EPA
United States
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Agency

High-Throughput Phenotypic Profiling with the Cell Painting Assay and Applications for Next Generation Risk Assessment

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Office of Research and Development

Michigan State University
Institute for Integrative Toxicology
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EPA United States Environmental Protection Agency

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Outline

- **Background**
  - **Computational Toxicology Research at EPA**
  - **New Approach Methods**

- **High-Throughput Phenotypic Profiling with Cell Painting**
  - **Assay Concept**
  - **Implementation at EPA**
    - *Laboratory workflows*
    - *Computational workflows*
    - *Study Design*
    - *Data infrastructure and accessibility*
  - **Bioactivity Screening Results**

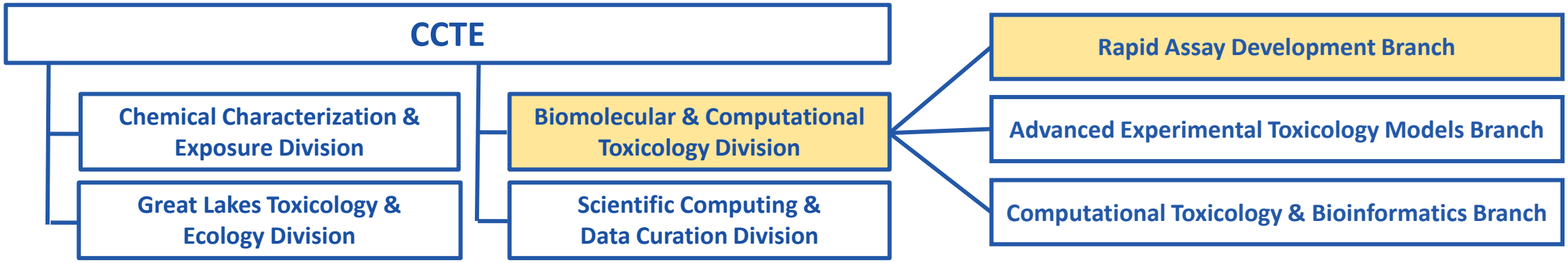
- **Applications for Chemical Risk Assessment**
  - **Bioactivity Exposure Ratio (BER) Analysis**
  - **Mechanistic Inference**
  - **Chemical Grouping**

Who is CCTE?

Research Triangle Park, NC

Center for Computational Toxicology and Exposure (CCTE)

A research organization at US EPA Office of Research and Development tasked with **developing** and **applying** cutting edge innovations in methods to rapidly evaluate chemical toxicity, transport and exposure to people and environments.



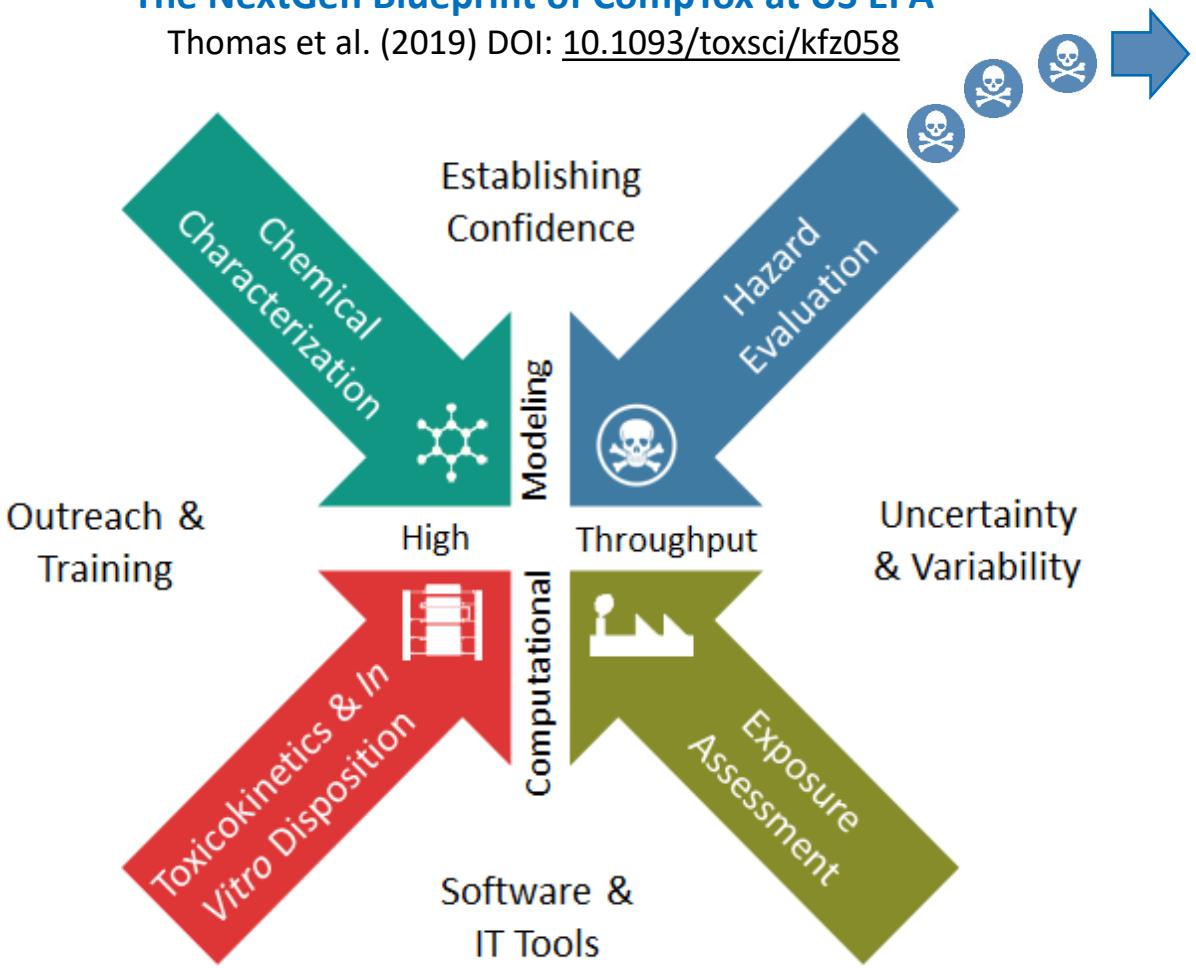
Rapid Assay Development Branch (RADB)

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.

EPA CompTox Blueprint Research Areas

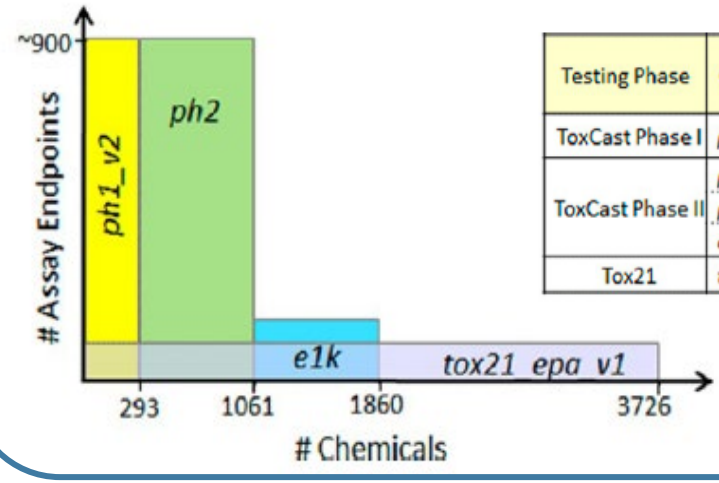
The NextGen Blueprint of CompTox at US EPA

Thomas et al. (2019) DOI: 10.1093/toxsci/kfz058



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

Richard et al. (2016) DOI: 10.1021/acs.chemrestox.6b00135



Testing Phase	Chemical Set	Unique Chemicals	Assay Endpoints
ToxCast Phase I	*ph1_v1*	310	~700
ToxCast Phase II	*ph1_v2*	293	~200
	ph2	768	~900
	e1k	799	~50
Tox21	*tox21_epa_v1*	3726	~80

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

New Strategy for Hazard Evaluation: Improve efficiency and increase biological coverage by using broad-based (i.e. non-targeted) assays that cast the broadest net possible for capturing the potential molecular and phenotypic responses of human cells in response to chemical exposures.

NAMs-Based Tiered Hazard Evaluation Approach

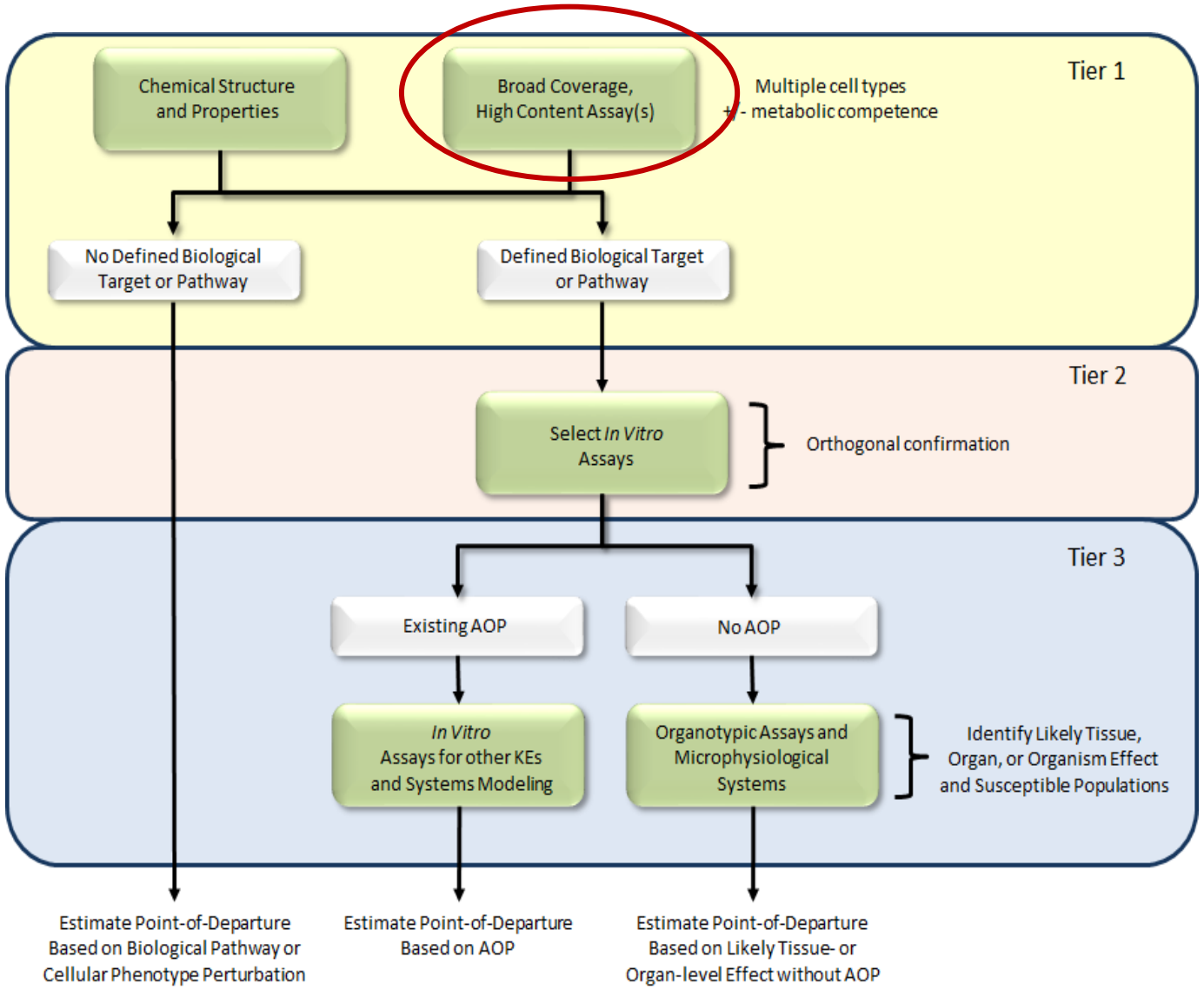
High throughput profiling (HTP) assays are proposed as the first tier in a NAMs-based hazard evaluation approach.

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.

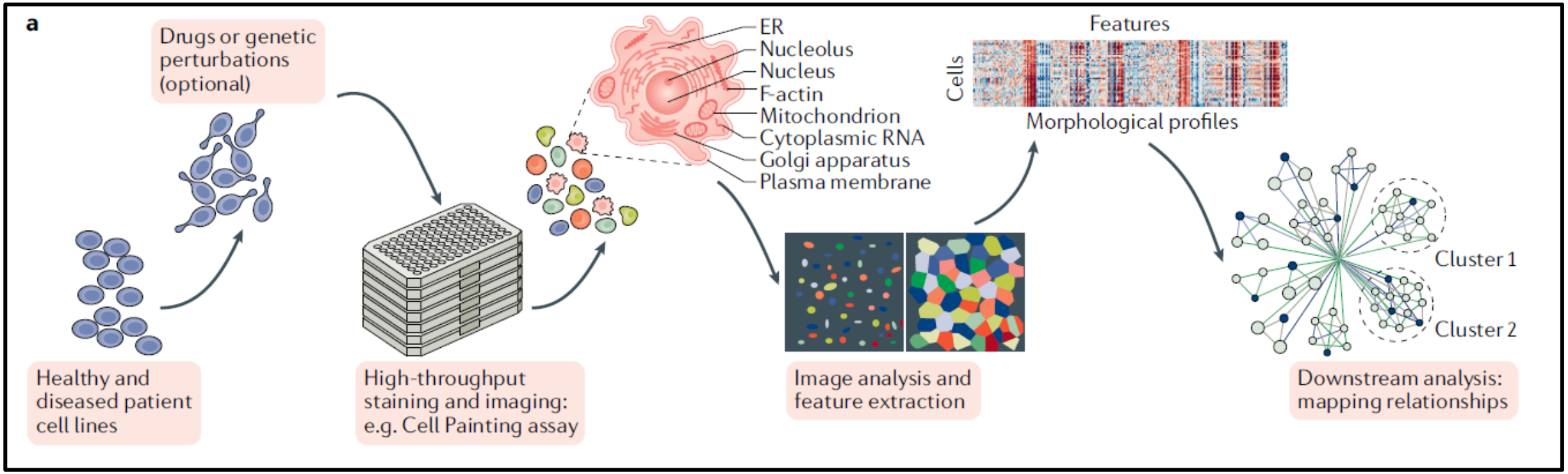
To date, EPA has identified and implemented two HTP assays that meet these criteria.

