# High-Throughput Phenotypic Profiling

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# Disclaimer

*The views expressed in this presentation are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency. Company or product names do not constitute endorsement by US EPA.*

# Research Drivers

- There are many chemicals in U.S. commerce with the potential to enter the environment that are poorly characterized in terms of human health hazards.

- Traditional toxicity testing approaches in laboratory animals are expensive and time-consuming and therefore cannot be used to efficiently address this large data gap.

- Animal-free New Approach Methods (NAMs) provide a means for accelerating the pace of chemical hazard assessment using models anchored in human biology.

- EPA has been tasked with and is committed to reducing the use of animals in toxicity testing and expanding the use of NAMs in chemical risk assessment

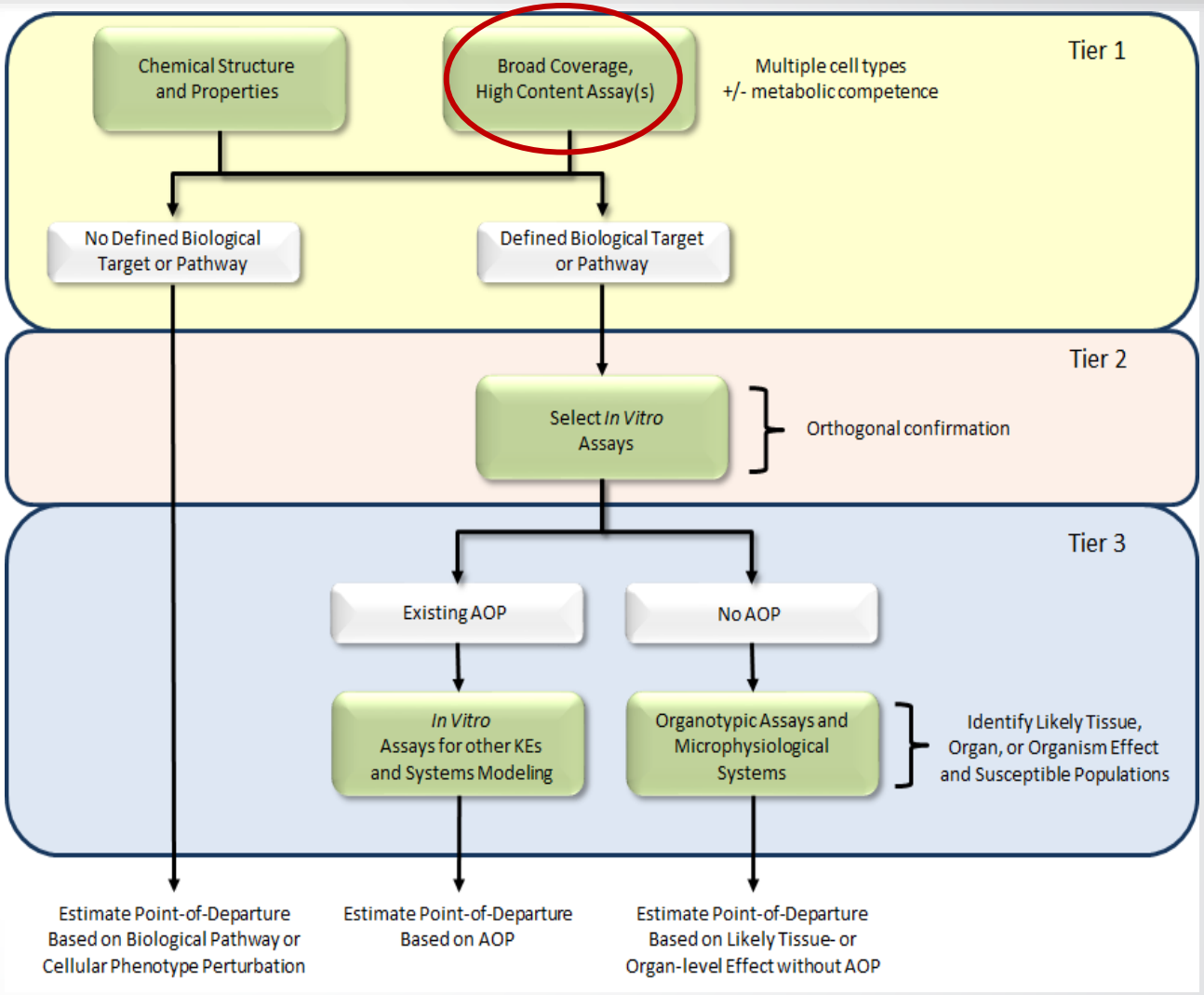
    - (**June '16**) Frank R. Lautenberg Chemical Safety for the 21st Century Act (*15 U.S.C. §2601*)
    - (**June '18**) US EPA Strategic Plan to Promote the Development and Implementation of Alternative Test Methods within the TSCA Program (*EPA-740-R1-8004*).
    - (**Sept '19**) Administrator's Directive to Prioritize Efforts to Reduce Animal Testing (*Wheeler 2019*)
    - (**June '20**) US EPA New Approach Methods Work Plan (*EPA 615B2000*)

# NAMs-Based, Tiered Hazard Evaluation Strategy

- **New Approach Methodologies (NAMs)** are any technology, methodology, approach or combination thereof that can be used to provide information on chemical hazard and risk that avoids the use of intact animals.

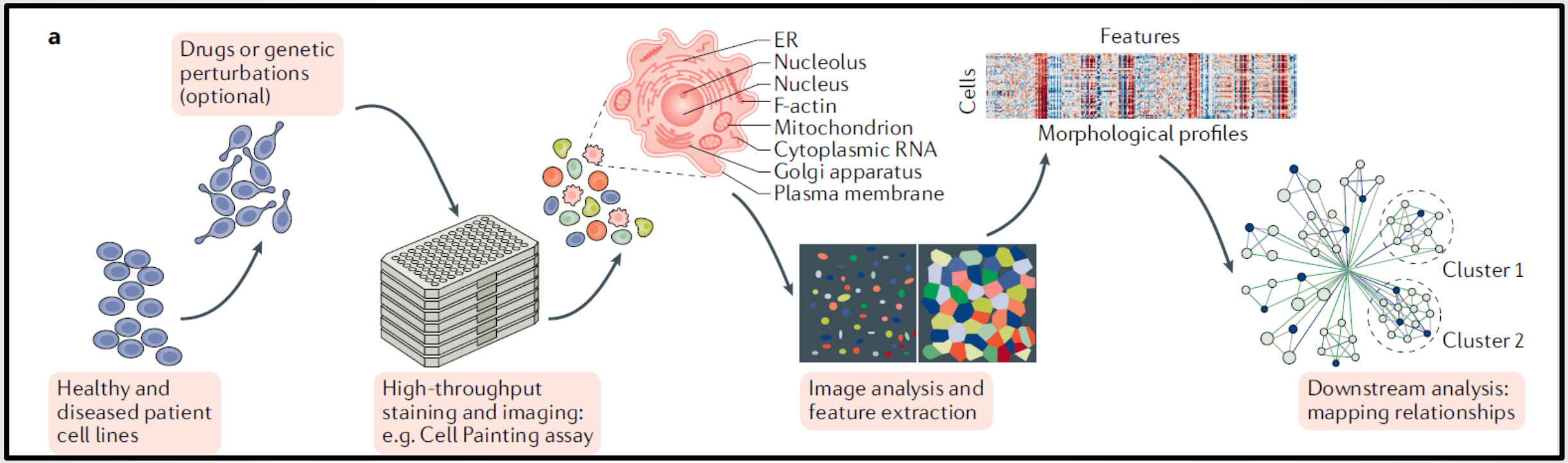
- US EPA CompTox Blueprint advocates the use of **high throughput profiling (HTP) assays** as the first tier in a NAMs-based hazard evaluation strategy.

- **HTP assay criteria:**
  1. Yield bioactivity profiles that can be used for **potency estimation, mechanistic prediction** and evaluation of **chemical similarity**.
  2. Compatible with multiple human-derived culture models.
  3. Concentration-response screening mode.




The NexGen Blueprint of CompTox at USEPA, Tox. Sci. 2019; 169(2):317-322

# Imaging-Based High-Throughput Phenotypic Profiling (HTPP)




*Chandrasekaran et al. Nat Rev Drug Discov. 2020 Dec 22:1–15*

- A high-throughput testing strategy where rich information present in biological images is reduced to multidimensional numeric profiles and mined for information characteristic to a chemical's biological activity.

- Originated in the pharmaceutical sector and has been used in drug development to understand disease mechanisms and predict chemical activity, toxicity and/or mechanism-of-action

# HTPP with the Cell Painting Assay

**Cell Painting** is a profiling method that measures a large variety of phenotypic features in fluoroprobe labeled cells *in vitro*.

