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Development and Use of a High-Throughput Phenotypic Profiling Assay at the USEPA NCCT

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Office of Research and Development
National Center for Computational Toxicology

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United States Environmental Protection Agency

Outline

- Background
  - Who is NCCT?
  - Aim
- Assay Development
  - Image Analysis Workflow
  - Data Analysis Pipeline
- Applications
  - *In vitro* bioactivity thresholds of nanoparticles
  - Margin-of-exposure analysis

Who is NCCT?

Research Triangle Park Campus

National Center for Computational Toxicology

Mission Statement:

A research organization tasked with advancing the science of toxicity testing through the **development and/or application of novel experimental and computational approaches** for rapidly characterizing the biological activity, exposure potential and potential human health risks associated with chemicals.

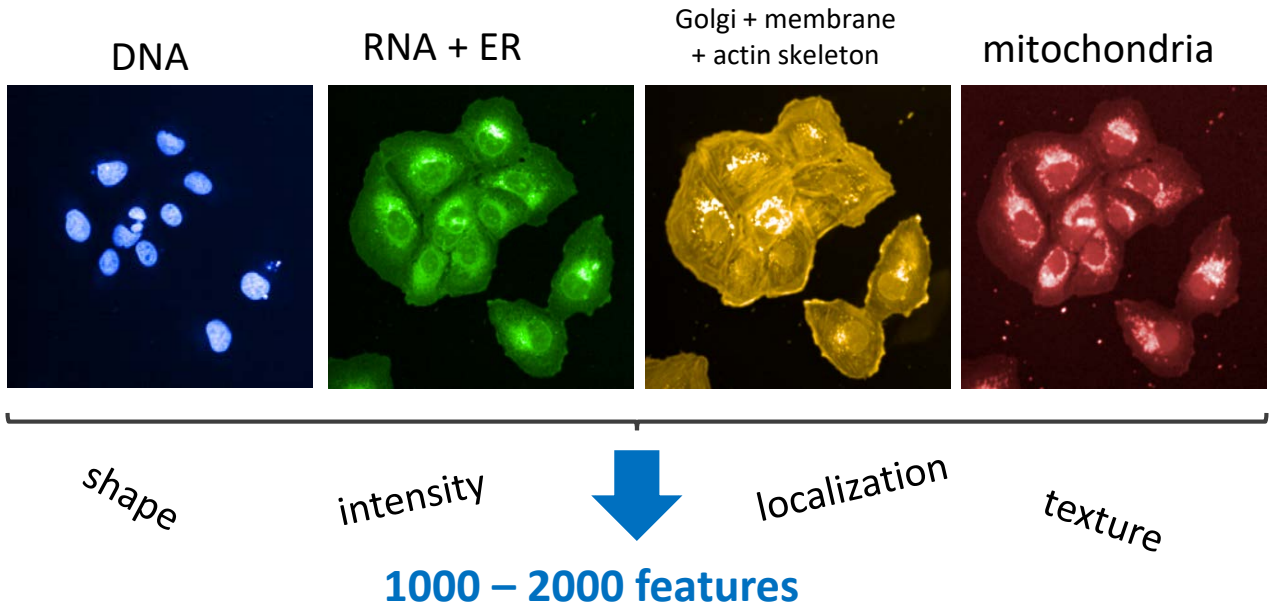
Scientific challenge

- *in vivo* toxicity testing is expensive, time-consuming and requires extrapolation to humans
- regulatory agencies (EPA, ECHA) have begun to explore the use of alternative methods (*in vitro* assays) for toxicity testing and risk assessment
- NCCT/EPA has previously performed high-throughput screening (HTS) using targeted assays to evaluate 1000s of chemicals → ToxCast
- Currently investigating broad-based, non-targeted screening assays as a compliment to targeted HTS

⇨ **Aim: Explore whether phenotypic profiling is a useful screening method for toxicology**

What is image-based phenotypic profiling?

- staining of various cell organelles with fluorescent dyes
- assessing a large variety of morphological features on individual cells in *in vitro* cultures



"Cell Painting"

- Developed by the BROAD institute (Bray et al. 2016, *Nature Protocols*)
- Multiplexing of six fluorescent "non-antibody" labels
- Imaged in five channels

- successfully used for functional genomic studies and in the pharmaceutical industry for compound efficacy and toxicity screening.

Cell Painting = Cytological Profiling = Phenotypic Profiling = High-Throughput Phenotypic Profiling = HTPP

Setup of laboratory workflow for high-throughput testing

Following the protocol of Bray *et al.* 2016 (*Nature Protocols*)

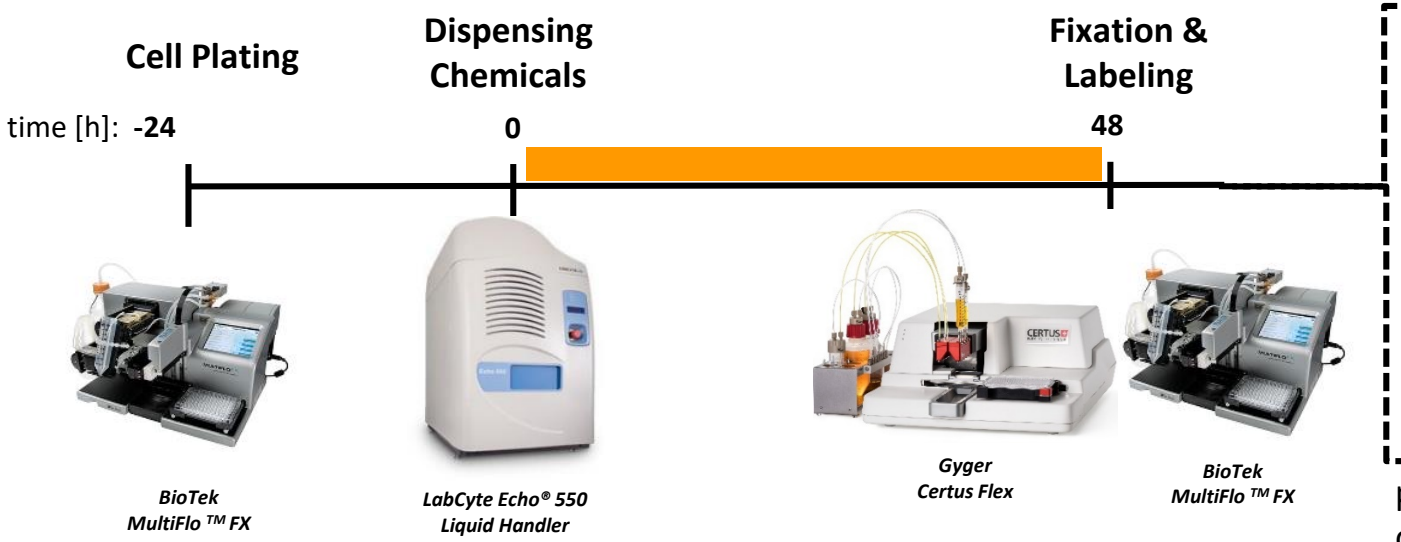


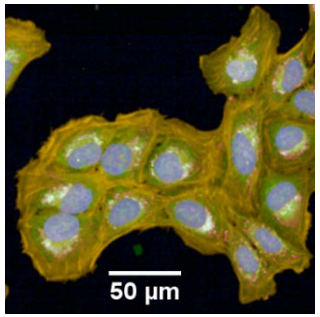
plate 1:
cell profiling

High Content Imaging & Analysis

Perkin Elmer Opera Phenix™ High Content Screening System Harmony Software

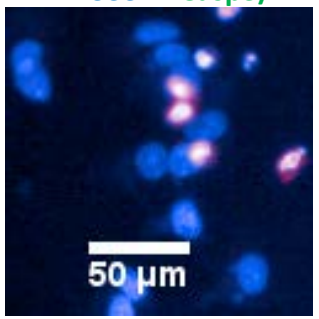
plate 2:
cell viability / cell count

DNA RNA/ER AGP Mito



Labels
DNA: H-33342
RNA: SYTO14
ER: Concanavalin A-488
Actin: Phalloidin-568
Golgi + Membrane: wheat germ agglutinin (WGA) -555
Mitochondria: MitoTracker

H-33342 Casp3/7 PI



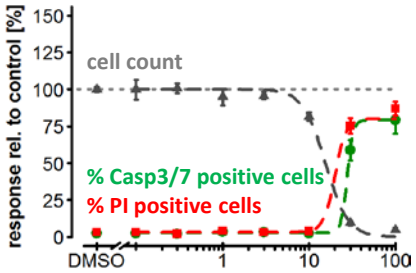


Image Acquisition

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

Image Analysis

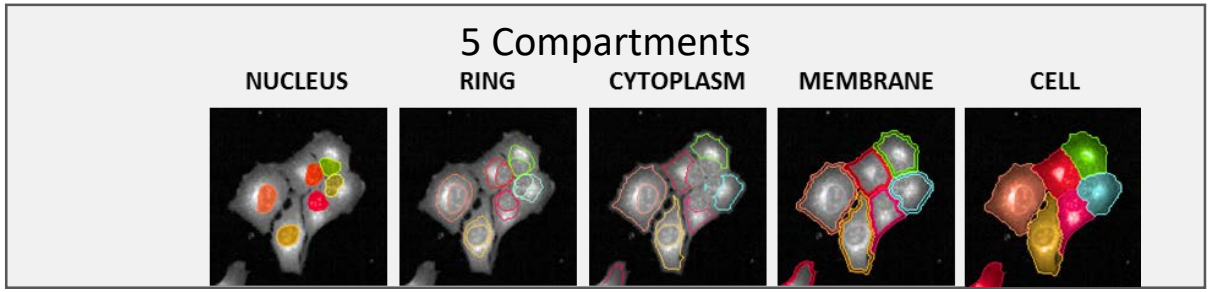
- Perkin Elmer Harmony Software

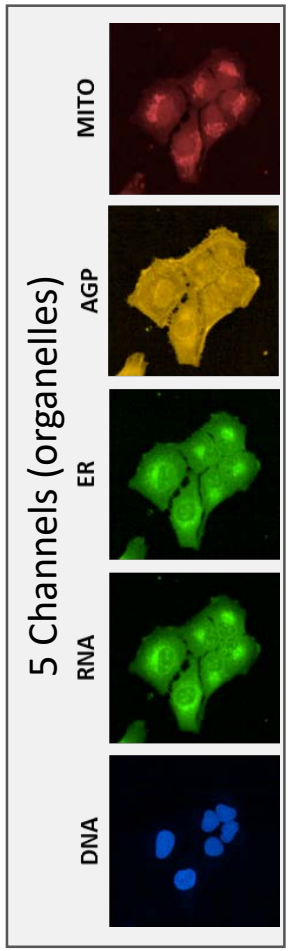
Data Processing

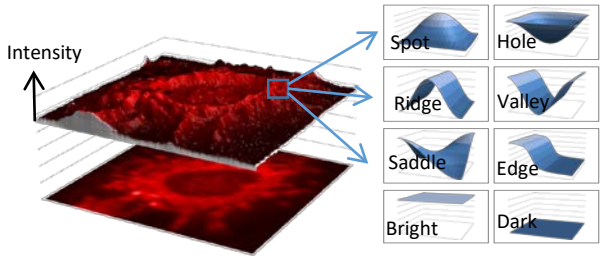
- R Statistical Computing Environment
- BMDExpress 2.0

Image processing for profiling plates

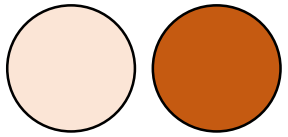
Profiling
with Perkin Elmer
Harmony Software





