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High-Throughput Chemical Screening Using High-Content Profiling Assays: Applications for Next Generation Risk Assessment

Joshua A. Harrill

USEPA Center for Computational Toxicology and Exposure (CCTE)

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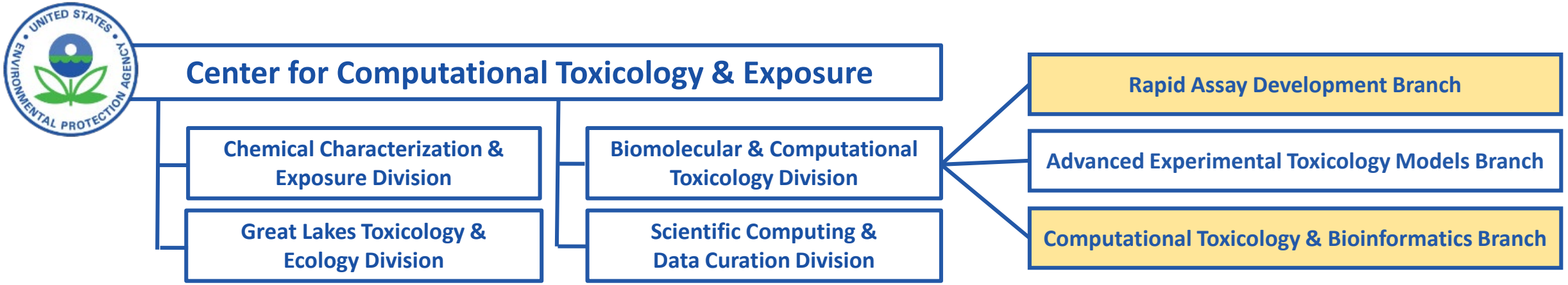
Outline

- **Background**
  - What is CCTE?
  - What does CCTE Do?
  - USEPA Computational Toxicology Blueprint
- **High-Throughput Profiling Assays**
  - High-Throughput Transcriptomics (HTTr) → TempO-Seq
  - High-Throughput Phenotypic Profiling (HTPP) → Cell Painting
- **Potential Applications for Next Generation Risk Assessment**
  - Bioactivity-to-Exposure Ratio (BER) Analysis
  - Profile Similarity for Chemical Read-Across

Who is CCTE ?

A research organization tasked with **developing** and **applying** cutting edge innovations in methods to rapidly evaluate chemical's toxicity, transport and exposure to people and environments.



Rapid Assay Development Branch (RADB)

Develops the next generation of **high-throughput toxicity assays** to comprehensively cover the potential **molecular and phenotypic responses** resulting from chemical exposure and **fill gaps** in biological pathways and processes not addressed using existing assays.

Computational Toxicology & Bioinformatics Branch (CTBB)

Utilizes computational and informatics approaches to **analyze** and **integrate** data from high-throughput toxicity assays, complex culture models, alternative species, toxicokinetics and chemistry to predict adverse effects of chemicals in humans and animal models.

Computational Toxicology Research Areas

CCTE research programs focus on developing the **tools, approaches and data** needed to accelerate the pace of chemical risk assessment and foster incorporation of non-traditional toxicity testing data into regulatory decision-making processes.

- **ToxCast:** Use of targeted high-throughput screening (HTS) assays to expose living cells or isolated proteins to chemicals and assess bioactivity and potential toxic effects.

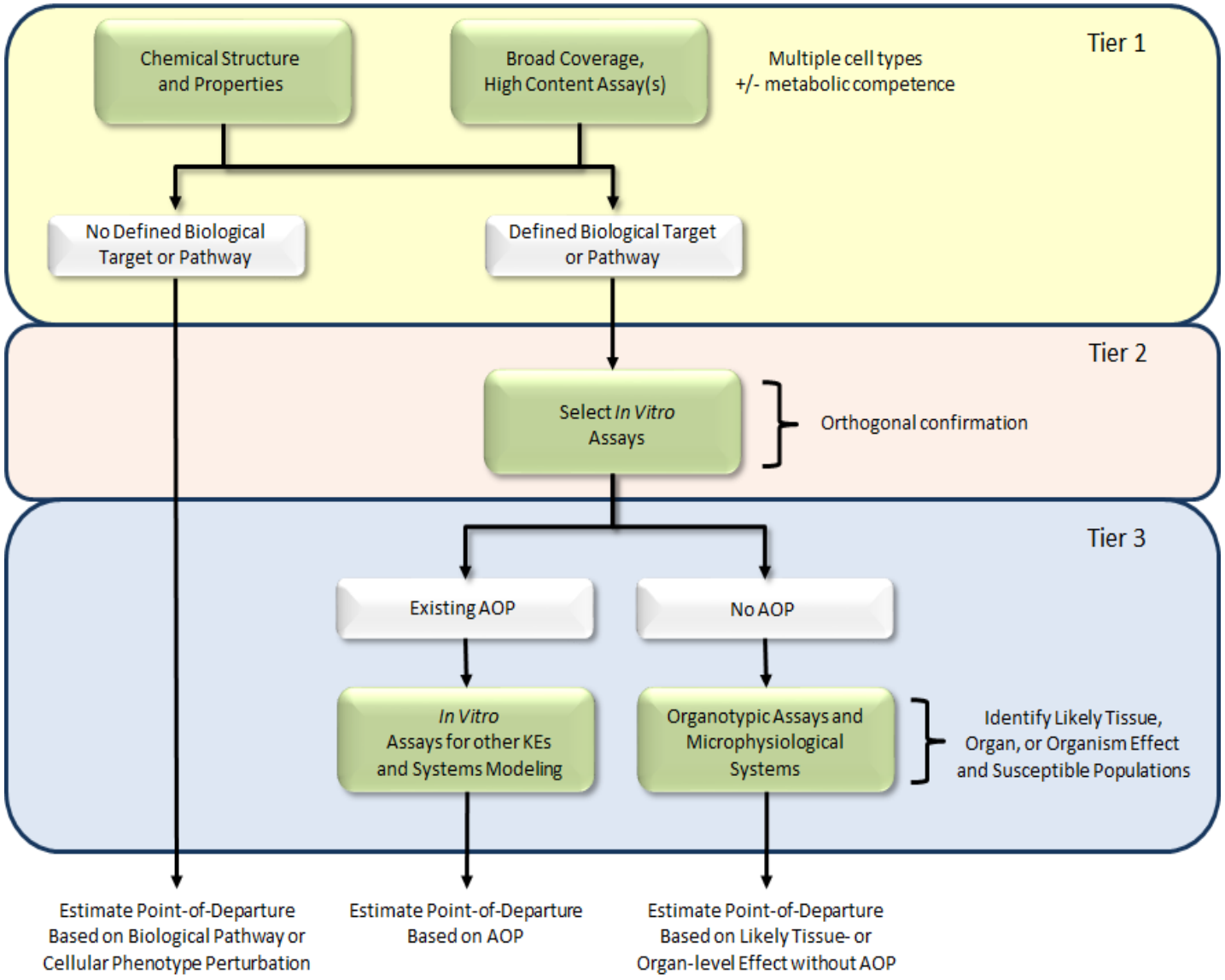
	# of assays	# of chemicals	Types of chemicals
Phase 1 *(2007 – 2009)*	500	300	Mostly pesticides
Phase 2 *(2009 – 2013)*	700	2,000	Industrial, consumer product, food use, "green"

- Mostly targeted assays (*chemical X → target Y*)
- Incomplete coverage of biological space.

- **New Approach for Hazard Evaluation:** Employ broad-based (i.e. non-targeted) **profiling assays** that cast the broadest net possible for capturing the potential molecular and phenotypic responses of human cells in response to chemical exposures.

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

Tiered Hazard Evaluation Approach



- Increasing efficiency and declining cost of generating whole transcriptome profiles has made **high-throughput transcriptomics (HTTr)** a practical option for *in vitro* chemical screening.
  - **Whole Transcriptome TempO-Seq**
- Imaging-based **high-throughput phenotypic profiling (HTPP)** provides a cost-effective means for characterizing the effects of chemicals on apical cellular morphology (i.e. cellular pathology).
  - **Cell Painting**
- Both methods are **complementary** to each other and can be used in **human-derived** *in vitro* models.
- The resulting bioactivity profiles can potentially be used for **potency estimation**, **mechanistic prediction** and evaluation of **chemical similarity**.

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

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High-Throughput Transcriptomics (HTTr)

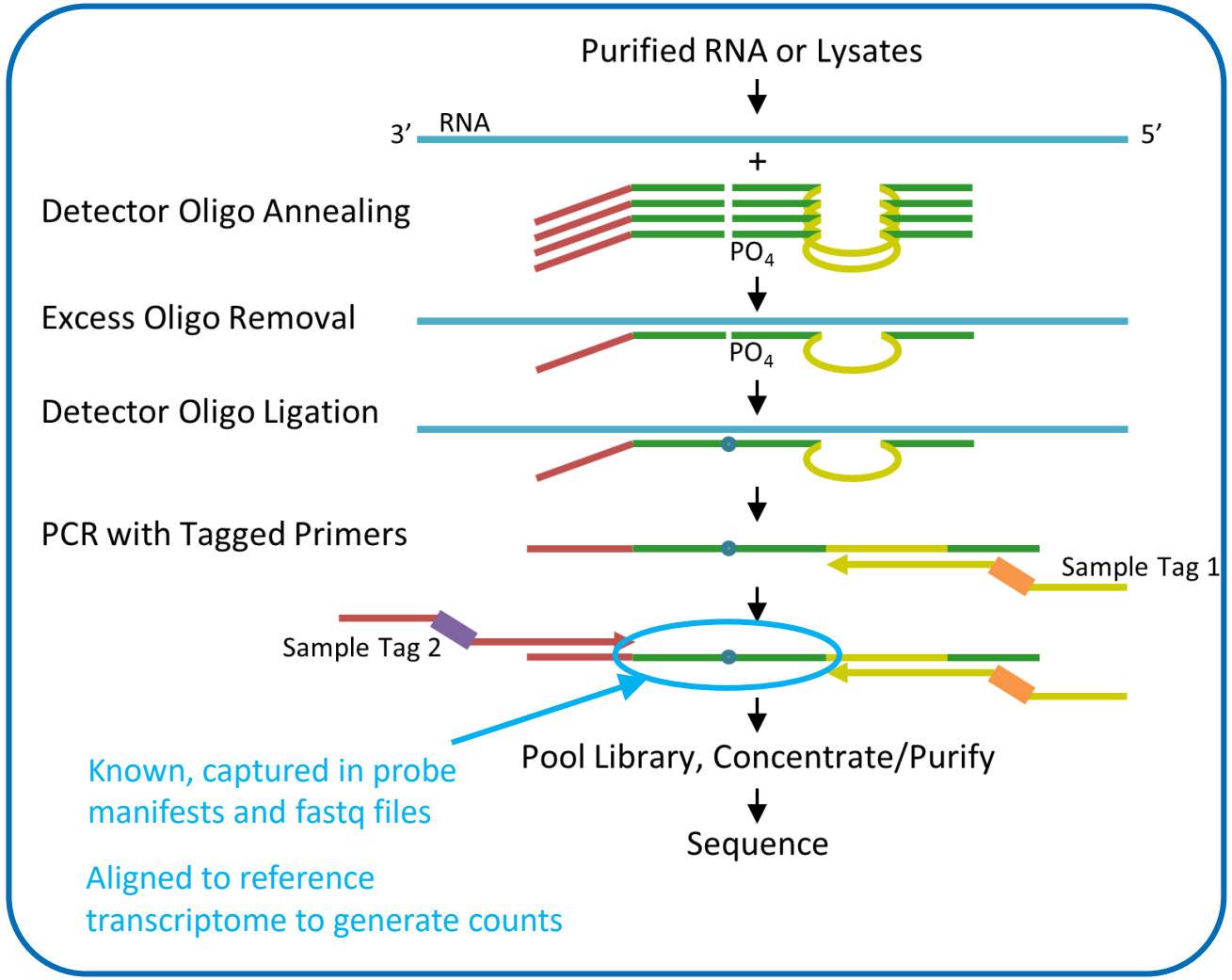
Whole Transcriptome TempO-Seq

Templated Oligo with Sequencing Readout (TempO-Seq)

Technology

- 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 generated in 384-well format are barcoded according to well position 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

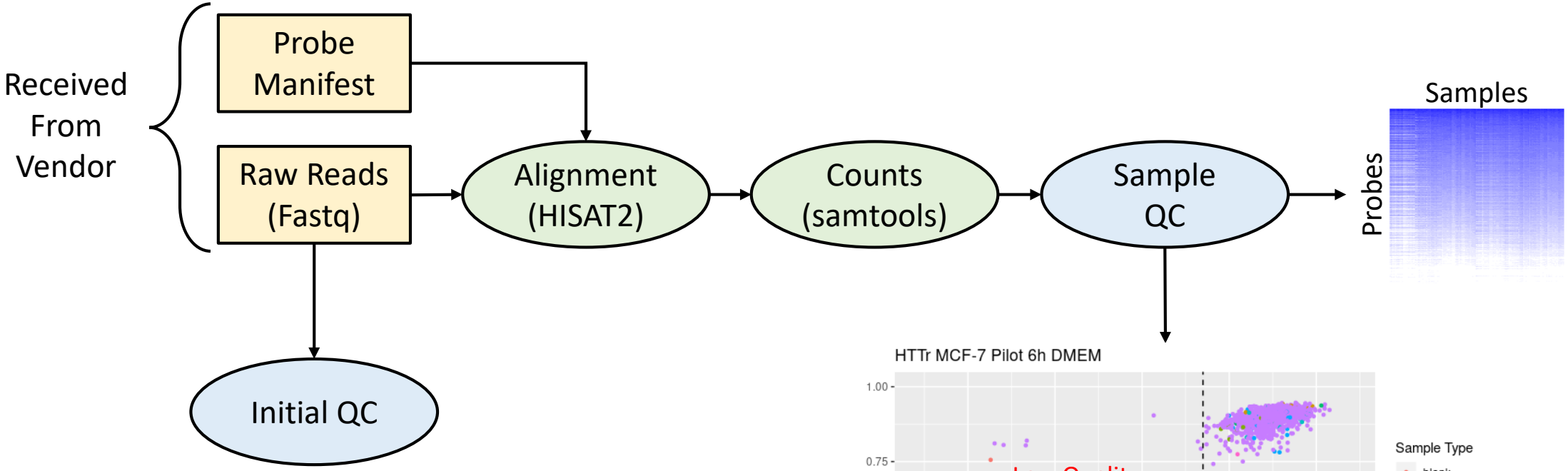
Slide courtesy of Logan Everett

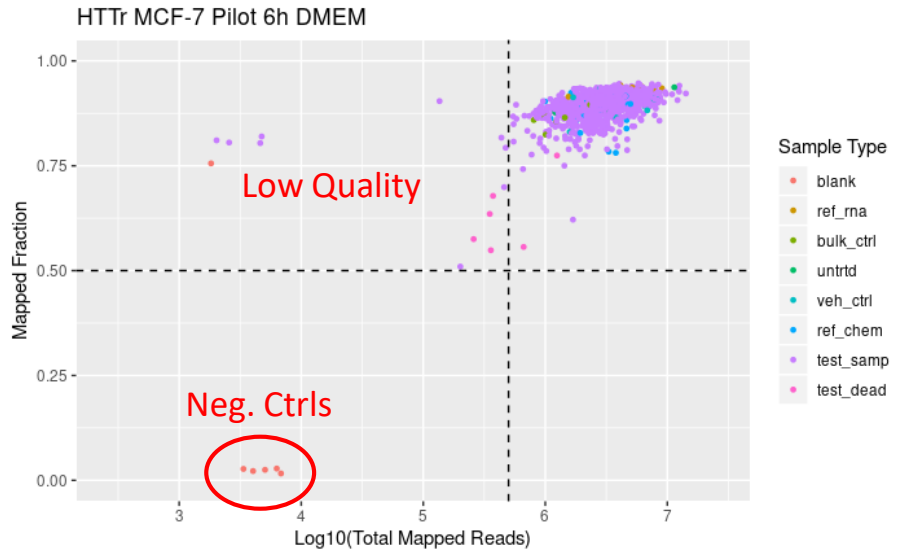
HTTr MCF-7 Screen: Experimental Design

Parameter	Multiplier	Notes
Cell Type(s)	1	MCF-7
Culture Condition	1	DMEM + 10% HI-FBS [a]
Chemicals	2,112	ToxCast ph1, ph2 Nominated chemicals from e1k / ph3
Time Points:	1	6 hours
Assay Formats:	2	TempO-Seq HCI Cell Viability & Apoptosis
Concentrations:	8	3.5 $\log_{10}$ units; ~half-$\log_{10}$ spacing
Biological Replicates:	3	--

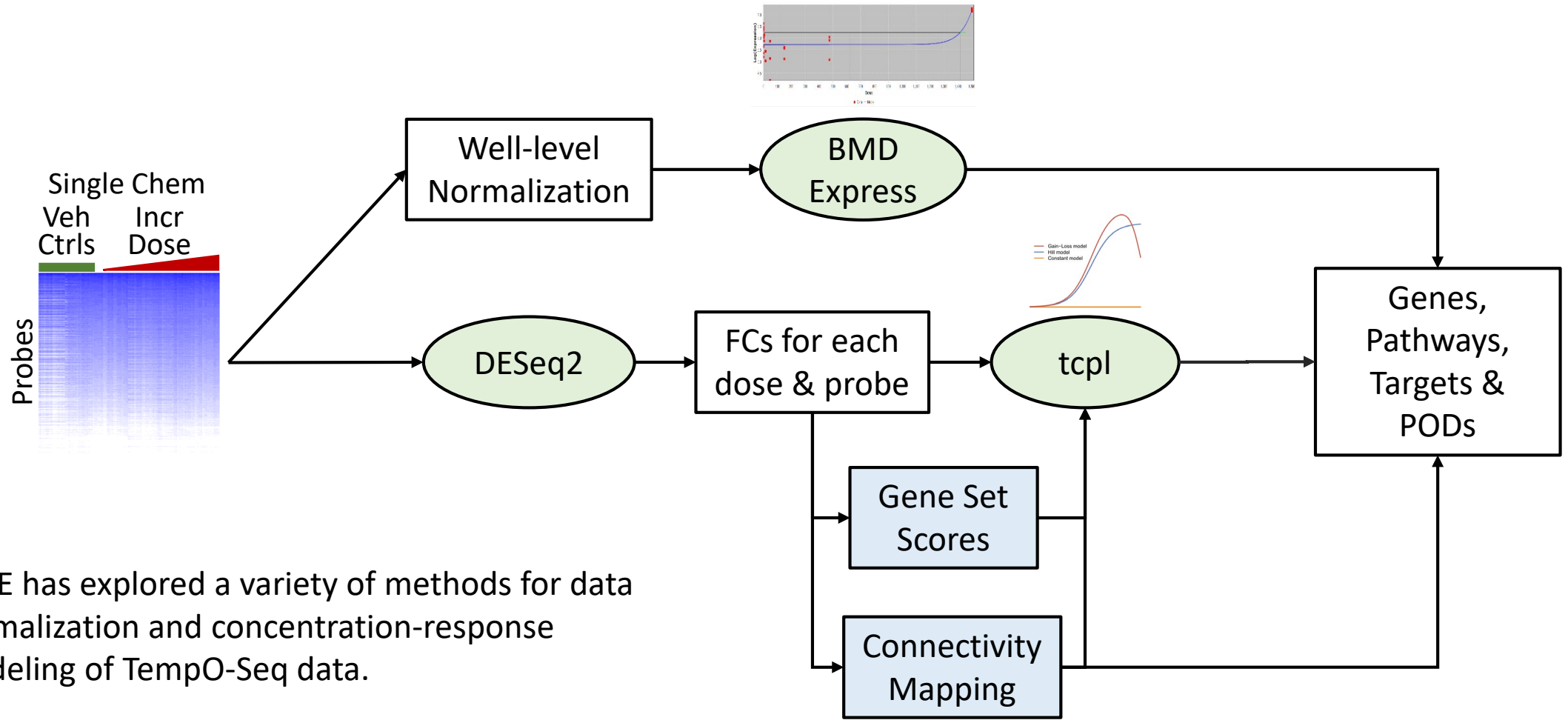
[a] MCF7 cells cultured in DMEM + 10% HI-FBS was selected as the test system to facilitate comparability to the Broad Institute Connectivity Map (CMAP) database (http://portals.broadinstitute.org/cmap/).

