EPA
United States
Environmental Protection
Agency

In Vitro Molecular Points-of-Departure (PODs) from High-Throughput Profiling Assays

Joshua A. Harrill

USEPA Center for Computational Toxicology and Exposure (CCTE)

Society of Toxicology (SOT) Annual Meeting
March 23rd, 2021

United States Environmental Protection Agency

Disclaimer

The views expressed in this presentation are those of the author(s) and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency, nor does mention of trade names or products represent endorsement for use.

EPA United States Environmental Protection Agency

Outline

- High Throughput Transcriptomics (HTTr)
- High Throughput Phenotypic Profiling (HTPP)
- Applications for Molecular PODs From HTP NAMs

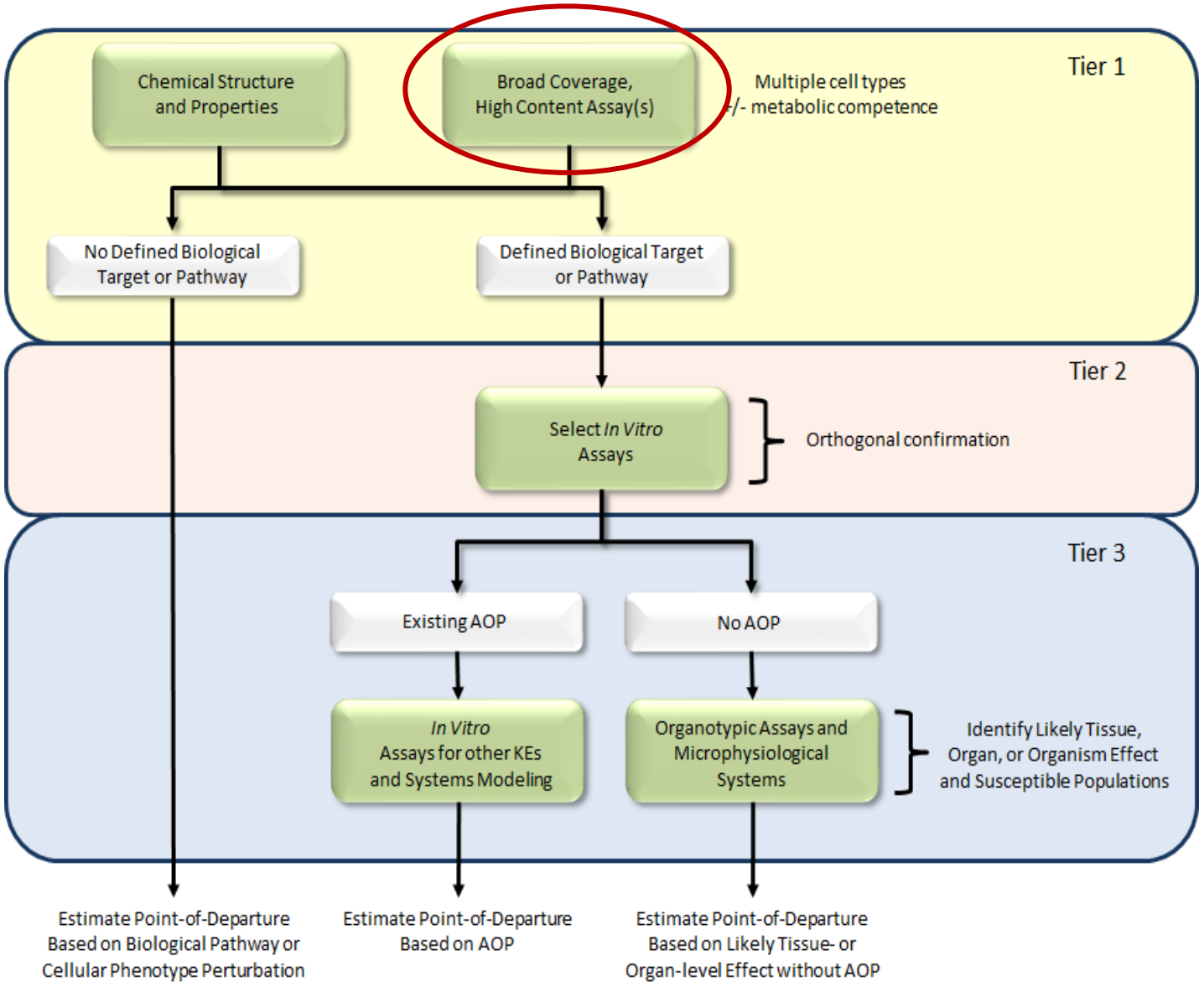
Tiered Hazard Evaluation Approach (1)

- **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.

- NAMs are a potential means to **reduce** the use of animals in toxicity testing and **accelerate** the pace of chemical risk assessment.

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

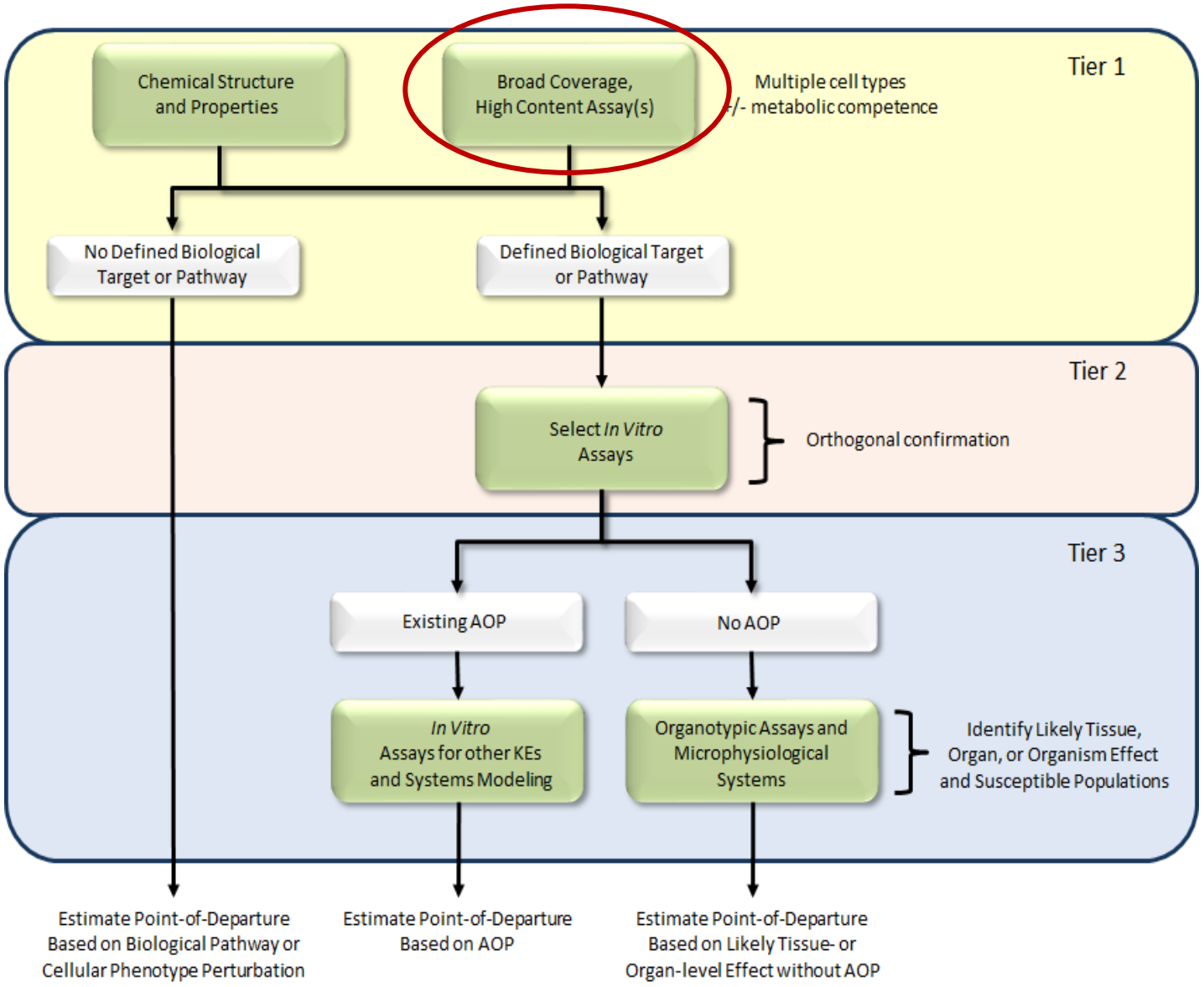
- **HTP assay criteria:**
  1. Yield bioactivity profiles that can be used for **potency estimation**, **mechanistic prediction** and evaluation of **chemical similarity**.
  2. Compatible with multiple human-derived culture models.
  3. Concentration-response screening mode.
  4. Cost-effective.



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

Tiered Hazard Evaluation Approach (2)

- To date, EPA has identified and implemented two HTP assays that meet this criteria.
- **High-Throughput Transcriptomics [HTTr]**
  - Whole Transcriptome TempO-Seq
- **High-Throughput Phenotypic Profiling [HTPP]**
  - Cell Painting
- Both methods are **complementary** to each other and can be used in many different human-derived cell types.
- EPA has established scalable laboratory and bioinformatics workflows for each assay.