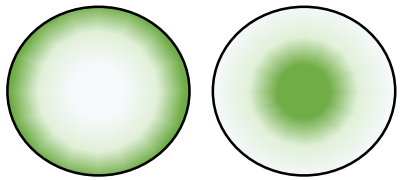


Texture

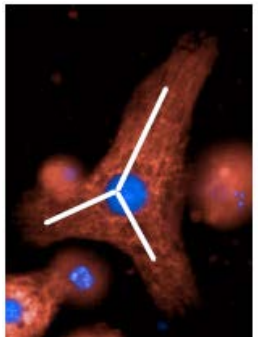


Intensity

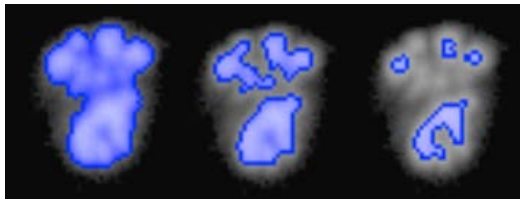
Axial



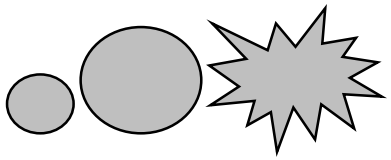
Radial distribution



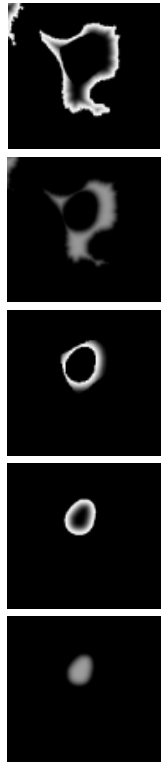
Symmetry



Compactness



Shape



Profile

= ~ 1300 endpoints

48 ontologies

Examples:

- AGP_Texture _Cytoplasm
- Mito_Compactness _Ring
- DNA_Intensity _Nuclei

Illustrations from Perkin Elmer

Data analysis

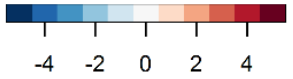
cell-level data

1293 endpoints
~500 cells/well
384 wells

Normalization

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

normalized cell-level data



Aggregation

median

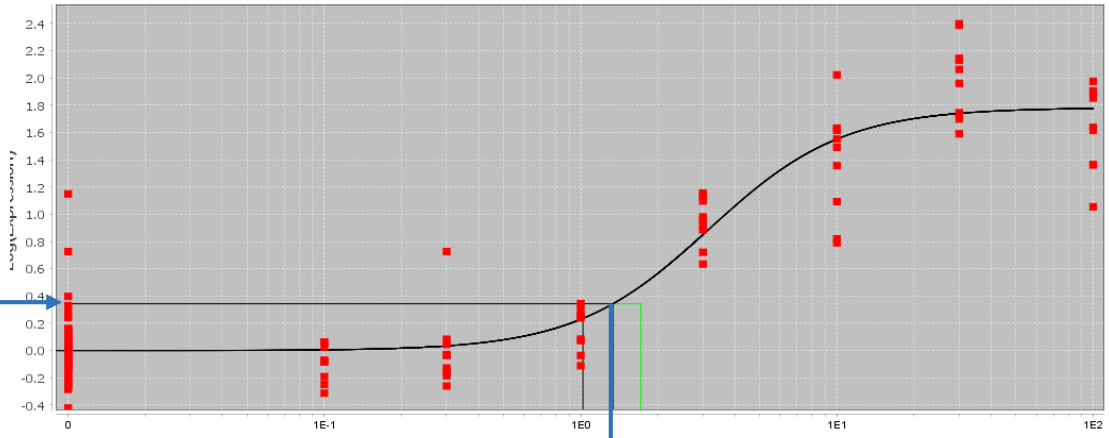
well-level data

1293 endpoints
384 wells

Benchmark dose (BMD) modelling

1293 endpoints
7 concentrations/chemical

Predefined effect level



Dose at which effect level is reached

Benchmark dose (BMD)

1293 endpoints

Point-of-departure (POD)
5% quantile of all BMDs

Profile

Initial findings

Replication of experiments of Gustafsdottir *et al.* 2013

- ⇨ Similar phenotypes were observed
- ⇨ Phenotypes could be quantified
- ⇨ Profiling BMDs were often below onset of cytotoxicity

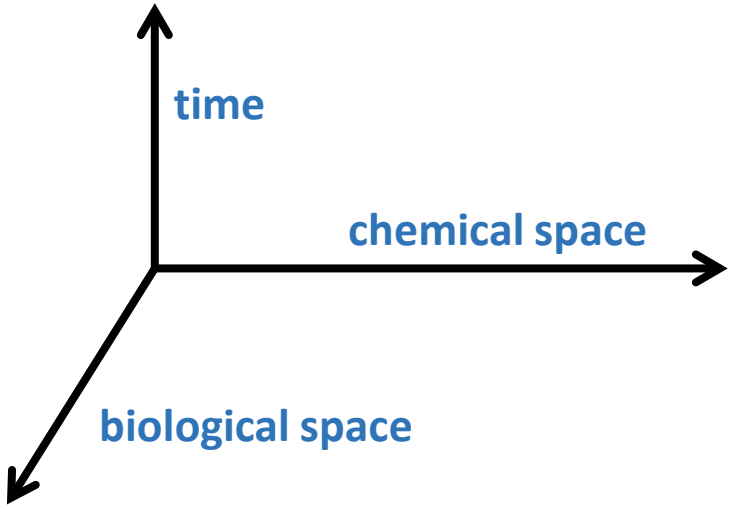
Reference
1 cell type: U-2 OS
48 h exposure
16 reference chemicals
7 concentrations (3 $\log_{10}$ units)
3 replicates / plate
3 biological replicates

Investigating time

- ⇨ Phenotypes measurable after 6 - 12 h

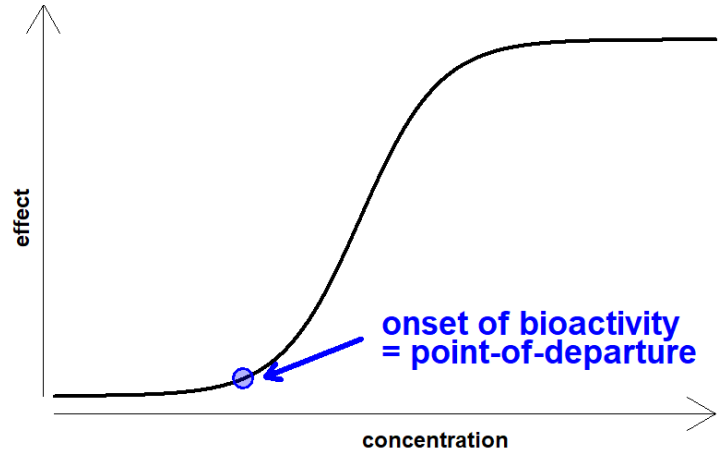
Expansion to 5 other cell lines

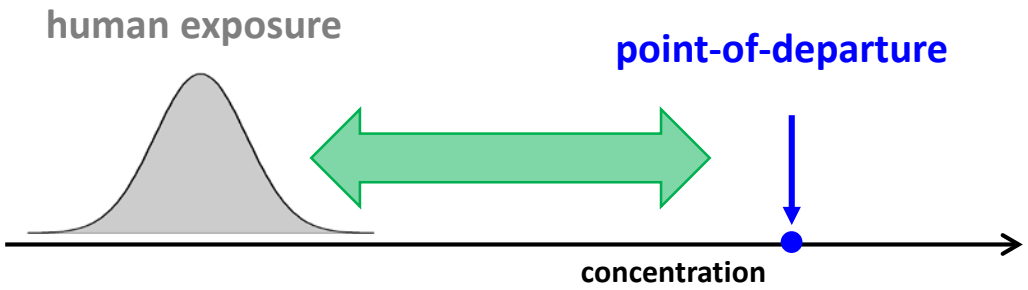
- ⇨ Reference chemicals give similar phenotypes in all cell lines
- ⇨ profiling BMDs were comparable among the cell lines



Potential applications

Estimation of *in vitro* point-of-departures (POD)





Profiles could provide mechanistic insights

Chemical 3
Profile 3:

Mechanism ???

Chemical 1
Profile 1:

Mechanism X

Chemical 2
Profile 2:

Mechanism Y

Image source: www.pixabay.com

Example 1: *In vitro* bioactivity thresholds of nanoparticles

Background:

- Nanoparticles (< 100 nm) have unique physical and chemical properties and produce effects that are different from the “bulk” material
- Toxicity of nanoparticles varies by size and coating, but these relationships are not well understood – particularly for sub-cytotoxic effects.

Experiment:

- Testing of 12 silver nanoparticles: 3 different coatings by 4 particle sizes

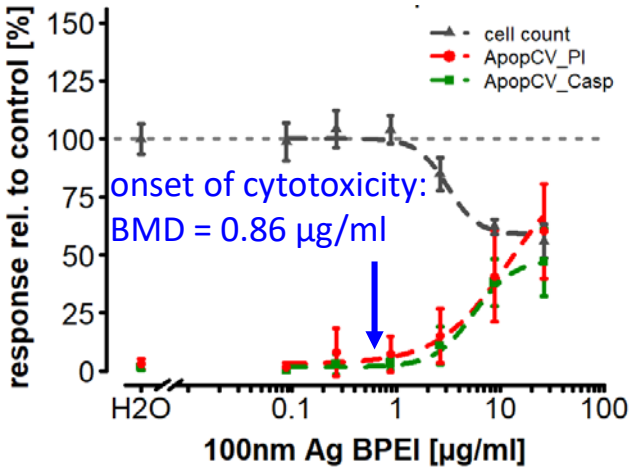
→ What is the relative potency of the different nanoparticles?
Where is the point-of-departure?

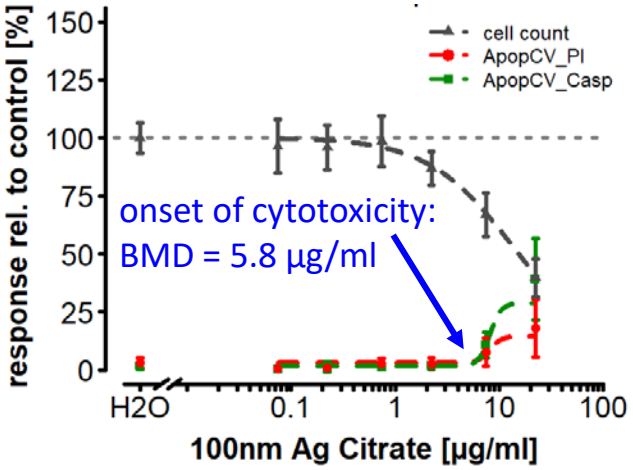
→ Can we obtain mechanistic information by investigating the profiles?

Example 1: *In vitro* bioactivity thresholds of nanoparticles

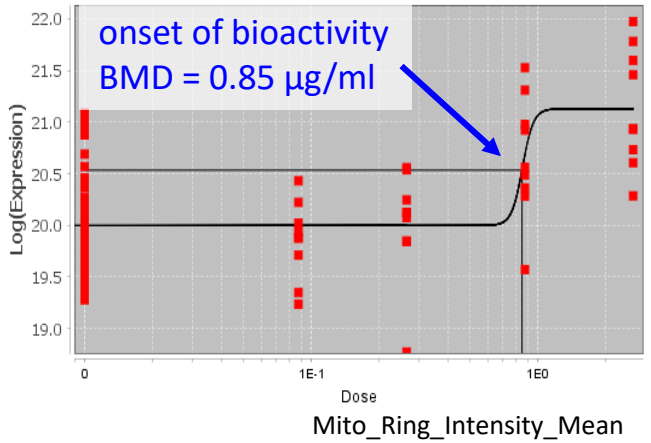
United States Environmental Protection Agency

Cytotoxicity testing:

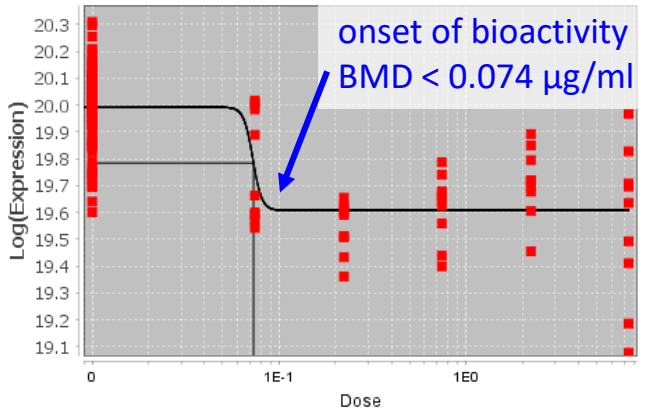




Phenotypic profiling:



Mito_Ring_Intensity_Mean



DNA_Nuclei_Intensity_Mean

Profiles:

	BMD median [µg/ml]
Mito_Intensity_Ring	0.85
Mito_Profile_Nuclei	0.88
Mito_Intensity_Cytoplasm	0.9
DNA_Radial_Cells	0.92
Mito_Profile_Cytoplasm	0.95
DNA_Profile_Cytoplasm	1.1
ER_Compactness_Cells	1.1
DNA_Radial_Nuclei	1.2
ER_Radial_Cells	1.3
DNA_Texture_Nuclei	1.4
DNA_Compactness_Nuclei	1.4
Mito_Radial_Cells	1.4
AGP_Radial_Cells	1.4
RNA_Compactness_Nuclei	1.5
DNA_Profile_Nuclei	1.5

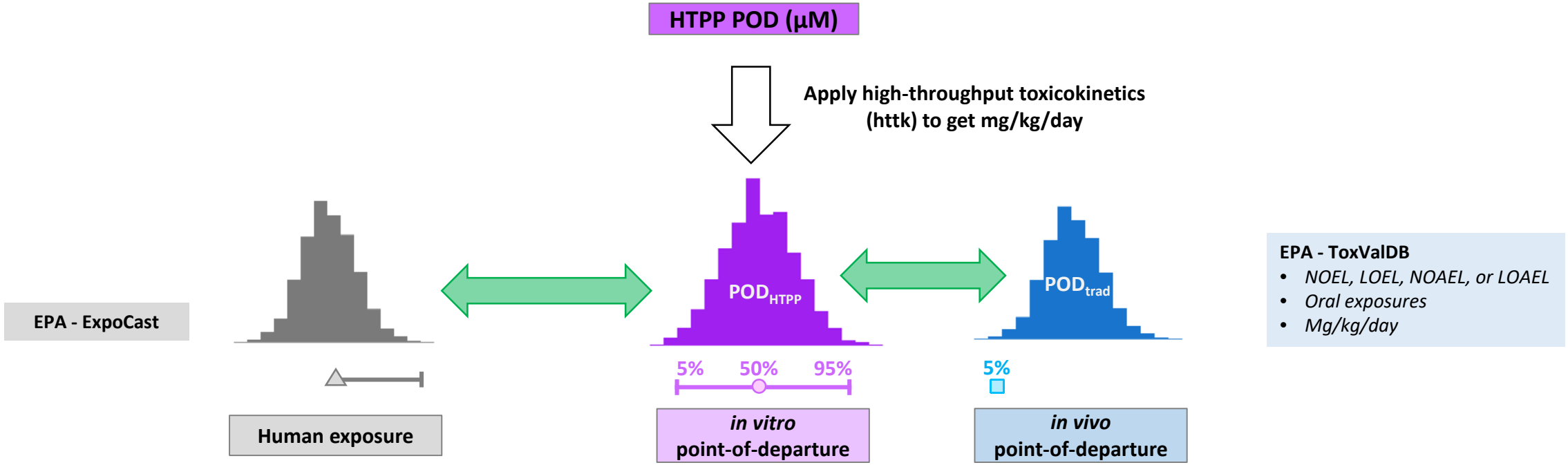
	BMD median [µg/ml]
DNA_Intensity_Nuclei	0.022
DNA_Profile_Nuclei	0.075
RNA_Intensity_Nuclei	0.086
DNA_Profile_Cytoplasm	0.12
DNA_Radial_Cells	0.58
ER_Radial_Cells	0.68
DNA_Radial_Nuclei	0.78
Mito_Radial_Cells	0.78
RNA_Radial_Nuclei	0.79
RNA_Compactness_Nuclei	0.85
RNA_Axial_Nuclei	0.92
DNA_Compactness_Nuclei	0.92
Mito_Compactness_Cells	0.98
DNA_Axial_Nuclei	1
RNA_Texture_Nuclei	1.1

⇨ **Profiling gave opposing information than cytotoxicity measurement**

⇨ **Profiles suggest different mechanisms of toxicity**

Example 2: Margin-of-exposure analysis

- Screen of 79 chemicals:
  - Subset of ToxCast chemicals
  - compounds had information about onset of bioactivity *in vivo* and human exposure data available

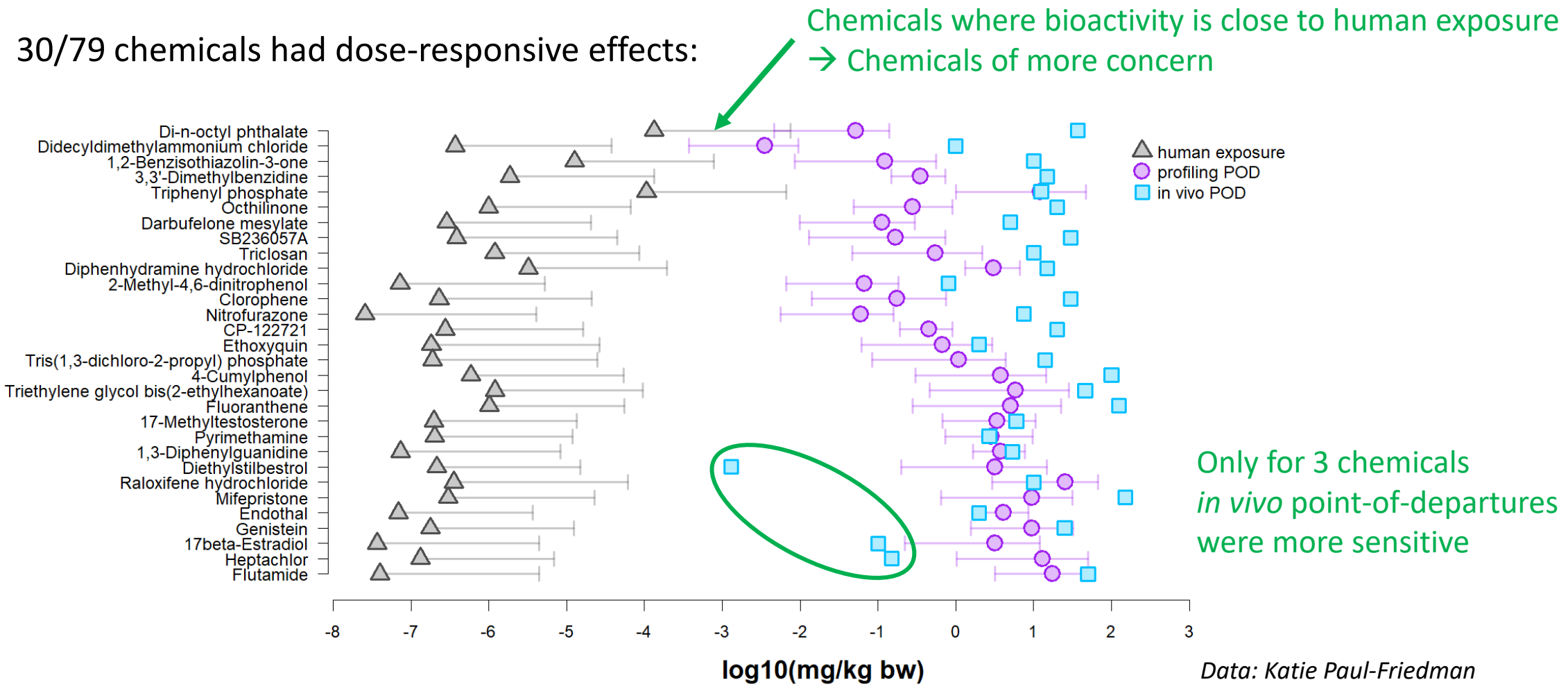


→ **How does this point-of-departure relate to *in vivo* data?**

→ **How does this point-of-departure relate to human exposure?**

Example 2: Margin-of-exposure analysis

- 30/79 chemicals had dose-responsive effects:



Only for 3 chemicals *in vivo* point-of-departures were more sensitive

Data: Katie Paul-Friedman

⇨ **Most chemicals' bioactivity occurred at concentrations above predicted human exposure levels**

⇨ **For 27/30 HTPP hits, the POD was at least as sensitive as in vivo data**

Take home messages

1. EPA is investigating the use of phenotypic profiling to screen chemicals for hazard identification
2. Microfluidics workflow and data analysis pipelines have been developed
3. Replication of published results confirmed assay performance and prompted exploration of biological space and time
4. The assay can be used to calculate point-of-departures that are comparable to *in vivo* toxicity studies
5. Potential use of the assay:
   - define an *in vitro* point-of-departure for hazard identification
   - profiles could give mechanistic information

Acknowledgment

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EPA
United States
Environmental Protection
Agency

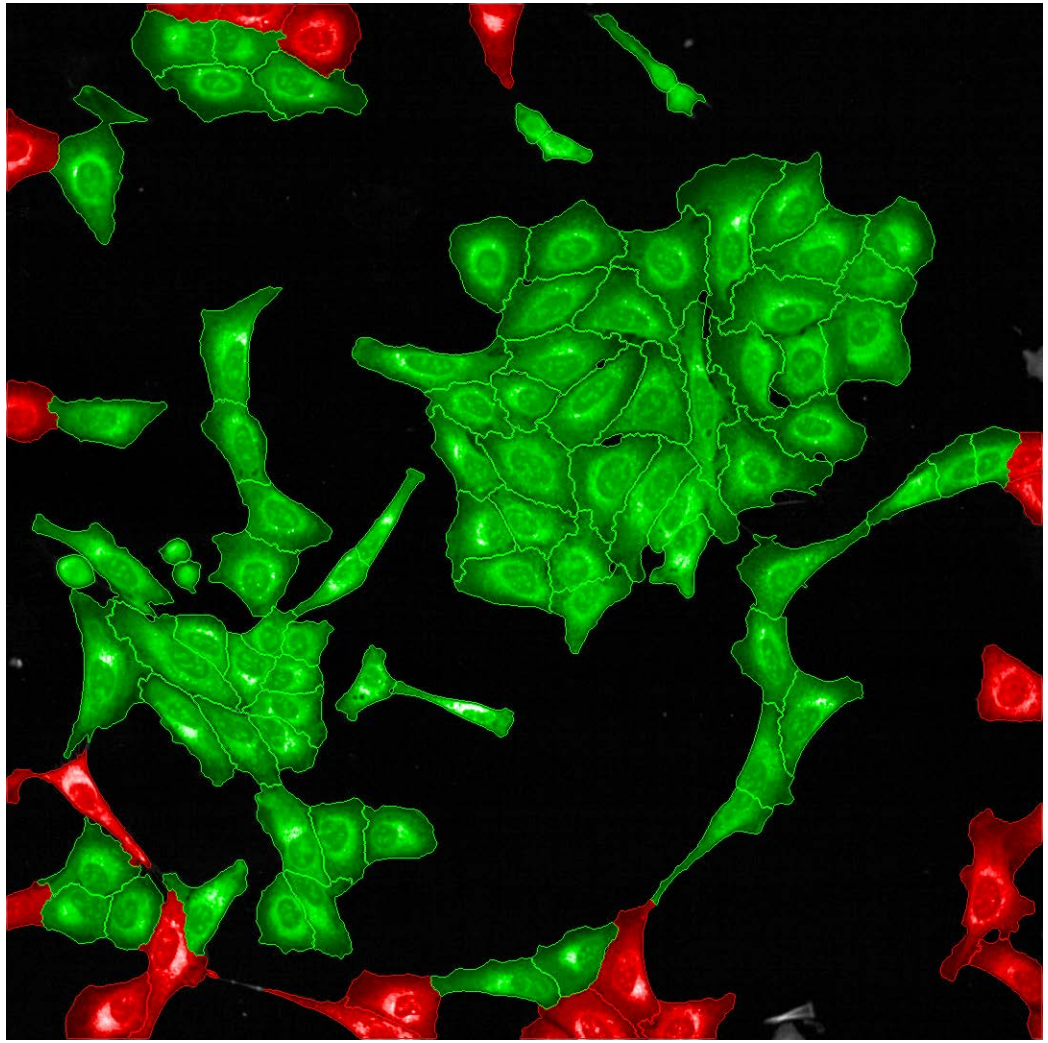
Thank you!

