EPA
United States
Environmental Protection
Agency

Cytological Profiling for Bioactivity Screening of Chemicals

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Office of Research and Development
National Center for Computational Toxicology

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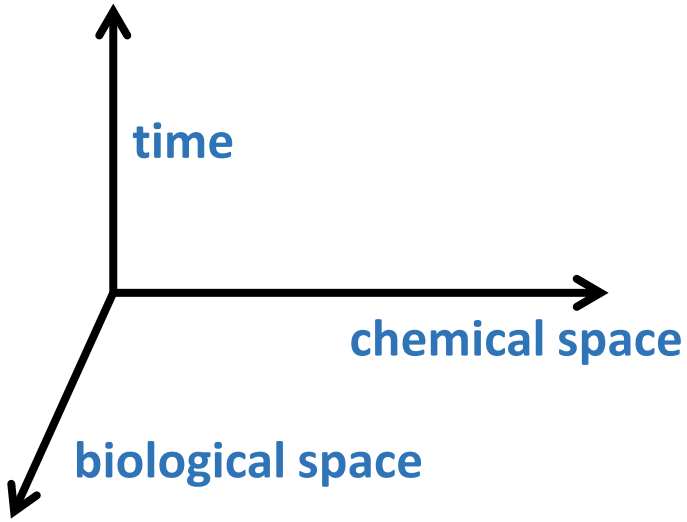
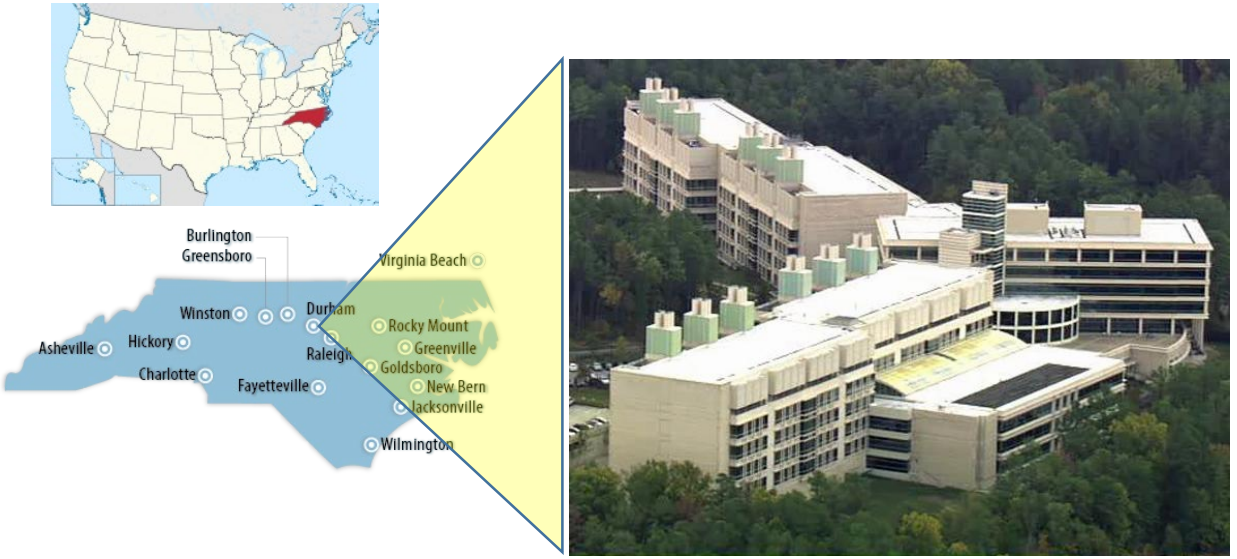
Outline

1. Who is NCCT
2. Introduction to phenotypic profiling
3. Methods:
   - Laboratory workflow
   - Image analysis with Harmony software
   - Data analysis and interpretation
4. Confirmation of published results:
   - Profiles of 16 reference chemicals in reference cell line
5. Profiles across
   - Time
   - Biological space
6. Applications



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 hazard identification and characterization**

Potential applications

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

Profiles could provide mechanistic insights

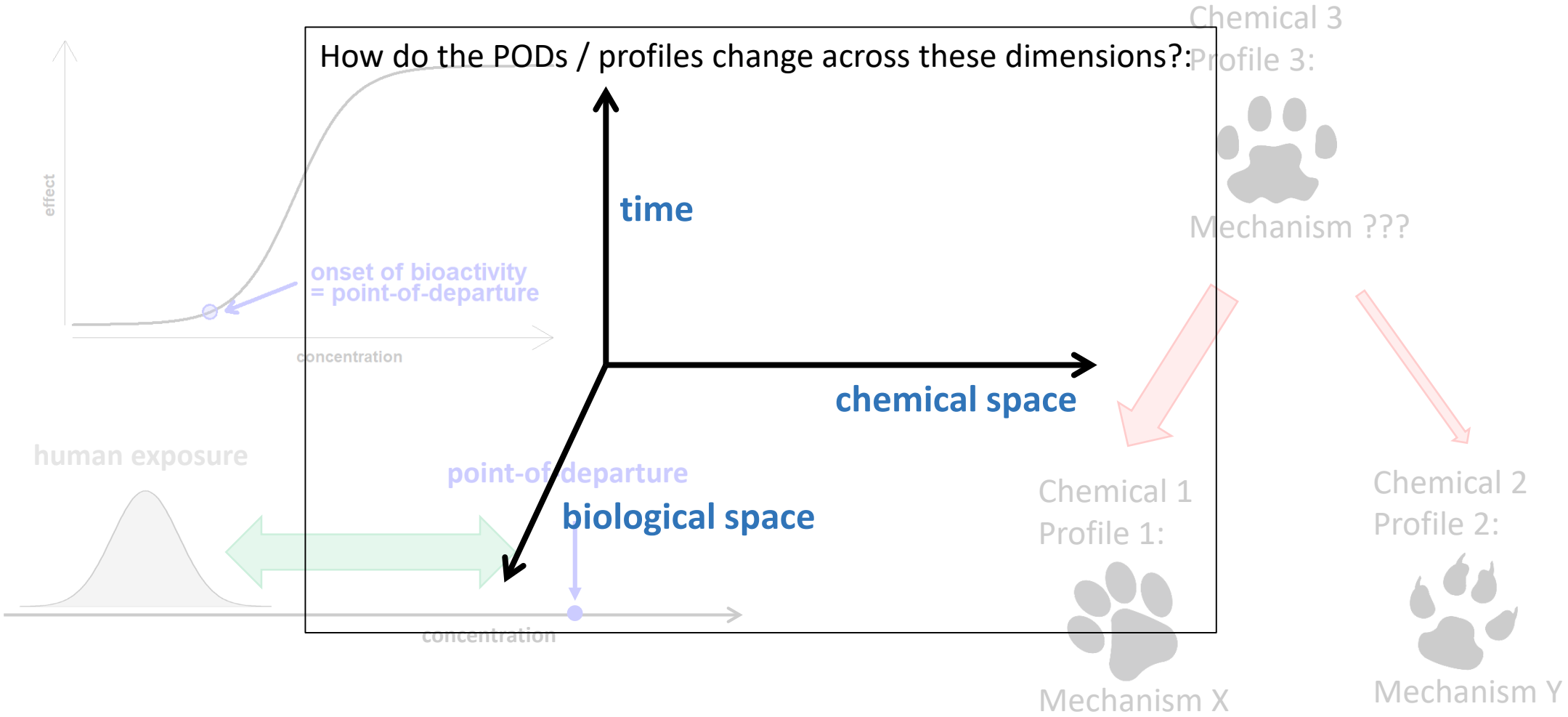
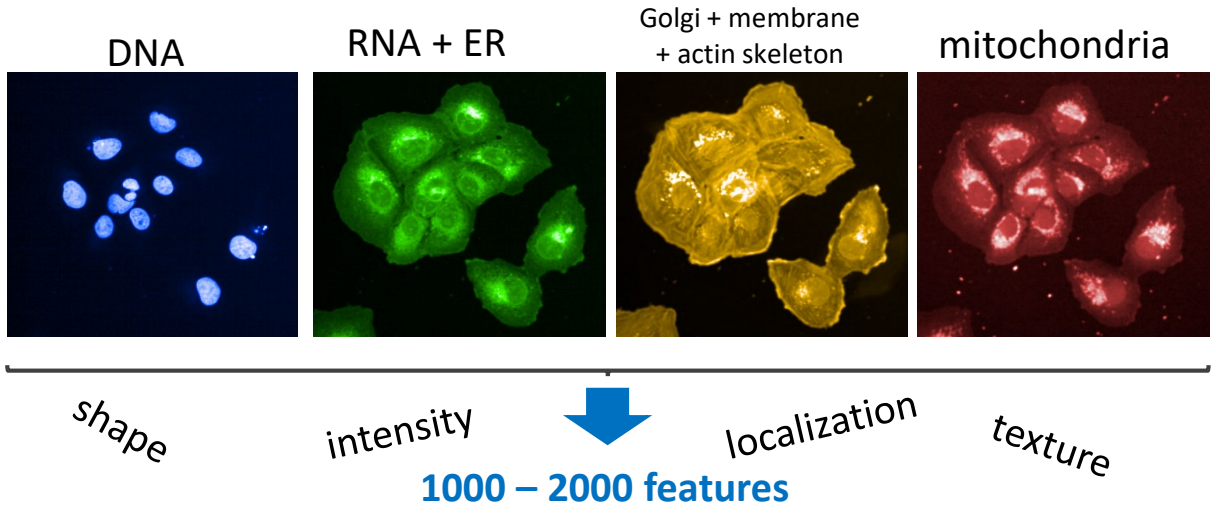


Image source: www.pixabay.com

What is imaging-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.

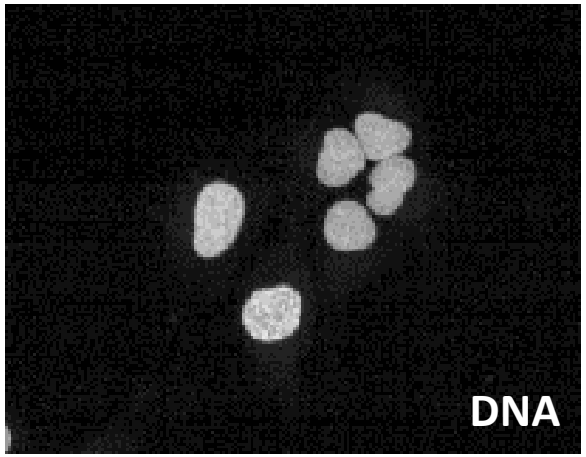
Advantages:

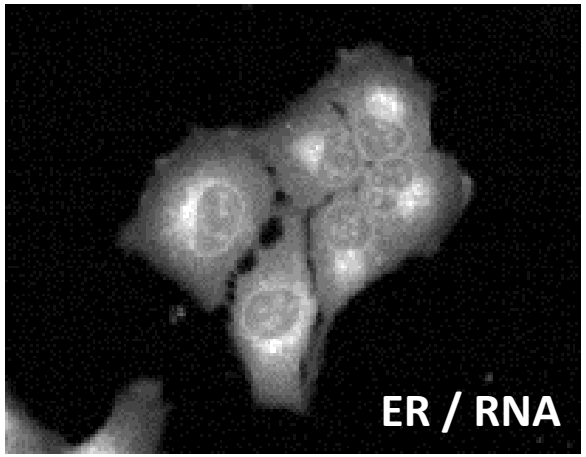
- No requirement for *a priori* knowledge of molecular targets.
- May be used to identify bioactivity thresholds for "dirty chemicals" (i.e. chemicals that affect many cellular proteins or processes simultaneously at a given test concentration).