HTTr Pipeline: Raw Data Processing





HTTr Pipeline: Targets & Concentration Response



- CCTE has explored a variety of methods for data normalization and concentration-response modeling of TempO-Seq data.
- Focus on one today → **Gene Set Scoring Analysis**

Gene Set Scoring Analysis Overview (1)

Step 1: Inputs

Chemical_Conc × Gene matrix of $\log_2$ (fold-change) (l2fc) values.

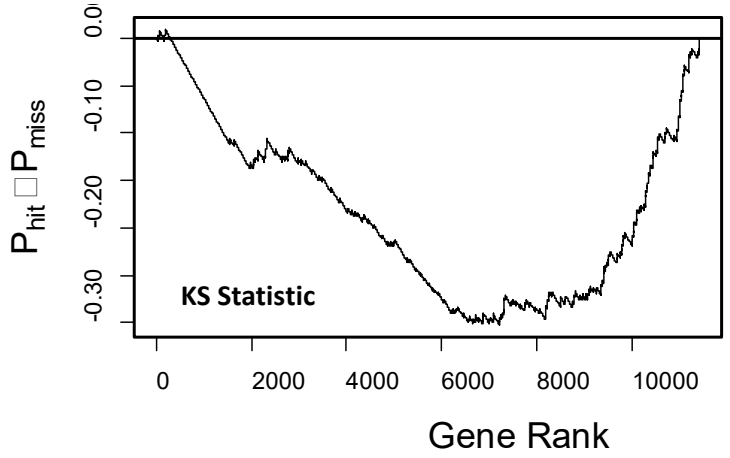
Collections of gene sets:

- MSigDB & BioPlanet "Response" (*n = 500*)
- Ryan et al. estrogen up/down (*n = 2*)
- MSigDB & BioPlanet "Random" (*n = 500*)
- CMAP gene sets for annotated chems (*n = 1775*)

Step 2: Pathway Scoring

FC } mean ($l2fc_{in}$) – mean ($l2fc_{out}$)

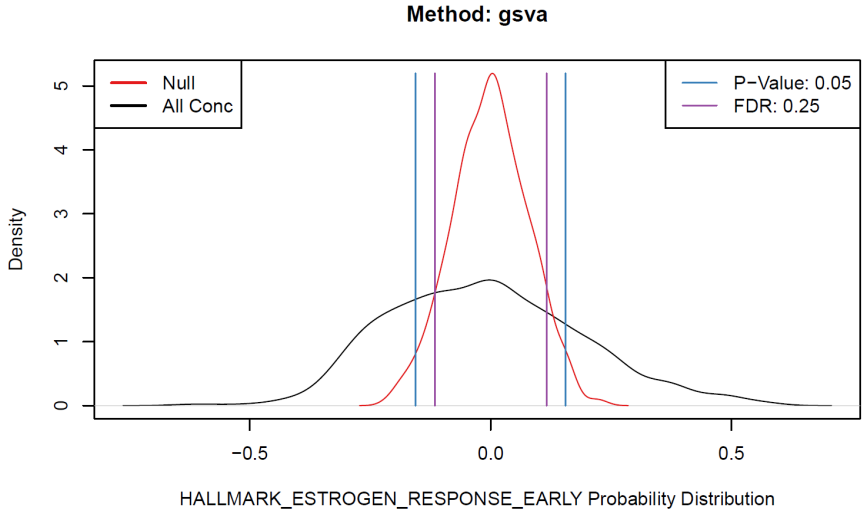
ssGSEA } scores based on K-S statistic



Chemical_Conc × Gene Set 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 **gene set scores for "null" data**
  - One null distribution (n = 1000 scores) / gene set



Prelimnary Analysis by Thomas Sheffield and Richard Judson

Gene Set Scoring Analysis Overview (2)

Step 4: Concentration-Response Modeling

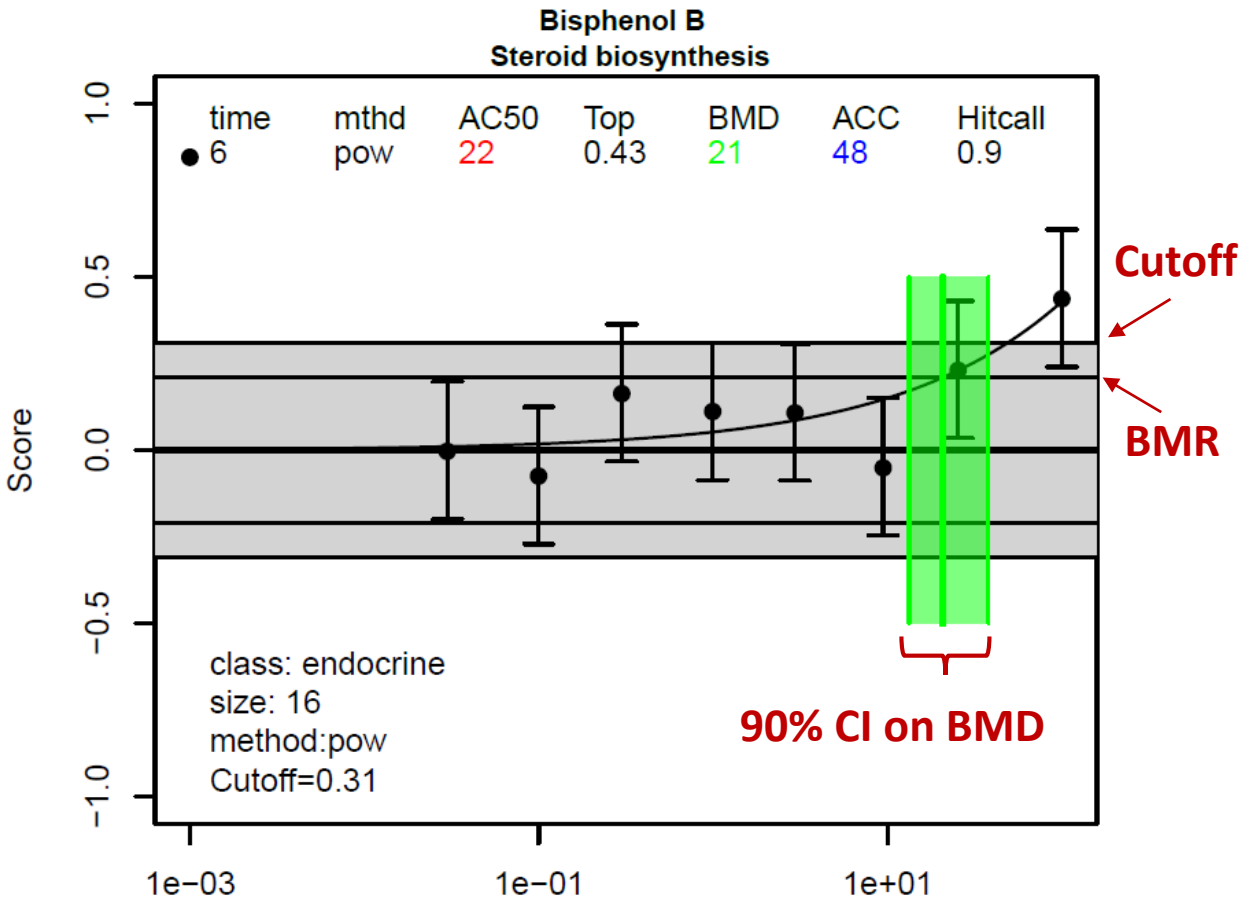
tcplFit2 fitting
- Uses maximum likelihood estimation
- Error modeled as 4 DF t-distribution

Models
- Constant, exp 2|3|4|5, hill, gnls, poly 1|2, power
- Winner → Lowest AIC

Benchmark Response
- 1.349*sd of NULL

Confidence Bounds
- Likelihood ratio test for 90% CI to give bounds

Continuous Hit Calls
- Multiply the following
  - Odds of at least one conc. > cutoff
  - Odds of top above cutoff using likelihood ratios
  - Winning model Akaike Weight (relative to constant)



Preliminary Analysis by Thomas Sheffield and Richard Judson

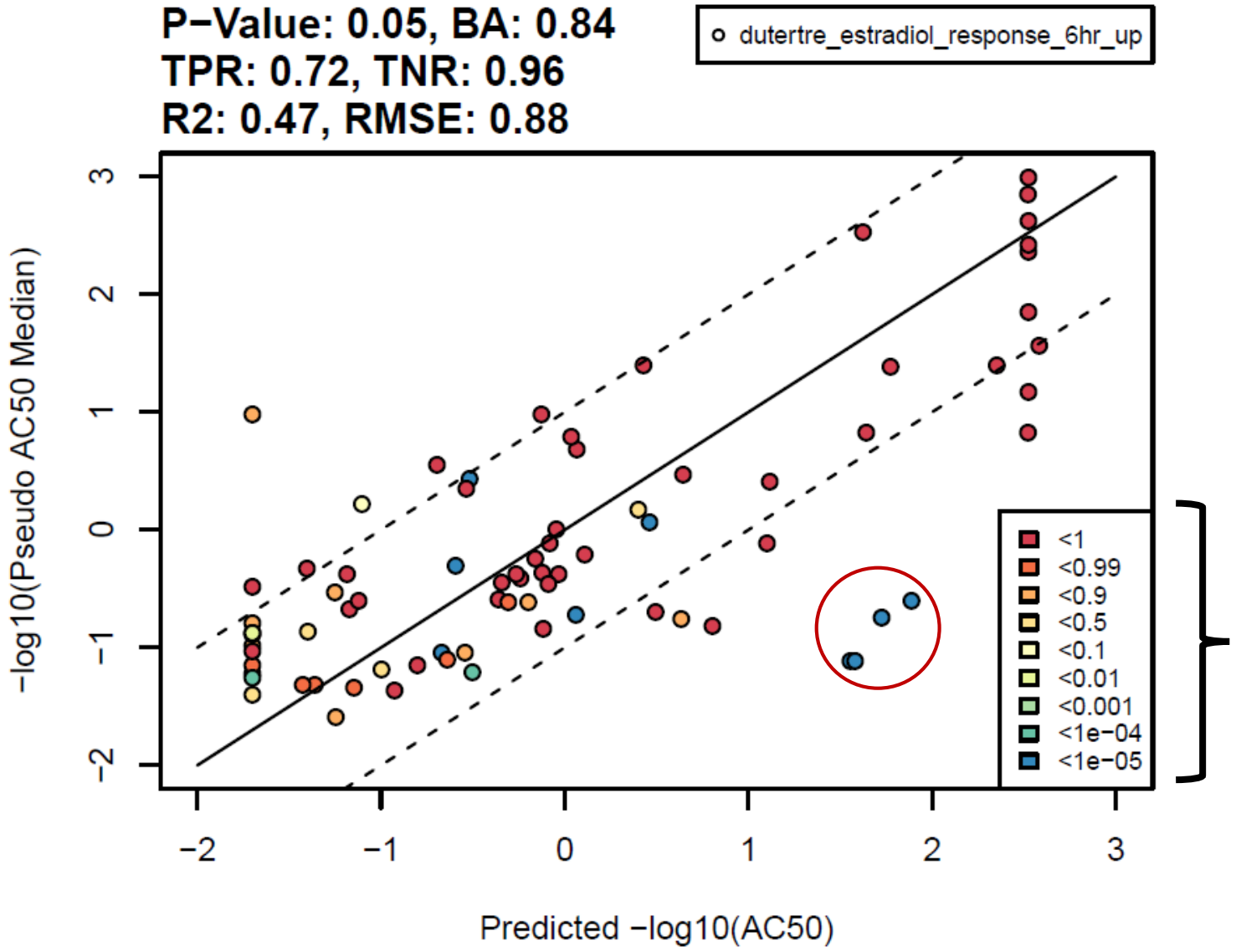
Comparison of Gene Set Scoring Analysis to ToxCast (1)

- EPA model of estrogen receptor (ER) activity uses 18 *in vitro* (ToxCast) assays.
- Provides a "pseudo AC_{50}", essentially a median estimate of potency.
- ER gene set potency in HTTR dataset should be correlated with ToxCast ER potency.

Approach:

- Select equal number of estrogen active & inactive chemicals according to ToxCast model.
- Focus on estrogen receptor activation gene set relevant to screening conditions
  - *MSigDB → Duertre Estradiol 6 HR UP*
- Compare at continuous and discrete levels:
  - *Balanced accuracy, RMSE, R^2 weighted by hitcall*

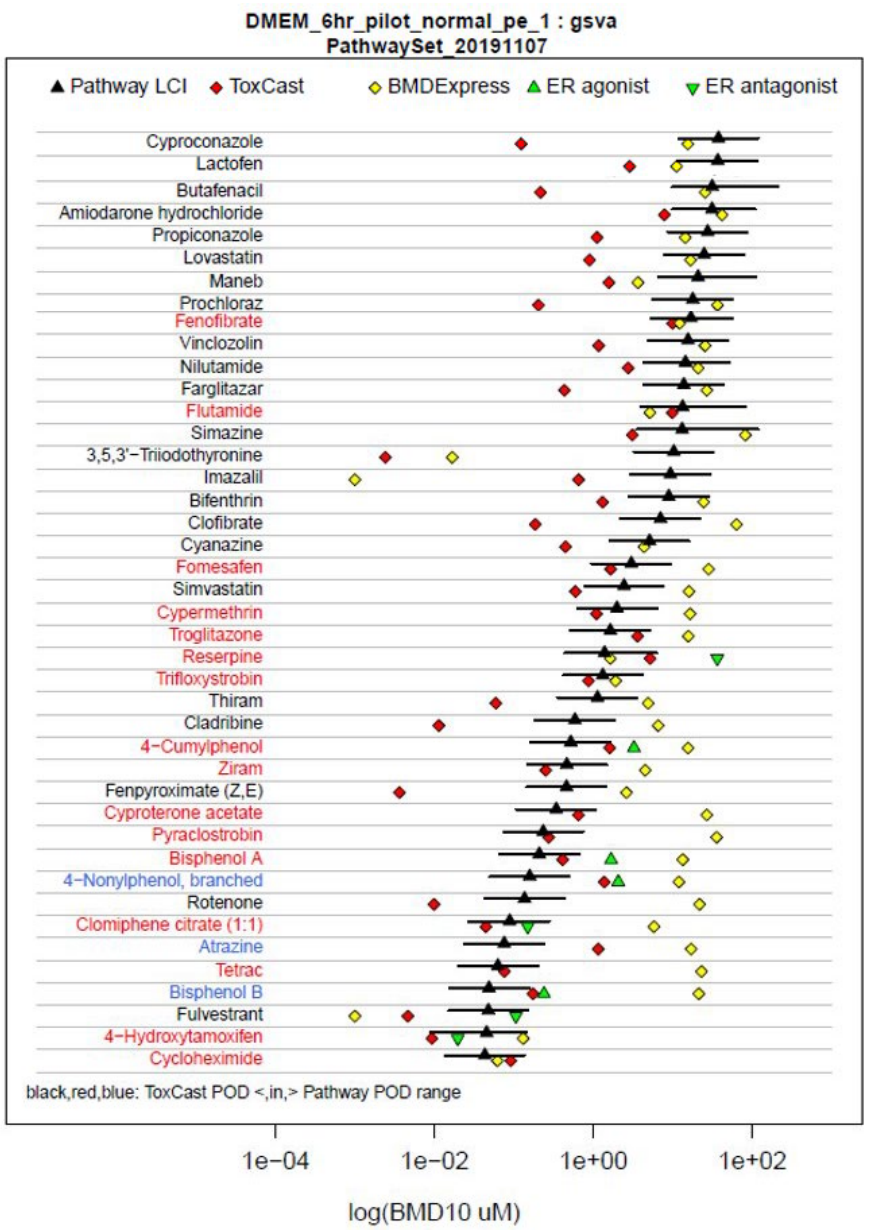
Comparison of Gene Set Scoring Analysis to ToxCast (2)