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

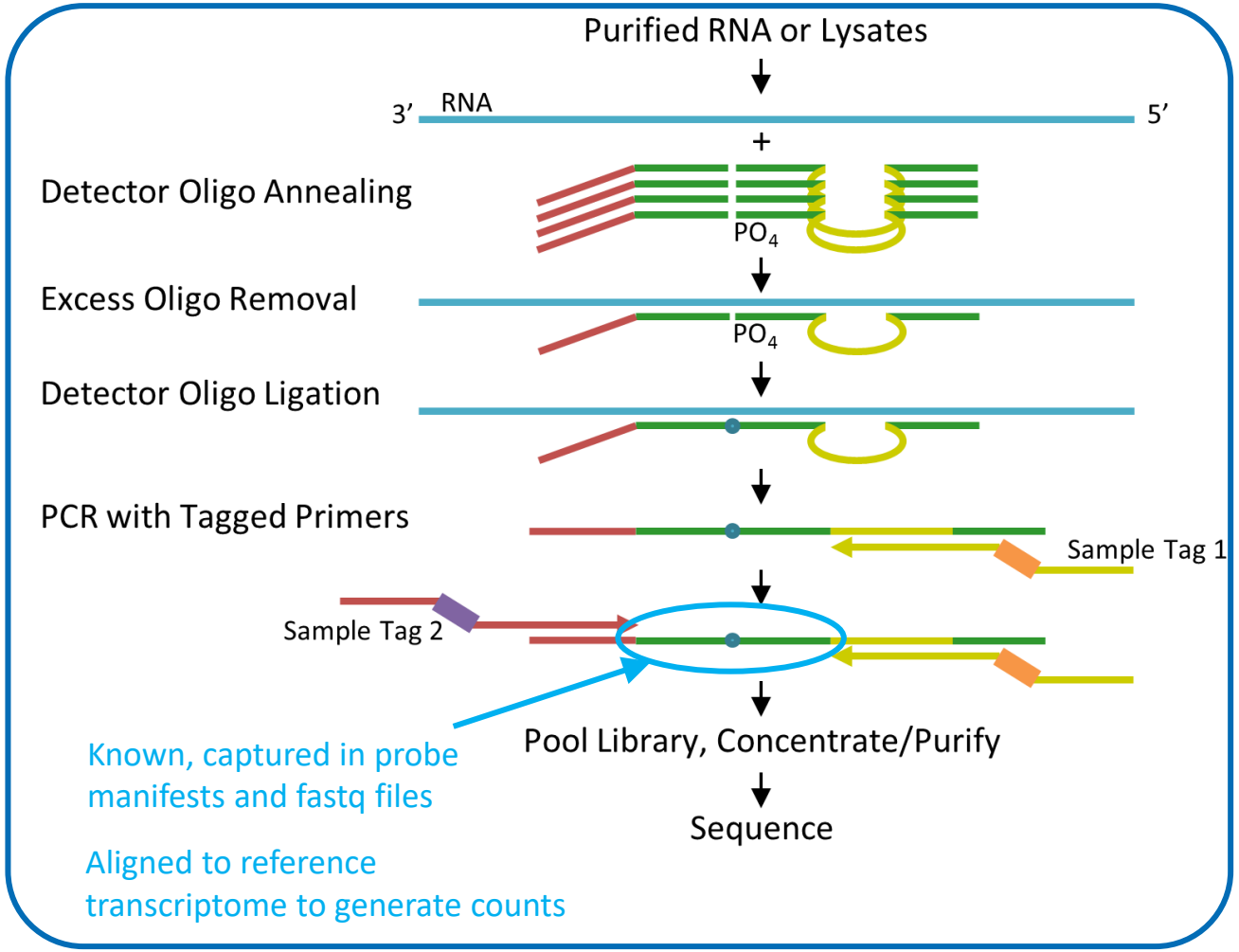
EPA United States Environmental Protection Agency

High-Throughput Transcriptomics (HTTr)

Templated Oligo with Sequencing Readout (TempO-Seq)

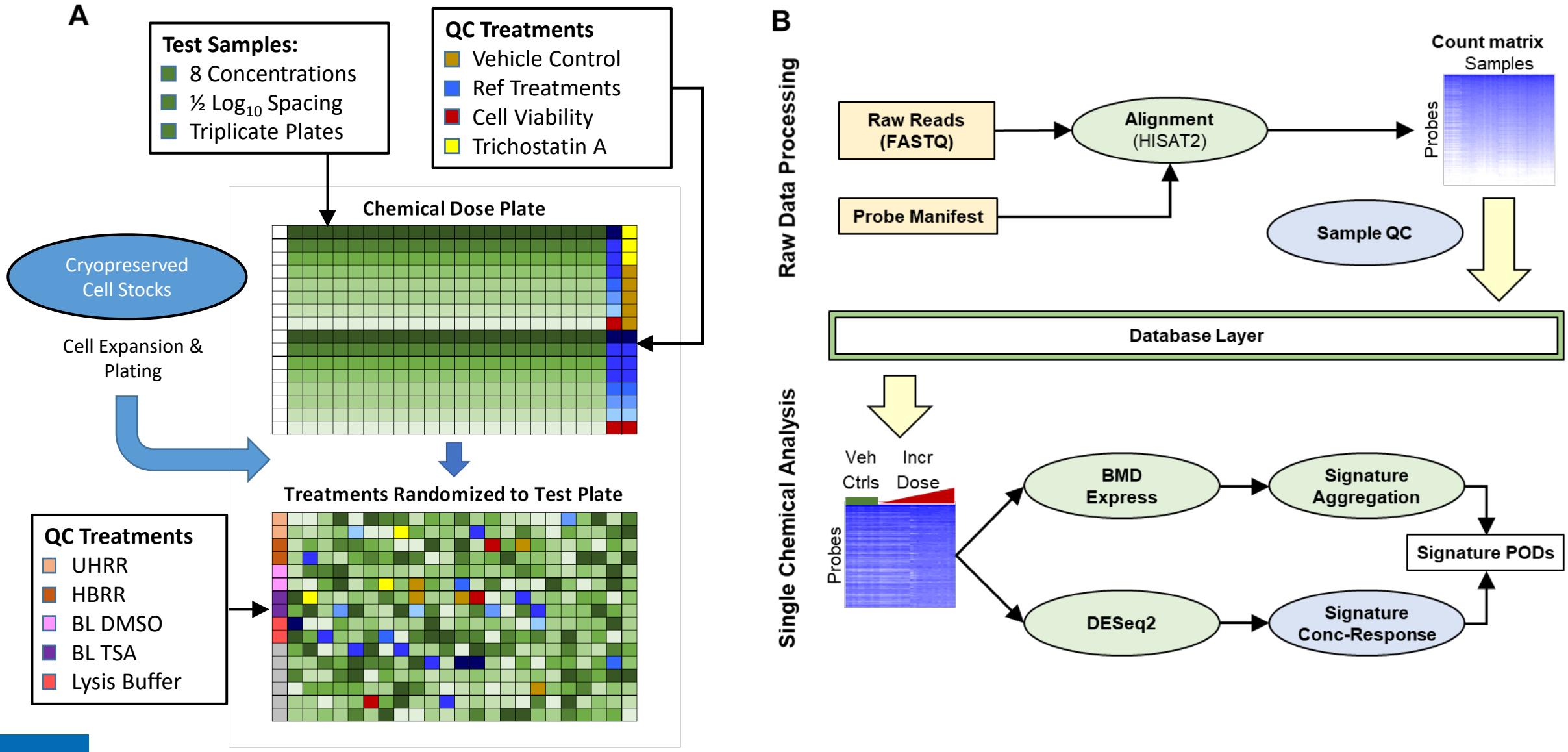
- The **TempO-Seq** human whole transcriptome assay measures the expression of greater than 20,000 transcripts.
- Requires only picogram amounts of total RNA per sample.
- Compatible with purified RNA samples or **cell lysates**.
- Lysates are barcoded according to sample identity and combined in a single library for sequencing using industry standard instrumentation.
- Scalable, targeted assay:
  - 1) specifically measures transcripts of interest
  - 2) ~50-bp reads for all genes
  - 3) requires less flow cell capacity than RNA-Seq

TempO-Seq Assay Illustration



Yeakley, et al. PLoS ONE 2017

HTTr Experimental Design and Bioinformatics Workflow



Concentration-Response Modeling of Signature Scores (1)

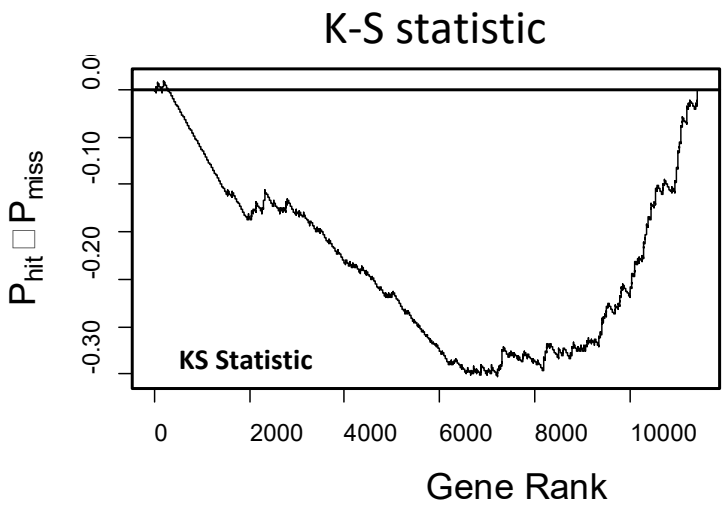
Step 1: Inputs

Experimental Data: Chemical_Conc × Gene matrix of DESeq2-moderated $\log_2$ [fold-change] values.

Signature Collections: MSigDB (*Liberzon et al. 2015*), BioPlanet (*Huang et al. 2019*), CMAP (*Subramanian et al. 2005*)

Step 2: Pathway Scoring

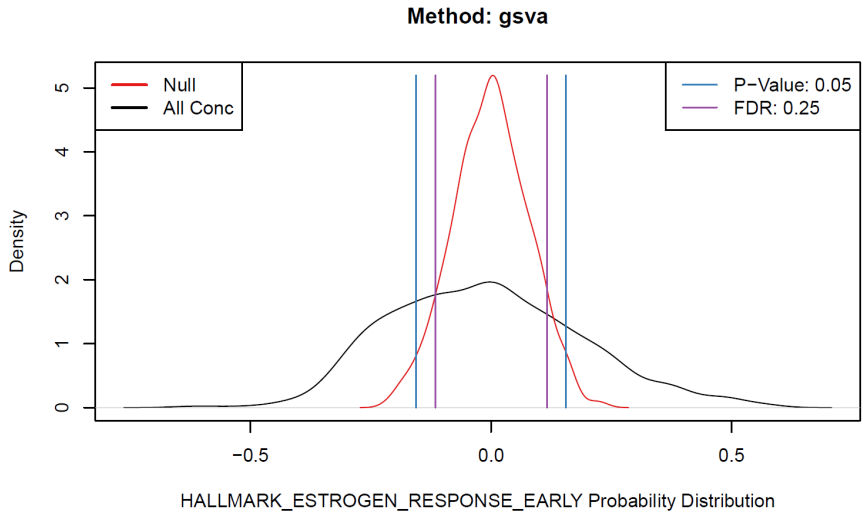
Scores based on single sample GSEA method (Barbie et al. 2009)



Chemical_Conc × Pathway matrix of scores.

