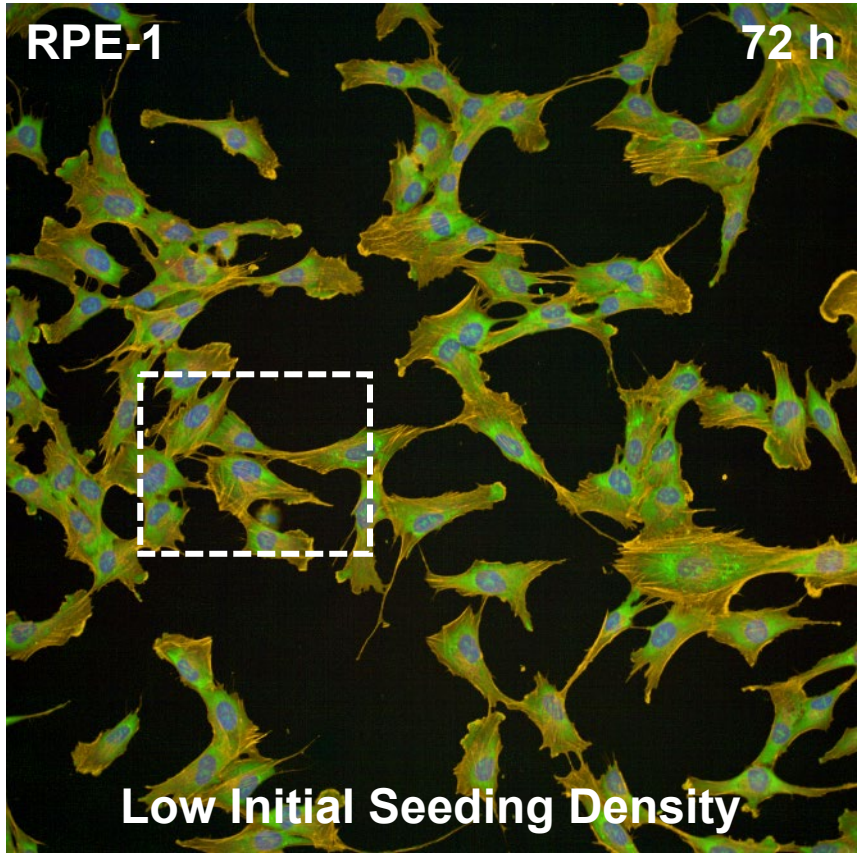
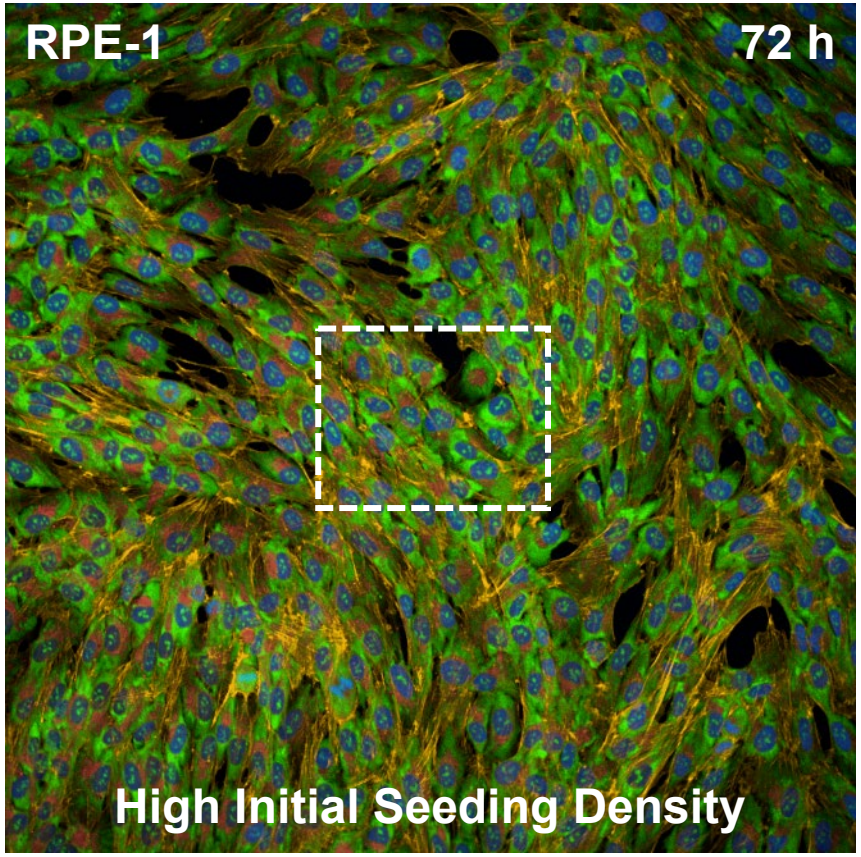
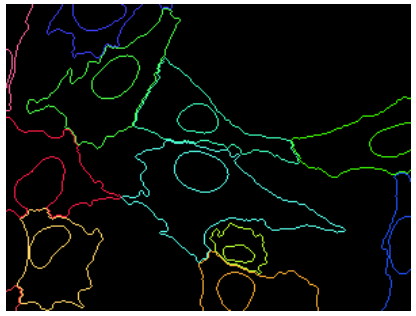
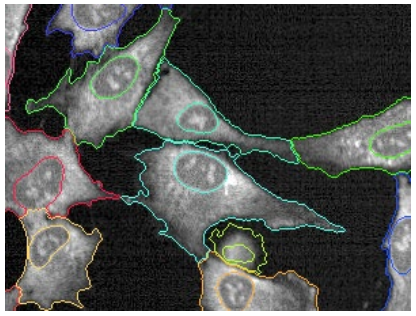
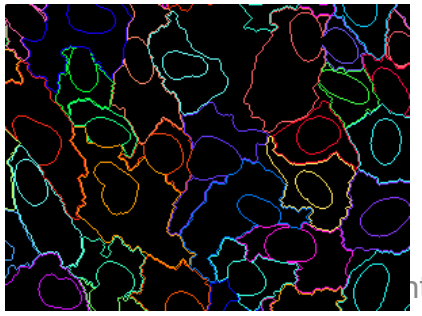
Cell Model Optimization (3)

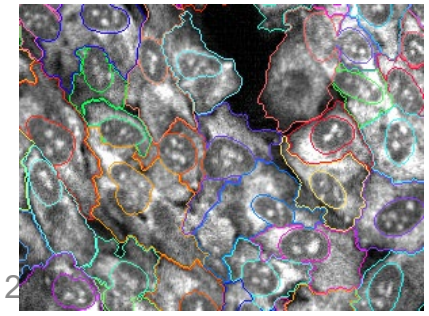












Overly confluent cultures:

- 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).