- High-throughput
- Cost-effective (¢ / well)
- Scalable
- Reproducible
- Amenable to lab automation
- Deployable across multiple human-derived cell types.
- Infrastructure investment
- High volume data management

**Laboratory & bioinformatics workflows** for conduct of this assay have been established at CCTE.

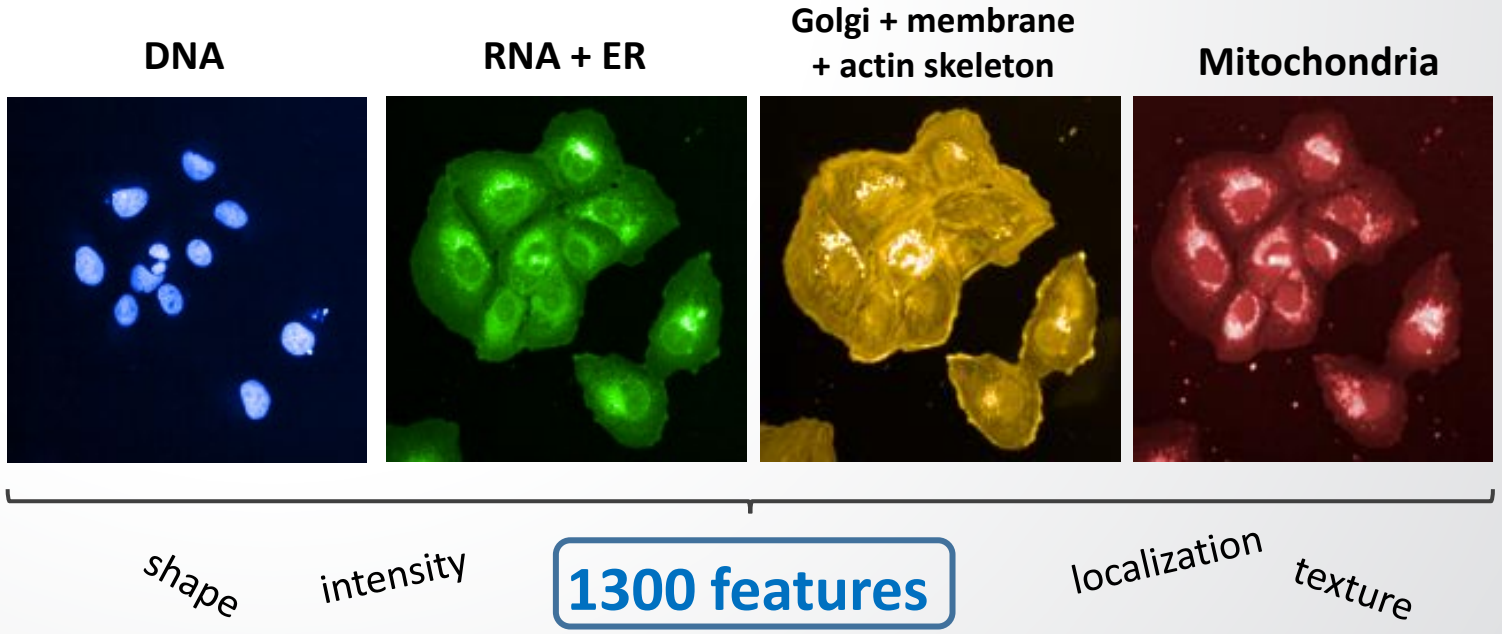
OPEN ACCESS Freely available online

PLOS ONE

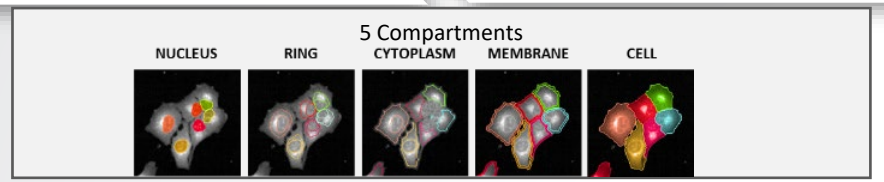
**Multiplex Cytological Profiling Assay to Measure Diverse Cellular States**

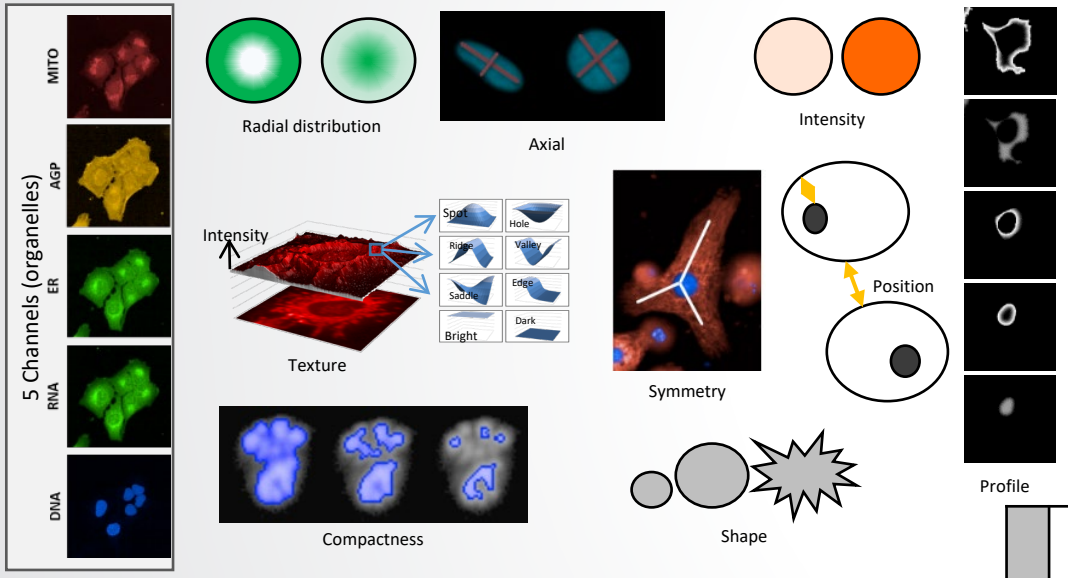
Sigrun M. Gustafsdottir, Vebjorn Ljosa, Katherine L. Sokolnicki, J. Anthony Wilson, Deepika Walpita, Melissa M. Kemp, Kathleen Petri Seiler, Hyman A. Carrel, Todd R. Golub, Stuart L. Schreiber, Paul A. Clemons, Anne E. Carpenter, Alykhan F. Shamji

Broad Institute of Harvard and MIT, Cambridge, Massachusetts, United States of America



# Imaging & Phenotypic Feature Extraction




49 Feature Categories
(ex. MITO_Texture_Cytoplasm)

1300 features / cell

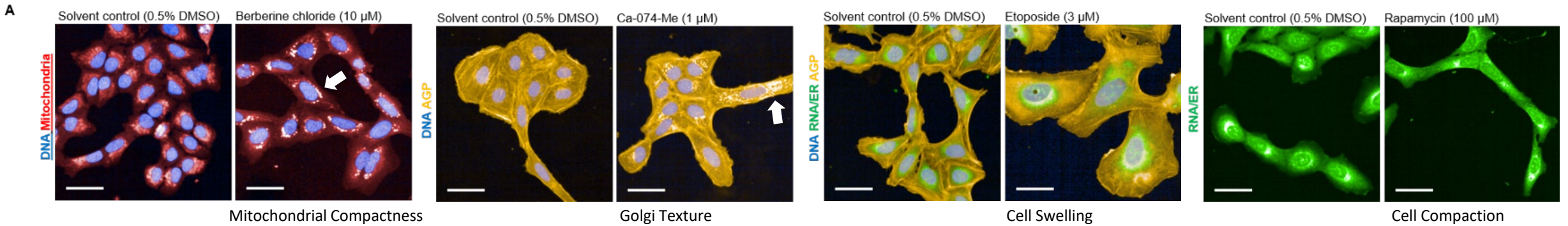
| | | Module | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Position [7] | Basic morph-ology [5] | SCARP morphology | | | | | Intensity [9] | Texture [14] |
| | | | | Symmetry [80] | Compactness [40] | Axial [20] | Radial [28] | Profile [20-30] | | |
| Channel | DNA | | | Nuclei | Nuclei | Nuclei | Nuclei Cell | Nuclei Cytoplasm | Nuclei | Nuclei |
| | RNA | | | Nuclei | Nuclei | Nuclei | Nuclei | Nuclei | Nuclei | Nuclei |
| | ER | | | Cell | Cell | Cell | Cell | Cytoplasm | Ring Cytoplasm | Ring Cytoplasm |
| | AGP | | | Cell | Cell | Cell | Cell | Nuclei Cytoplasm | Ring Cytoplasm Membrane | Ring Cytoplasm Membrane |
| | Mito | | | Cell | Cell | Cell | Cell | Nuclei Cytoplasm | Ring Cytoplasm | Ring Cytoplasm |
| | Not associated with a channel | Nuclei Cell | Nuclei Cell | | | | | | | |