Step 3: Cut-off Estimation via NULL Modeling

- For each gene, **resample** l2fc based on the cross-sample gene distribution → breaks gene correlation
- Calculate **pathway scores for "null" data**
  - One null distribution (n = 1000 scores) / pathway



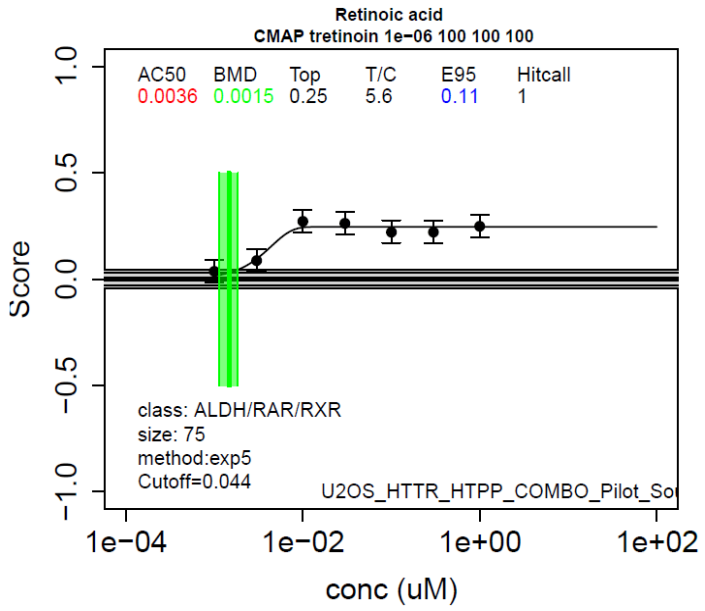
Analysis by Thomas Sheffield and Richard Judson

Biological Pathway Altering Concentrations (BPACs)

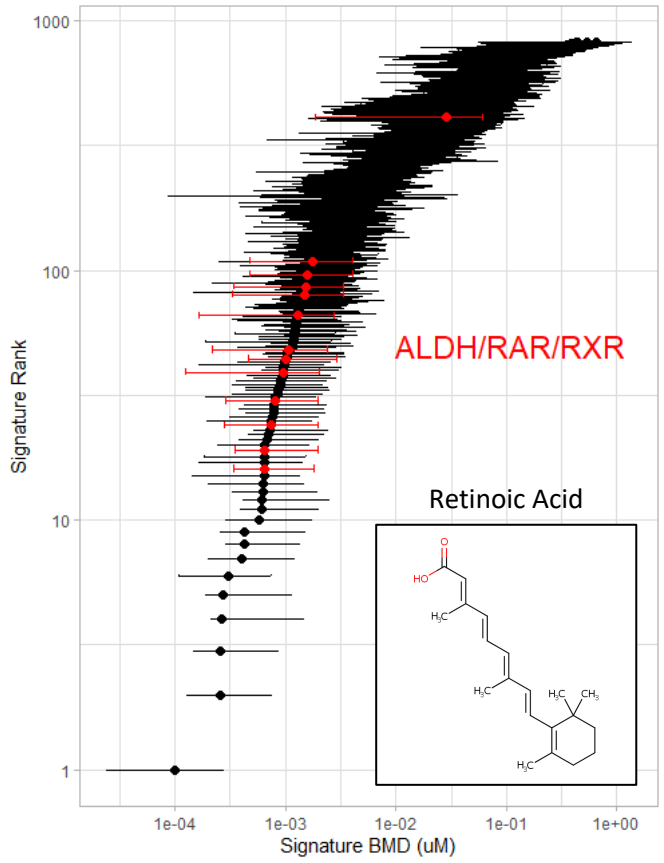
Step 4: Concentration-Response Modeling (*tcplfit2*)

Signature-Level:

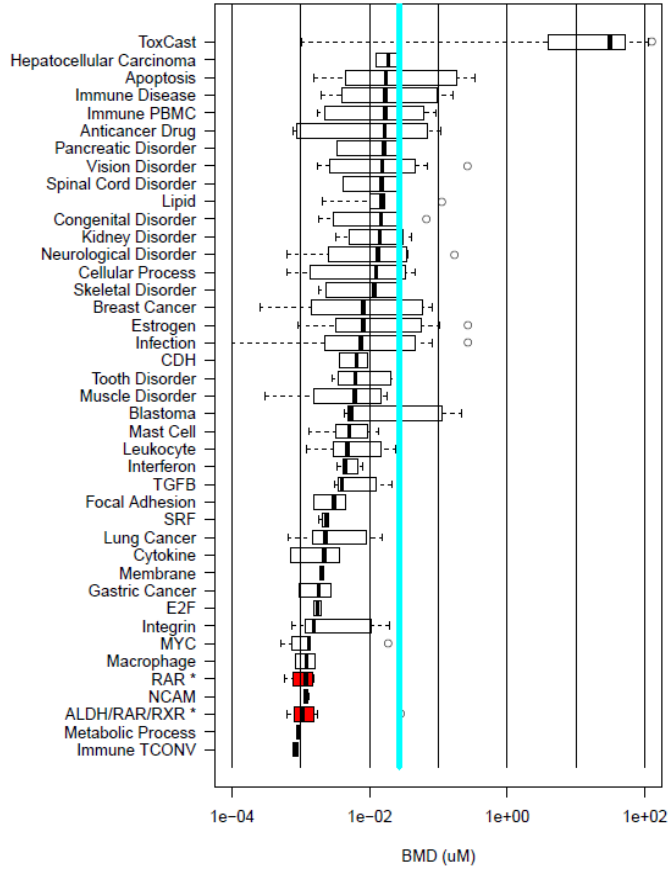
- Benchmark Dose (BMD)
- Confidence Interval on BMD
- Hit Call Probability



Step 5: Ranking of Signatures



Step 6: Signature Aggregation

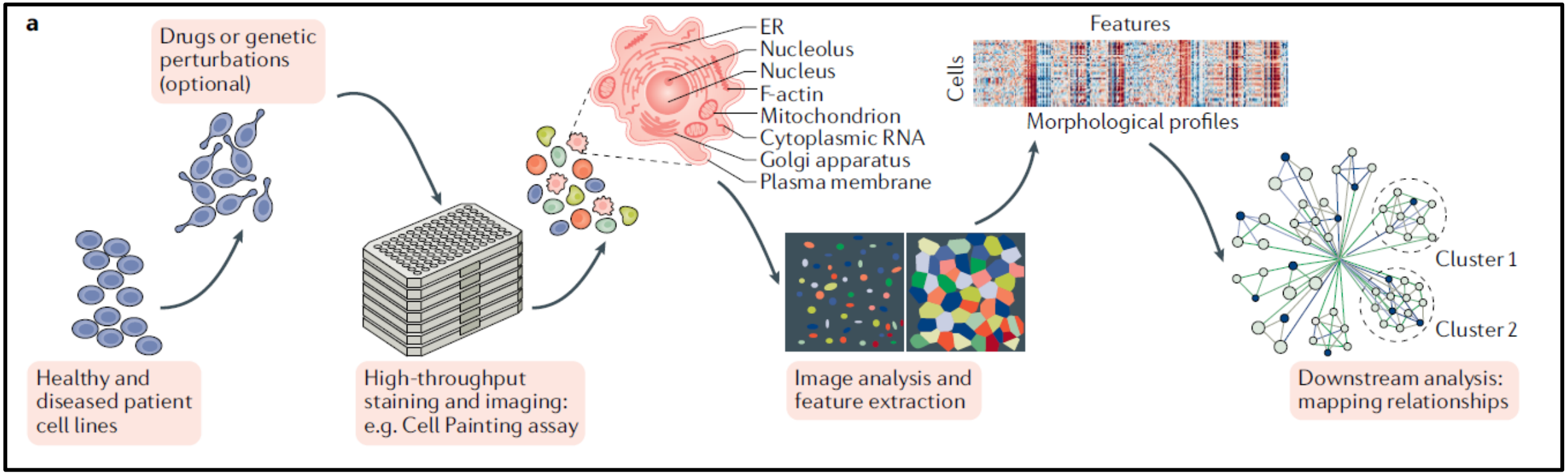


- Molecular PODs based on **biological pathway altering concentrations (BPACs)** may be derived in several ways.
- Most sensitive signature **OR** statistic based on distribution of active signatures (5th %ile) **OR** by target class.

EPA United States Environmental Protection Agency

High-Throughput Phenotypic Profiling (HTPP)

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
- Scalable
- Amenable to lab automation
- Deployable across multiple human-derived cell types.
- Reproducible
- Cost-effective (¢ / well)
- 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[a], J. Anthony Wilson[ab], Deepika Walpita, Melissa M. Kemp, Kathleen Petri Seiler[ac], Hyman A. Carrel[ad], 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