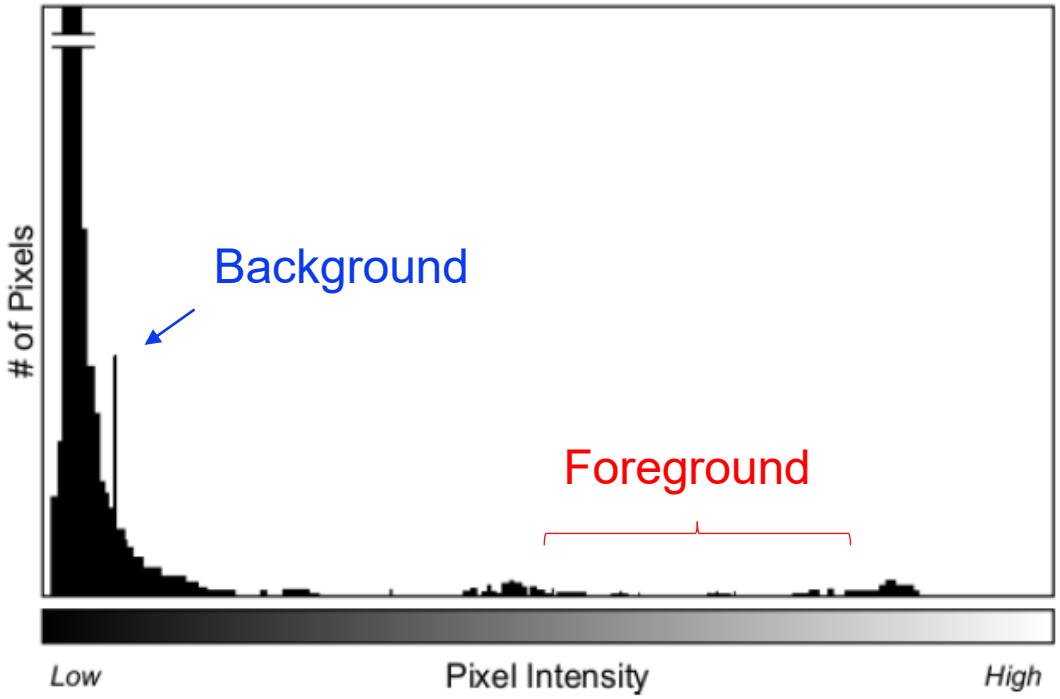
- In HCS, the assay concept can be designed to provide **intuitive measurements** of the biology of interest.

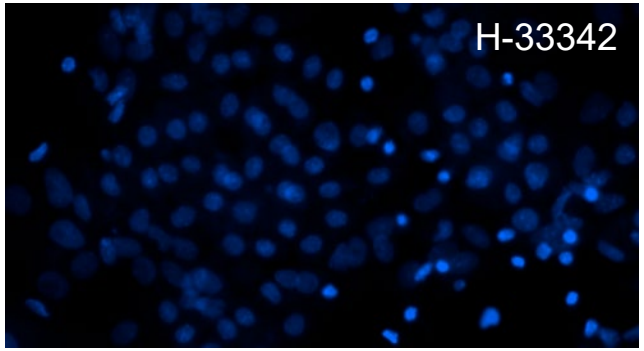
Concept	Notes
Nuclear Receptor Activation	• Ratio of fluorescent intensity inside vs. outside nucleus
Apoptosis	• Nucleus area, Nucleus shape, % of caspase positive cells
Steatosis	• Number of lipid droplets per cell
Neurite Outgrowth	• Total neurite length per neuron

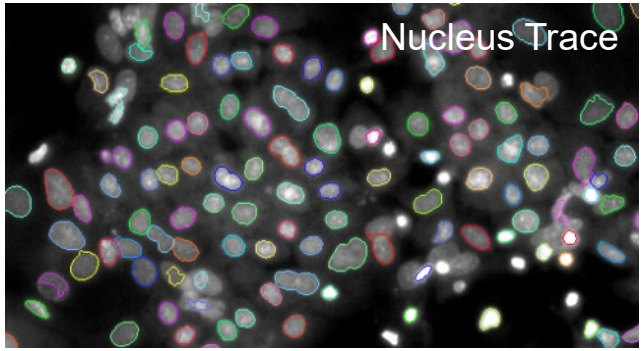
- Multiplexing of reagents and/or measurement of different features can provide information on different aspects of the biology of interest.

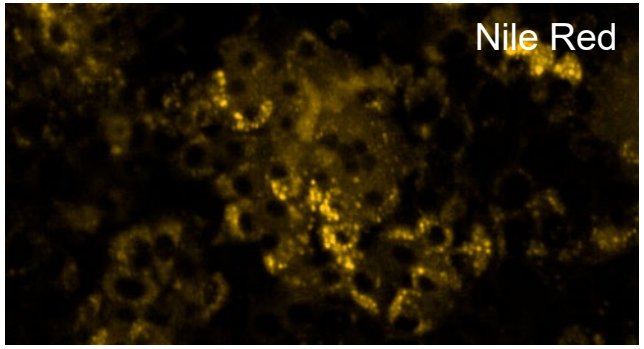
Identification of Labeled Objects

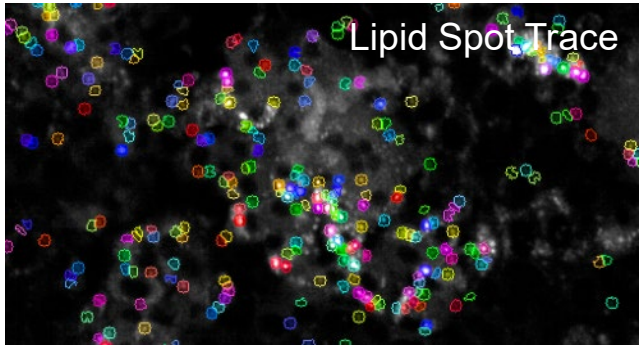
- **Segmentation:** Separation of signal from background











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)

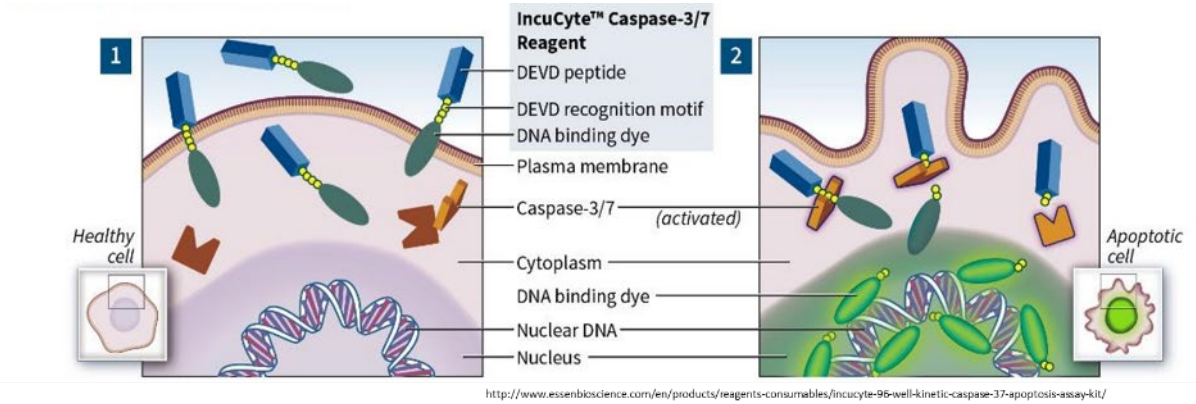
Cell Model:
- MCF-7 Adenocarcinoma cells

Biology of Interest:
- Apoptosis

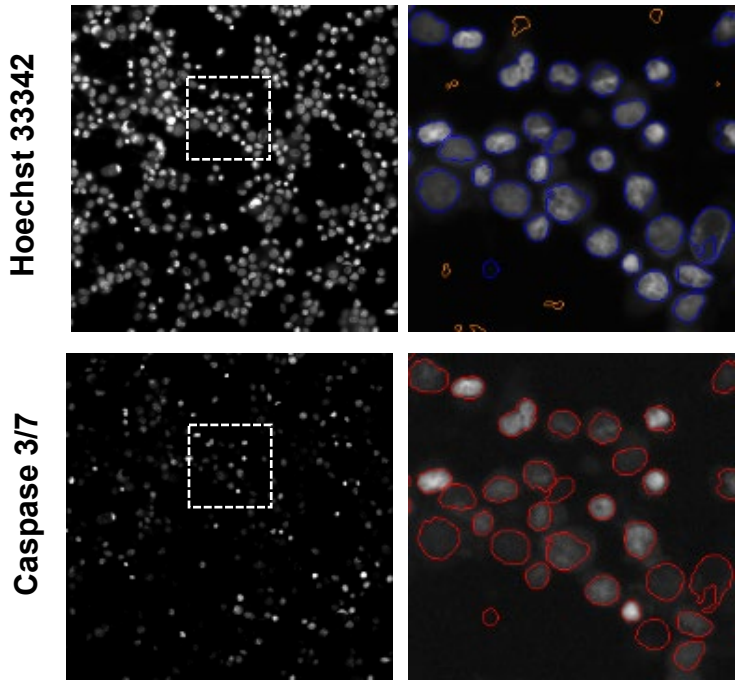
Visualization Approach:
- Caspase cleavage site peptide (DEVD) conjugated to fluorophore and quencher
  - ThermoFisher CellEvent™ Caspase 3/7 OR
  - IncuCyte™ Caspase-3/7 Reagent
- Nucleus counter-stain (Hoechst-33342)

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.