- **High-Throughput Phenotypic Profiling [HTPP]**
- **High-Throughput Transcriptomics [HTTr]**



The NexGen Blueprint of CompTox at US EPA
Thomas et al. (2019) DOI: 10.1093/toxsci/kfz058

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.
- **Cell Painting** originated at the Broad Institute of Harvard & MIT as a non-targeted approach to characterize chemical- and disease-associated cell states and support future probe (i.e. drug) discovery.

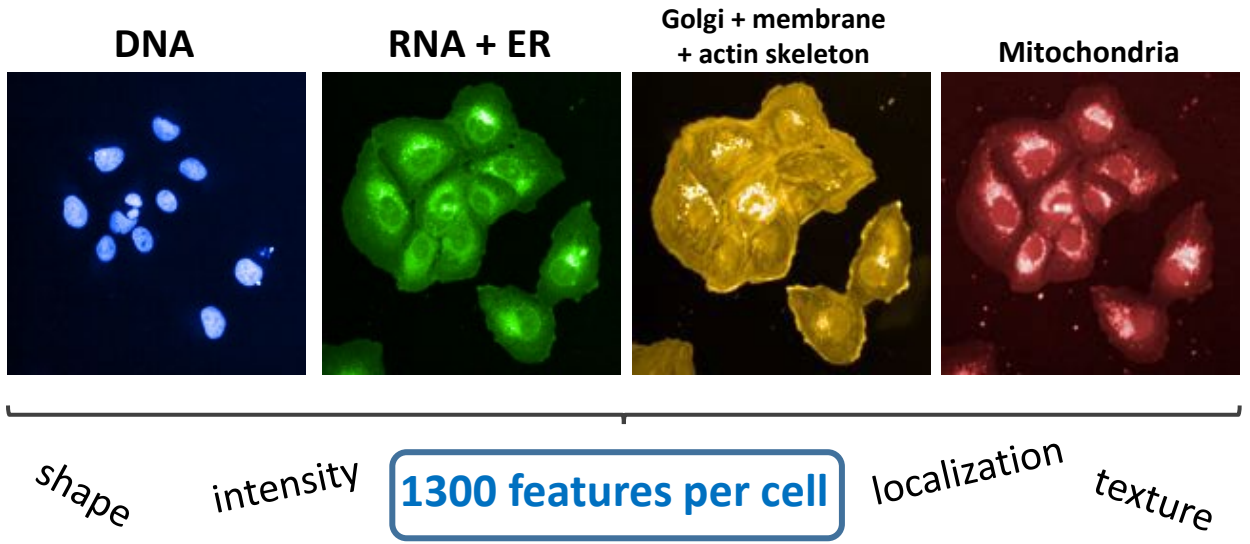
High Throughput Phenotypic Profiling with Cell Painting

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

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

- 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



Cell Painting Method & Implementation at EPA

Cell Painting, a high-content image-based assay for morphological profiling using multiplexed fluorescent dyes

Mark-Anthony Bray[1], Shantanu Singh[1], Han Han[2], Chadwick T Davis[2], Blake Borgeson[2], Cathy Hartland[3], Maria Kost-Alimova[3], Sigrun M Gustafsdottir[3], Christopher C Gibson[2] & Anne E Carpenter[1]

[1]Imaging Platform, Broad Institute of Harvard and MIT, Cambridge, Massachusetts, USA. [2]Recursion Pharmaceuticals, Salt Lake City, Utah, USA. [3]Center for the Science of Therapeutics, Broad Institute of Harvard and MIT, Cambridge, Massachusetts, USA. Correspondence should be addressed to C.C.G. (chris.gibson@recursionpharma.com) or A.E.C. (anne@broadinstitute.org).

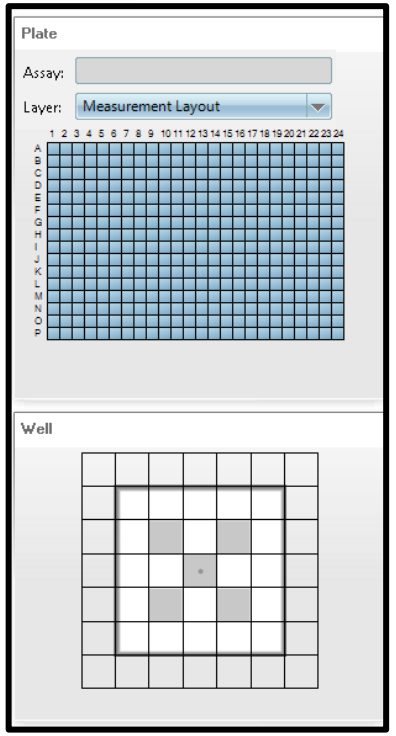
NATURE PROTOCOLS | VOL.11 NO.9 | 2016 | 1757

The Cell Painting assay was implemented at EPA based on Bray et al. (2016) (PMID: 27560178), with modifications:

- **Sample preparation** using in house microfluidics / lab automation.
- **Image acquisition** on in house high content imaging platform.
- **Image analysis** on Harmony® analysis software.
- **Experimental optimizations**:
  - Chemical exposure duration (24 hr).
  - U-2 OS seeding density (600[a] or 3,000[b] cells / well)

PerkinElmer Opera Phenix

Modality:	Confocal (single *z*)
Objective:	20X Water
Plate:	PhenoPlate 384
Fields / well:	5



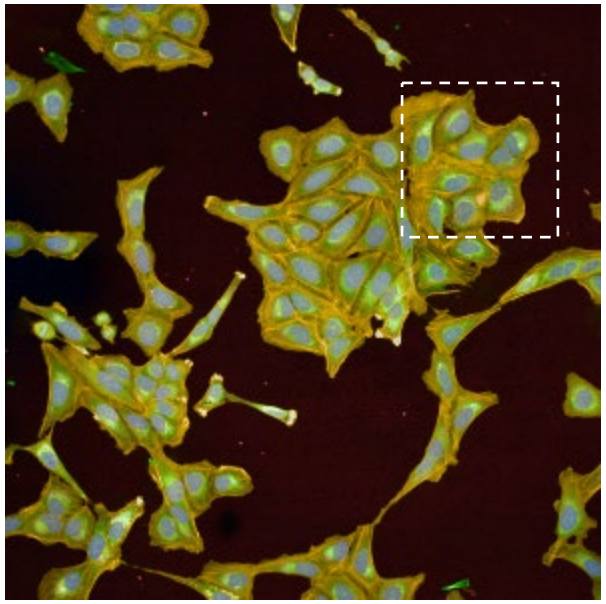
[a] *Nyffeler et al. 2020 (PMID:31899216)* [b] *Nyffeler et al. 2023 (PMID:37044265)*

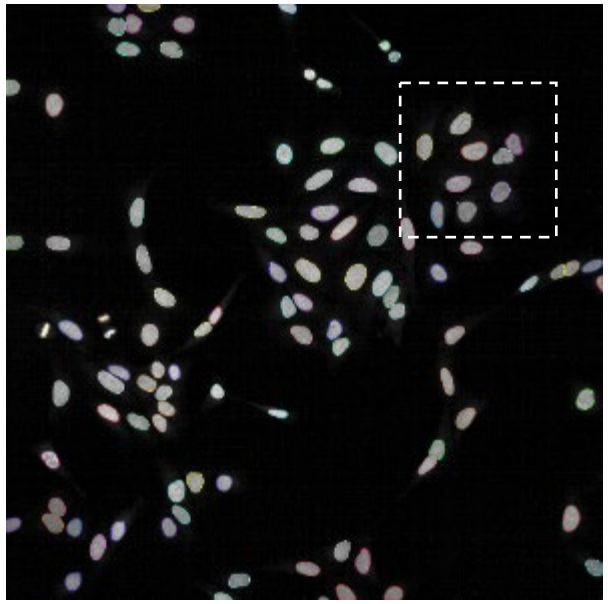
Image Analysis Workflow → Image Segmentation