Questions?

Image processing for profiling plates

Segmentation & Object definition

1. Find nuclei
2. Find cell outline
3. Reject border objects



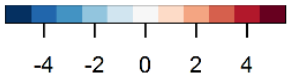
Data processing for profiling plates

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

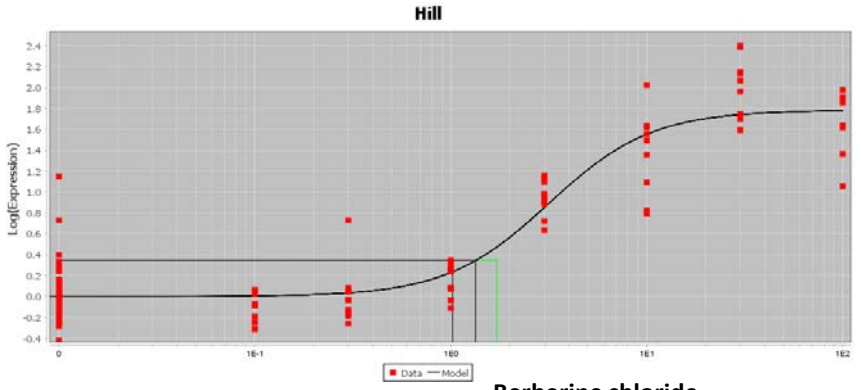
Benchmark dose (BMD) modelling
using BMDExpress 2.2

1293 endpoints

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

BMD modelling — 4 models: Hill, Linear, Poly2, Power

Model selection — Model with best AIC



Berberine chloride
Mito_Cells_Morph_STAR

BMD_10%

Experimental design

Goal:

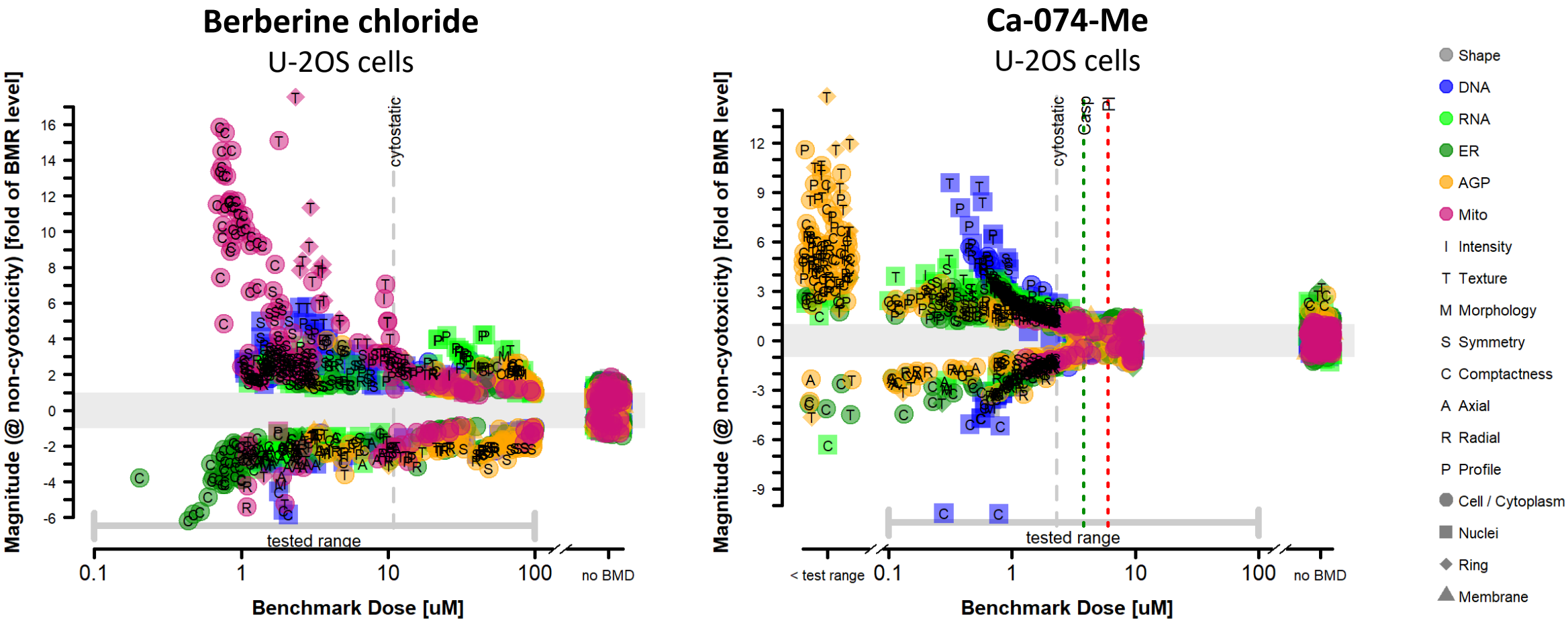
Replicate data from a published study (Gustafsdottir et al. 2013) using
- same cell line
- same chemical set
- same exposure time

Reference
1 cell type: U-2 OS
48 h exposure
16 reference chemicals
7 concentrations (3 $\log_{10}$ units)
3 replicates / plate
3 biological replicates

Reference chemical set:

Compound Name	Phenotype in Gustafsdottir et al. 2013
Amperozide	Toroid **nuclei**
Berberine Chloride	Redistribution of **mitochondria**
Ca-074-Me	Bright, abundant **Golgi** staining
Etoposide	Large, flat **nucleoli**
Fenbendazole	Giant, **multi-nucleated** cells
Fluphenazine	Enhanced **Golgi** staining and some cells with fused **nucleoli**
Latrunculin B	**Actin** breaks
Metoclopramide	Enhanced **Golgi** staining and some cells with fused **nucleoli**
NPPD	Redistribution of **ER** to one side of the nucleus
Oxibendazole	Large, **multi-nucleated** cells with fused **nucleoli**
Rapamycin	Reduced **nucleolar** size
Rotenone	**Mitochondrial** stressor
Saccharin	Negative control
Sorbitol	Negative control
Taxol	Large, **multi-nucleated** cells with fused **nucleoli**
Tetrandrine	Abundant **ER**

Can we quantify different profiles?



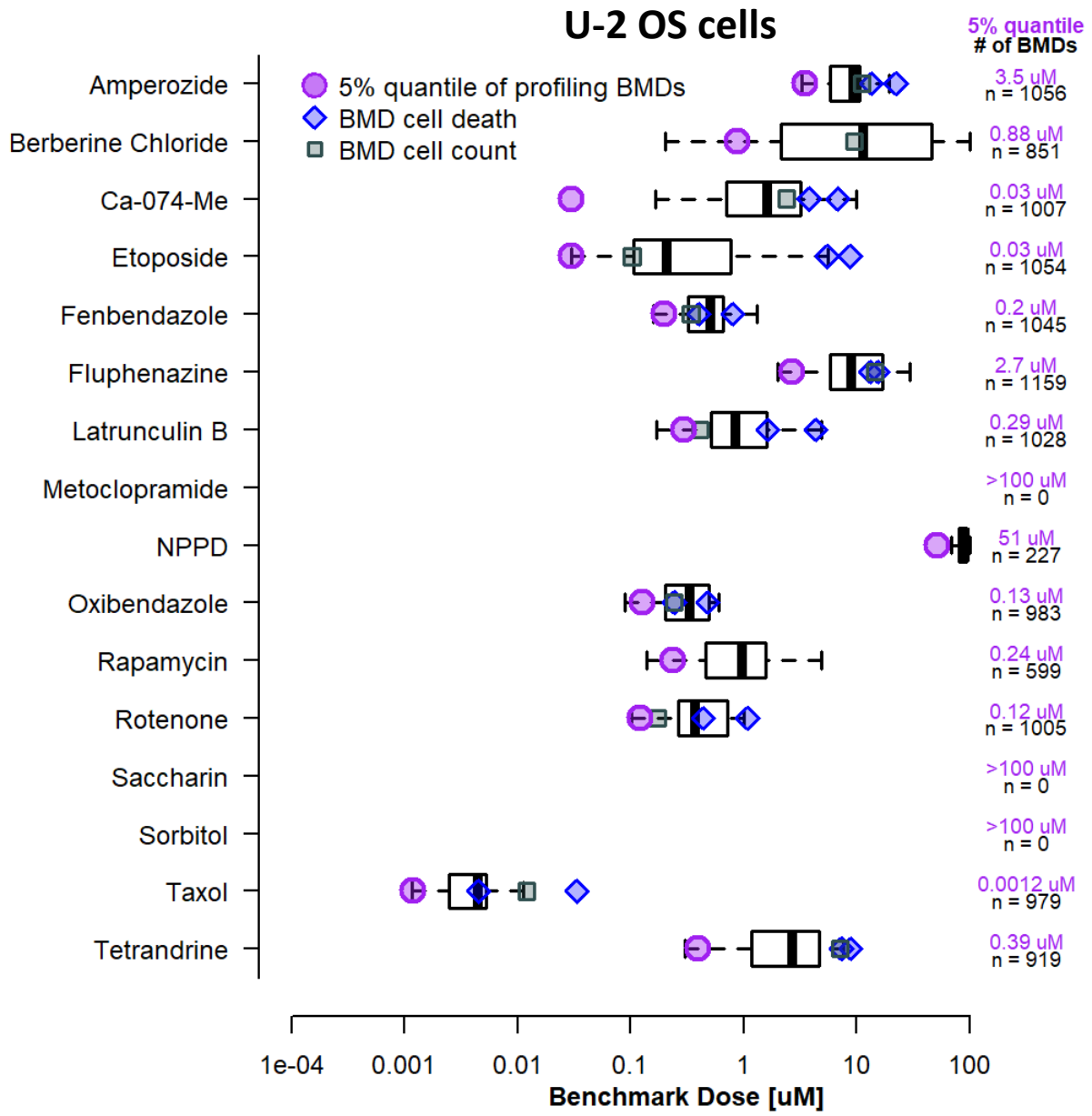
⇨ **Different compounds lead to different profiles**

In vitro point-of-departure (POD) determination

Point of departure definition

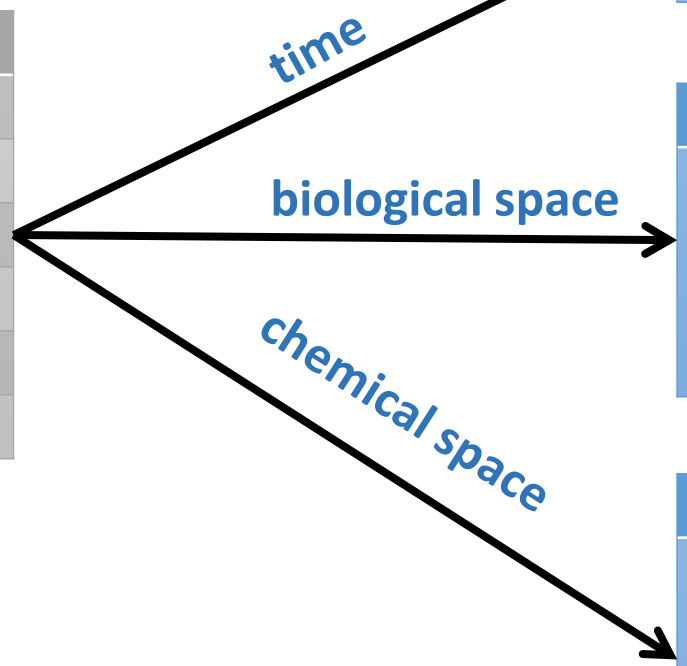
- **POD** = 5% quantile of all profiling BMDs

⇨ **Profiling POD is often more sensitive than cell death BMDs**



Experimental design

Reference
1 cell type: U-2 OS (Bone)
48 h exposure
16 reference chemicals
7 concentrations (3 $\log_{10}$ units)
3 replicates / plate
3 biological replicates