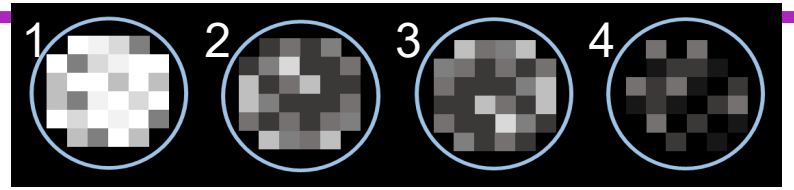
http://www.essenbioscience.com/en/products/reagents-consumables/incucyte-96-well-kinetic-caspase-37-apoptosis-assay-kit/

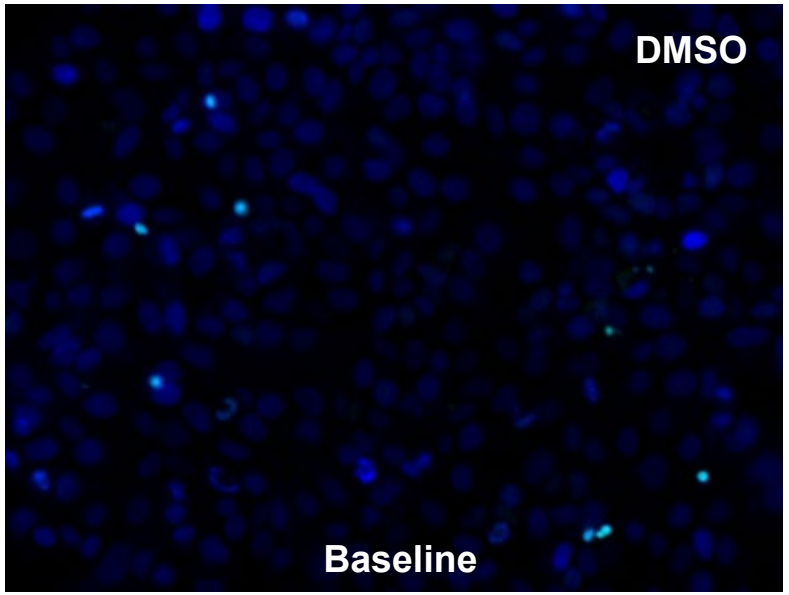


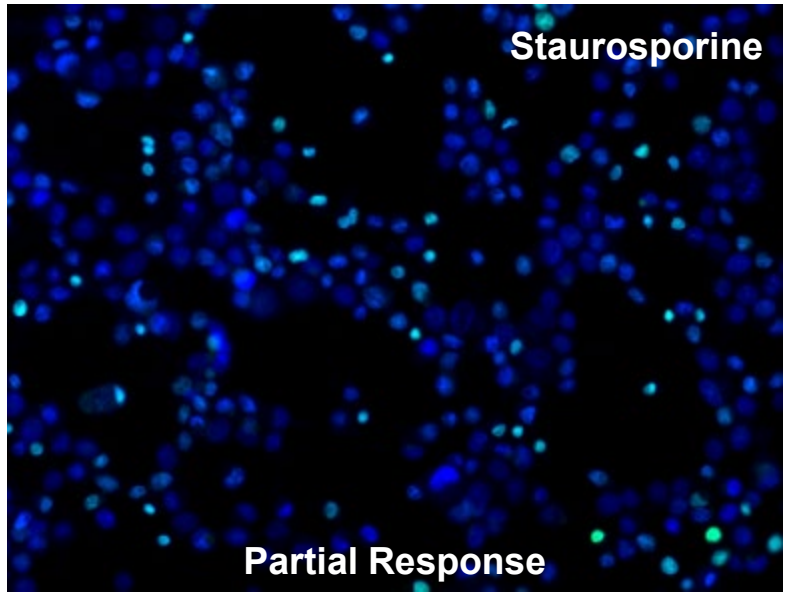
Willis, Nyffeler & Harrill, Unpublished Results

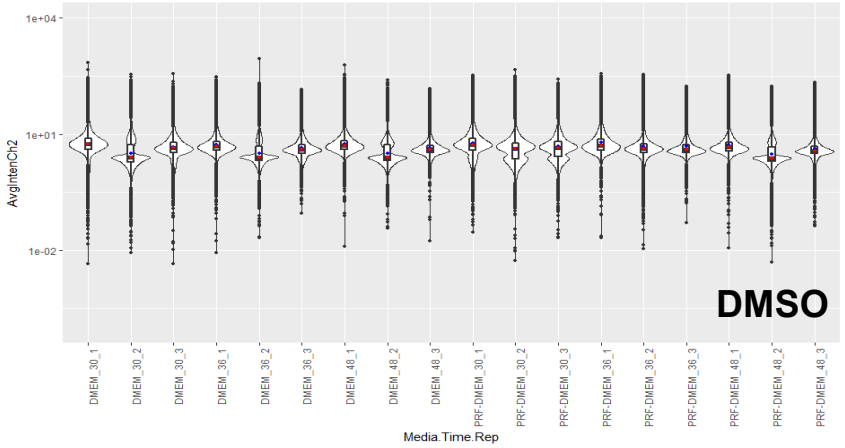
Assay Concepts – Apoptosis (2)

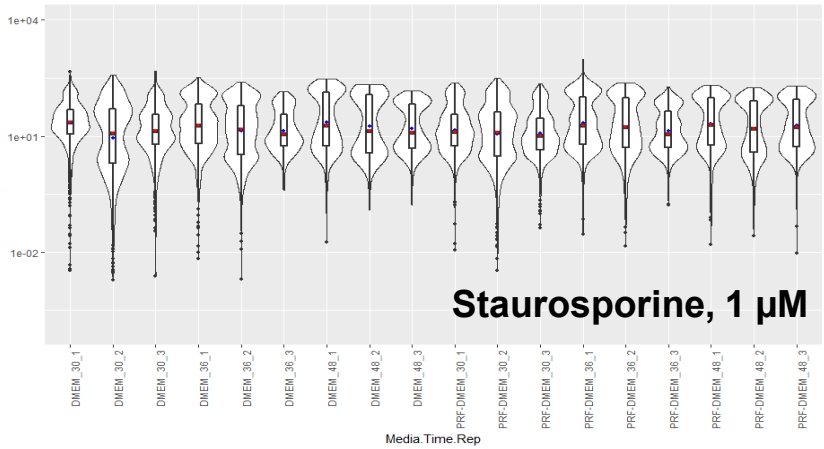
- Average fluorescent pixel intensity within each selected object
  - 1 > 2 ≈ 3 > 4

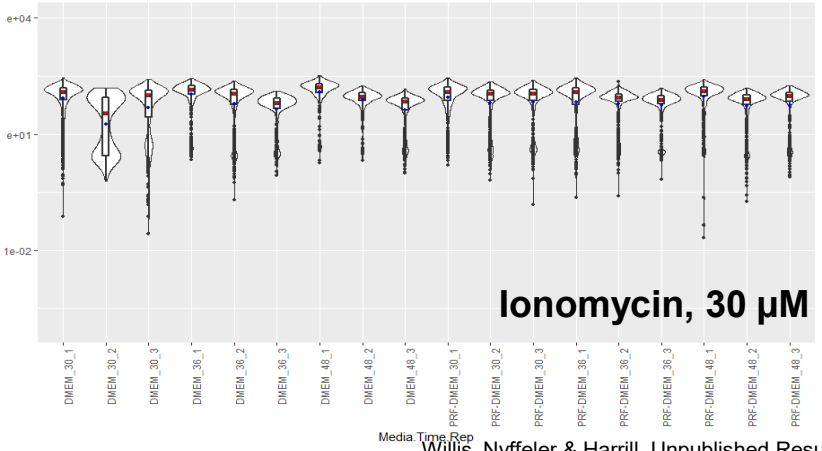












Willis, Nyffeler & Harrill, Unpublished Results

Assay Concepts – Apoptosis (3)