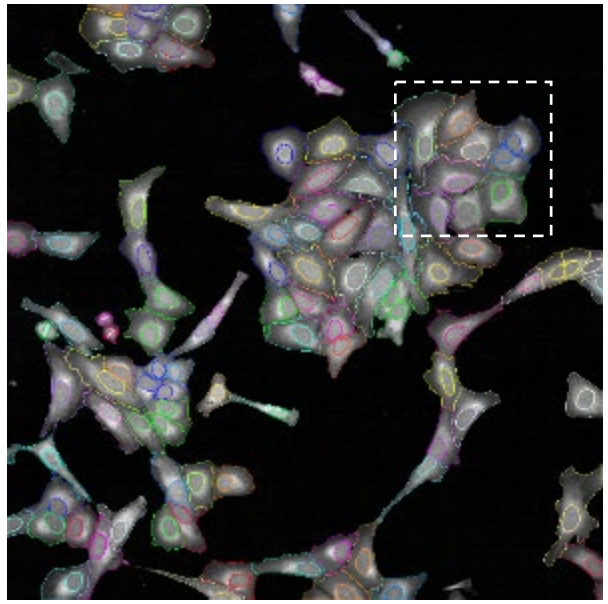
1. find nuclei

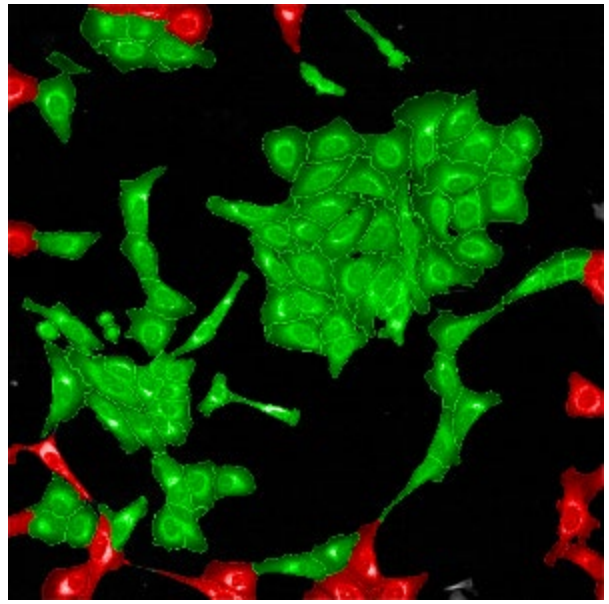
2. find cell outline

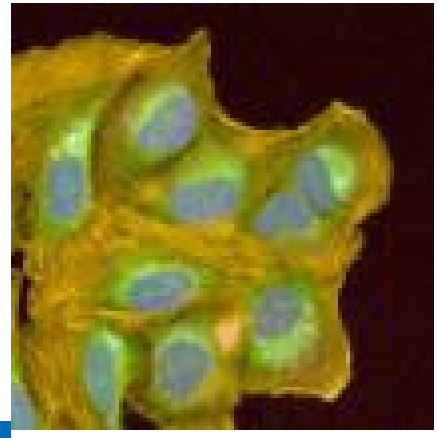
3. reject border objects

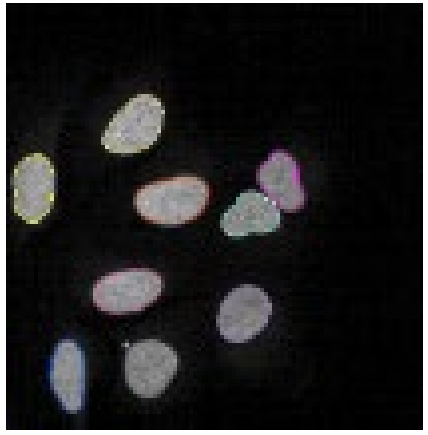












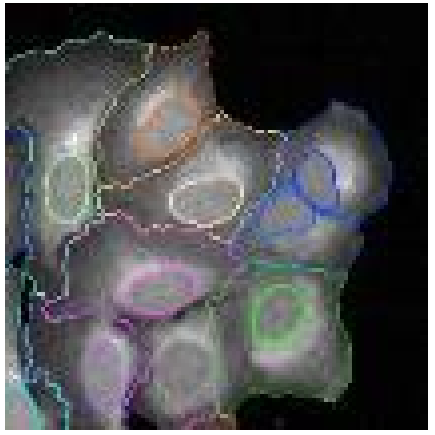
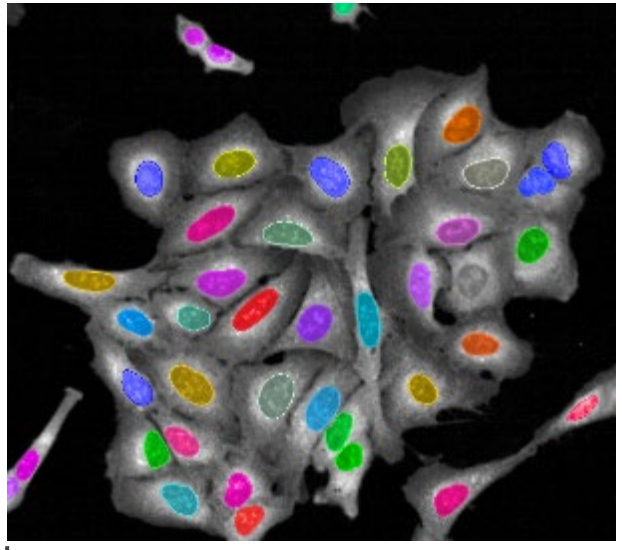
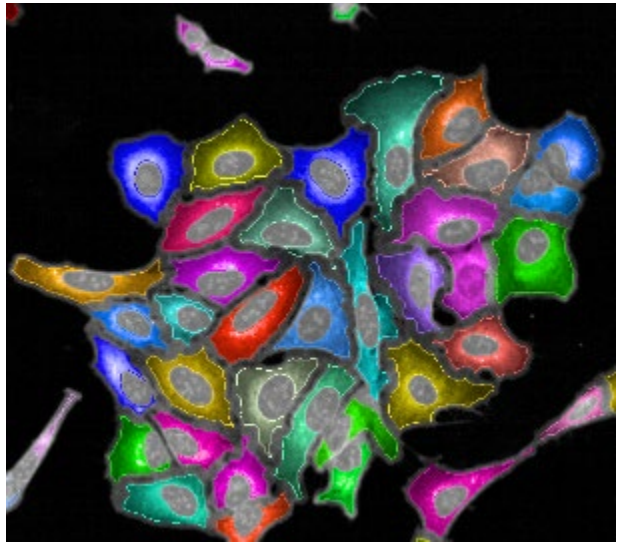


Image Analysis Workflow → Define Cellular Compartments

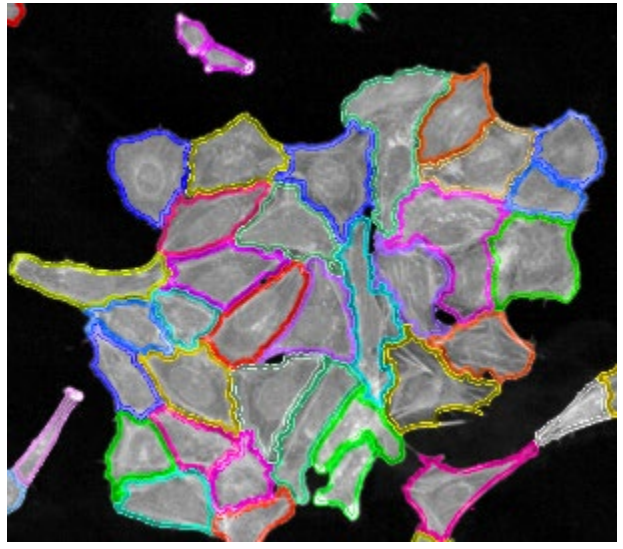
nuclei



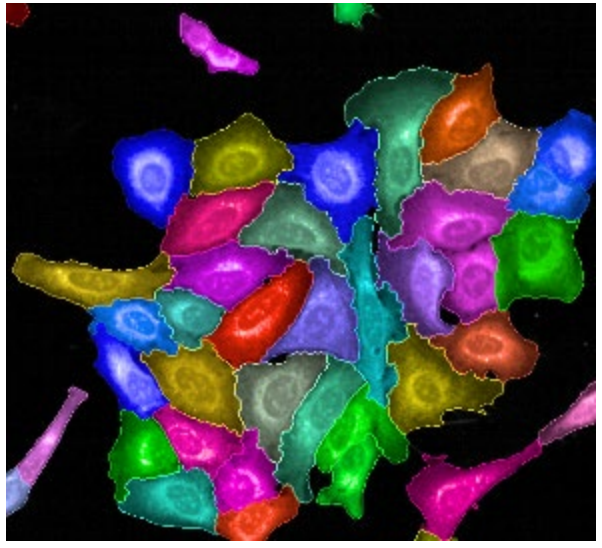
cytoplasm



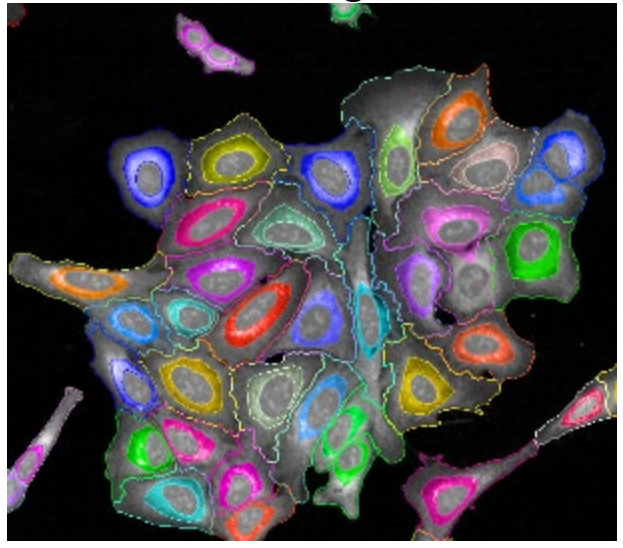
membrane



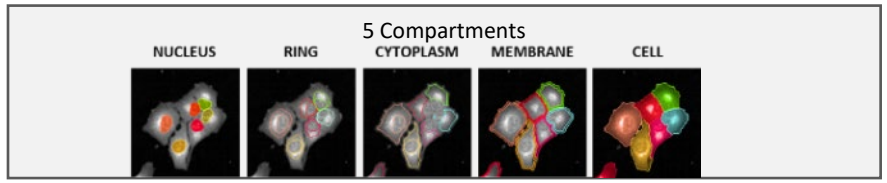
cell

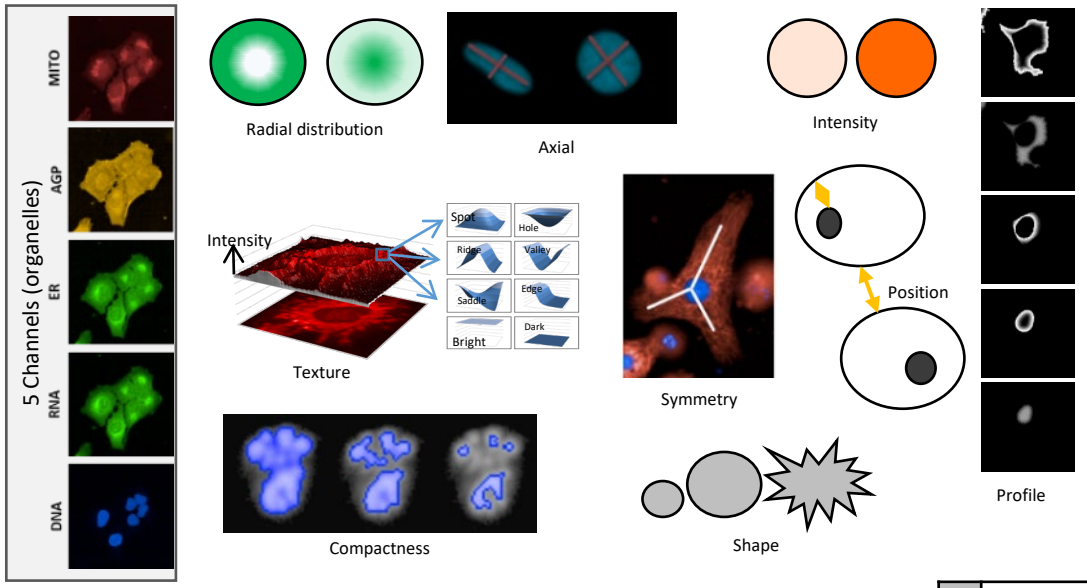


ring



Phenotypic Feature Extraction





49 Feature Categories
(ex. MITO_Texture_Cytoplasm)

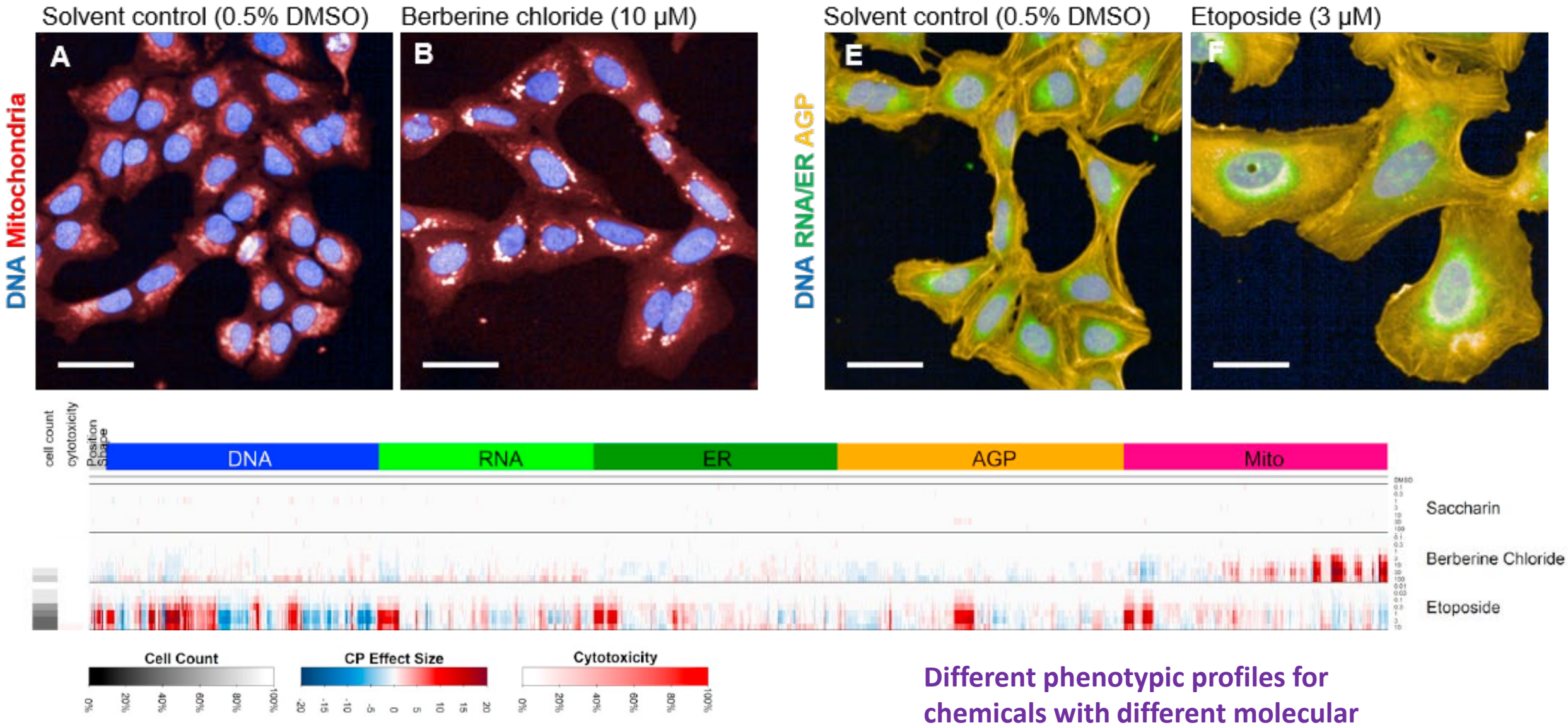
1300 features / cell

PerkinElmer Harmony®

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							

With illustrations from Perkin Elmer

Phenotypic Changes Measured Quantitatively with HTPP



Different phenotypic profiles for chemicals with different molecular targets!

