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Development and Use of a High Content Imaging-Based Phenotypic Profiling Assay for Chemical Bioactivity Screening

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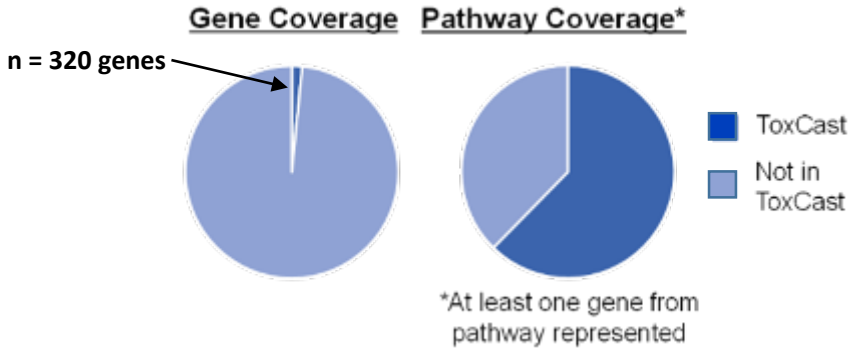
Outline

- **Background**
  - Computational Toxicology Strategic Vision
  - Phenotypic Profiling via Cell Painting
- **Study Design & Methodology**
  - Objectives
  - Experimental Design
  - Reference Chemicals
  - Laboratory Workflows
  - Data Analysis Pipeline
- **Results**
  - Building Phenotypic Profiles of Cell Morphology
  - Comparison of Reference Chemical Profiles
    - To Scientific Literature
    - Within Cell LInes
    - Across Cell Lines
- **Application**
    - In vitro bioactivity thresholds / PODs
    - Bioactivity – Exposure Ratio

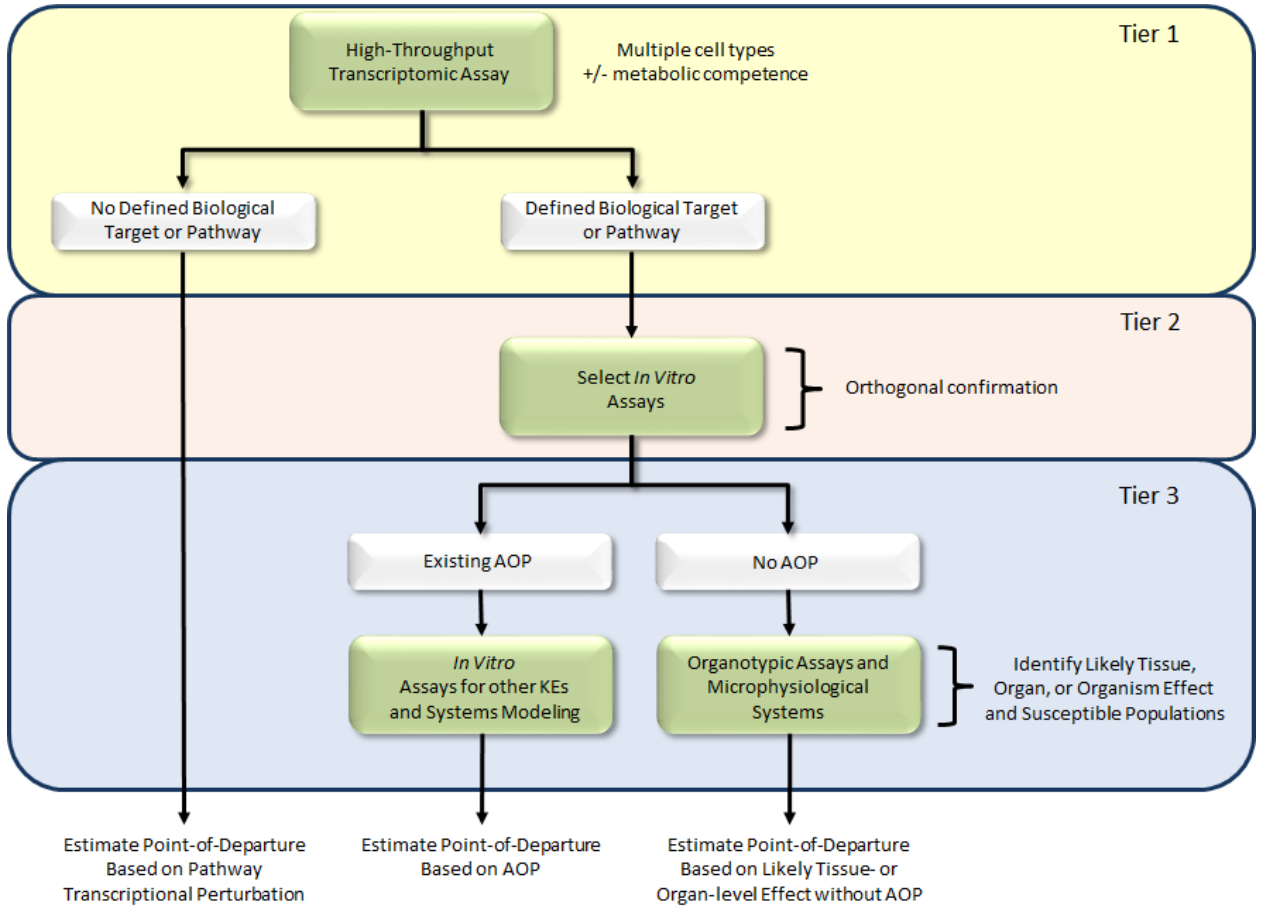
Background

- ToxCast assays cover many genes and pathways, but do not provide complete coverage of biological space.



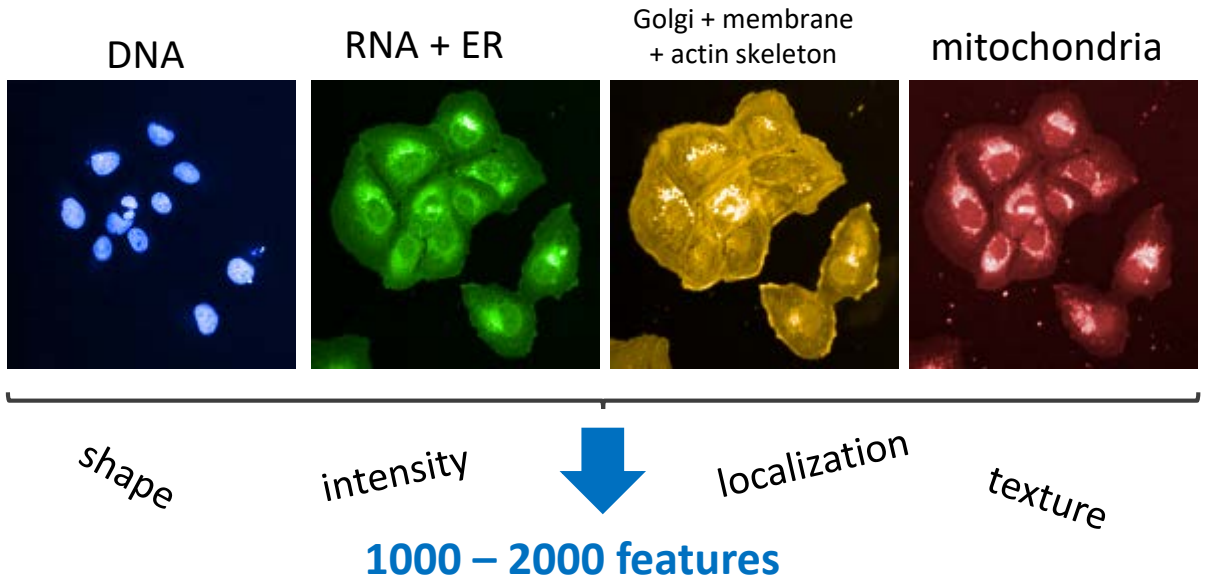
- **USEPA Strategic Vision and Operational Roadmap:**
  - Tier 1 strategy must cast the broadest net possible for capturing hazards associated with chemical exposure.
  - **Form follows function** → activation or inhibition of protein targets by chemicals may manifest as changes in cellular morphology.
  - Certain types of **high content imaging (HCI)** provides a cost effective means for profiling the effects of chemicals and identifying thresholds for chemical bioactivity.

A strategic vision and operational road map for computational toxicology at the U.S. Environmental Protection Agency



High Content Imaging-Based Phenotypic Profiling

- A chemical screening method that measures a large variety of morphological features of individual cells in *in vitro* cultures.
- Successfully used for functional genomic studies and in the pharmaceutical industry for compound efficacy and toxicity screening.
- No requirement for *a priori* knowledge of molecular targets.
- May be used to identify bioactivity thresholds for "dirty chemicals" (i.e. chemicals that affect many cellular proteins or processes simultaneously at a given test concentration).



- **Cell Painting (Bray et al., 2016, *Nature Protocols*):** A cell morphology-based phenotypic profiling assay multiplexing six fluorescent "non-antibody" labels, imaged in five channels, to evaluate multiple cellular compartments and organelles.

Study Objectives

1. Develop a microfluidics-based laboratory workflow for cell plating, chemical exposures and fluorophore labeling based on the Cell Painting assay (Bray et al. 2016).

2. Develop image acquisition protocols, analysis workflows and a data processing pipeline for highly-multiplexed measurements of cellular morphology

3. Identify a small set of phenotypic reference chemicals and:
   a. Screen in concentration-response mode in multiple cell types.
   b. Evaluate reproducibility of observed phenotypes as compared to literature
   c. Identify chemicals & test concentrations for use as reference chemical controls in screening applications.

4. Evaluate the applicability of the Cell Painting assay for:

   a. Identification and/or grouping chemicals with similar biological effects
   b. Derivation of *in vitro* bioactivity thresholds
   c. Use in screening level risk assessments → bioactivity exposure ratio (BER) approach.

Experimental Design

Parameter	Multiplier	Notes	
Cell Type(s)	6	U-2 OS [a] MCF-7 [b] A549 [b] HTB-9 [b] ARPE-19 HepG2	Bone Breast Lung Urinary bladder Retina Liver
Culture Condition	1	DMEM + 10% HI-FBS	
Chemicals	16	14 phenotypic reference chemicals 2 negative control chemicals	
Time Points:	1	48 hours	
Assay Formats:	2	Cell Painting HCI Cell Viability & Apoptosis	
Concentrations:	8	3.5 $\log_{10}$ units; semi $\log_{10}$ spacing	
Biological Replicates:	3	--	

[a] Reference cell line (Bray et al. 2016).
[b] Previously characterized using Cell Painting (Gustafdottir et al. 2013).

