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Optimization and Application of Cell Painting, a High Content Imaging-Based Phenotypic Profiling Assay for Chemical Bioactivity Screening

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Disclaimer

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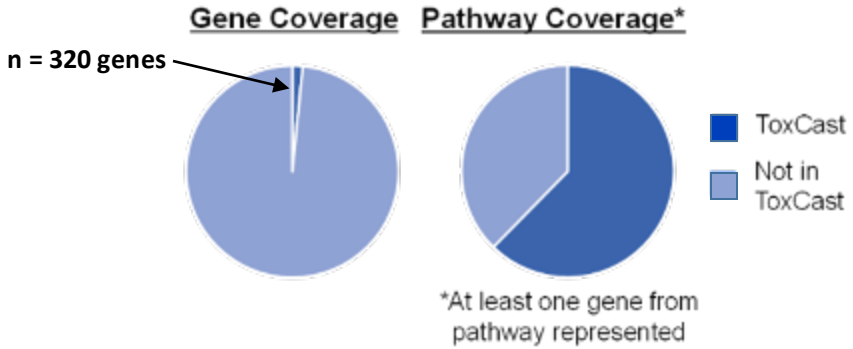
Outline

- **Background**
  - Computational Toxicology Strategic Vision
  - Phenotypic Profiling via Cell Painting
  - Project Objectives
- **Assay Development**
- **Identification and Screening of Phenotypic Reference Chemicals**
- **Explore Phenotypic Responses Across:**
  - Biological Space (i.e. cell types)
  - Exposure Duration
  - Chemical Space (i.e. ToxCast pilot)
- **Summary and Conclusions**

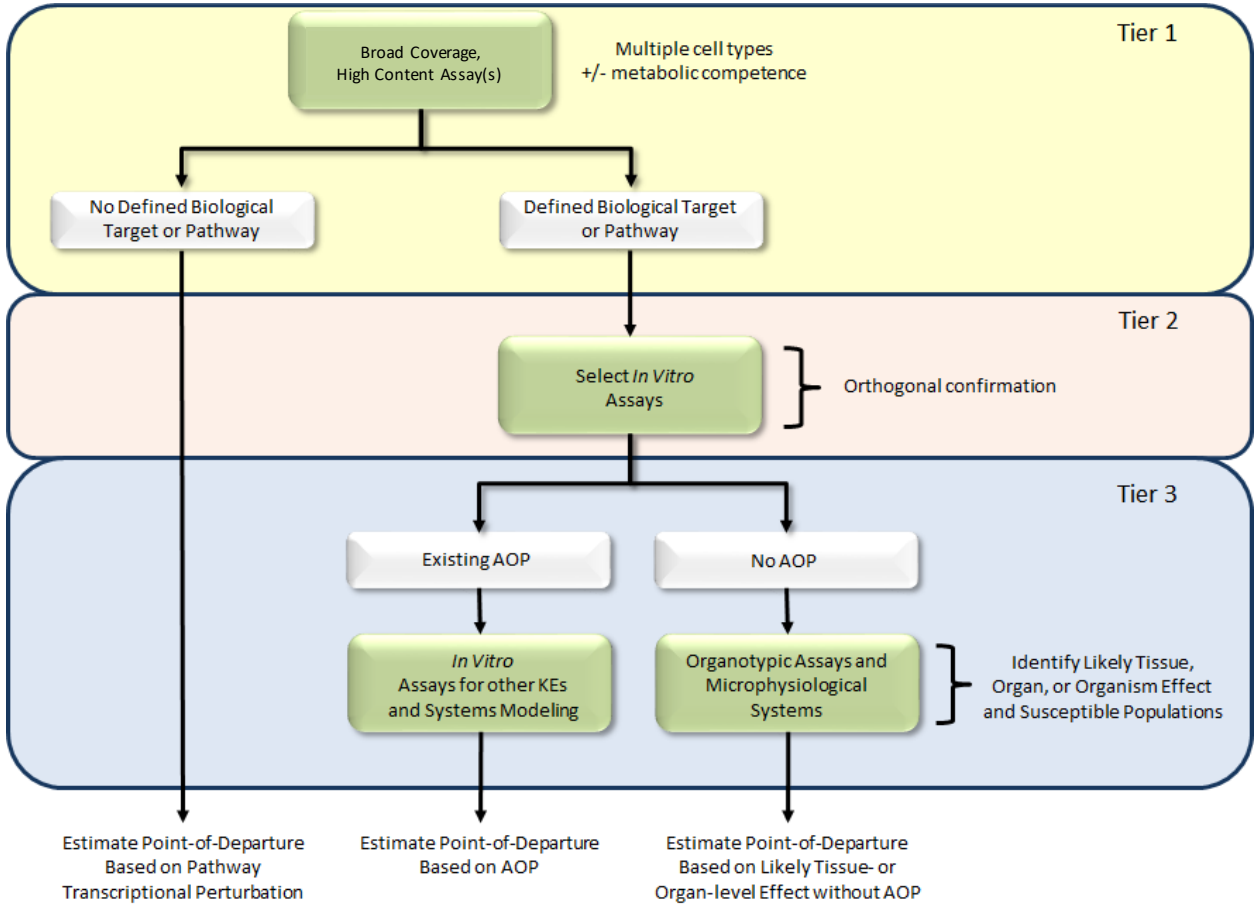
Background

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

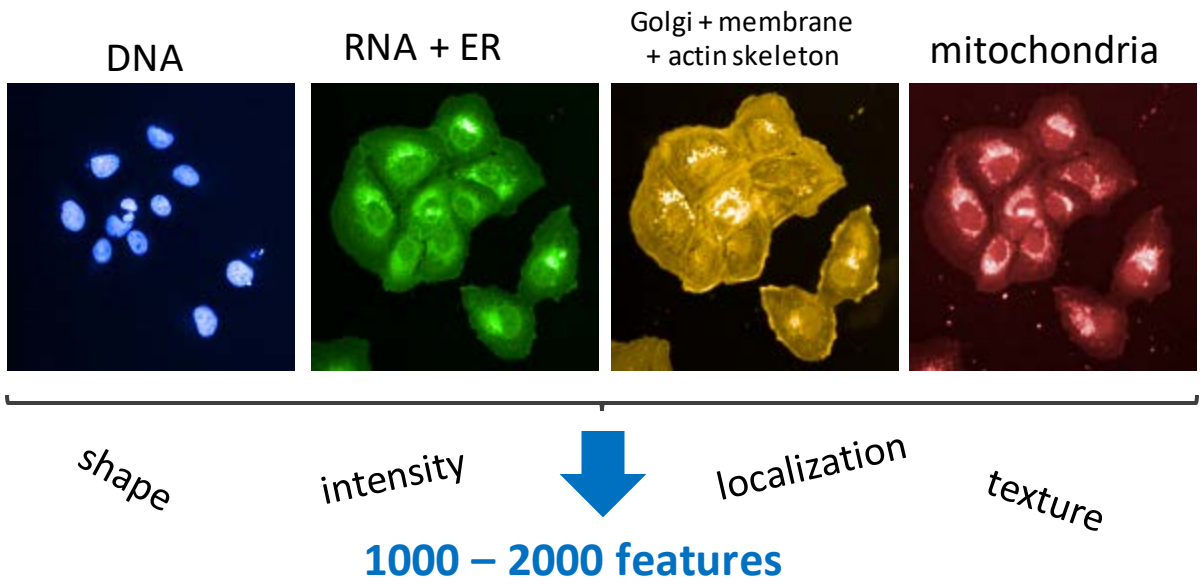


- **USEPA Strategic Vision and Operational Roadmap:**
  - Tier 1 strategy must cast the broadest net possible for capturing hazards associated with chemical exposure.
  - **Form follows function** → activation or inhibition of protein targets by chemicals may manifest as changes in cellular morphology.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

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



High Content Imaging-Based Phenotypic Profiling



- **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.

- 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 and MOA prediction.
- 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).

Project Objectives

Phase 1: **Methods development**

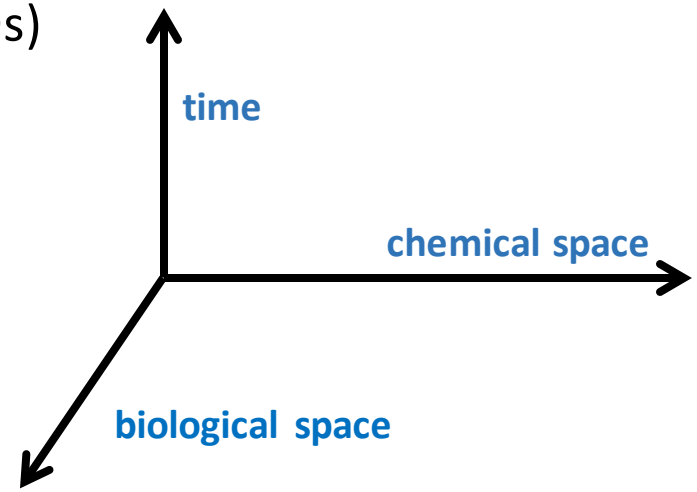
- Microfluidics-based laboratory workflow for cell plating, chemical exposures and fluorophore labeling based on the Cell Painting assay (Bray et al. 2016).
- Image acquisition protocols, analysis workflows and a data processing pipeline for highly-multiplexed measurements of cellular morphology

Phase 2: **Identify a small set of phenotypic reference chemicals and:**

- Screen in concentration-response mode in a reference cell type
- Evaluate reproducibility of observed phenotypes as compared to literature
- Identify reference chemicals for use in screening applications.
- Explore ways to calculate in vitro point-of-departures (PODs)

Phase 3: **Use phenotypic profiling to explore responses across:**

- Time
- Biological space (i.e. cell types)
- Chemical space (i.e. ToxCast)



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Phase 1: Assay Development

Laboratory Workflow

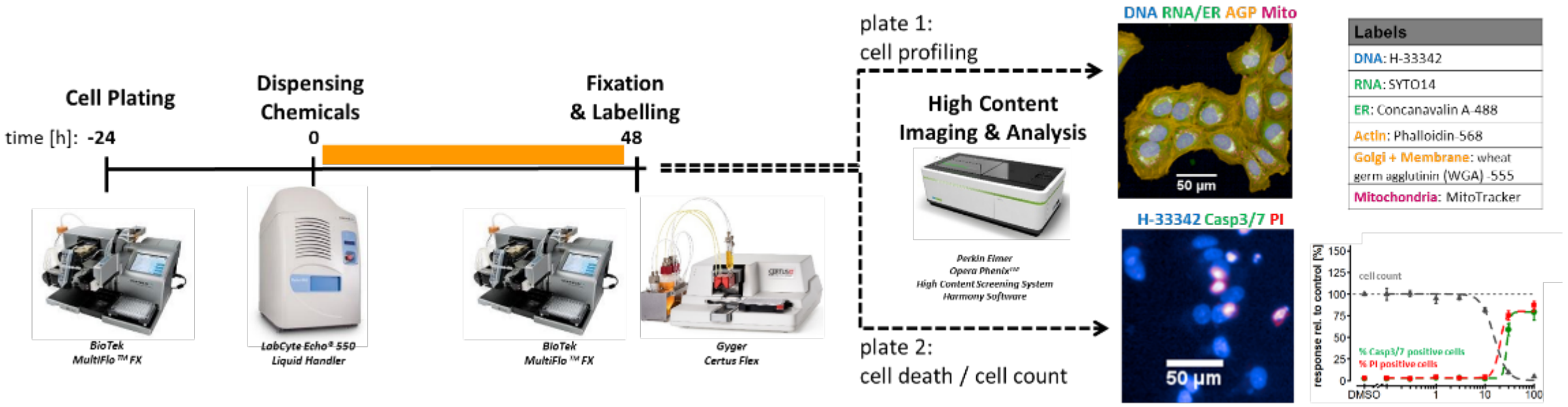

Image Acquisition

- 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

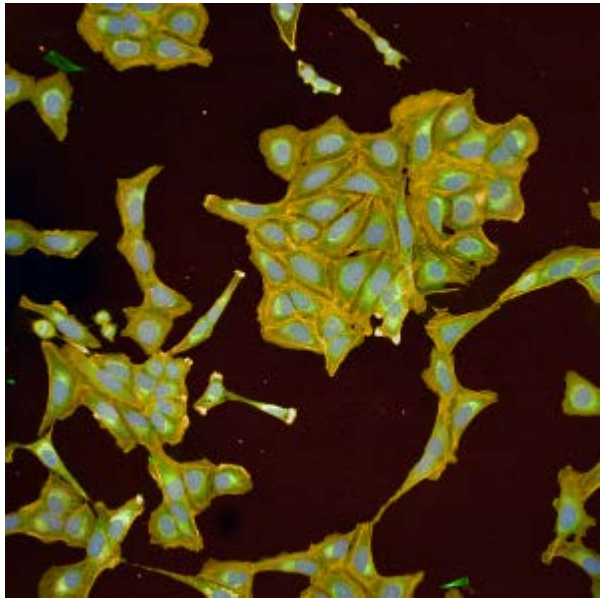
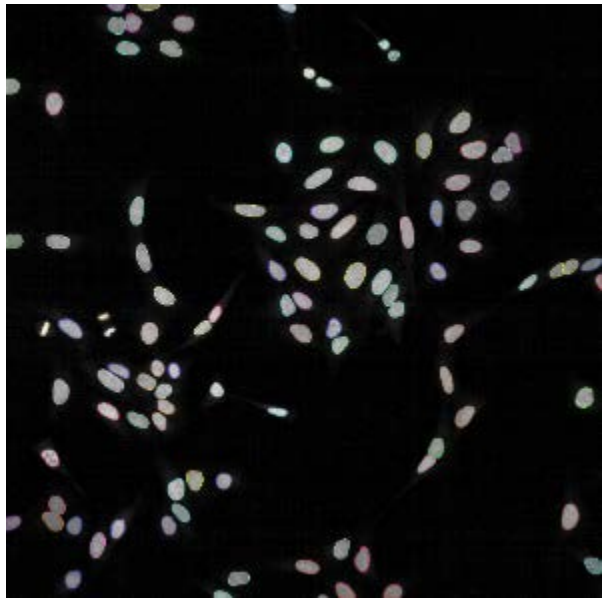
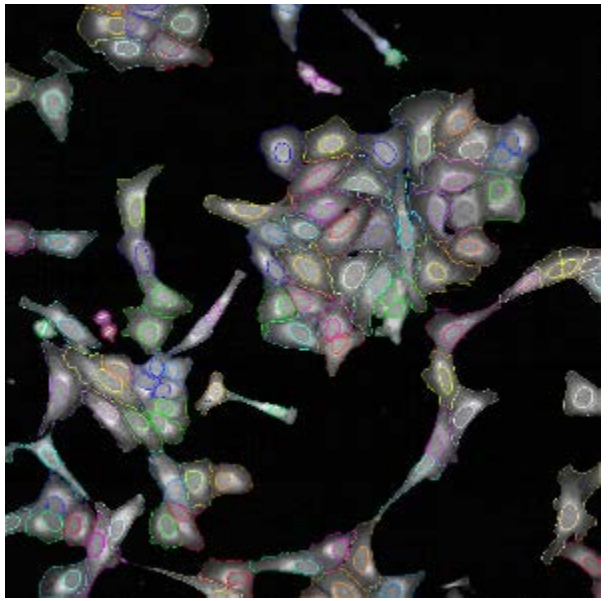
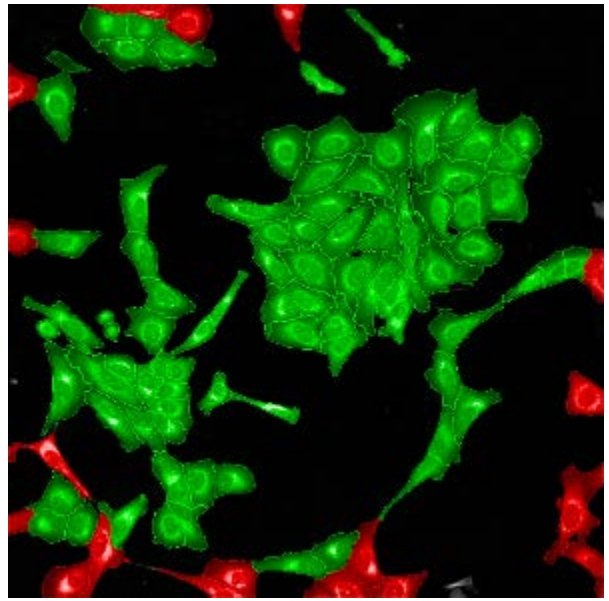
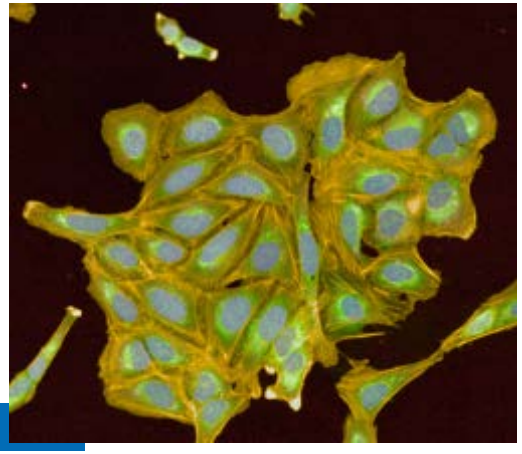
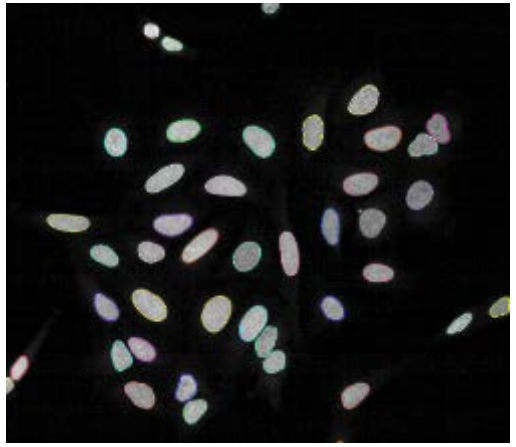
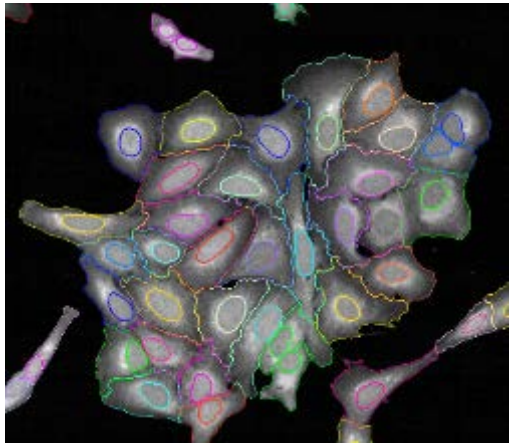