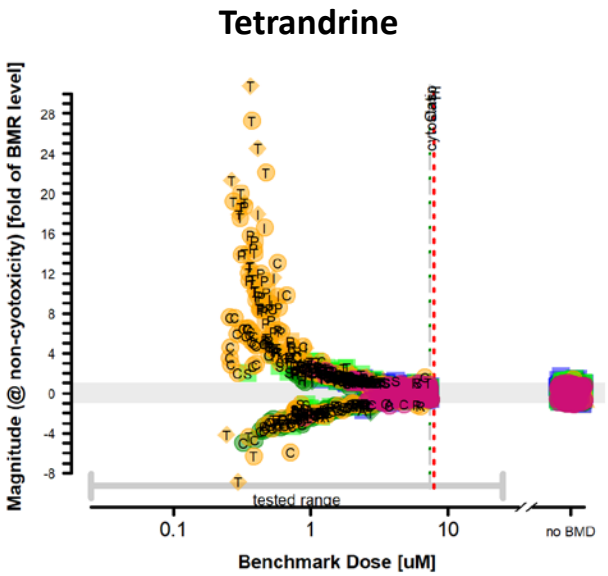
Time
3 / 6 / 12 / 24 / 48 h exposure
2 biological replicates

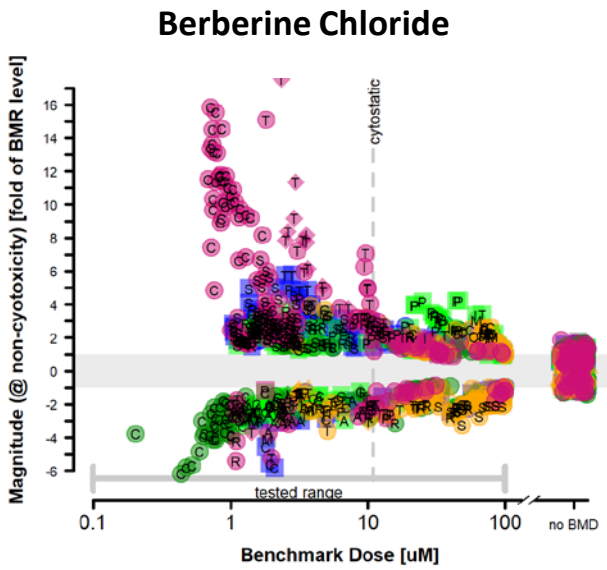
Biological space
+ 5 cell types: MCF-7 (Breast) A549 (Lung) HTB-9 (Urinary bladder) ARPE-19 (Retina) HepG2 (Liver)

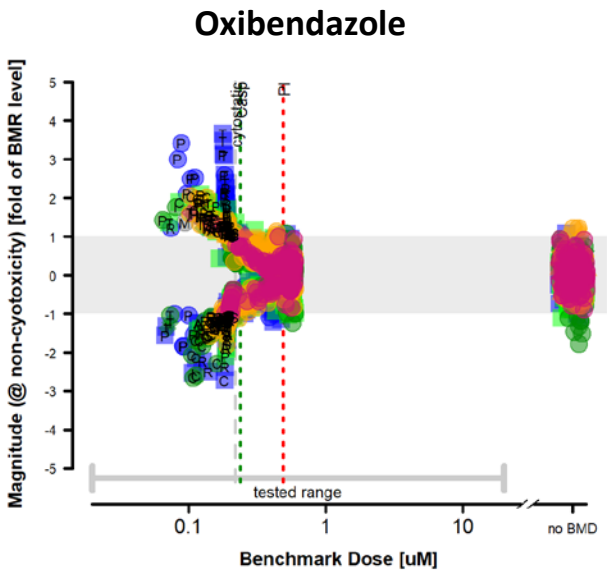
Chemical space
79 chemicals Selected from ToxCast to contain HTTK information
8 concentrations (3.5 $\log_{10}$ units)
1 replicate / plate & 3 plates
1 biological replicate

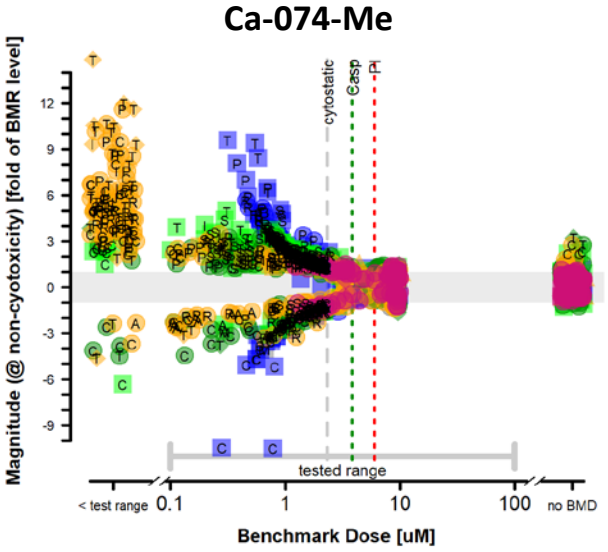
preliminary data!

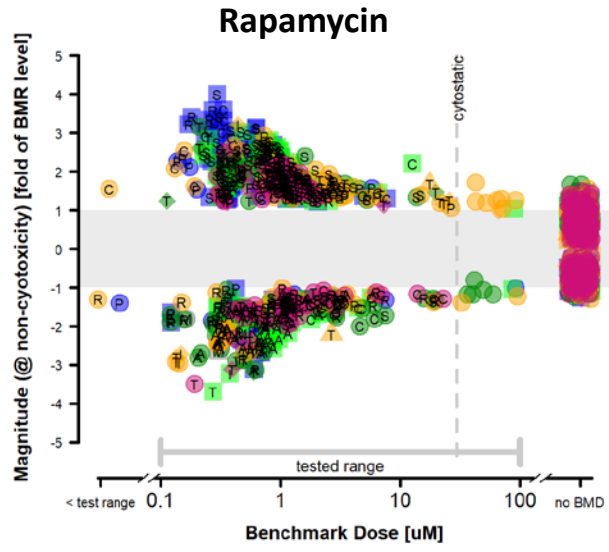
Visualizing Phenotypic Profiles: Potency vs. Efficacy Plots