Reference Chemical Set

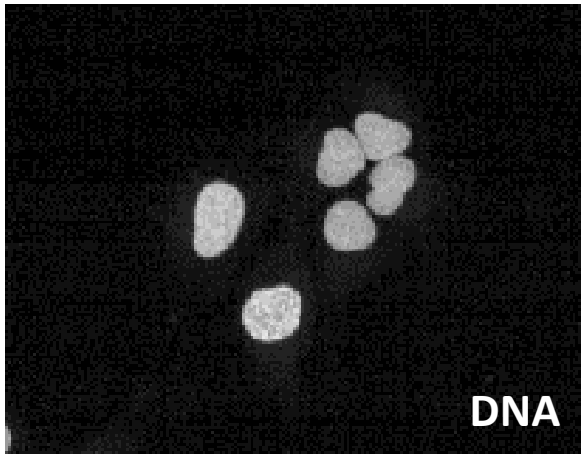
- Reference chemicals (n=14) with narrative descriptions of observed phenotypes were identified from Gustafdottir et al. 2013.
- Candidate negative control chemicals (n=2) with no anticipated effect on cell phenotype were included in the reference set.

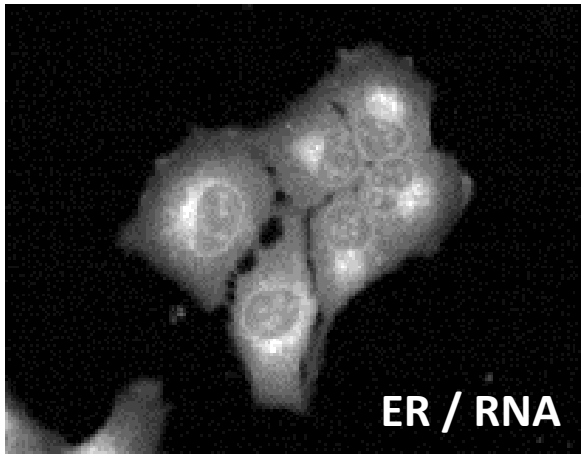
Compound Name	Chemical Use	Expected Phenotype
Amperozide	Atypical antipsychotic	Toroid nuclei
Berberine Chloride	Tool Compound (Mitochondria complex I inhibitor)	Redistribution of mitochondria
Ca-074-Me	Tool Compound (Cathepsin B inhibitor)	Bright, abundant golgi staining
Etoposide	Chemotherapeutic	Large, flat nucleoli
Fenbendazole	Anthelmintic	Giant, multi-nucleated cells
Fluphenazine	Typical antipsychotic	Enhanced golgi staining and some cells with fused nucleoli
Latrunculin B	Tool compound (Actin polymerization inhibitor)	Actin breaks
Metoclopramide	Anti-nausea medication	Enhanced golgi staining and some cells with fused nucleoli
NPPD	Spy Dust	Redistribution of ER to one side of the nucleus
Oxibendazole	Anthelmintic	Large, multi-nucleated cells with fused nucleoli
Rapamycin	Macrolide antibiotic / antifungal	Reduced nucleolar size
Rotenone	Insecticide	Mitochondrial stressor
Saccharin	Artificial Sweetener	Negative Control
Sorbitol	Artificial Sweetener	Negative Control
Taxol	Chemotherapeutic (Microtubule Stabilizer)	Large, multi-nucleated cells with fused nucleoli
Tetrandrine	Antiinflammatory	Abundant ER

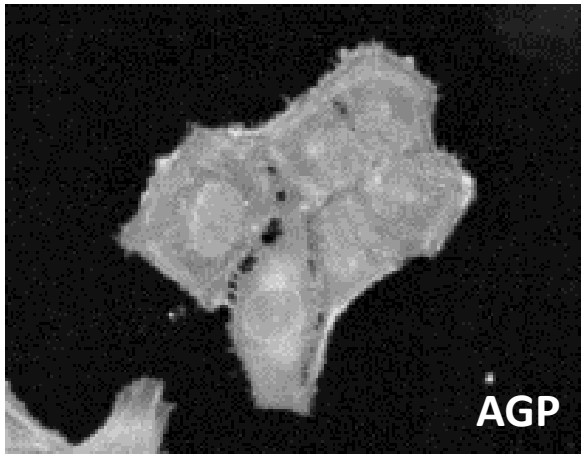
Gustafsdottir, et al. 2013

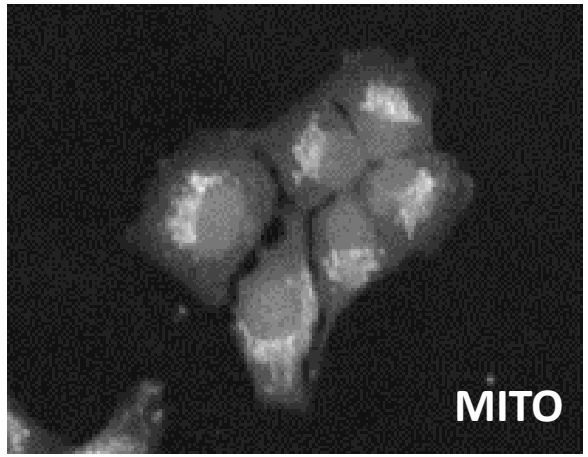
Fluorescent Labeling Scheme

Marker	Cellular Component	Labeling Chemistry	Labeling Phase	Opera Phenix	
				Excitation	Emission
Hoechst 33342	Nucleus	Bisbenzamide probe that binds to dsDNA	Fixed	405	480









Laboratory Workflow

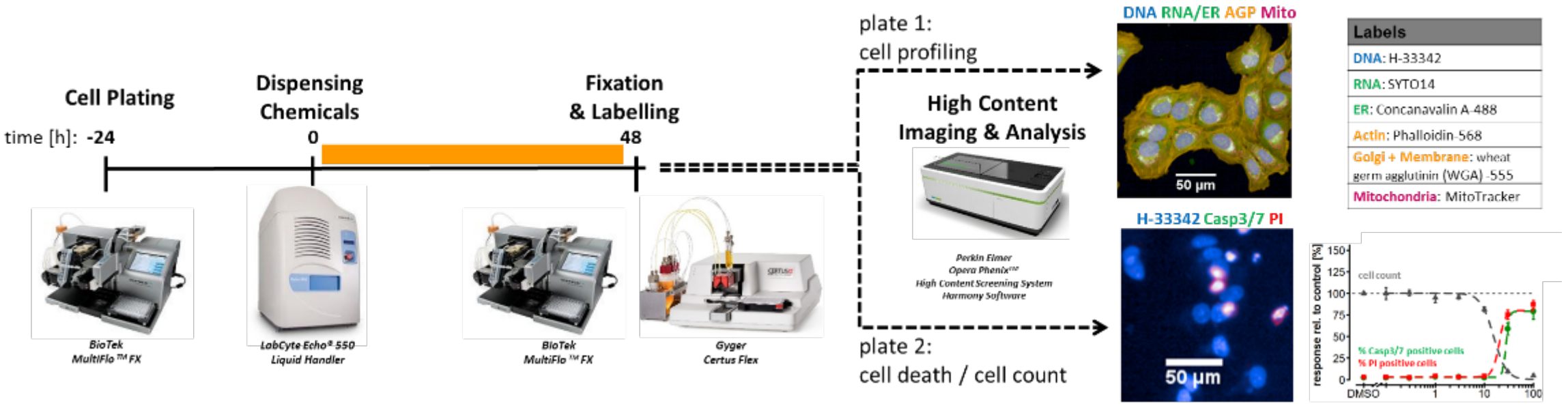


Image Acquisition

- Perkin Elmer Opera Phenix
- 20x Water Immersion Objective
- Confocal Mode, Single Z
- CellCarrier-384 Ultra Microplates