Adapted from Nyffeler et al. 2020 (PMID:31899216)

Data Analysis Pipeline

Jo **Nyffeler**

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

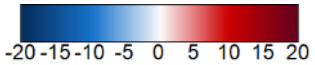
well-level data

Standardization
Z transformation

Cell Count Info
Conc. > 50% cell loss

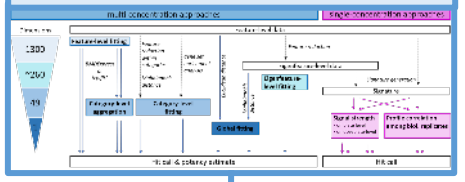
scaled well-level data

clipped well-level data



Concentration Response Modeling

Calculate Response Metrics



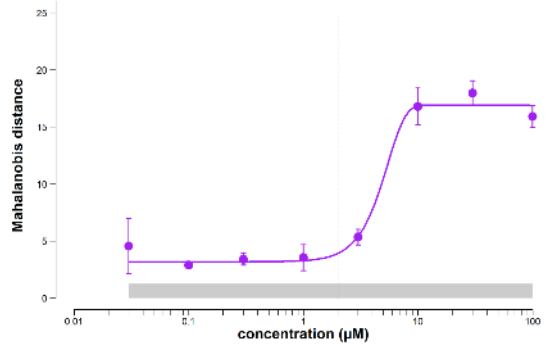
Nyffeler et al. (2021) SLAS Discov. (PMID: 32862757)

Fit Multiple Curve Shapes

tcplfit2

Sheffield et al. (2022) Bioinformatics (PMID: 34791027)

Best Model Selection



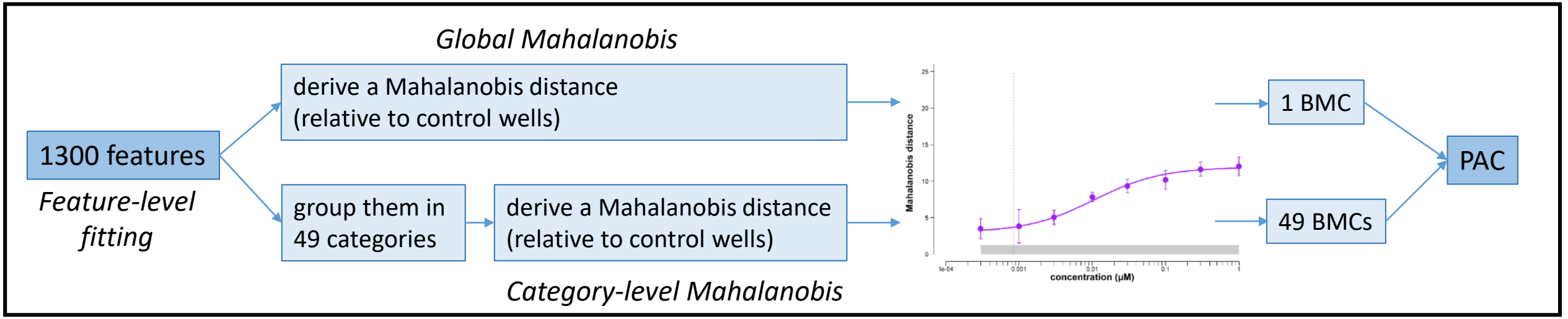
BMC

Slide courtesy of Jo Nyffeler

Phenotype Altering Concentrations (PACs)

Mahalanobis Distance (D_M):

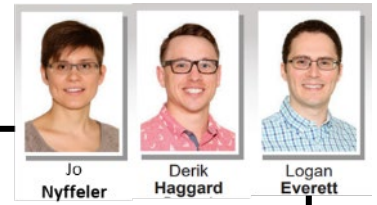
- A multivariate metric that measures the distance between a treatment and a distribution of controls in feature space.
- Accounts for unpredictable changes in cell states across test concentrations and inherent correlations in profiling data.

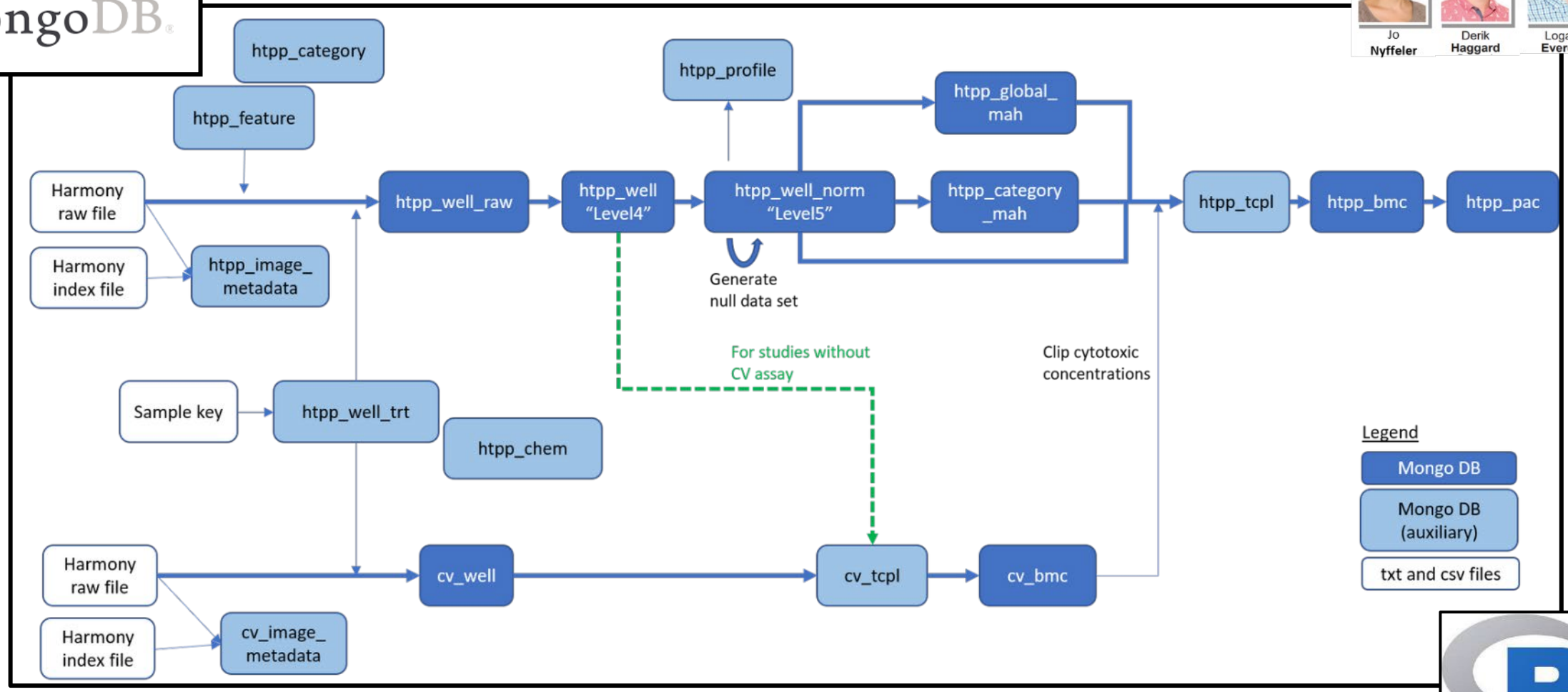


- Chemicals where a BMC can be determined using either the global or category D_M approach are considered **active**.
- The minimum of the global or most sensitive category BMC is the **Phenotype Altering Concentration (PAC).**
- The PAC is the **molecular point-of-departure (mPOD)** used for downstream NGRA applications.

Slide courtesy of Jo Nyffeler

Data Management Using Open Source Tools

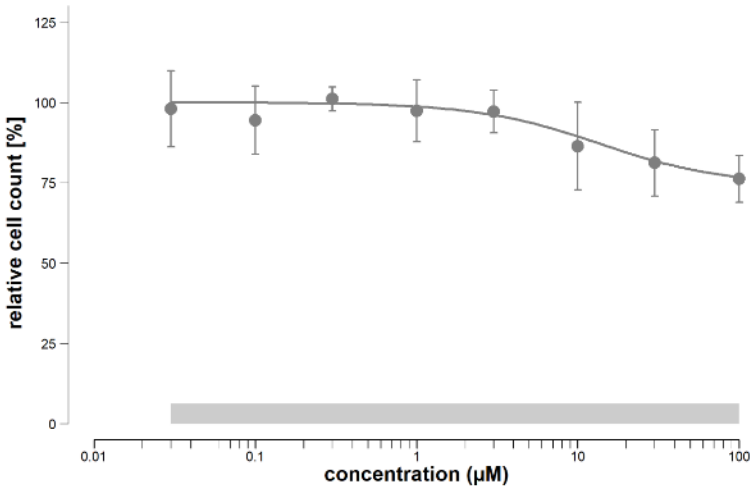




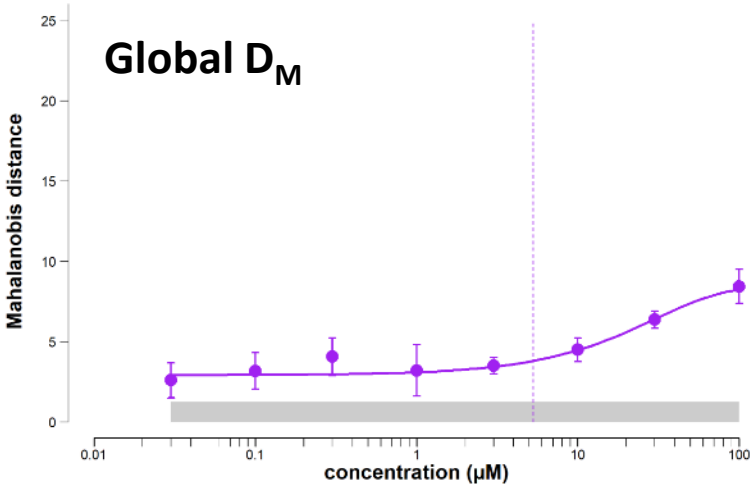
- MongoDB used to house, access and selectively query analysis results stored in collections.
- Working to establish public-facing GitHub repository with pipelining scripts.