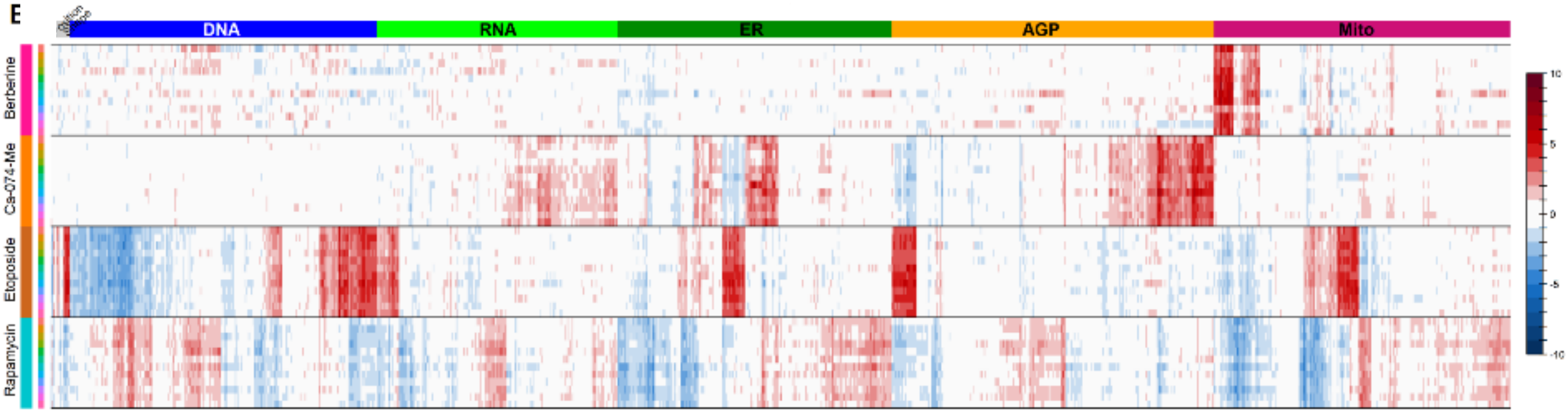
**PerkinElmer Opera Phenix**

**Modality:** Confocal (single *z*)
**Objective:** 20X Water
**Plate:** CellCarrier-384 Ultra
**Fields:** 5 or 9

*With illustrations from Perkin Elmer*

# Examples of Chemical Induced Phenotypes




- Strong phenotypes are observed qualitatively and produce distinct profiles when measured quantitatively.

*Adapted from Nyffeler et al. Toxicol Appl Pharmacol. 2020 Jan 15;389:114876*

# HTPP Data Analysis Pipeline

## Data reduction

cell-level data

**Normalization**
MAD normalization

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

normalized cell-level data

**Aggregation**
median

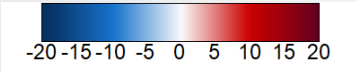
well-level data

**Standardization**
Z transformation

Cell Count Info
Conc. > 50% cell loss

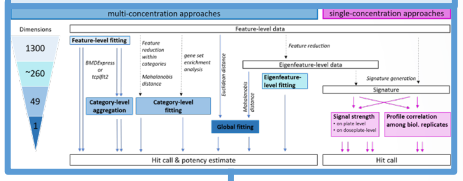
scaled well-level data

clipped well-level data




## Concentration Response Modeling

Calculate Response Metrics

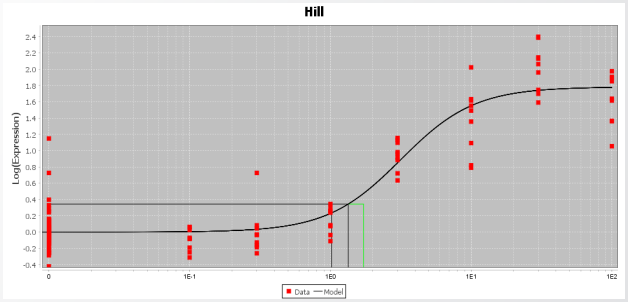



*See Nyffeler et al. SLAS Discov. 2020 Aug 29: doi: 10.1177/2472555220950245*

Fit Multiple Curve Shapes

Best Model Selection

BMC

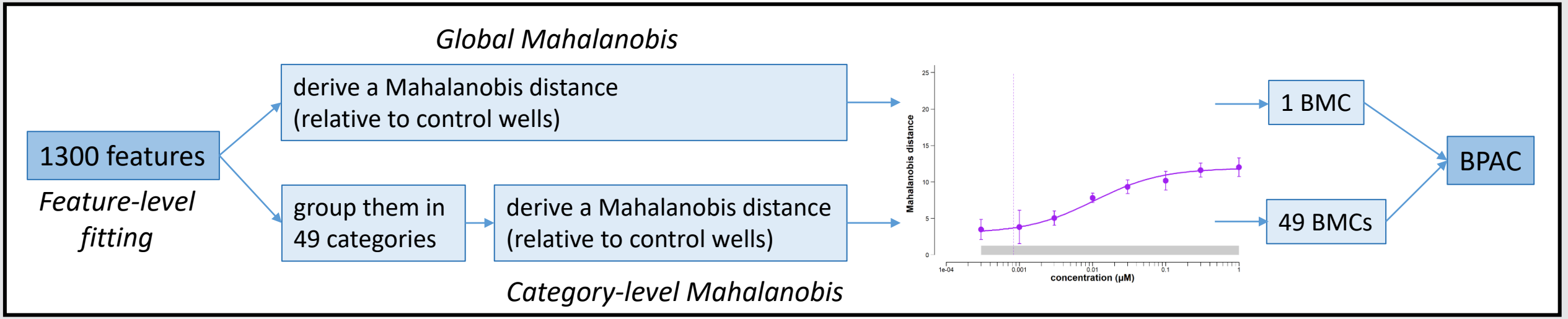



**Berberine chloride**
Mito_Cells_Morph_STAR

# Phenotype Altering Concentration (PACs)

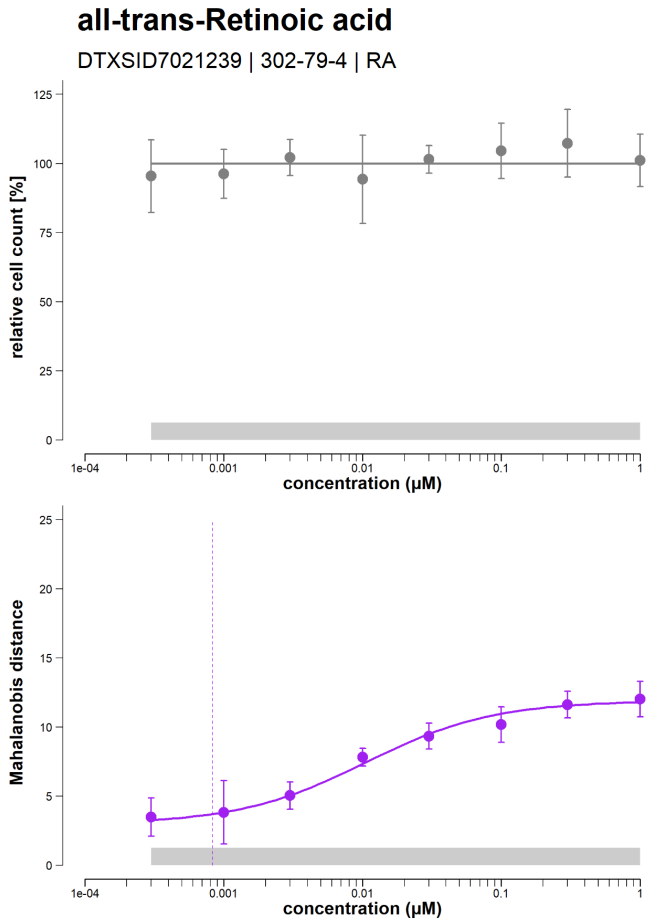
**Mahalanobis Distance ($D_M$):**

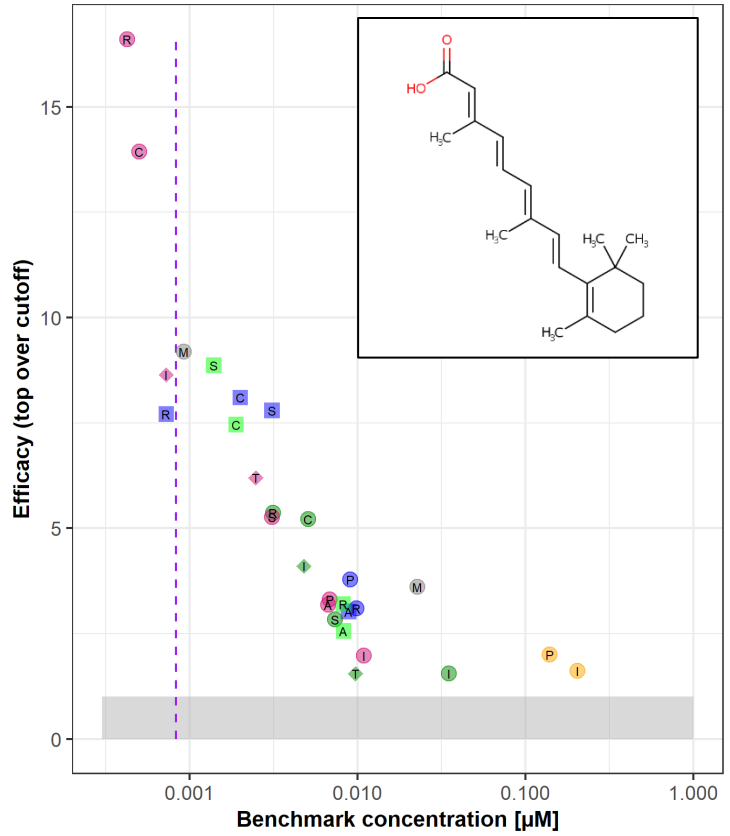
- A multivariate distance metric that measures the distance between a point (vector) and a distribution.
- Accounts for unpredictable changes in cell states across test concentrations and inherent correlations in profiling data.