Image Analysis

- Perkin Elmer Harmony Software

Data Processing

- R Statistical Computing Environment
- BMDExpress 2.0

Image Analysis Workflow:
Nucleus and Cell Segmentation

1. find nuclei

2. find cell outline

3. reject border objects

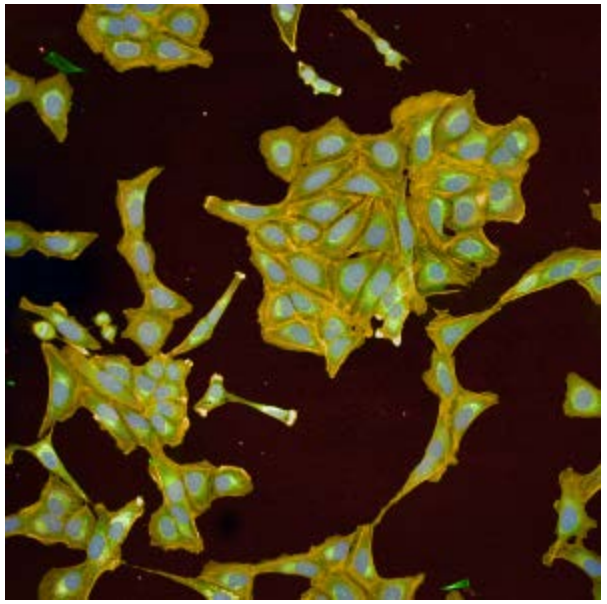
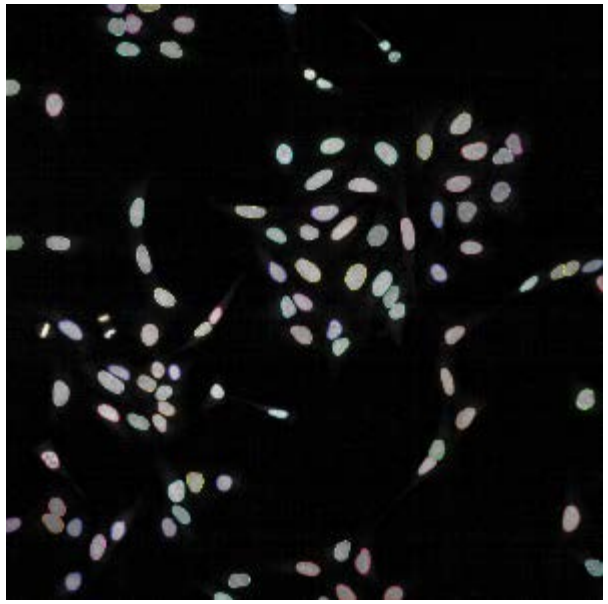
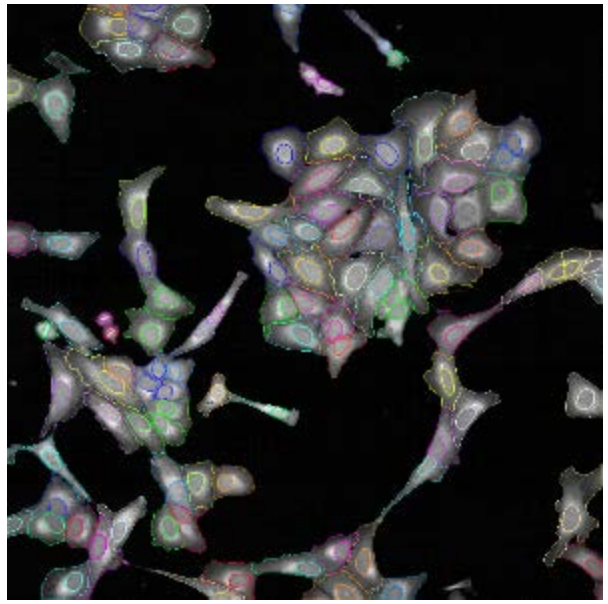
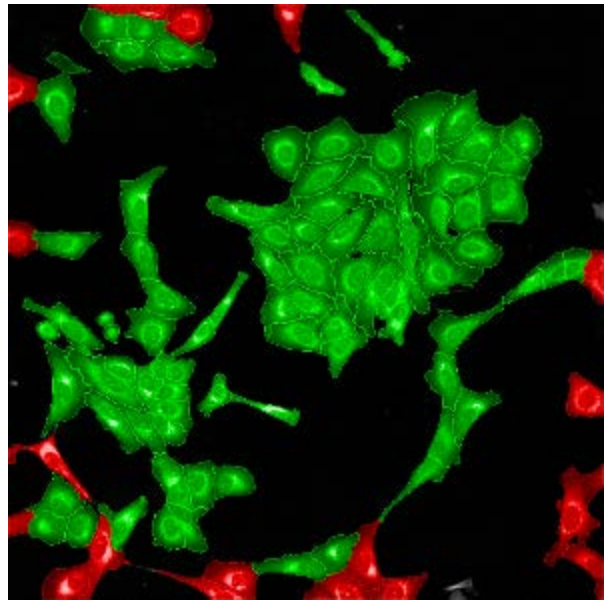
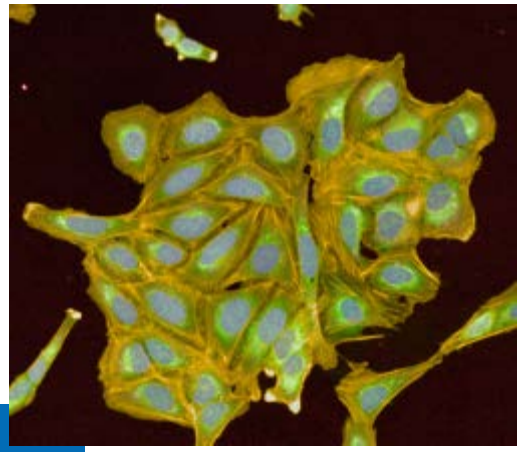
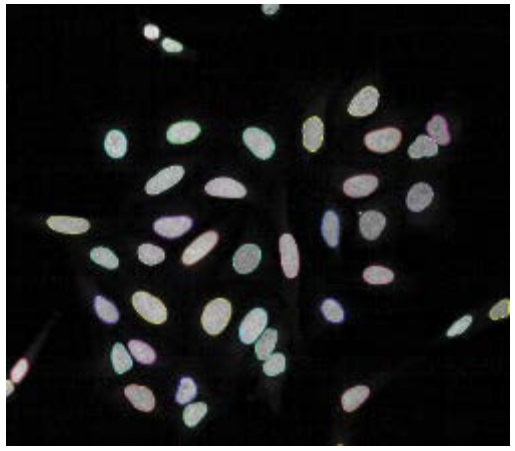
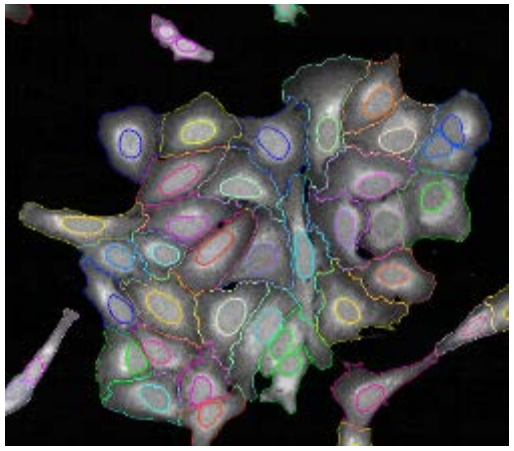
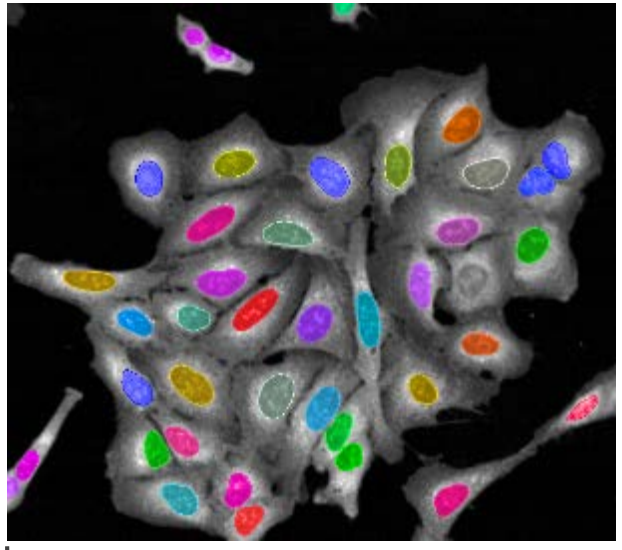








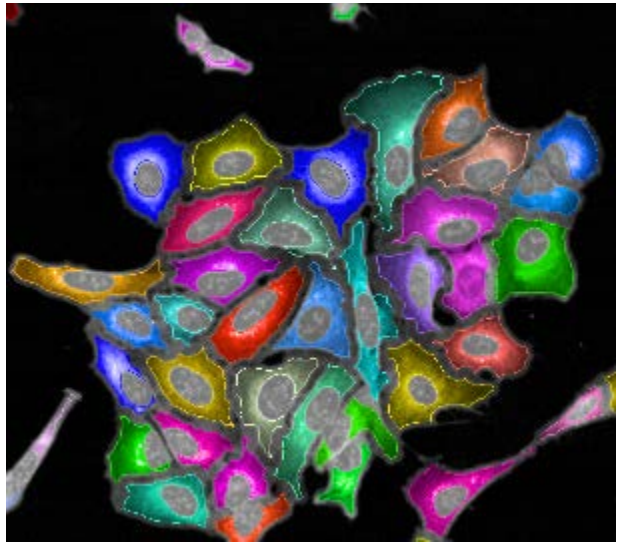
Image Analysis Workflow

Define Cellular Compartments

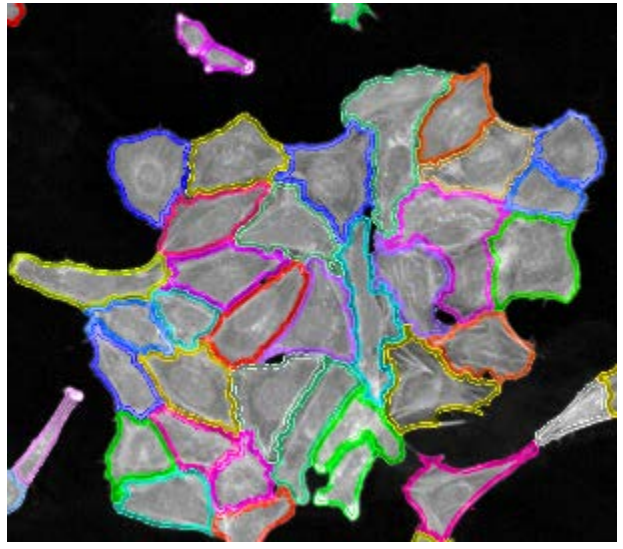
nuclei



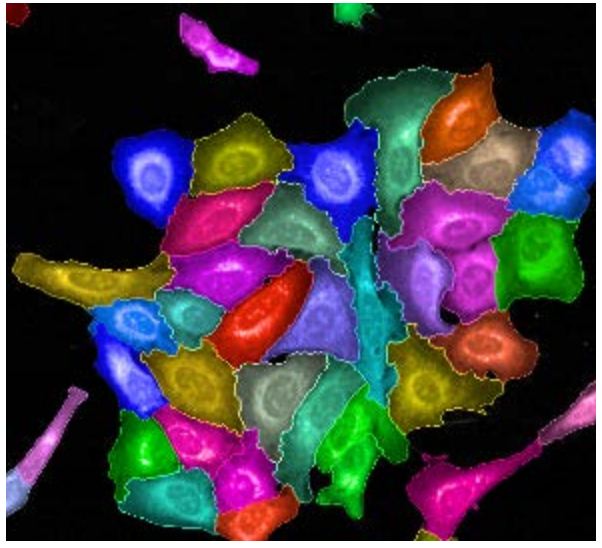
cytoplasm



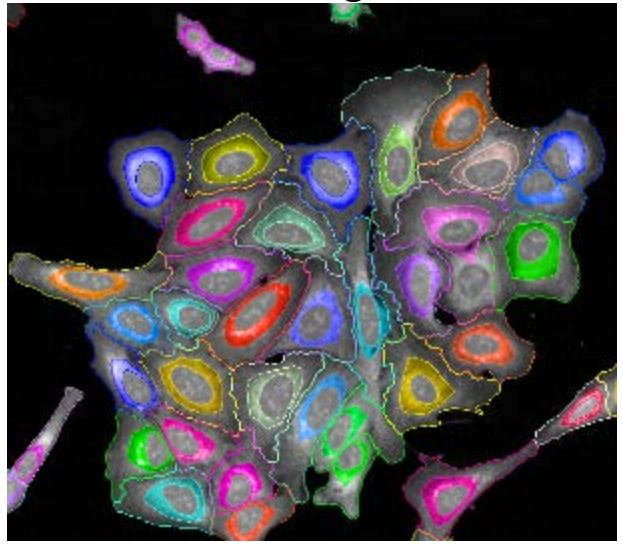
membrane



cell



ring



Morphological Profiling with Harmony Software

Compartments:
- cell
- nuclei
- ring
- cytoplasm
- membrane

X

Channels (organelles):
- DNA
- RNA
- ER
- Golgi + cytoskeleton + membrane
- mitochondria

X

Domains:
- shape / morphology
- intensity
- texture

= **1669 Measurements Per Cell**

	Cell	Nuclei	Ring	Cytoplasm	Membrane
"Shape"	M	M			
DNA	S, C, A R, P	S, C, A R, P, I, T			
RNA	S, C, A R, P	S, C, A R, P, I, T			
ER	S, C, A R, P		I, T	I, T	I
AGP	S, C, A R, P		I, T	I, T	I, T
Mito	S, C, A R, P		I, T	I, T	I

Features:

M = basic morphology [5 features]

M* = STAR morphology:

Symmetry [80]

Threshold **C**ompactness [40]

Axial [20]