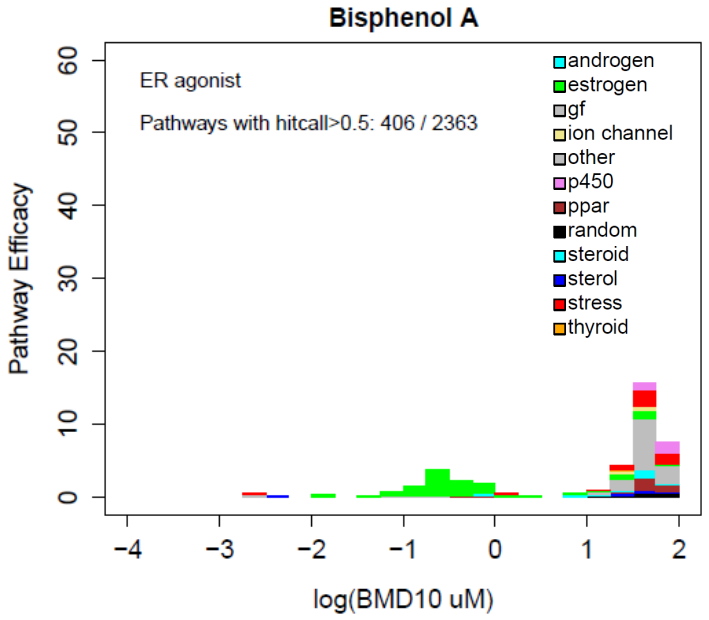


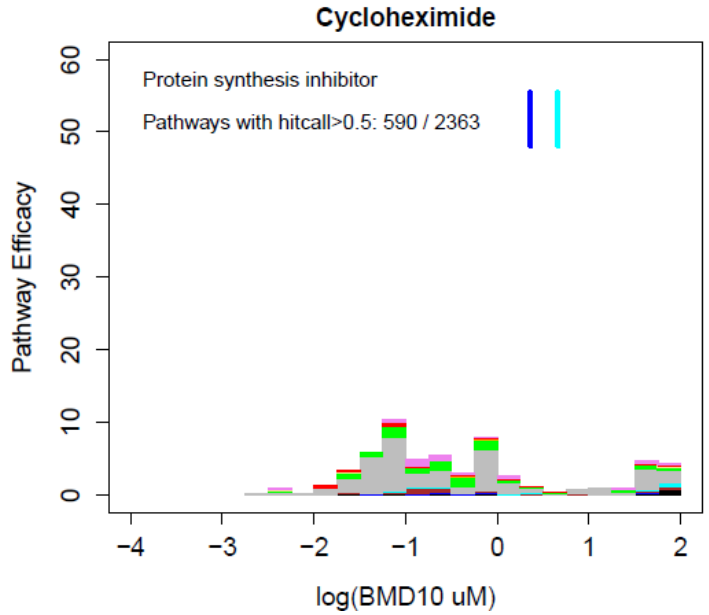
Continuous hit calls with tcplFit2

Preliminary Analysis by Thomas Sheffield and Richard Judson

Comparison of Gene Set Scoring Analysis to ToxCast (3)

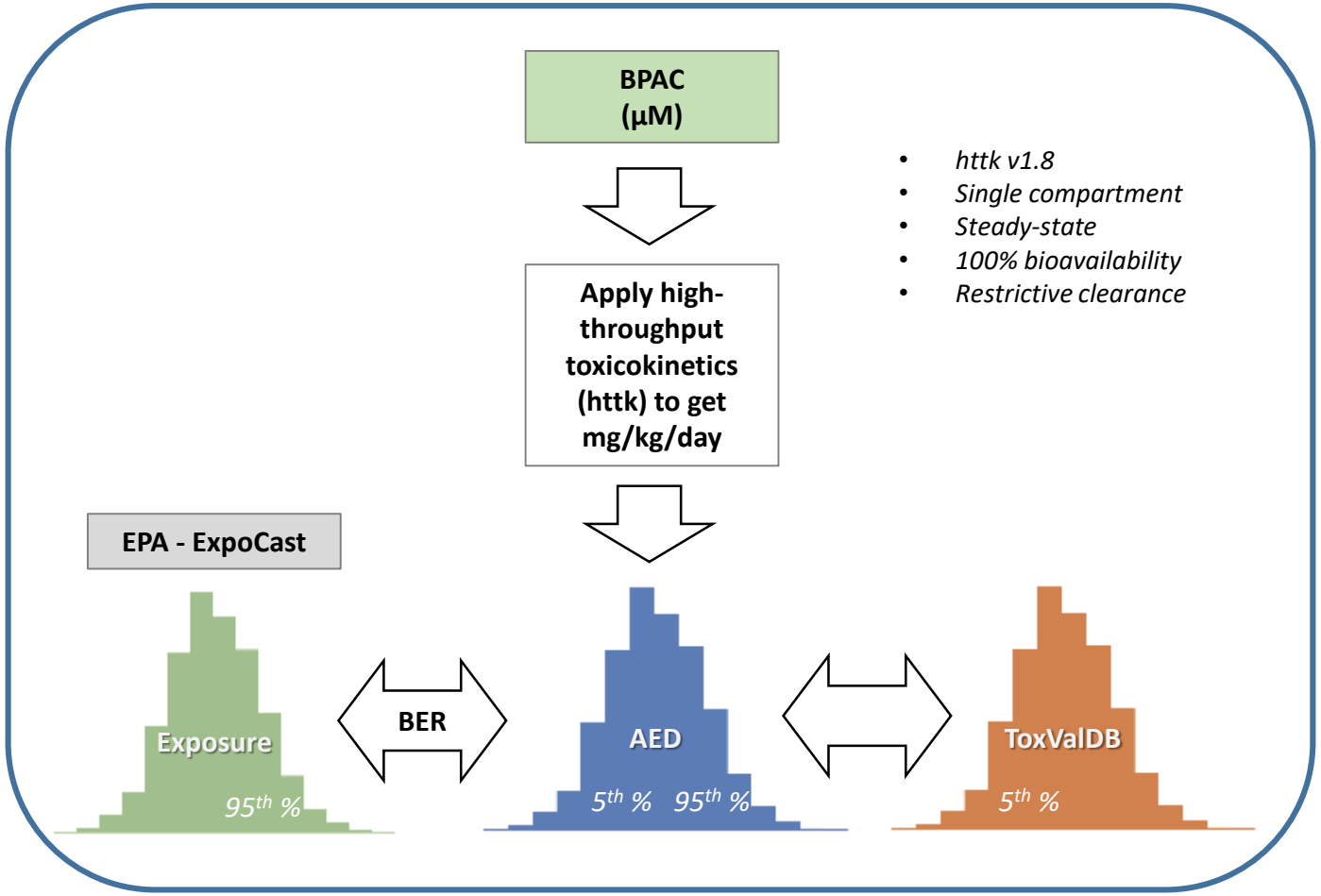


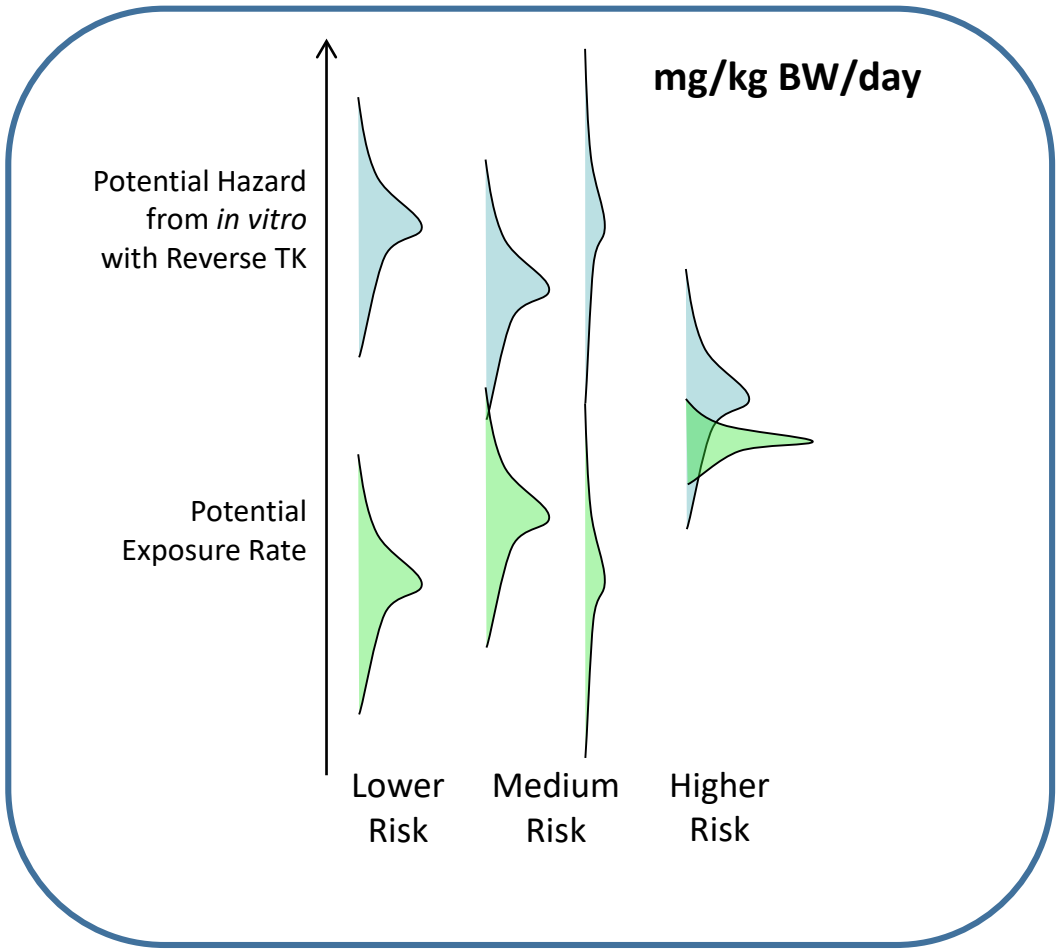




Preliminary Analysis by Richard Judson

In Vitro-in-*In Vivo* Extrapolation (IVIVE) & Bioactivity Exposure Ratios (BER)



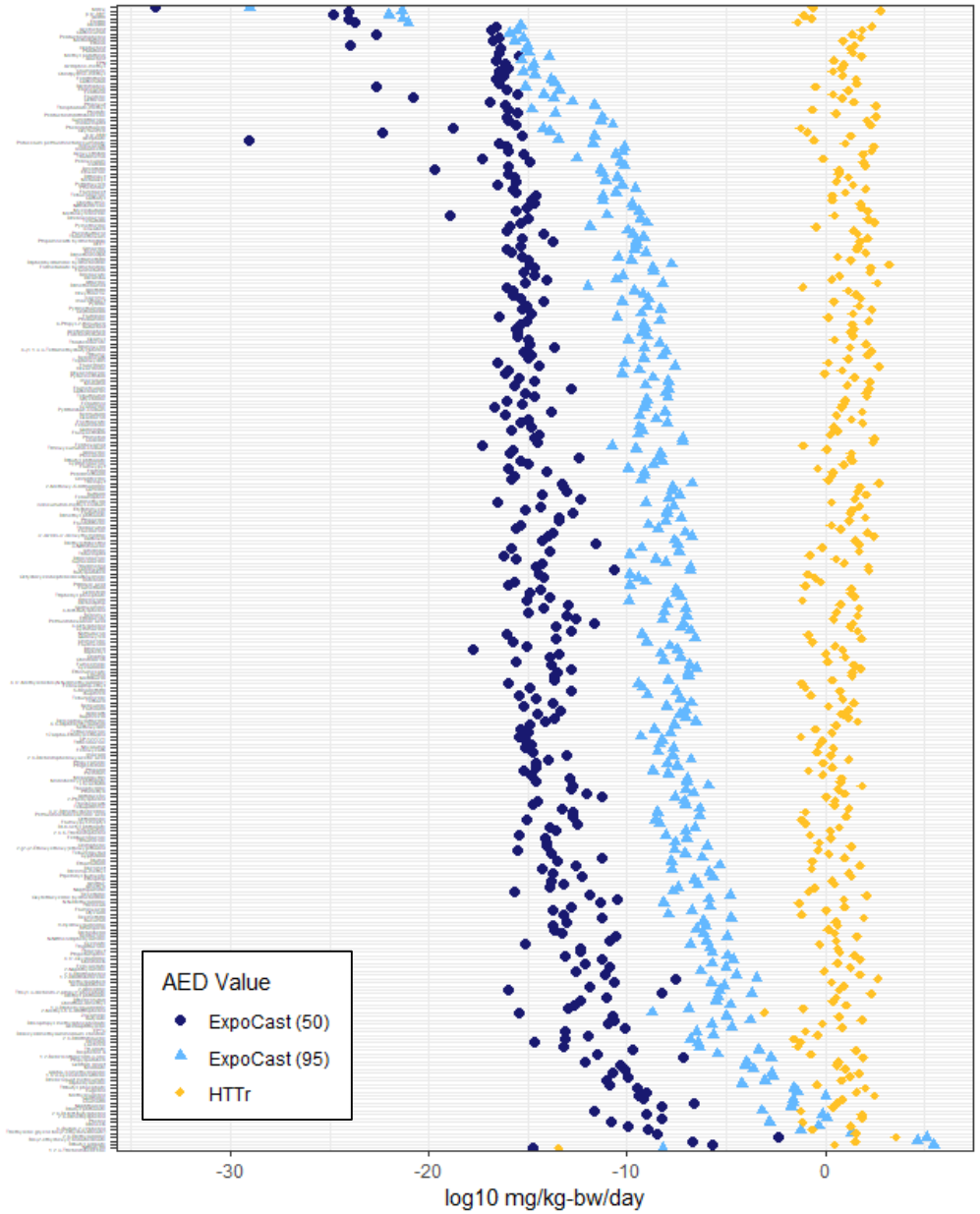


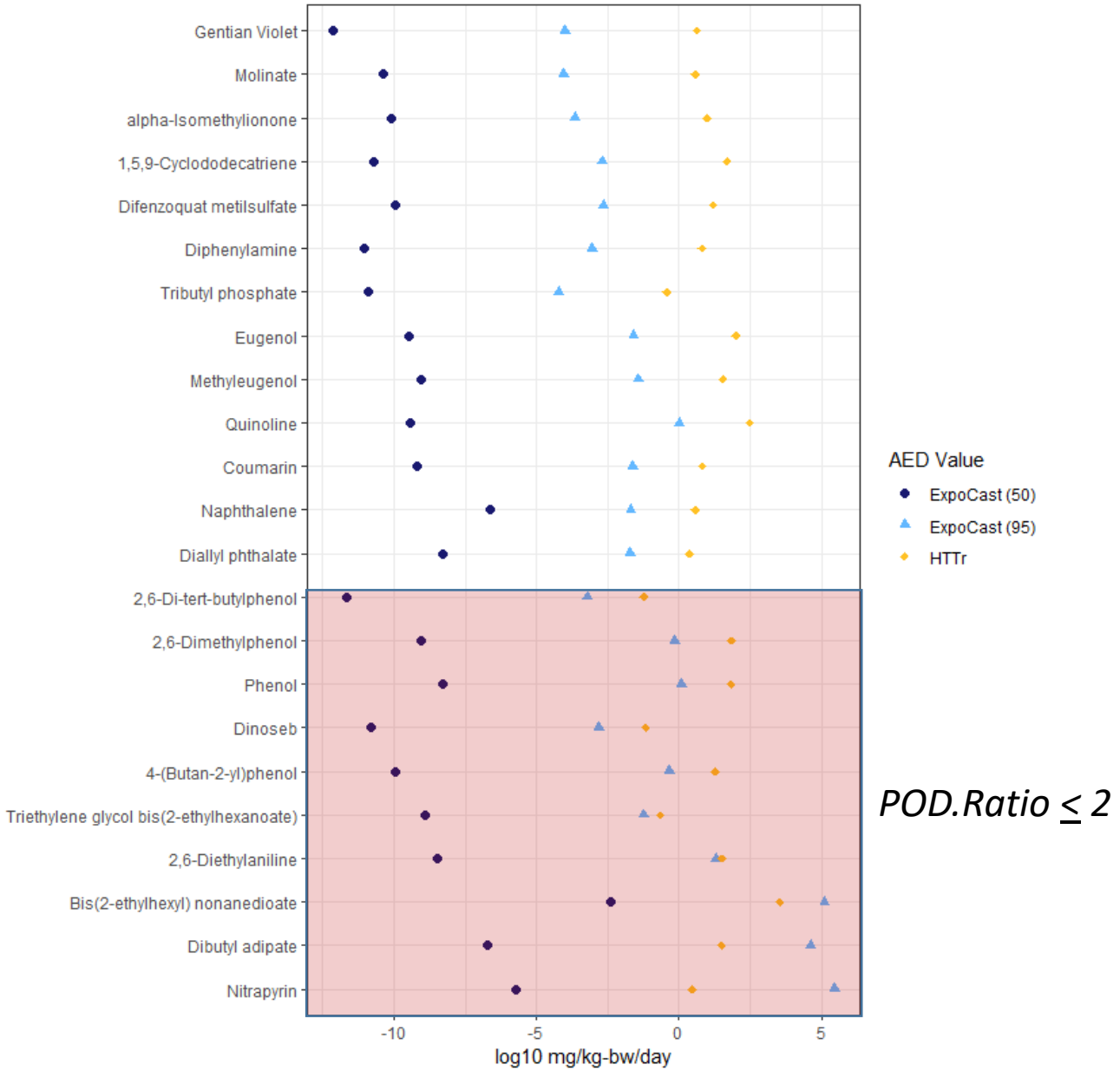
High-throughput toxicokinetic (httk) modeling: Conversion of *in vitro* bioactivity to *in vivo* steady state concentration (C_{ss})

Reverse dosimetry: Conversion of predicted C_{ss} to an administered equivalent dose (AED)