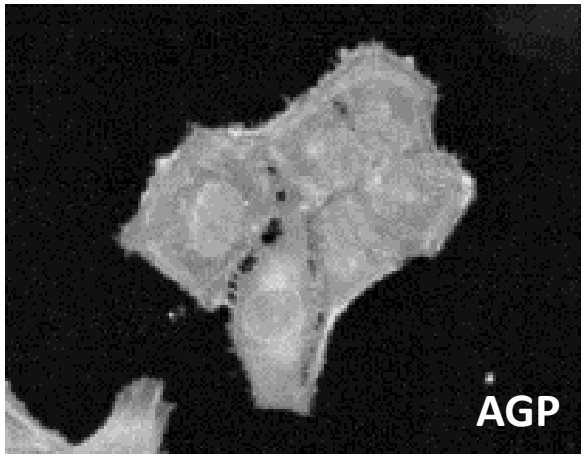
Cell Painting = Cytological Profiling = Phenotypic Profiling = high-throughput Phenotypic Profiling = HTPP

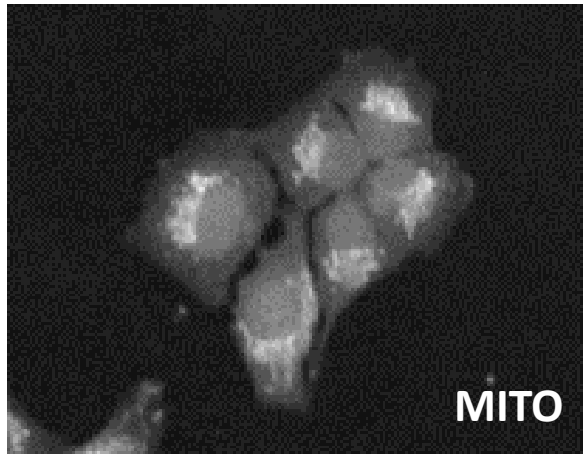
Fluorescent labeling scheme

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









Setup of laboratory workflow for high-throughput testing

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

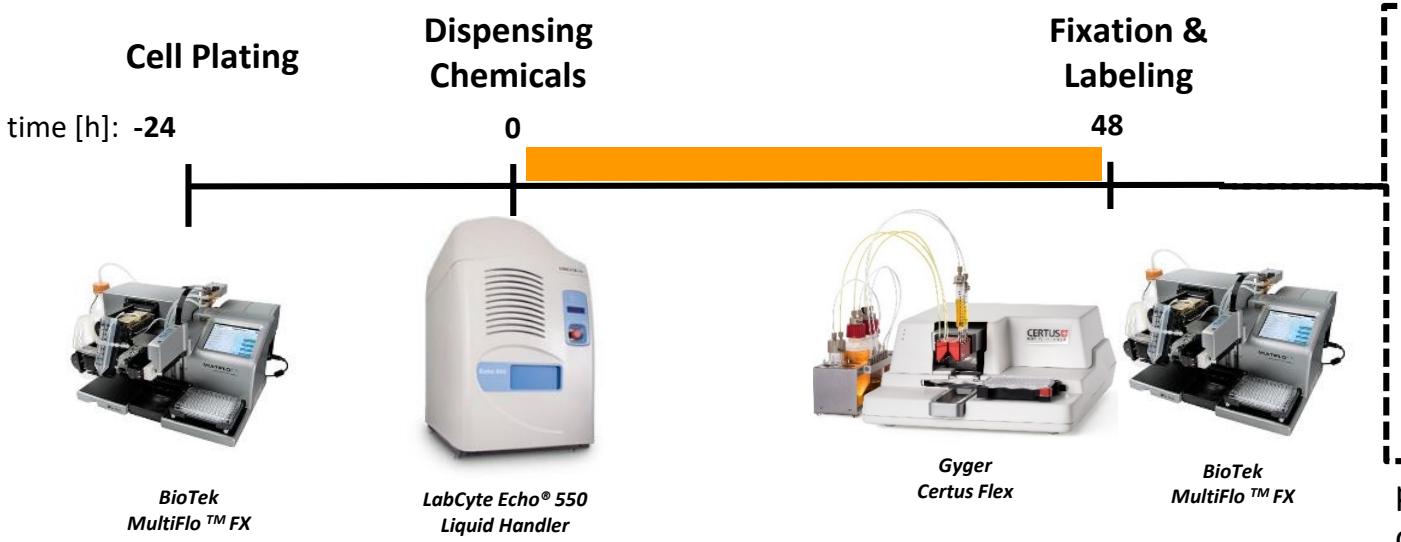


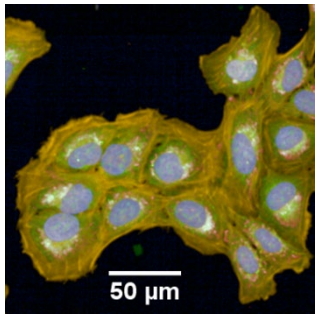
plate 1:
cell profiling

High Content Imaging & Analysis

Perkin Elmer Opera Phenix™ High Content Screening System Harmony Software

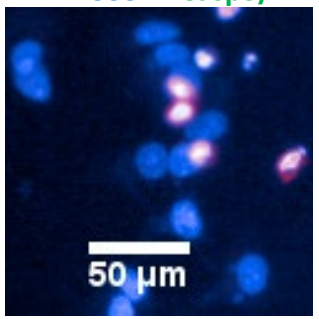
plate 2:
cell viability / cell count

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



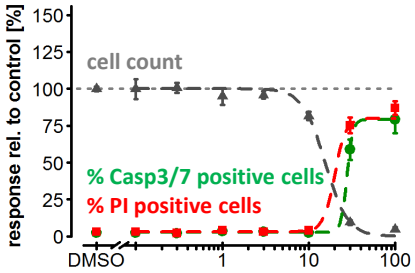


Image Acquisition

- 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 analysis workflow

Nucleus and cell segmentation

1. find nuclei

2. find cell outline

3. reject border objects

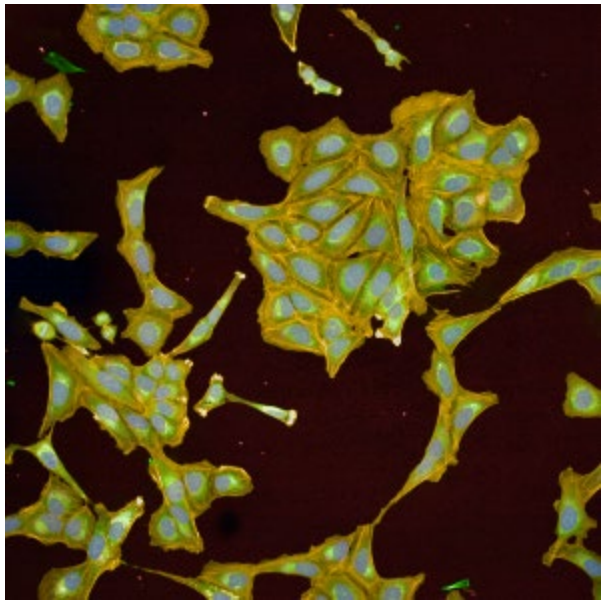
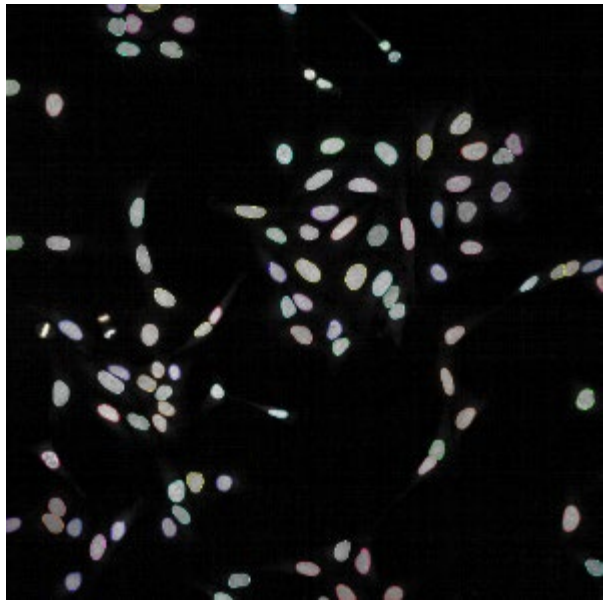
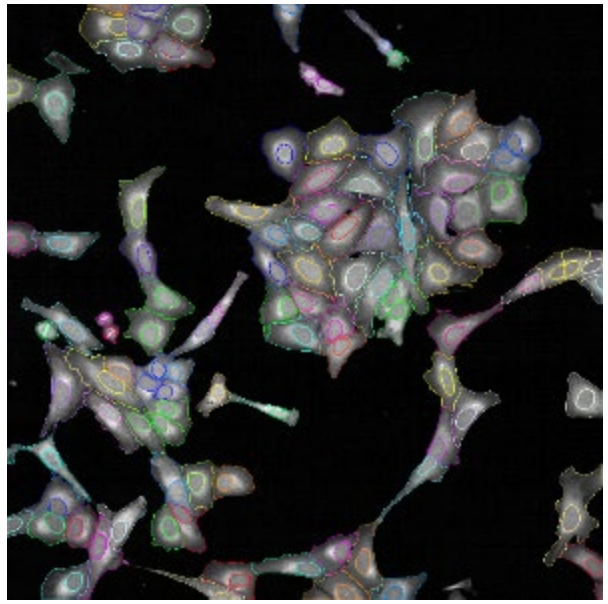
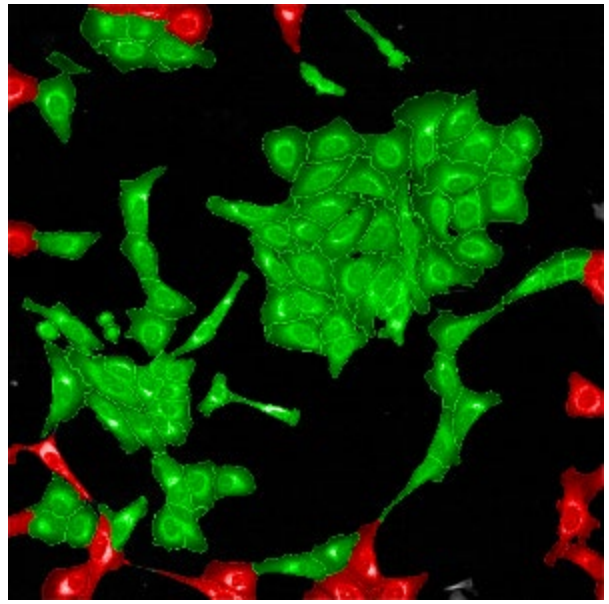
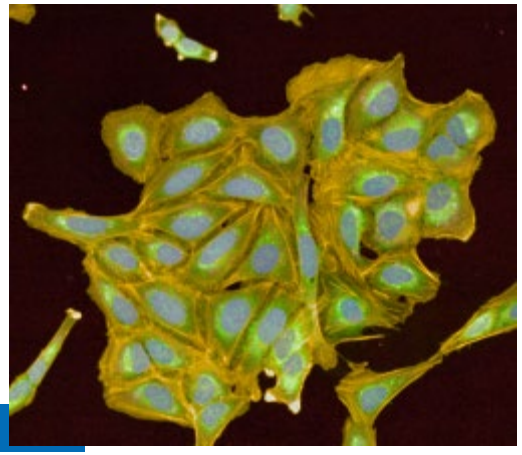
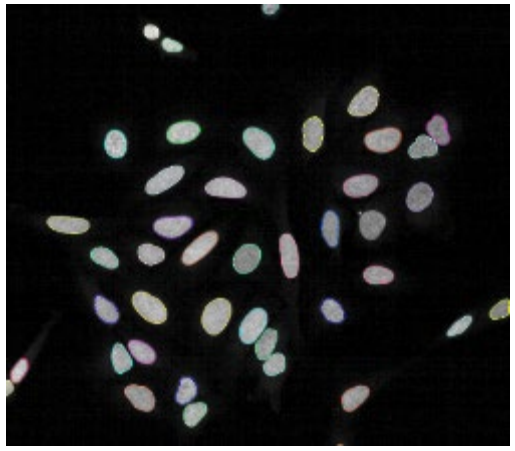
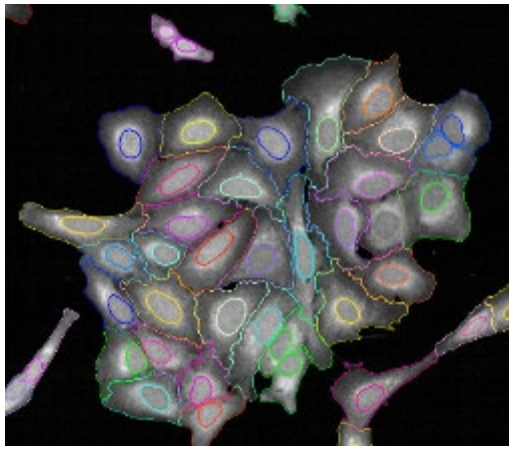
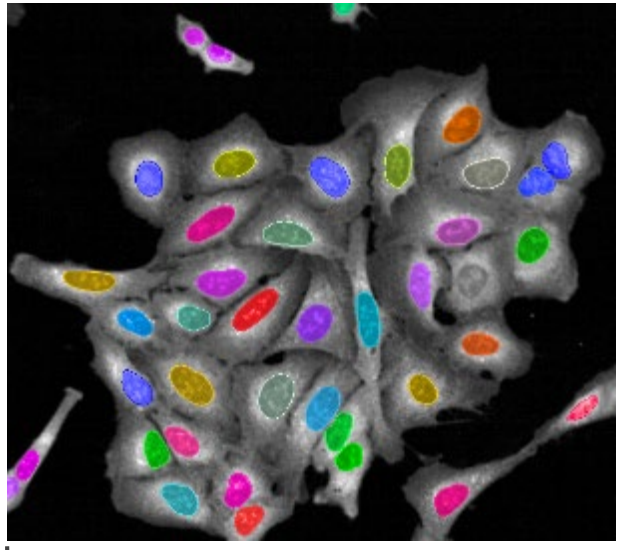