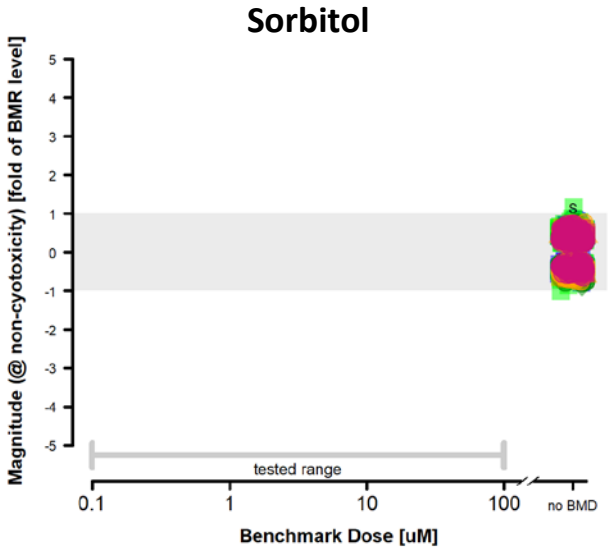




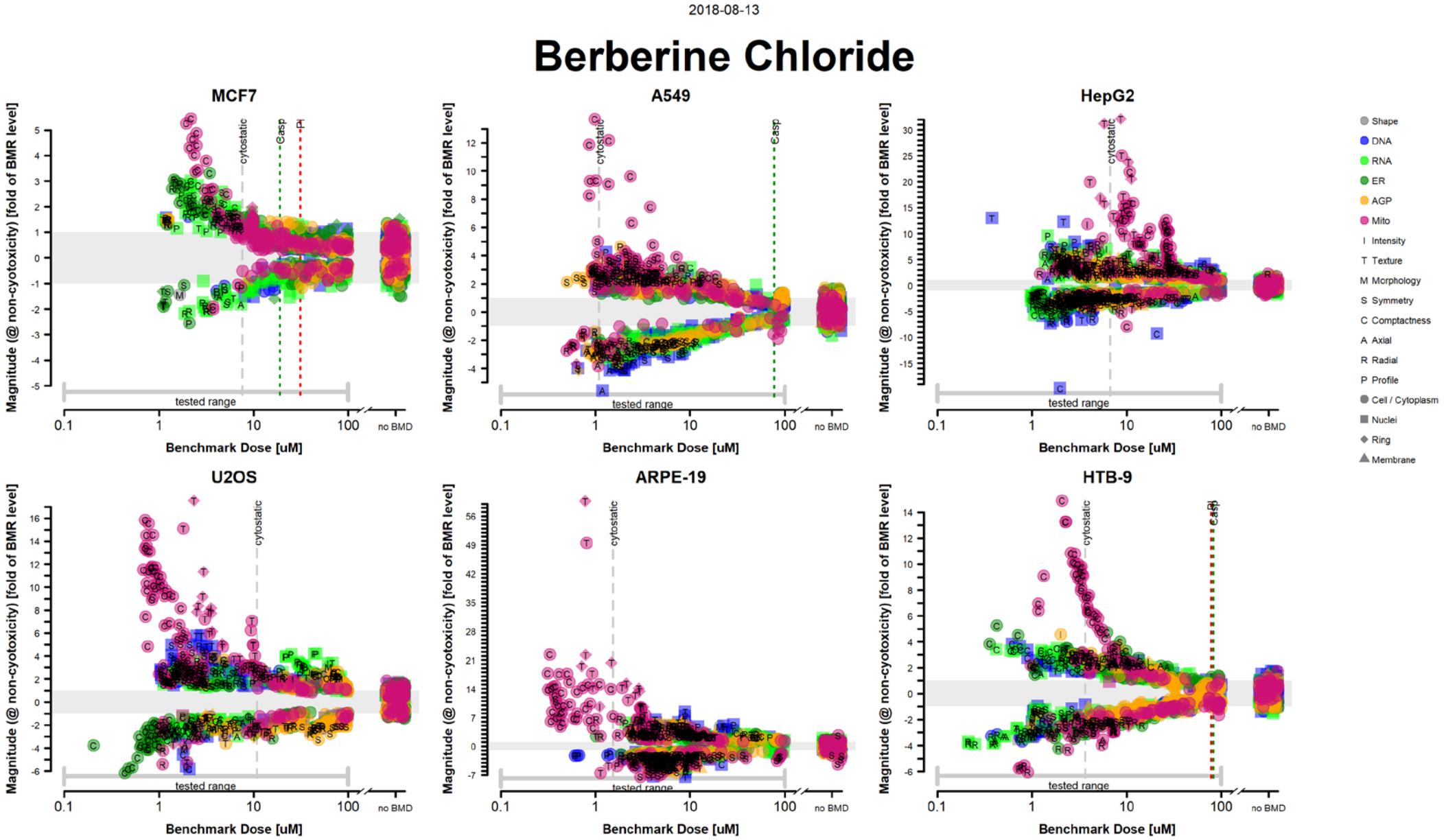




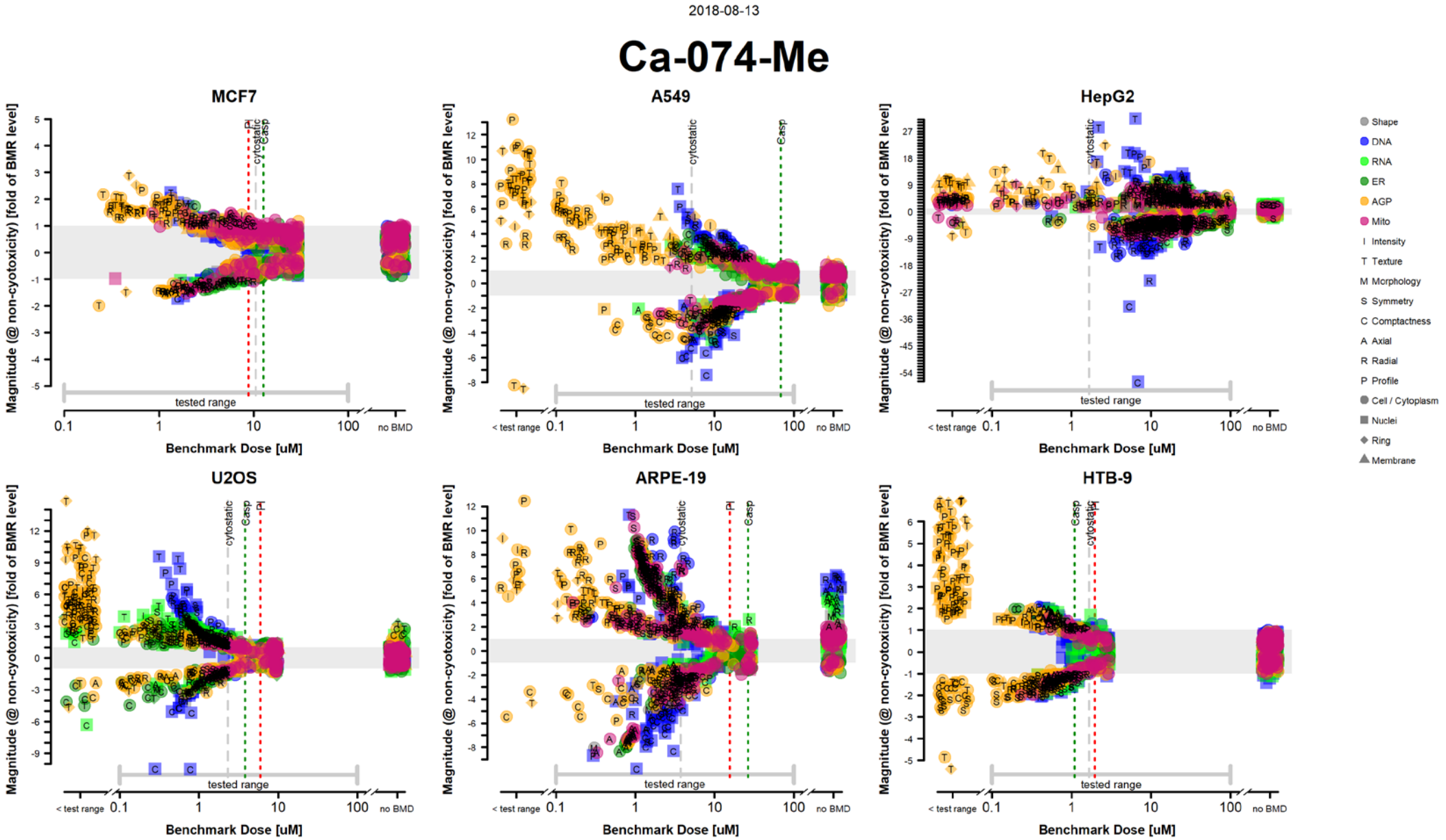




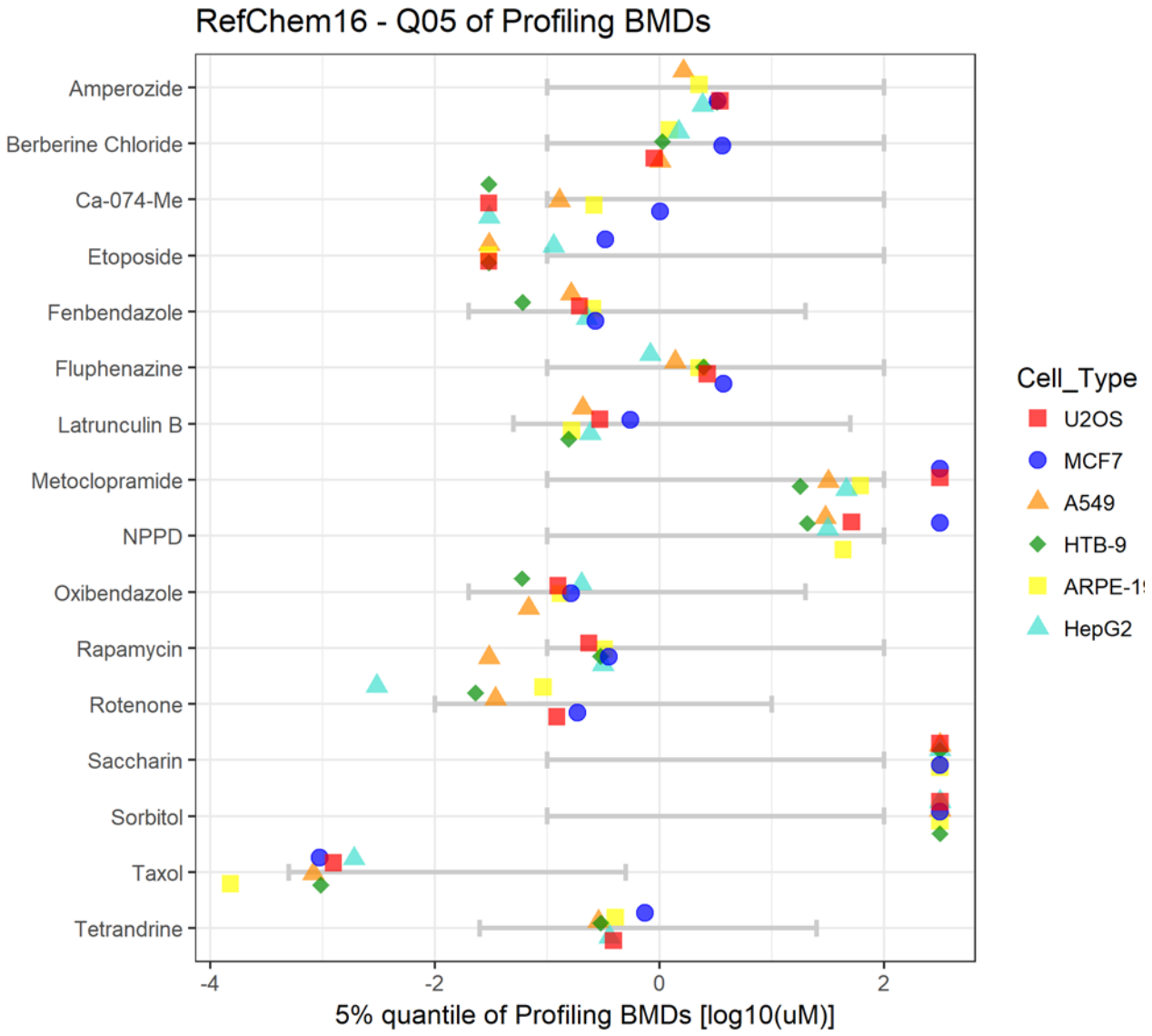
Comparable Response Profiles Across Cell Types (1)

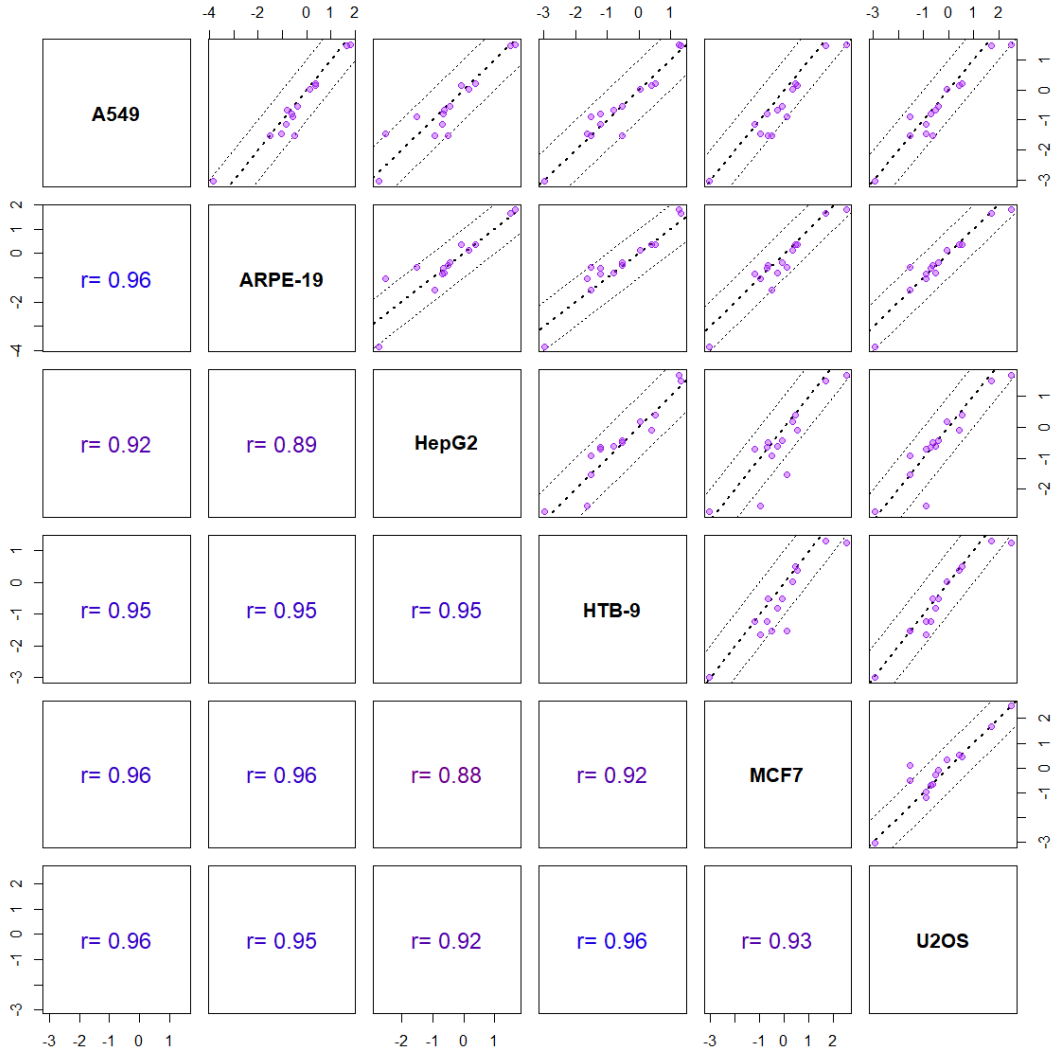


Comparable Response Profiles Across Cell Types (2)