Figure courtesy of Katie Paul-Friedman & John Wambaugh

HTTr BER Results



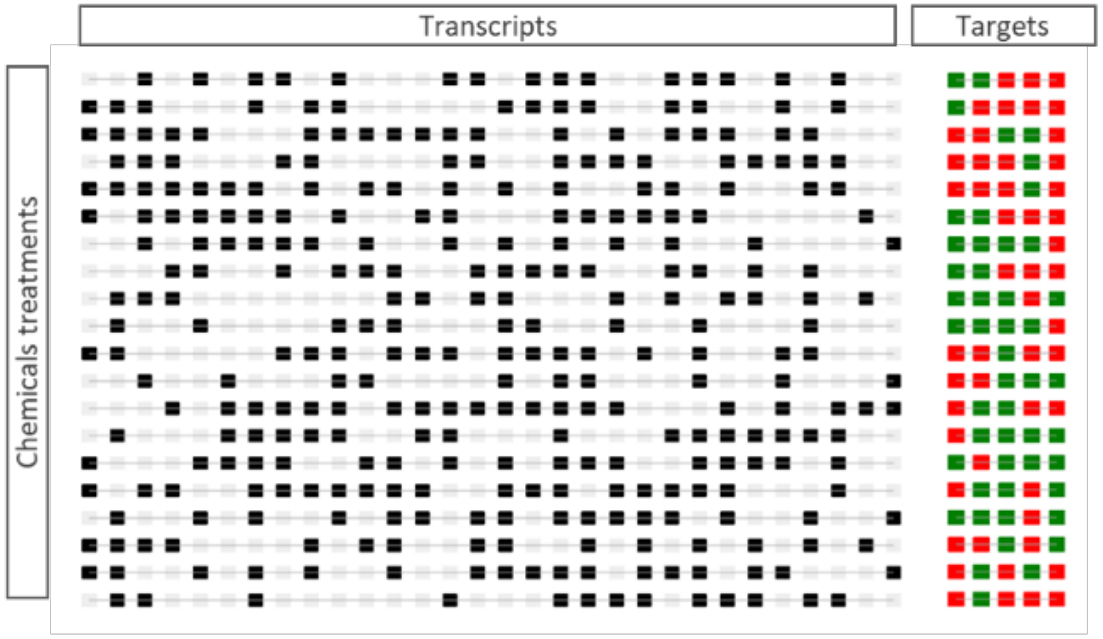


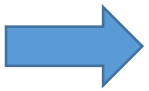
POD.Ratio ≤ 2

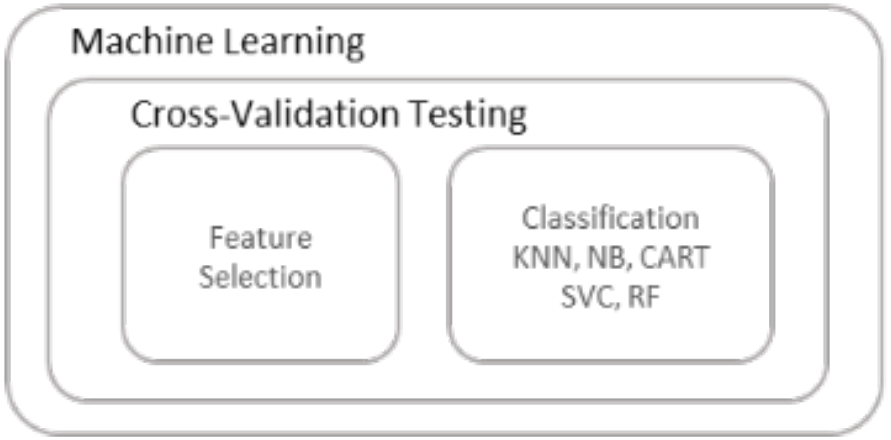
Signatures/Classifiers For Putative Target Prediction

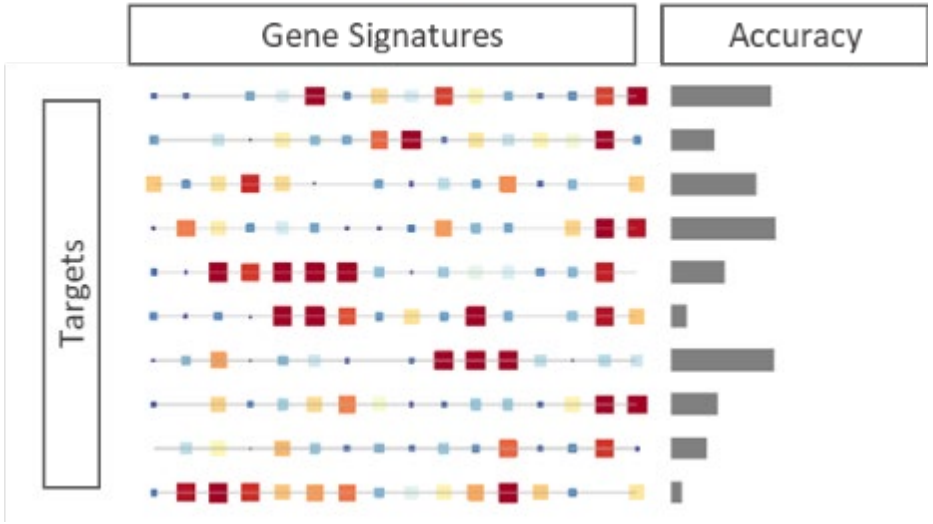
Transcriptomic Reference Database

Chemicals / Profiles Annotated with Respect to Known Biological Targets









BROAD INSTITUTE

- Manually curated a sub-set of the Connectivity Map (v2) MCF7 database with target associations.
- Created a series of target-centric signatures.
- Queried against both CMAP and TempO-Seq HTTr database

Slide by Imran Shah

ER Model (any Mode) Derived from CMAP

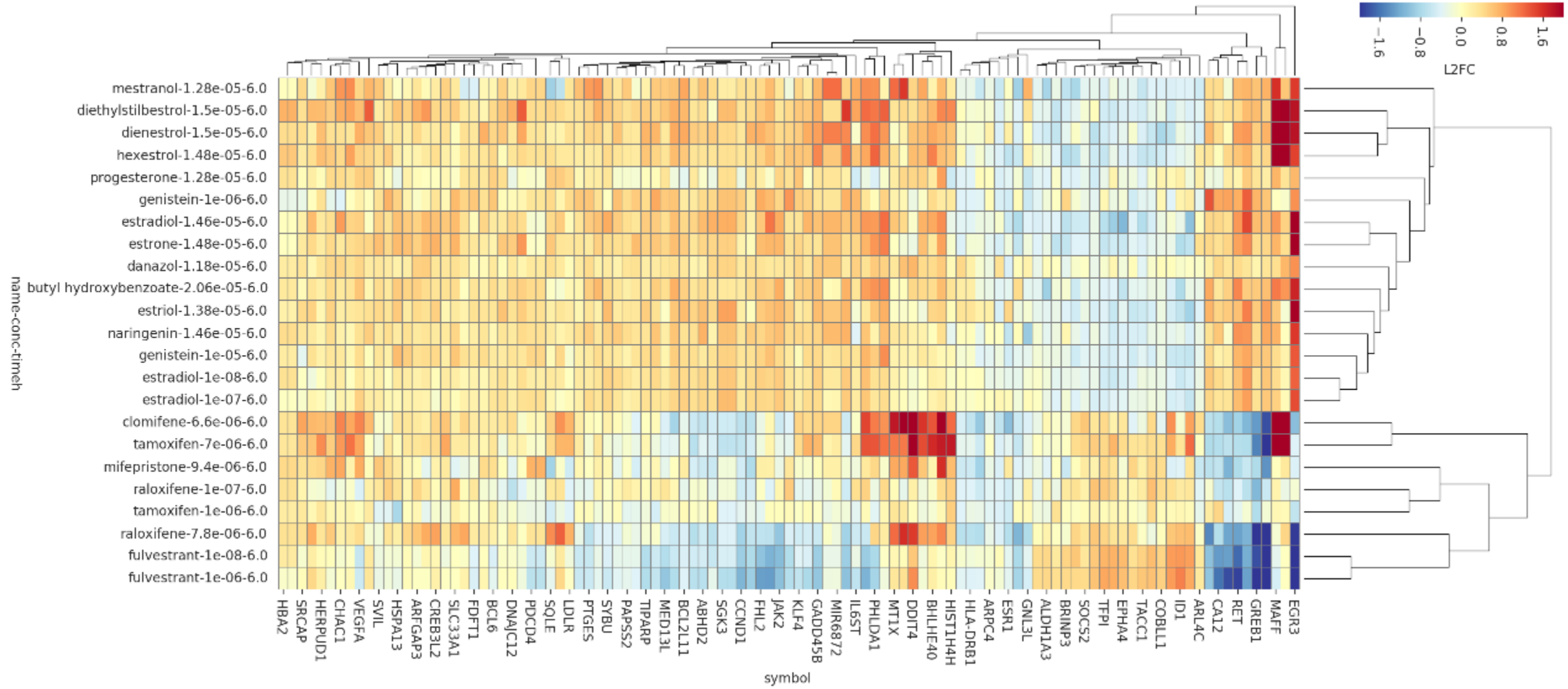


Figure Courtesy of Imran Shah

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Performance of Signatures for Putative Target Prediction in HTTr Data

Putative Target	CMap v2 / Affymetrix	BioSpyder HTTr-Phase I (n = 352)			
	Signature size	PPV	Positives	Positive Chemicals found (Curated)	Top 5 Prediction (Uncurated)
CYP2C9	131	1	1	Fluconazole	Emodin, Phenazopyridine hydrochloride, Lactofen, Hexachlorophene, 2-Amino-5-azotoluene
ESR1	257	1	11	o,p'-DDT, Genistein, 4-Nonylphenol, 4-Hydroxytamoxifen, Diethylstilbestrol, Raloxifene hydrochloride, Bisphenol A, 17beta-Estradiol, 5alpha-Dihydrotestosterone, Mifepristone, 4-(1,1,3,3-Tetramethylbutyl)phenol	dl-Norgestrel, SSR504734, Haloperidol, Cyclosporin A, Astemizole
HDAC1	124	1	2	Trichostatin A, Valproic acid	2-(Thiocyanomethylthio)benzothiazole, Azinphos-methyl, Sodium (2-pyridylthio)-N-oxide, 3,3'-Dichlorobenzidine dihydrochloride
DHFR	215	1	2	Pyrimethamine, Methotrexate	Adriamycin hydrochloride, PharmaGSID_48505, Etoposide, Resveratrol, Nisoldipine
NR1I2	139	1	2	17beta-Estradiol, Bisphenol A	dl-Norgestrel, Endosulfan, Isodrin, Genistein, 17alpha-Estradiol
PGR	115	1	1	Mifepristone	Flurandrenolide, Fluorometholone, Dexamethasone, Melengestrol acetate, Betamethasone
HMGCR	236	1	1	Lovastatin	Resveratrol, dl-Norgestrel, o,p'-DDT, Tamoxifen, Chlorhexidine
ABCC2	357	1	1	Methotrexate	4-Nitrosodiphenylamine, Resveratrol, Adriamycin hydrochloride, Nisoldipine, 8-Hydroxyquinoline sulfate
TYMS	329	1	1	Methotrexate	Etoposide, Resveratrol, 4-Nitrosodiphenylamine, Cytarabine hydrochloride, PharmaGSID_48505
ESR2	281	0.86	7	Genistein, Diethylstilbestrol, 4-Nonylphenol, Bisphenol A, 4-Hydroxytamoxifen, 17beta-Estradiol	dl-Norgestrel, 17alpha-Estradiol, Haloperidol, Cyclosporin A, Isodrin
AR	261	0.78	9	o,p'-DDT, 17beta-Estradiol, 5alpha-Dihydrotestosterone, Flutamide, Bisphenol A, Mifepristone, 17-Methyltestosterone	dl-Norgestrel, Melengestrol acetate, Dehydroepiandrosterone, 8-Hydroxyquinoline, Genistein
NR3C2	352	0.5	2	Mifepristone	Fluocinolone acetonide, Bexarotene, 1-Naphthol, Dexamethasone, dl-Norgestrel
ABCB1	117	0.5	2	Reserpine	Fabesetron hydrochloride, Abamectin, SAR115740, SSR69071, Chlorobenzilate
NR3C1	148	0.5	4	Triamcinolone, Mifepristone	Medroxyprogesterone acetate, Fluorometholone, Melengestrol acetate, Dexamethasone, Prednisolone
CA1	176	0.5	4	Phenol, Sodium nitrite	Triclopyr, Triclopyr butotyl, p-Bromodiphenyl ether, 2-Fluoroacetamide, 1-Ethyl-2-methylbenzene
CA2	341	0.5	4	Celecoxib, Phenol	PharmaGSID_48509, Acenaphthylene, CP-105696, Aloe-emodin, 2-Fluoroacetamide
PTGS1	307	0.25	4	Indomethacin	SSR69071, 17alpha-Estradiol, Chlordane, Cetylpyridinium bromide, Zoxamide

Slide by Imran Shah

Table Courtesy of Imran Shah

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High-Throughput Phenotypic Profiling (HTPP)

Cell Painting

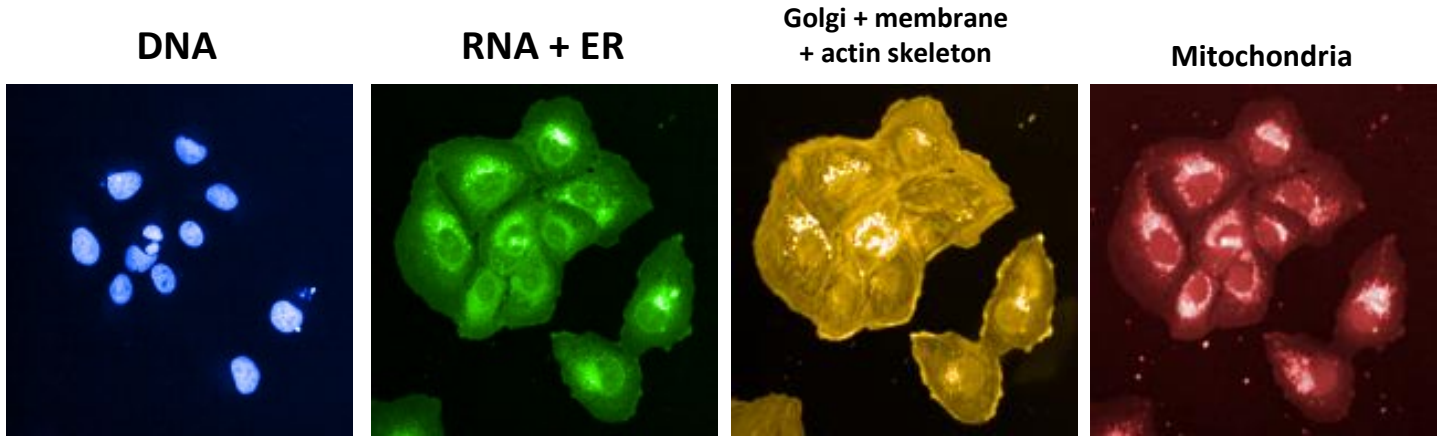
Cell Painting

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

- No requirement for *a priori* knowledge of molecular targets.

- Uses:
  - Functional genomics
  - Drug discovery
  - Compound efficacy and toxicity screening
  - Mechanism-of-action identification
  - Chemical grouping

- **Hypothesis:** **Cell Painting may be an efficient and cost-effective method for evaluating the bioactivity of environmental chemicals.**