Radial [28]

Profile [50, nuclei: 4]

I = Intensity [9]

T = Texture [14] (Haralick, Gabor & SER)

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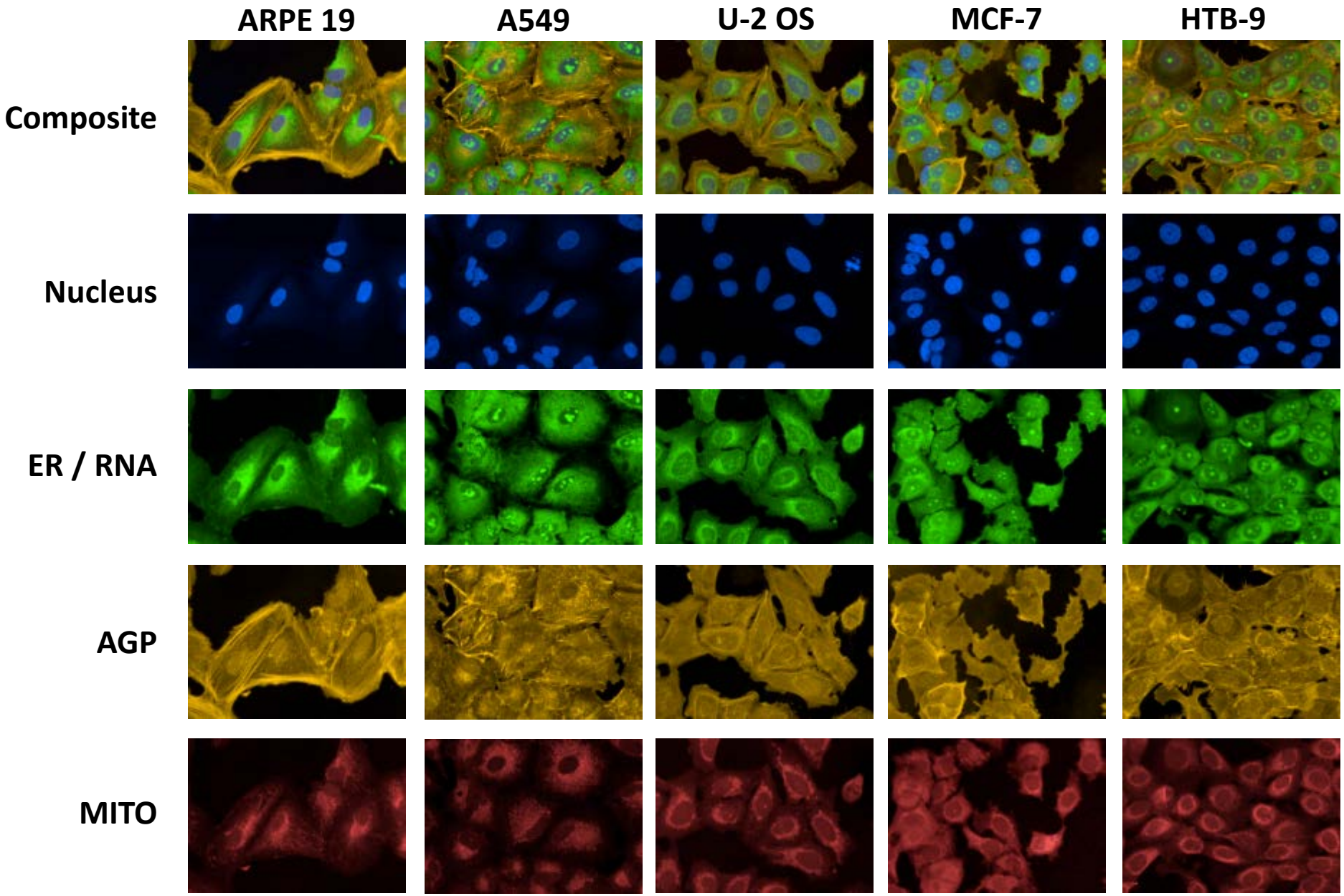
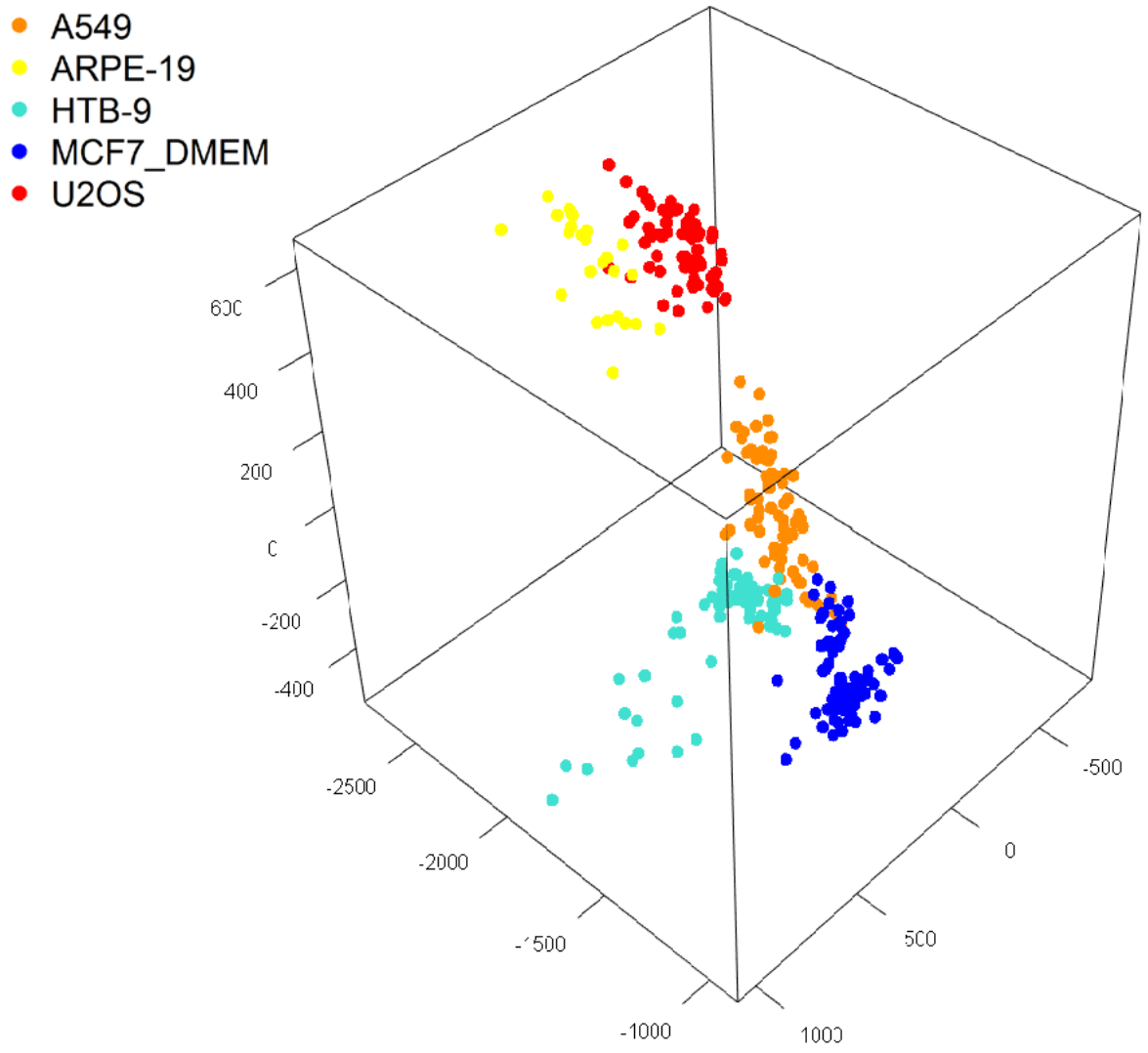
Morphological Heterogeneity Across Cell Lines



Morphological Heterogeneity Across Cell Line Panel



***Excludes intensity domain*

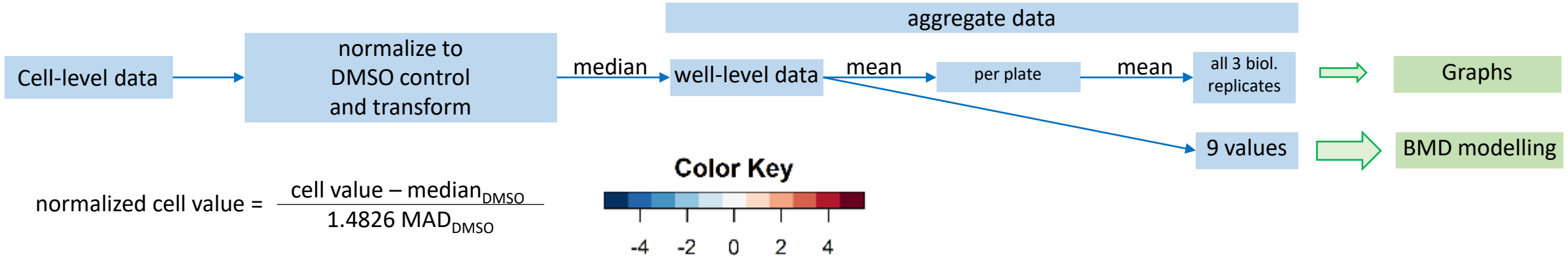
- Quantification of morphological features can distinguish different cell types.

Question:

- If the morphology of different cell lines is distinct, would response to a chemical treatment be similar or different across cell types?