Strong Correlation of Cell Painting PODs Across Cell Types

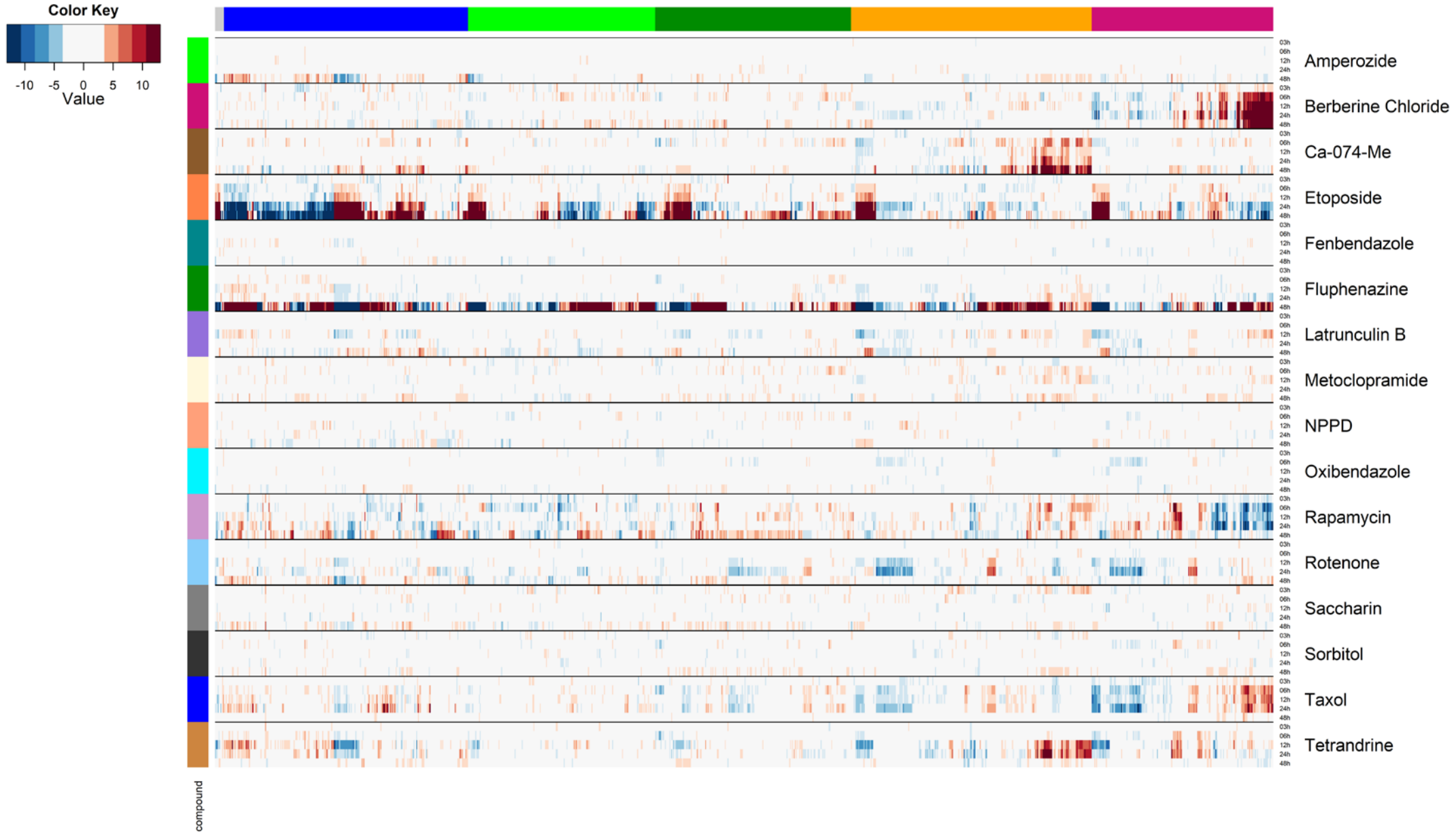




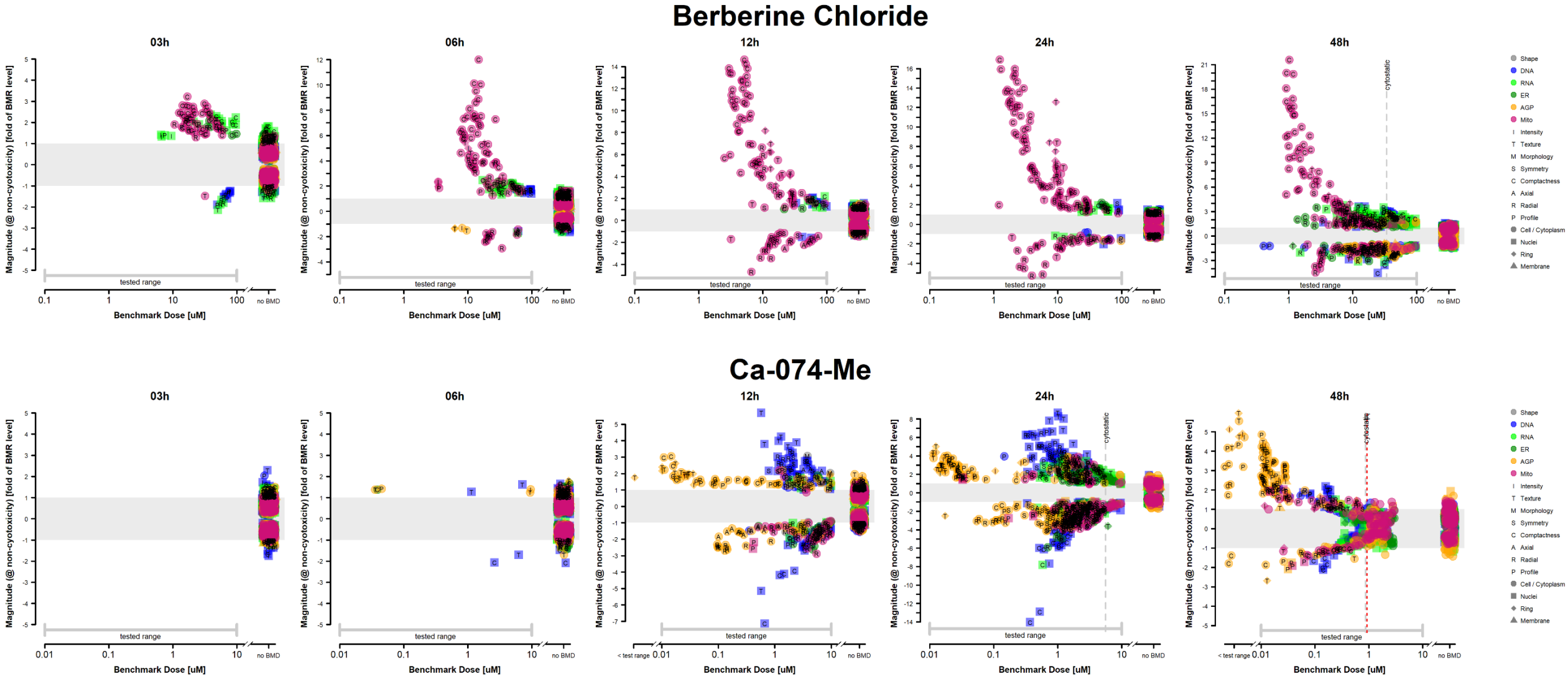
- Different cell lines correlate to ~ 90%.

EPA United States Environmental Protection Agency

Qualitative Similarity in Response Profiles Over Time

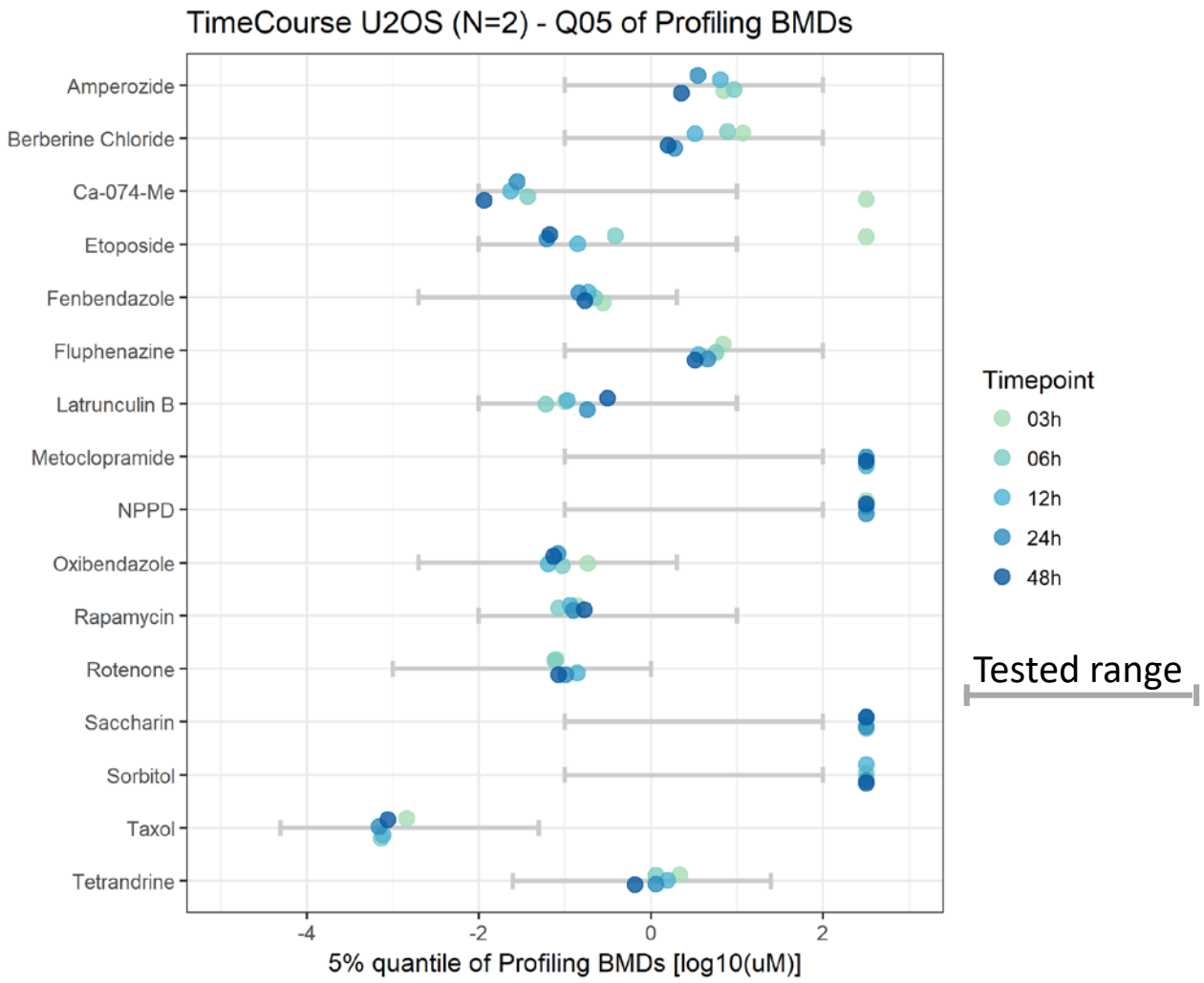


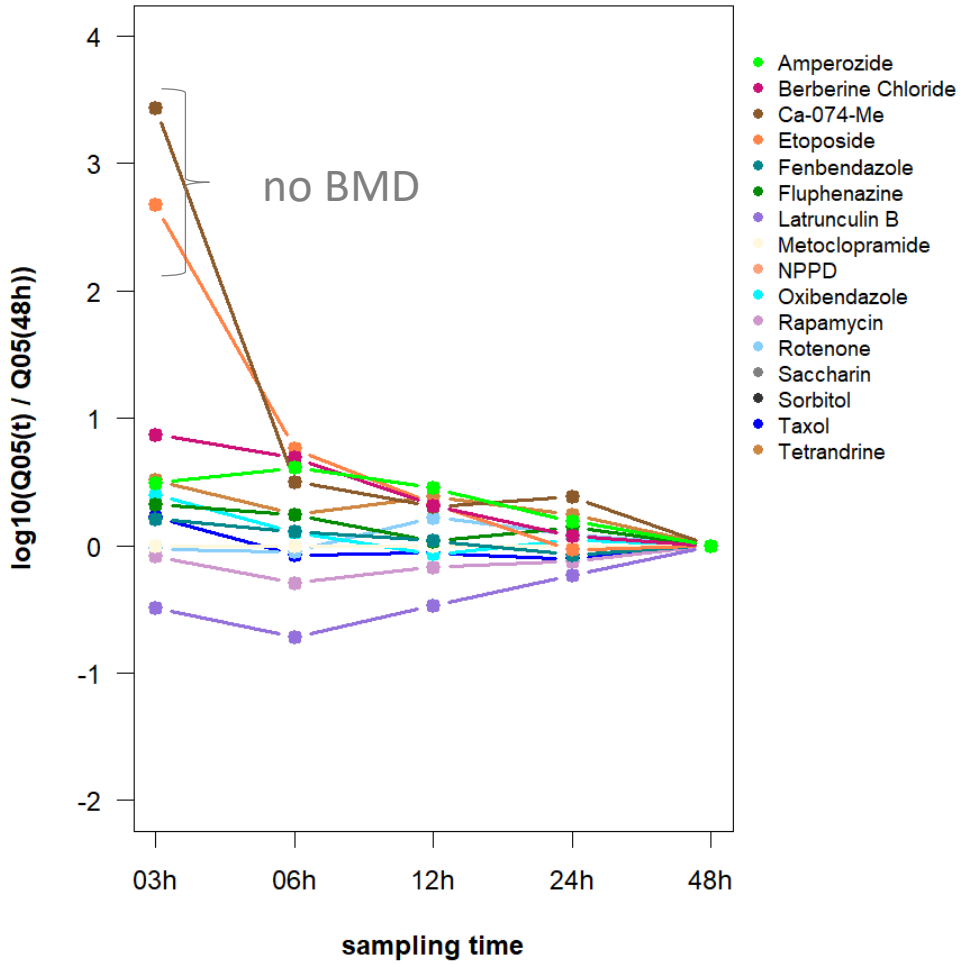
How do the profiles vary across sampling times?