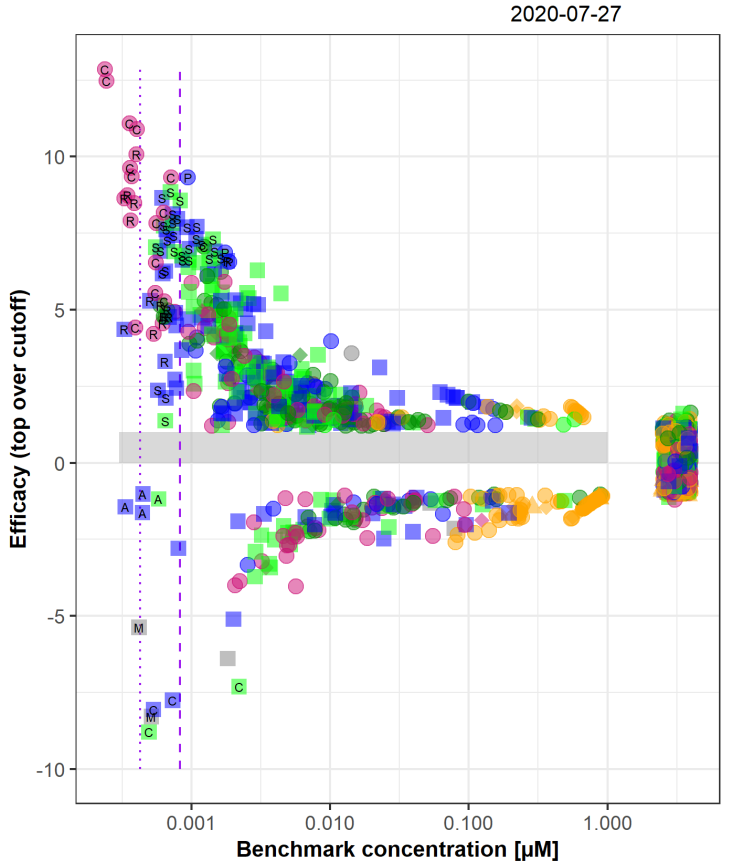


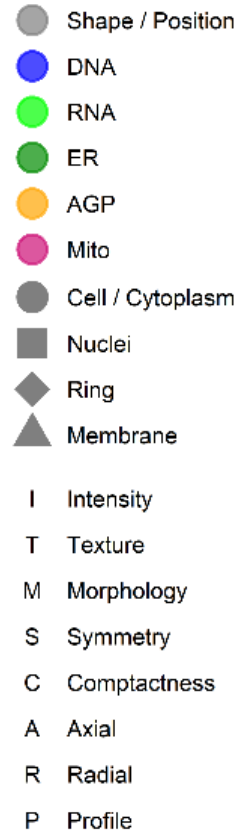

- Chemicals where a BMC can be determined using either the global or category $D_M$ approach are considered active.
- The minimum of the global or most sensitive category BMC is the **Phenotype Altering Concentration (PAC)**

# Concentration-Response Modeling Example



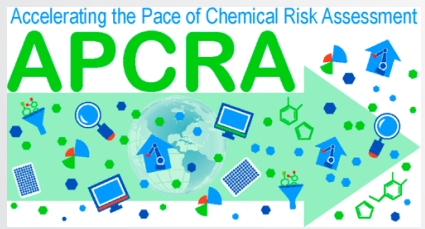




- Phenotypic effects can be observed below the threshold for cytotoxicity and in the absence of cytostatic effects.
- Category and feature-level modeling can reveal which organelles exhibit treatment-related changes in morphology.

# ToxCast Chemical Screen – Experimental Design (1)

| Parameter | Multiplier | Notes |
|---|---|---|
| Cell Type(s) | 1 | U-2 OS |
| Culture Condition | 1 | DMEM + 10% HI-FBS |
| Chemicals | 1,202 | Selected from US EPA ToxCast chemical collection<br>**Includes 179 chemicals with annotated molecular targets**<br>**Includes 462 APCRA case study chemicals** |
| Time Points: | 1 | 24 hours |
| Assay Formats: | 2 | High Throughput Phenotypic Profiling (Cell Painting)<br>*High Throughput Transcriptomics (TempO-Seq)* |
| Concentrations: | 8 | 3.5 $\log_{10}$ units; ~half-$\log_{10}$ spacing |
| Biological Replicates: | 4 | -- |

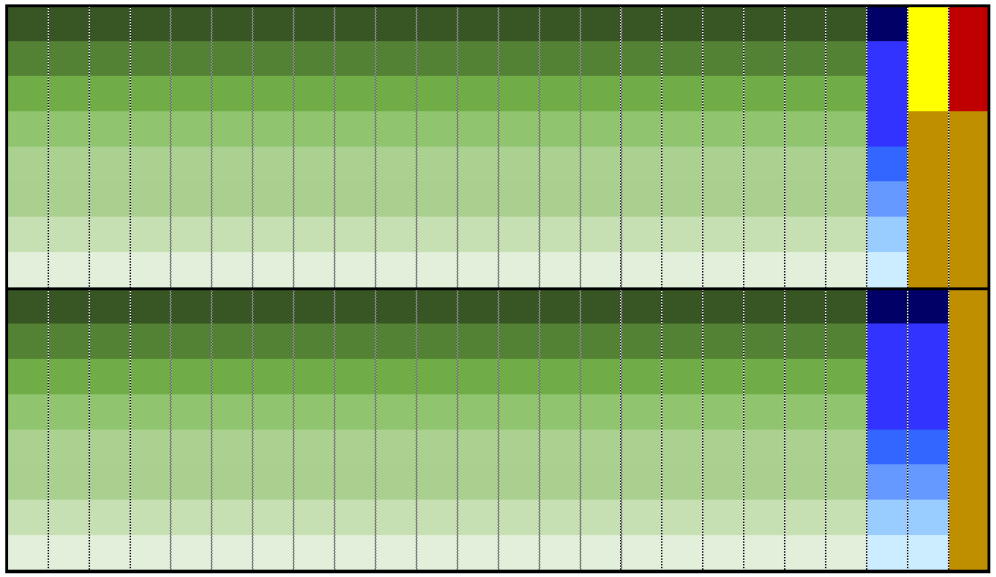



*Kavlock et al. (2018)*
*Chem. Res. Tox; 31(5): 287-290*

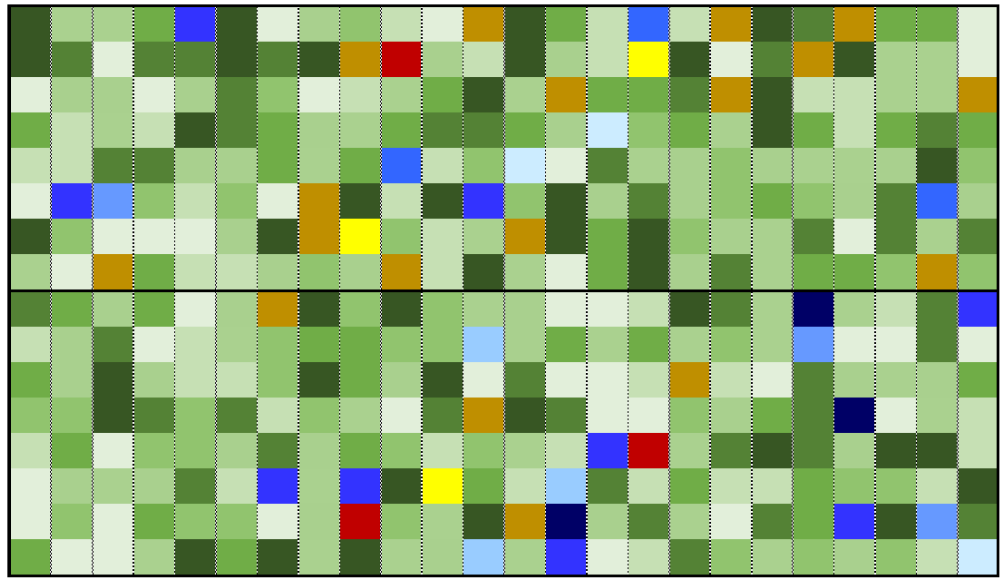
International collaboration of regulatory scientists focused on next generation chemical risk assessment including **deriving quantitative estimates of risk based on NAM-derived potency information and computational exposure estimates.**