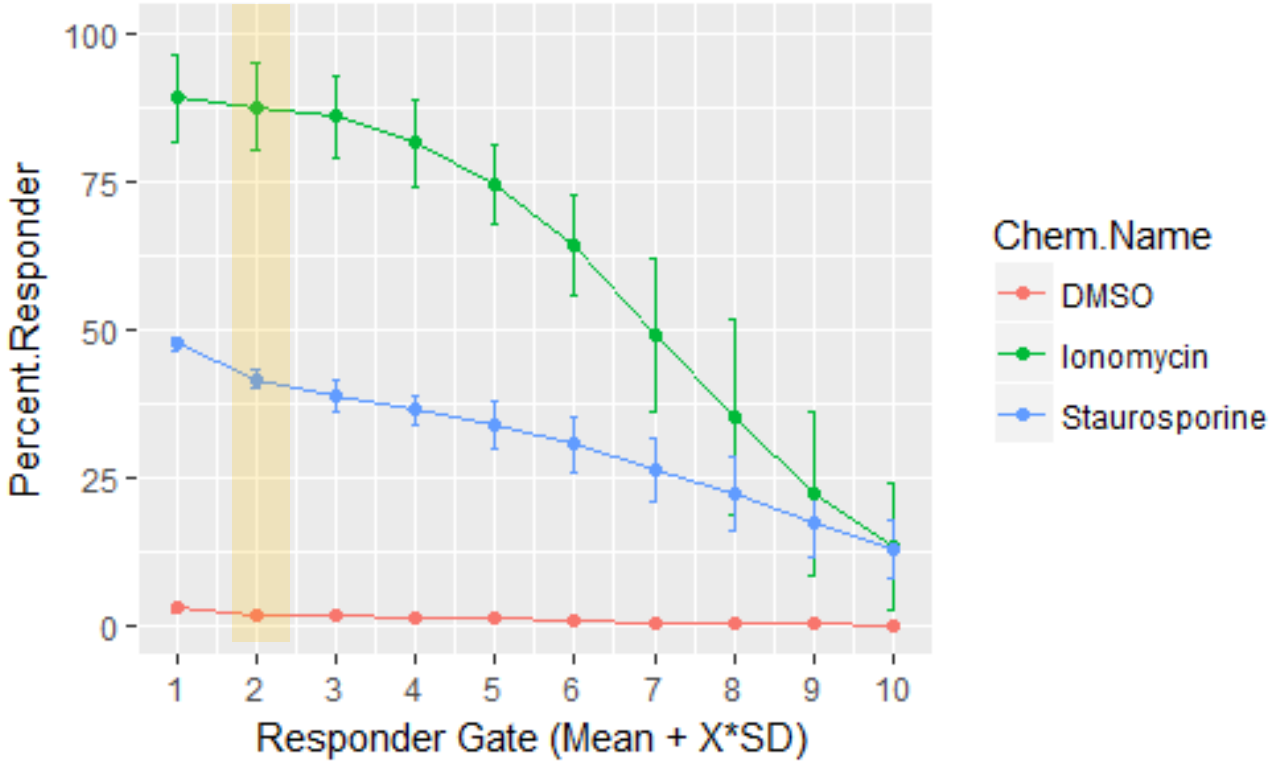
Two Approaches →	Mean Intensity	Percent Responder
Description	Well level mean of AvgIntenCh2 values.	Percent of cells with AvgIntenCh2 values above range of control.
Example	Breier et al. (2008), Culbreth et al. (2012)	Vogt et al. (2005), Foldes et al. (2011)
Detect Rare Events ?	NO	YES

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

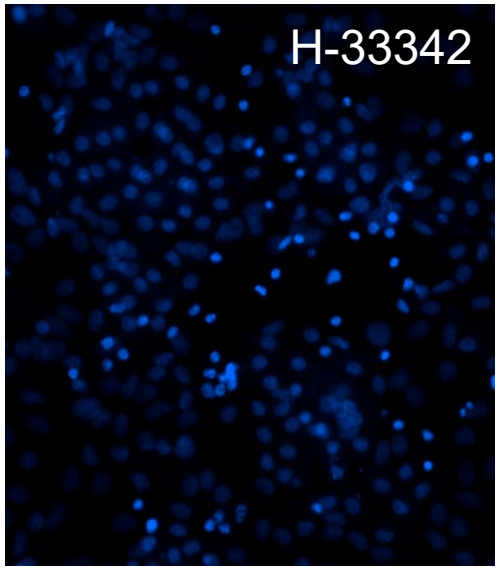
Cell Model:
- Differentiated (2-D) HepaRG.
- Mixed culture:
  - “Hepatocyte-like” cells
  - “Cholangiocyte-like” cells.

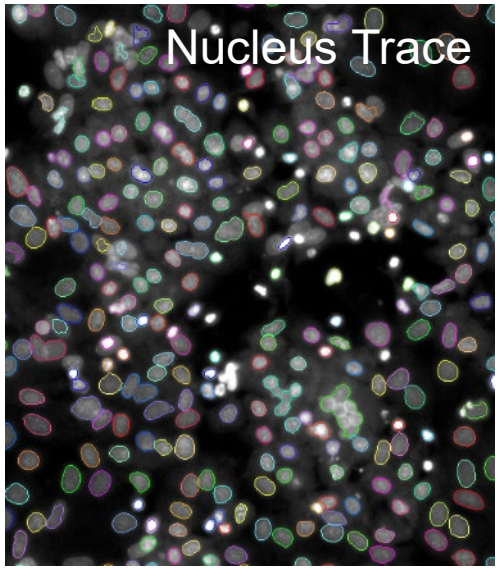
Biology of Interest:
- Lipid accumulation in hepatocyte-like cells is biology of interest.

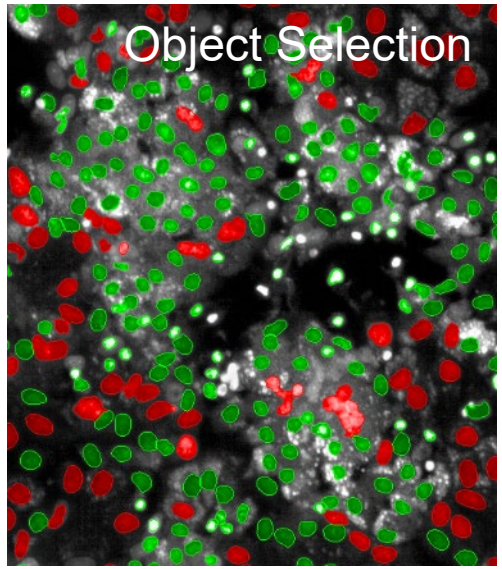
Visualization Approach:
- Nile Red labeling of lipids.
- Nucleus counterstain

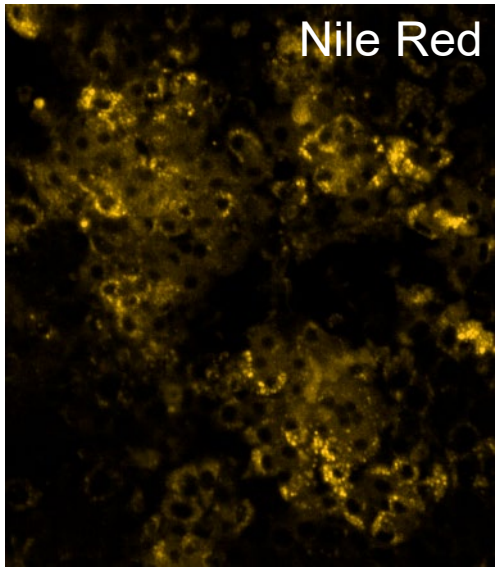
Image Analysis Approach:
- Identify labeled nuclei
- Select nuclei of interest based on morphology
- Define a Region-of-Interest (ROI) around each cell
- Identify lipid droplets within each ROI.