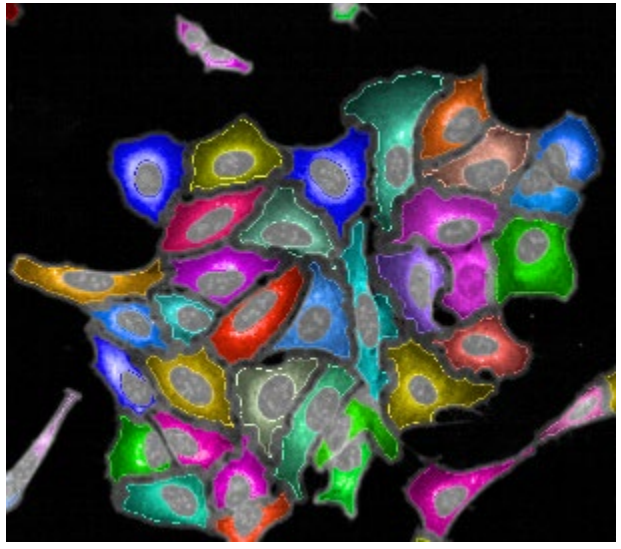
Image analysis workflow

Define cellular compartments

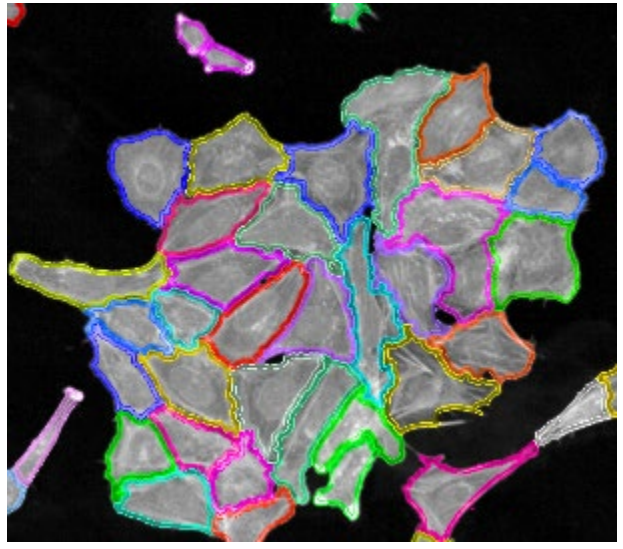
nuclei



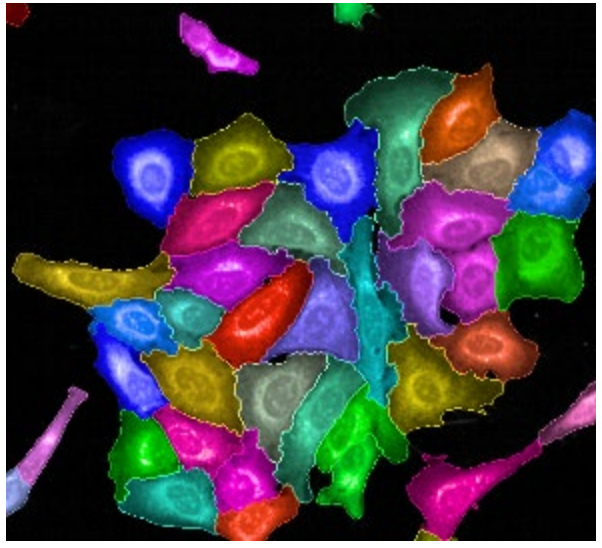
cytoplasm



membrane



cell



ring

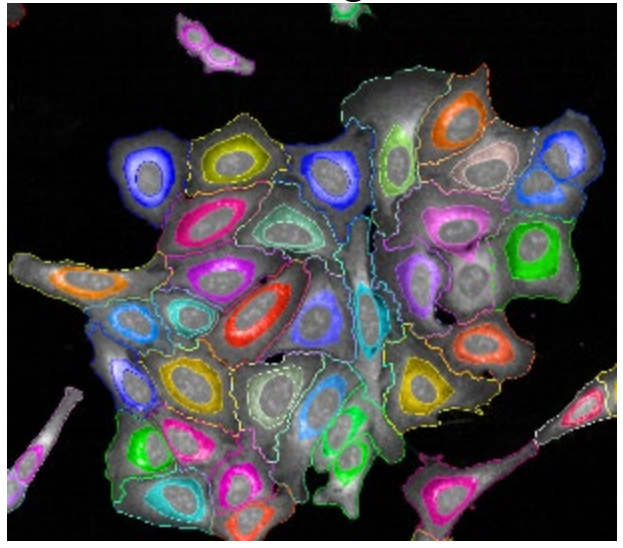
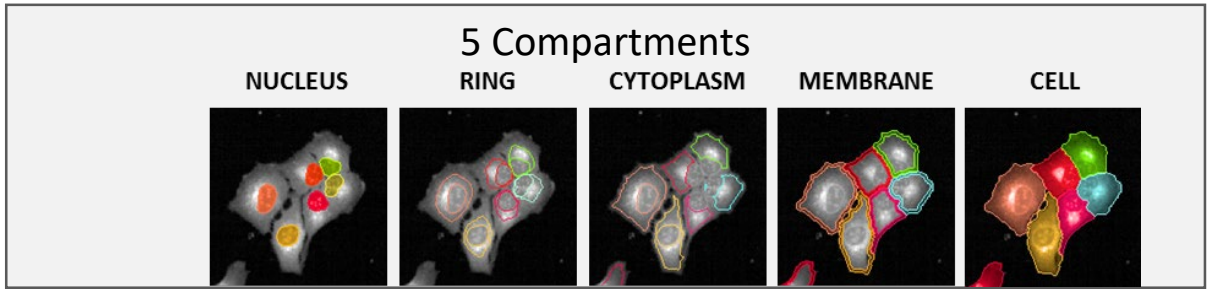
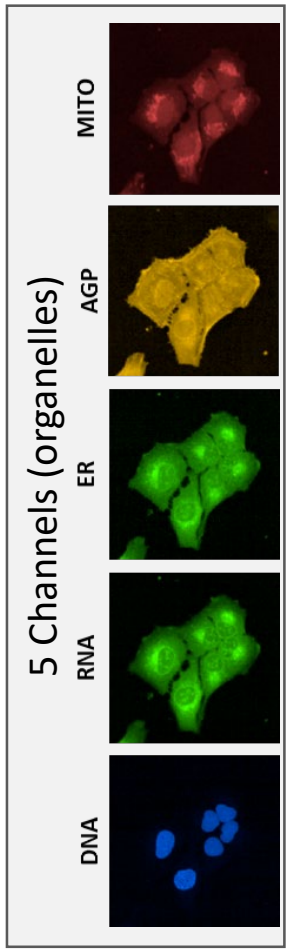
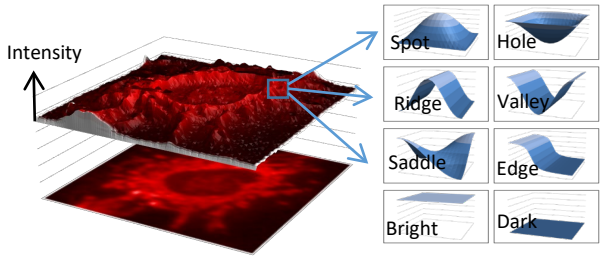


Image processing for profiling plates

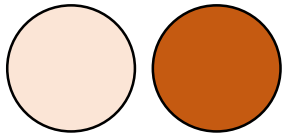
Profiling
with Perkin Elmer
Harmony Software





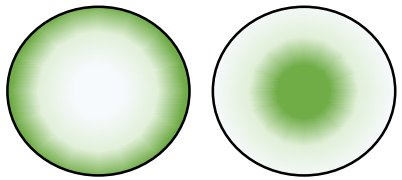


Texture

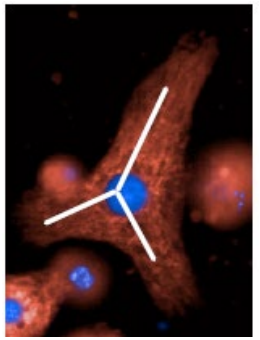


Intensity

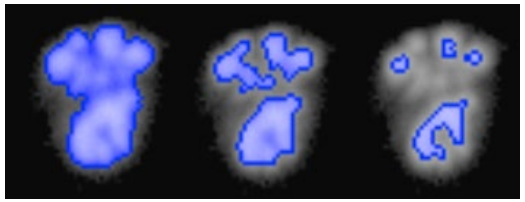
Axial



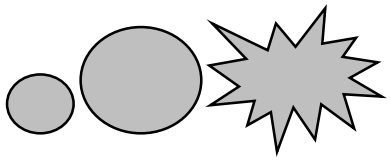
Radial distribution



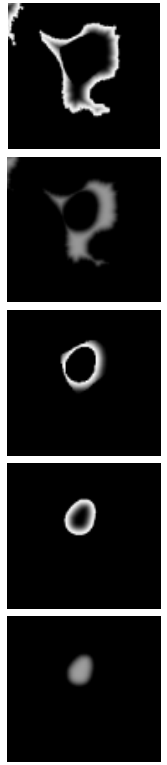
Symmetry



Compactness



Shape



Profile

= 1293 endpoints

Illustrations from Perkin Elmer

Rational for selection of endpoints

	Intensity	Texture	Morphology					
			Symmetry	Compactness	Axial	Radial	Profile	Basic
Endpoints:	9	14	80	40	20	28	20-30	5
DNA	Nuclei	Nuclei	Nuclei	Nuclei	Nuclei	Nuclei Cell	Nuclei Cytoplasm	
RNA	Nuclei	Nuclei	Nuclei	Nuclei	Nuclei	Nuclei	Nuclei	
ER	Ring Cytoplasm	Ring Cytoplasm	Cell	Cell	Cell	Cell	Cytoplasm	
AGP	Ring Cytoplasm Membrane	Ring Cytoplasm Membrane	Cell	Cell	Cell	Cell	Nuclei Cytoplasm	
Mito	Ring Cytoplasm	Ring Cytoplasm	Cell	Cell	Cell	Cell	Nuclei Cytoplasm	
"Shape"								Nuclei Cell

1293 endpoints grouped in 48 categories ("ontologies")

Examples:

- AGP_Texture _Cytoplasm
- Mito_Compactness _Ring
- DNA_Intensity _Nuclei

Data analysis

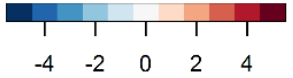
cell-level data

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

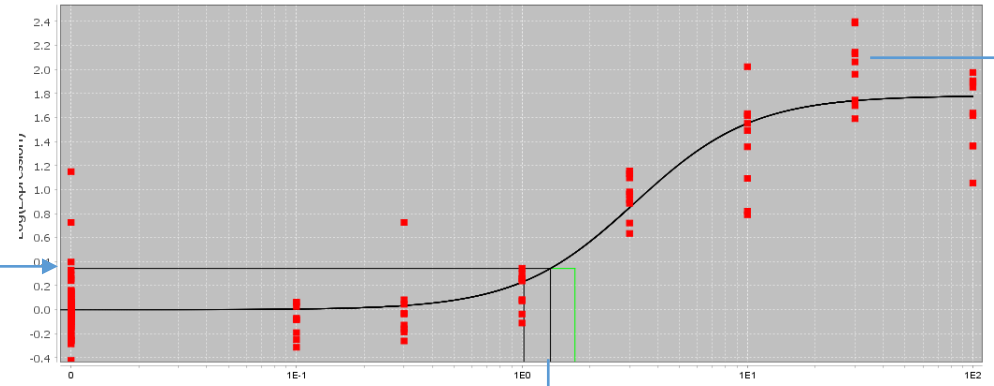
median

well-level data

1293 endpoints
384 wells

Benchmark dose (BMD) modelling

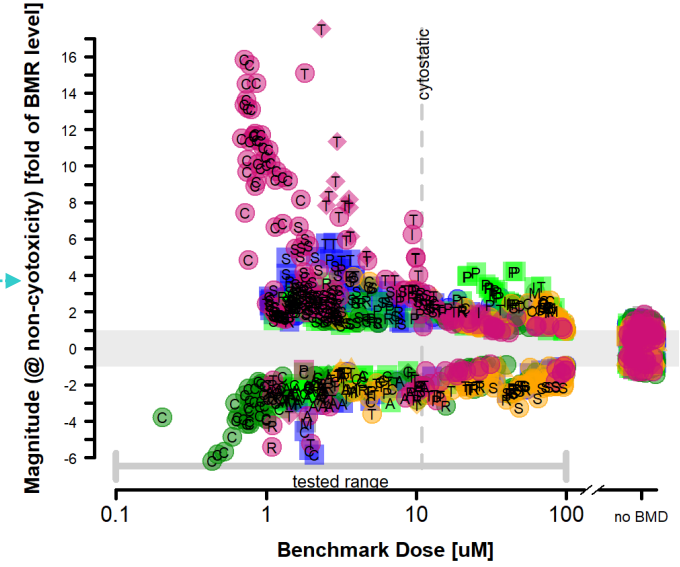
1293 endpoints
7 concentrations/chemical



Predefined effect level

Dose at which effect level is reached

Effect size (Magnitude) at non-cytotoxic concentration



Benchmark dose (BMD)

1293 endpoints

Experimental design

Goal:

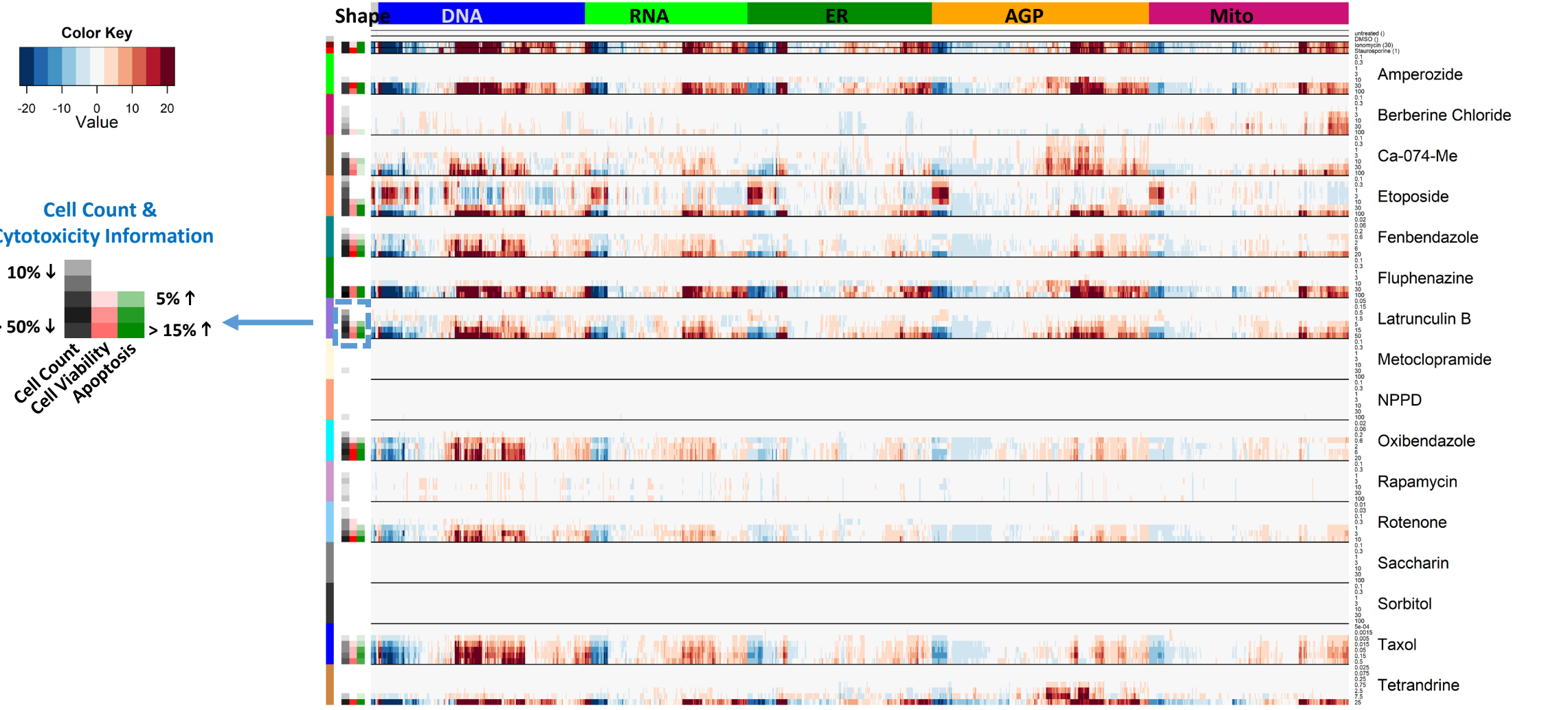
- Replicate data from a published study (Gustafsdottir et al. 2013) using
  - same cell line
  - same chemical set
  - same exposure time
- Run in concentration-response mode

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
Beta-dihydrorotenone	extensive **mitochondrial** fission
Saccharin	Negative control
Sorbitol	Negative control
Taxol	Large, **multi-nucleated** cells with fused **nucleoli**
Tetrandrine	Abundant **ER**

Phenotypic profiles for reference chemicals [U-2 OS]



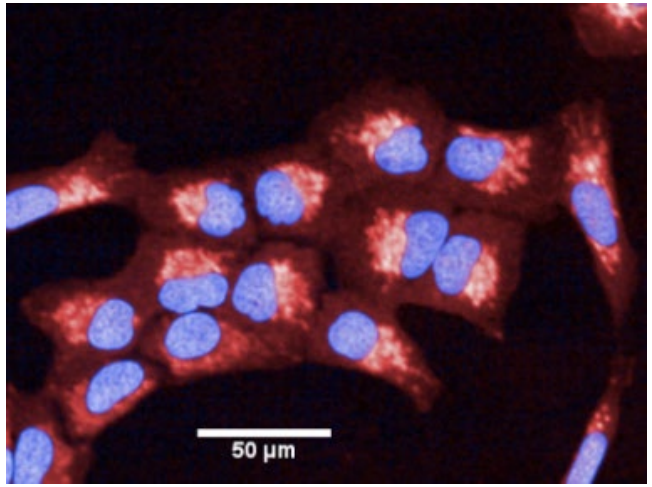
- ⇨ **Effects on morphology observed at sub-cytotoxic concentrations.**
- ⇨ **Some chemicals did not produce any effects.**
- ⇨ **Unique phenotypic profiles observed across the reference chemical set.**

Example 1: Berberine Chloride

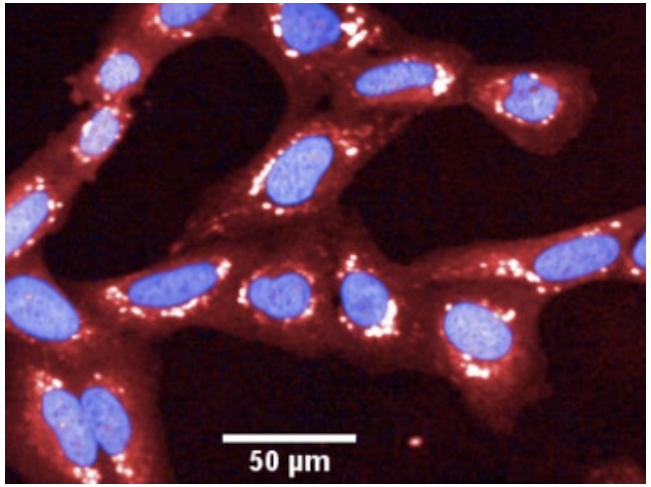
Gustafsdottir et al. 2013: Redistribution of **mitochondria**

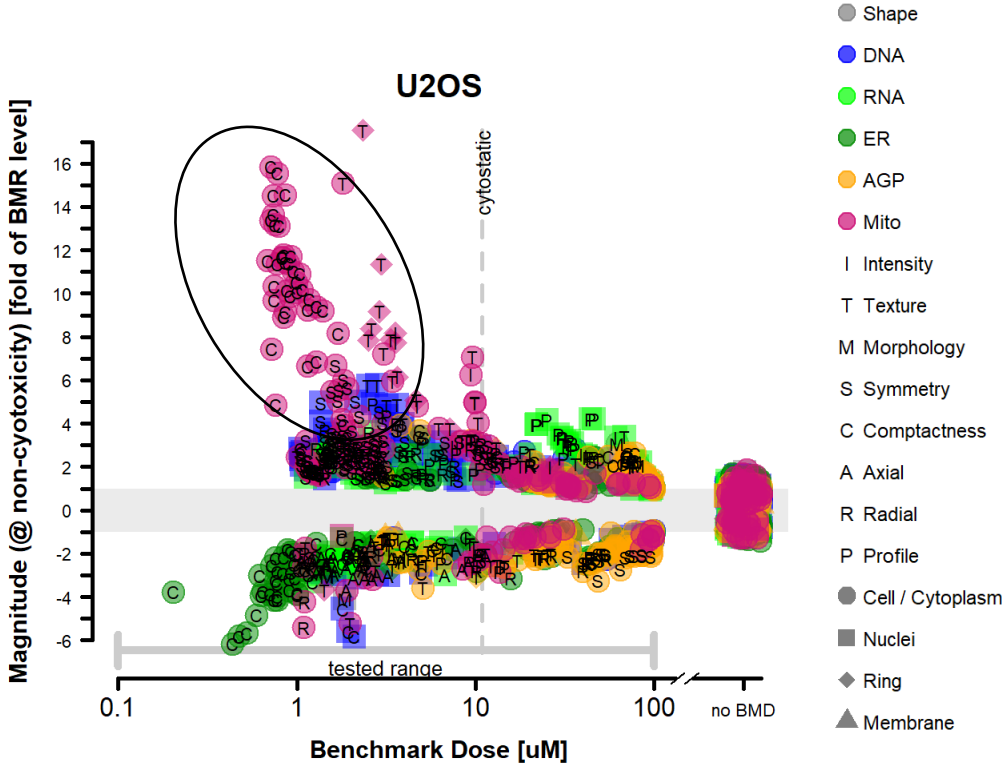
DNA Mitochondria

solvent control (0.5% DMSO)



Berberine chloride (10 uM)