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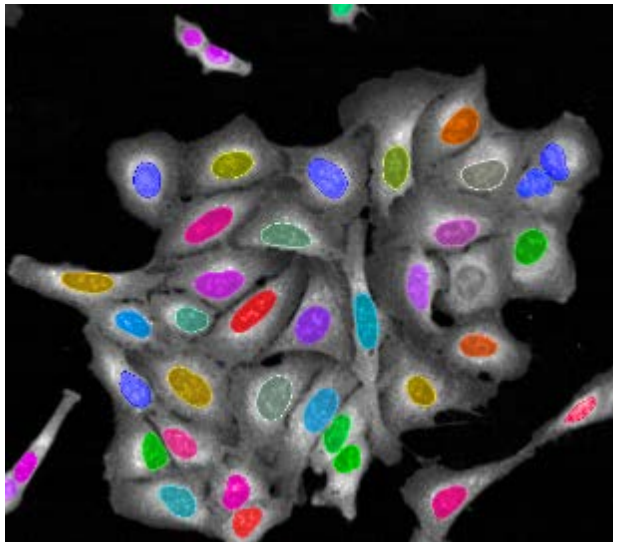
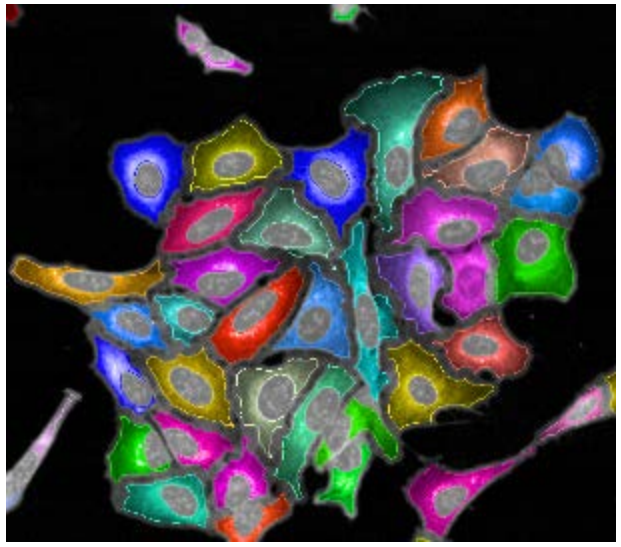
Image Analysis Workflow

Define Cellular Compartments

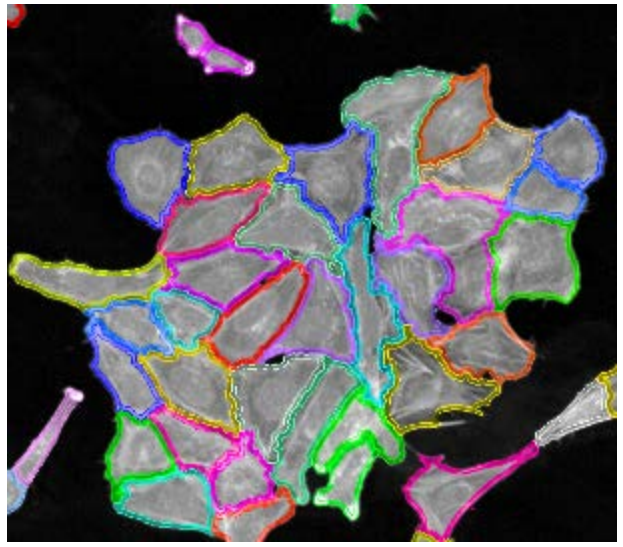
nuclei



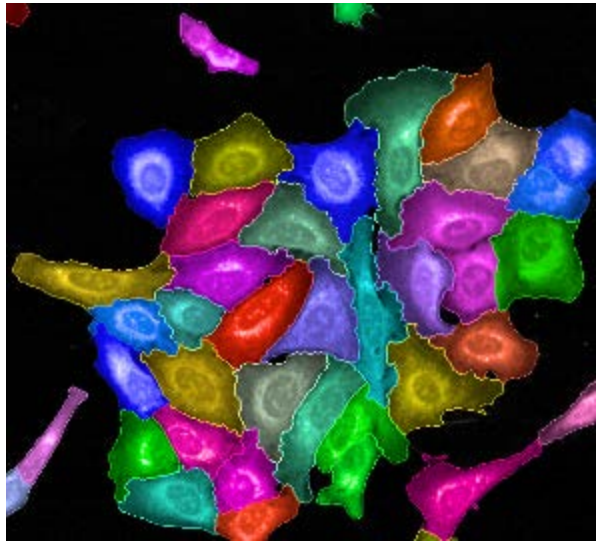
cytoplasm



membrane



cell



ring

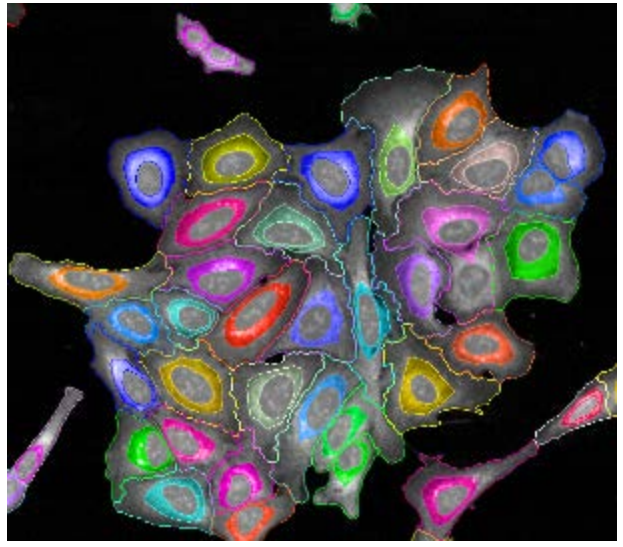


Image Analysis Workflow

Endpoints

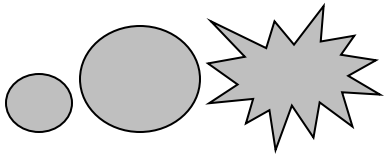
Profiling
with Harmony Software

	NUCLEUS	RING	CYTOPLASM	MEMBRANE	CELL
DNA	S,C,A,R, P,I,T,M	--	--	--	S,C,A,R, P,M
RNA	S,C,A,R, P,I,T	--	--	--	S,C,A,R, P
ER	S,C,A,R, P,I,T	I,T	I,T	I	S,C,A,R, P
AGP	S,C,A,R, P,I,T	I,T	I,T	I,T	S,C,A,R, P
MITO	S,C,A,R, P,I,T	I,T	I,T	I	S,C,A,R, P

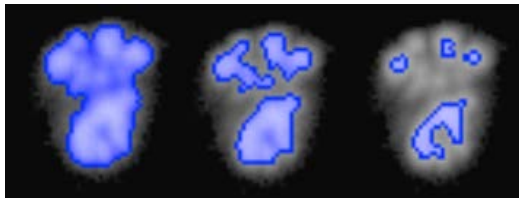
~ 1300 endpoints

Ontologies:

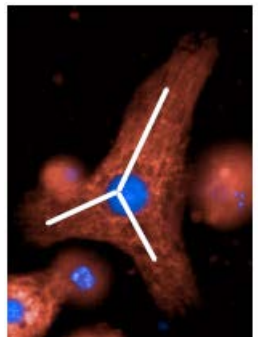
- AGP_Texture _Cytoplasm
- Mito_Compactness _Ring
- DNA_Intensity _Nuclei



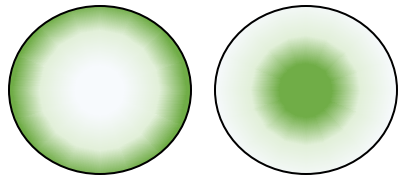
Shape (M)



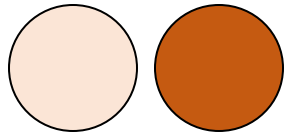
Threshold Compactness (C)



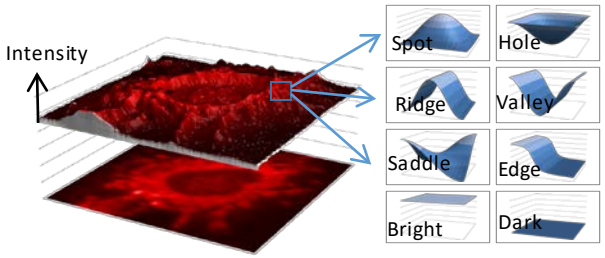
Symmetry (S)



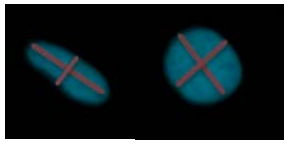
Radial distribution (R)



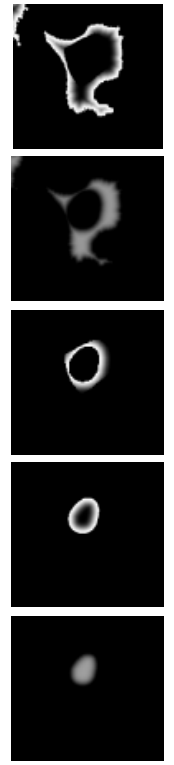
Intensity (I)



Texture (T)



Axial (A)



Profile (P)

Image Analysis Workflow

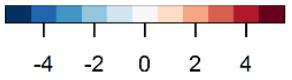
Concentration-Response Modeling

Data Reduction
in R

cell-level data (~500 cells/well)

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

normalized cell-level data



median

well-level data

Caspase/PI BMD
BMD_3SD
Conc. above LEC removed

clipped well-level data

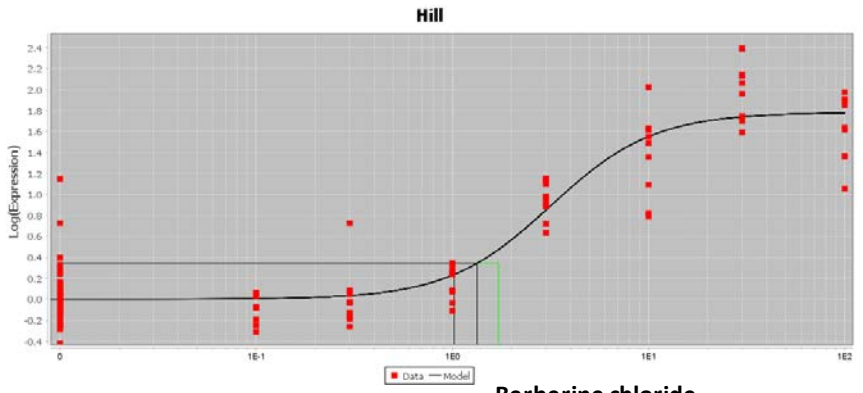
Benchmark dose (BMD) modelling
using BMDExpress 2.2

1293 endpoints

ANOVA filtering — $p \leq 0.01$, FDR adjusted

BMD modelling — 6 models: Hill, Linear, Poly2, Power, Exp2, Exp5

Model selection — Model with best AIC



Berberine chloride
Mito_Cells_Morph_STAR

BMD_10%

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Phase 2: Phenotypic Reference Chemicals in U-2 OS Cells

Experimental Design (Phase 1)

Parameter	Multiplier	Notes	
Cell Type(s)	1	U-2 OS [a]	Bone
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).

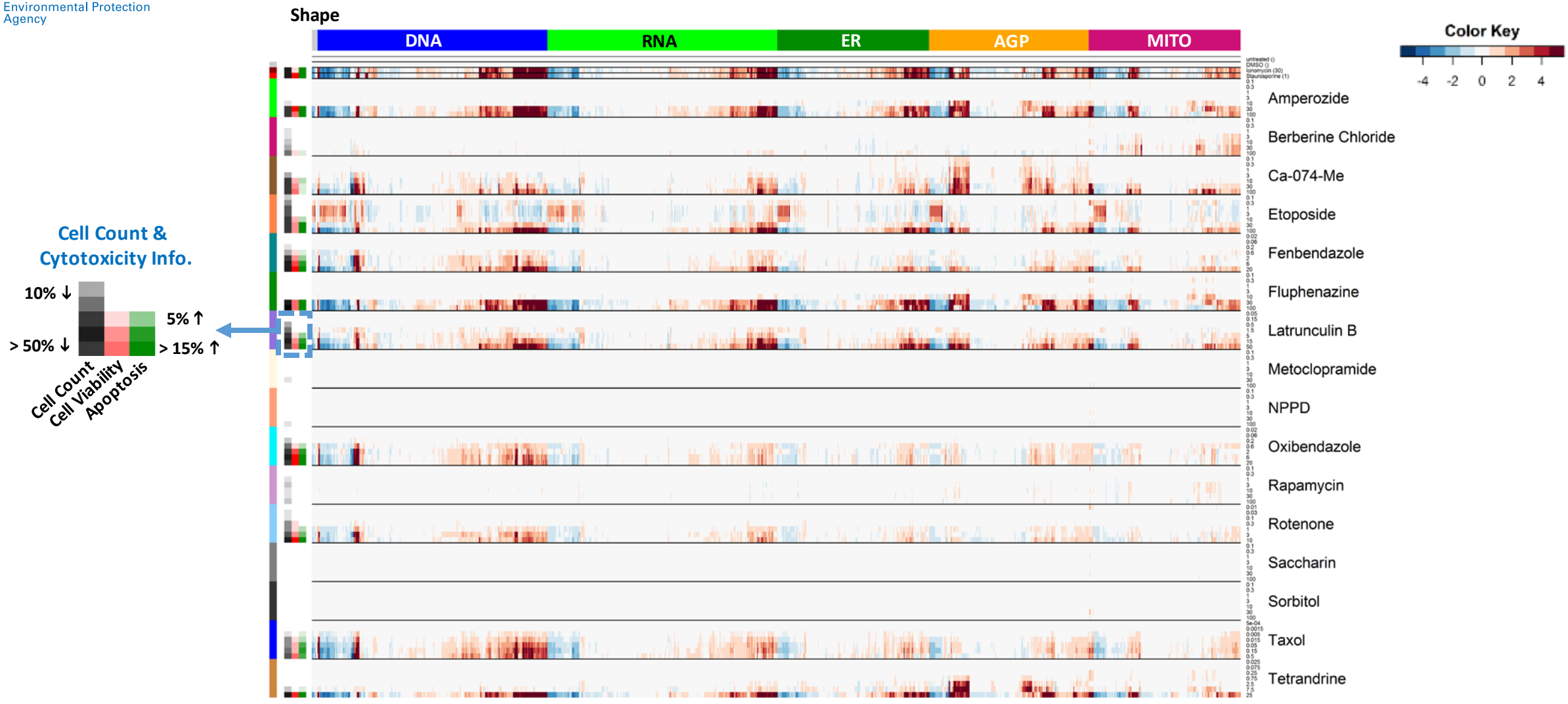
Reference Chemical Set

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

Compound Name	Chemical Use	Expected Phenotype
Amperozide	Atypical antipsychotic	Toroid nuclei
Berberine Chloride	Mitochondria complex I inhibitor	Redistribution of mitochondria
Ca-074-Me	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	Actin cytoskeleton disruptor	Actin breaks
Metoclopramide	D_2 dompaine receptor antagonist	Enhanced golgi staining and some cells with fused nucleoli
NPPD	Chloride channel blocker	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	Mitochondria complex I inhibitor	Mitochondrial stressor
Saccharin	Artificial Sweetener	Negative Control
Sorbitol	Artificial Sweetener	Negative Control
Taxol	Microtubule Stabilizer	Large, multi-nucleated cells with fused nucleoli
Tetrandrine	Calcium channel blocker	Abundant ER