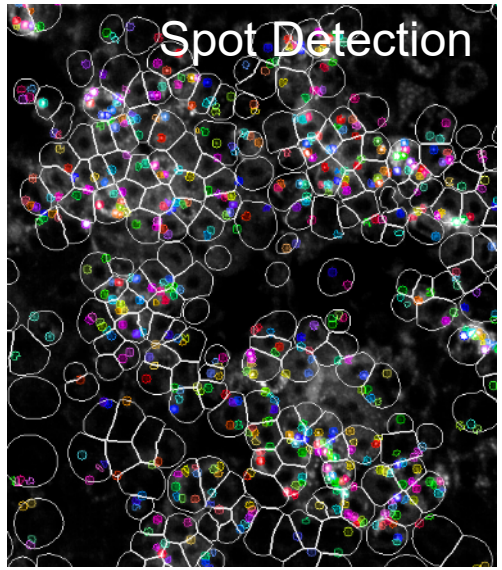
Intuitive Output:
- Average number of lipid droplets per hepatocyte-like cell











Tucker, Nelson, Harrill, Chorley: Unpublished Results

Assay 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)

	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

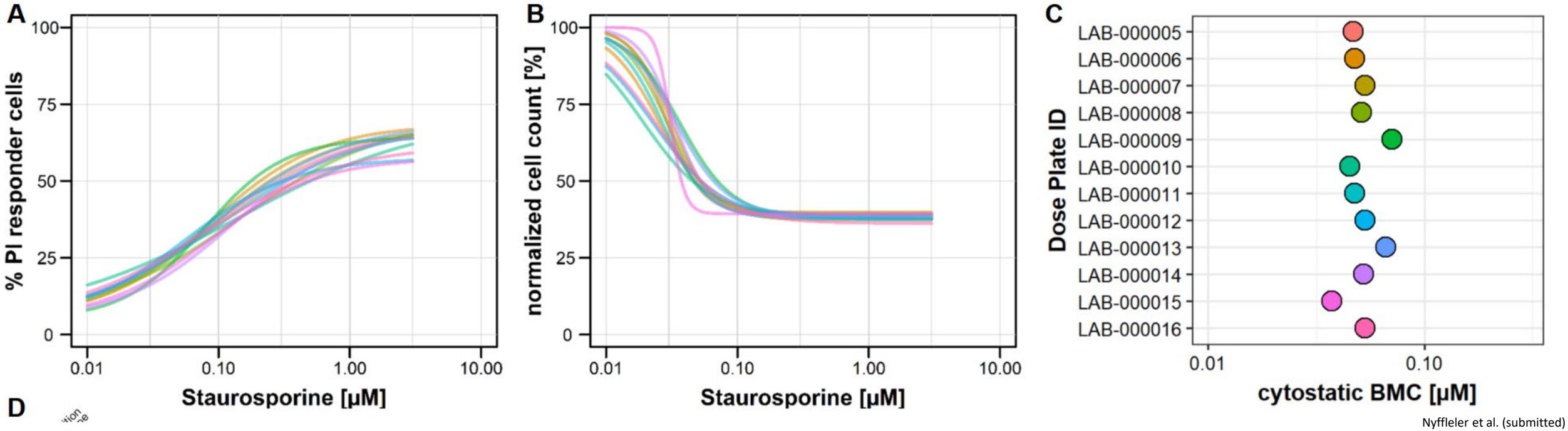
= Positive Control

= Negative Control

= Test Chemicals

Assay Controls for HCS (3)

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

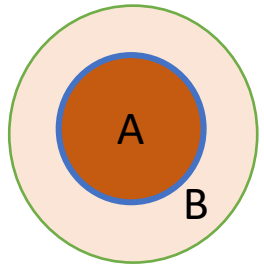


Nyffleler et al. (submitted)

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 ????

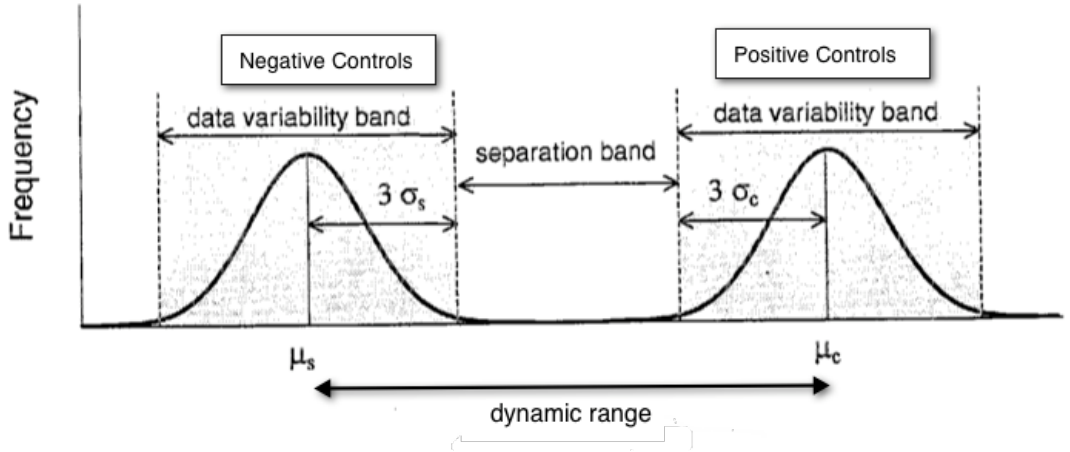


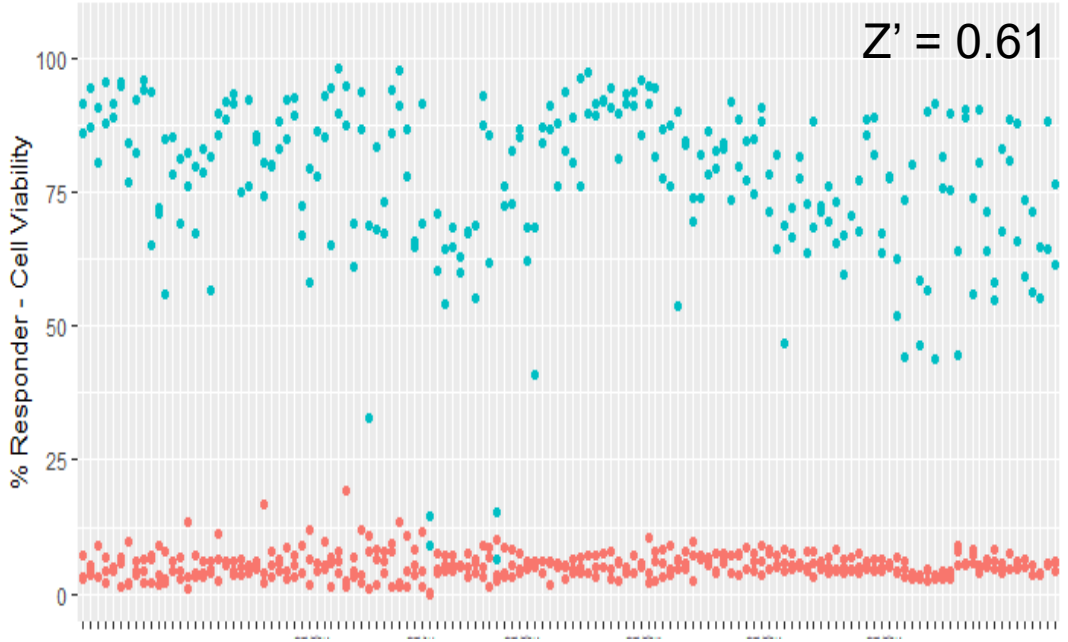
- **Empirical Dynamic Range:** The difference in values between control conditions and the most efficacious positive control condition.