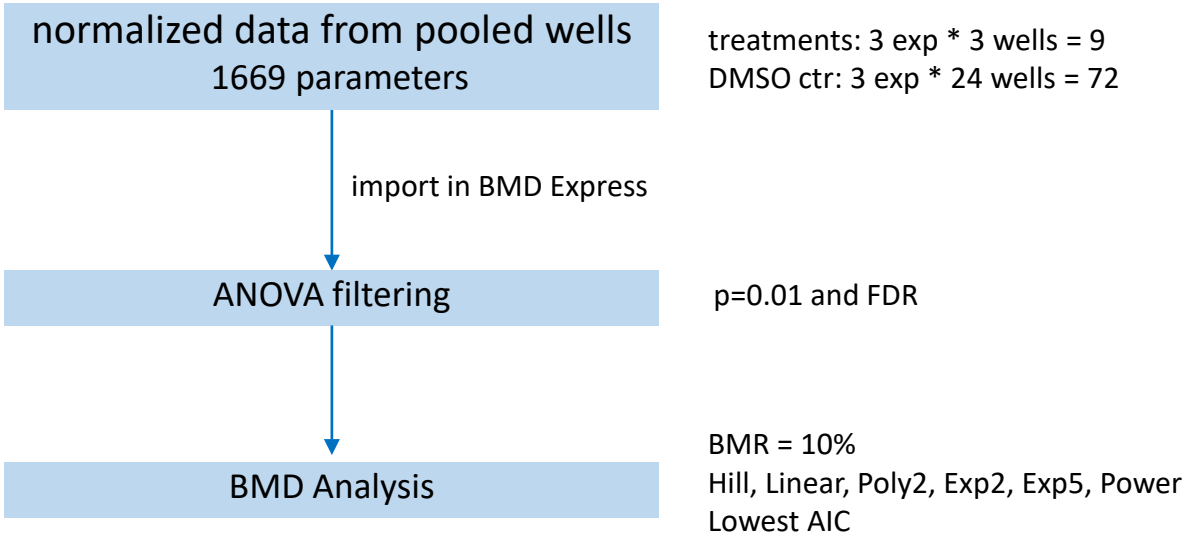
Data Analysis Pipeline

Part 1: Data Normalization and Summarization



$$\text{normalized cell value} = \frac{\text{cell value} - \text{median}_{DMSO}}{1.4826\ \text{MAD}_{DMSO}}$$

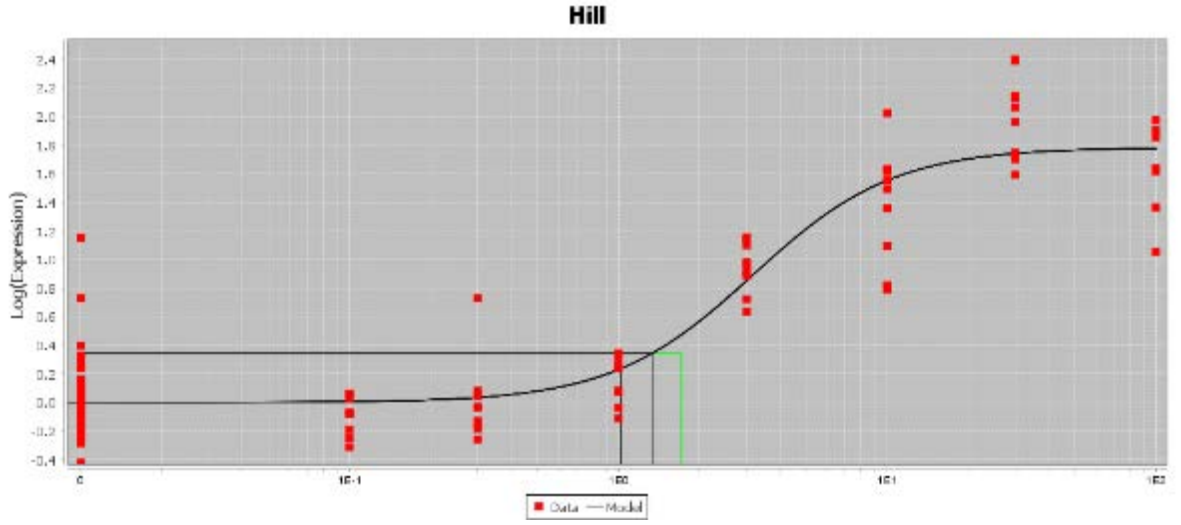
Part 2: Concentration-Response Modeling



treatments: 3 exp * 3 wells = 9
DMSO ctr: 3 exp * 24 wells = 72

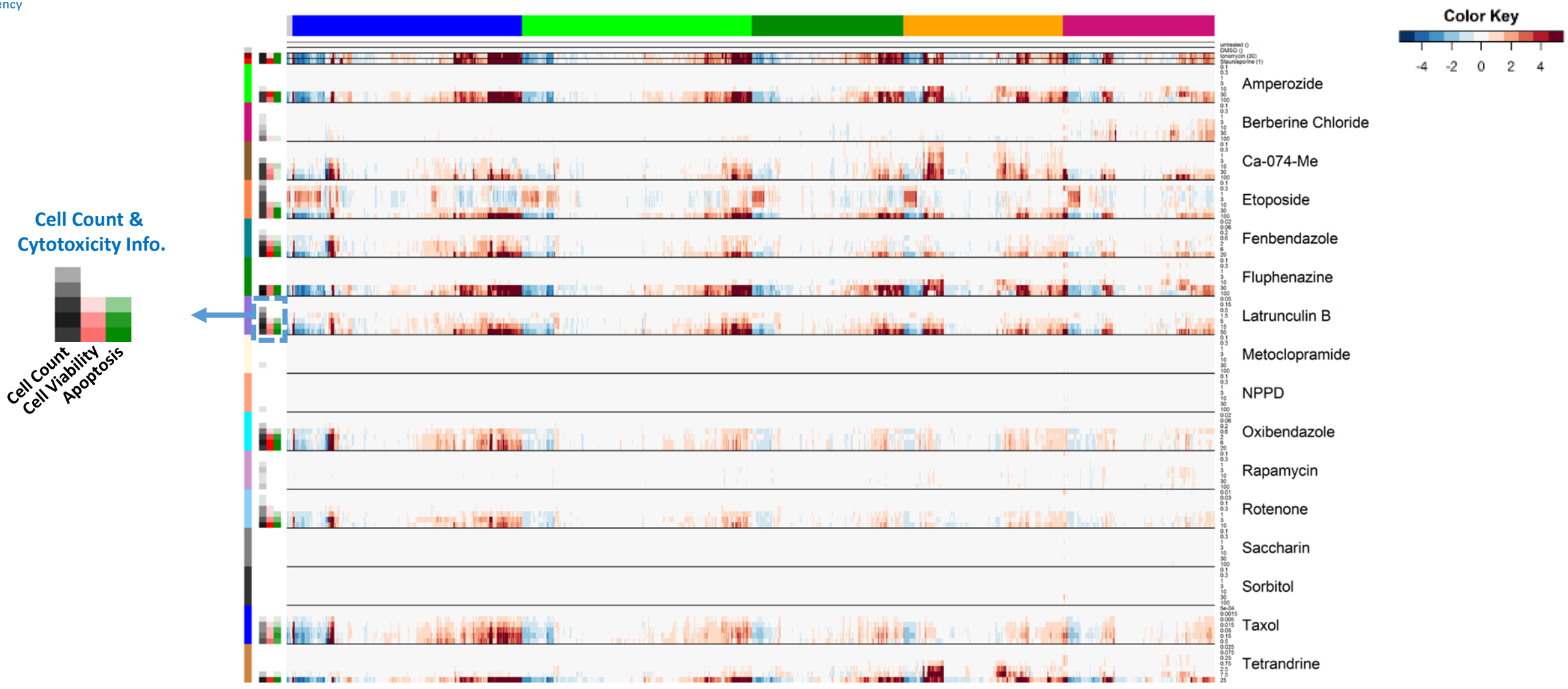
p=0.01 and FDR

BMR = 10%
Hill, Linear, Poly2, Exp2, Exp5, Power
Lowest AIC



Berberine chloride
Mito_Cells_Morph_STAR

Phenotypic Profiles for Reference Chemicals [U-2 OS]



- Unique phenotypic profiles observed across the reference chemical set.
- Some chemicals did not produce any effects.
- Effects on morphology observed at sub-cytotoxic concentrations.

Phenotypic Profiles Are Consistent with Previous Literature Studies

Parameters with marked effects:

Channel	Compartment	Domain
Mito	Cytoplasm	Texture
Mito	Cytoplasm + Ring	Intensity: Maximum
Mito	Entire Cell	Compactness (of the bright spots)

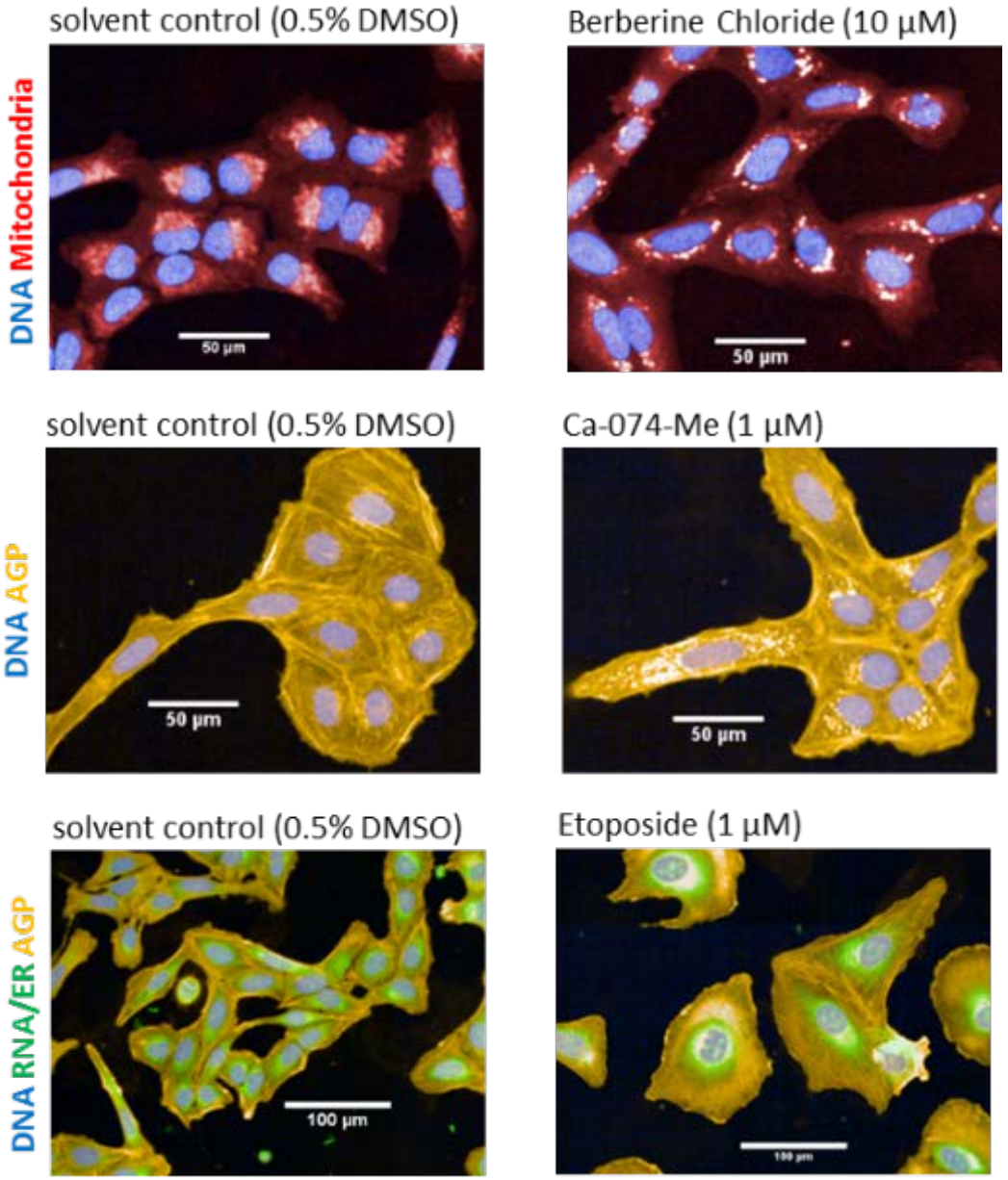
Literature: redistribution of mitochondria