Gustafsdottir, et al. 2013

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	Morphology: Compactness

Literature: redistribution of mitochondria

Channel	Compartment	Domain
AGP	Cytoplasm + Ring	Texture
AGP	Cytoplasm + Ring	Intensity Maximum
AGP	Entire Cell	Morphology/Texture

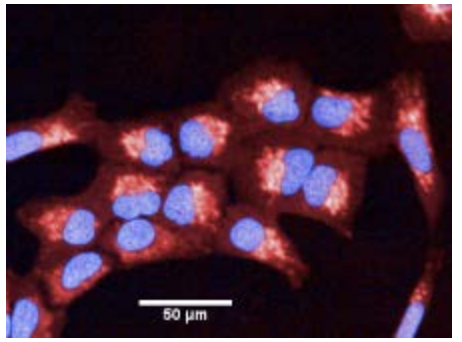
Literature: bright, abundant Golgi stain

Channel	Compartment	Domain
"Shape"	Entire Cell	Morphology: Area
DNA + RNA	Nuclei	Morphology: Compactness Texture
ER + AGP	Cytoplasm + Ring	Intensity: Sum
all	Entire Cell	Morphology

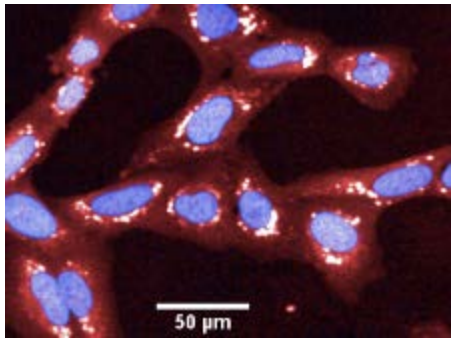
Literature: large, flat nucleoli

DNA Mitochondria

solvent control (0.5% DMSO)

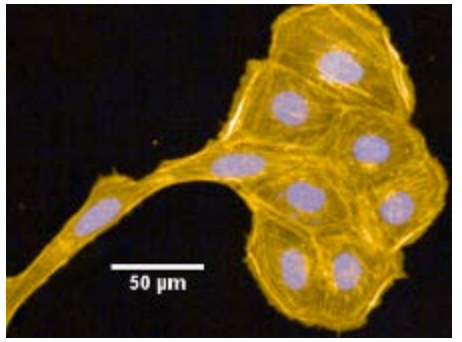


Berberine Chloride (10 µM)

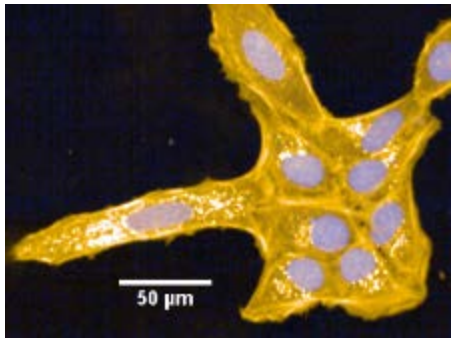


DNA AGP

solvent control (0.5% DMSO)

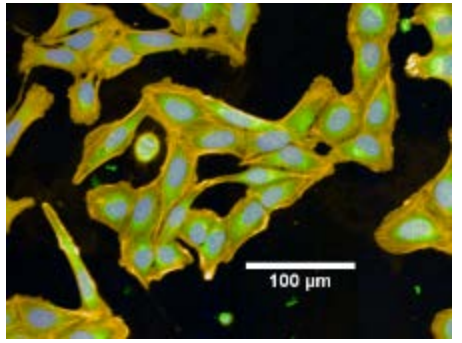


Ca-074-Me (1 µM)

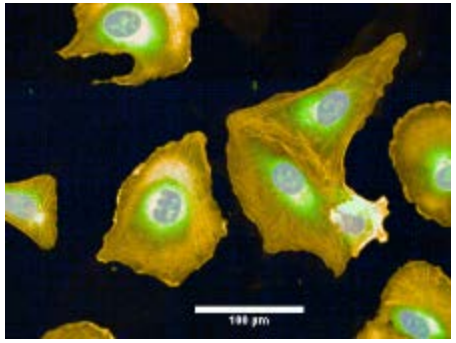


DNA RNA/ER AGP

solvent control (0.5% DMSO)

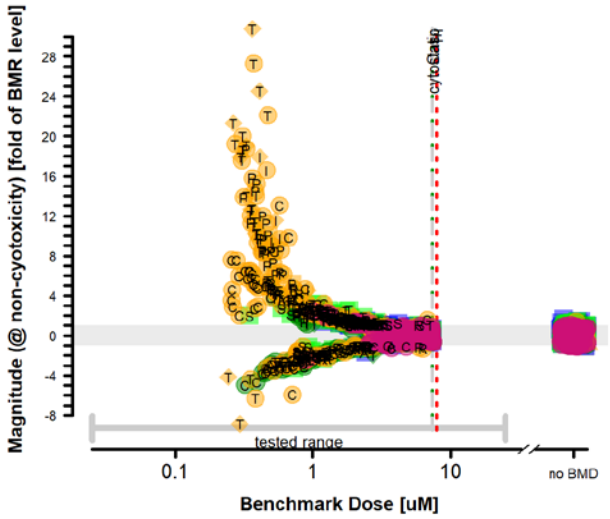


Etoposide (1 µM)

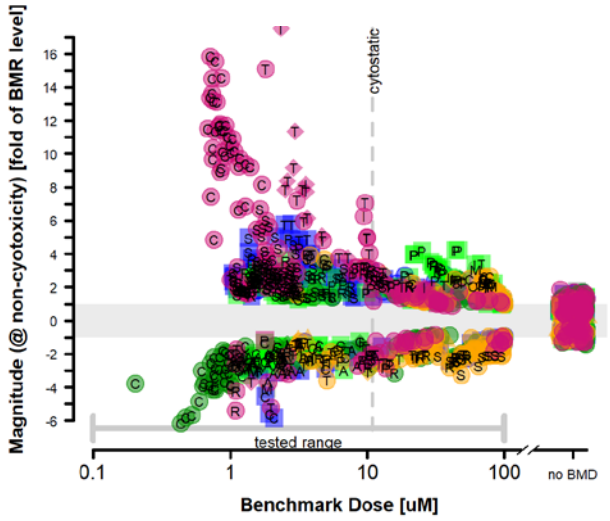


Visualizing Phenotypic Profiles: Potency vs. Efficacy Plots

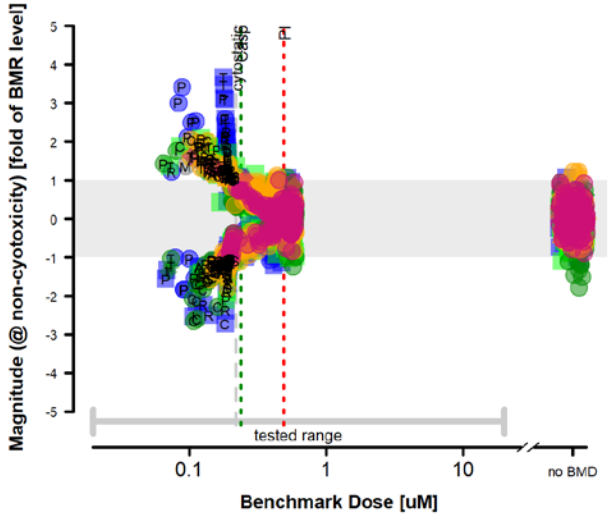
Tetrandrine



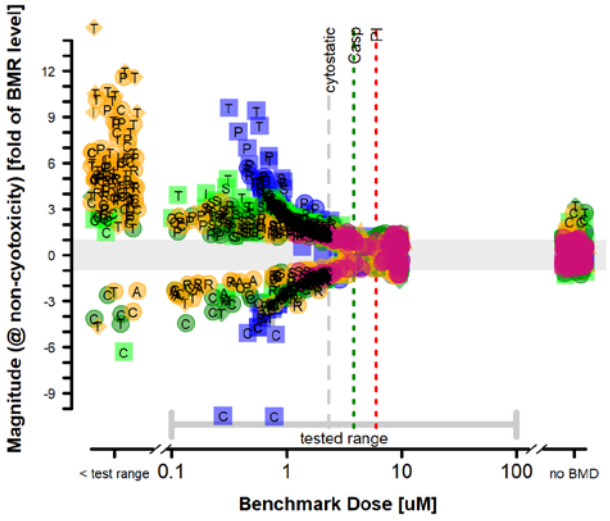
Berberine Chloride



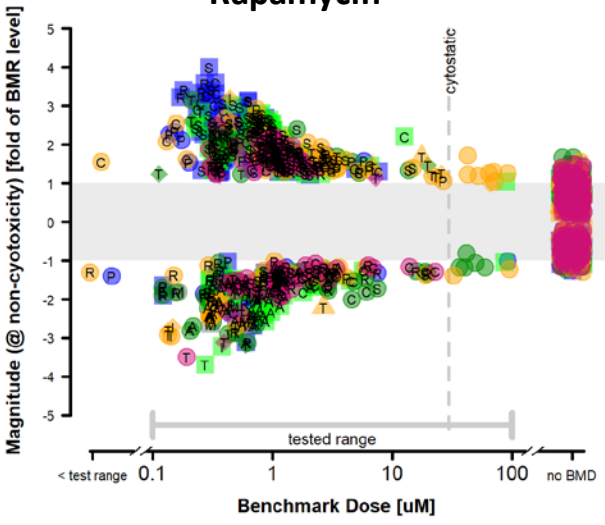
Oxibendazole



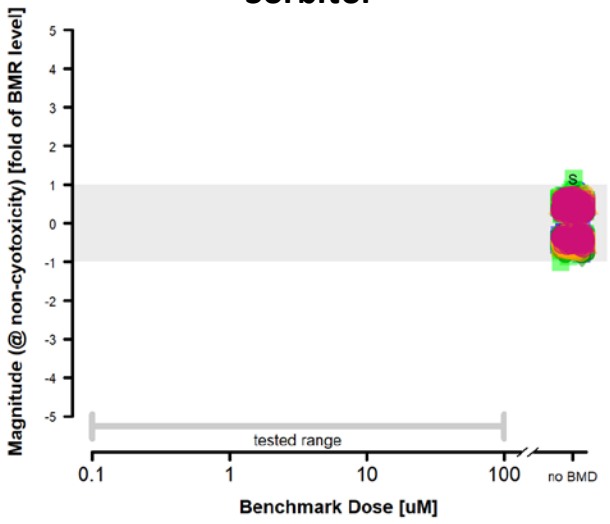
Ca-074-Me



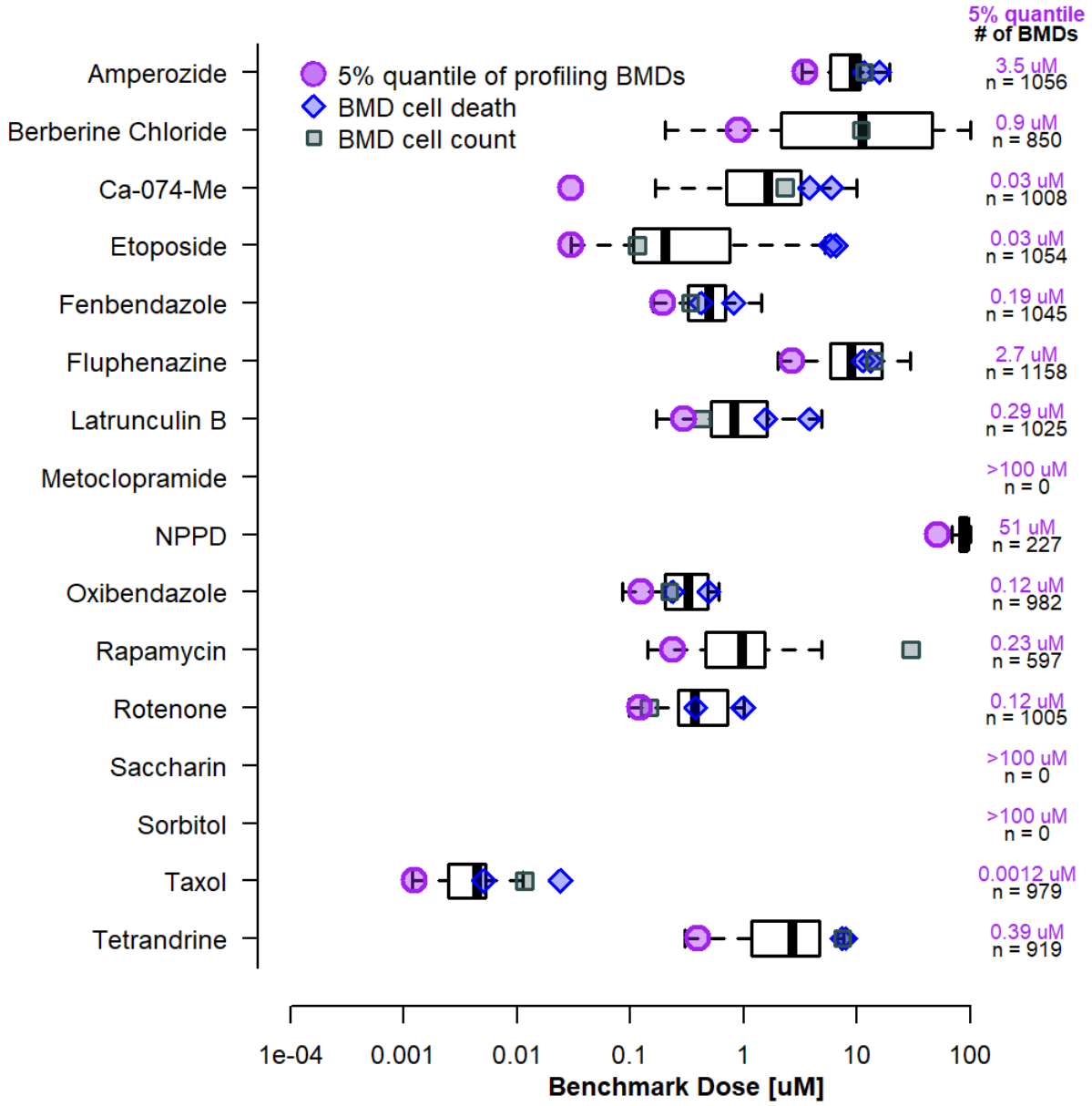
Rapamycin



Sorbitol

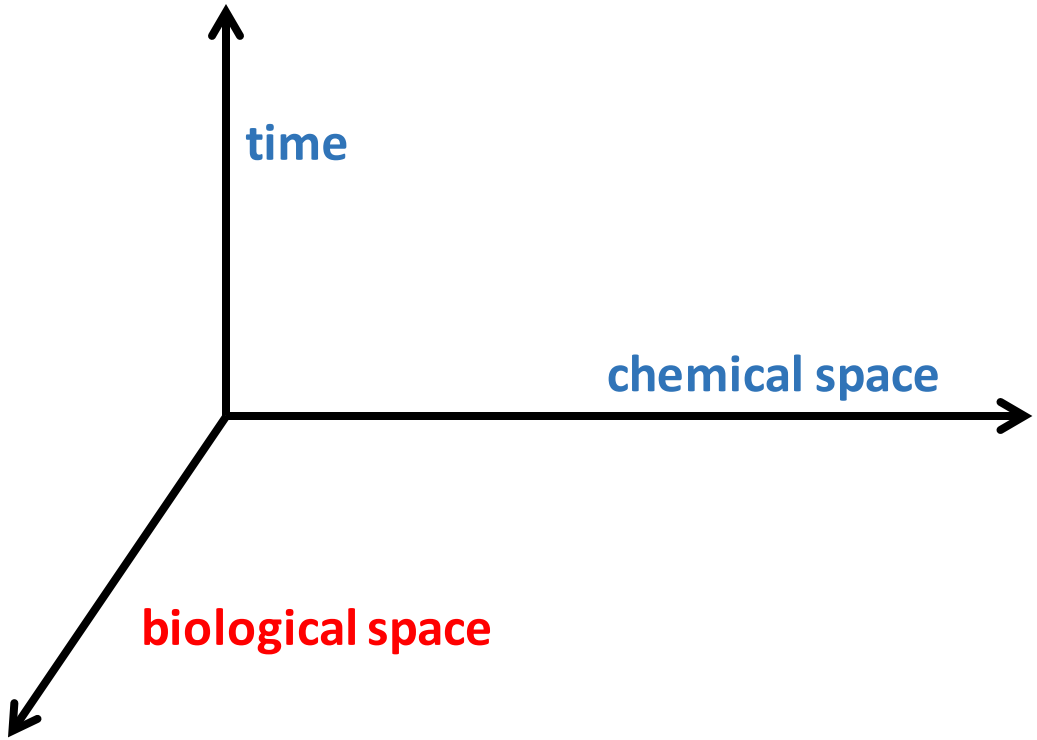


In Vitro Point-of-Departure (POD) Determination



- *In vitro* PODs calculates as lower 5th percentile of affected endpoints
- Effects on cell morphology observed at concentrations well below cytotoxicity.
- Potency varies across reference chemical set