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

HCS Assay Performance

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

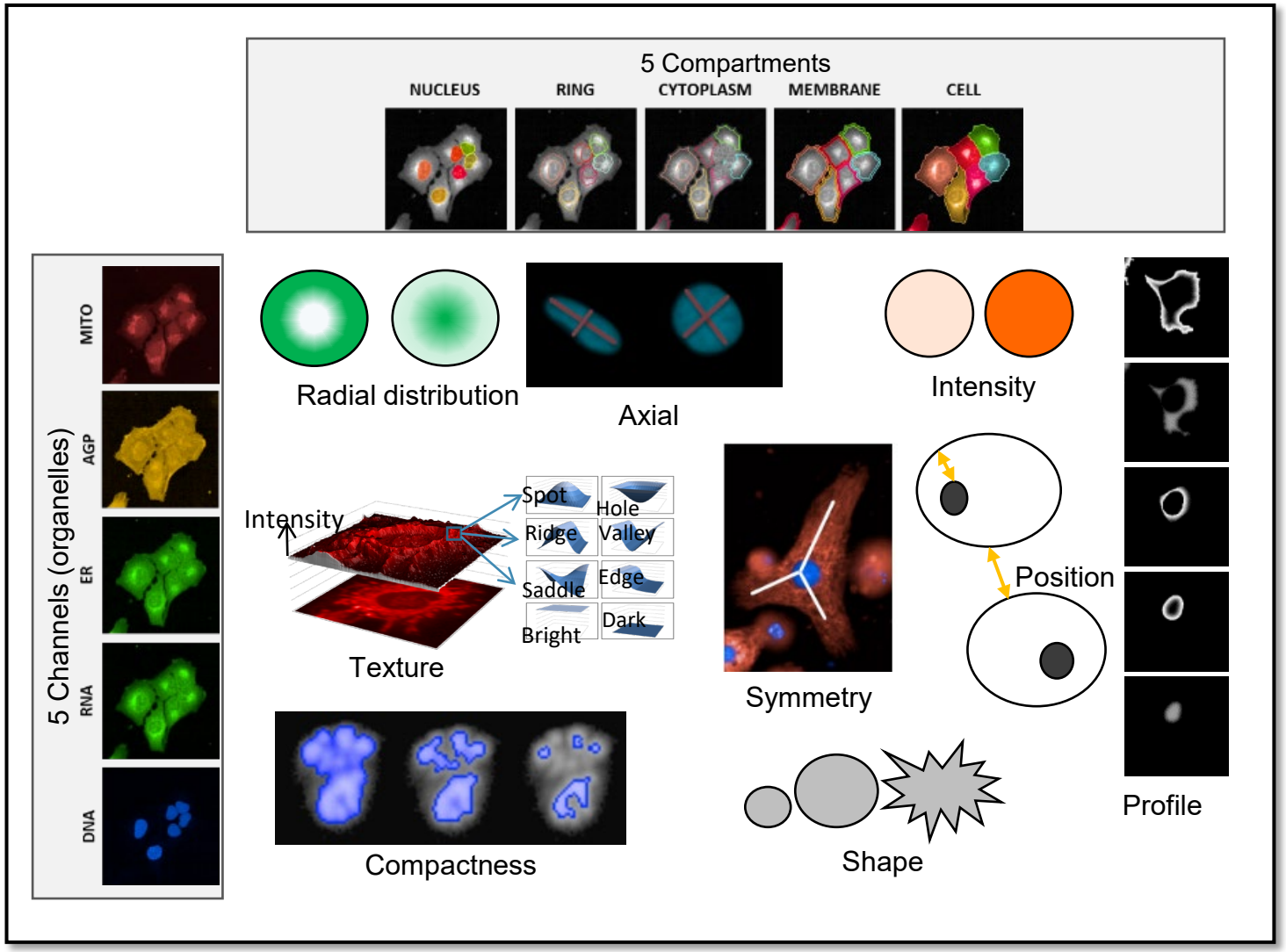




Z-factor	Interpretation
1.0	Ideal. Z-factors can never exceed 1.
between 0.5 and 1.0	An excellent assay.
between 0 and 0.5	A marginal assay.
less than 0	There is too much overlap between the positive and negative controls for the assay to be useful.

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

High Throughput Profiling (HTP)

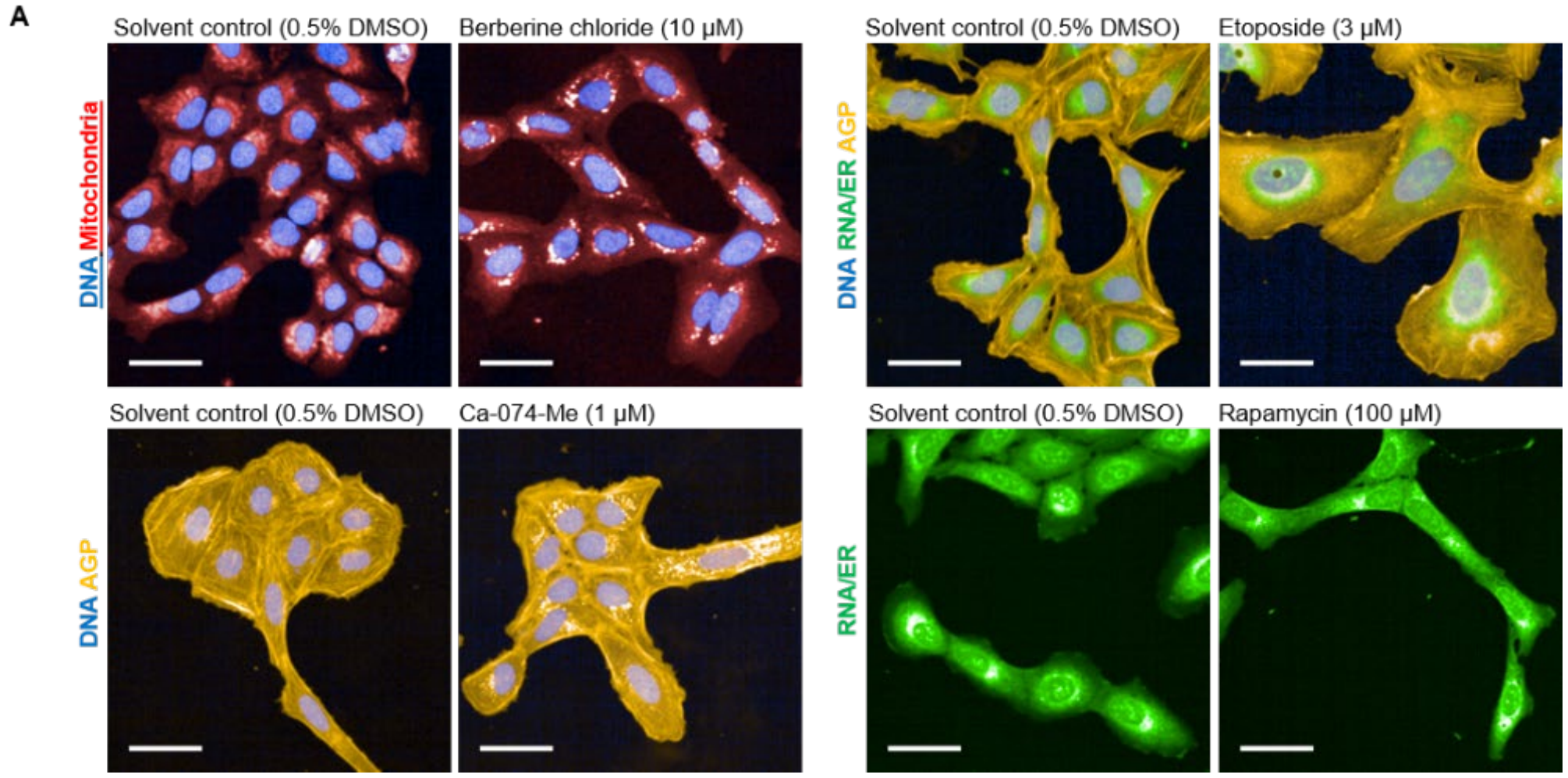


~ 1300 endpoints

- 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)