Channel	Compartment	Domain
AGP	Cytoplasm + Ring	Texture
AGP	Cytoplasm + Ring	Intensity: Maximum
AGP	Entire Cell	Profile (=distribution of intensity)

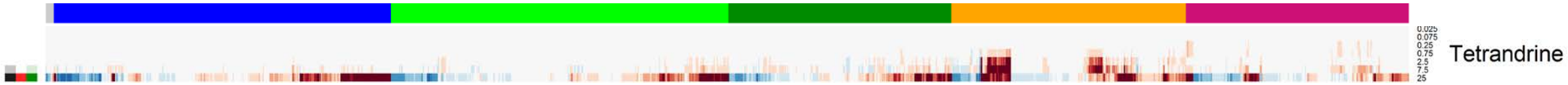
Literature: bright, abundant Golgi stain

Channel	Compartment	Domain
"Shape"	Entire Cell	Morphology: Area, Length, Width
DNA + RNA	Nuclei	Compactness (of the bright spots) Texture
ER + AGP	Cytoplasm + Ring	Intensity: Sum
all	Entire Cell	Morphology: intensity distribution

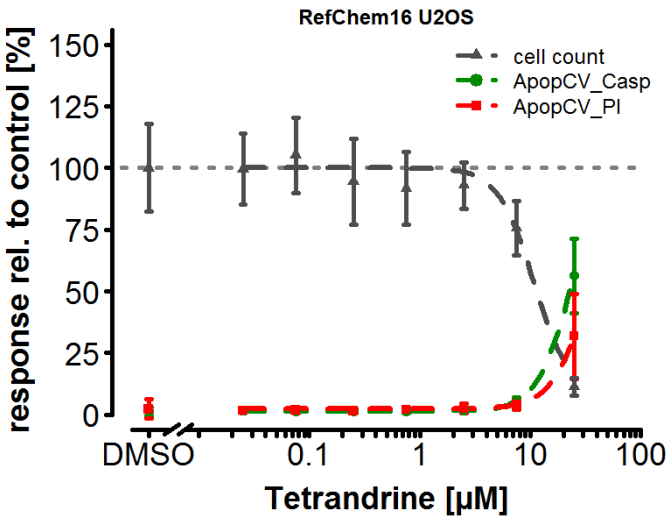
Literature: large, flat nucleoli



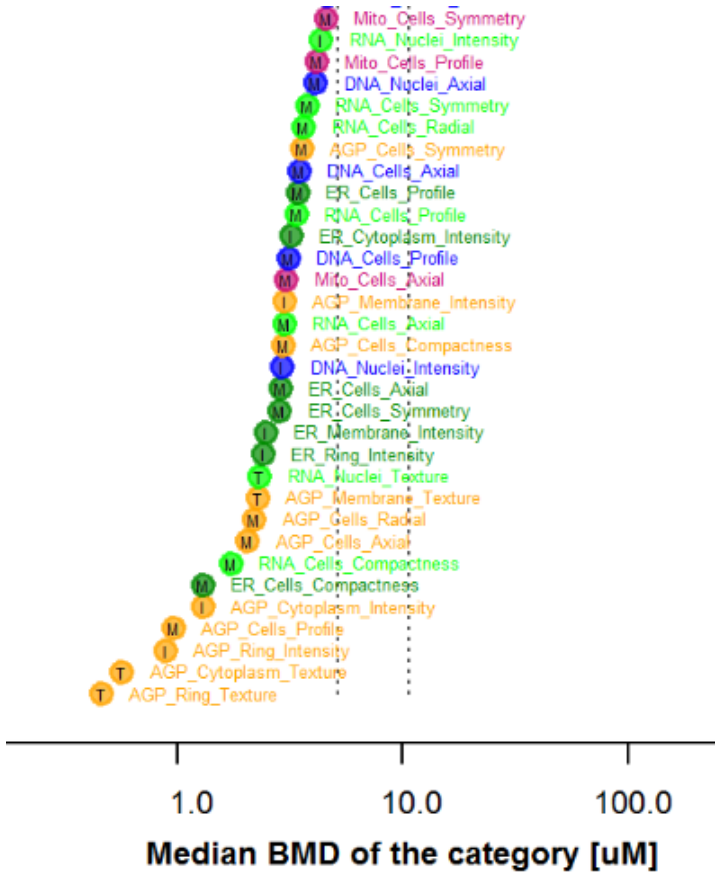
Visualizing Phenotypic Profiles