Phase 3: Biological Space (i.e. Cell Types)

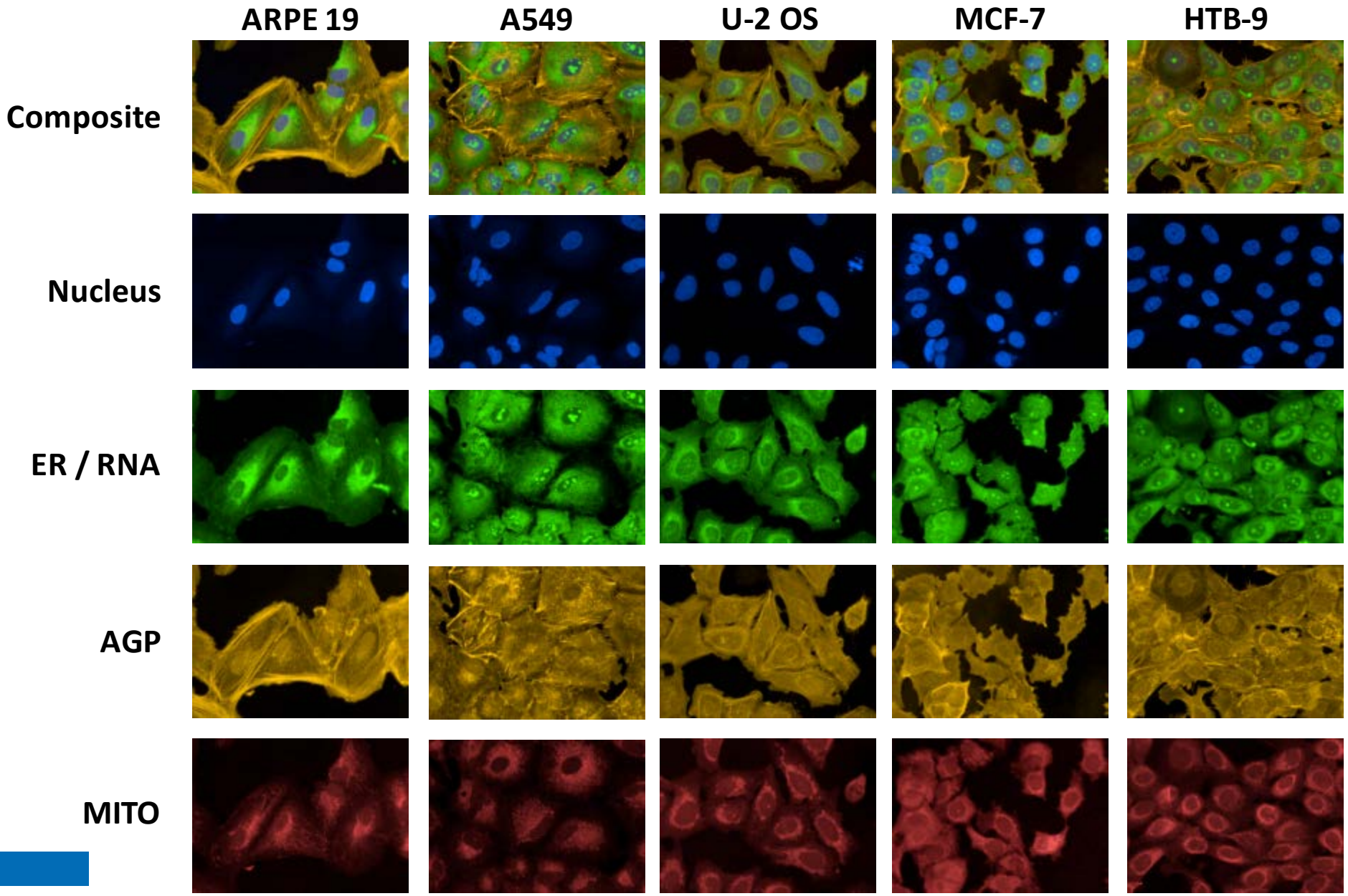


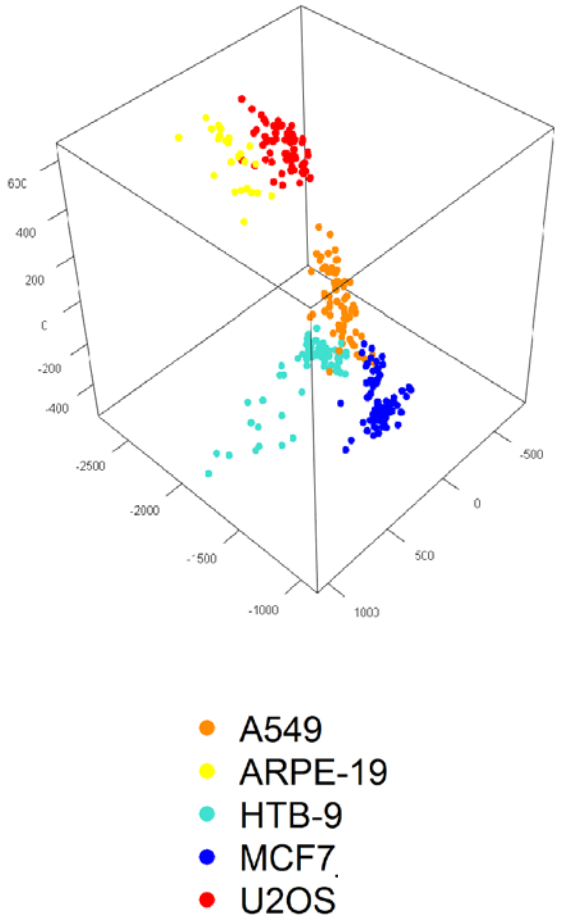
Experimental Design: Biological Space

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	--	

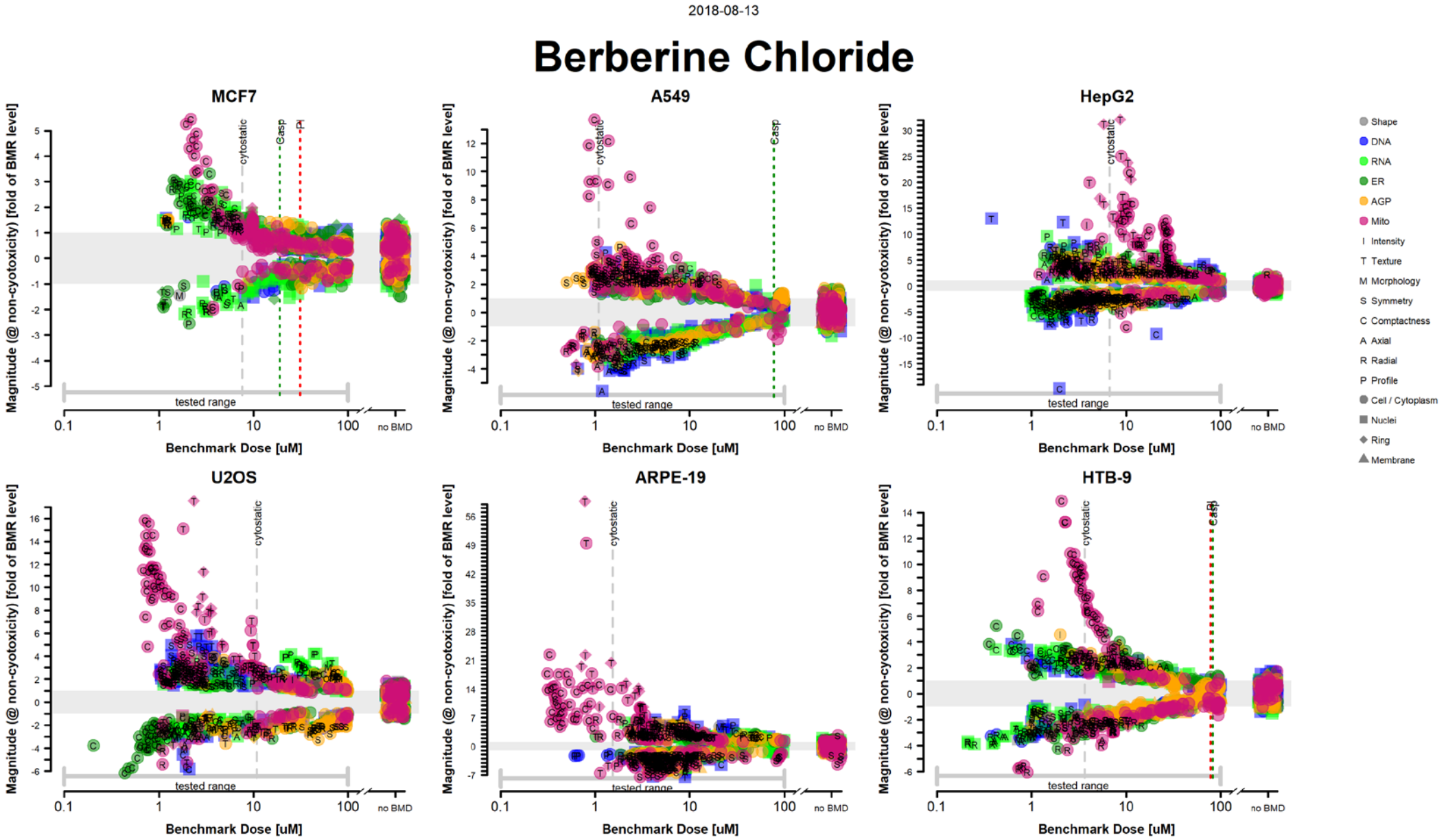
[a] Reference cell line (Bray et al. 2016).
[b] Previously characterized using Cell Painting (Gustafdottir et al. 2013).