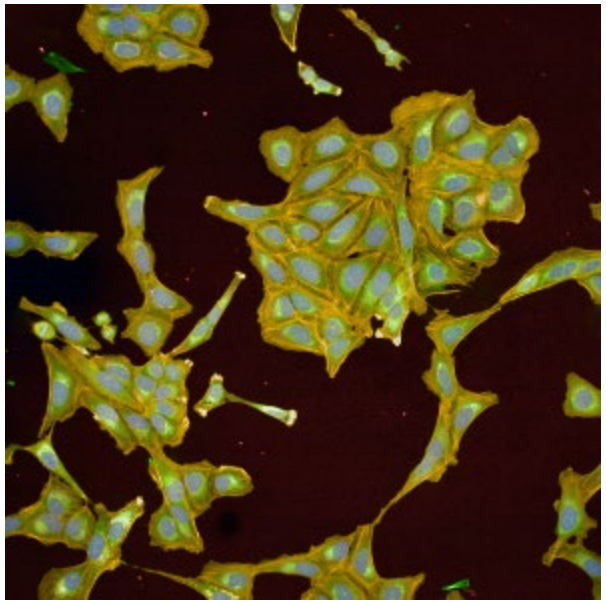
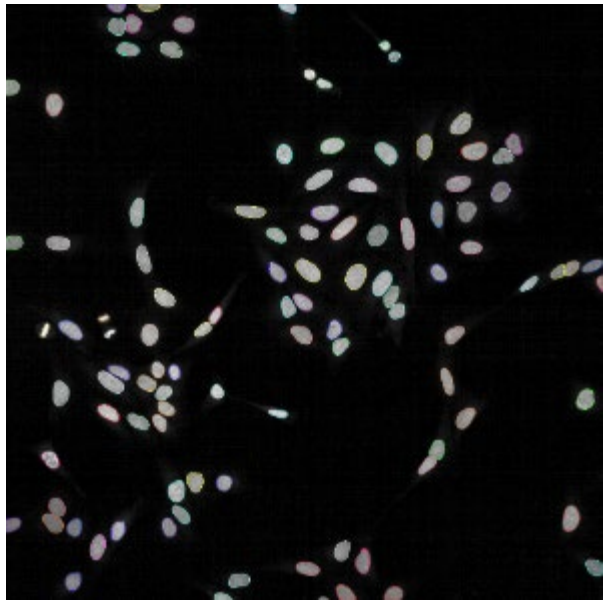
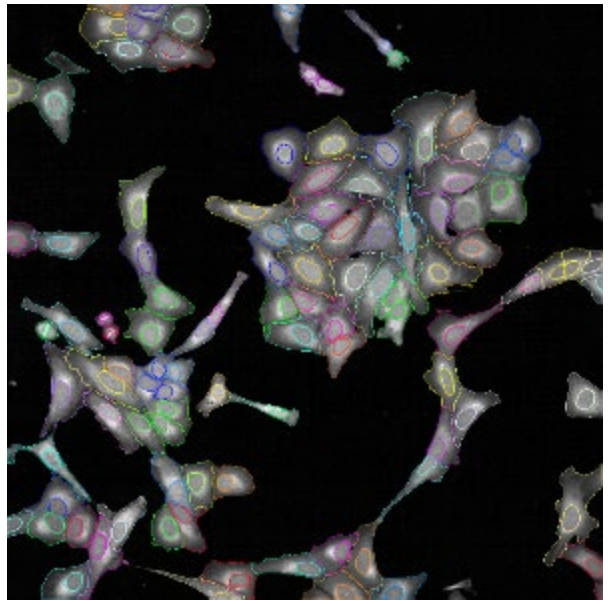
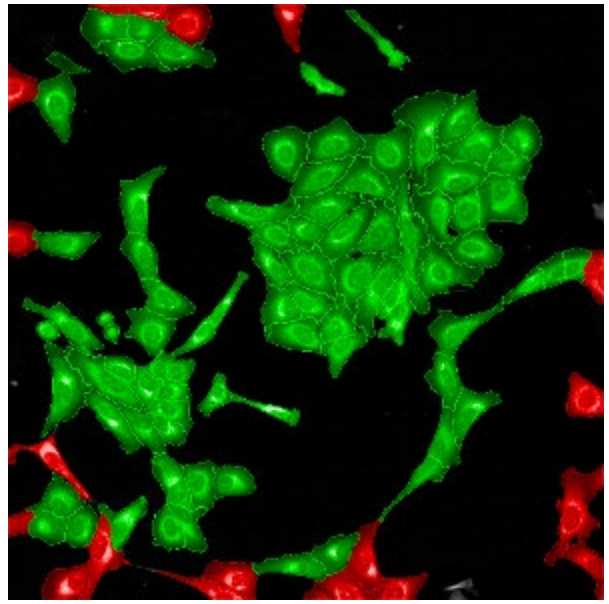
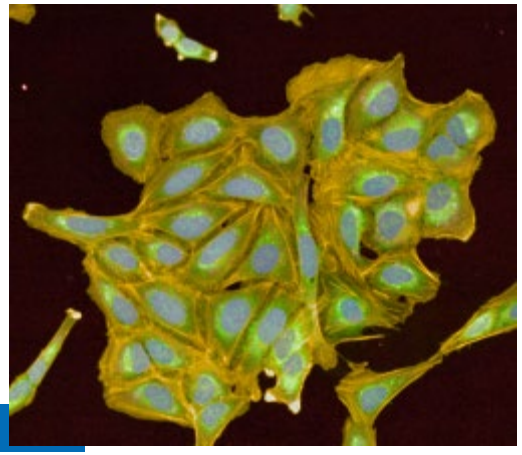
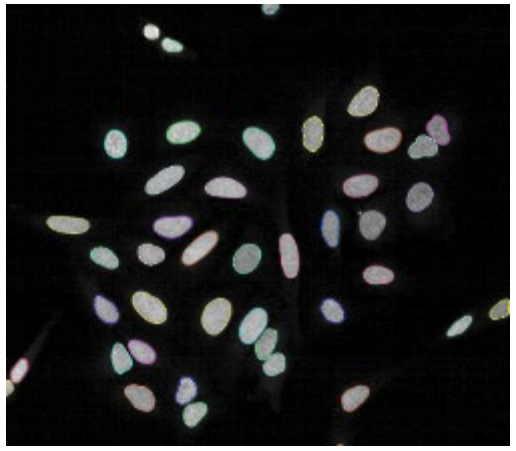
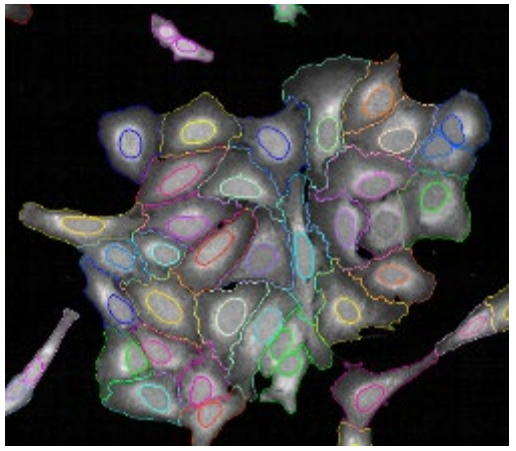
Marker	Cellular Component	Labeling Chemistry	Labeling Phase	Opera Phenix	
				Ex.	Em.
Hoechst 33342	Nucleus	Bisbenzamide probe that binds to dsDNA	Fixed	405	480
Concanavalin A – AlexaFluor 488	Endoplasmic reticulum	Lectin that selectively binds to α-mannopyranosyl and α-glucopyranosyl residues enriched in rough endoplasmic reticulum		435	550
SYTO 14 nucleic acid stain	Nucleoli	Cyanine probe that binds to ssRNA		435	550
Wheat germ agglutinin (WGA) – AlexaFluor 555	Golgi Apparatus and Plasma Membrane	Lectin that selectively binds to sialic acid and N-acetylglucosaminyl residues enriched in the trans-Golgi network and plasma membrane		570	630
Phalloidin –AlexaFluor 568	F-actin (cytoskeleton)	Phallotoxin (bicyclic heptapeptide) that binds filamentous actin			
MitoTracker Deep Red	Mitochondria	Accumulates in active mitochondria	Live	650	760

Image Analysis Workflow → Image Segmentation

1. find nuclei

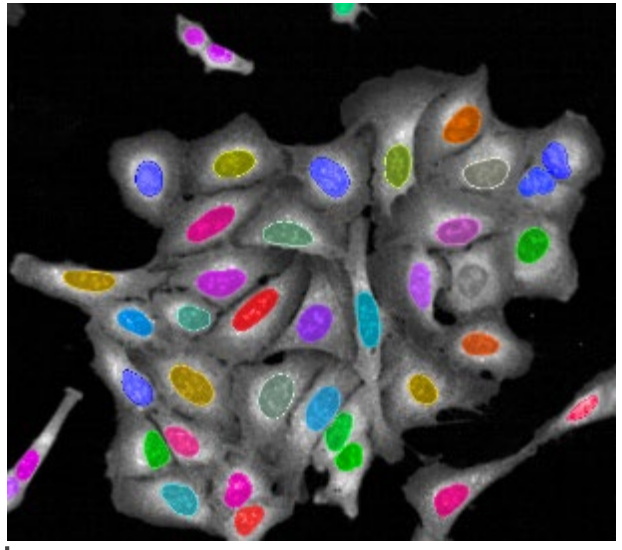
2. find cell outline

3. reject border objects

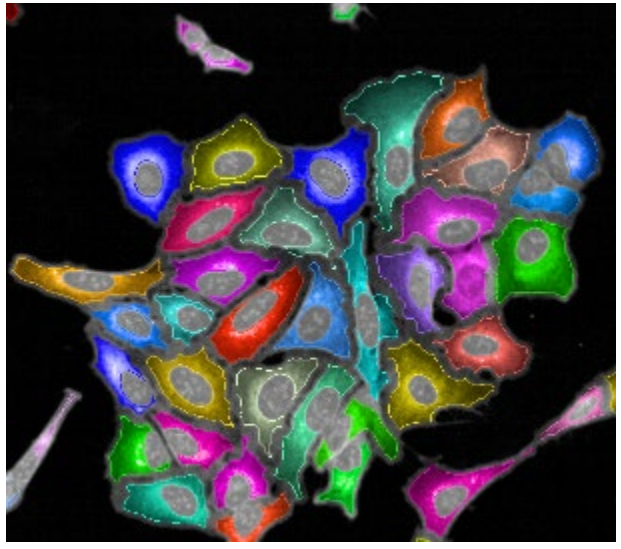








Define Cellular Compartments

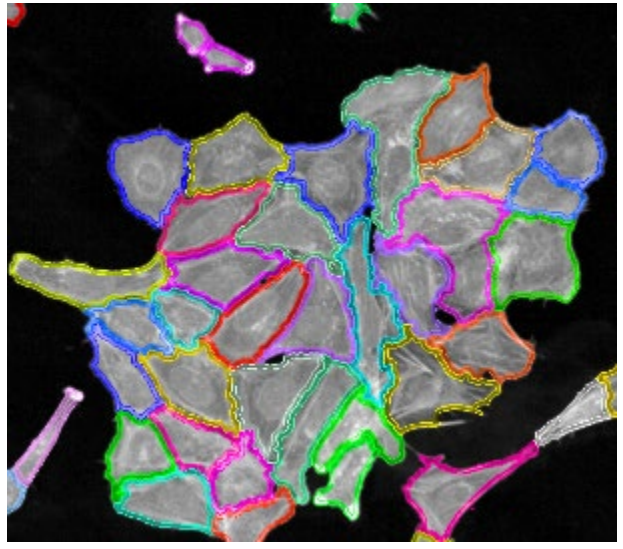
nuclei



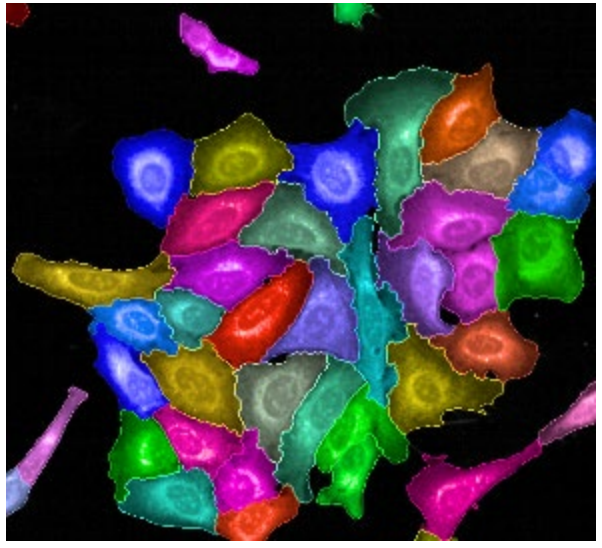
cytoplasm



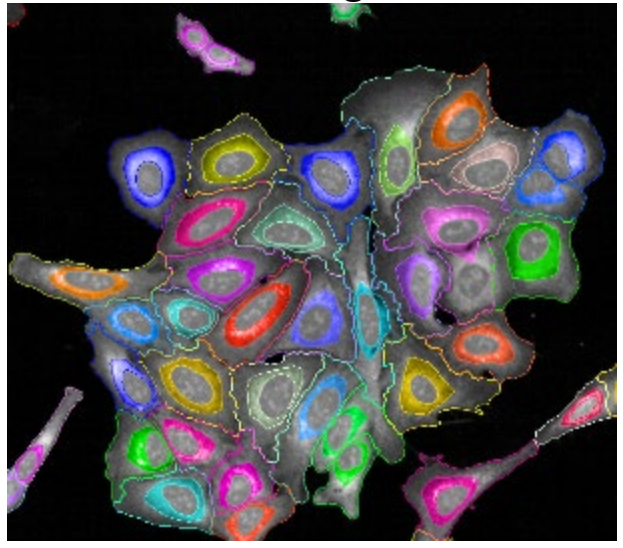
membrane



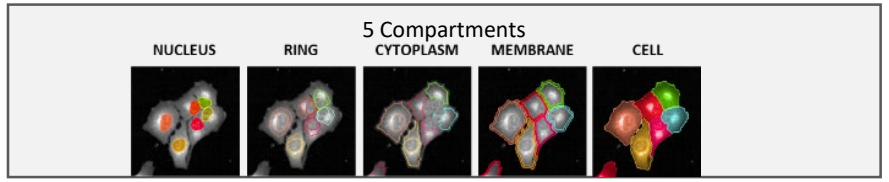
cell

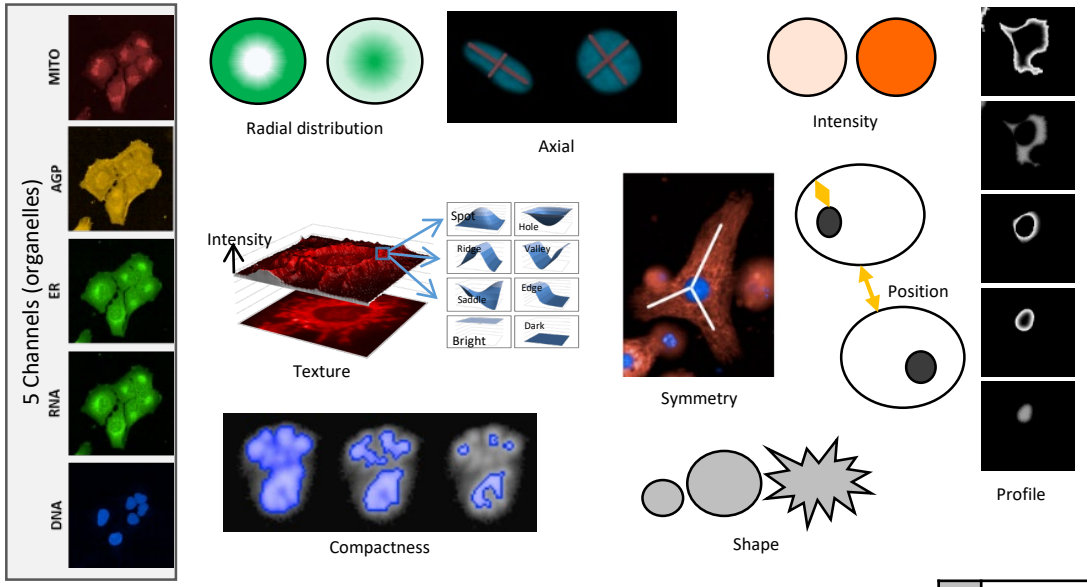


ring



Phenotypic Feature Extraction





1300 features / cell

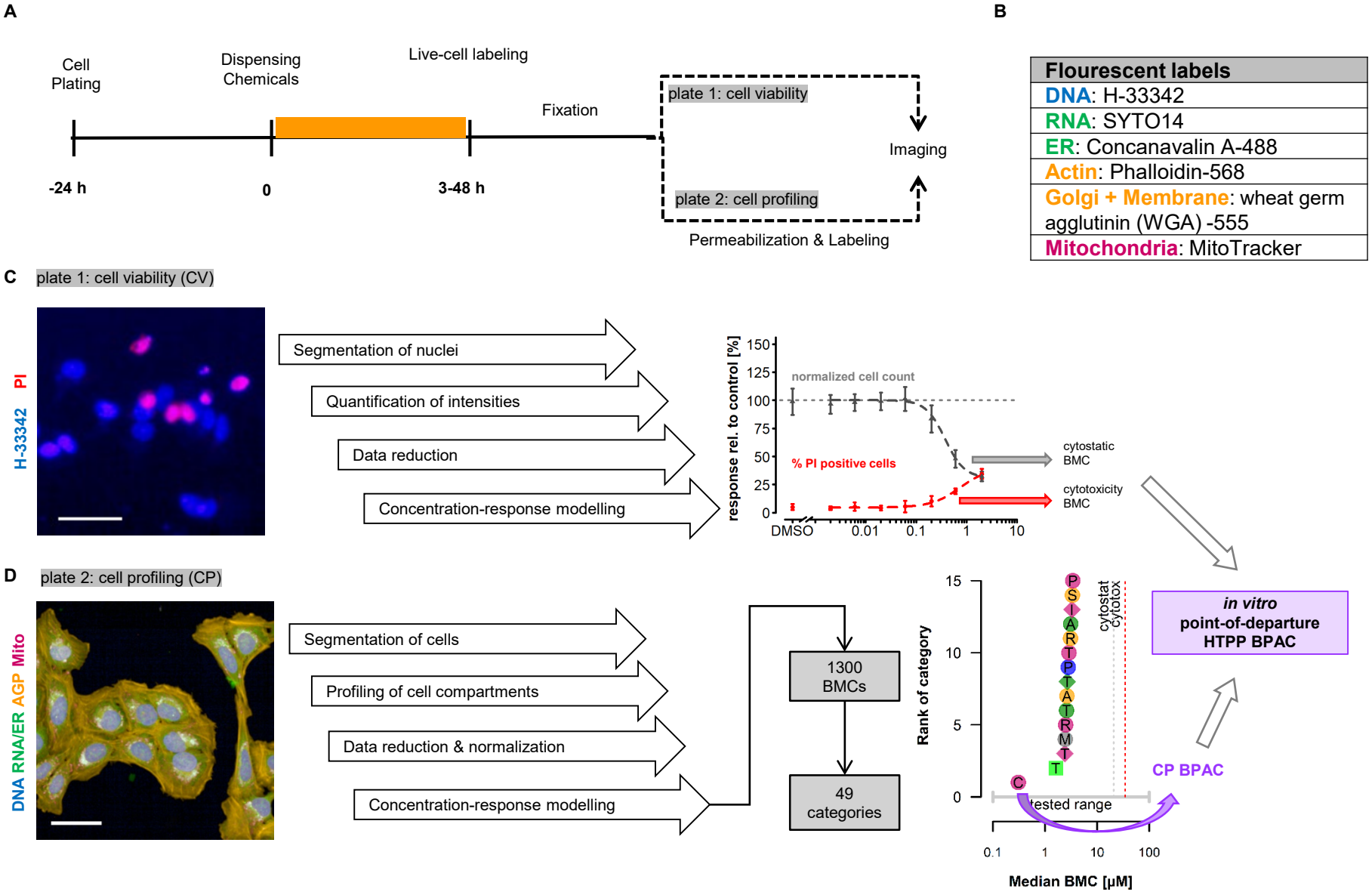
		Module								
		Position [7]	Basic morph-ology [5]	SCARP morphology					Intensity [9]	Texture [14]
				Symmetry [80]	Compactness [40]	Axial [20]	Radial [28]	Profile [20-30]		
Channel	DNA			Nuclei	Nuclei	Nuclei	Nuclei Cell	Nuclei Cytoplasm	Nuclei	Nuclei
	RNA			Nuclei	Nuclei	Nuclei	Nuclei	Nuclei	Nuclei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

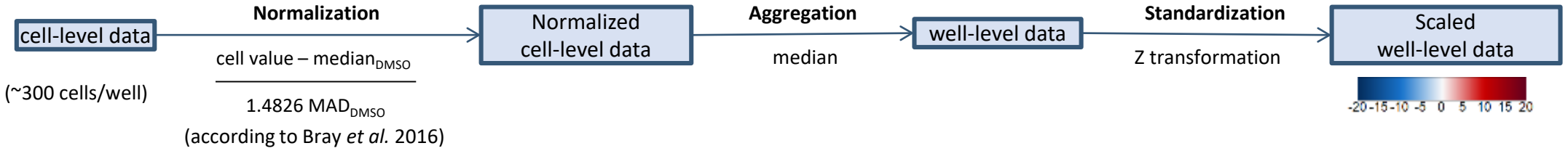
Modality: Confocal (single *z*)
Objective: 20X Water
Plate: CellCarrier-384 Ultra
Fields: 5 or 9