Data Visualization

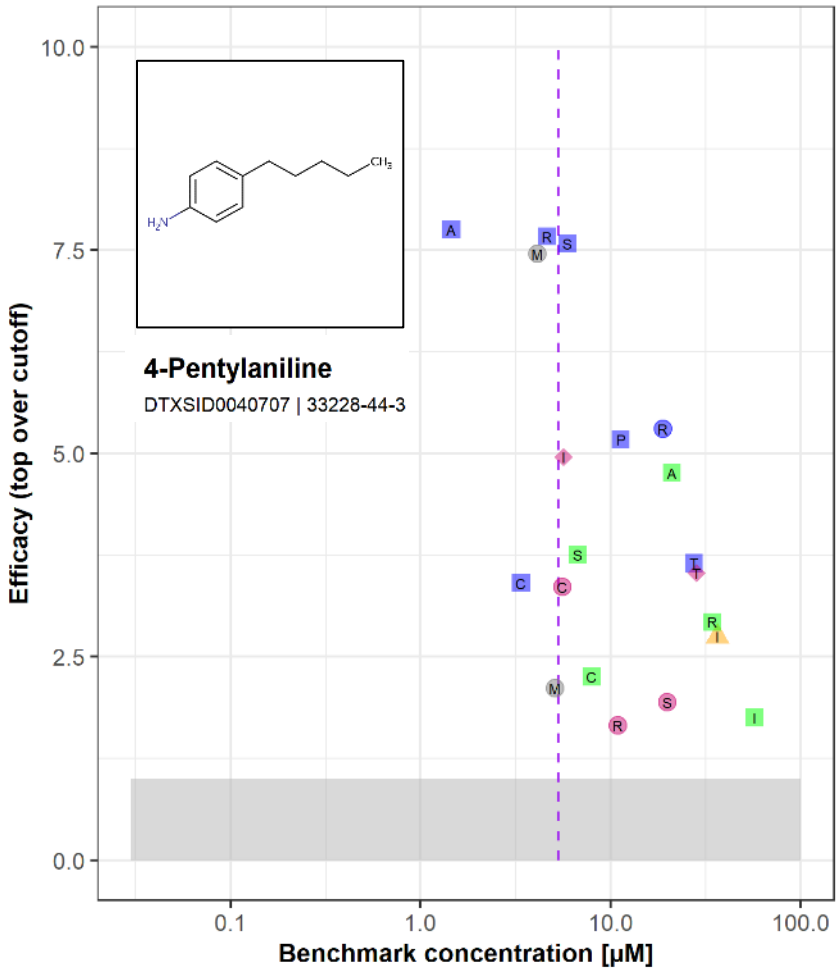
Cell Count



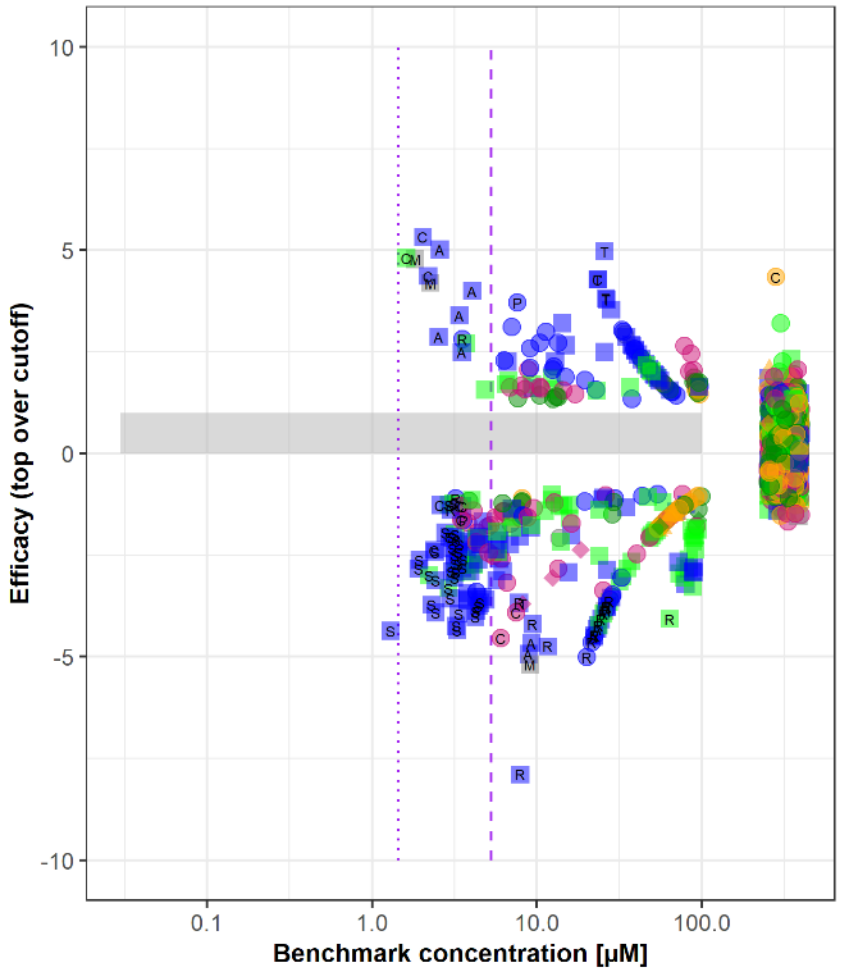
Global D_M



Categories



Features

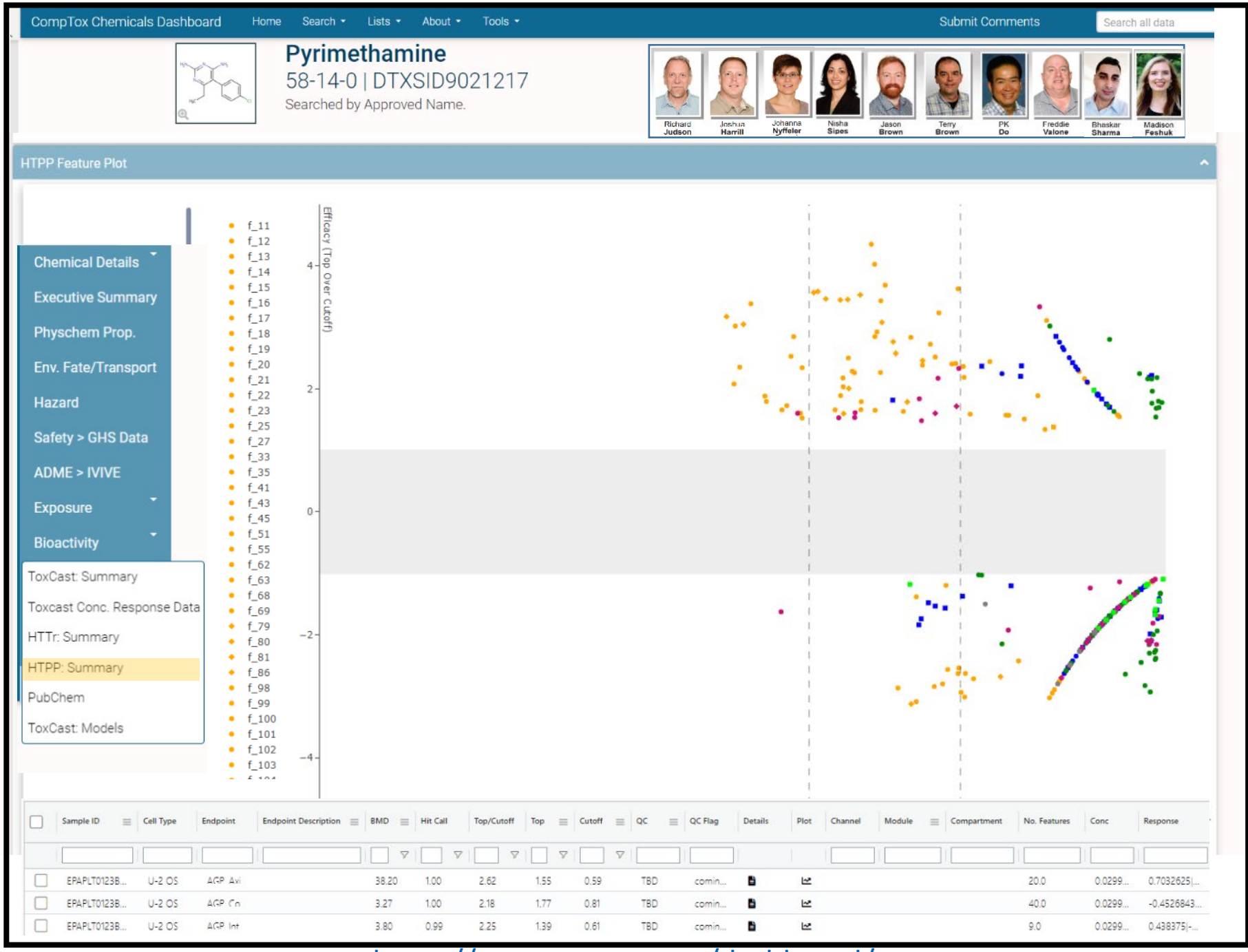


- Potency magnitude plots aid in visualization of phenotypic effects that are observed below threshold for cytotoxicity.
- Nucleus most profoundly affected by 4-Pentylaniline, followed by nucleolus an mitochondria.

Preliminary results. Do not cite or quote.

Public Data Accessibility

- Feature (and category) conc-response modeling results displayed as potency-magnitude plots.
- Features categorized according fluorescent channels / organelles.
- Filter according to multiple parameters.
- Data summarized and searchable in interactive table.