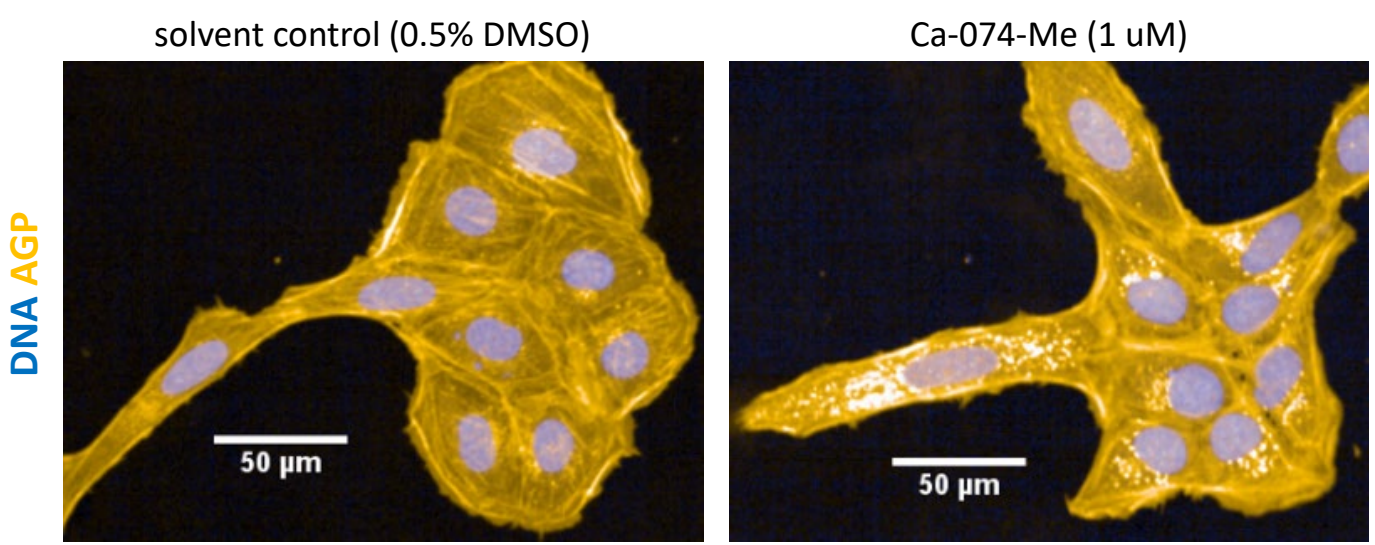


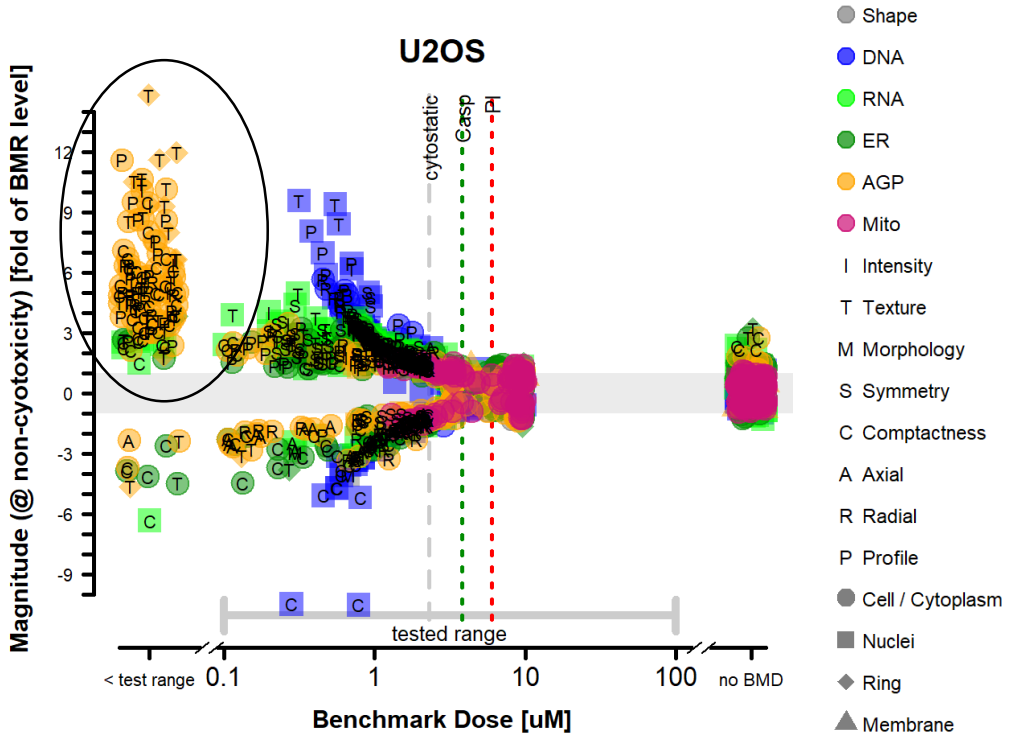


⇨ **Mitochondrial compactness is affected**

Example 2: Ca-074-Me

Gustafsdottir et al. 2013: Bright, abundant **Golgi** staining

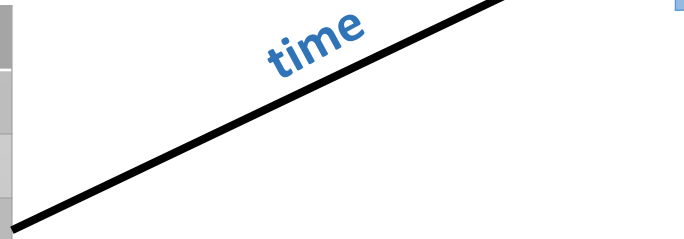




⇨ **Texture, Compactness and Profile is affected in the Ring/Cytoplasm compartment (Golgi)**