Morphological Heterogeneity Across Cell Lines

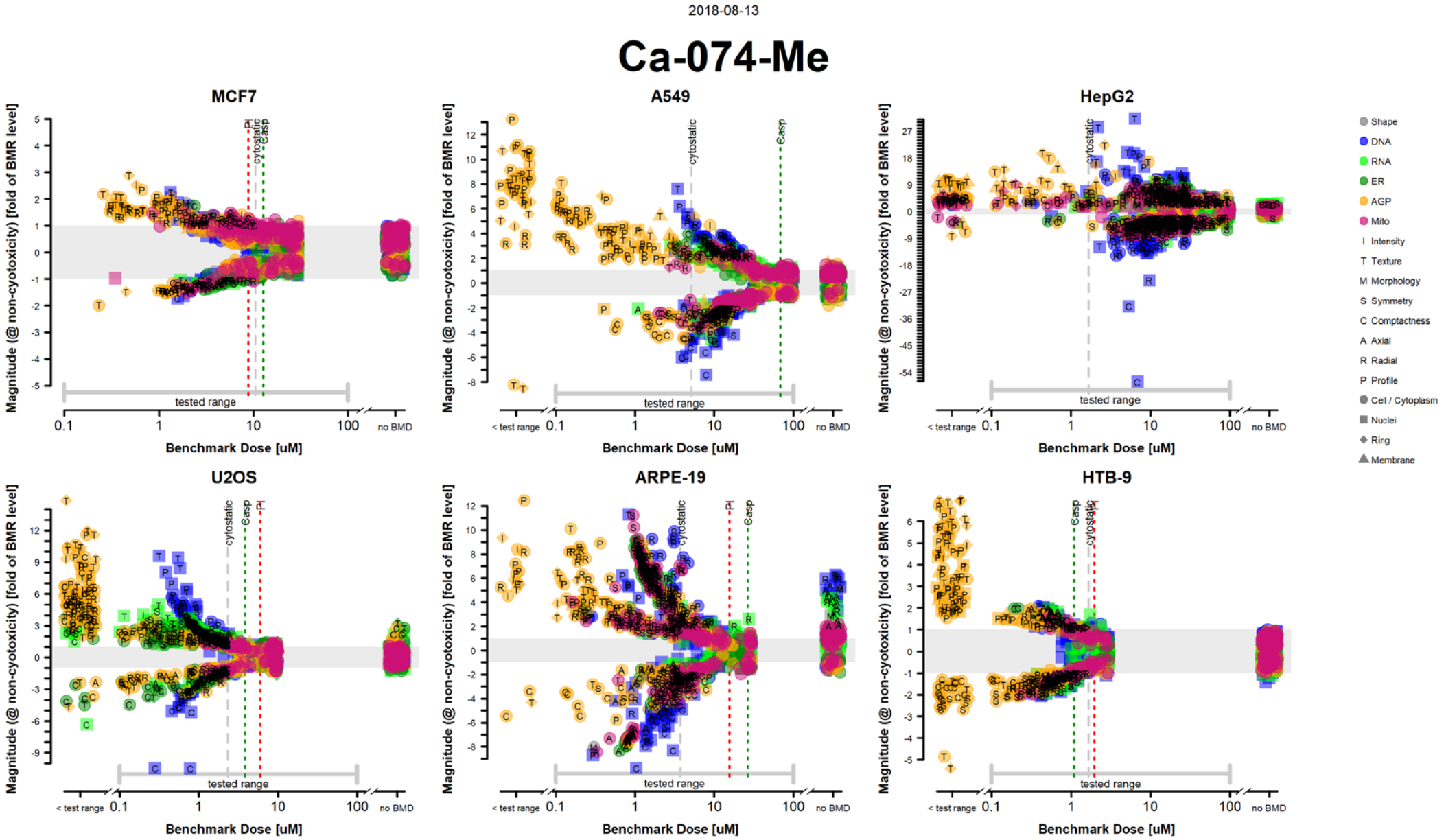




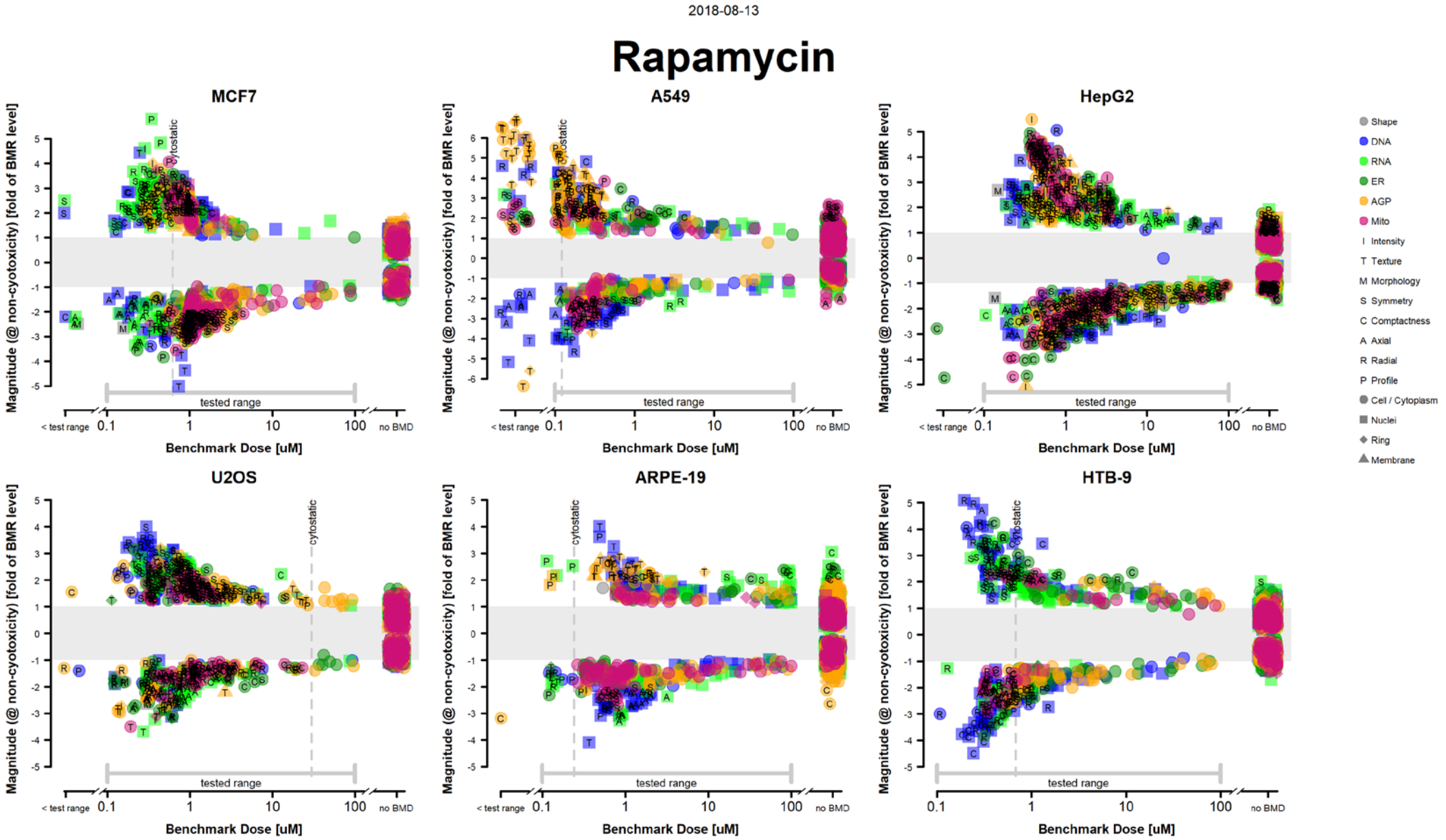
Comparable Response Profiles Across Cell Types (1)



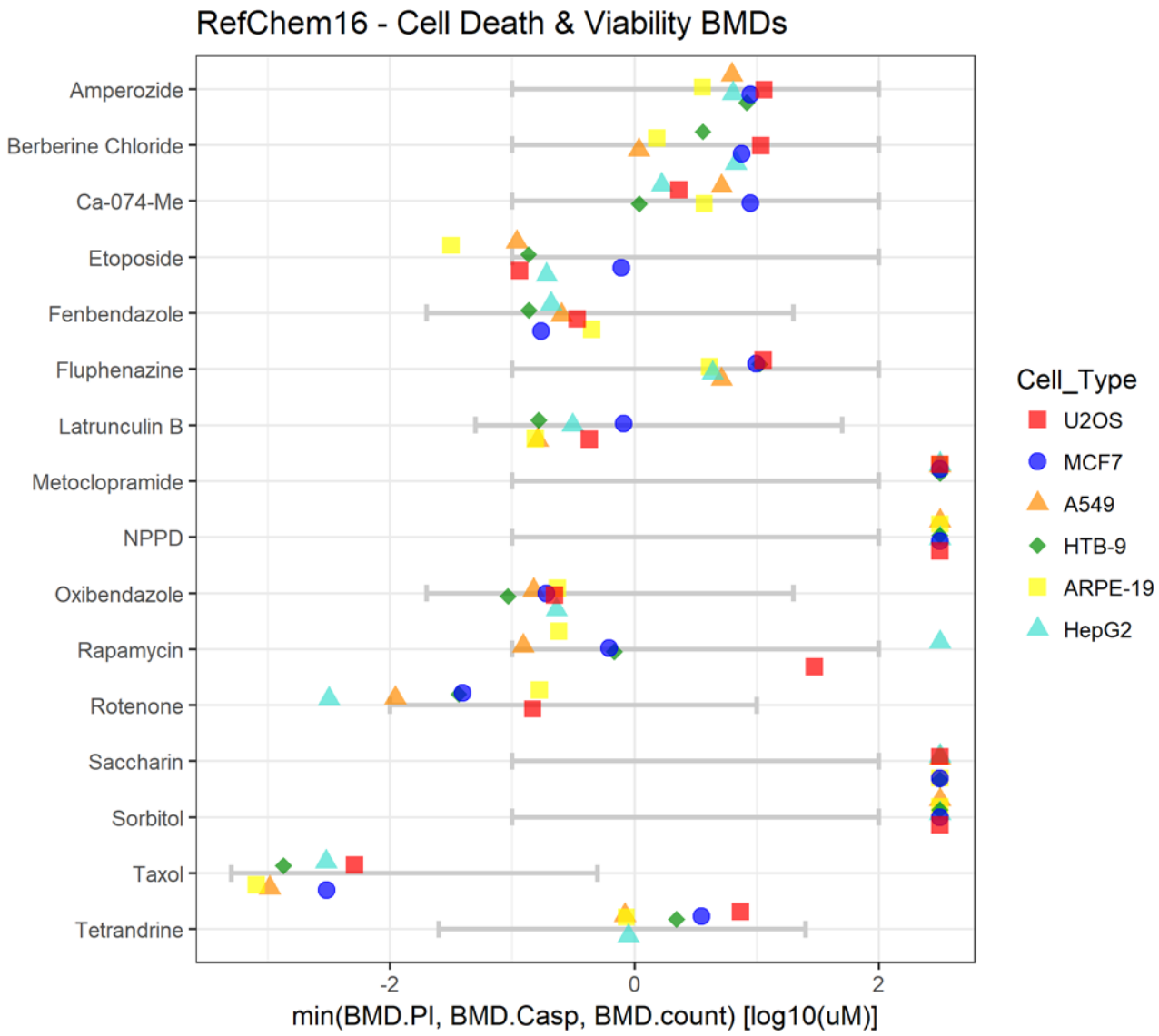
Comparable Response Profiles Across Cell Types (2)

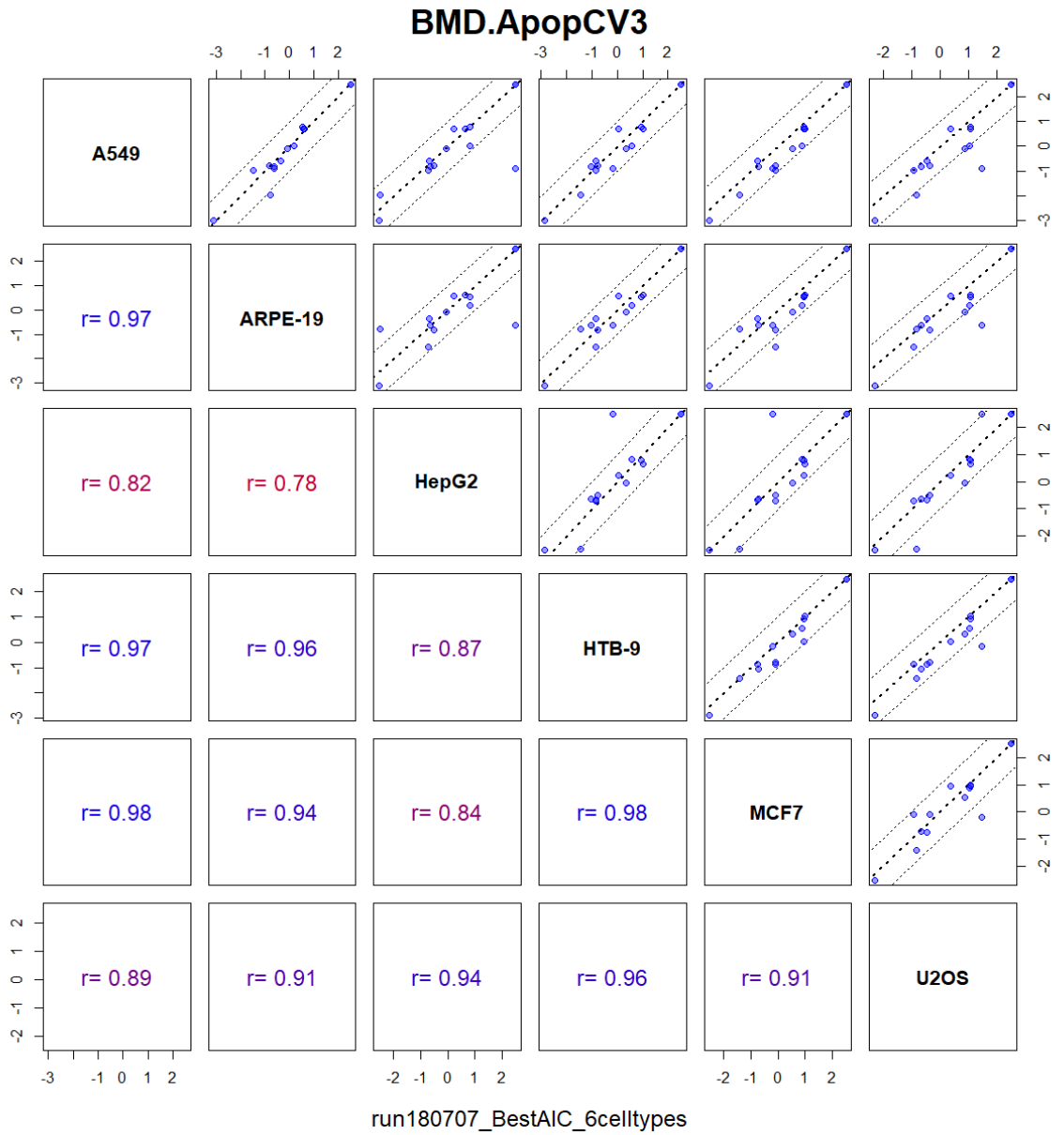


Comparable Response Profiles Across Cell Types (3)

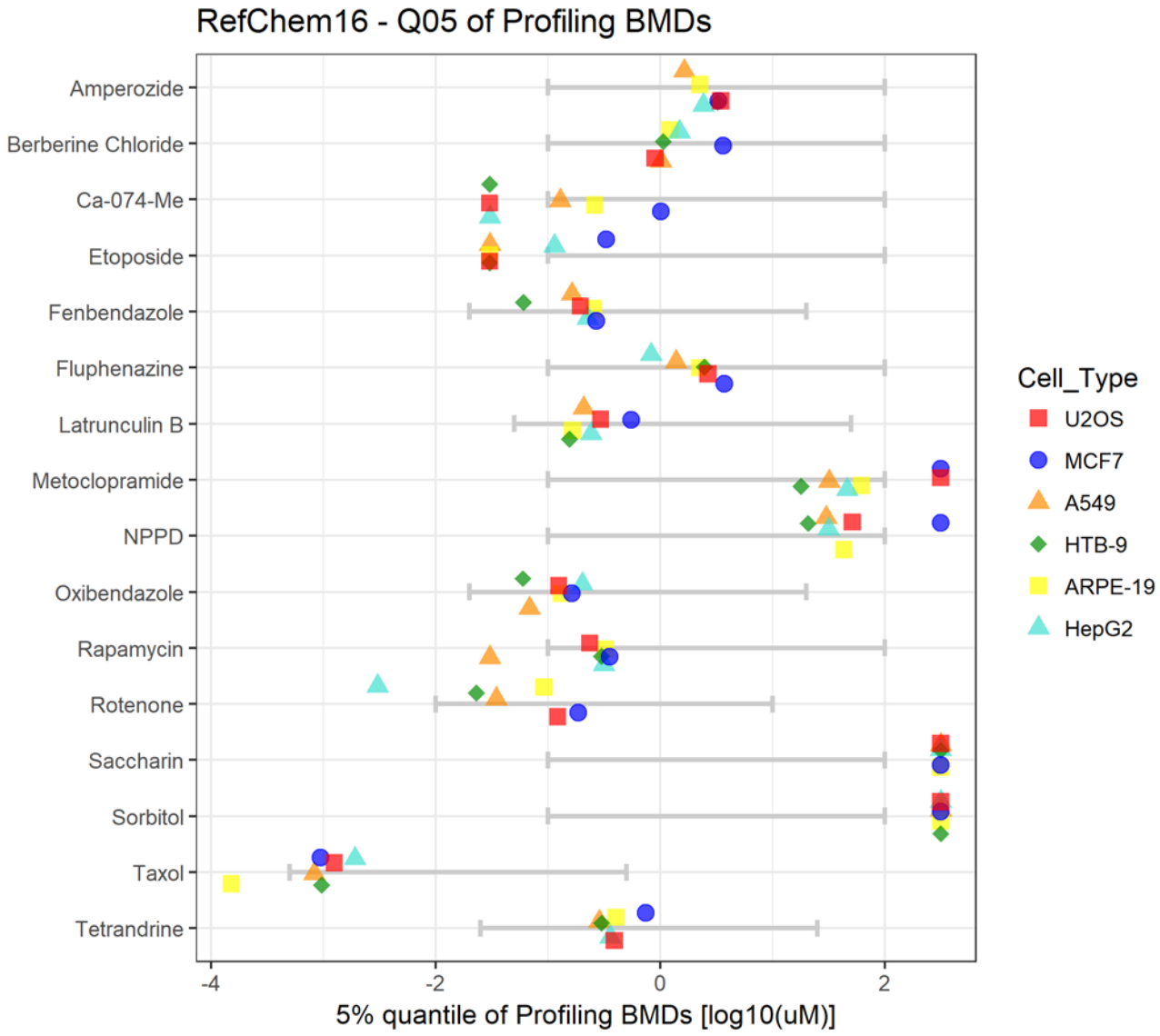


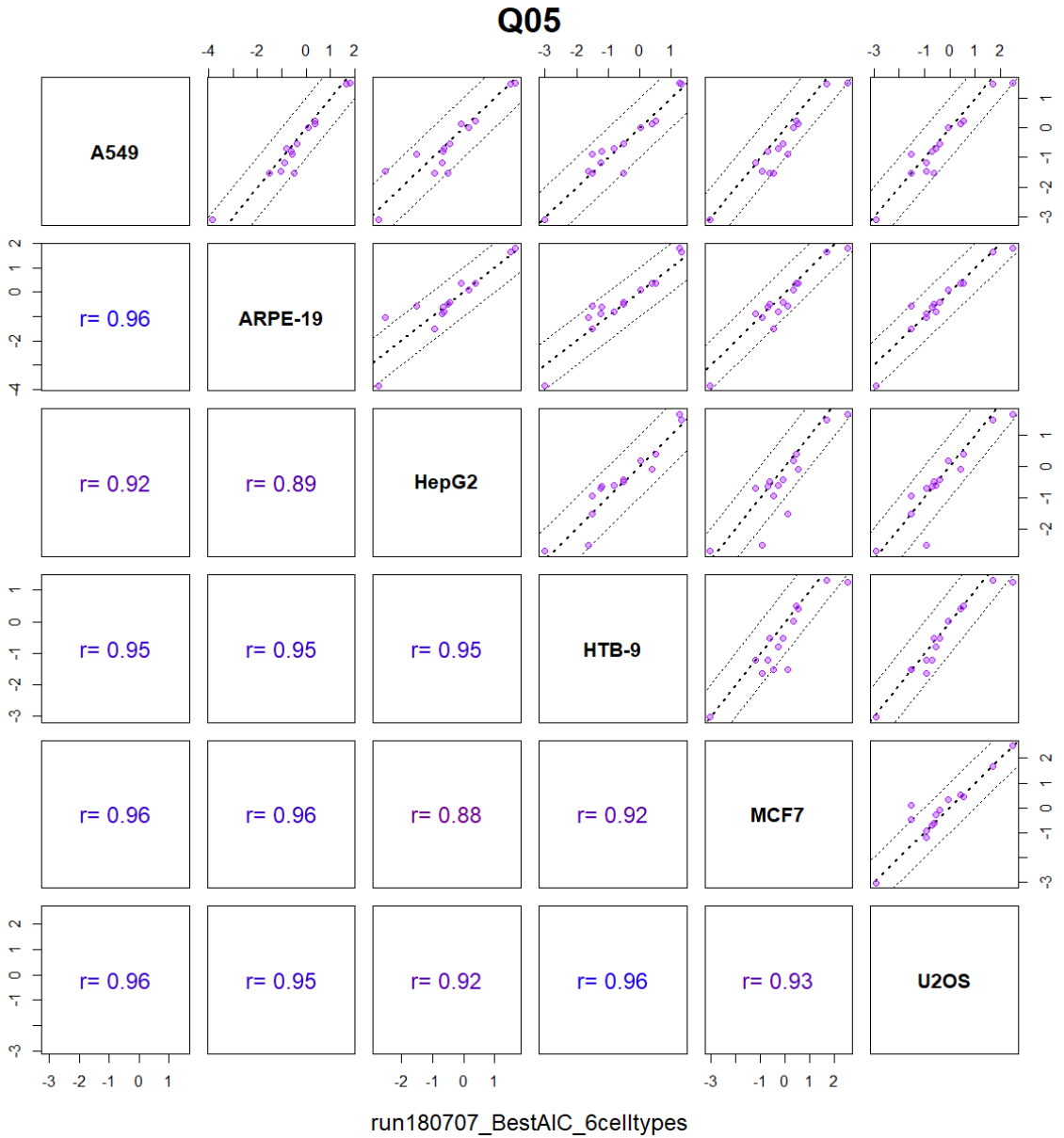
Correlation of Cell Viability BMDs Across Cell Types