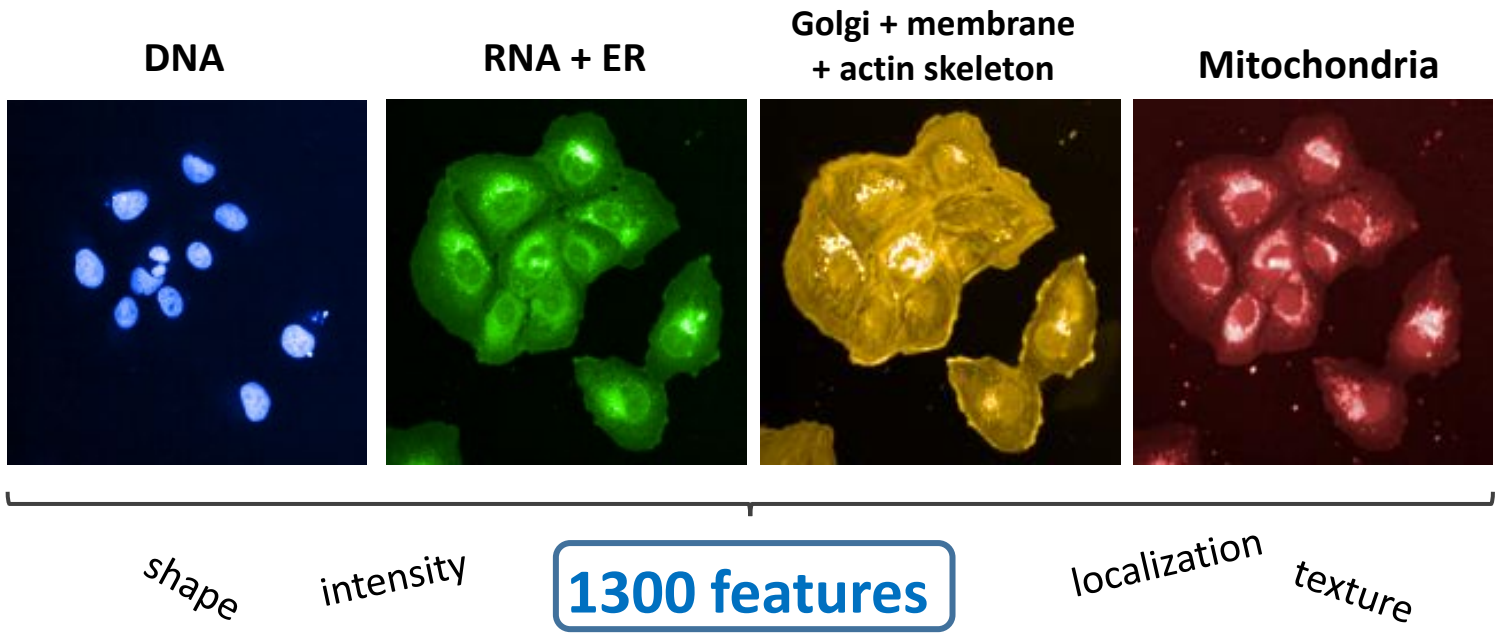
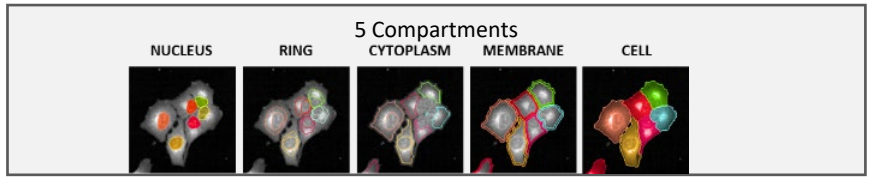
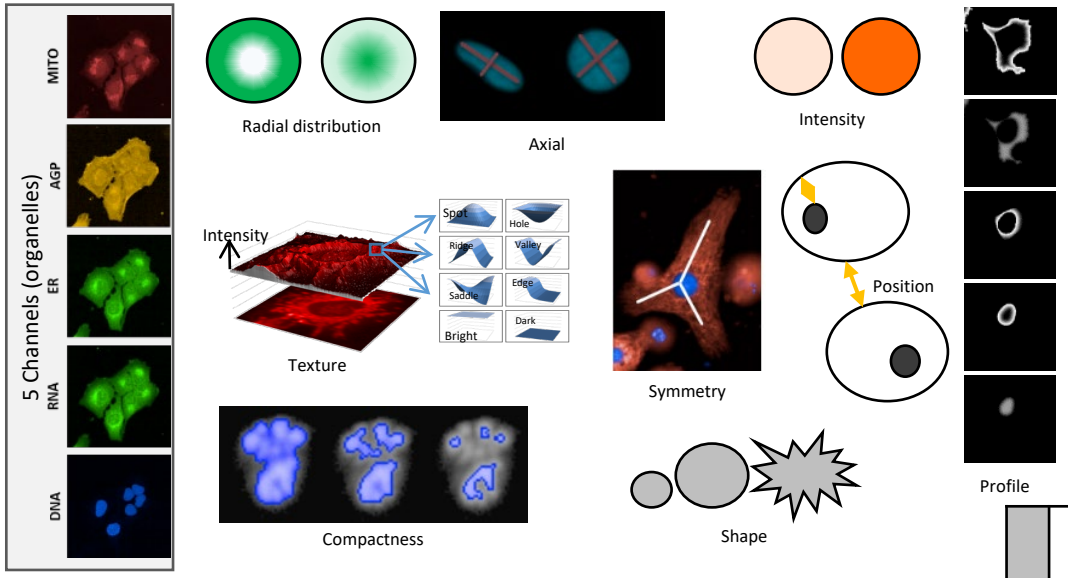


Image Acquisition & Phenotypic Feature Extraction





1300 features / cell

49 Feature Categories
(ex. MITO_Texture_Cytoplasm)

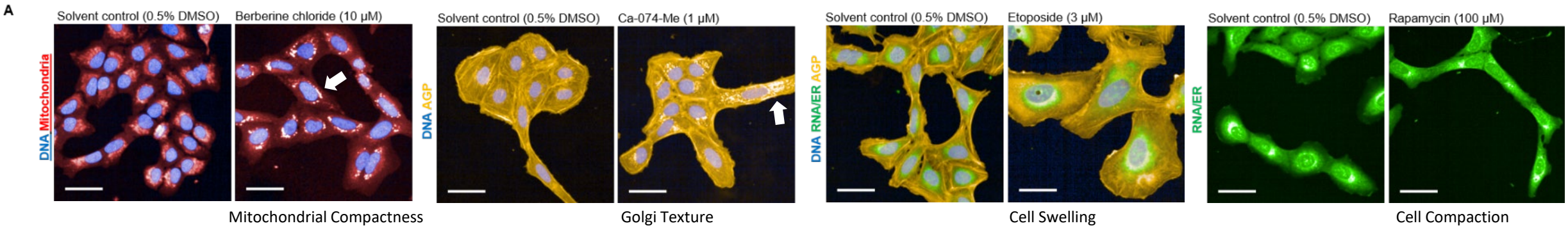
Channel	Module: Position [7]	Module: Basic morph-ology [5]	Module: SCARP morphology: Symmetry [80]	Module: SCARP morphology: Compactness [40]	Module: SCARP morphology: Axial [20]	Module: SCARP morphology: Radial [28]	Module: SCARP morphology: Profile [20-30]	Module: Intensity [9]	Module: Texture [14]
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							

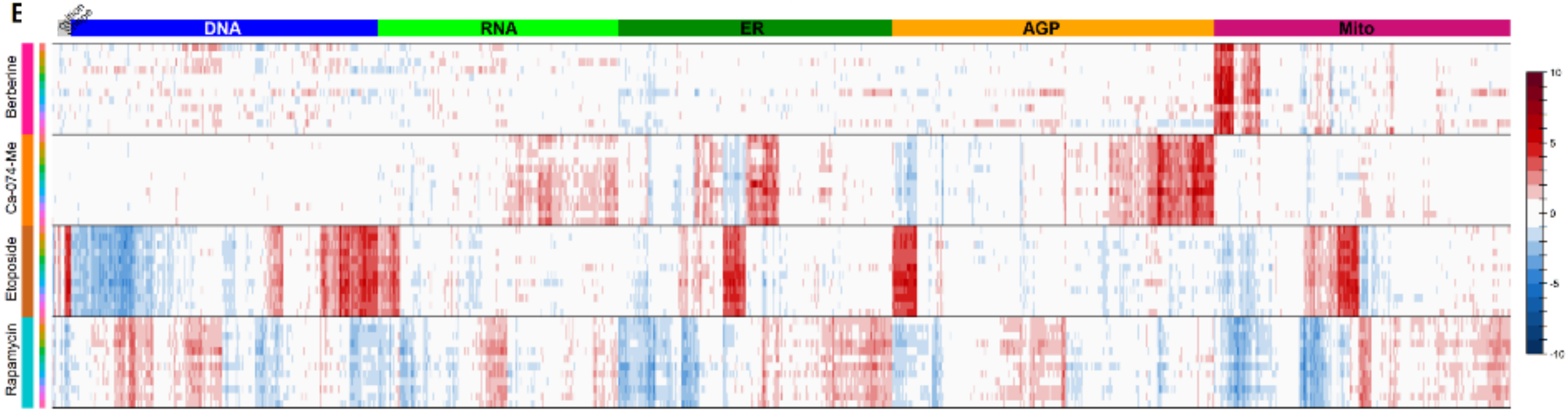
PerkinElmer Opera Phenix

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

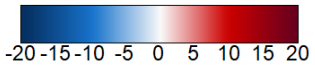
well-level data

Standardization
Z transformation

Cell Count Info
Conc. > 50% cell loss

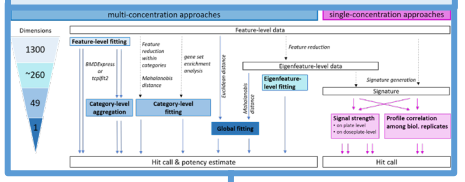
scaled well-level data

clipped well-level data



Concentration Response Modeling

Calculate Response Metrics

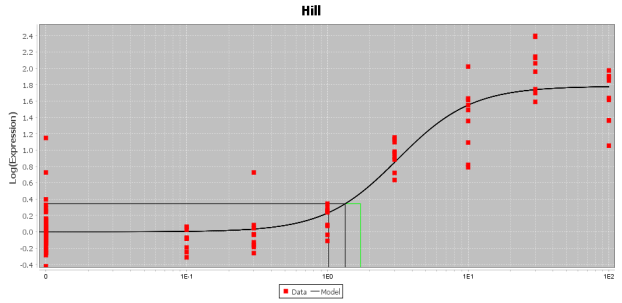


See Nyffeler et al. SLAS Discov. 2021 Feb;26(2):292-308.