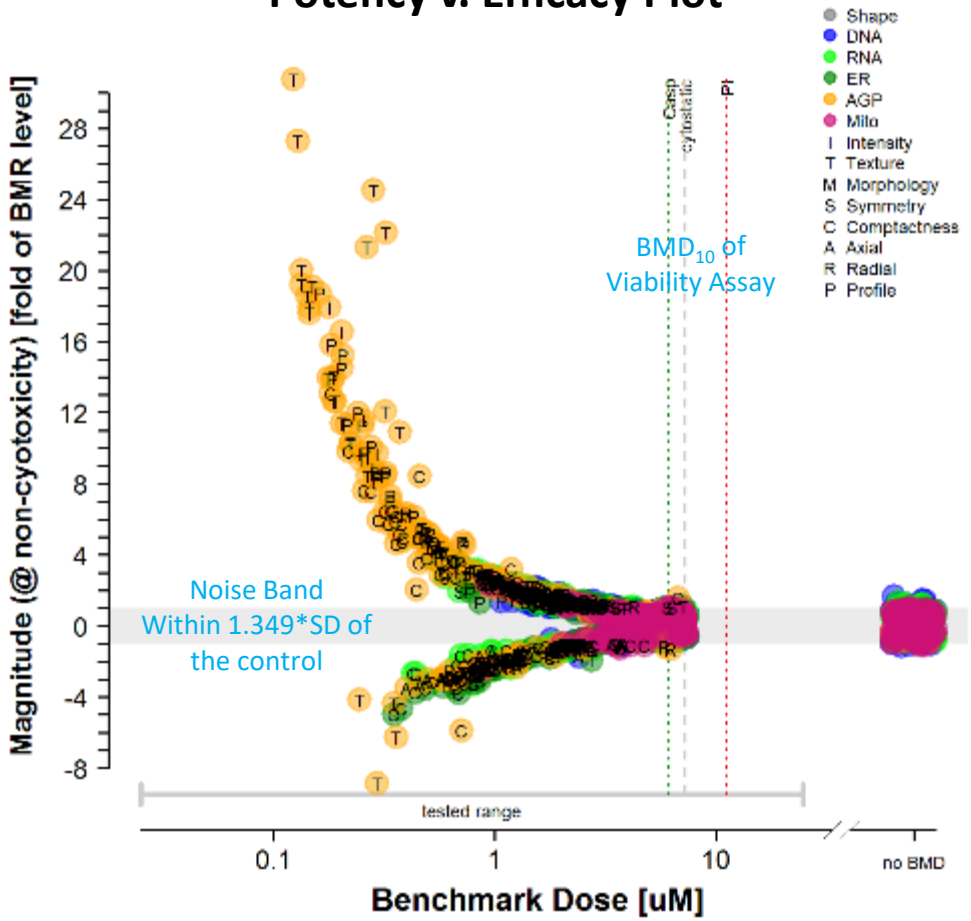
Cell Viability Assay



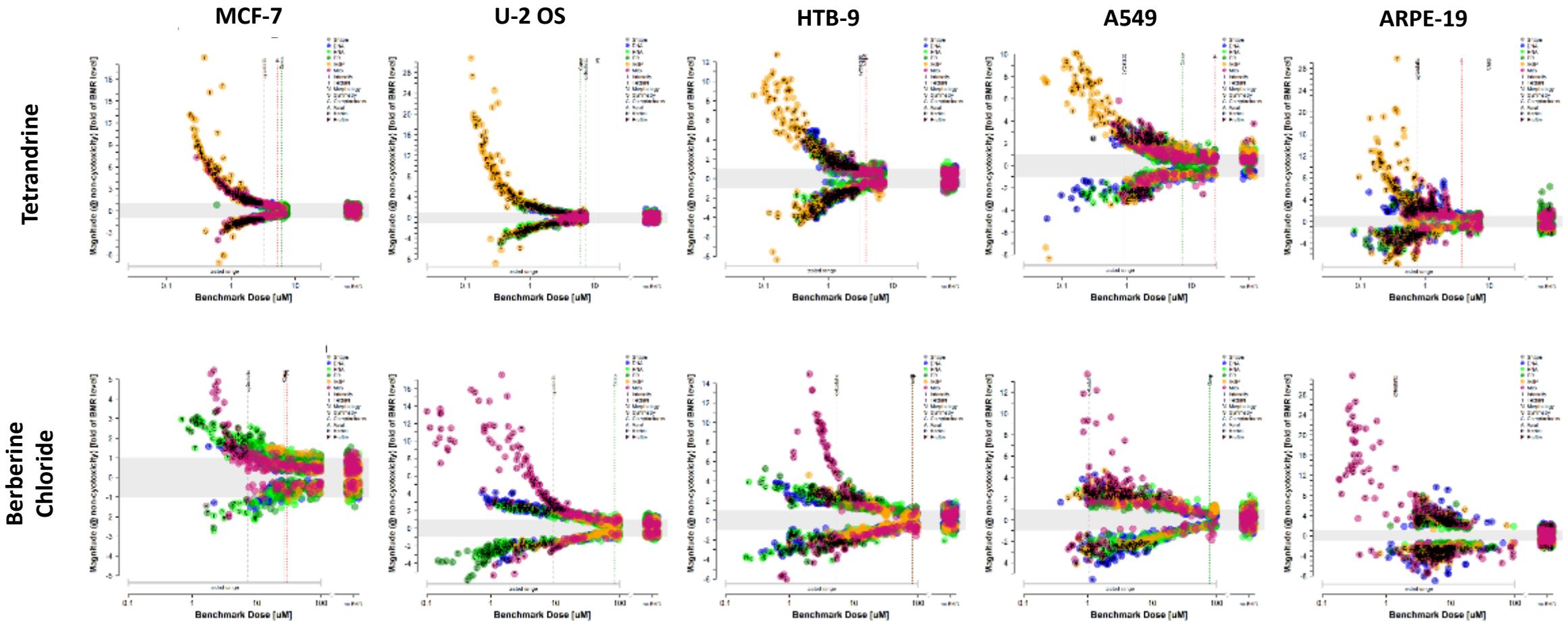
Phenotypic Onotology Accumulation Plot



Phenotypic Onotology Potency v. Efficacy Plot

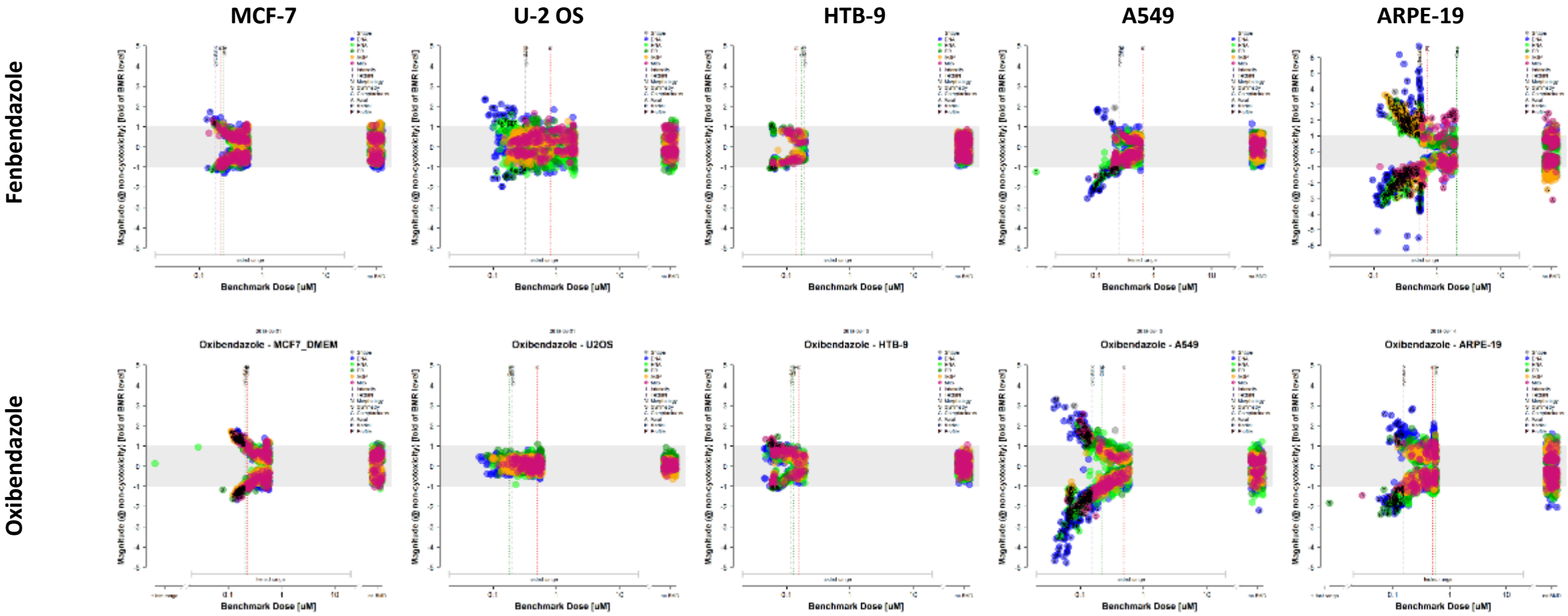


Comparing Phenotypic Profiles Across Cell Lines for a Chemical



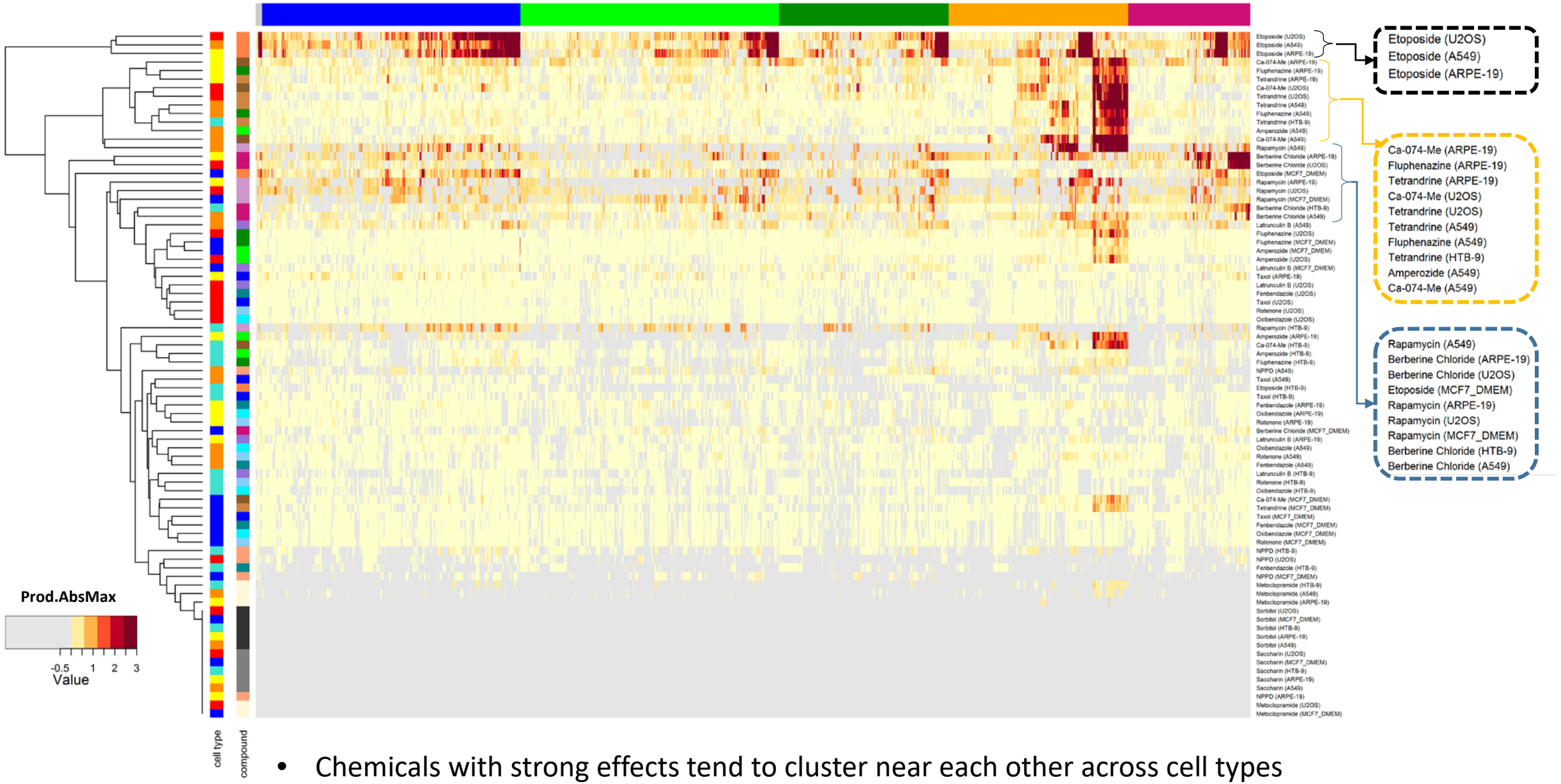
- Some chemicals produce similar phenotypes across the cell line panel

Comparing Phenotypic Profiles Across Cell Lines for a Chemical



- At times, a particular cell type is more sensitive to a chemical or greater magnitude of effects is observed.