**APCRA Chemicals** → PK parameters necessary for *in vitro* to *in vivo* extrapolation (IVIVE)
*in vivo* toxicity data

# ToxCast Chemical Screen – Experimental Design (2)

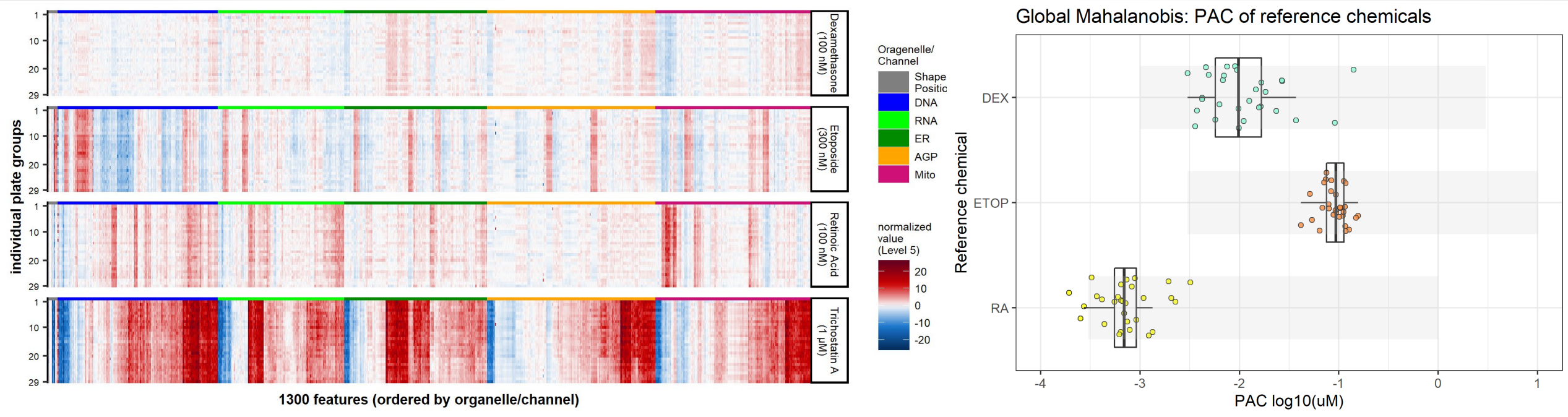


Treatment
Randomization



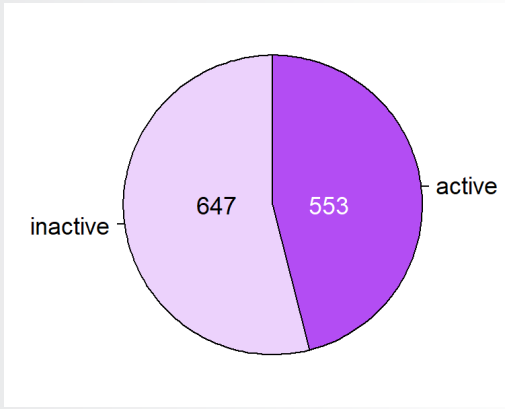
| Label | Reference Chemicals: | Molecular Mechanism-of-Action | Test Concentrations |
|---|---|---|---|
| A | Etoposide | DNA topoisomerase inhibitor | 0.03 - 10 µM |
| B | all-trans-Retinoic Acid | Retinoic acid receptor agonist | 0.0003 – 1 µM |
| C | Dexamethasone | Glucocorticoid receptor agonist | 0.001 – 3 µM |
| D | Trichostatin A | Histone deacetylase inhibitor | 1 µM |
| E | Staurosporine | Cytotoxicity control | 1 µM |
| F | DMSO | Vehicle control | 0.5 % |

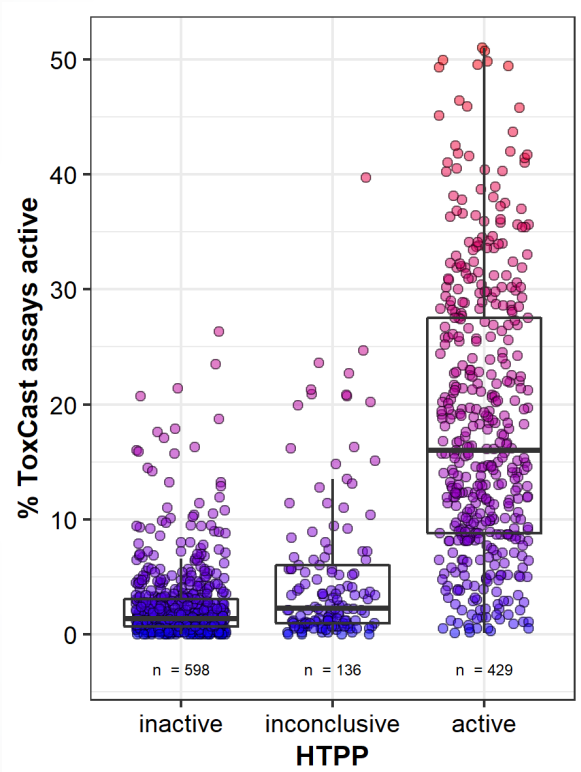
# Assay Performance / Reproducibility

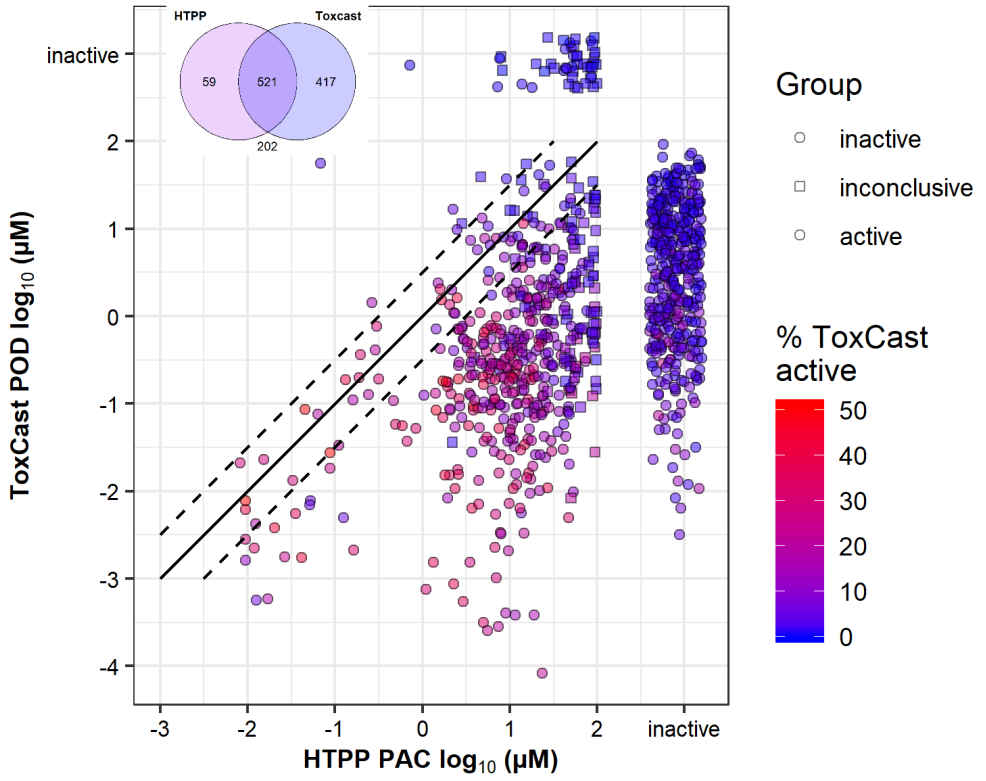



- Reference chemicals produce **reproducible** and **distinct** profiles.
- Reference chemicals produce reproducible **potency** estimates (PACs).

# ToxCast Chemical Screening Results




- Chemicals active in HTPP are more often ‘promiscuous’ in ToxCast.
- Chemicals active in HTPP produce less potency PACs compared to ToxCast.