Fit Multiple Curve Shapes

Best Model Selection

BMC

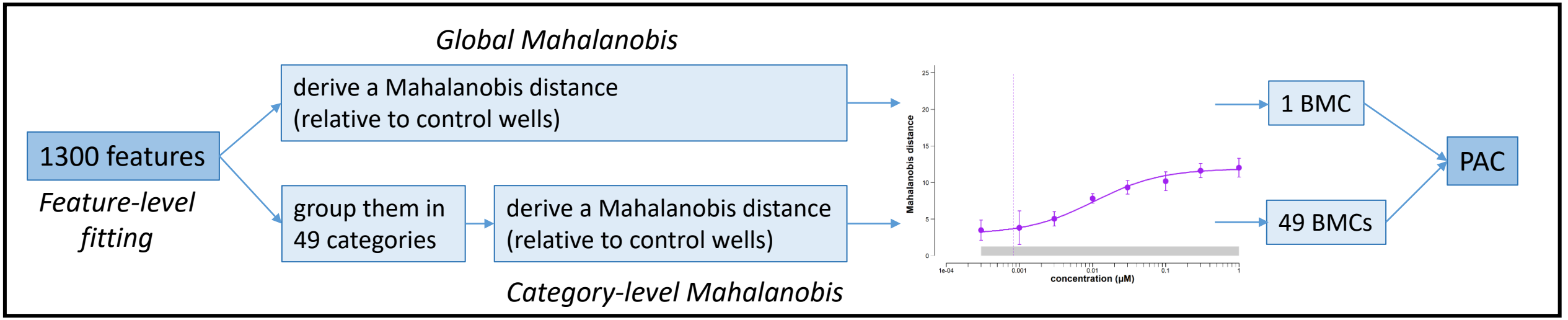


Berberine chloride
Mito_Cells_Morph_STAR

Phenotype Altering Concentrations (PACs)

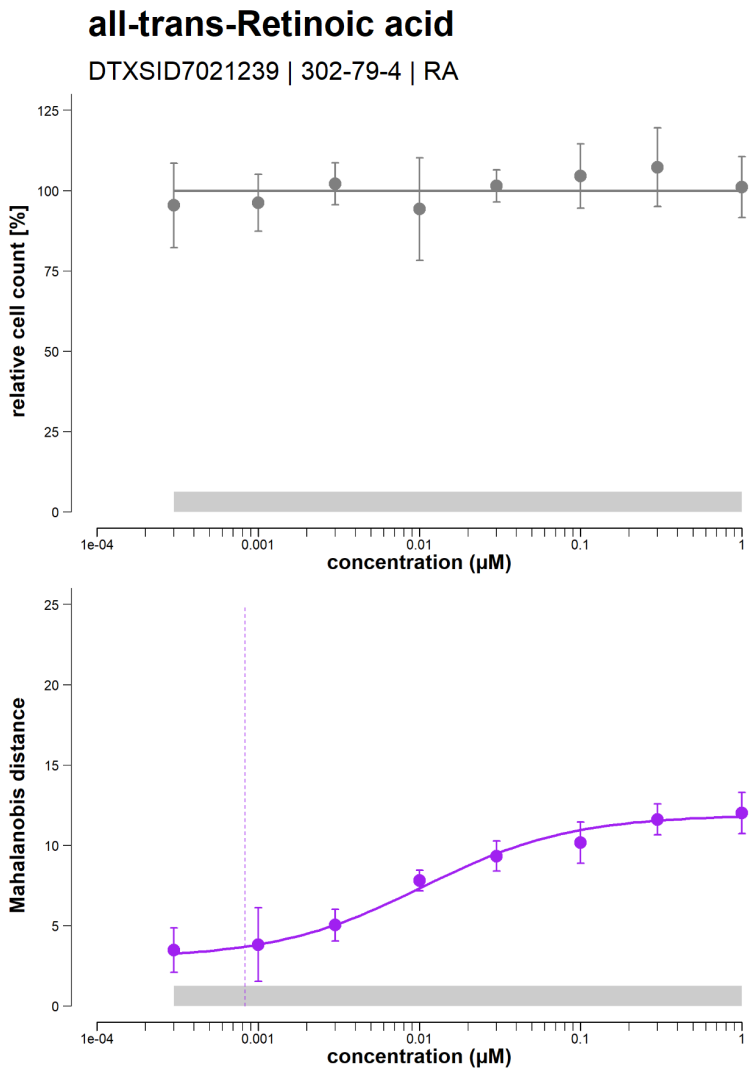
Mahalanobis Distance (D_M):

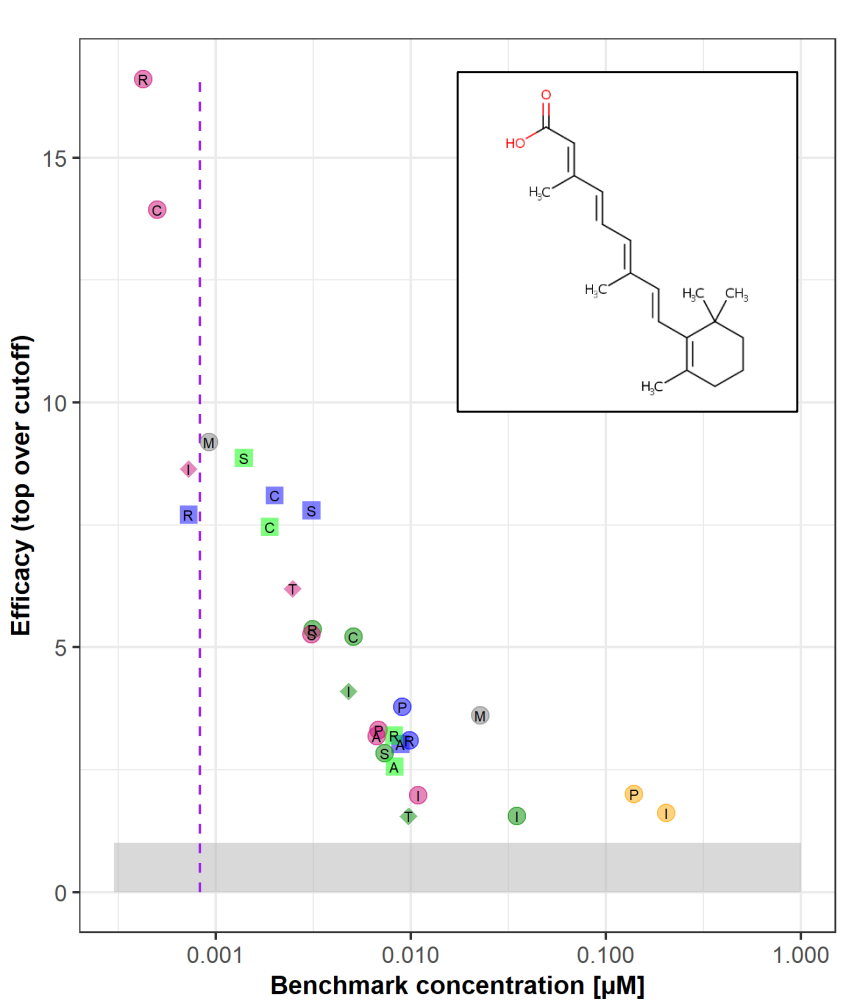
- A multivariate metric that measures the distance between a treatment and a distribution of controls in feature space.
- Accounts for unpredictable changes in cell states across test concentrations and inherent correlations in profiling data.

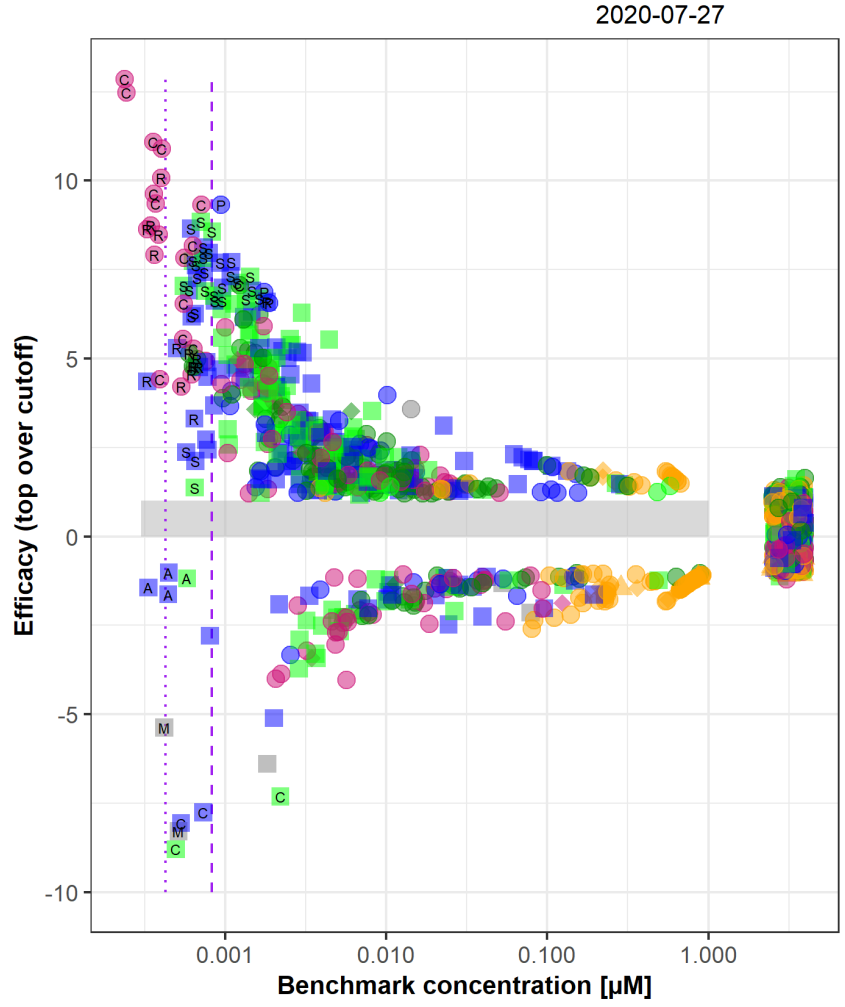


- Chemicals where a BMC can be determined using either the global or category D_M approach are considered active.
- The minimum of the global or most sensitive category BMC is the **Phenotype Altering Concentration (PAC)**

Concentration-Response Modeling Example Chemical







- Changes in cell morphology observed at concentrations below the threshold for cytotoxicity or cytostatic effects

EPA United States Environmental Protection Agency

Applications for Molecular PODs From HTP NAMs

HTP Screening Experimental Designs

Parameter	Multiplier	Notes			
Chemicals	462	APCRA case study chemicals			
Cell Types	4	U-2 OS		HepaRG-2D	MCF-7
Assay Formats	2	HTPP	HTTr	HTTr	HTTr
Exposure Durations	Variable	24 HR	24 HR	24 HR	6 HR
Concentrations:	8	3.5 log_{10} units; ~half-log_{10} spacing			
Biological Replicates:	Variable	4	3	3	3