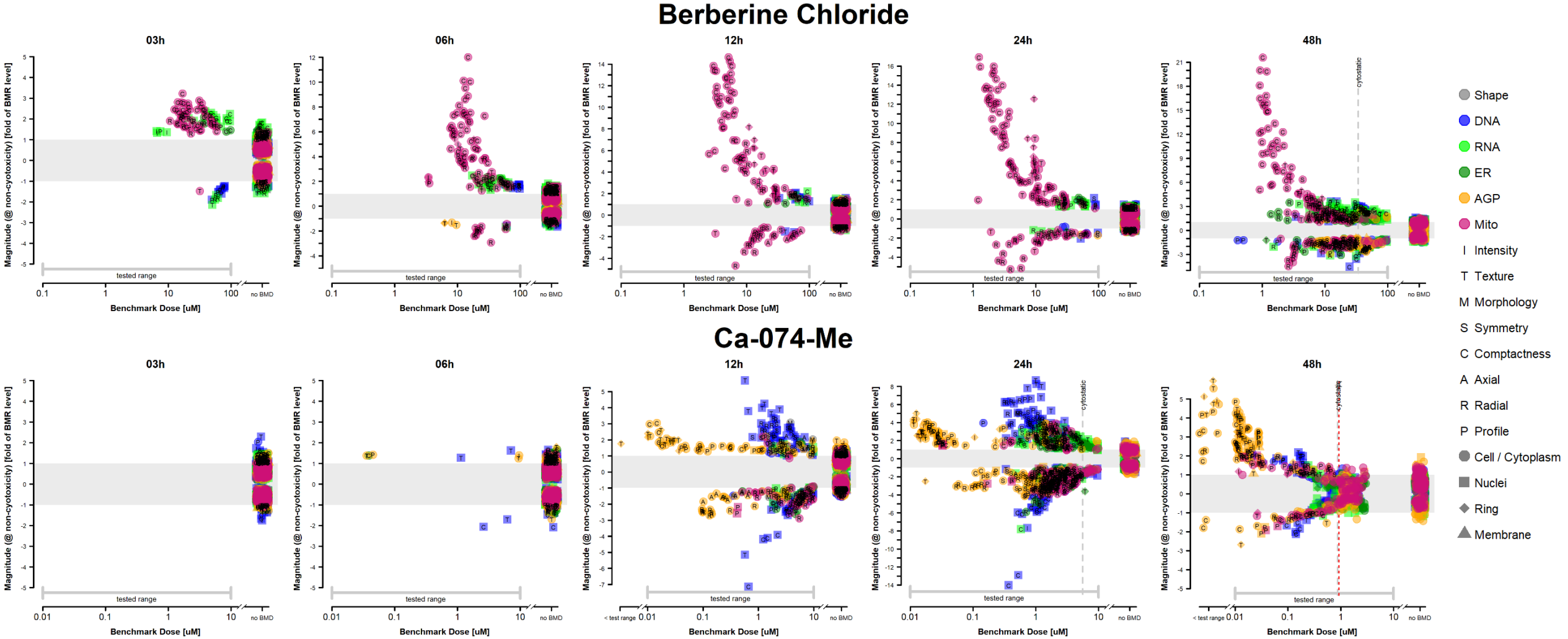
Experimental design

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

Profiles across time



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

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

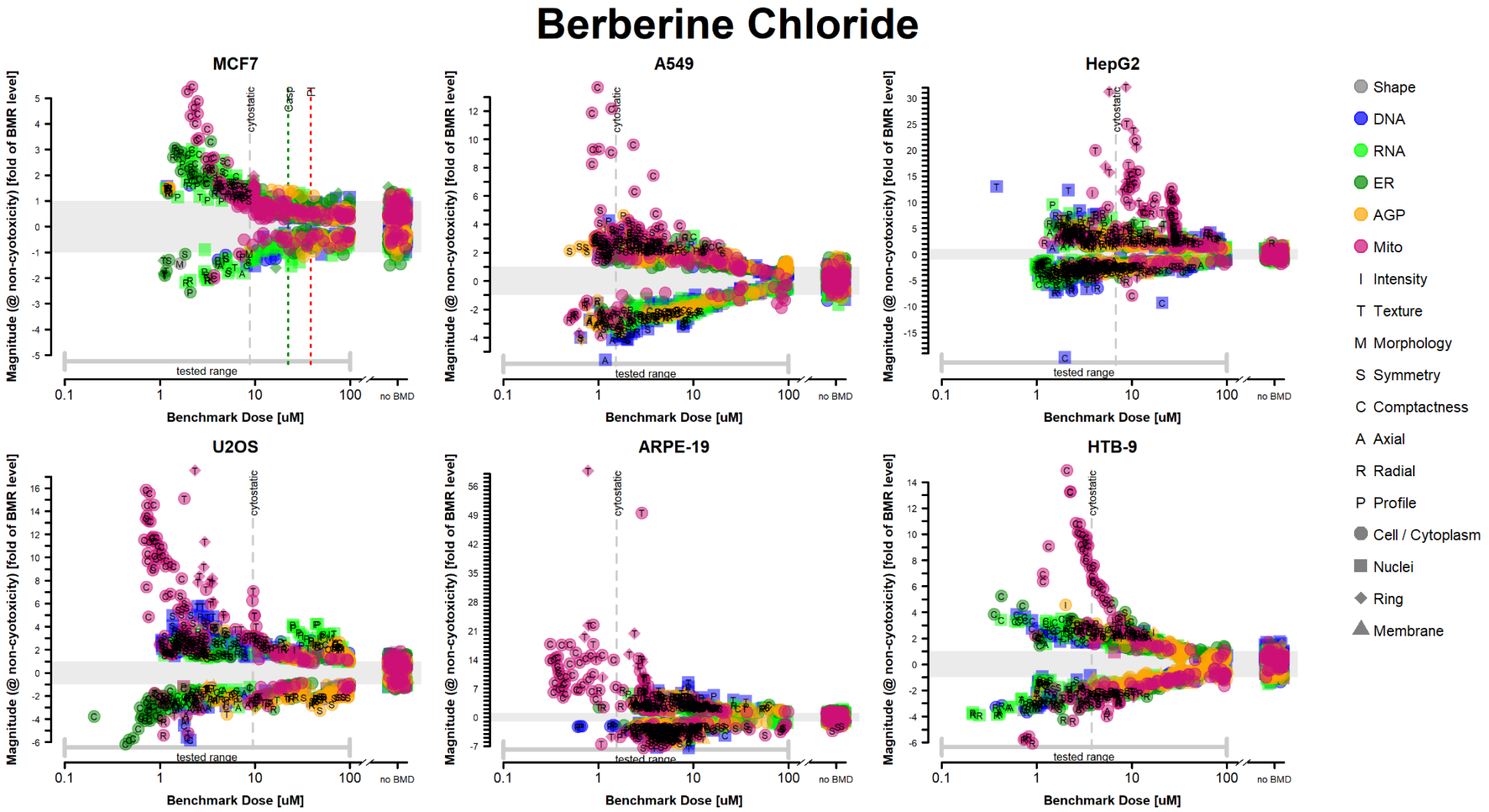
time

biological space

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

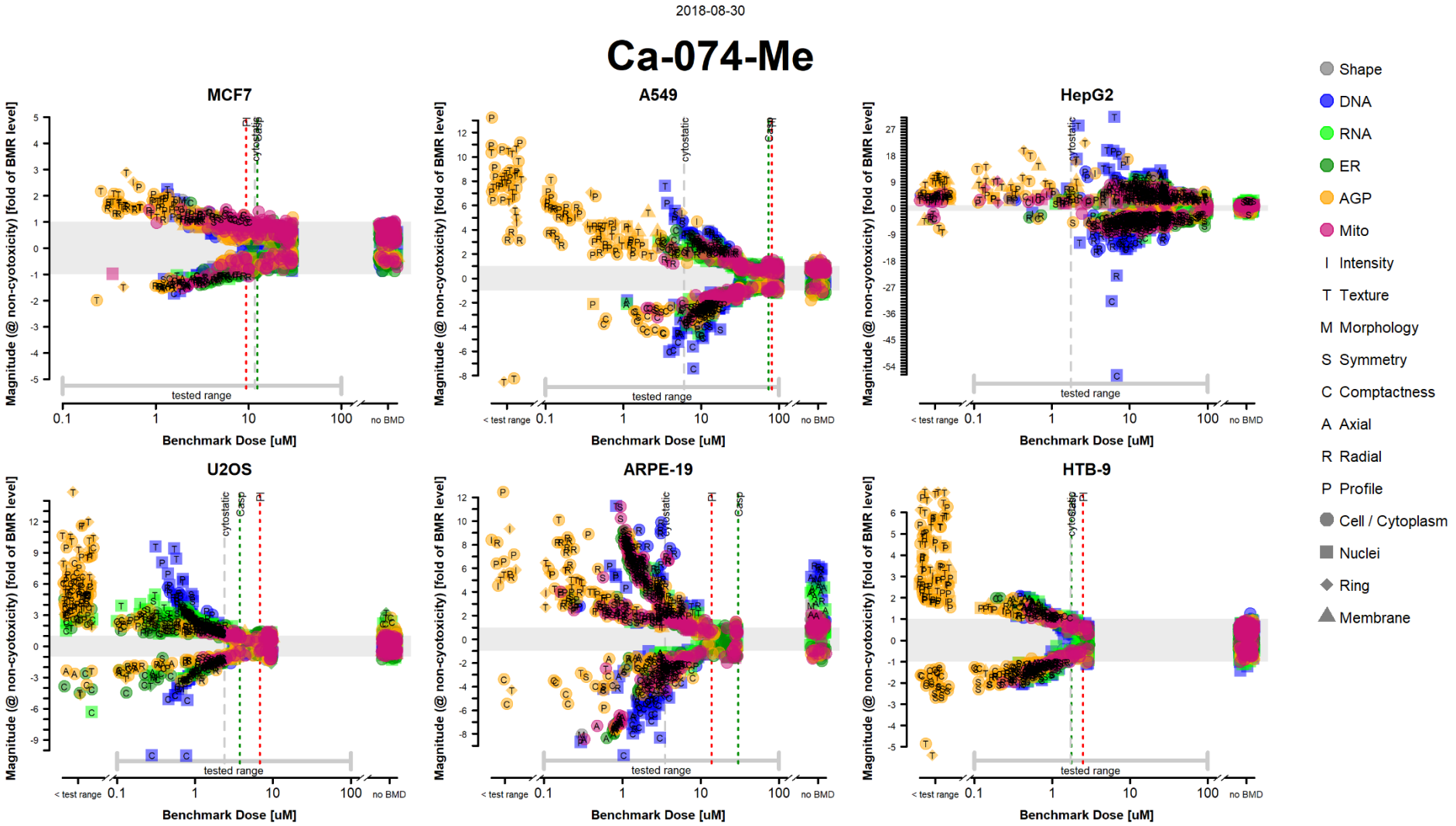
Biological space
+ 5 cell types: MCF-7 (Breast) A549 (Lung) HTB-9 (Urinary bladder) ARPE-19 (Retina) HepG2 (Liver)

Profiles across biological space (I)



⇨ **Profiles are often similar in different cell lines...**

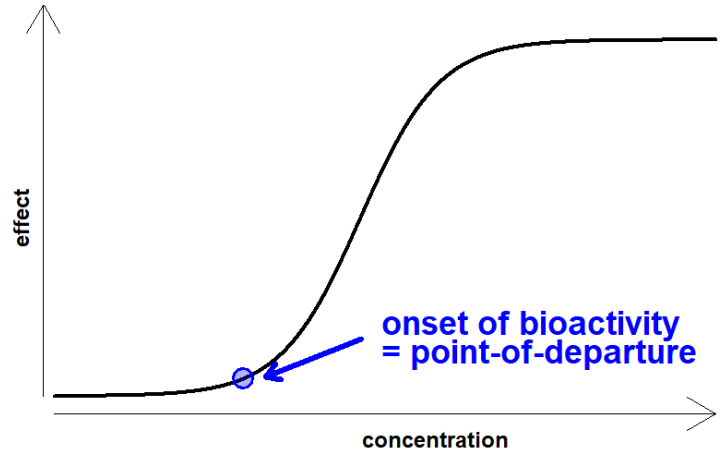
Profiles across biological space (II)

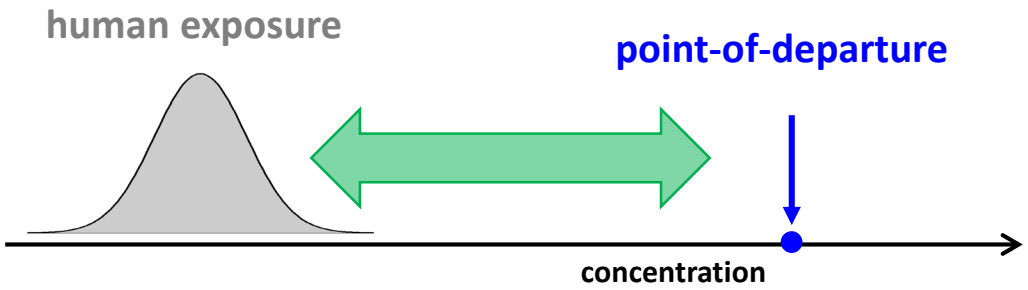


⇨ **… but not identical.**

Potential applications

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





Profiles could provide mechanistic insights

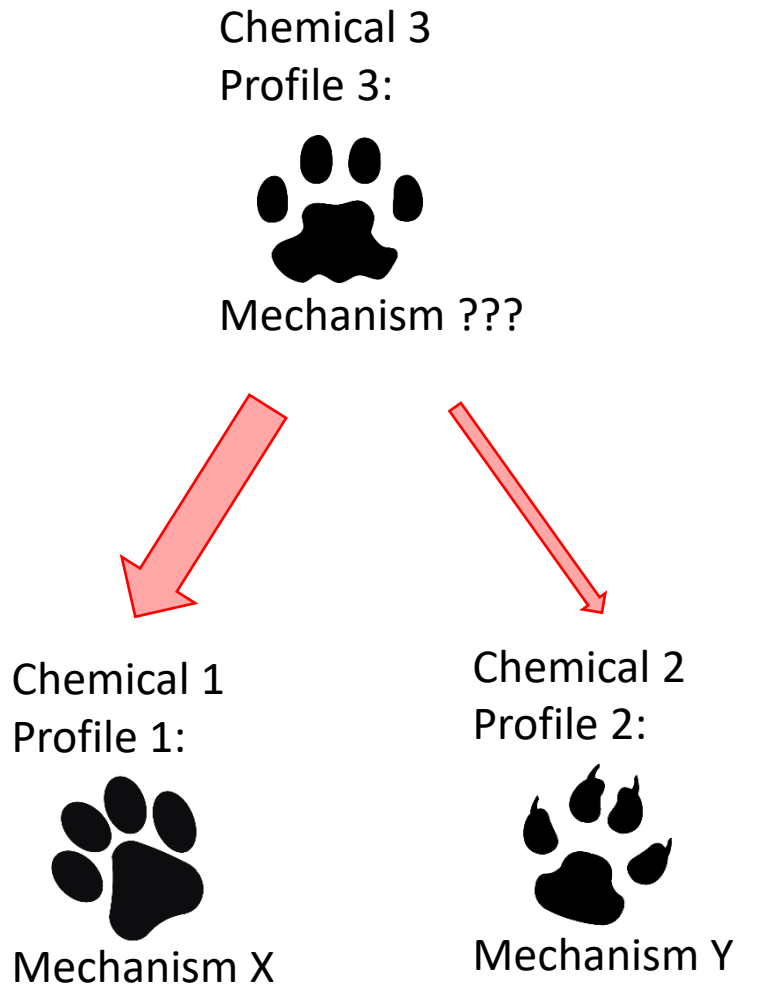


Image source: www.pixabay.com

Application : *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:

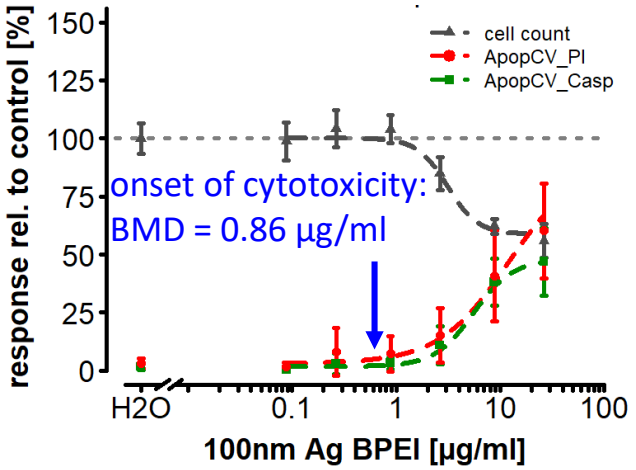
- Testing of 12 silver nanoparticles: 3 different coatings by 4 particle sizes

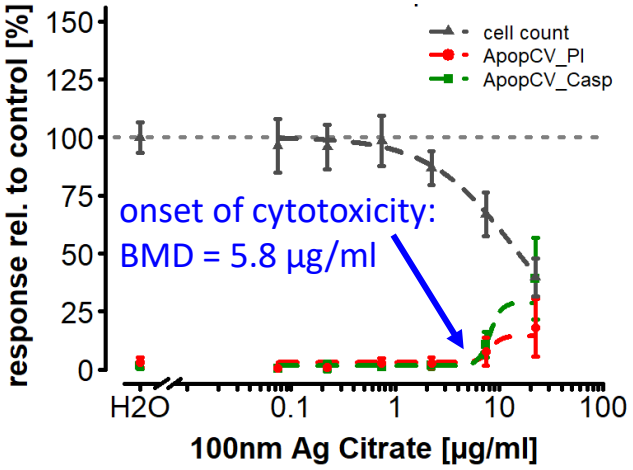
→ What is the relative potency of the different nanoparticles?
Where is the point-of-departure?

→ Can we obtain mechanistic information by investigating the profiles?