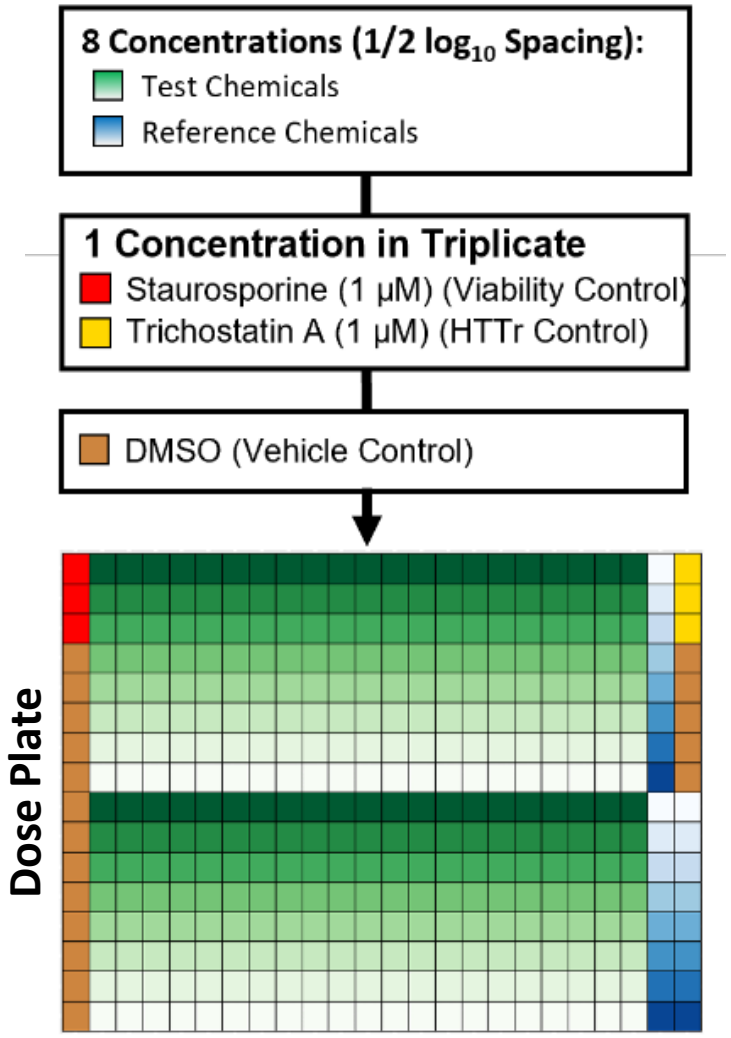
https://comptox.epa.gov/dashboard/

EPA United States Environmental Protection Agency

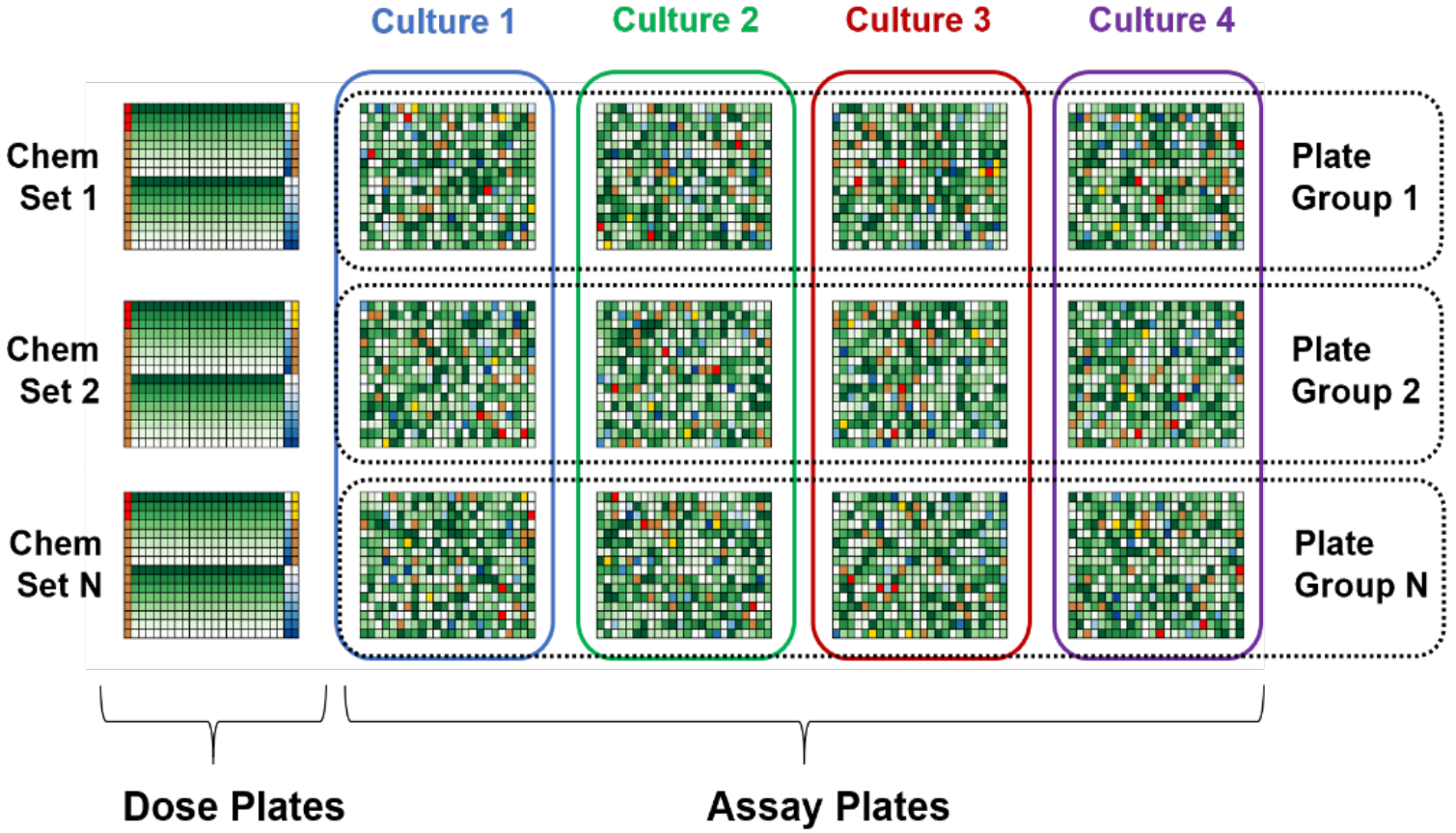
HTPP Bioactivity Screening Results

Experimental Design

Dose Plate Configuration



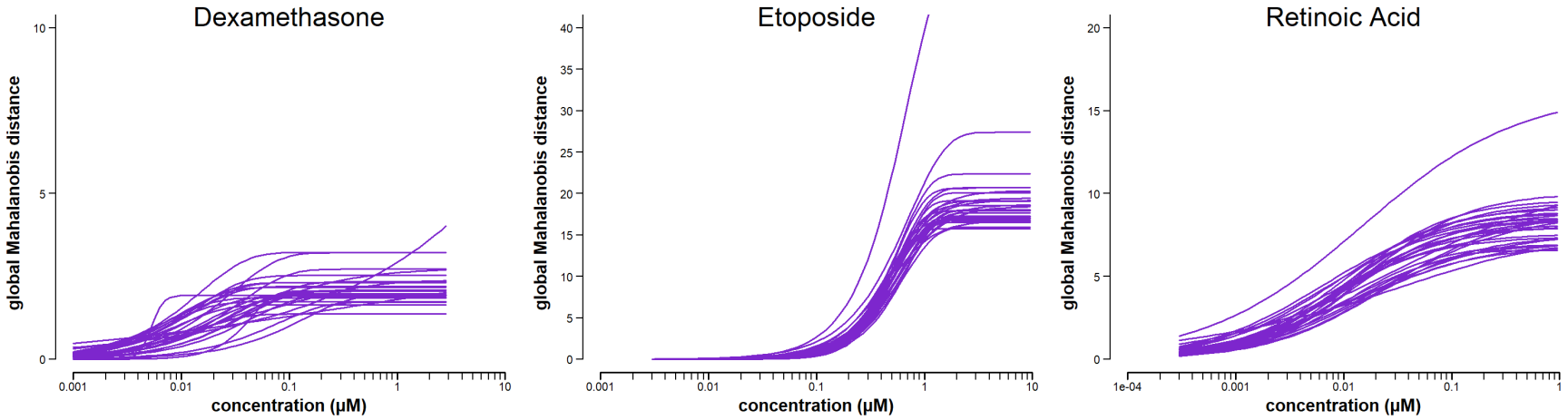
Screening Study Design

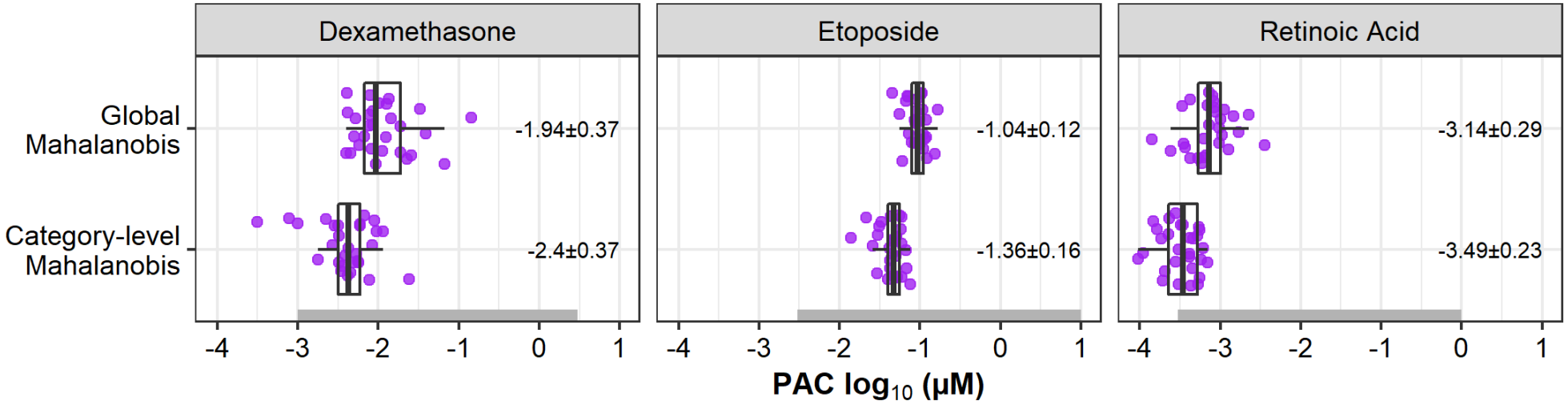


- Treatment randomization
- Biological replication across independent cultures
- Reference chemicals for tracking assay performance

Reproducibility of PACs

Chemicals screened 29 times over many months / weeks





- Reference chemicals tested over many plate groups (weeks / months) yield reproducible potency values.
- PACs vary by < 1/2 order of magnitude.

Preliminary results. Do not cite or quote.

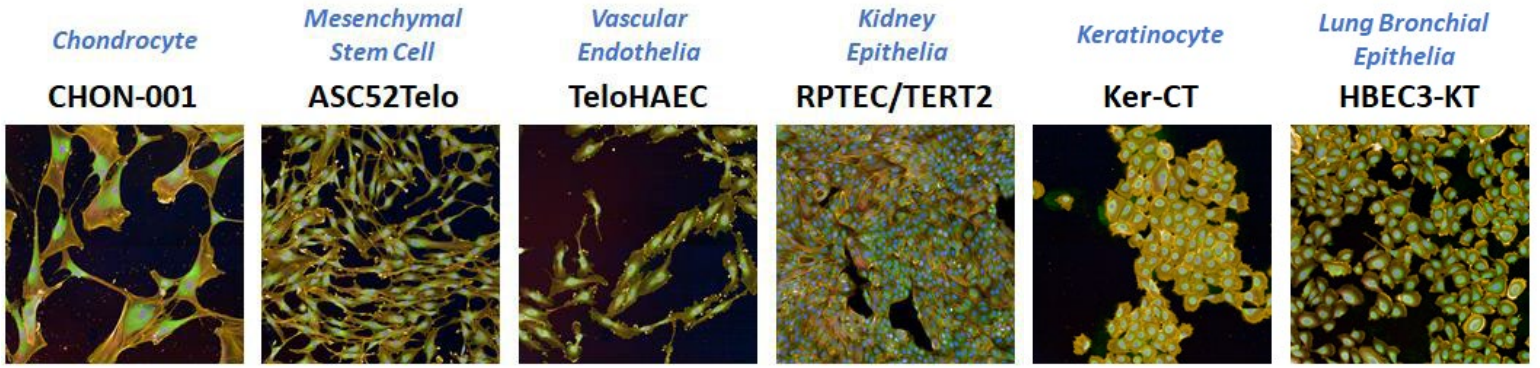
Surveying Biology Using Complementary Cell Lines

Clinton Willis

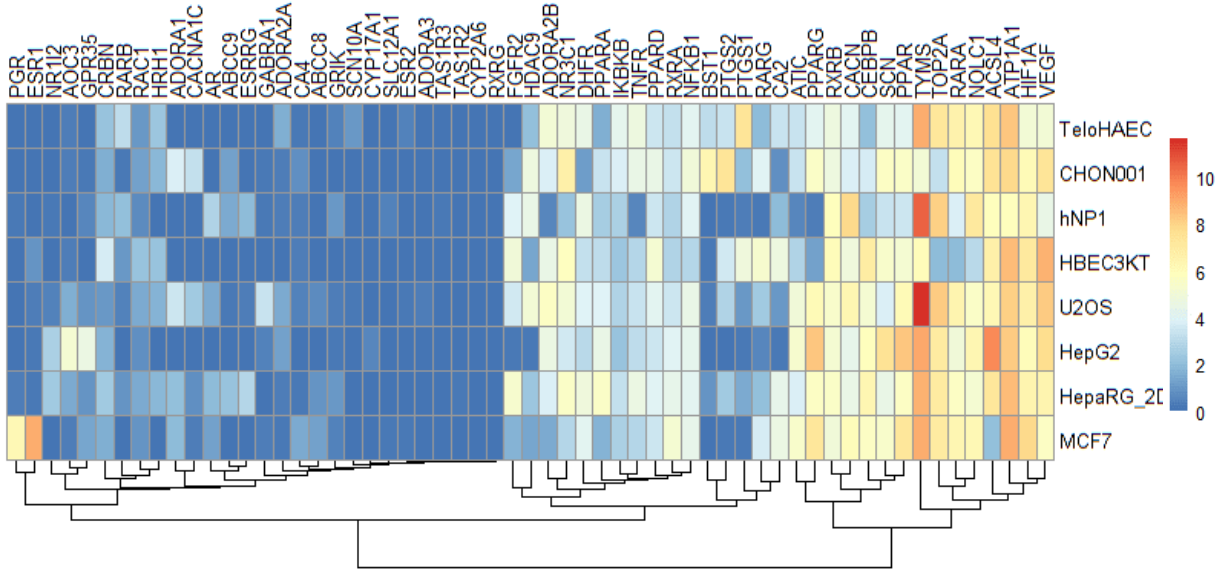
Ranked List of Biologically Diverse Cell Lines Determined by Data Driven Cell Line Selection (hTERT Immortalized Cells & Seeds)

Cell_Type	Tissue_Origin	Derivation	Growth_Mode
MCF-7	Breast	Cancer	Adherent
U-2 OS	Bone	Cancer	Adherent
HBEC3-KT	Lung	Immortalized	Adherent
TeloHAEC	Vascular	Immortalized	Adherent
RPTEC	Kidney	Immortalized	Adherent
Ker-CT	Skin	Immortalized	Adherent
hNP1	CNS	Immortalized	Adherent
CHON-001	Fibroblast	Immortalized	Adherent
ARPE-19	Retina	Immortalized	Adherent
CCD-18Co	Fibroblast	Immortalized	Adherent
ASC52telo	Mesenchymal Stem Cell	Immortalized	Adherent
BJ-5ta	Fibroblast	Immortalized	Adherent
HME-1	Breast	Immortalized	Adherent
HPNE	Pancreas	Immortalized	Adherent
TIME	Vascular	Immortalized	Adherent
RPE-1	Retina	Immortalized	Adherent
HUVEC	Vascular	Immortalized	Adherent
HSAEC-1	Lung	Immortalized	Adherent

Morphological Heterogeneity Across Diverse Cell Lines

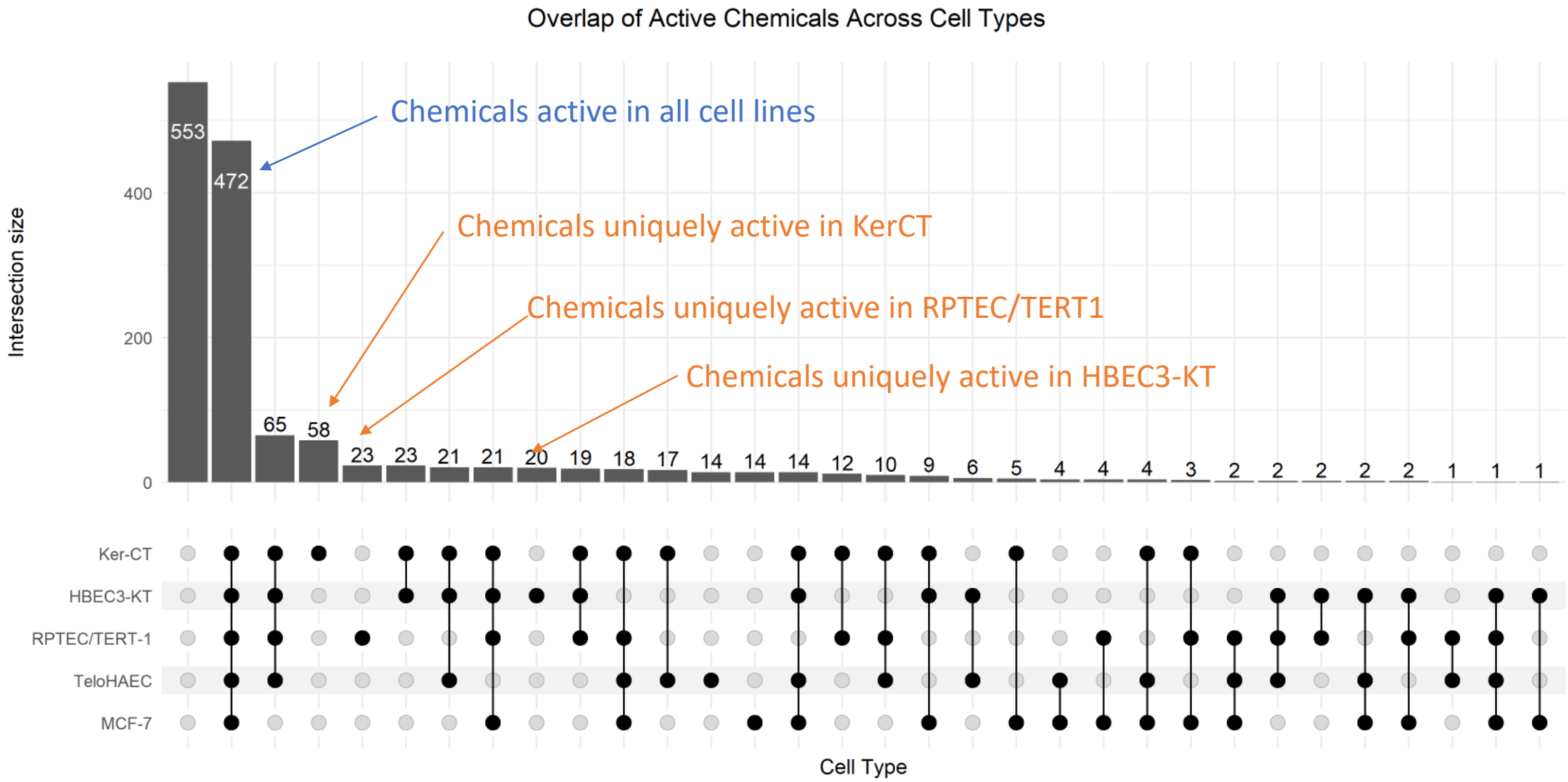


Variation in Molecular Target Expression Across Diverse Cell Lines

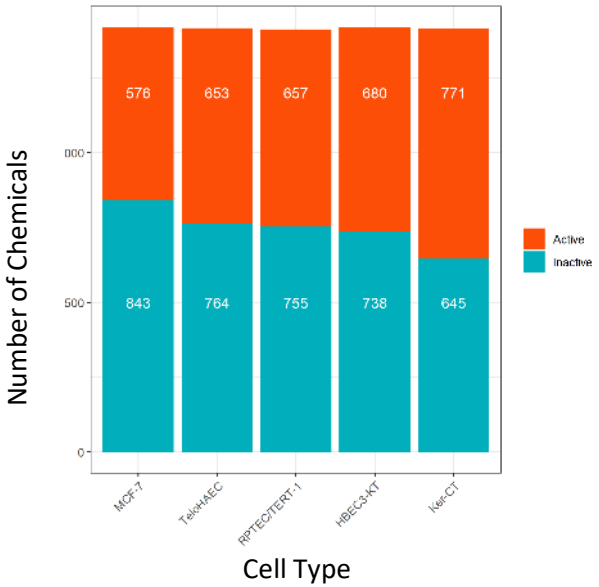


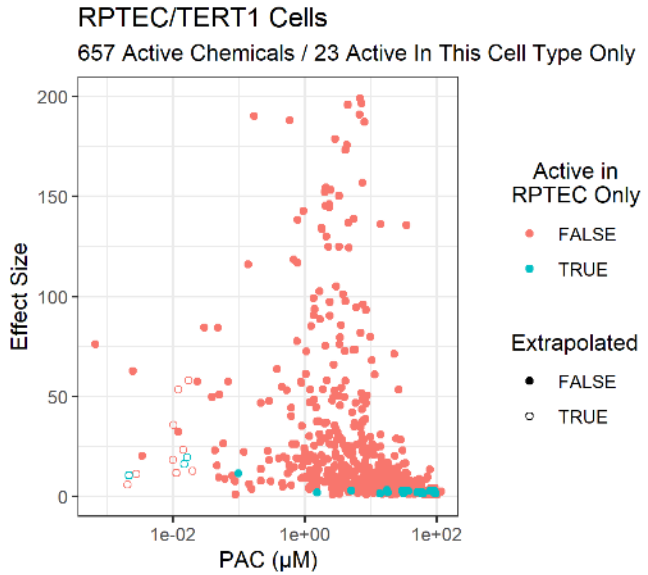
Preliminary results. Do not cite or quote.