*Preliminary results. Do not cite or quote.*

# *In Vitro* to *In Vivo* Extrapolation (IVIVE)

**Predicted exposure**

**Exposure predictions (EPA ExpoCast)**

- Systematic Empirical Evaluation of Models (SEEM) version 3
- Inferred from human biomonitoring data, production volume and use categories (industrial / consumer use)

**New approach methodologies (NAMs)**

**Toxicological threshold of concern (TTC)**

**Toxcast BPAC (µM)**

**HTPP BPAC (µM)**

***In vitro*-to-*in vivo* extrapolation (IVIVE)**

high-throughput toxicokinetics (httk)

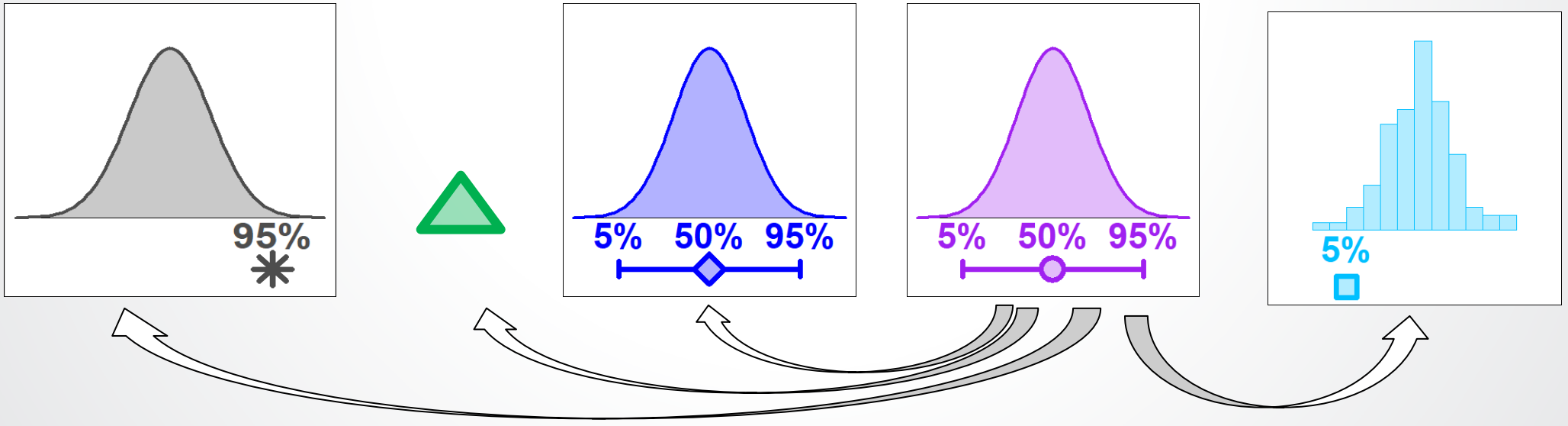
**Toxcast AED (mg/kg bw/day)**

**HTPP AED (mg/kg bw/day)**

***in vivo* point-of-departure**

**Database of *in vivo* effect values (EPA – ToxValDB)**

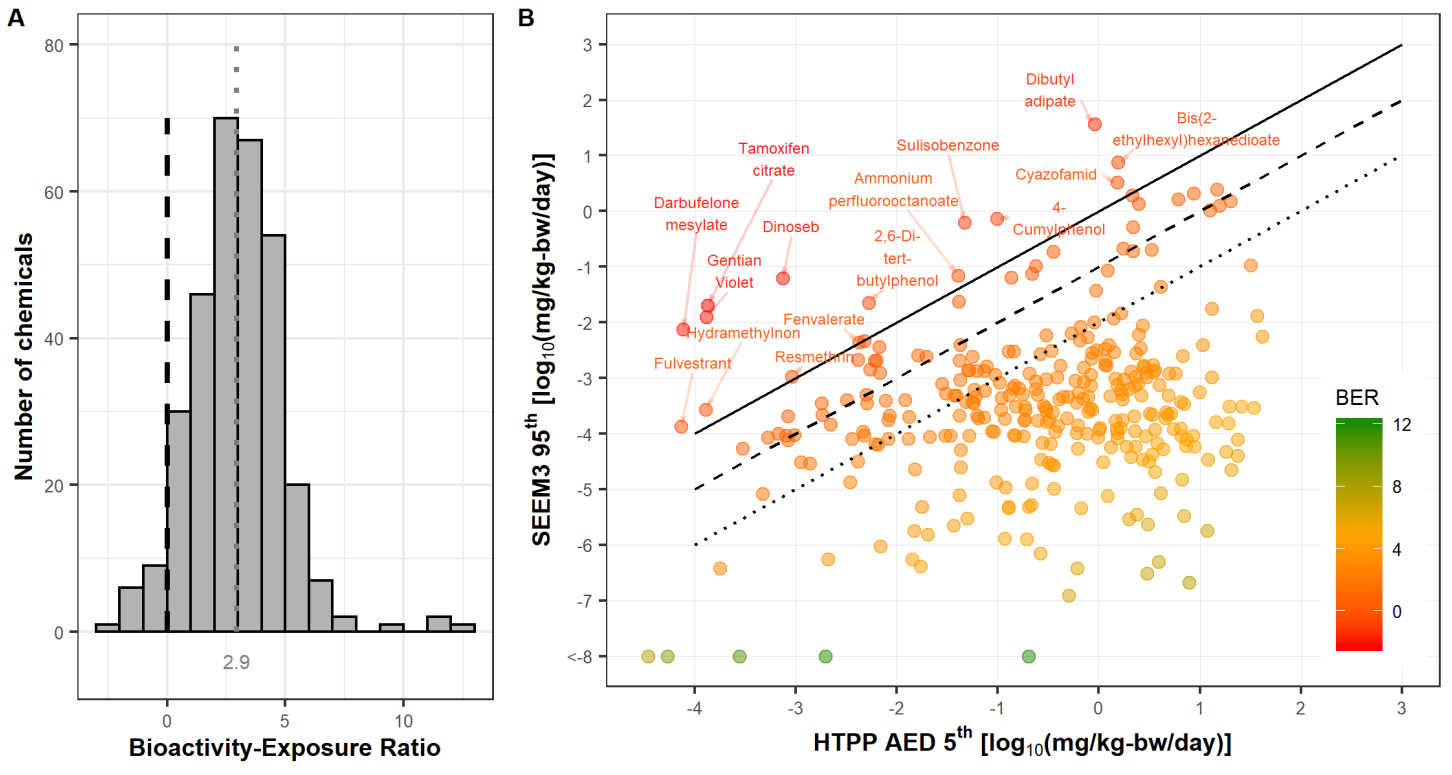
- Mammalian species
- oral exposures
- Various study types
- NOEL, LOEL, NOAEL, LOAEL
- mg/kg/day




POD: point-of-departure
AED: administered equivalent dose

# Bioactivity to Exposure Ratio (BER) Analysis

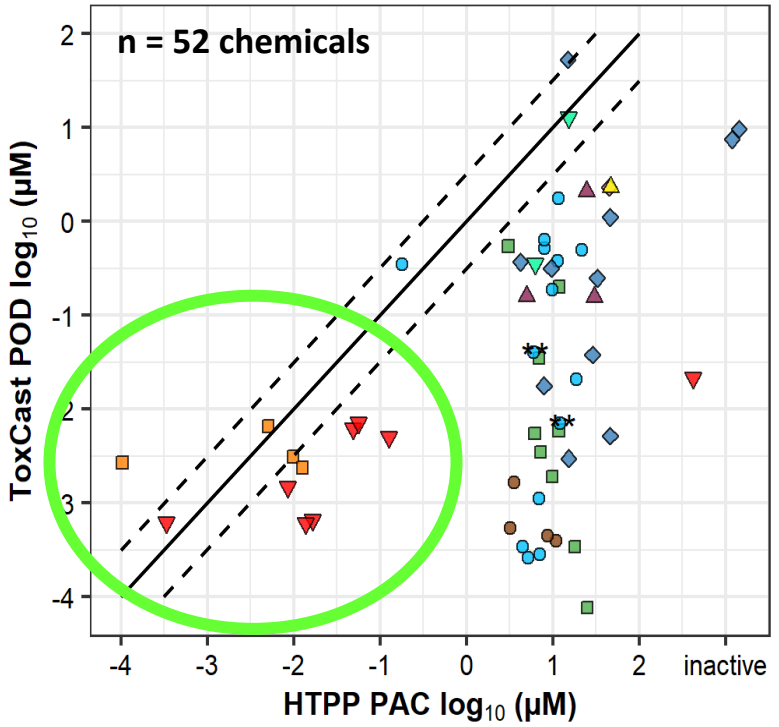
$$\text{Bioactivity exposure ratio (BER)} = \frac{\text{lower bound of HTPP bioactivity}}{\text{upper bound of exposure estimate}} = \log_{10}\left(\frac{\text{HTPP AED } 5^{th}}{\text{SEEM3 } 95^{th}}\right)$$