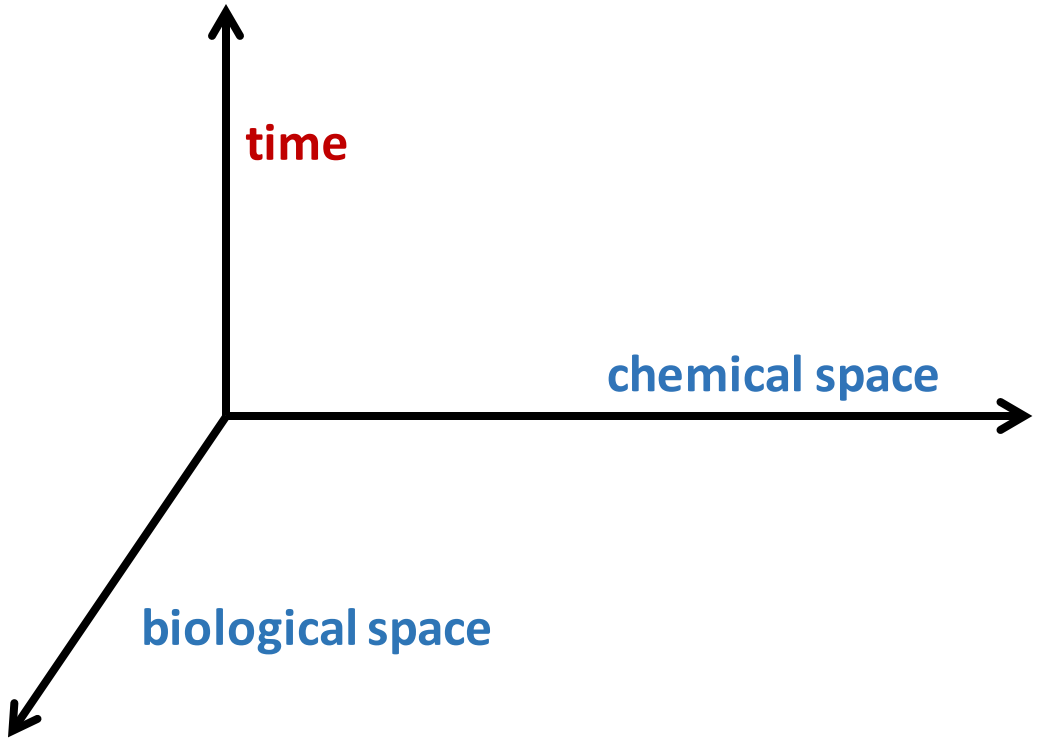


Strong Correlation of Cell Painting PODs Across Cell Types





Phase 3: Time Course (U-2 OS)

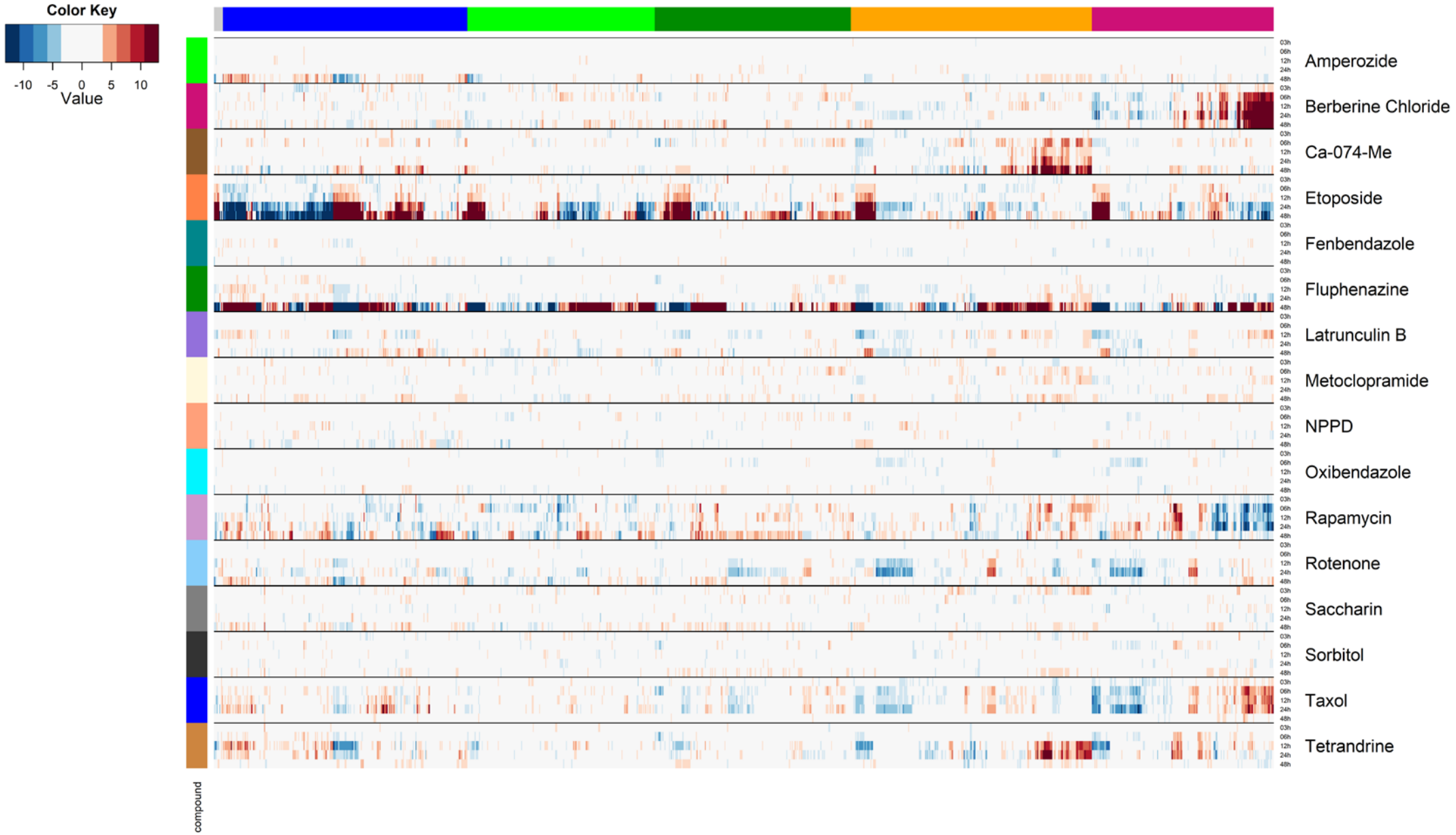


Experimental Design: Time Course

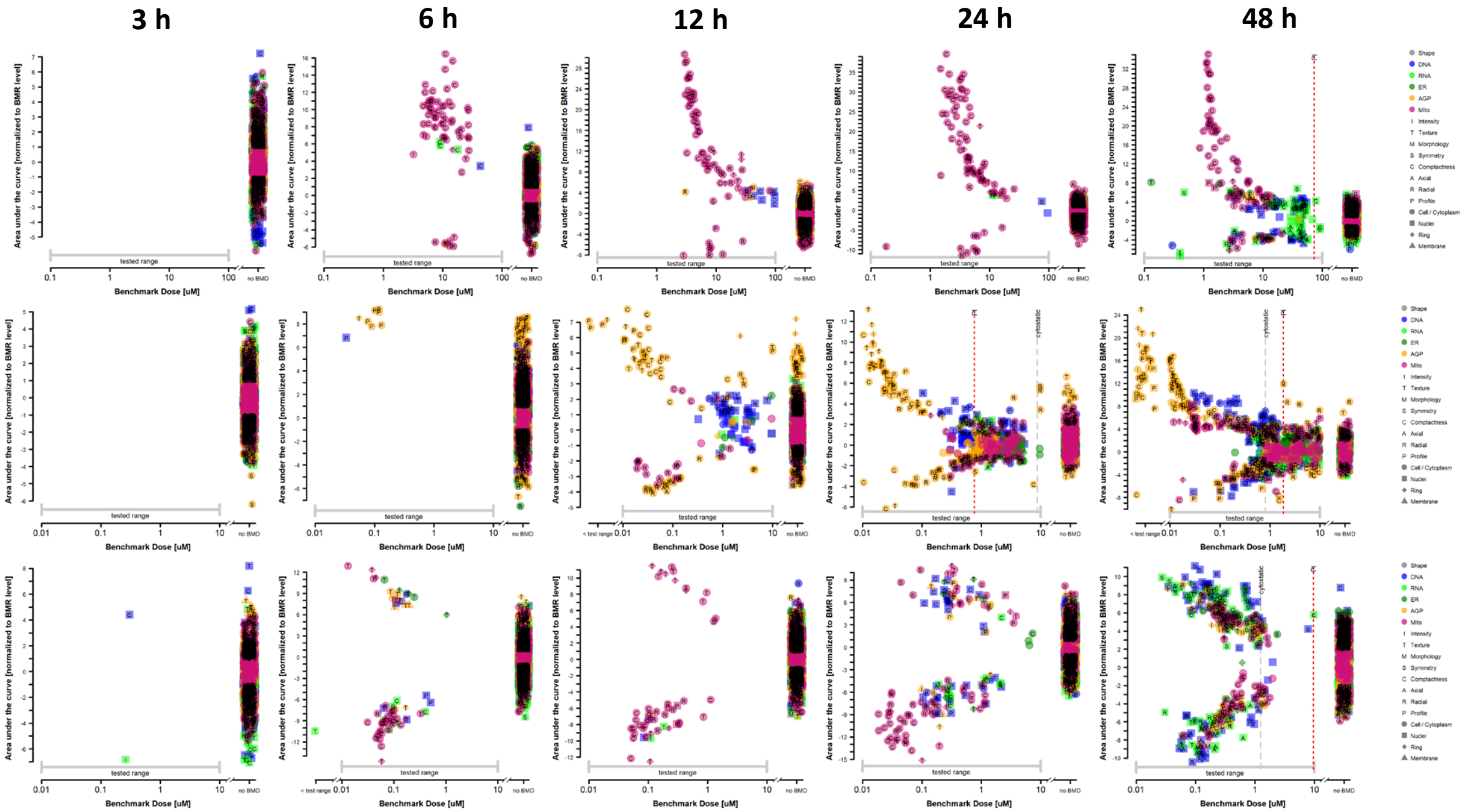
Parameter	Multiplier	Notes	
Cell Type(s)	1	U-2 OS [a]	Bone
Culture Condition	1	DMEM + 10% HI-FBS	
Chemicals	16	14 phenotypic reference chemicals 2 negative control chemicals	
Time Points:	5	3,6,12,24,48 hours	
Assay Formats:	2	Cell Painting HCI Cell Viability & Apoptosis	
Concentrations:	8	3.5 $\log_{10}$ units; semi $\log_{10}$ spacing	
Biological Replicates:	3	--	

[a] Reference cell line (Bray et al. 2016).

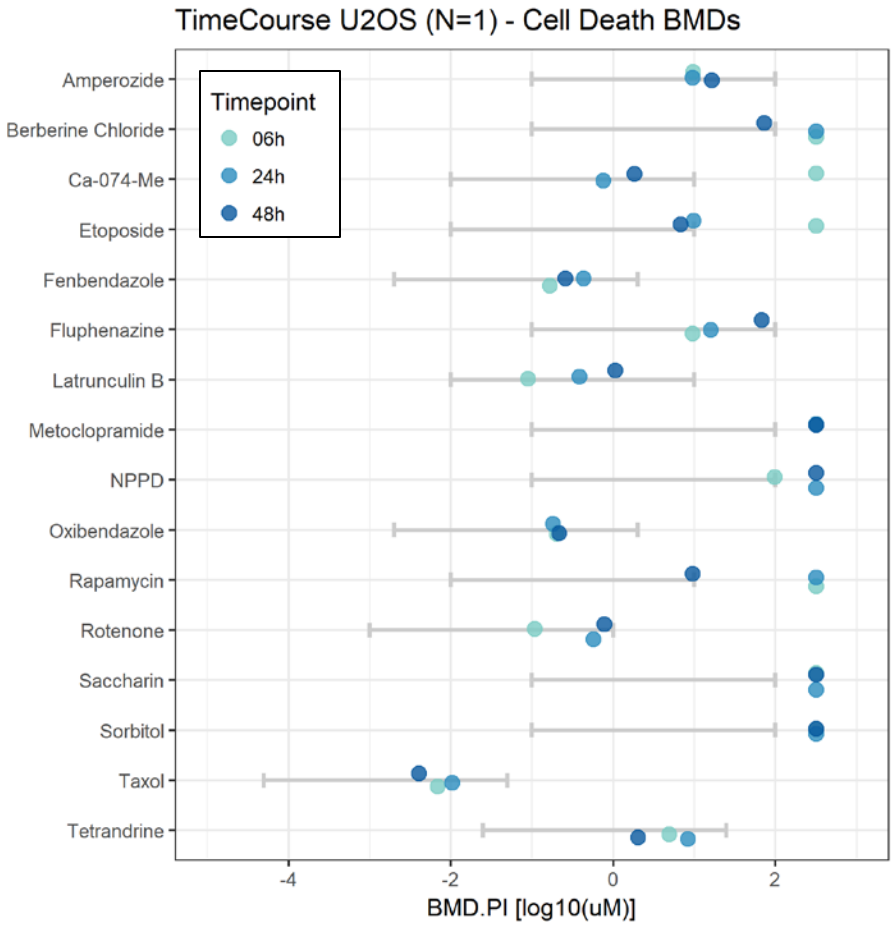
Qualitative Similarity in Response Profiles Over Time