With illustrations from Perkin Elmer

HTPP Assay Overview



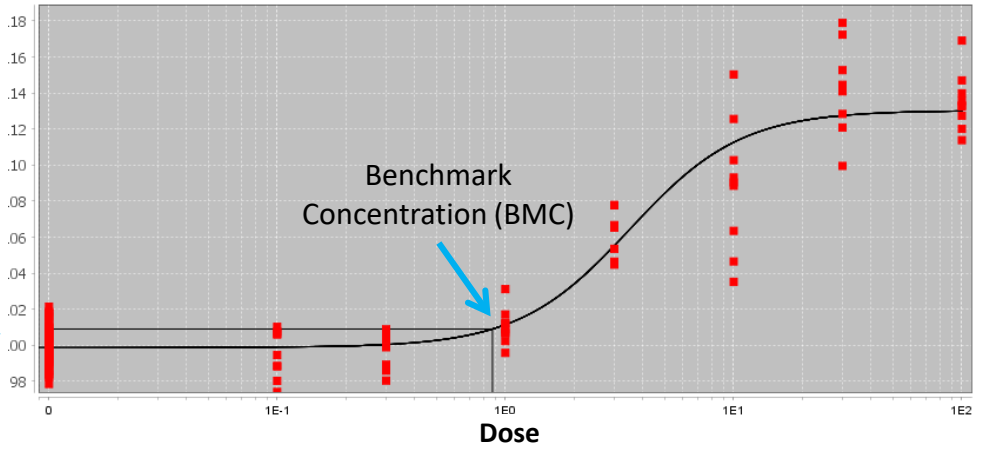
Analysis of Phenotypic Features



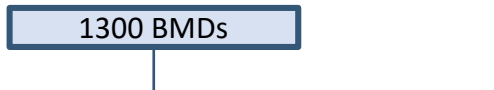
Dose-response modelling

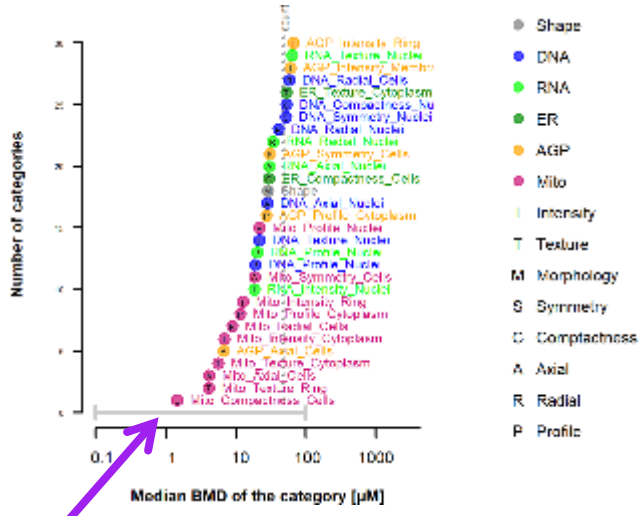
- Scaled well-level data is clipped above the first cytotoxic dose (determined by the CV assay)
- Software: BMDExpress 2.2
- 4 models: Hill, Linear, Poly2, Power
- Best model selection:
  1. nested χ^2 to select the better polynomial (Linear vs Poly2)
  2. best AIC (Hill, Power, Poly)
- BMCs above the tested range are reported as NA
  BMCs below the tested range are assigned $\log_{10}$(min dose)-0.5

Benchmark response (BMR): 1 SD



Phenotypic Category Analysis

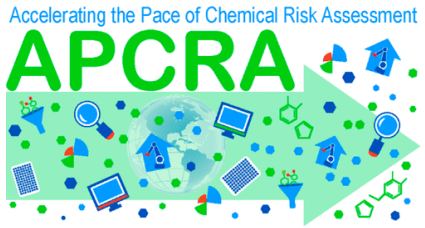




Biological Phenotype Altering Concentration (BPAC):
Median BMC of the most sensitive category
(where ≥ 30% ontology elements affected)

U-2 OS APCRA Screen Experimental Design

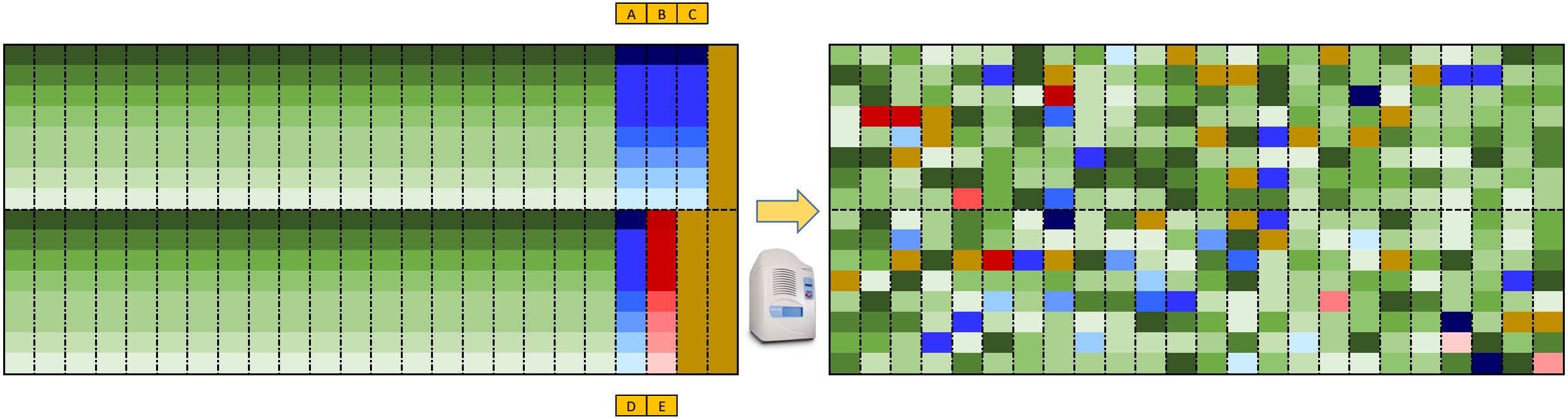
Parameter	Multiplier	Notes
Cell Type(s)	1	U-2 OS
Culture Condition	1	DMEM + 10% HI-FBS
Chemicals	462	APCRA Case Study Chemicals + Duplicates Unilever CRADA Consensus Chemicals HTTr Pilot Chemicals
Time Points:	1	24 hours
Assay Formats:	2	Cell Painting Cell Viability
Concentrations:	8	3.5 $\log_{10}$ units; semi $\log_{10}$ spacing
Biological Replicates:	4	Independent cultures



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

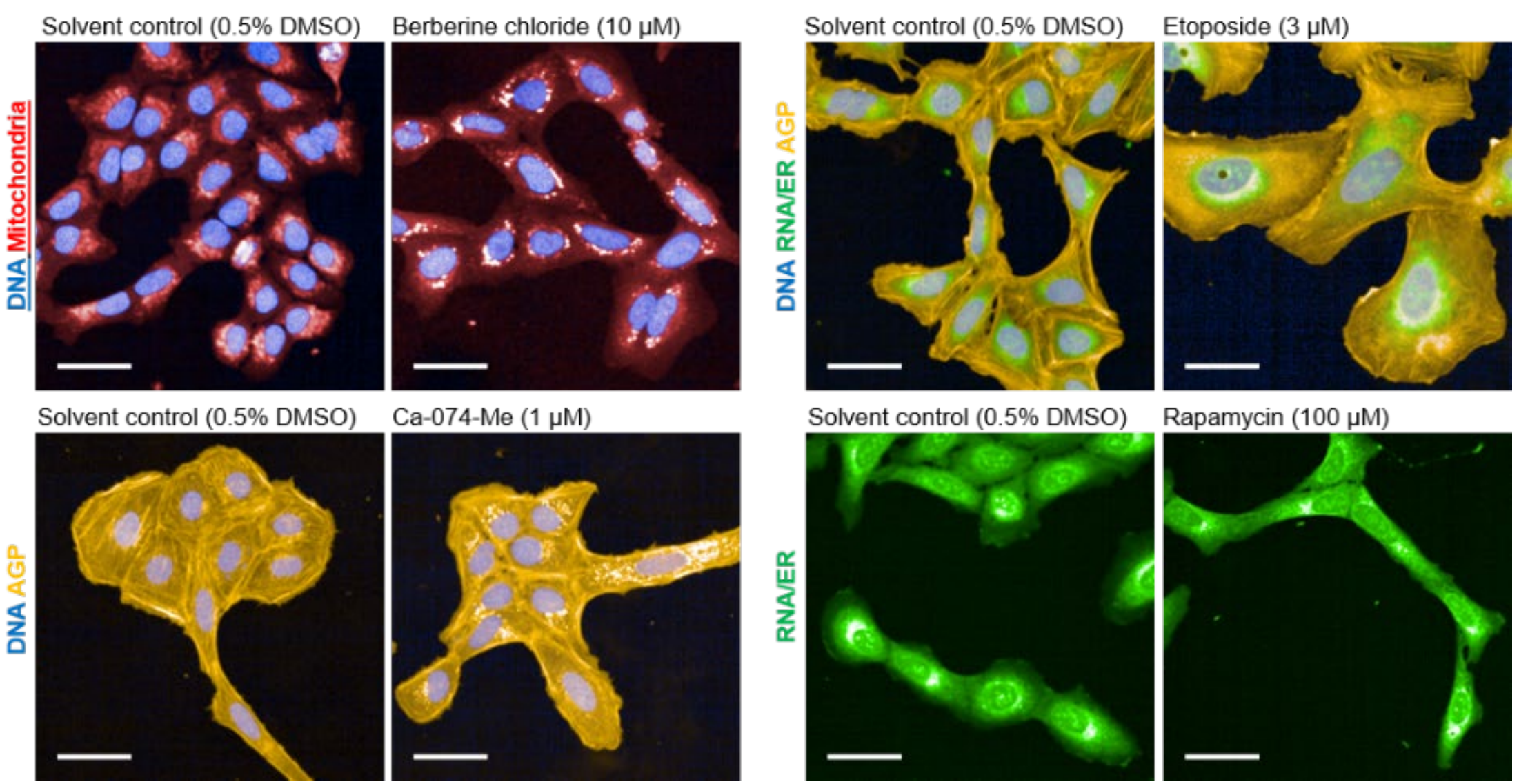
- International collaboration of regulatory scientists focused on developing case studies for evaluating the use of New Approach Methodologies (NAMs) in chemical risk assessment.
- ECHA Workshop (2017) case study focuses on **deriving quantitative estimates of risk based on NAM-derived potency information and computational exposure estimates**

U-2 OS APCRA Screen: Dose Plate Design

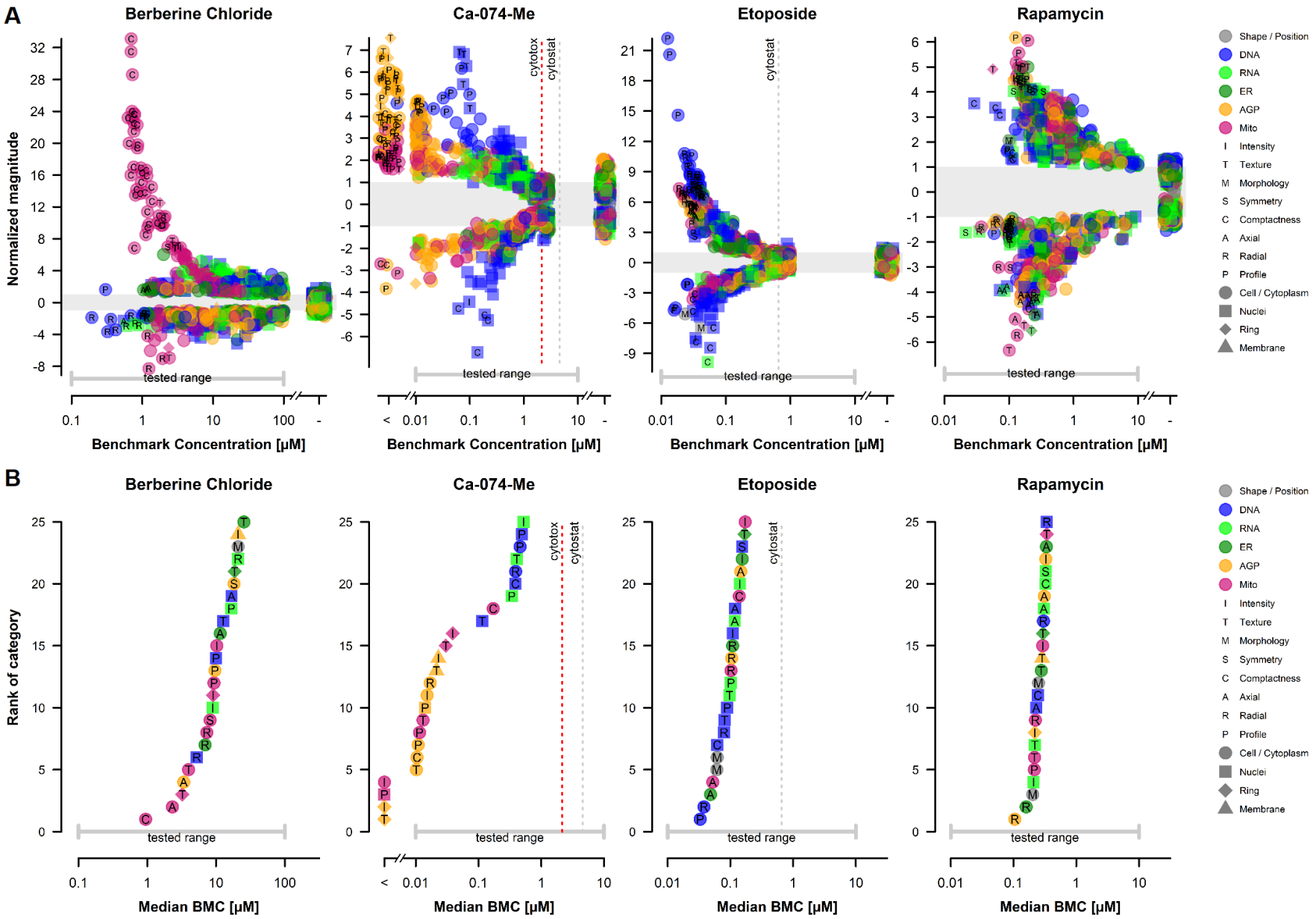


Label	Reference Chemicals:	Phenotypic Observations	Test Concentrations
A	Berberine Chloride	Specific mitochondrial effects	0.03 – 10 µM
B	Etoposide	Cell hypertrophy control that produces effects in every channel / organelle	0.03 - 10 µM
C	Ca-074-Me	Effects on AGP channel at sub-cytotoxic doses	0.03 -10 µM
D	Rapamycin	Effects on RNA and DNA channels	0.03 - 10 µM
E	Staurosporine	Cytotoxicity Control	0.01 -3 µM

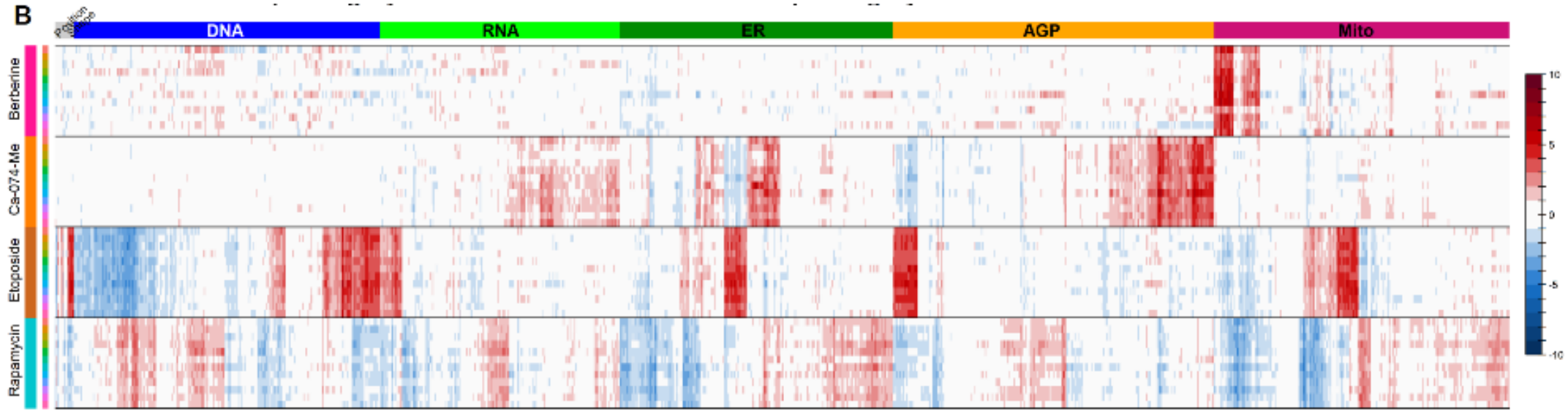
Reference Chemical Phenotypes (1)