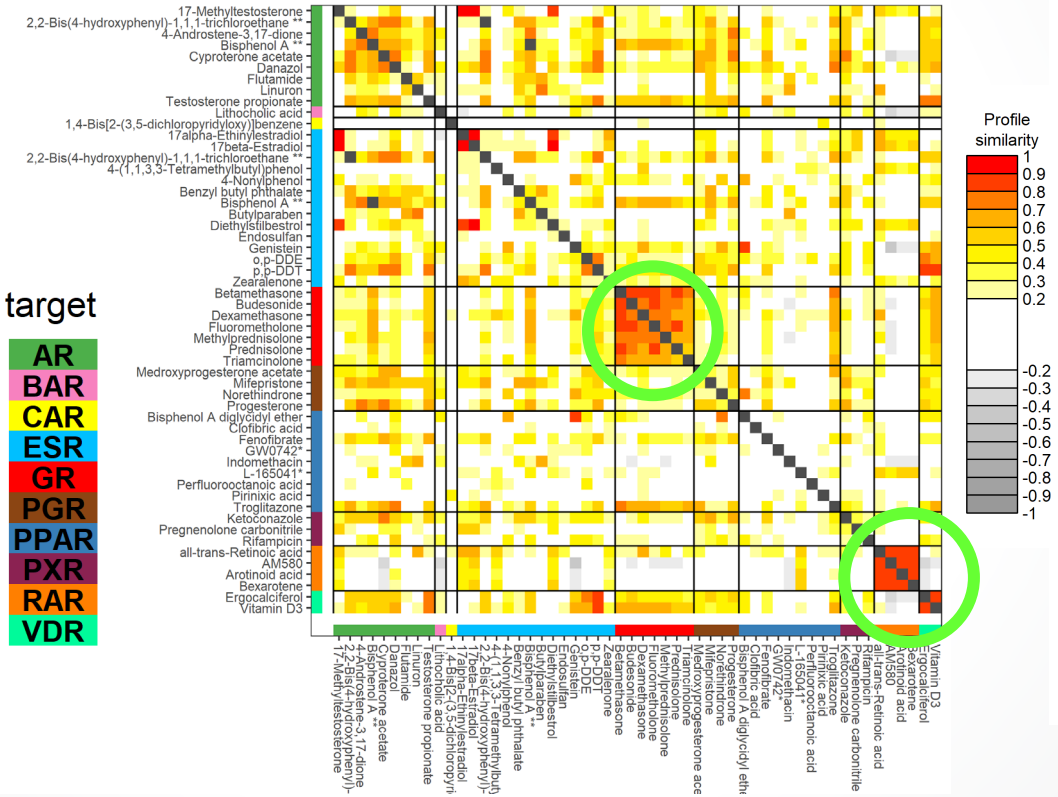

For some chemicals, the BER was negative, indicating a potential for humans to be exposed to bioactive concentrations of these chemicals

# Contextual Response of Nuclear Receptor Modulators

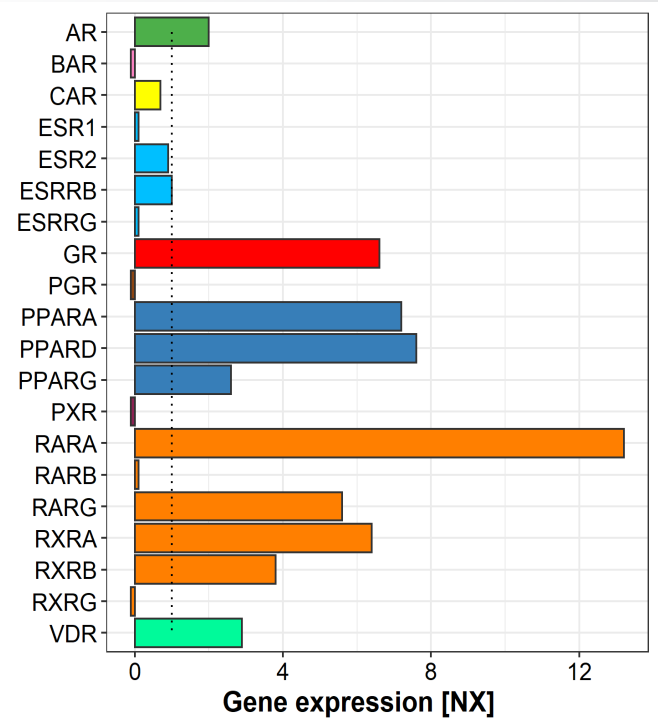
## Comparison to ToxCast potencies




## Profile Similarity

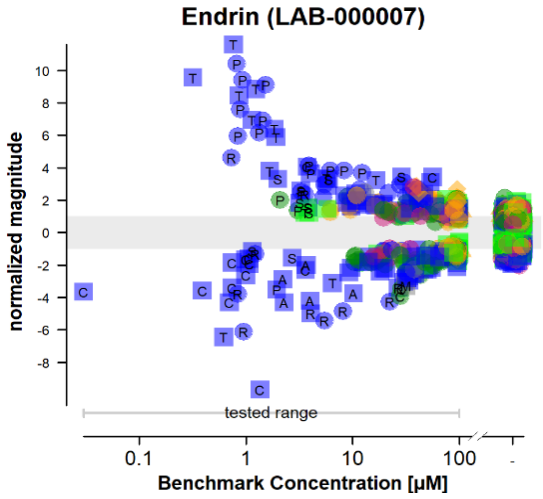


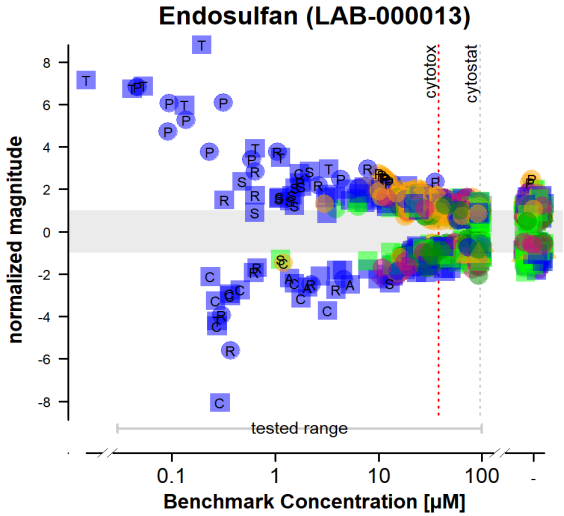

## Gene expression in U-2 OS

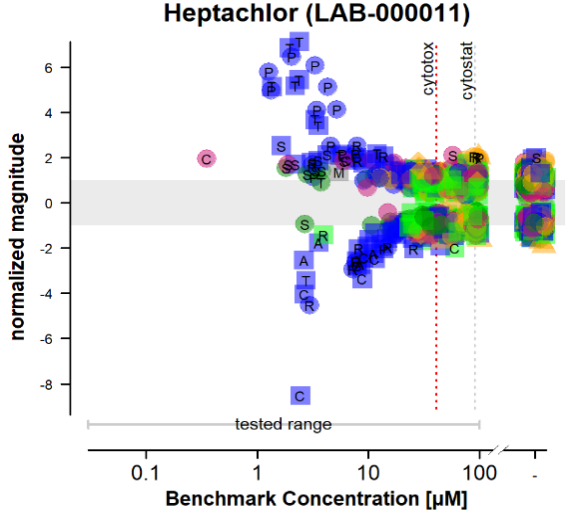


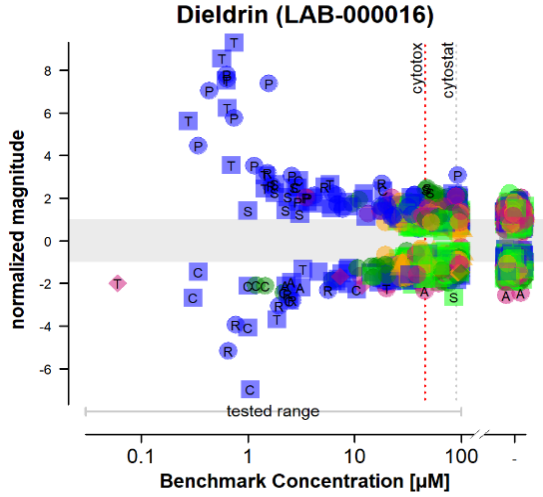

- For three receptor systems that are expressed in U-2 OS cells (GR, RAR/RXR, VDR) potencies were comparable with ToxCast.
- Phenotypic profiles for chemicals that affect these receptor systems are similar.

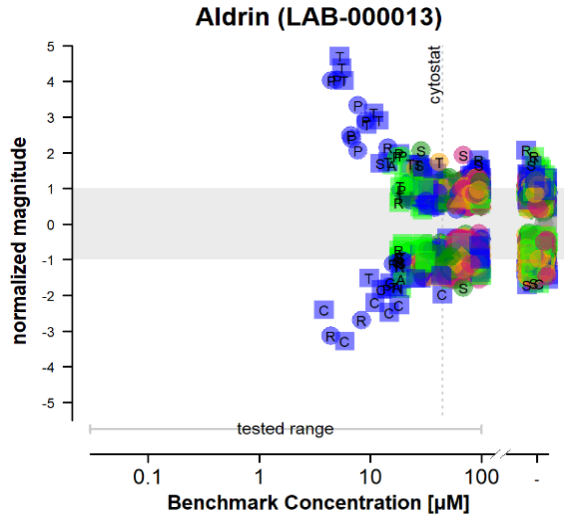
# Structurally Similar Environmental Chemicals Can Produce Similar HTPP Profiles