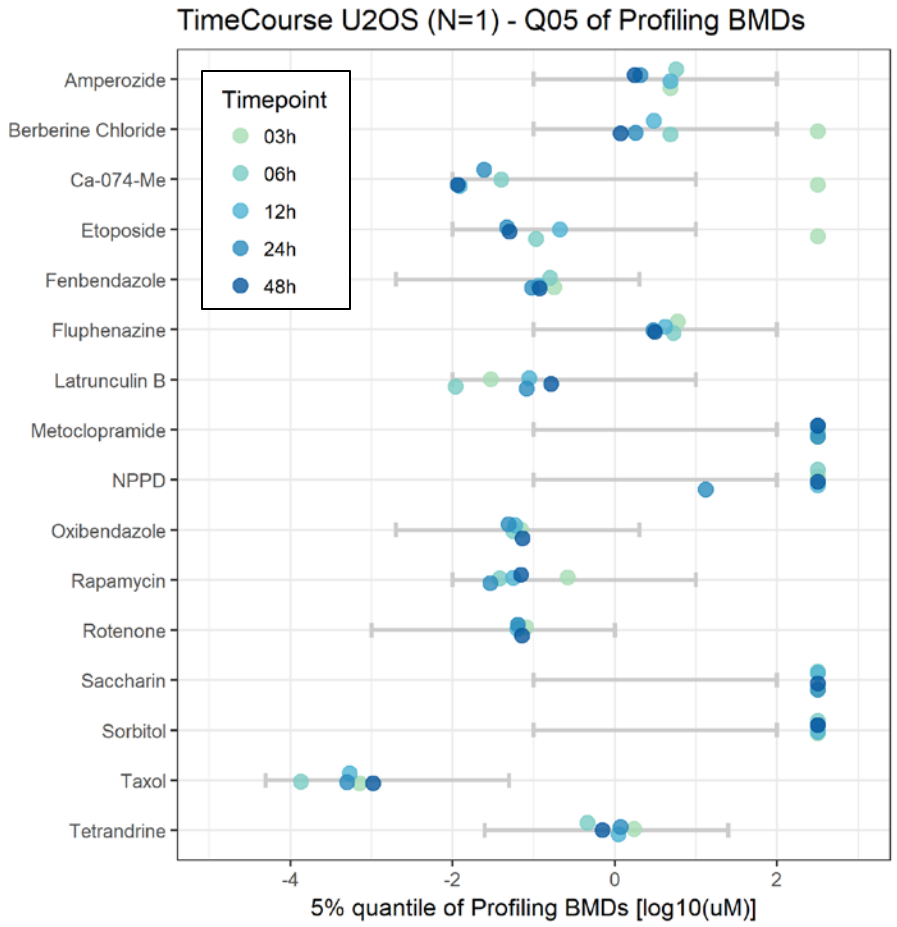


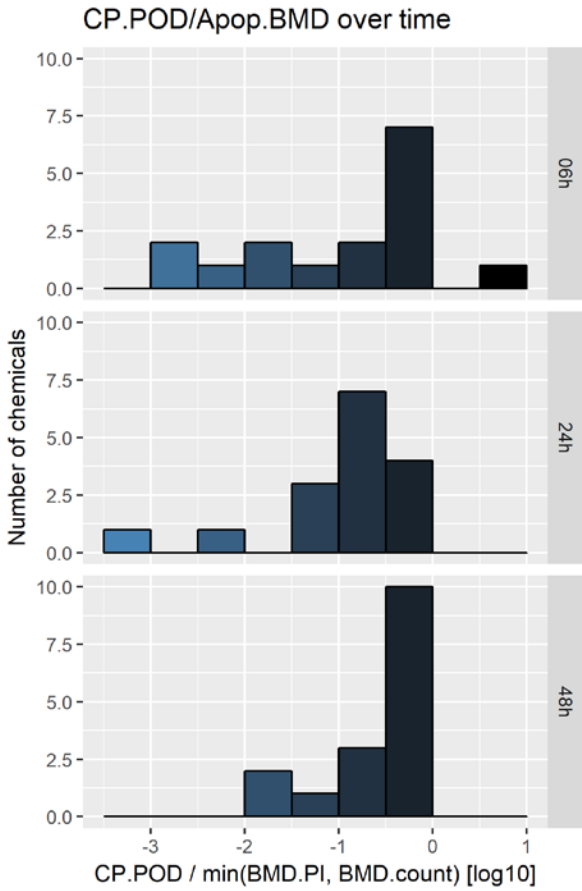
Greater Specificity Observed at Shorter Exposure Durations



Cell Painting PODs Are Stable Over Time



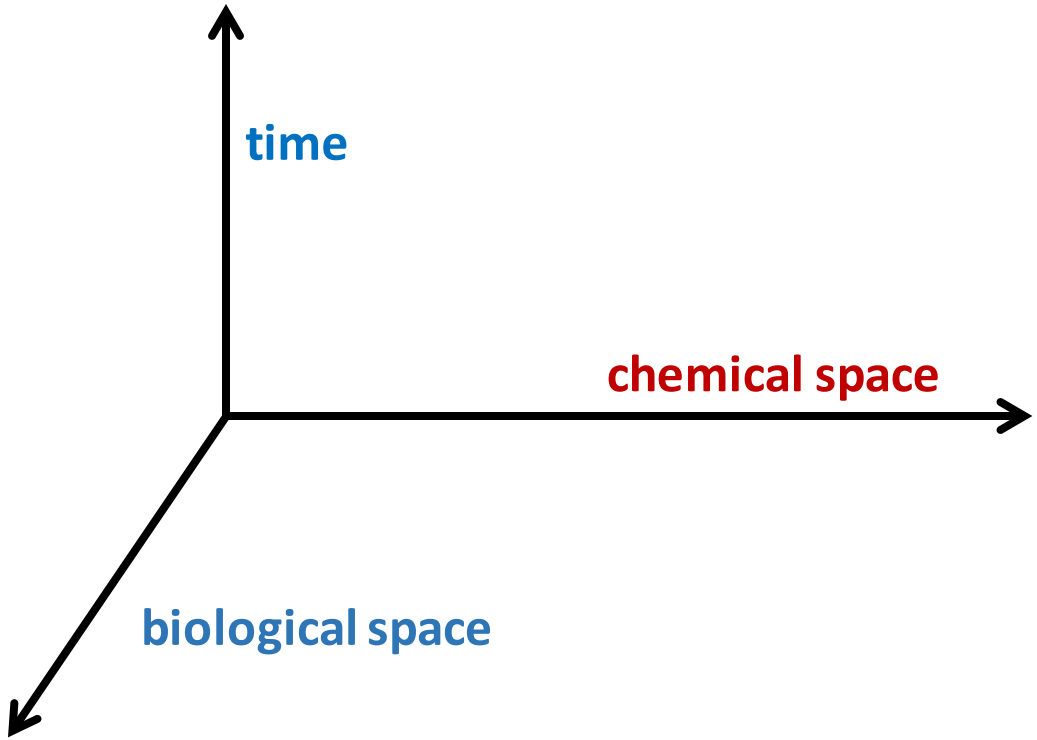




- Cell Painting PODs (Q05) are quantitatively similar after ~6 hr exposure duration.
- Cell viability PODs show greater variation across exposure durations.

EPA United States Environmental Protection Agency

Phase 3: Chemical Space

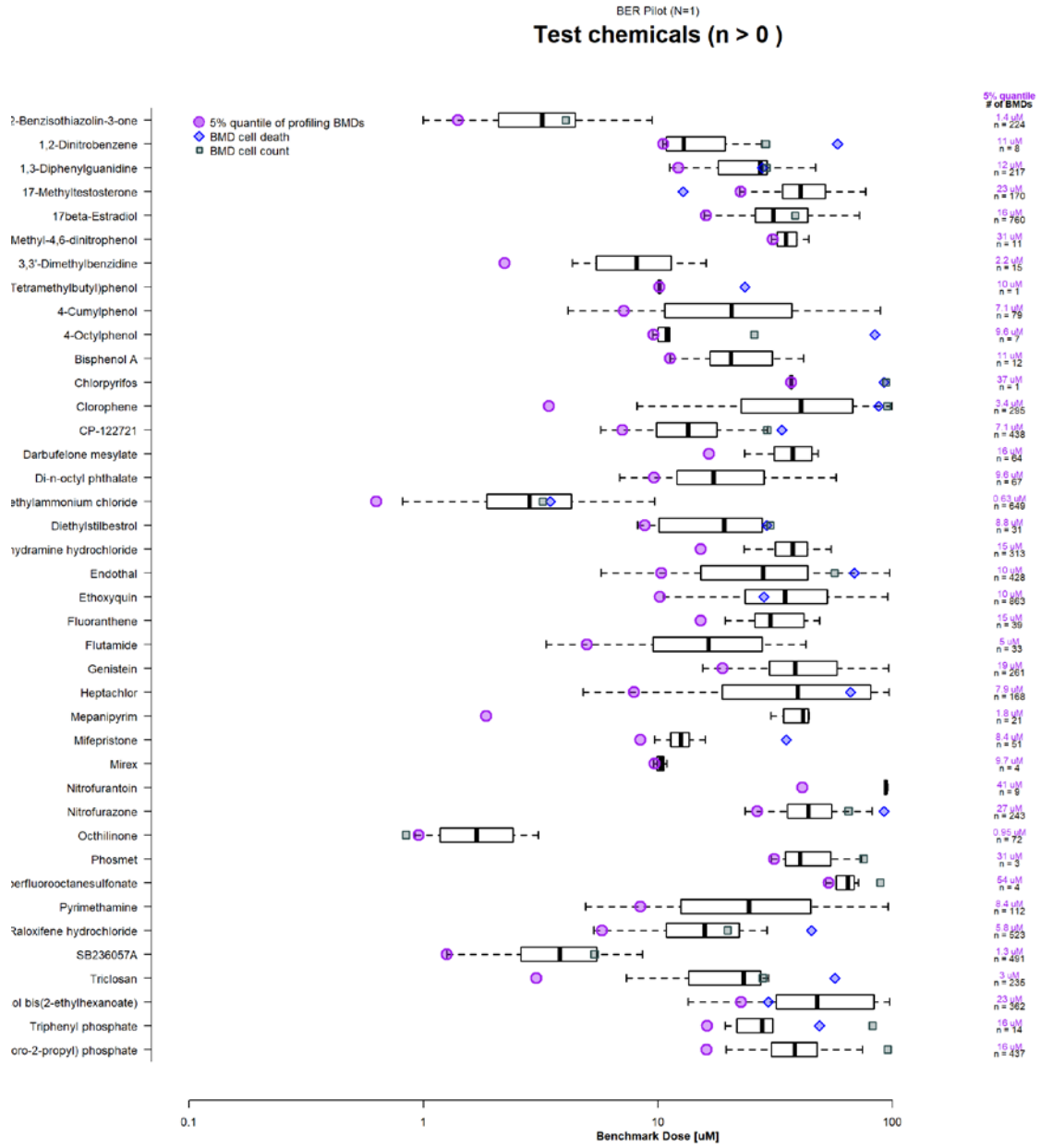


Experimental Design (Chemical Space)

Parameter	Multiplier	Notes	
Cell Type(s)	1	U-2 OS [a]	Bone
Culture Condition	1	DMEM + 10% HI-FBS	
Chemicals	80	Selected from ToxCast HTTK parameters	
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	--	

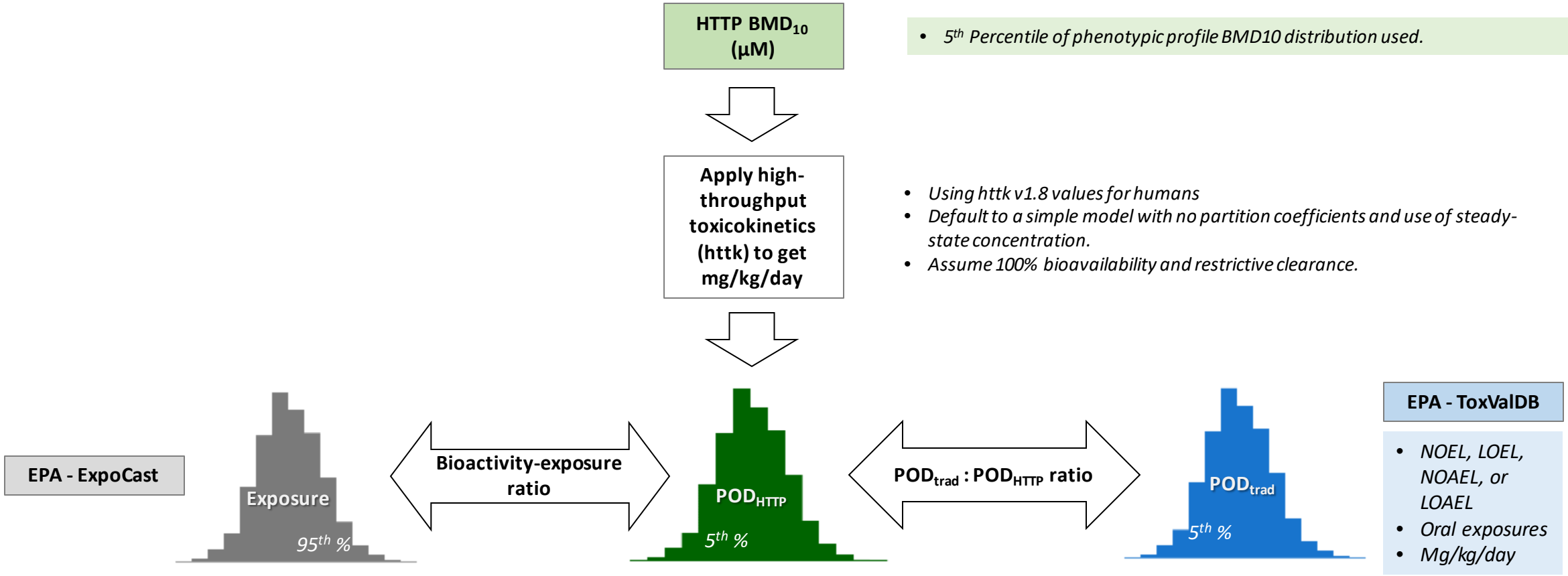
[a] Reference cell line (Bray et al. 2016).

In Vitro PODs, ToxCast Chemicals



- 43 out of 80 (54%) of chemicals tested produced concentration-dependent changes in cell morphology.
- In most cases, the Cell Painting in vitro POD (Q05) was well below the threshold for cytotoxicity.

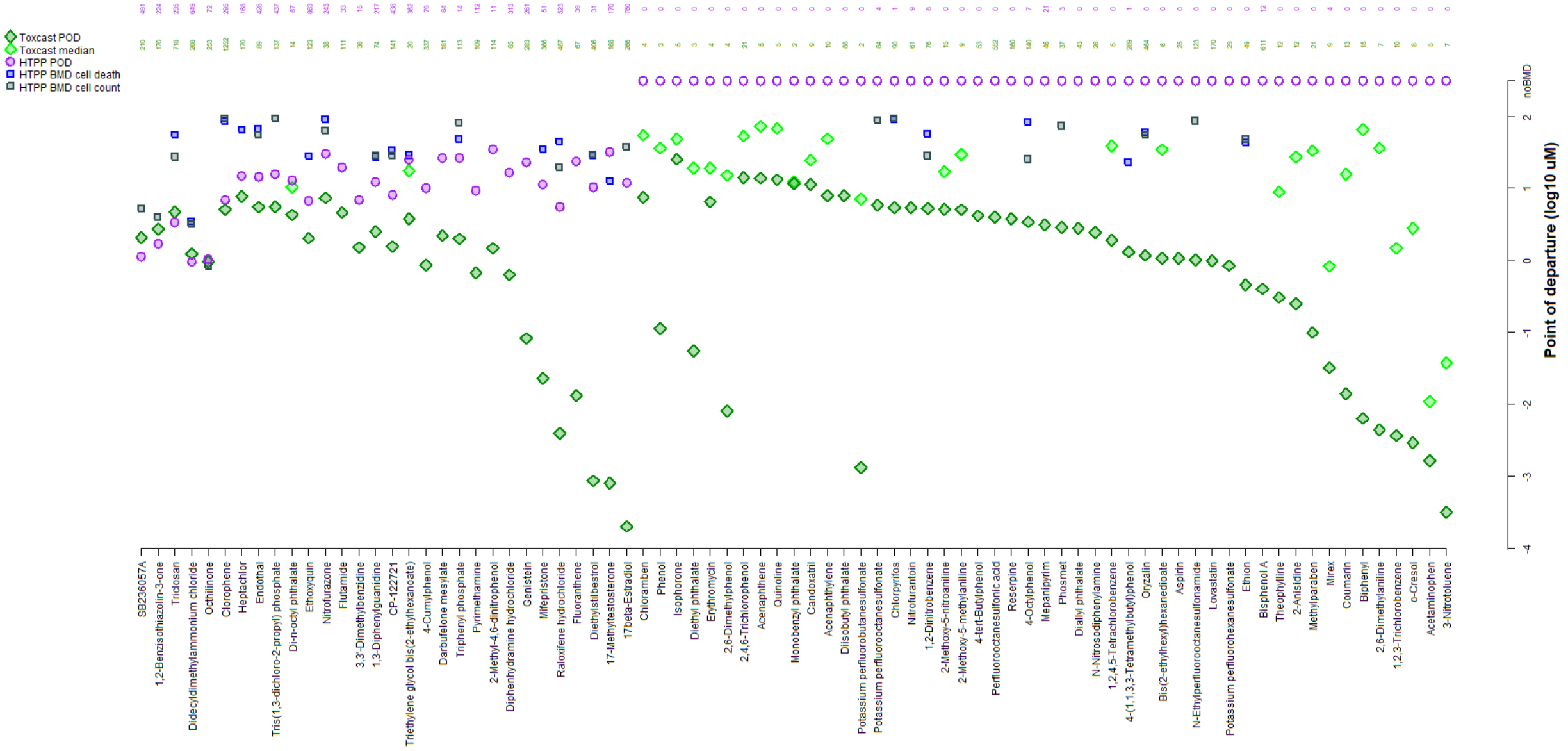
Bioactivity & Exposure Ratio Comparisons Using Reverse Dosimetry