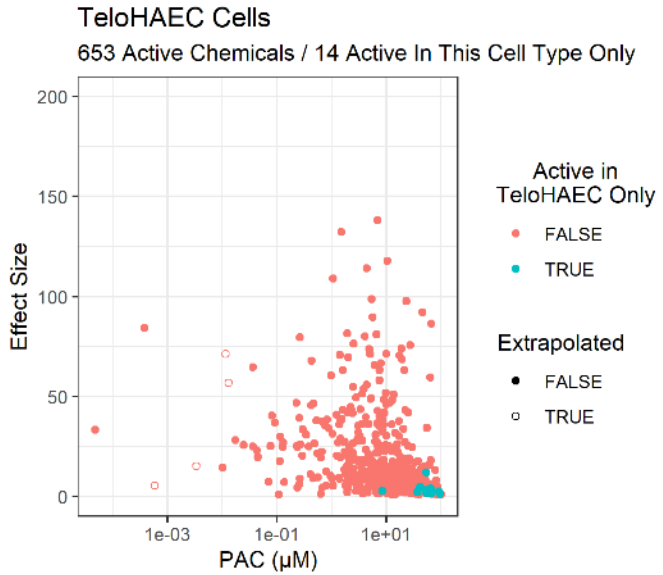
Comparison of HTPP Bioactivity Hits Across Cell Lines (1)

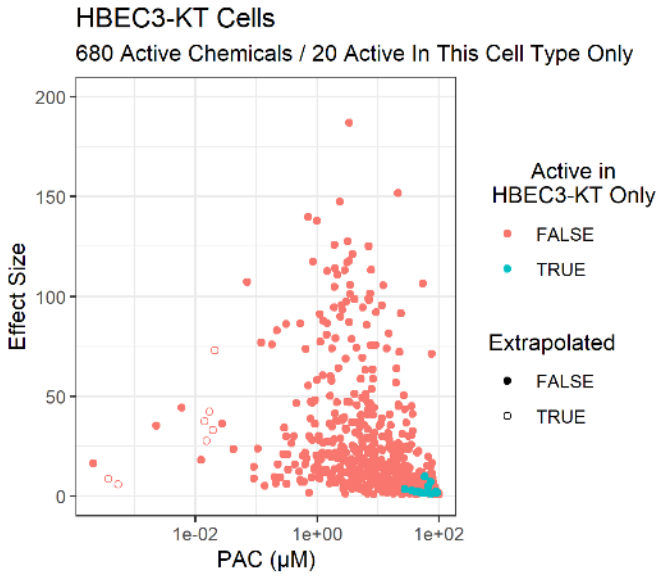


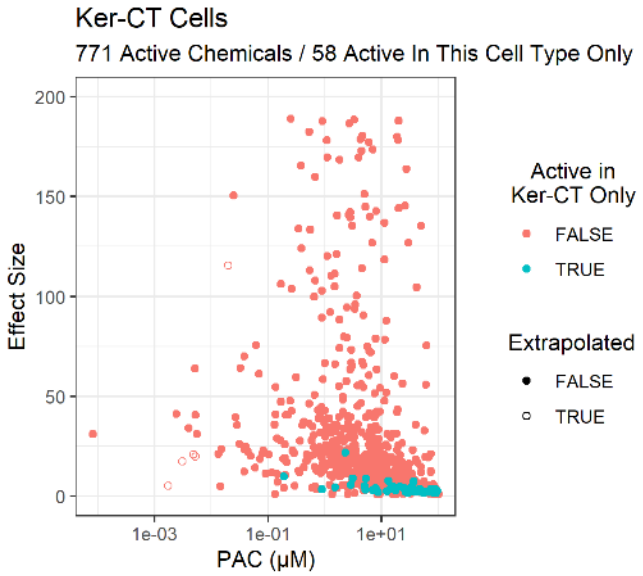
Comparison of HTPP Bioactivity Hits Across Cell Lines (2)

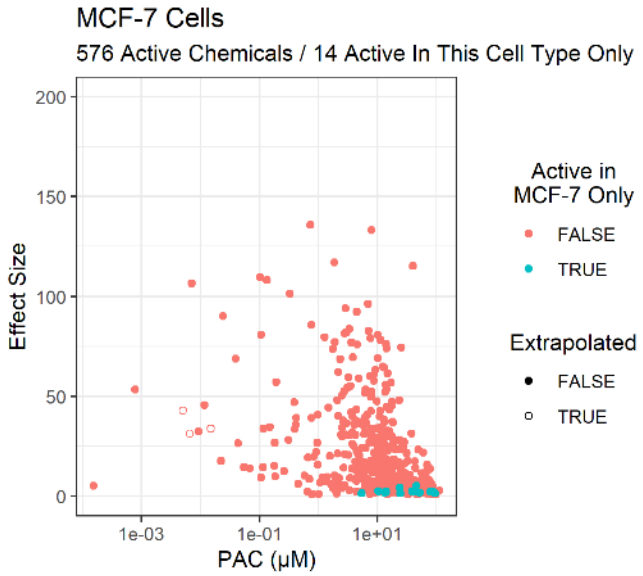




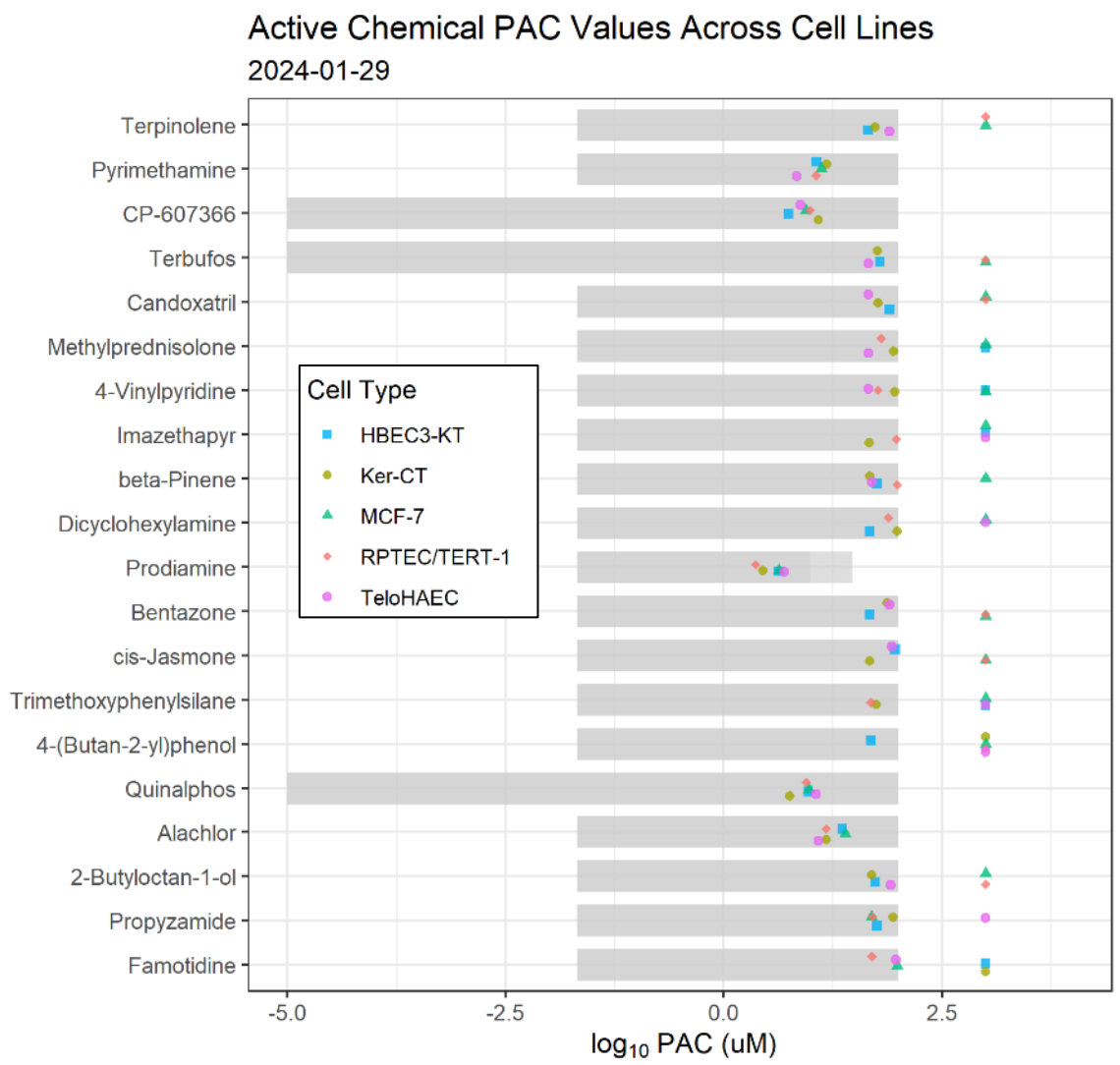






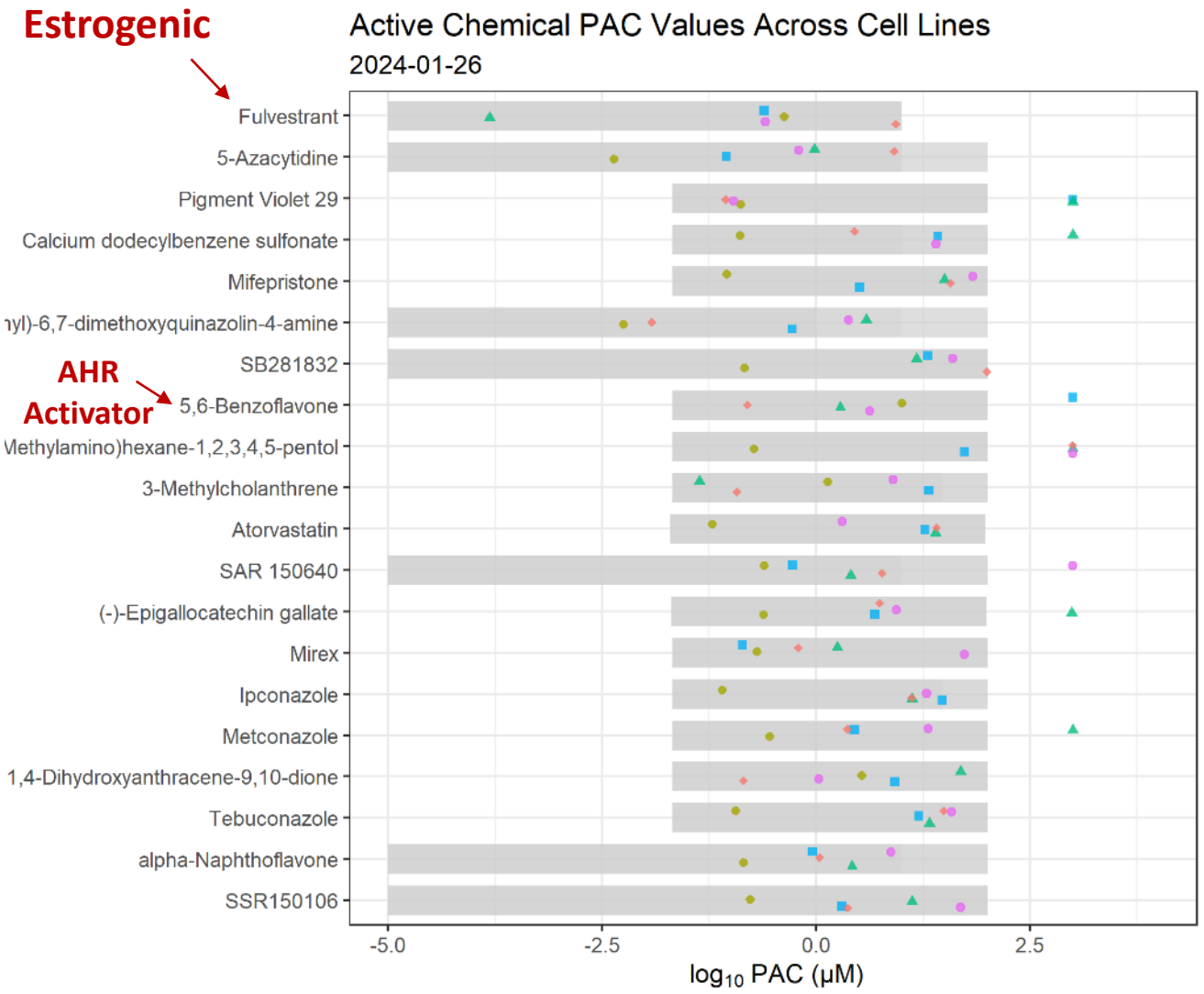


Comparison of HTPP PACs Across Cell Lines



Estrogenic

AHR Activator



- PACs can vary by several orders of magnitude across biologically diverse cell lines.