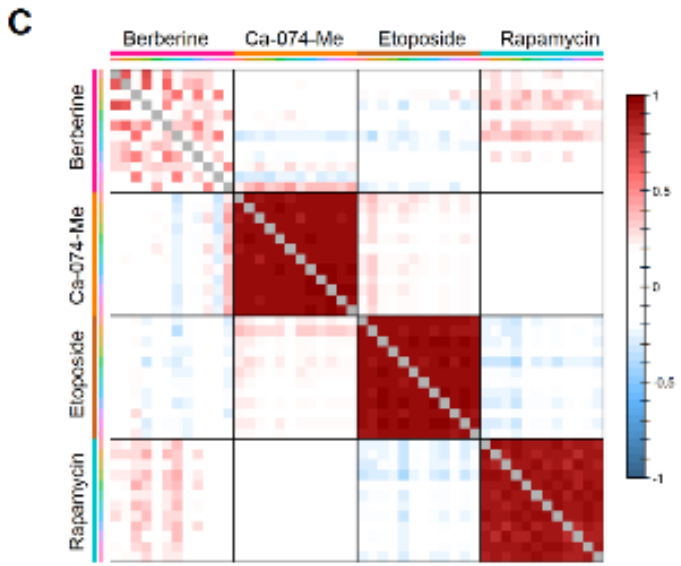


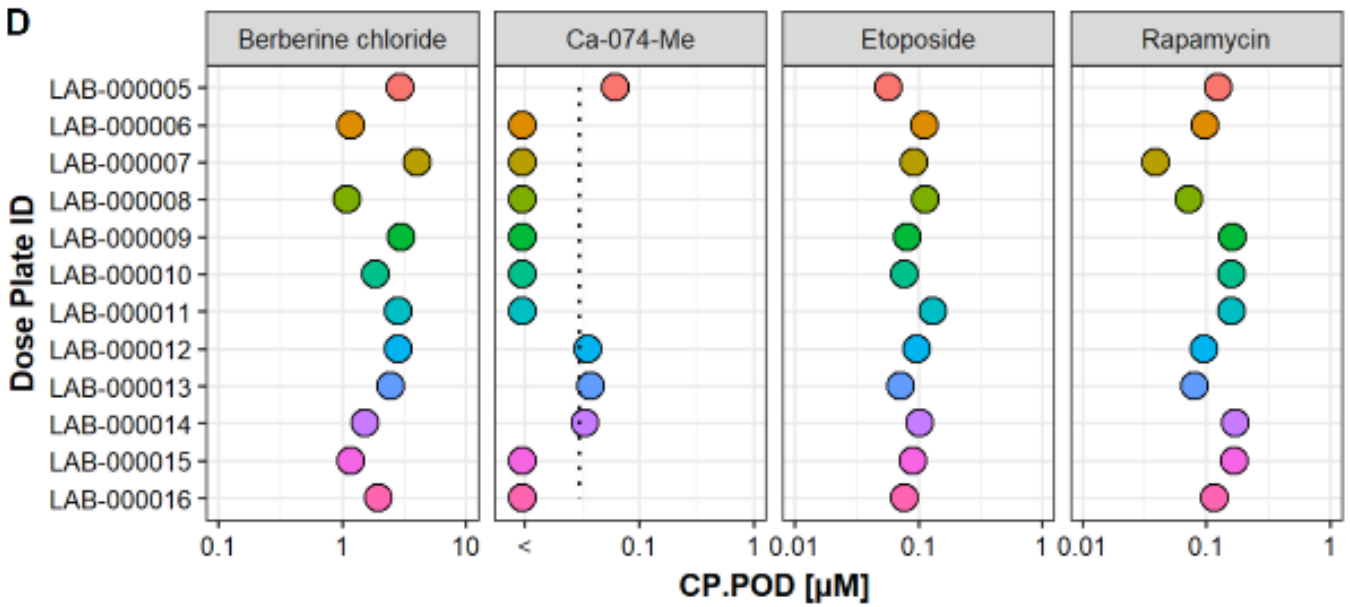
Reference Chemical Phenotypes (2)



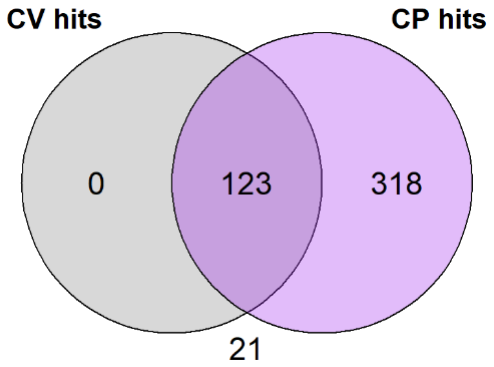
Assay Reproducibility

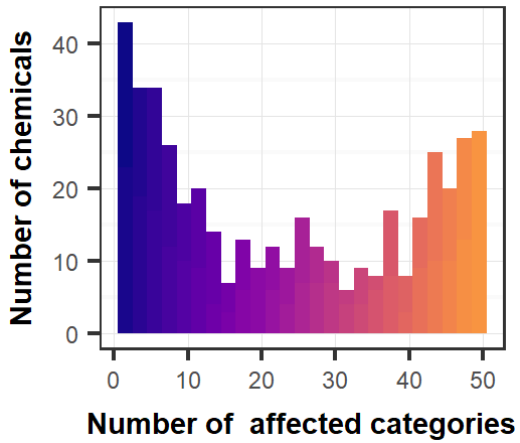


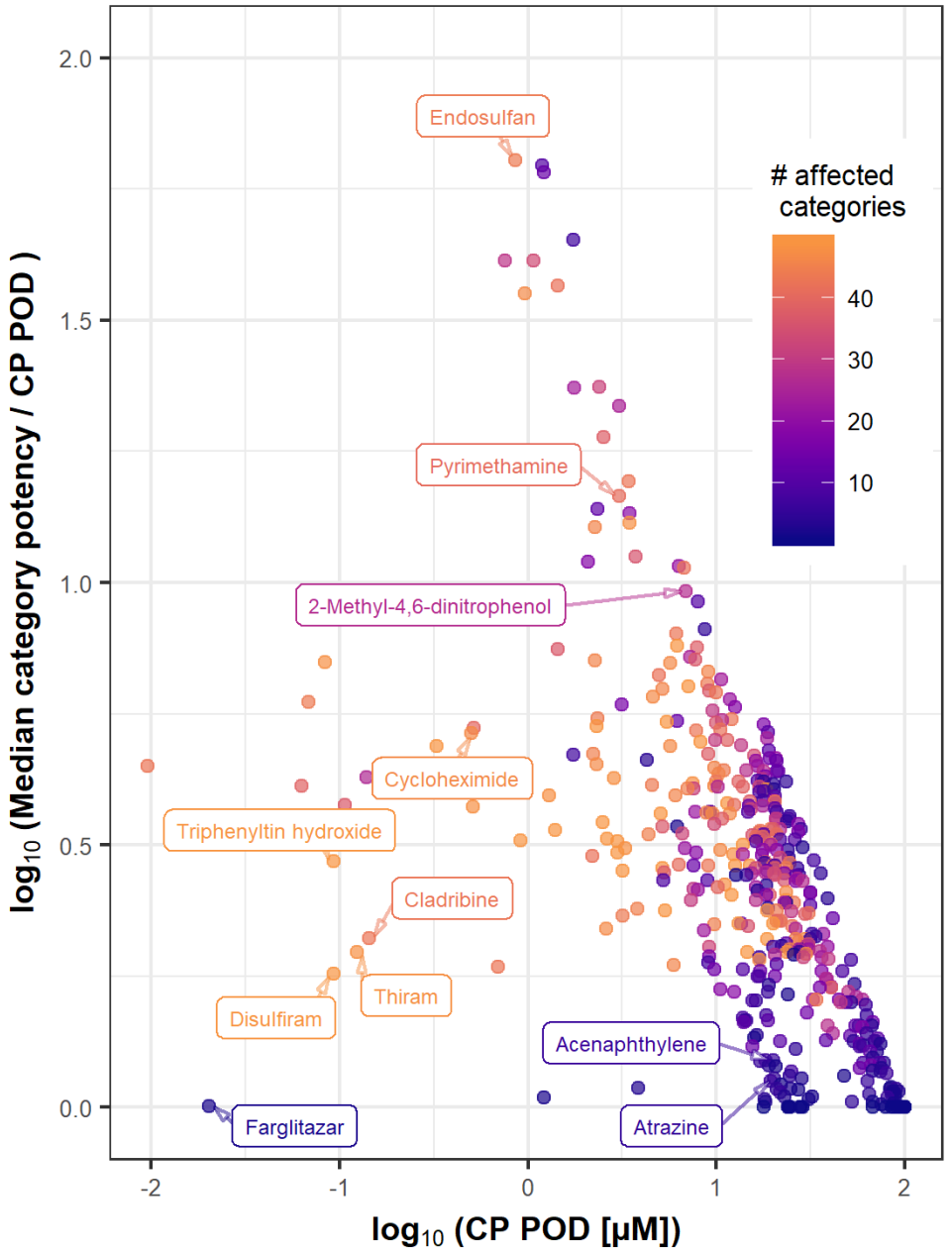


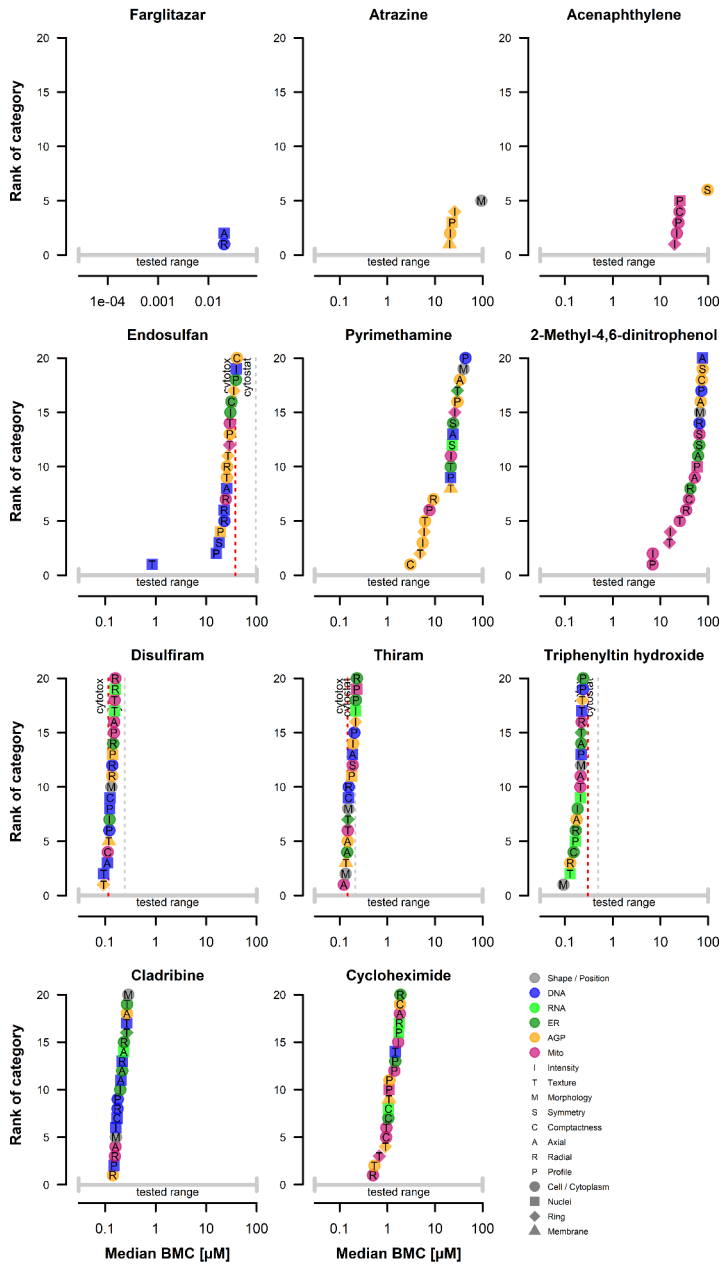


APCRA Screening Results

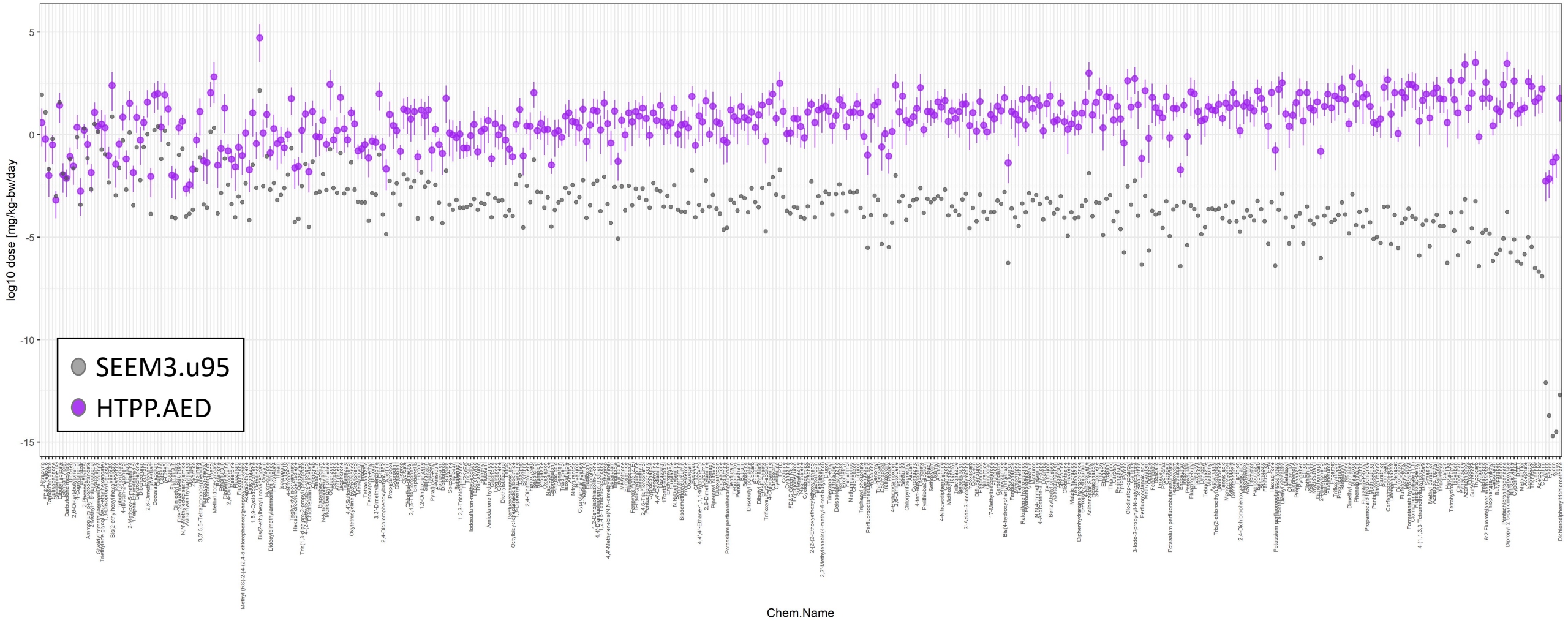








BER Results



- Chemicals with small BER ratio would be of higher priority than chemicals with a large BER ratio.

Read Across Pilot, Experimental Design

Parameter	Multiplier	Notes
Cell Type(s)	1	U-2 OS
Culture Condition	1	DMEM + 10% HI-FBS
Chemicals	120	Pharmacological Tool Compounds Model Toxicants Structure Series
Time Points:	1	24 hours
Assay Formats:	2	Cell Painting Cell Viability
Concentrations:	8	3.5 $\log_{10}$ units; ~half-$\log_{10}$ spacing
Biological Replicates:	4	Independent cultures

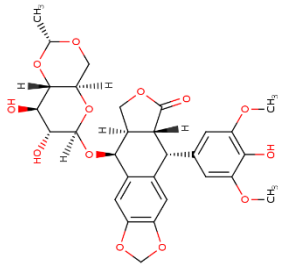
Actin cytoskeleton modulators
Actin stabilizers
ER modulator
Golgi modulator
Mitochondrial fission
Microtuble modulator
Microtuble stabilizer

DNA toxicants: alkylators
DNA toxicants: topoisomerase
DNA toxicants: antimetabolites
DNA toxicants: genotoxic
Oxidative stress
Proteosome inhibitors
Oxidative phosphorylation uncoupler

Mito. Respiratory complex inhibitor
Autophagy inhibitor
Autophagy activator
RNA polymerase inhibitor
Benzimidazole structure series
Rapamycin analogues
Ca-074-Me analogues

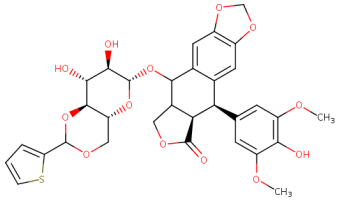
Read Across Pilot, Results

Profile: Feature level response magnitudes
Signature: Response threshold @ 1.5
Similarity Metric: Pearson Correlation
Comparisons: All test chemicals against each other
“Hit” Criteria: Correlation > 0.5

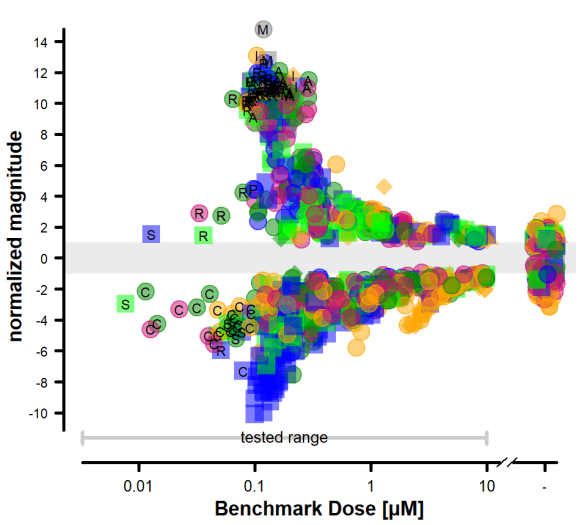


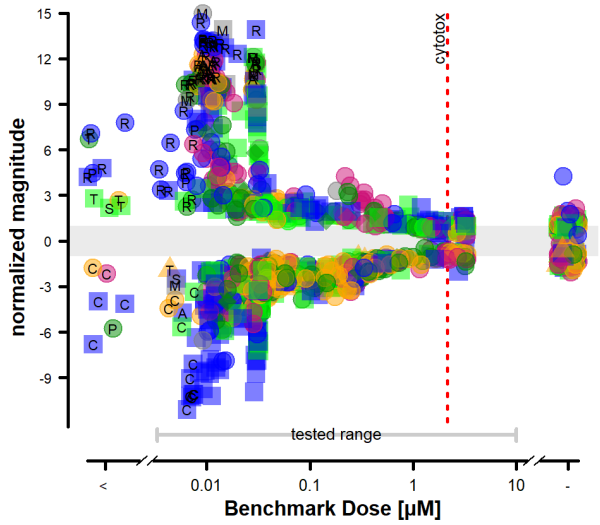
Etoposide | DTXSID5023035

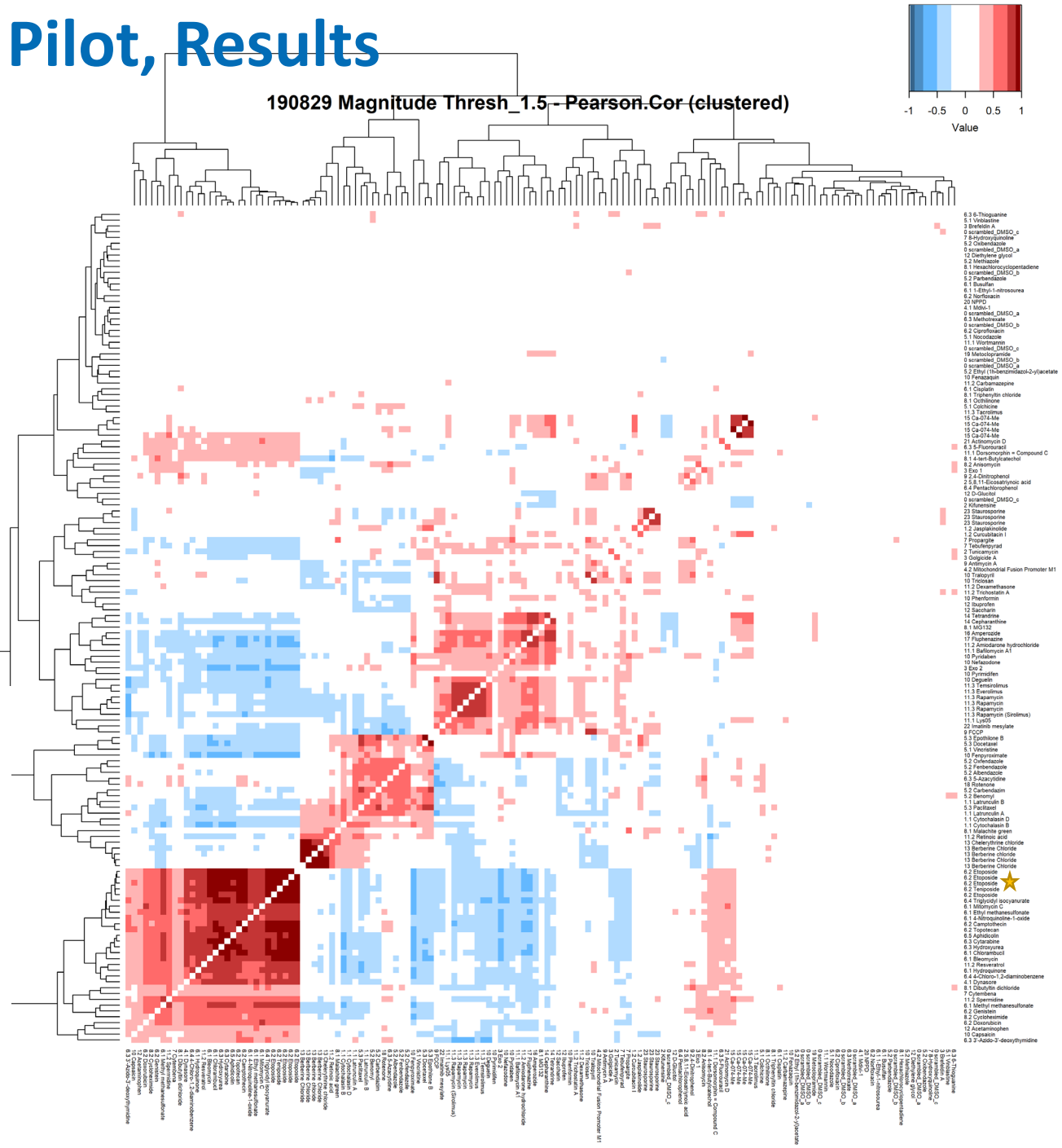
0.966



Teniposide | DTXSID8023638







Read Across Example (1)