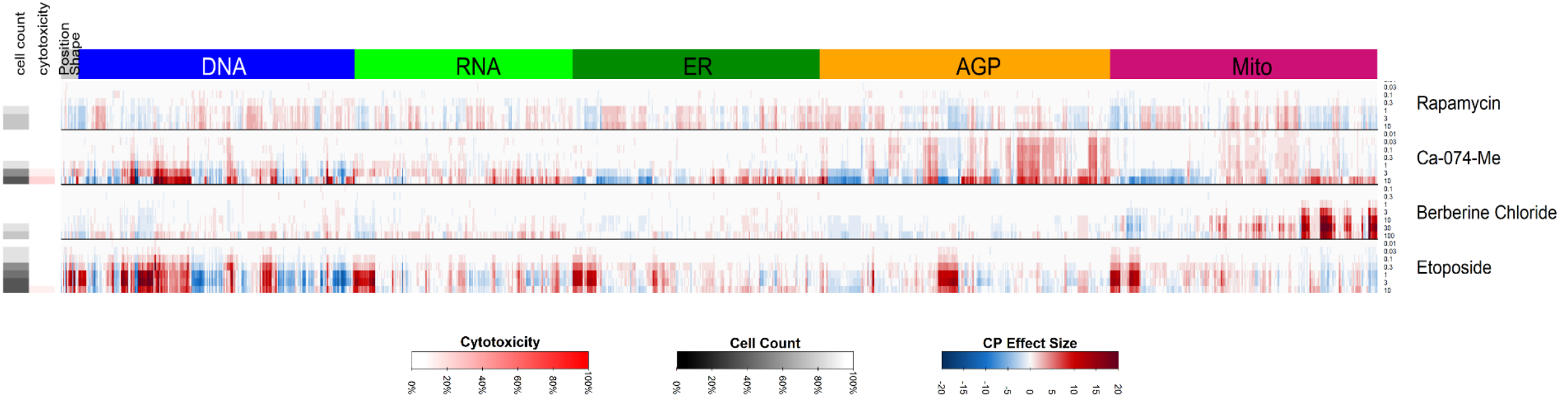
- A set of chemicals that elicit reproducible but distinct profiles of phenotypic effects.
- The profile may be specific for a particular channel / organelle or affect many components of the cell.



Adapted from Nyffleler et al. (2020)