APCRA
ACCELERATING THE PACE OF CHEMICAL RISK ASSESSMENT

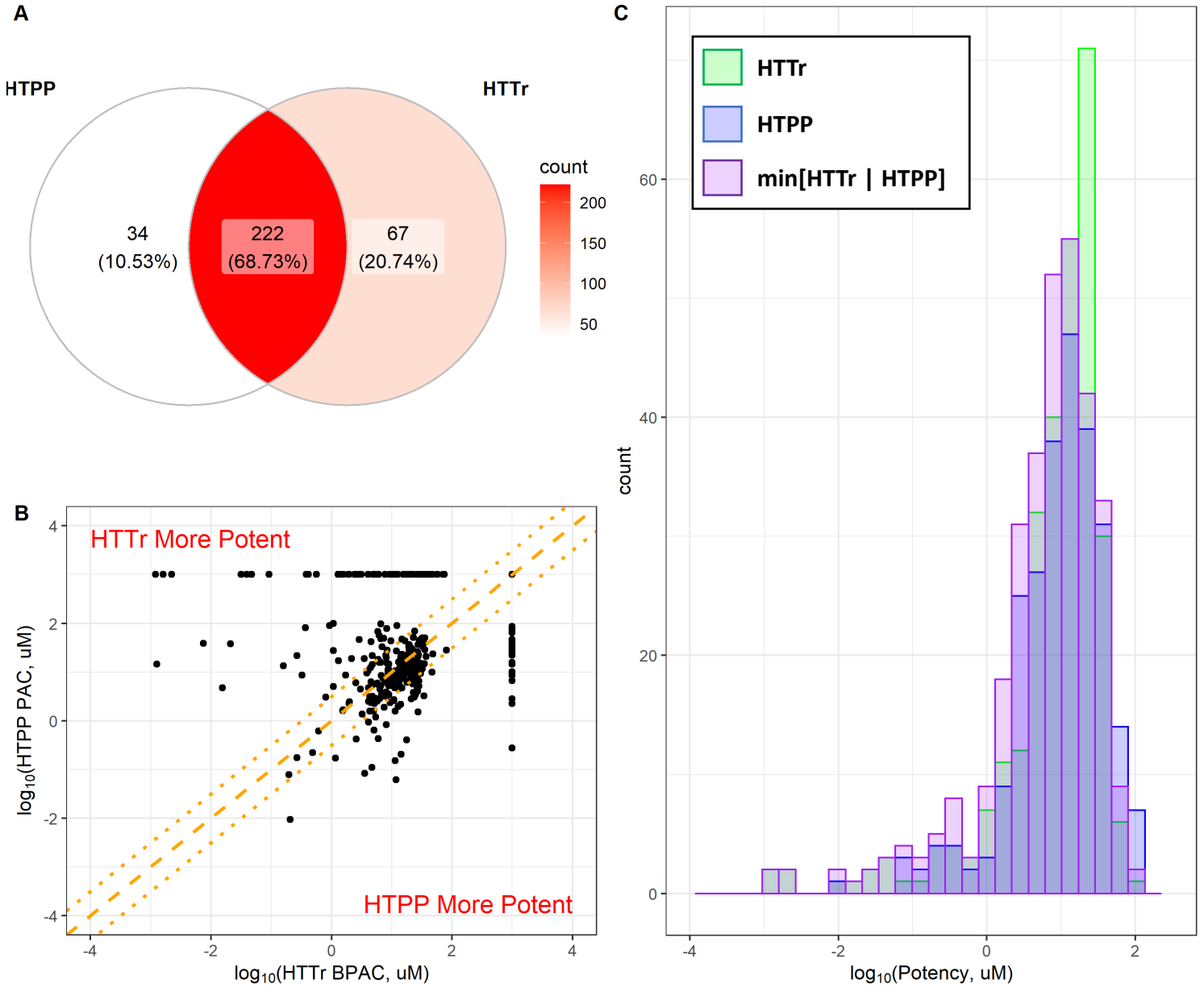
International collaboration of regulatory scientists focused on next generation chemical risk assessment including **deriving quantitative estimates of risk based on NAM-derived potency information and computational exposure estimates.**

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

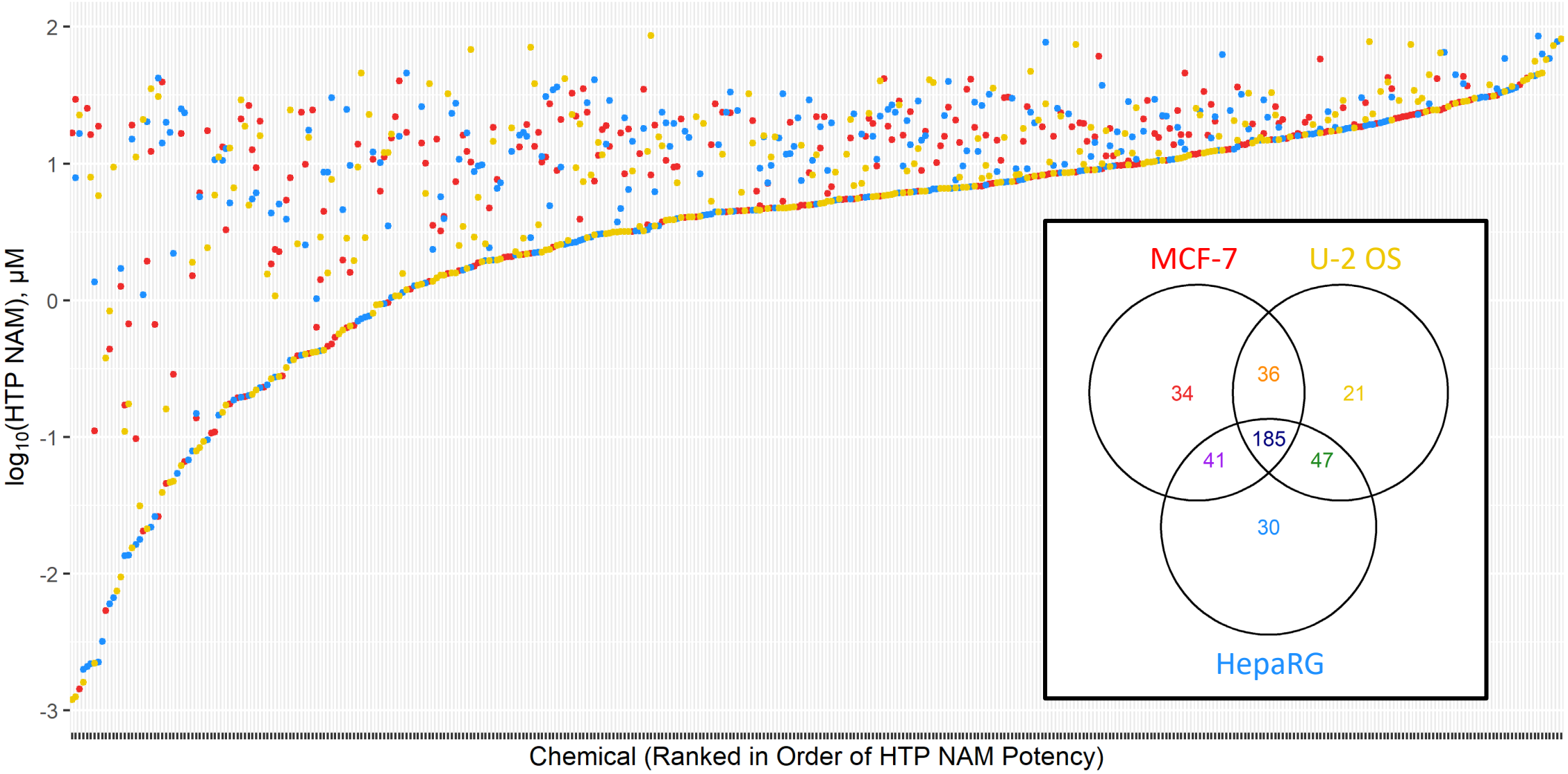
Kavlock et al. (2018)
Chem. Res. Tox; 31(5): 287-290

U-2 OS Screening Results

- A majority of chemicals were active in both the HTTr and HTPP assays.
- There were a larger number of chemicals active in HTTr only versus HTPP only.
- Most biological activity was observed between 1 and 10 uM.
- A few chemicals with HTTr PACs < 1 uM had HTPP BPACs > 10 uM or were inactive.



Comparison of Screening Results Across Cell Lines



- Molecular POD defined as the minimum potency observed in HTP NAM assays across three cell types.

In Vitro to *In Vivo* Extrapolation (IVIVE) Using High-Throughput Toxicokinetic (httk) Modeling

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)

HTP Potency Estimate (µM)

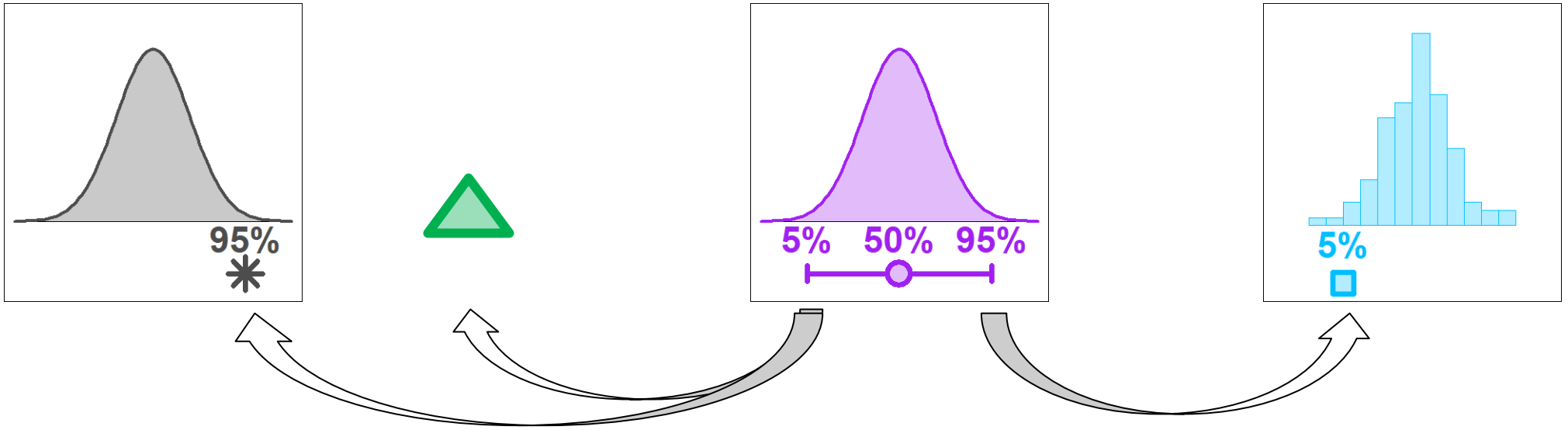
***In vitro*-to-*in vivo* extrapolation (IVIVE)**
high-throughput toxicokinetics (httk)