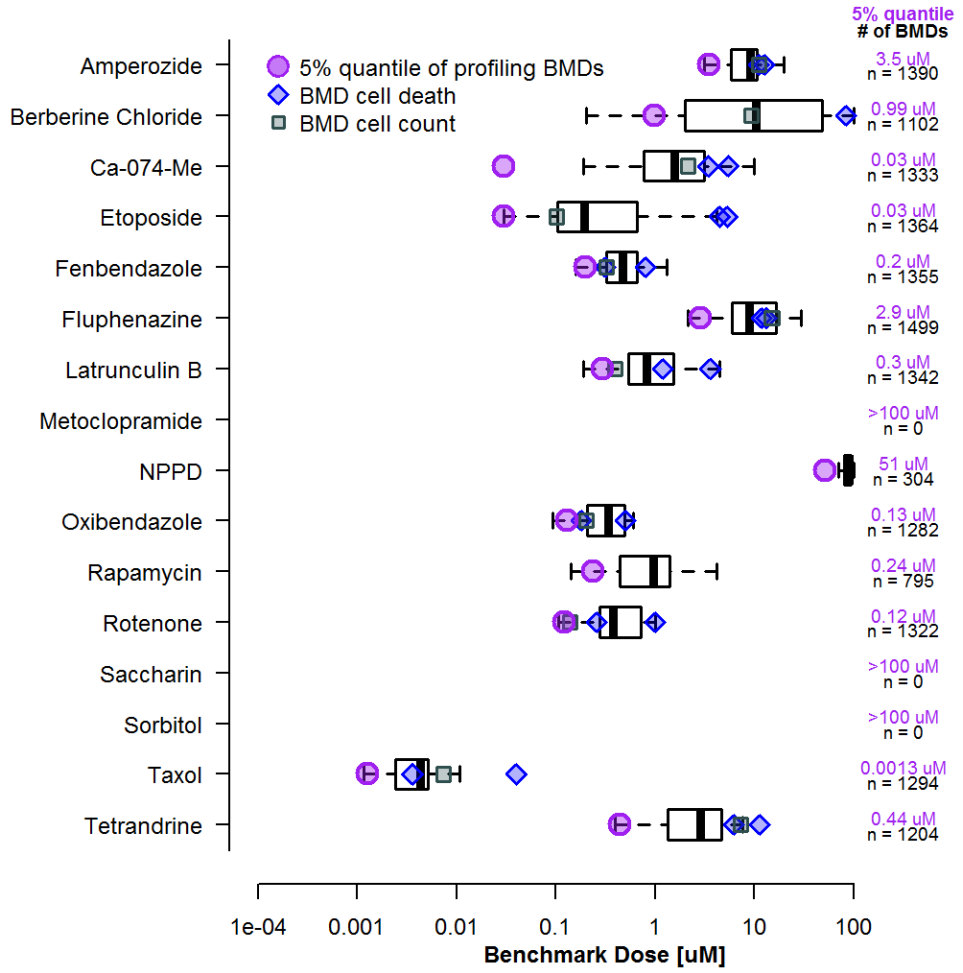
Clustering Based on Phenotypic Profiles



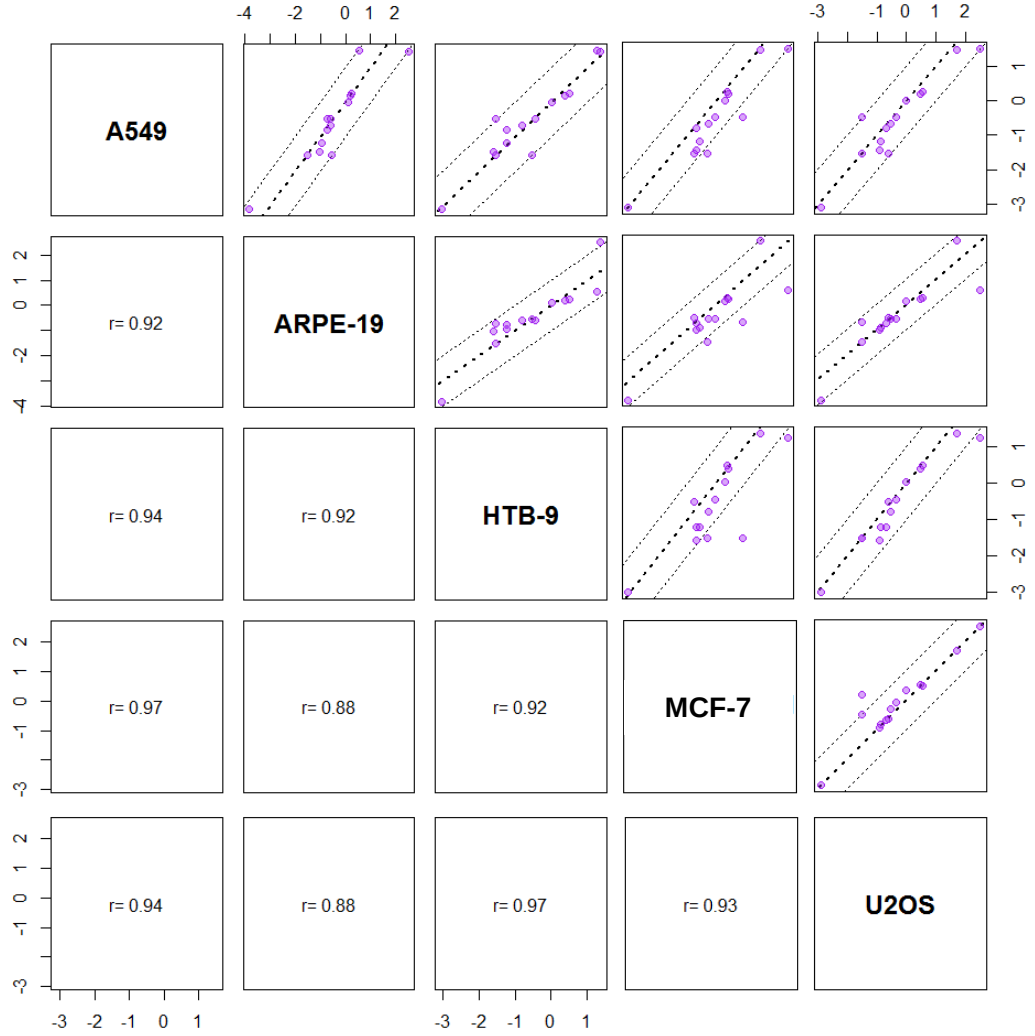
- Chemicals with strong effects tend to cluster near each other across cell types

Comparison of Bioactivity Thresholds Across Cell Lines

U-2 OS



5th Percentile of BMD Distribution

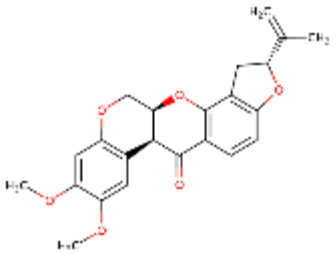


- Bioactivity estimates are strongly correlated across the cell line panel.

Bioactivity & Exposure Ratio Comparisons Using Reverse Dosimetry

BER Example Chemical

Rotenone
83-79-4 | DTXSID6021248



- Broad spectrum pesticide, insecticide and piscicide.
- Most uses in U.S. and Canada phased out.
- Known mitochondrial toxicant

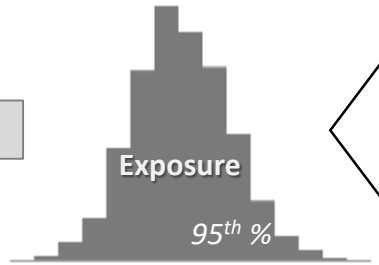
HTTP BMD_{10} (µM)

- *5th Percentile of phenotypic profile BMD10 distribution used.*

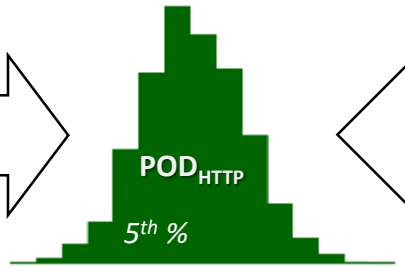
Apply high-throughput toxicokinetics (httk) to get mg/kg/day

- *Using httk v1.8 values for humans*
- *Default to a simple model with no partition coefficients and use of steady-state concentration.*
- *Assume 100% bioavailability and restrictive clearance.*

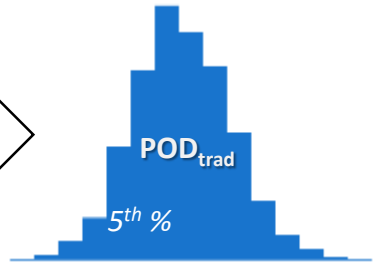
EPA - ExpoCast



Bioactivity-exposure ratio



POD_{trad} : POD_{HTTP} ratio



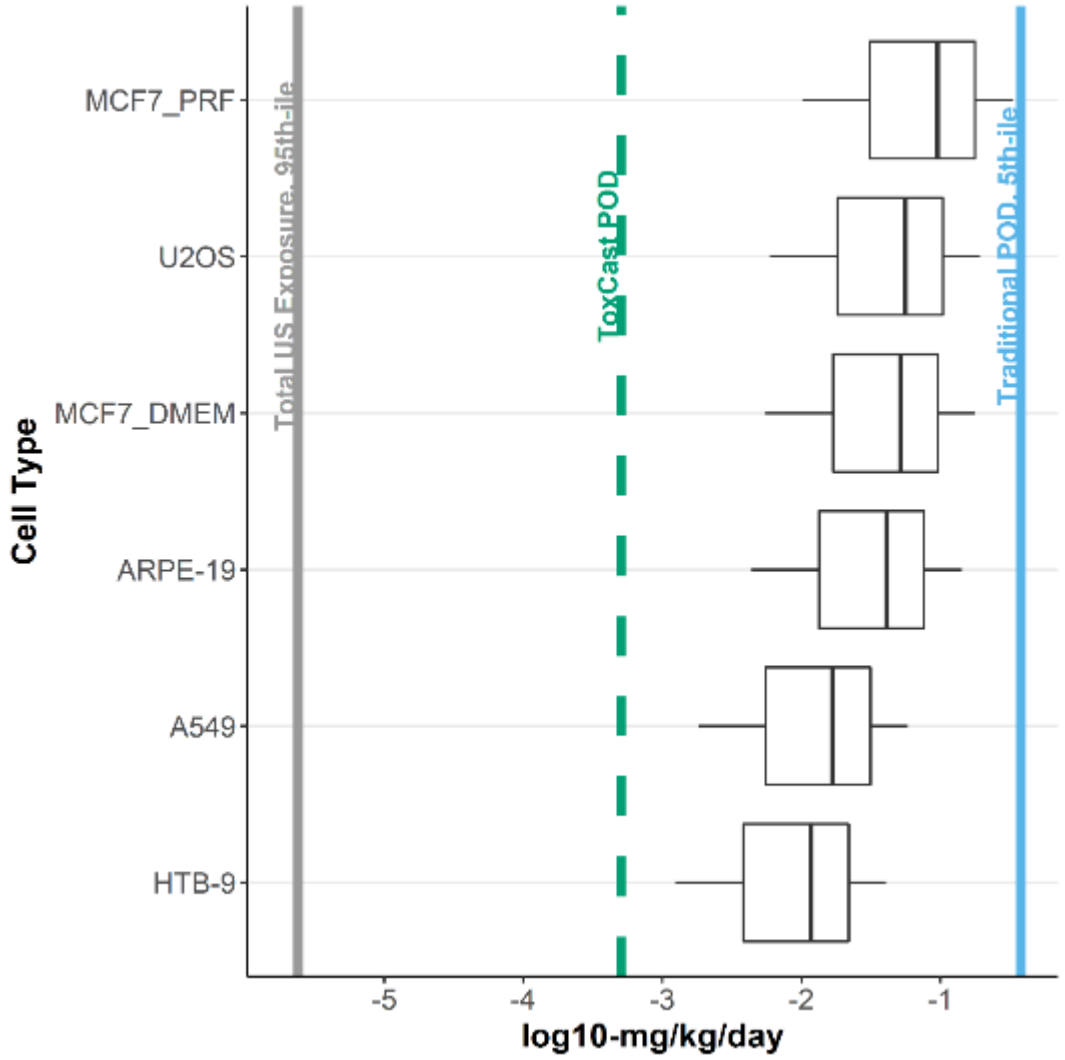
EPA - ToxValDB

- *NOEL, LOEL, NOAEL, or LOAEL*
- *Oral exposures*
- *Mg/kg/day*

- **Reverse dosimetry:** Conversion of a bioactivity value to an in vivo steady state concentration using high-throughput toxicokinetic (httk) modeling.
- Facilitates comparisons of biologically active in vitro concentrations to predicted human exposures and/or points-of-departure (PODs) from *in vivo* toxicology studies

Figure Courtesy of Katie Paul-Friedman

Bioactivity & Exposure Ratio Comparisons Using Reverse Dosimetry



For Rotenone:

- POD_{HTTP} slightly more conservative than POD_{trad}.
- POD_{HTTP} less conservative than $POD_{ToxCast}$.
- POD_{HTTP} well above upper limit of predicted exposures (ExpoCast) in the U.S. population.

Summary & Future Directions

Summary:

- Developed a microfluidics-based laboratory workflow for cell plating, chemical screening and fluorescent labeling of cells to evaluation of organelle morphology.
- Developed a high-content image analysis workflow (Harmony) and data analysis pipeline (R & BMDExpress2.0).
- Demonstrated that phenotypic profiles of reference chemicals tested in five cancer cell lines are comparable to observed phenotypes from the scientific literature.
- Demonstrated strong correlation of bioactivity thresholds (i.e. potency) of reference chemicals across cell lines.
- Identified reference chemicals for use in high throughput screening mode.

Future Directions:

- Concentration-response screening of chemicals from the ToxCast library in multiple cell types.
- Continue evaluating the utility of bioactivity exposure ratio (BER) analysis using HTTP data in the context of screening level risk assessments.
- Conduct time-course studies and evaluate the utility of HTTP data for determining tipping points (i.e. the transition from an adaptive to an adverse response).

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BONUS SLIDES

Comparing Phenotypic Profiles Across Chemicals [U-2 OS]