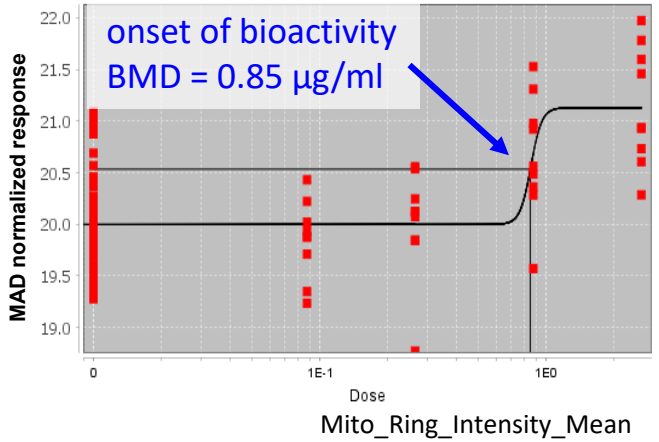
Application : *In vitro* bioactivity thresholds of nanoparticles

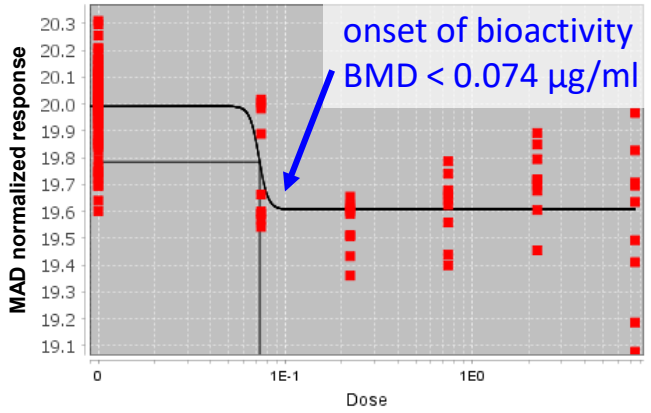
Cytotoxicity testing:





Phenotypic profiling:





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 opposite potency ranking as compared to cytotoxicity assay**

⇨ **Profiles suggest different mechanisms of toxicity**

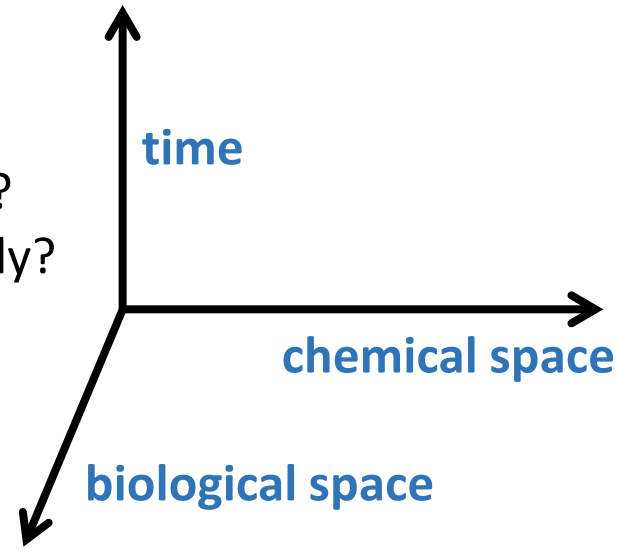
Take home messages

1. Microfluidics workflow and data analysis pipelines were setup
2. Replication of published results to confirm that the assay is working
3. Profiles arise at 6-12 h and become less specific at 48 h
   Profiles are similar (but not identical) among cell lines
4. EPA is evaluating the use of cytological profiling to test chemicals to find
   - onset of bioactivity
   - mechanistic information

Outlook

Chemical space

- Screen chemicals of interest to the agency
  → *hear more on Wednesday*
- How do the results compare to other HTS methods?
- Are the results relevant? How do they compare to *in vivo* toxicity data?
- Potential for evaluating chemicals that could not be analyzed previously?
  - (Water soluble chemicals, mixtures, etc.,)



Time

- How do the results change with exposure time?
- Tipping points

Biological space

- How do results change across different cell lines?
- Complementary to high-throughput transcriptomics (HTTr) screening approach?
- Is there a cell line more useful for toxicology? Can we define a battery of cell lines to use for testing?

Acknowledgment

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Agency

Thank you!

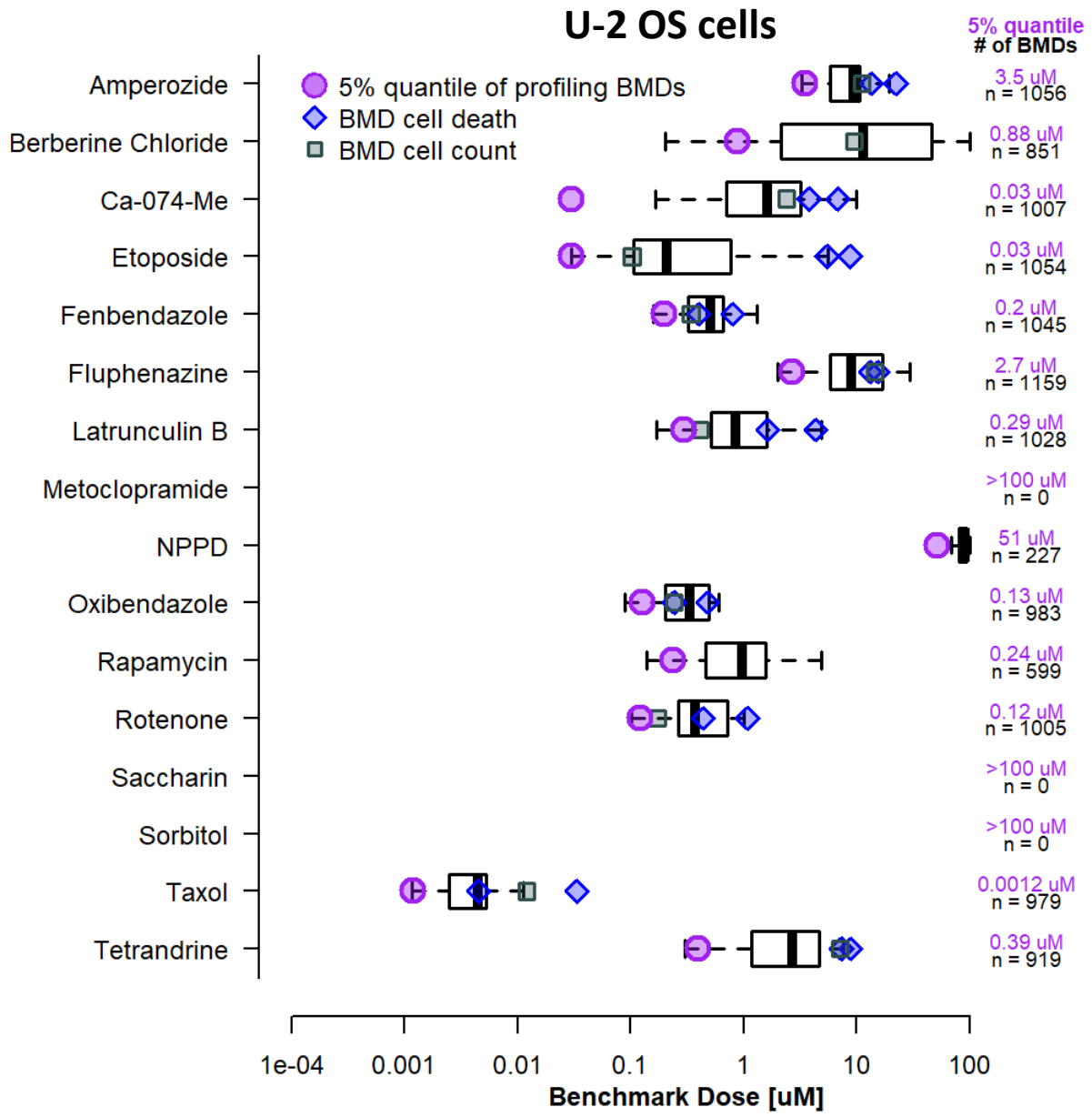
Questions?

In vitro point-of-departure (POD) determination

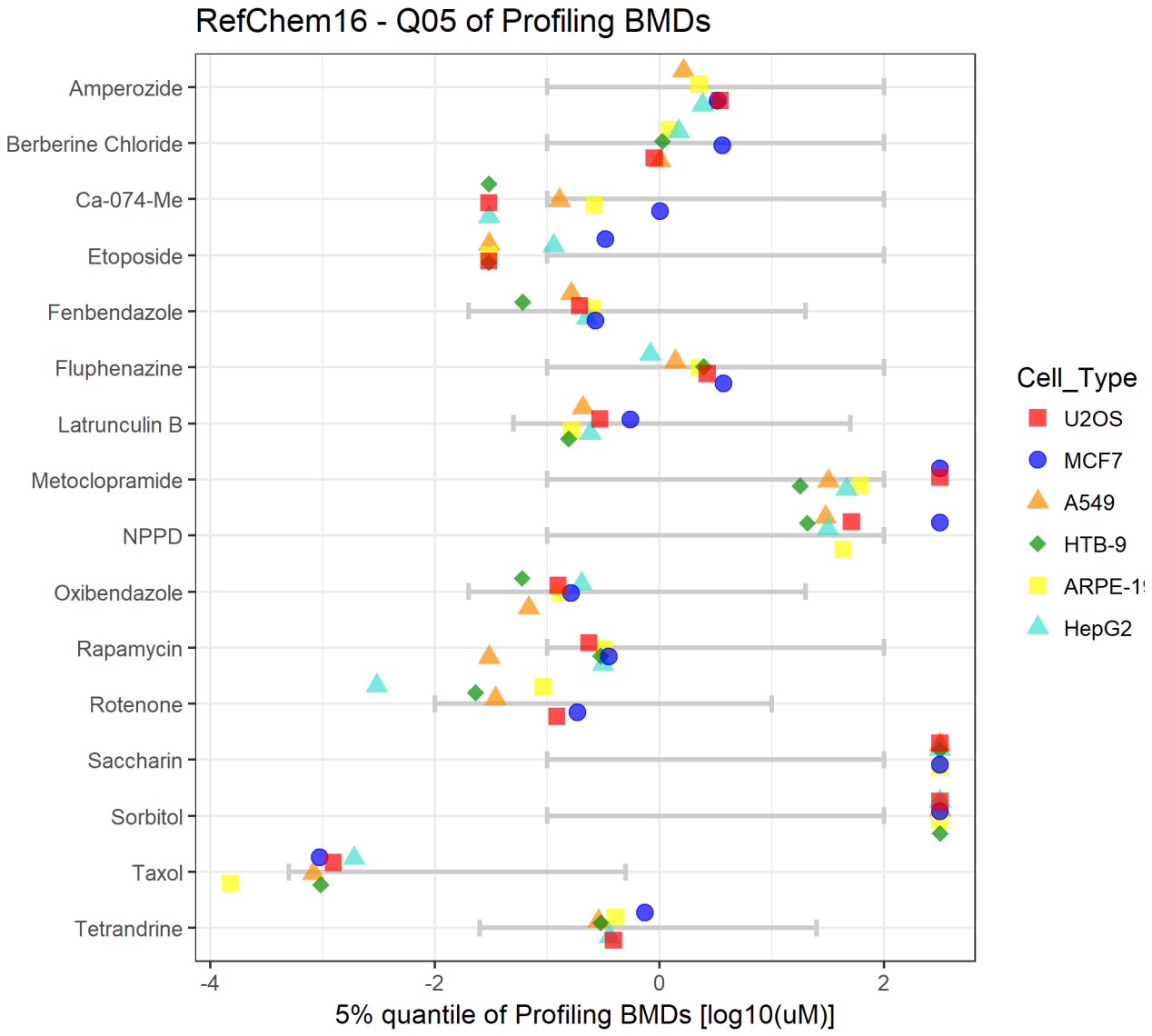
Point of departure definition

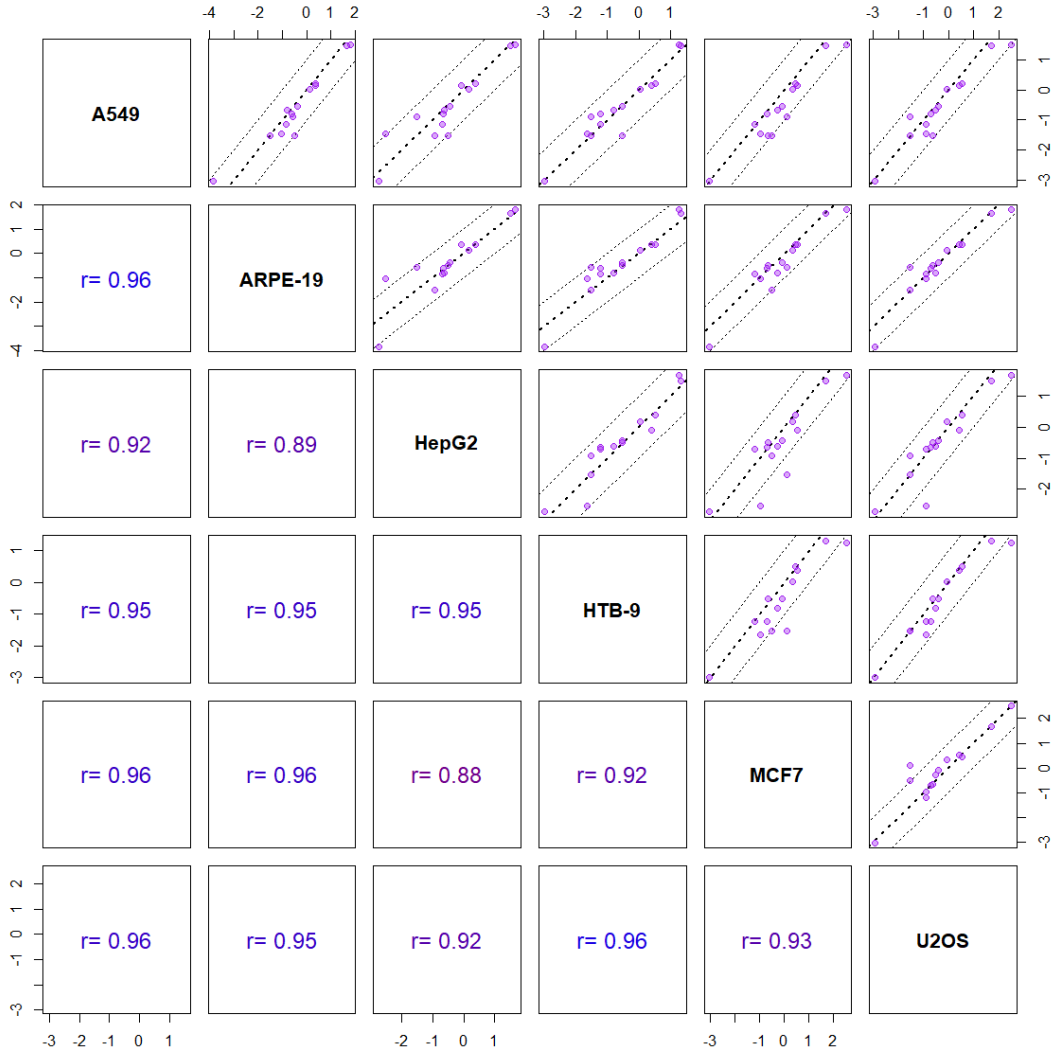
- **POD** = 5% quantile of all profiling BMDs