HTPP AED (mg/kg bw/day)

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

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

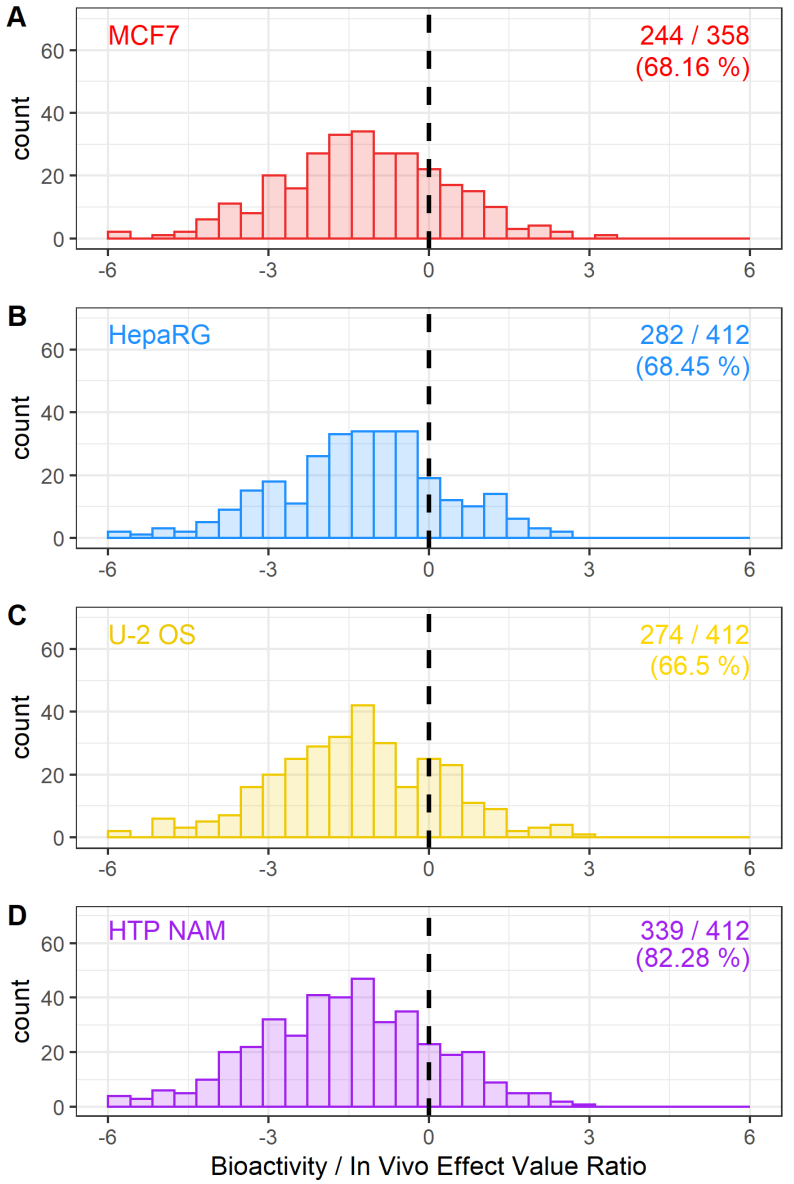
- Mammalian species
- oral exposures
- Various study types
- NOEL, LOEL, NOAEL, LOAEL
- mg/kg/day

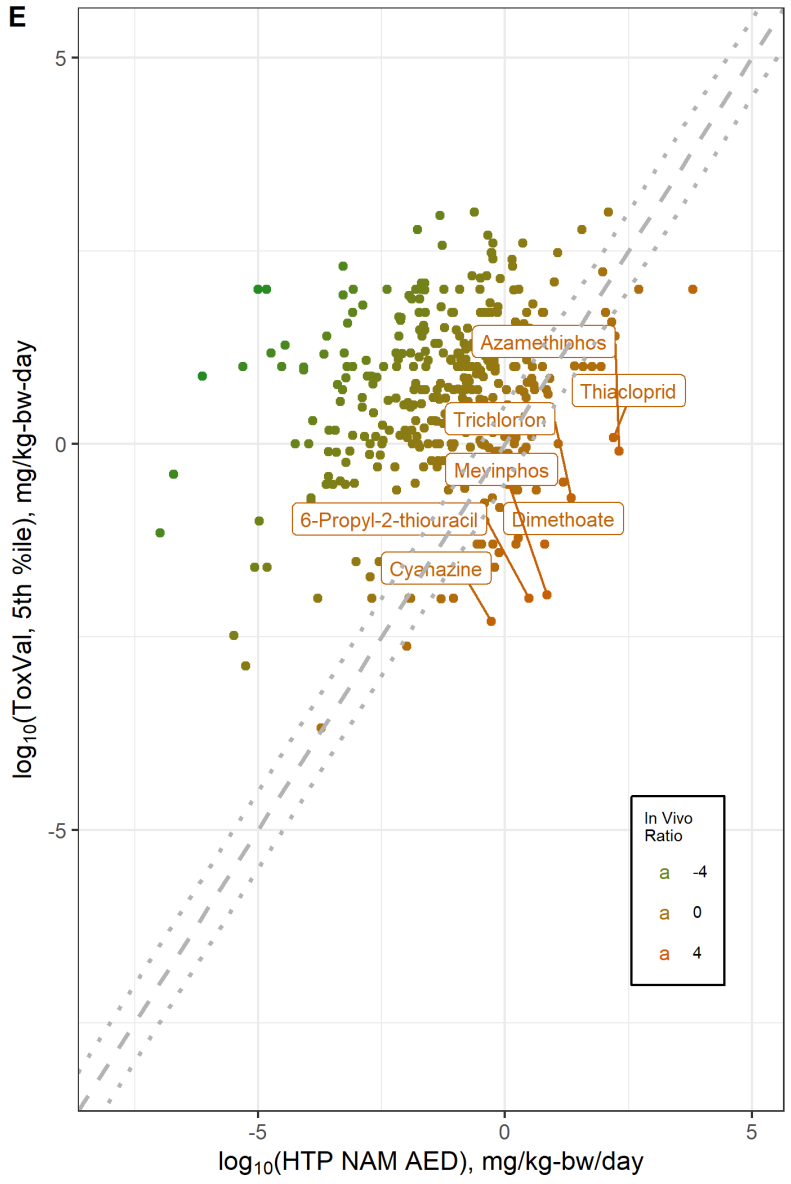


POD: point-of-departure
AED: administered equivalent dose

Bioactivity / *In Vivo* Effect Value Ratio Analysis

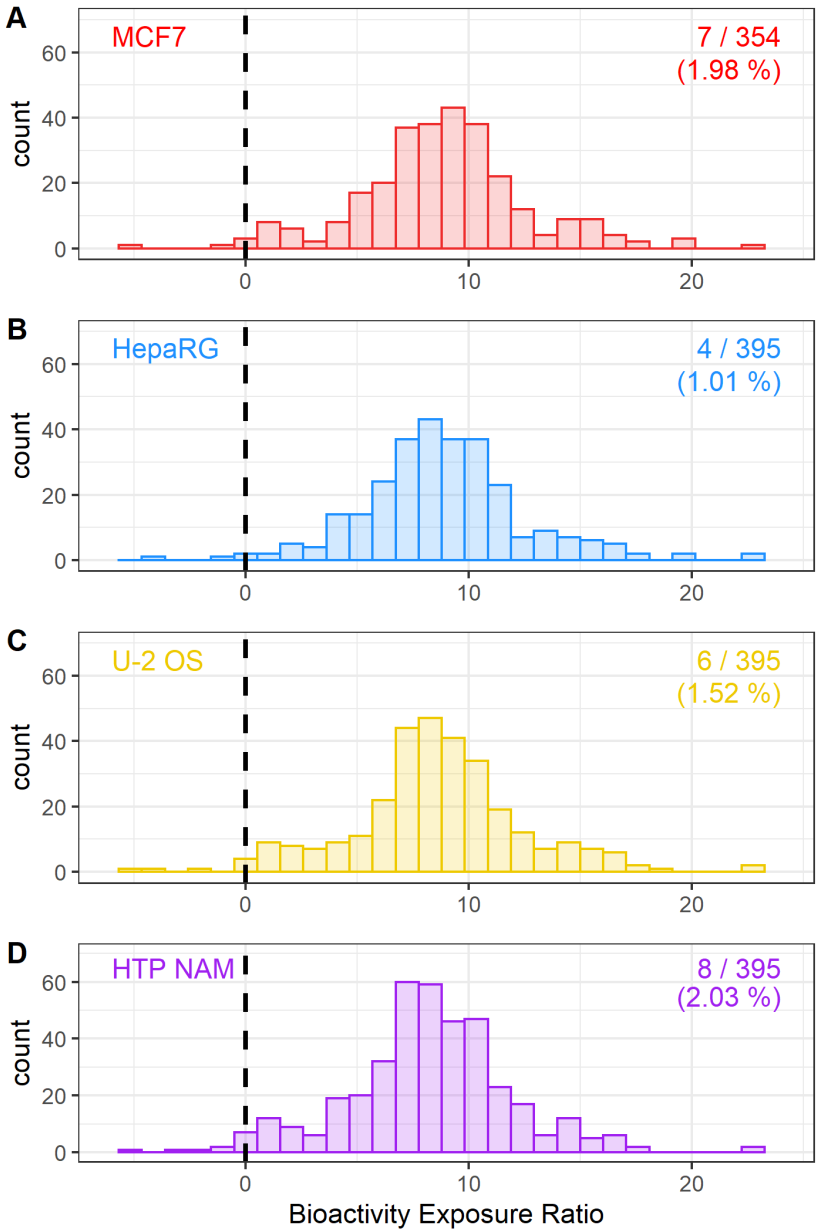
- **Negative ratios** indicate that AEDs derived from HTP NAMs molecular PODs are **conservative** surrogates for traditional *in vivo* PODs.
- When cell lines are considered individually, **~66-68%** of chemicals had negative ratios.
- When considered in combination, the number and percentage of chemicals with negative ratios **increased (82.3 %)**.
- Paul-Friedman et al. (2020)[a]:
  - Using ToxCast, **89 %** of APCRA chemicals had negative ratios.
- Positive ratios observed for several organophosphate and carbamate pesticides.