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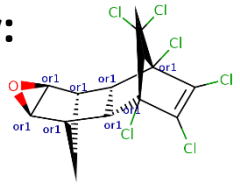
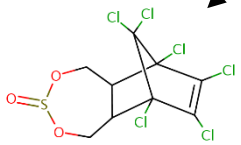


Endrin
72-20-8 |
DTXSID6020561

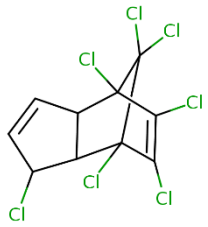
Matches:

0.71



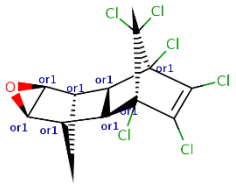
Endosulfan
115-29-7 |
DTXSID1020560

0.70



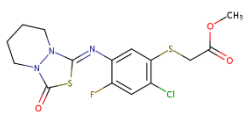
Heptachlor
76-44-8 |
DTXSID3020679

0.61



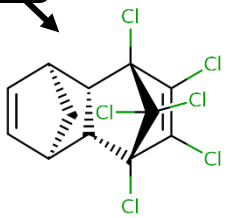
Dieldrin
60-57-1 |
DTXSID9020453

0.59

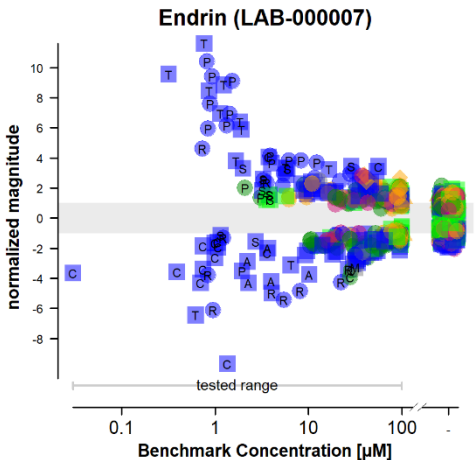


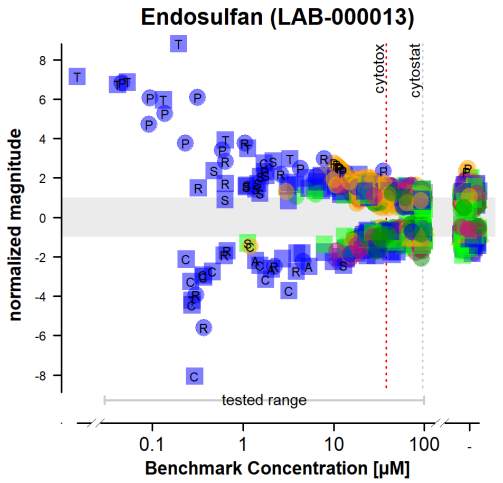
Fluthiacet-methyl
117337-19-6 |
DTXSID2032556

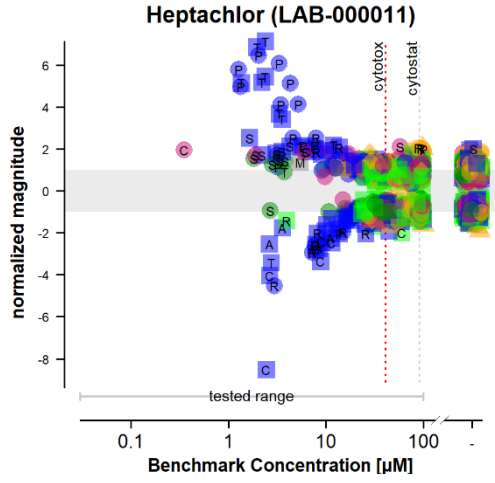
0.59

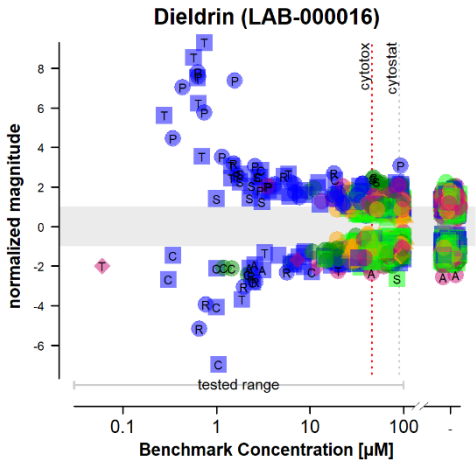


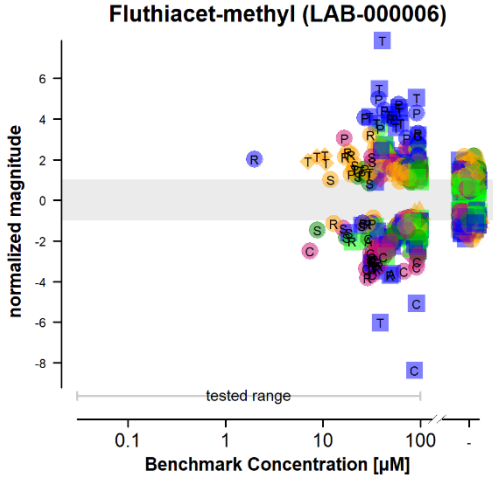
Aldrin
309-00-2 |
DTXSID8020040

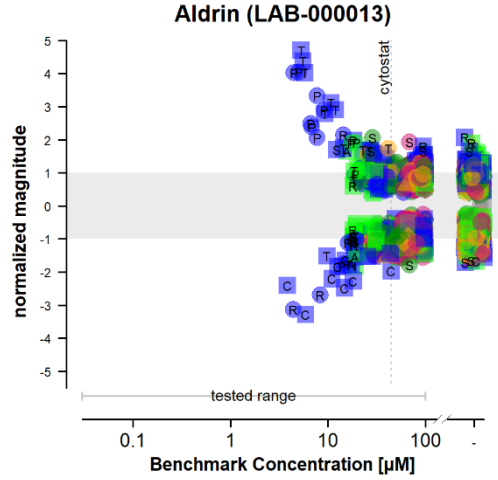






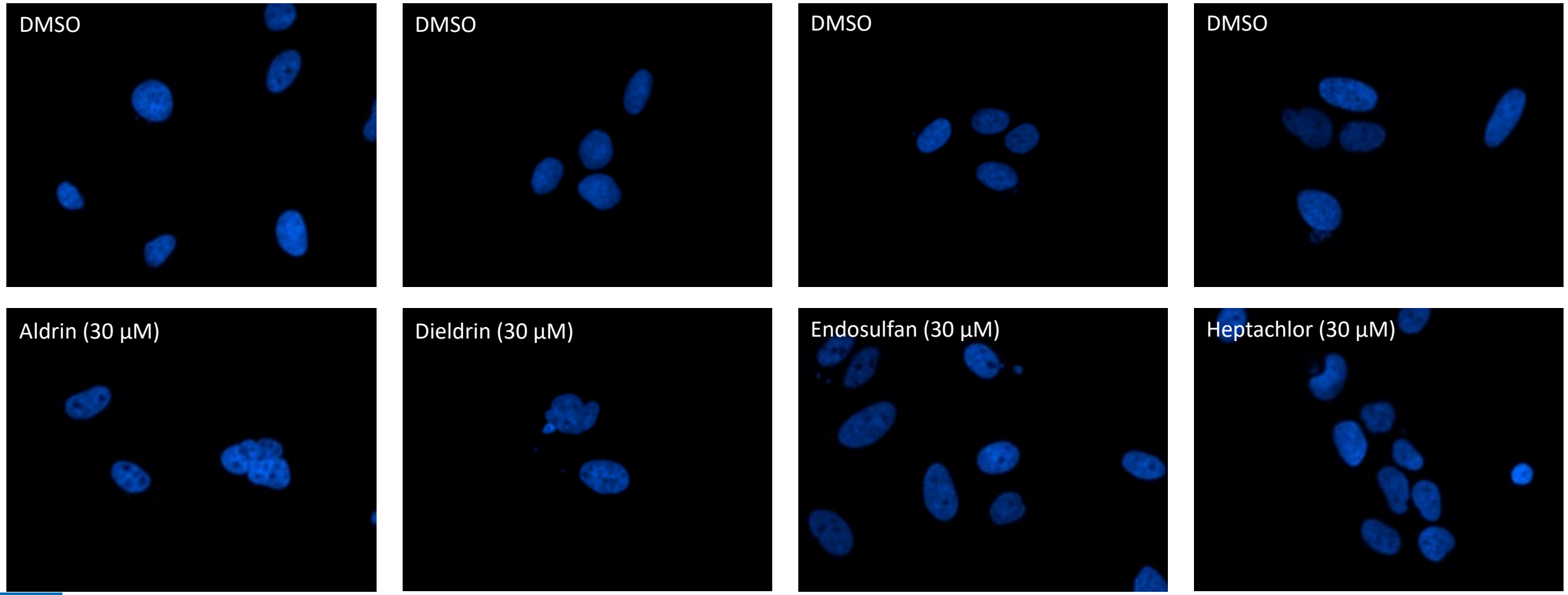






Organochlorine Pesticides

- Changes in nuclear texture manifest as “holes”.



Read Across Example (2)

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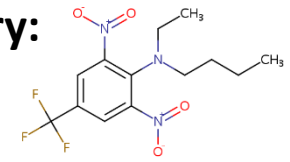
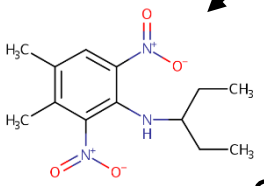


Matches:

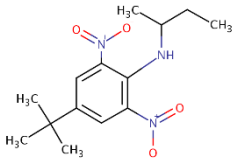
Benfluralin
1861-40-1 |
DTXSID3023899

0.75



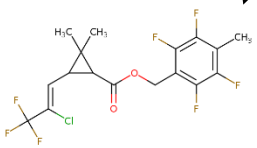
Pendimethalin
40487-42-1 |
DTXSID7024245

0.68



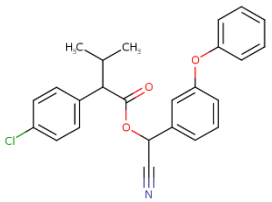
Butralin
33629-47-9 |
DTXSID3032337

0.75



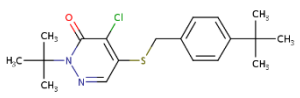
Tefluthrin
79538-32-2 |
DTXSID5032577

0.70



Fenvalerate
51630-58-1 |
DTXSID101017940

0.69



Pyridaben
96489-71-3 |
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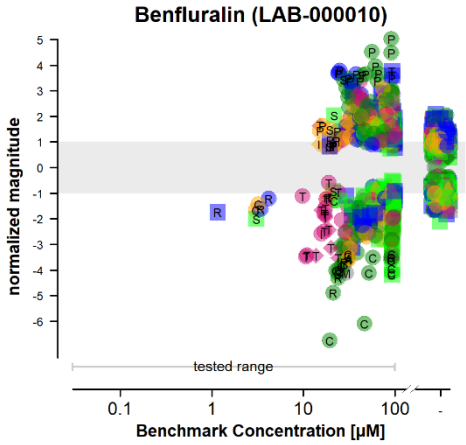

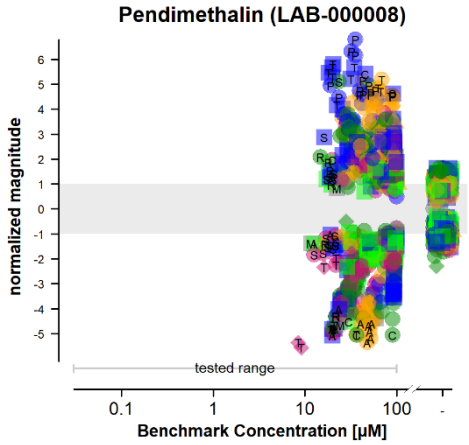

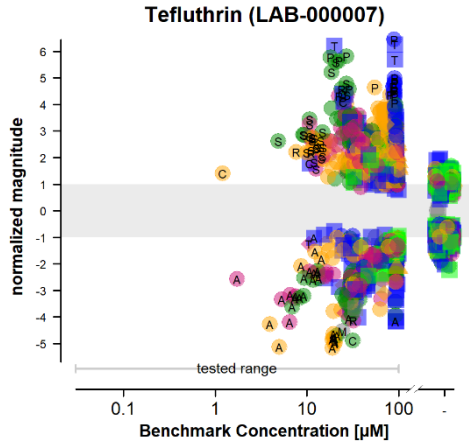

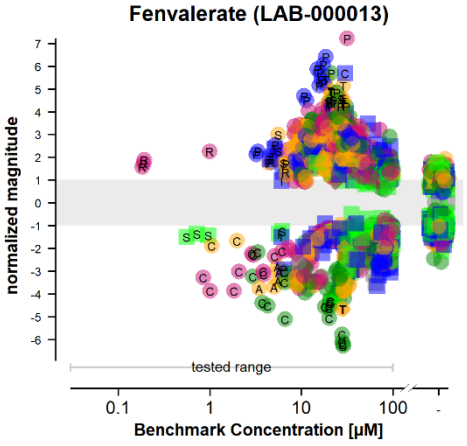

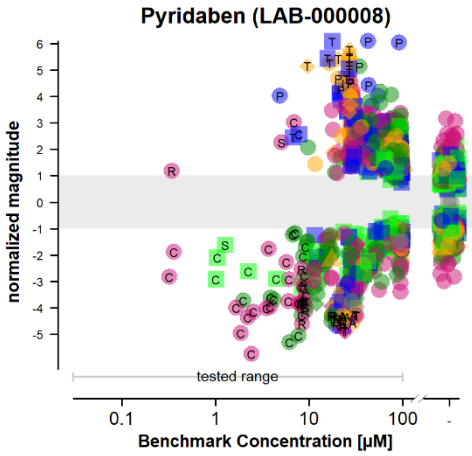

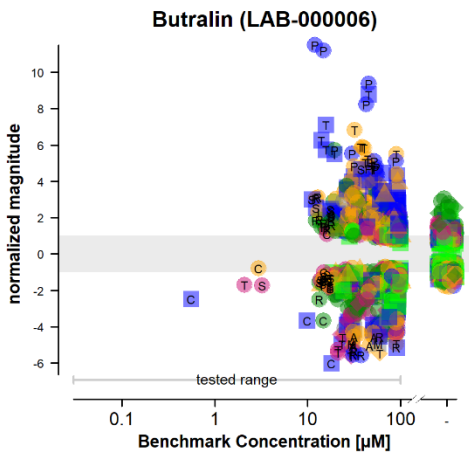

EPA United States Environmental Protection Agency

Summary

- **Workflows:** We have established wet lab and computational workflows for high-throughput transcriptomics (HTTr) and high-throughput phenotypic profiling (HTPP) of environmental chemicals in human-derived cell lines.
- **HTTr:** Evaluated a variety of alignment, normalization and concentration-response modeling approaches for TempO-Seq data, including a novel approach for pathway-level concentration-response modeling. Aggregating signal at the pathway level improves reproducibility and reduces uncertainty in screening results.
- **HTPP:** Developed a novel phenotypic categorization approach for analysis of Cell Painting data. Demonstrated reproducibility of potency estimates and phenotypic profiles using reference chemicals.
- **Screening:** Performed concentration-response screening of sets of environmental and reference chemicals using both technologies.
- **Bioactivity Exposure Ratio (BER):** HTTr and HTPP data may be used in combination with IVIVE and ExpoCast estimates to identify chemicals with bioactivity thresholds in relevant human exposure ranges.
- **Chemical Read Across:** Connectivity mapping and vector-based similarity approaches were able to identify chemicals with similar response profiles using data from the respective technologies.

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