⇨ **Profiles arise at 6-24 h and become less specific at 48 h.**

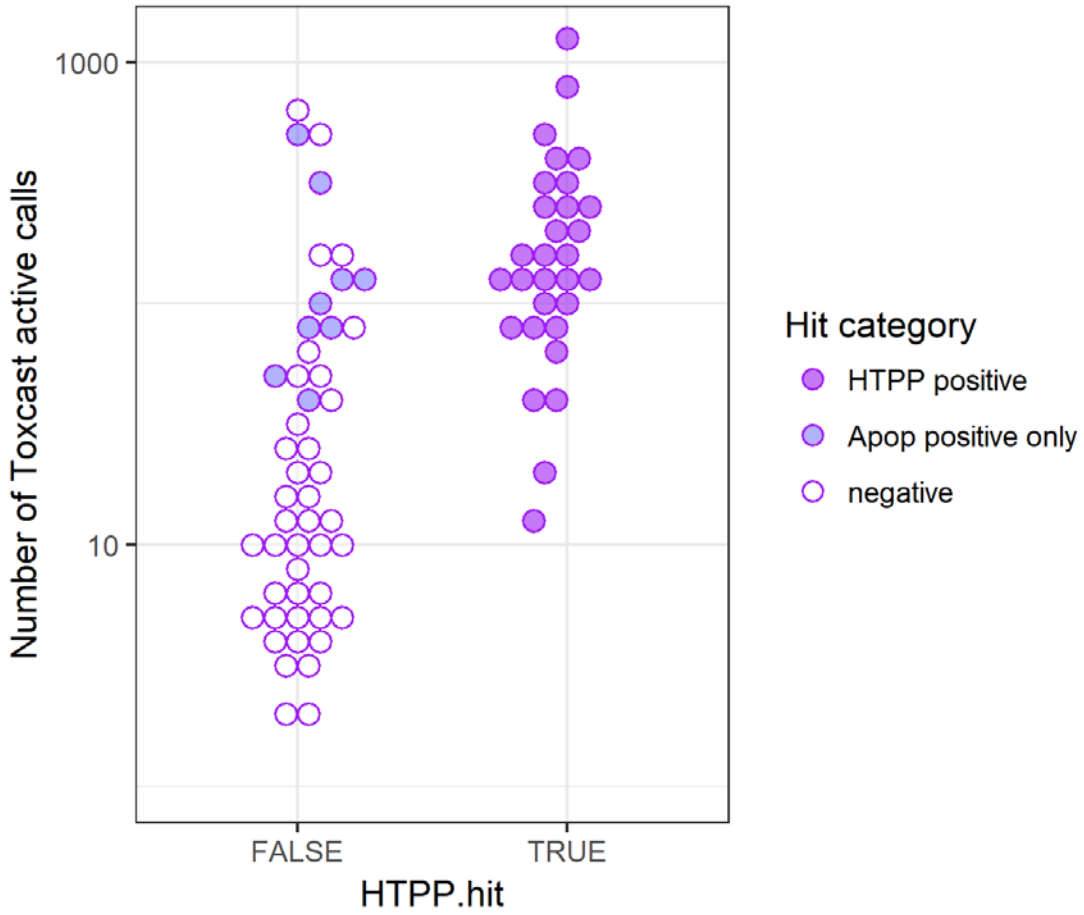
How do PODs vary across sampling times?

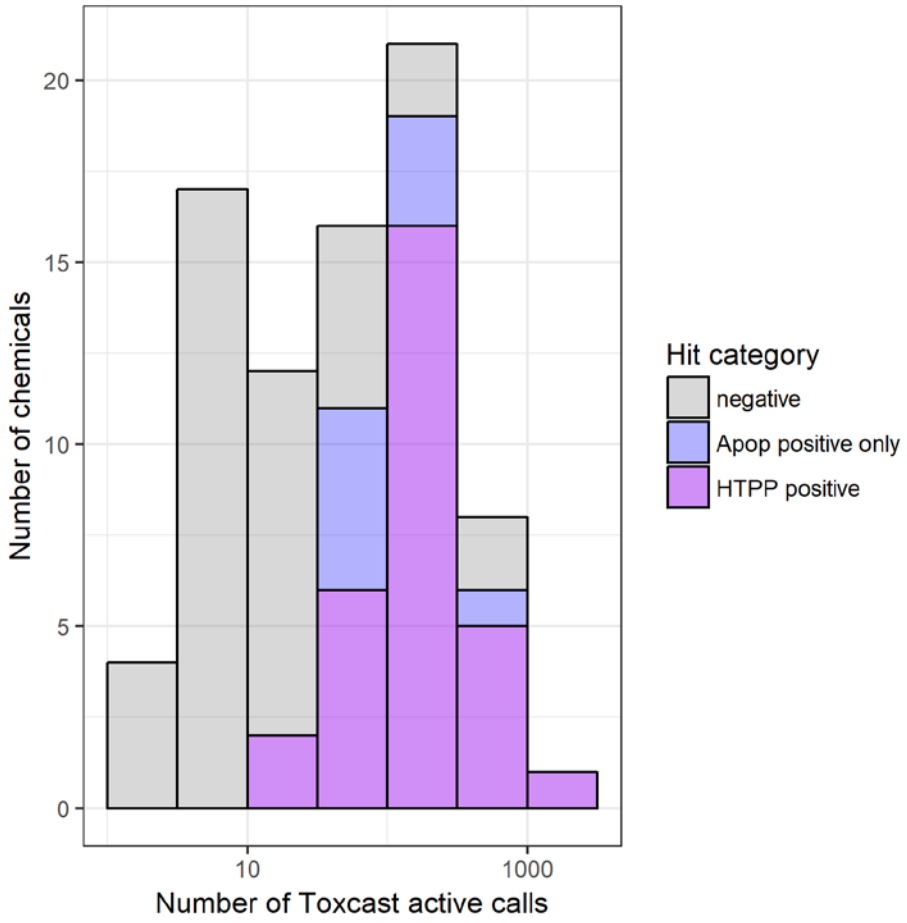




⇨ **PODs are stable over time (vary less than 1 order of magnitude)**

Comparative Sensitivity of Cell Painting and ToxCast



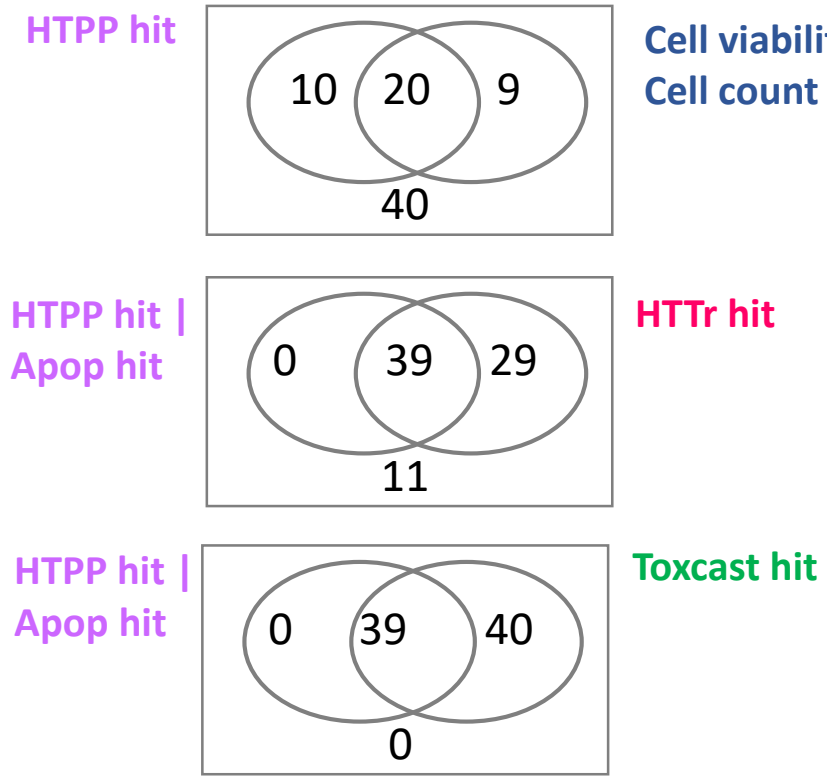


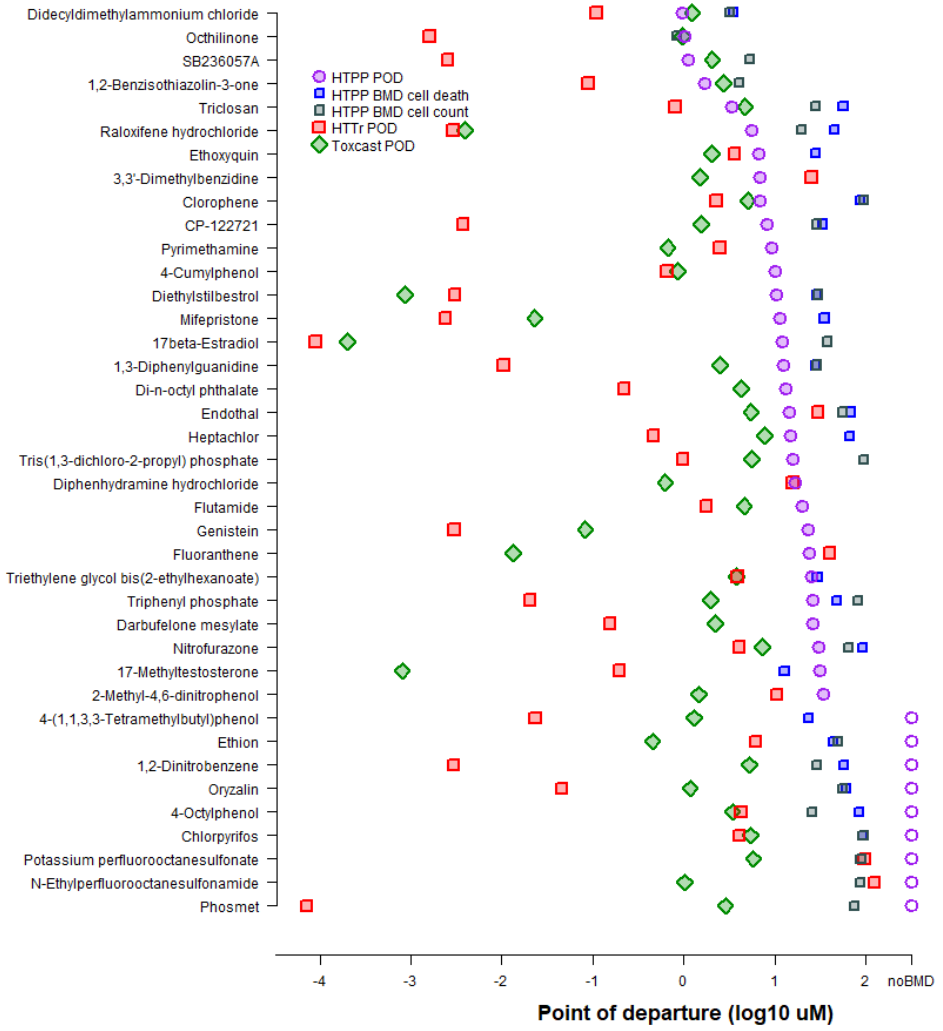
- Preliminary analysis indicates that ToxCast is more sensitive than Cell Painting.
- **Caveats:** To date, only one cell type evaluated in Cell Painting.
  Cell Painting perform in intact cells with adaptive mechanisms.

Preliminary Data –
DO NOT CITE OR QUOTE

Screen of a 79 chemical test set: *in vitro* comparison

- 30/79 chemicals had a POD (i.e. are HTPP hits),
  9 chemicals had a cell viability/cell count BMD only:





HTTr: Data from Josh (preliminary analysis)
Toxcast: Data and POD definition by Katie

⇨ **HTPP POD are higher than ToxCast and HTTr**

⇨ **HTPP hits seem to be promiscuous chemicals**