- **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

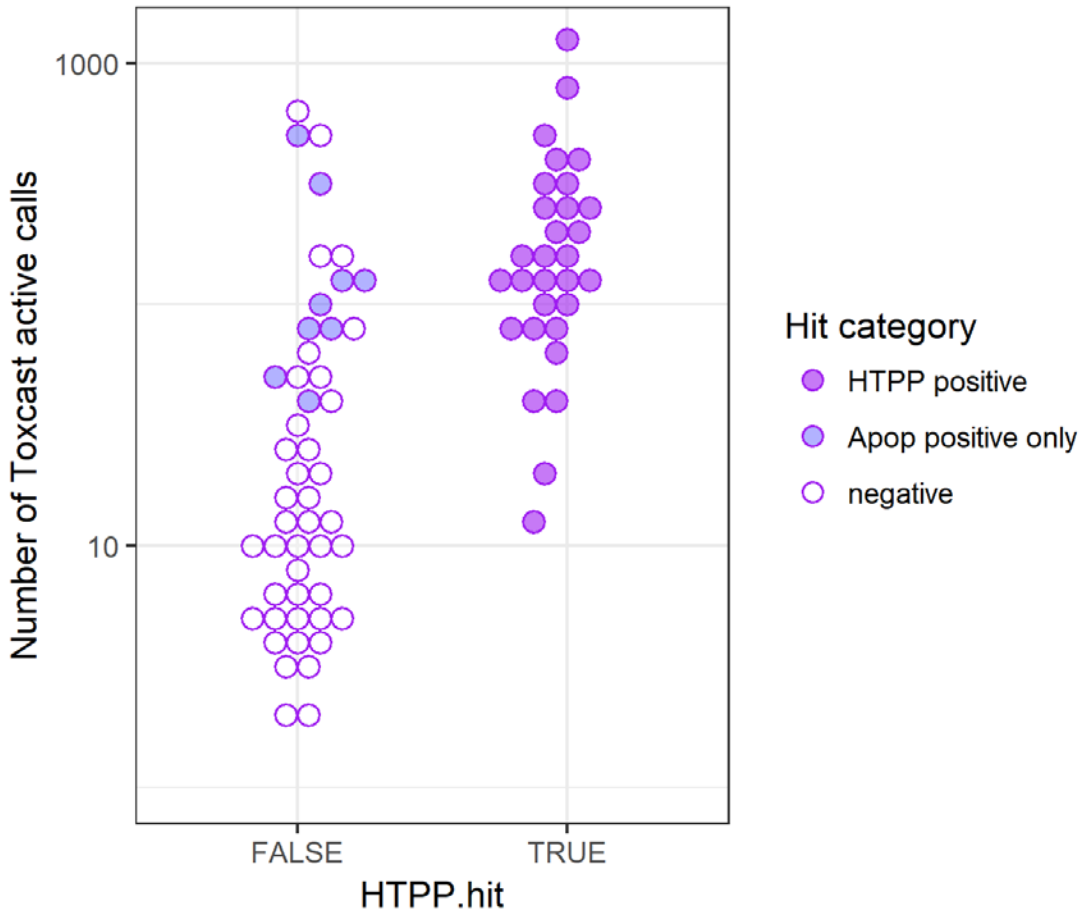
Figure Courtesy of Katie Paul-Friedman

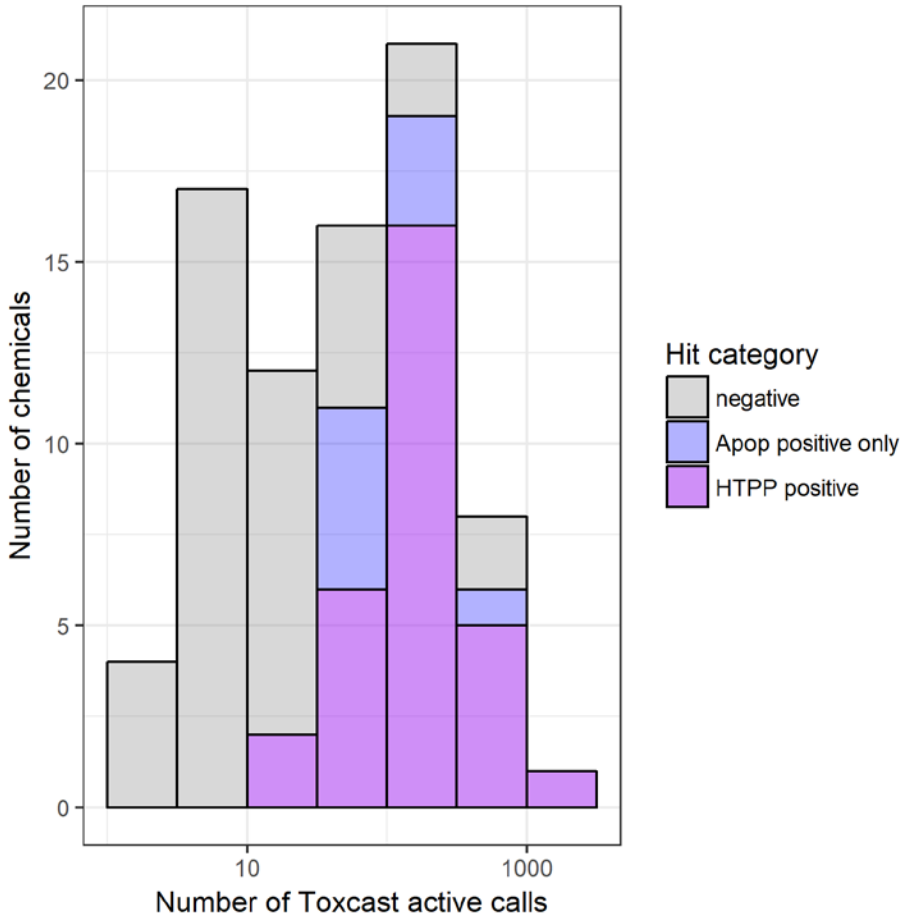
Preliminary Comparison with ToxCast Data



Preliminary Data – DO NOT CITE OR QUOTE

Comparative Sensitivity of Cell Painting and ToxCast





- Preliminary analysis indicates that ToxCast is more sensitive than Cell Painting.
- **Caveats:** To date, only one cell type evaluated in Cell Painting.
  Cell Painting perform in intact cells with adaptive mechanisms.

Preliminary Data –
DO NOT CITE OR QUOTE

Summary

- **Workflow:** Developed a microfluidics-based laboratory workflow for cell plating, chemical screening and fluorescent labeling of cells for measurements of organelle morphology.

- **Concentration-Response Analysis:** Developed a high content image analysis workflow (Harmony) and data analysis pipeline that incorporates concentration-response modeling (R & BMDExpress 2.2).

- **Reference Chemicals:** Replicated profiles described in previous publications and identified candidate chemicals for use as reference controls for screening applications.

- **Sensitivity:** Effects on cell morphology were often observed at concentrations well below the threshold for cytotoxicity both with reference chemicals and a subset of the ToxCast library.

- **Biological Space:** Cell Painting BMDs for the reference chemical set were strongly correlated across six cancer cell lines.

- **Time Course:** As exposure duration increases, a greater number of morphological endpoints are affected, however, the in vitro POD (Q05) remains stable across time points. More specific effects observed at earlier times.

- **Chemical Space:** Screening of 80 ToxCast chemicals in U-2 OS cells produced ~50% hit rate. Comparison with ToxCast data indicates that there is a positive association between the number of ToxCast assays affected by a chemical and likelihood of a “hit” in the Cell Painting assay.

Acknowledgments

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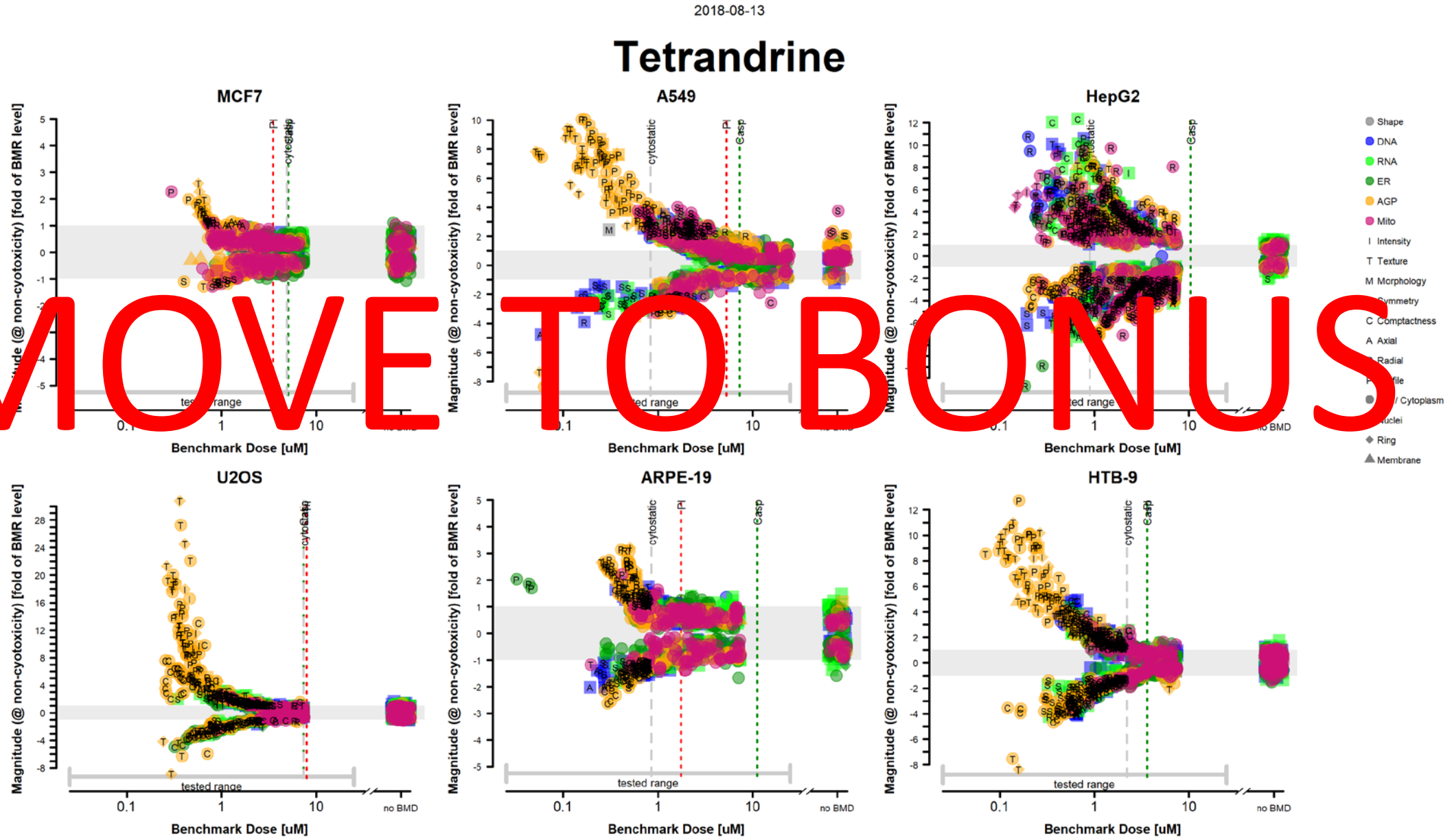
Scott Auerbach

National Center for Computational Toxicology

EPA United States Environmental Protection Agency

Bonus Slides

Comparing Response Profiles Across Cell Types (1)

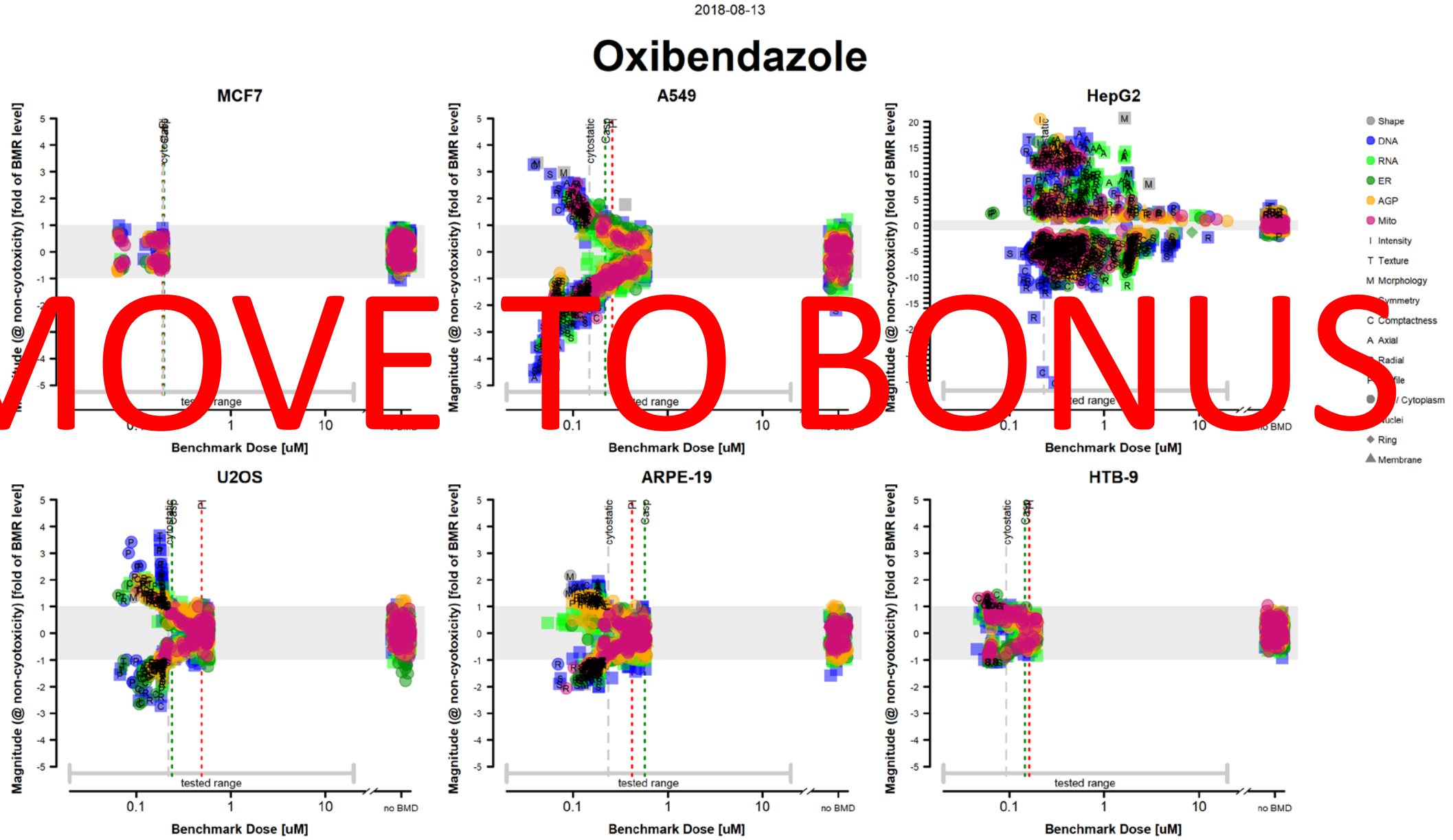


MOVE TO BONUS

Comparing Response Profiles Across Cell Types (5)

MOVE TO BONUS

Comparing Response Profiles Across Cell Types (3)



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