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



Strong Correlation of Cell Painting PODs Across Cell Types

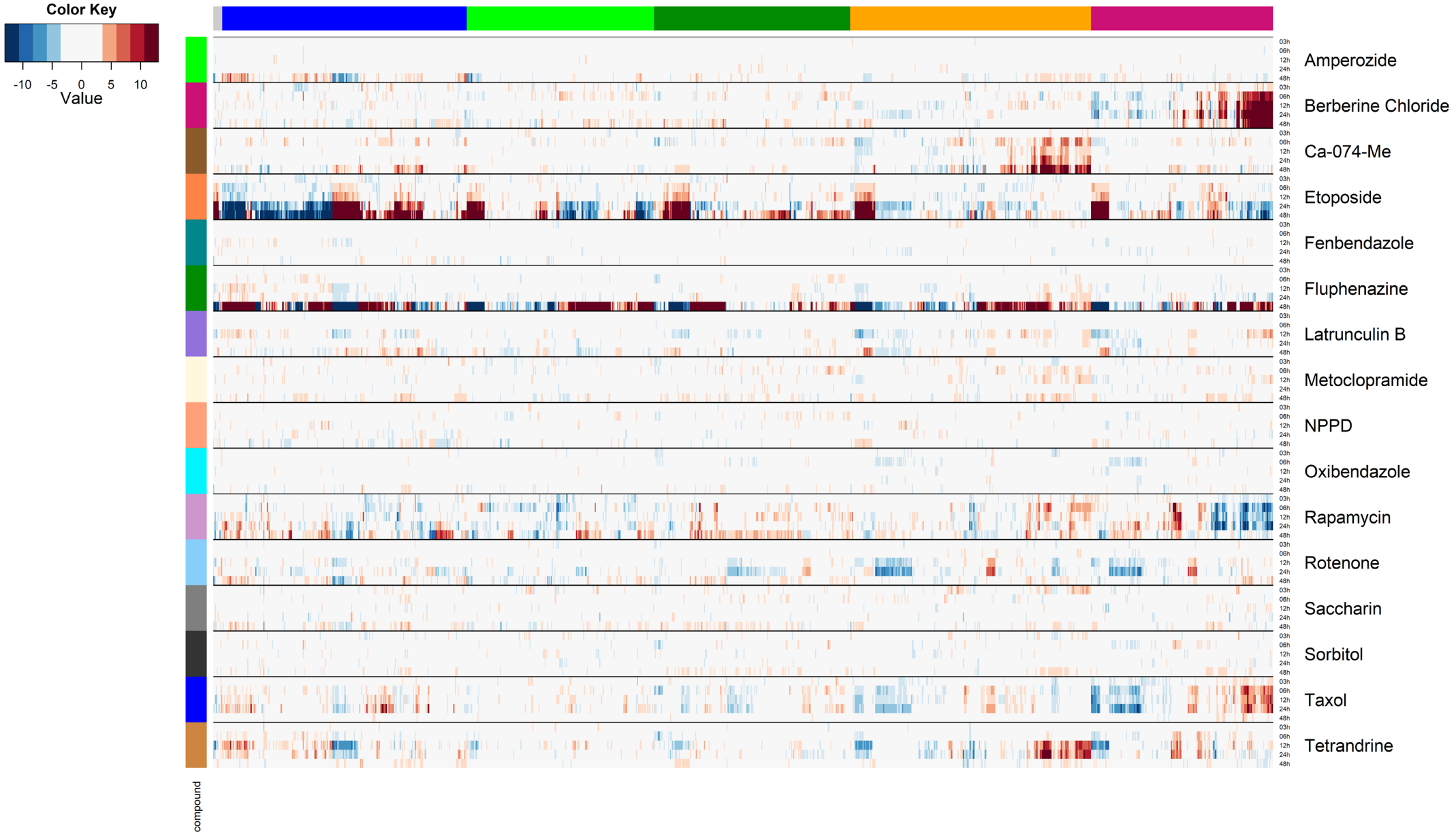




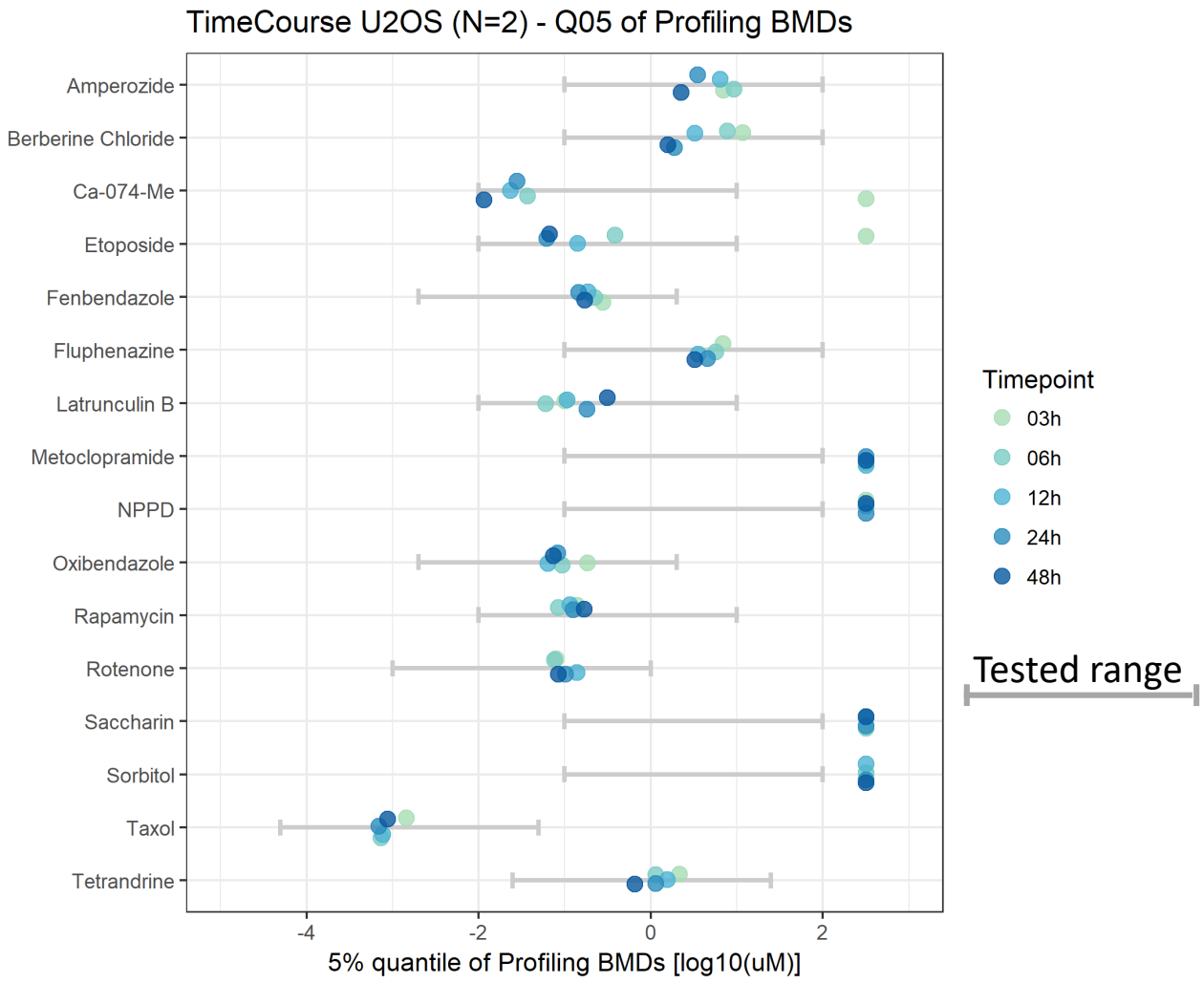
- Different cell lines correlate to ~ 90%.

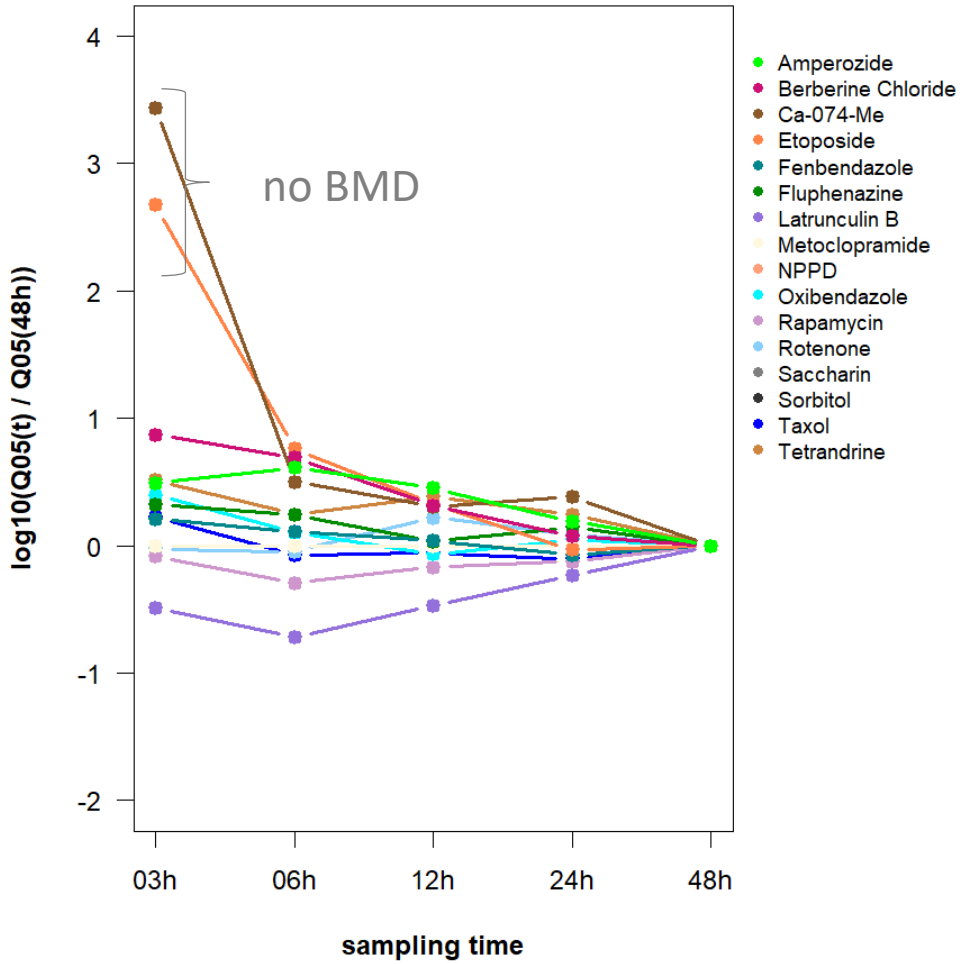
EPA United States Environmental Protection Agency

Qualitative Similarity in Response Profiles Over Time



How do PODs vary across sampling times?





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