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

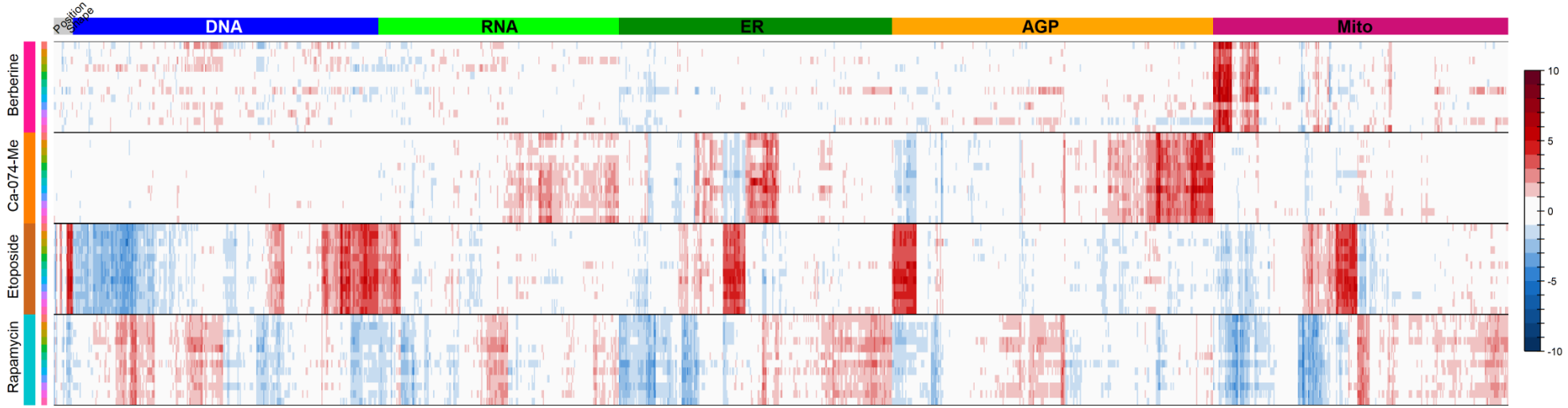
= Vehicle Control

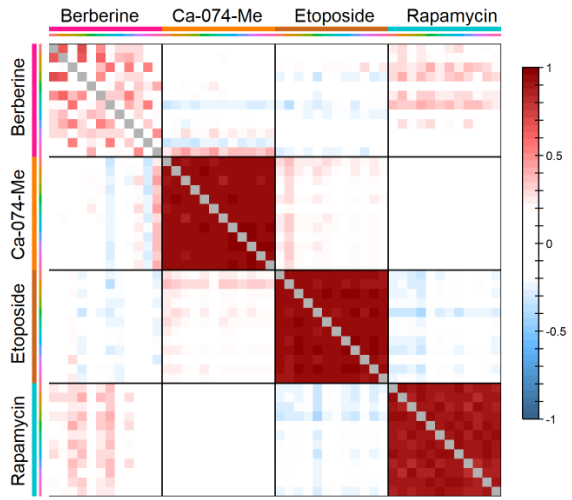
= Cytotoxicity Reference Chemical

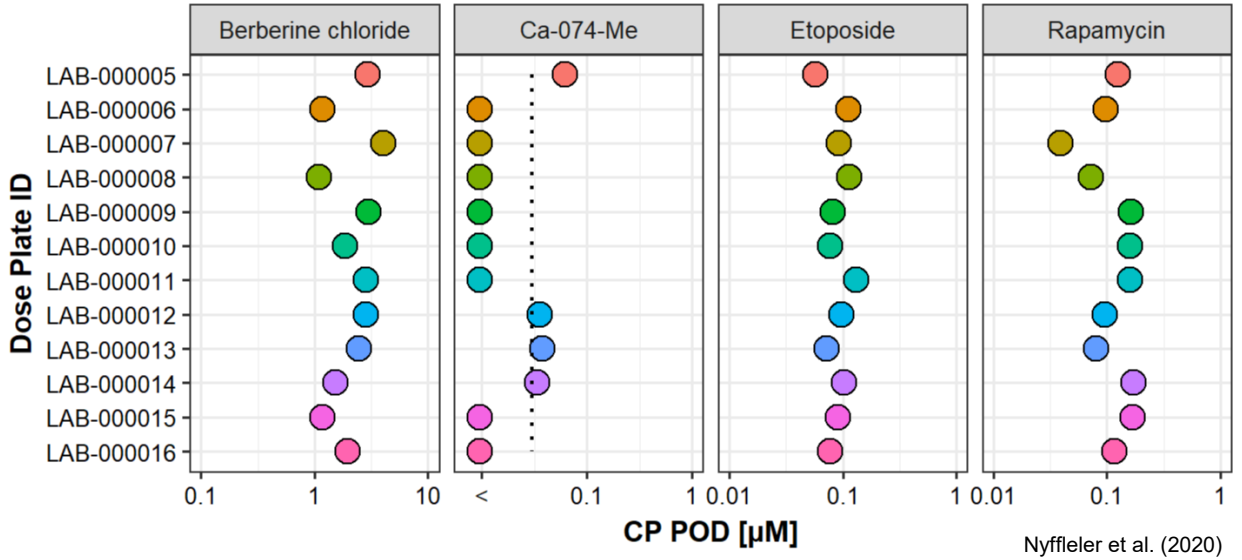
= Phenotypic Reference Chemical

= Test Chemicals

Evaluating HTP Assay Performance

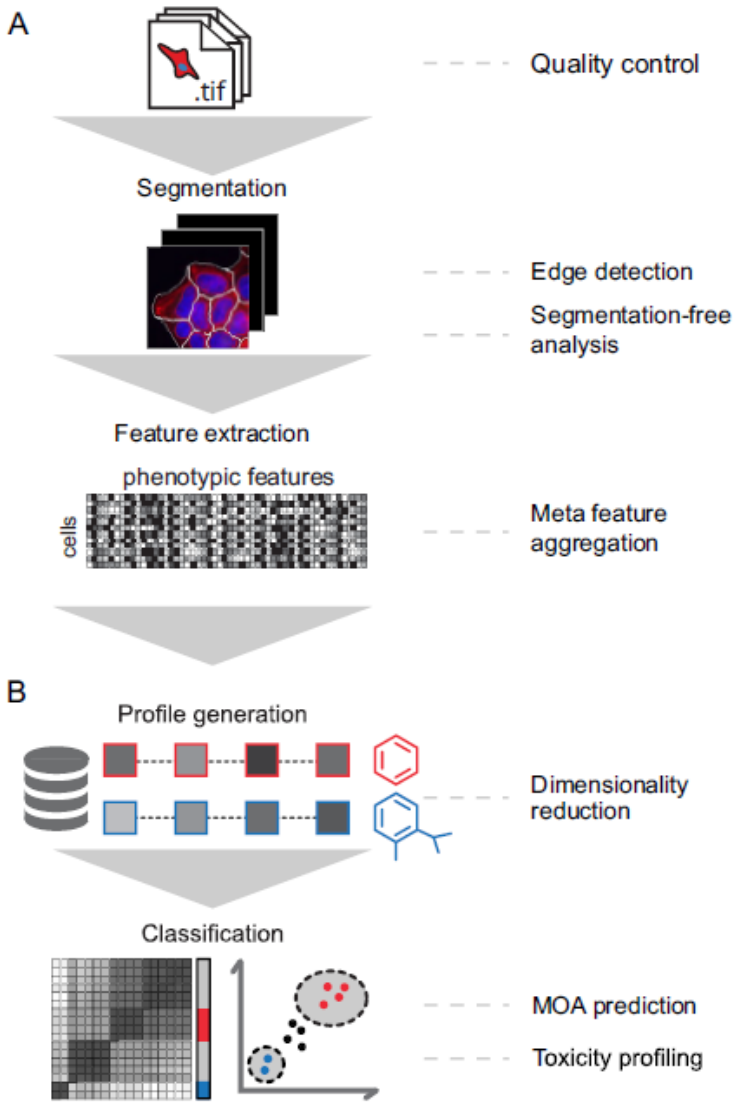




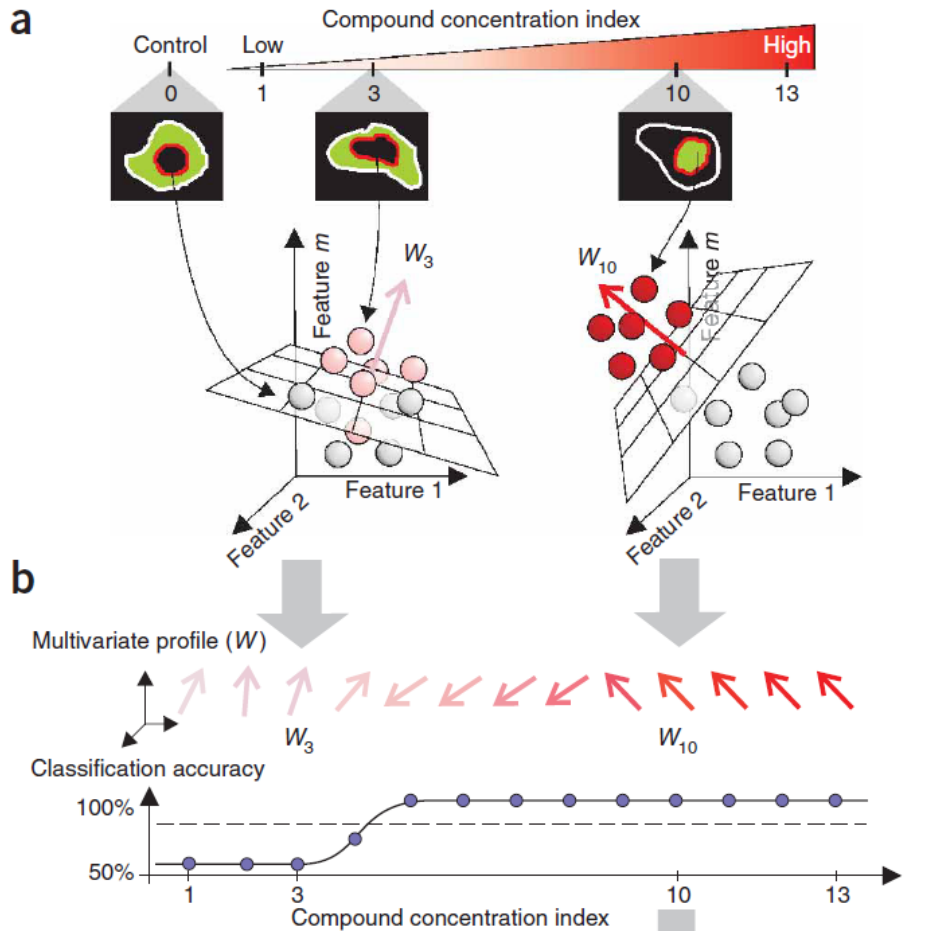


Nyffleler et al. (2020)

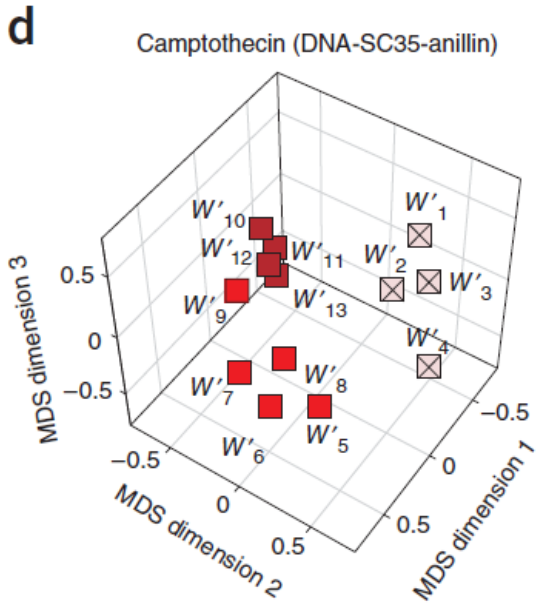
Machine Learning in HCS & HTP Assays

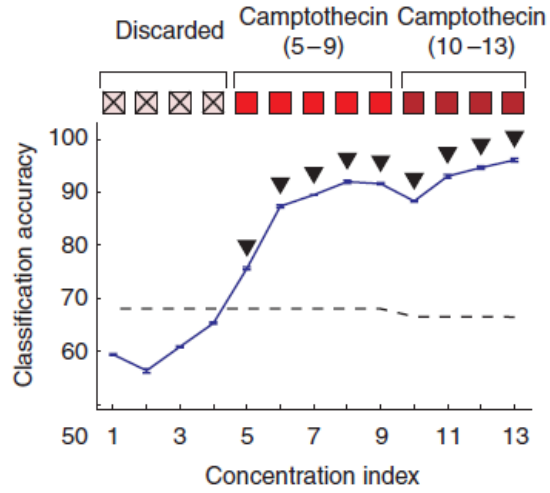


Scheeder et al. (2018)



Loo et al. (2007) Nature Methods.





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.

THANK YOU!