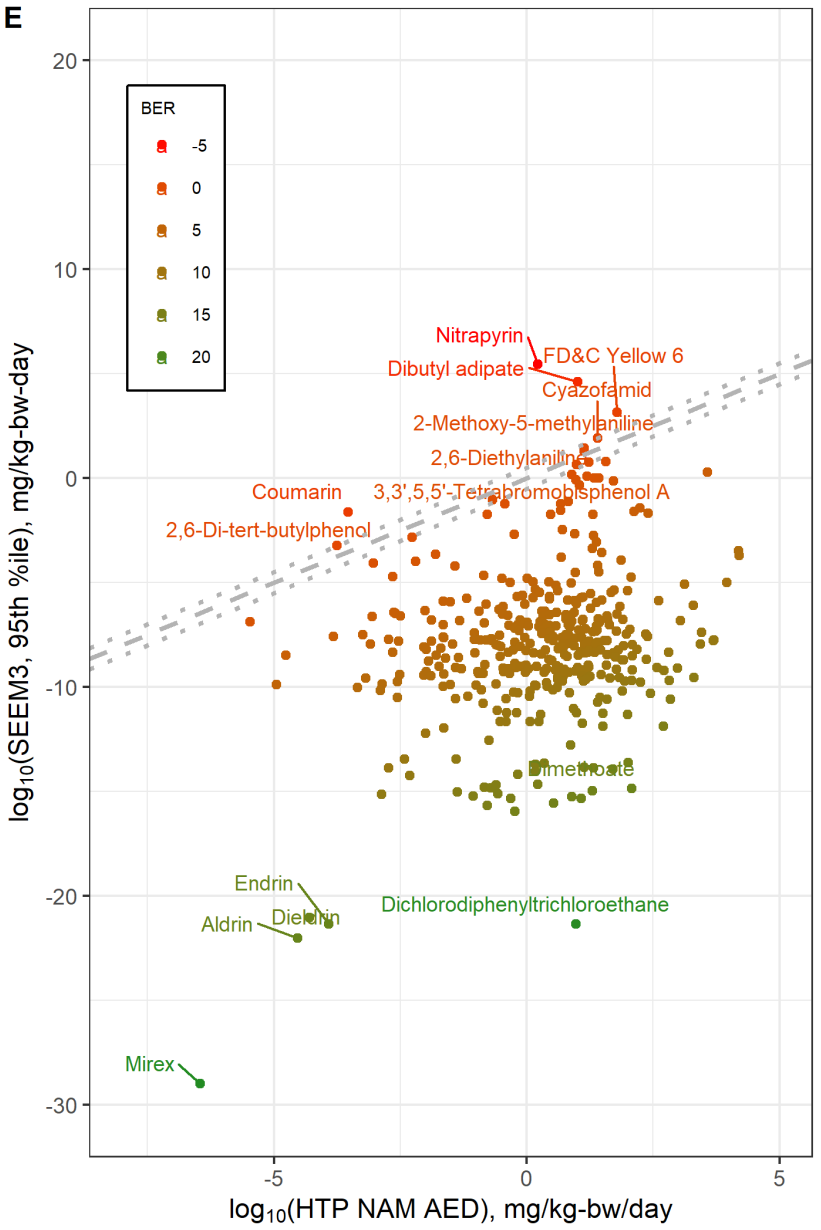




Bioactivity Exposure Ratio (BER) Analysis

- **Negative ratios** indicate a potential for human exposure to chemicals in a range that is bioactive in vitro.
- When cell lines are considered individually, **~1-2%** of chemicals had negative ratios.
- When considered in combination, the percentage of chemicals with negative ratios **did not appreciably change**.
- Positive ratios observed for several chemicals found in consumer products.
- Most extreme negative ratios associated with banned or limited use organochlorine pesticides.





Summary and Conclusions

- **High-Throughput Profiling:** Developed experimental designs and scalable laboratory workflows for high-throughput transcriptomics and high-throughput phenotypic profiling of environmental chemicals that can be used in multiple human-derived cell types.
- **Potency Estimation:** Developed high-throughput concentration-response modeling workflows to identify thresholds for perturbation of gene expression (e.g. BPACs) and cell morphology (e.g. PACs).
- **IVIVE:** Potency estimates can be converted to administered equivalent doses (AEDs) using high-throughput toxicokinetic modeling.
- **Bioactivity to *In Vivo* Effect Value Ratio Analysis:** AEDs derived from HTP assays were conservative compared to traditional PODs a majority of the time. Performance improved to ~80% when results from multiple cell types were considered in combination.
- **Bioactivity to Exposure Ratio (BER) Analysis:** AEDs derived from HTP assays were compared to high-throughput exposure predictions. There were very few chemicals where AEDs were within the range of exposure predictions.
- **Comparison to ToxCast:** Applications using HTP NAMs potencies as input yielded comparable results compared to the use of ToxCast NAMs potencies.

Acknowledgements

Office of Research and Development (ORD)
Center for Computational Toxicology and Exposure (CCTE)

- **Johanna Nyffeler**
- Clinton Willis
- Rick Brockway
- Megan Culbreth
- Dan Hallinger
- Terri Fairley

- Ann Richard
- Kathy Coutros
- Maureen Gwinn
- Russell Thomas

- Logan Everett
- Imran Shah
- Richard Judson
- Derik Haggard
- Thomas Sheffield
- Joseph Bundy
- Woody Setzer

- Katie Paul-Friedman
- John Wambaugh

PerkinElmer

- Joe Trask
- Dana Hanes
- Jim Hostetter

National Toxicology Program
U.S. Department of Health and Human Services

- Scott Auerbach

BioSpyder™

- Jo Yeakley
- Bruce Seligmann
- Joel McComb
- Pete Shepherd
- Milos Babic
- Dalia Gonzalez
- Kyle LeBlanc
- Garrett McComb

Publications

- ## Computational Toxicology
  - **Thomas RS,** Bahadori T, Buckley TJ, Cowden J, Deisenroth C, Dionisio KL, Frithsen JB, Grulke CM, Gwinn MR, Harrill JA, Higuchi M, Houck KA, Hughes MF, Hunter ES, Isaacs KK, Judson RS, Knudsen TB, Lambert JC, Linnenbrink M, Martin TM, Newton SR, Padilla S, Patlewicz G, Paul-Friedman K, Phillips KA, Richard AM, Sams R, Shafer TJ, Setzer RW, Shah I, Simmons JE, Simmons SO, Singh A, Sobus JR, Strynar M, Swank A, Tornero-Valez R, Ulrich EM, Villeneuve DL, Wambaugh JF, Wetmore BA, Williams AJ. The Next Generation Blueprint of Computational Toxicology at the U.S. Environmental Protection Agency. Toxicol Sci. 2019 Jun 1;169(2):317-332. doi: 10.1093/toxsci/kfz058. PMID: 30835285
  - **Paul Friedman K,** Gagne M, Loo LH, Karamertzanis P, Netzeva T, Sobanski T, Franzosa JA, Richard AM, Lougee RR, Gissi A, Lee JJ, Angrish M, Dorne JL, Foster S, Raffaele K, Bahadori T, Gwinn MR, Lambert J, Whelan M, Rasenberg M, Barton-Maclaren T, Thomas RS. Utility of In Vitro Bioactivity as a Lower Bound Estimate of In Vivo Adverse Effect Levels and in Risk-Based Prioritization. Toxicol Sci. 2020 Jan 1;173(1):202-225. doi: 10.1093/toxsci/kfz201. PMID: 31532525.

- ## High-Throughput Transcriptomics (HTTr):
  - **Harrill J,** Shah I, Setzer RW, Haggard D, Auerbach S, Judson R, Thomas RS. Considerations for Strategic Use of High-Throughput Transcriptomics Chemical Screening Data in Regulatory Decisions. Curr Opin Toxicol. 2019;15:64-75. doi: 10.1016/j.cotox.2019.05.004. PMID: 31501805.
  - **Harrill JA**, Everett LJ, Haggard DE, Sheffield T, Bundy J, Willis CM, Thomas RS, Shah I, Judson RS. High-Throughput Transcriptomics Platform for Screening Environmental Chemicals. Toxicol Sci. 2021 Feb 4:kfab009. doi: 10.1093/toxsci/kfab009. Epub ahead of print. PMID: 33538836.

- ## High-Throughput Phenotypic Profiling (HTPP)
  - **Nyffeler J,** Willis C, Lougee R, Richard A, Paul-Friedman K, Harrill JA. Bioactivity screening of environmental chemicals using imaging-based high-throughput phenotypic profiling. Toxicol Appl Pharmacol. 2020 Jan 15;389:114876. doi: 10.1016/j.taap.2019.114876. Epub 2019 Dec 30. PMID: 31899216.
  - **Willis C**, Nyffeler J, Harrill J. Phenotypic Profiling of Reference Chemicals across Biologically Diverse Cell Types Using the Cell Painting Assay. SLAS Discov. 2020 Aug;25(7):755-769. doi: 10.1177/2472555220928004. Epub 2020 Jun 17. PMID: 32546035.
  - **Nyffeler J**, Haggard DE, Willis C, Setzer RW, Judson R, Paul-Friedman K, Everett LJ, Harrill JA. Comparison of Approaches for Determining Bioactivity Hits from High-Dimensional Profiling Data. SLAS Discov. 2021 Feb;26(2):292-308. doi: 10.1177/2472555220950245. Epub 2020 Aug 29. PMID: 32862757.