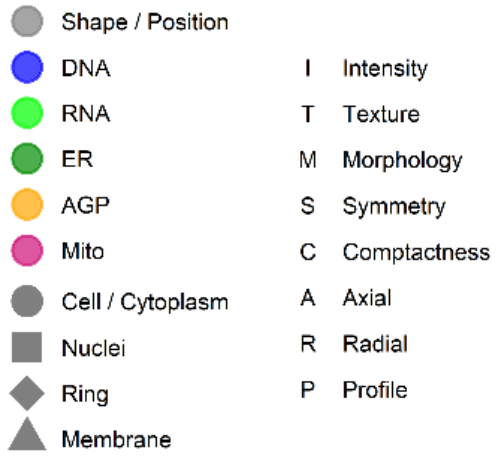


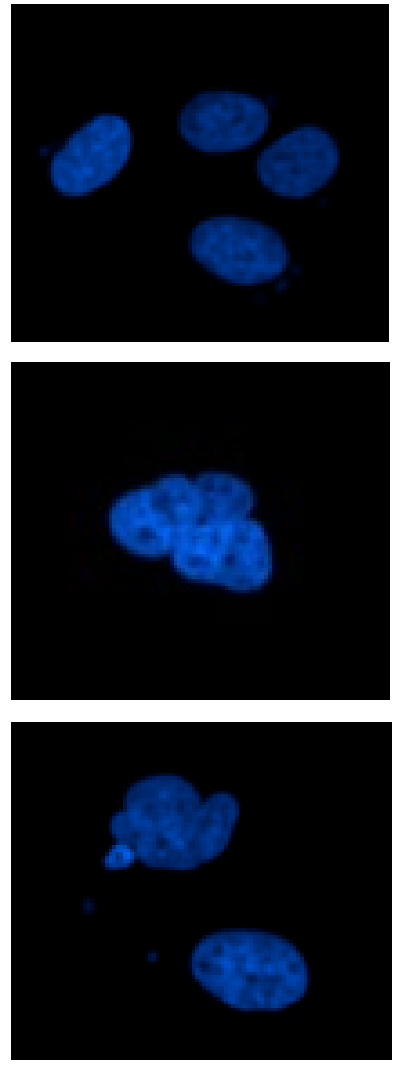



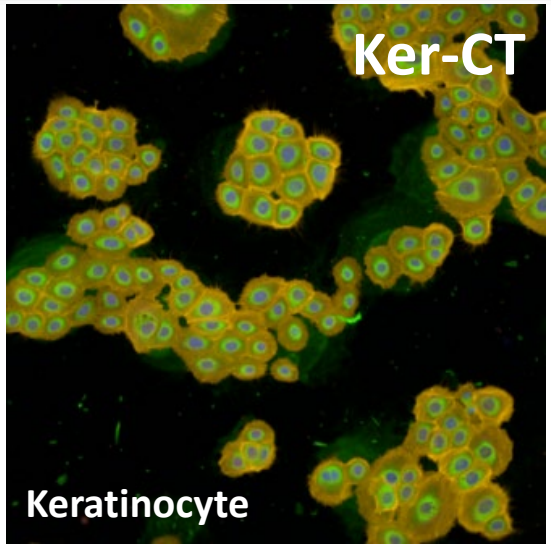



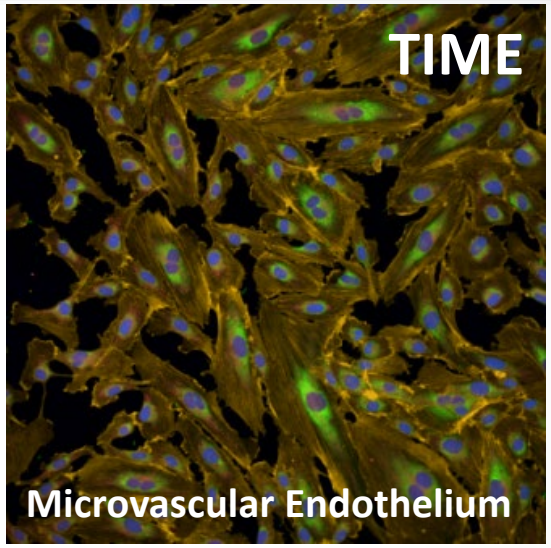



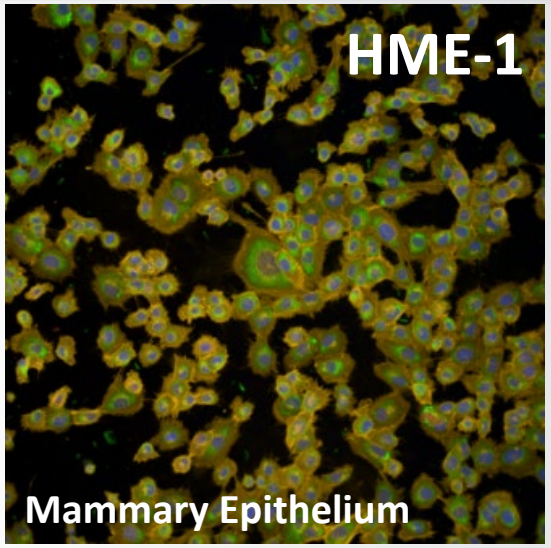

*Preliminary results. Do not cite or quote.*

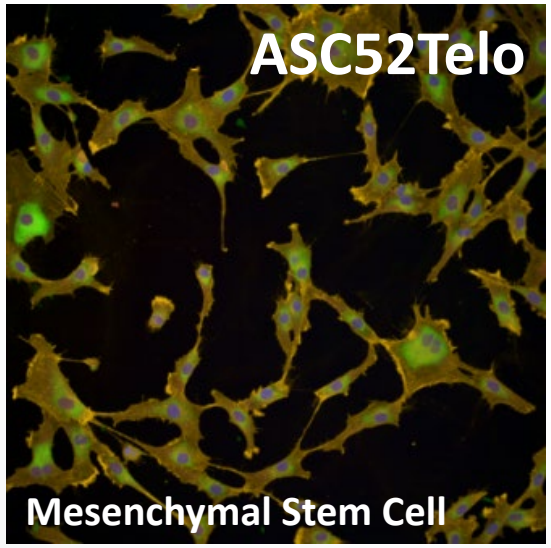
# HTPP is Compatible with Biologically Diverse Cell Lines

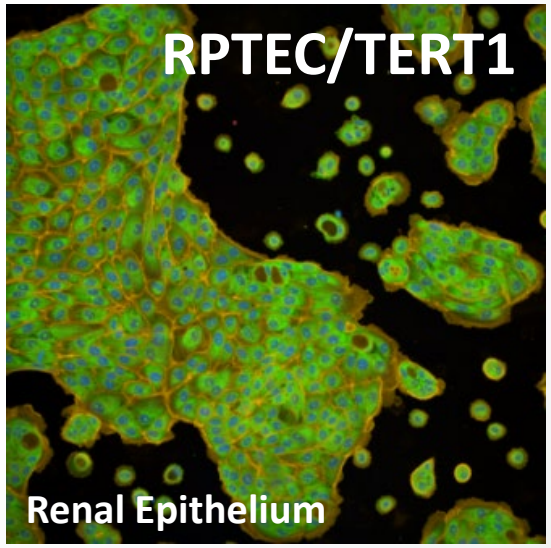
- HTPP is compatible with many human-derived cell culture models.
- Enables characterization of chemical effects across different domains of human biology.

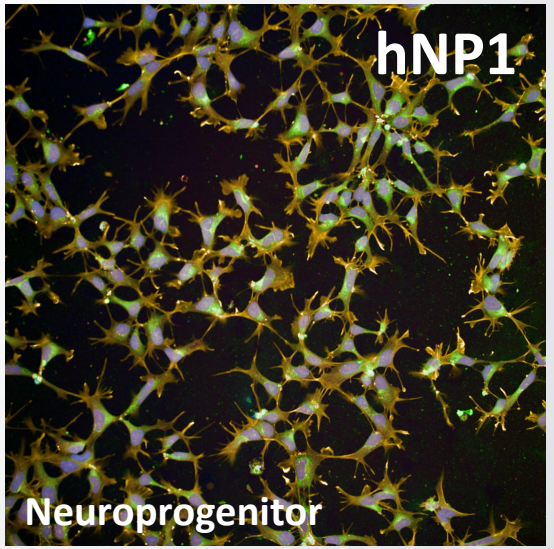



*Preliminary results. Do not cite or quote.*

# Summary

- **Assay Reproducibility:** Demonstrated high assay reproducibility through the use of phenotypic reference chemicals and developed experimental designs that allow for evaluation of assay performance throughout large-scale screening campaigns.
- **Potency Estimation:** Developed a concentration-response modeling workflow to identify concentration thresholds for perturbation of cell morphology (e.g. phenotypic altering concentration, PAC).
- **Mechanistic Prediction:** Chemicals with strong and specific target mode associations can produce similar phenotypic profiles in U-2 OS cells. Strength of similarity varies according to baseline target expression.
- **Chemical Similarity:** Chemicals with similar chemical structures can also produce similar phenotypic profiles in U-2 OS cells.
- **Bioactivity to Exposure Ratio:** Phenotype altering concentrations (PACs) can be converted to administered equivalent doses (AEDs) and compared to human exposure predictions for chemical ranking and prioritization.
- **Biologically Diverse Cell Lines:** Compatibility of HTPP with many human-derived cell models permits characterization of chemical bioactivity across different domains of human biology.

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