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Chemical Prioritization Using Bioactivity Exposure Ratio (BER)

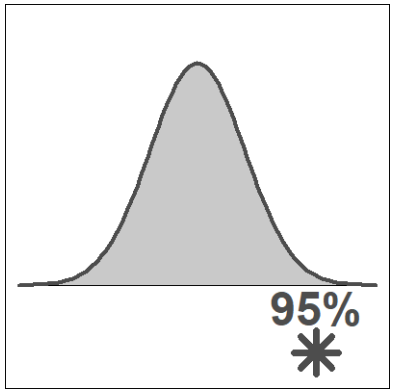
Chemical Prioritization Using Bioactivity Exposure Ratio (BER) Analysis

EPA ExpoCast
Exposure inferred from human biomonitoring data, production volume and use categories (industrial / consumer use)

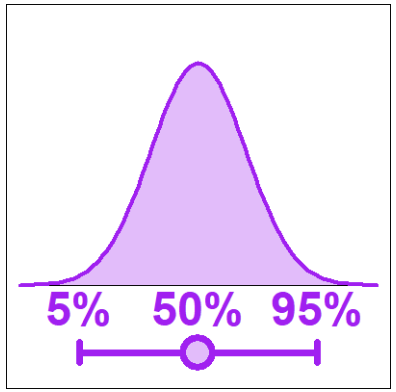
Phenotype Altering Concentration (PAC) (µM)

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

SEEM3 Human Exposure Prediction (mg/kg bw/day)

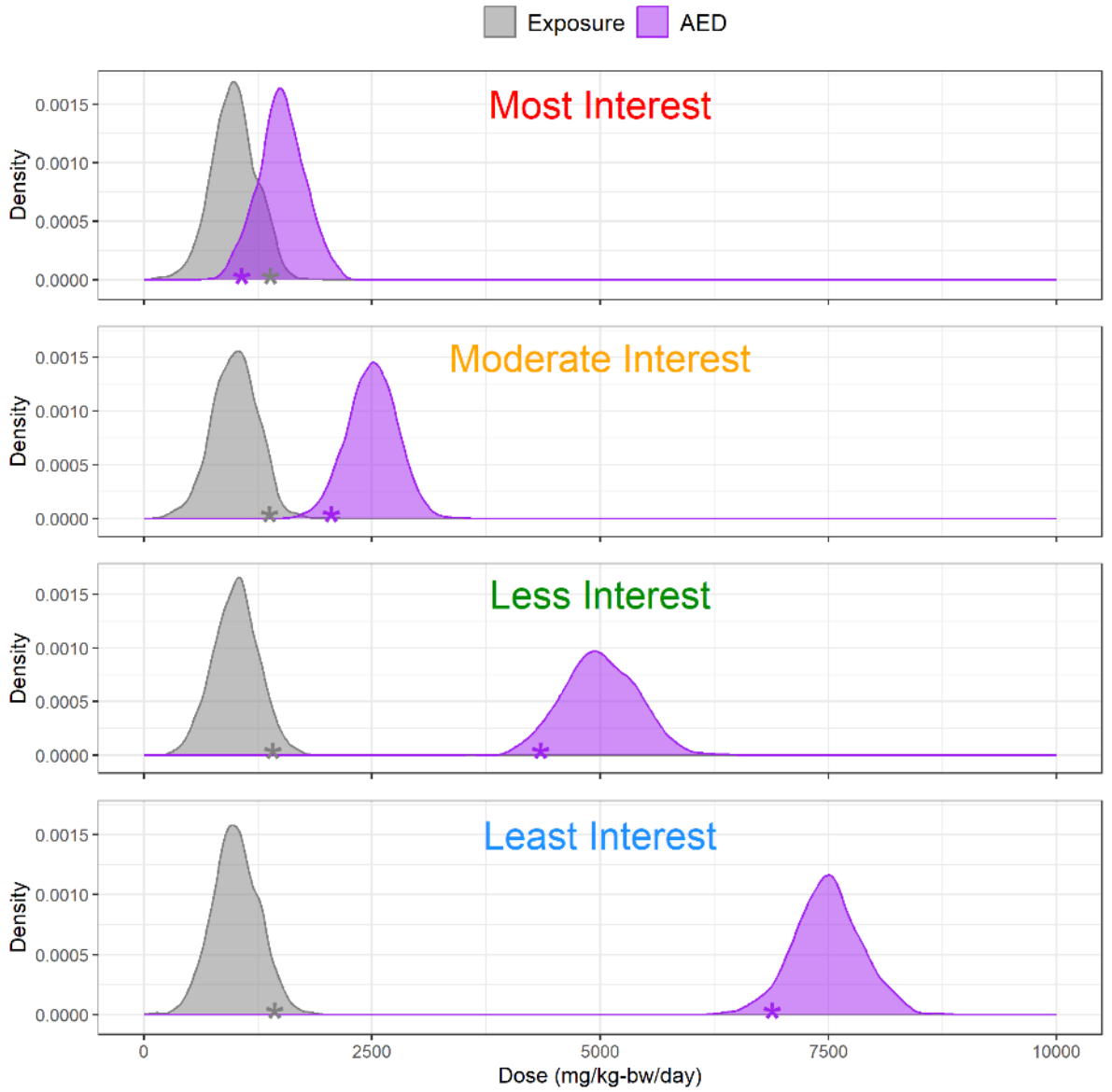


Administered Equivalent Dose (mg/kg bw/day)



Bioactivity Exposure Ratio (BER)

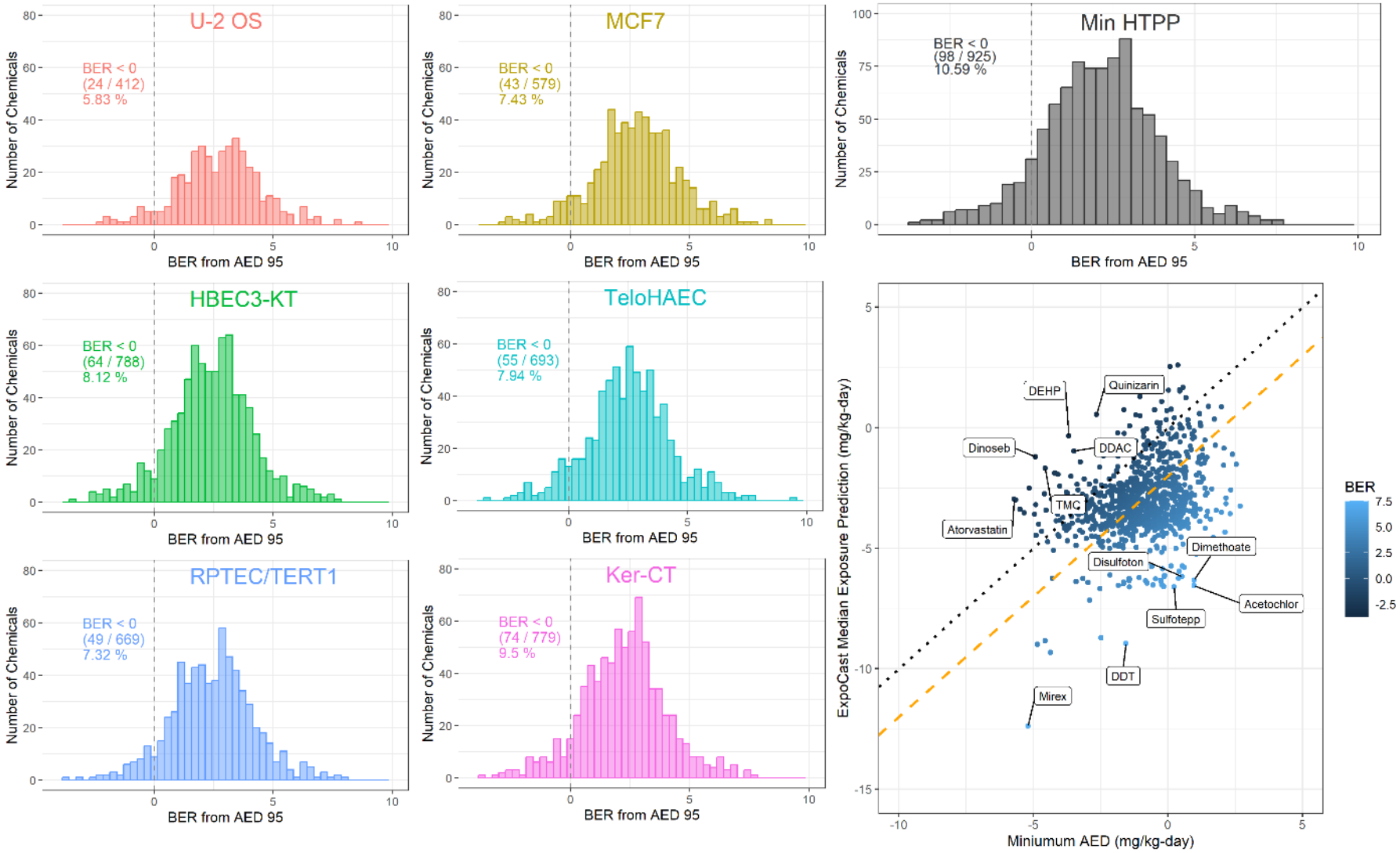
$Log_{10}(Exposure_{95}) - log_{10}(AED_{05})$



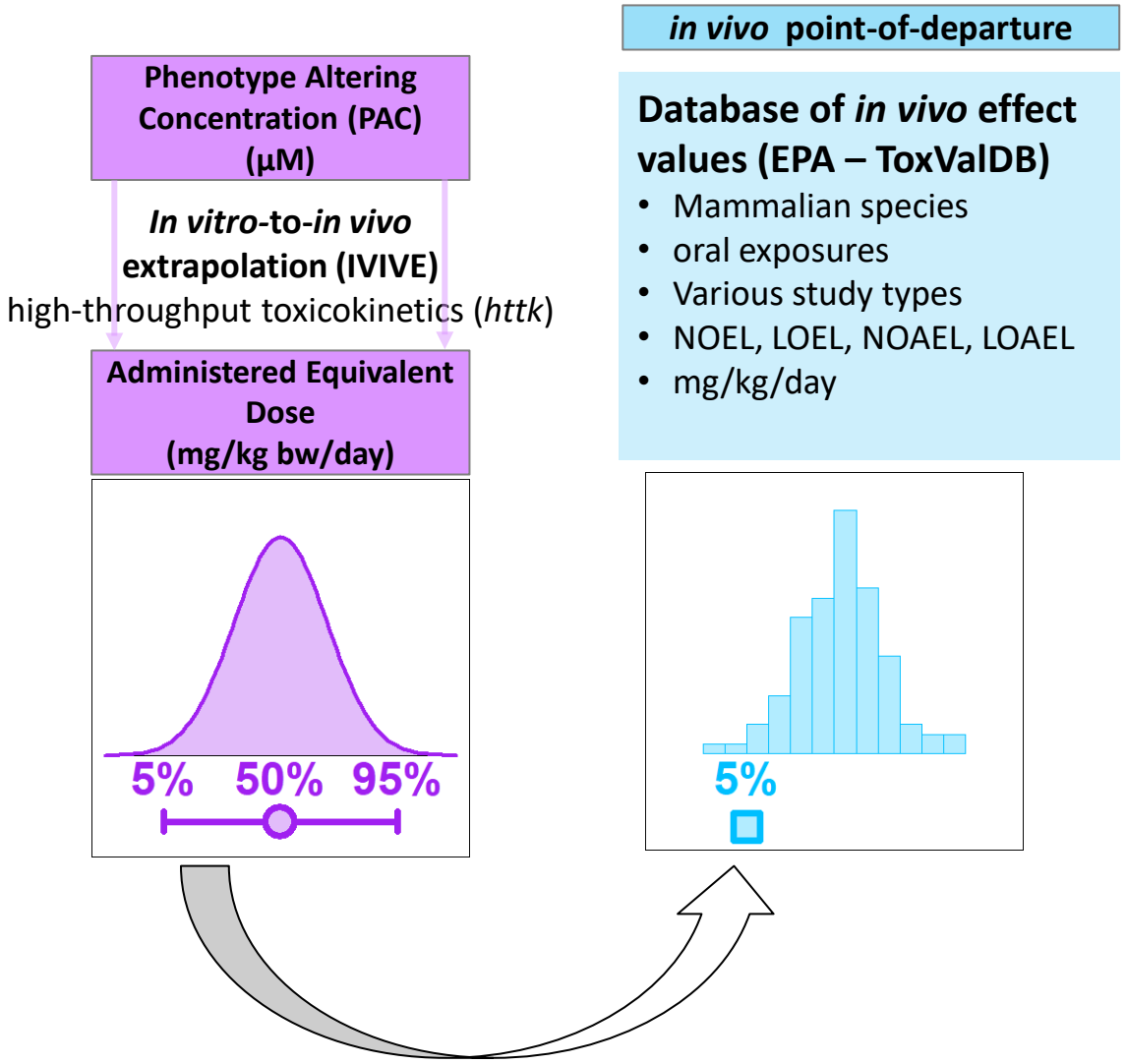
Approach Adapted from Paul Friedman et al. (2020) Tox Sci (PMID: 31532525)

BER with Cell Painting

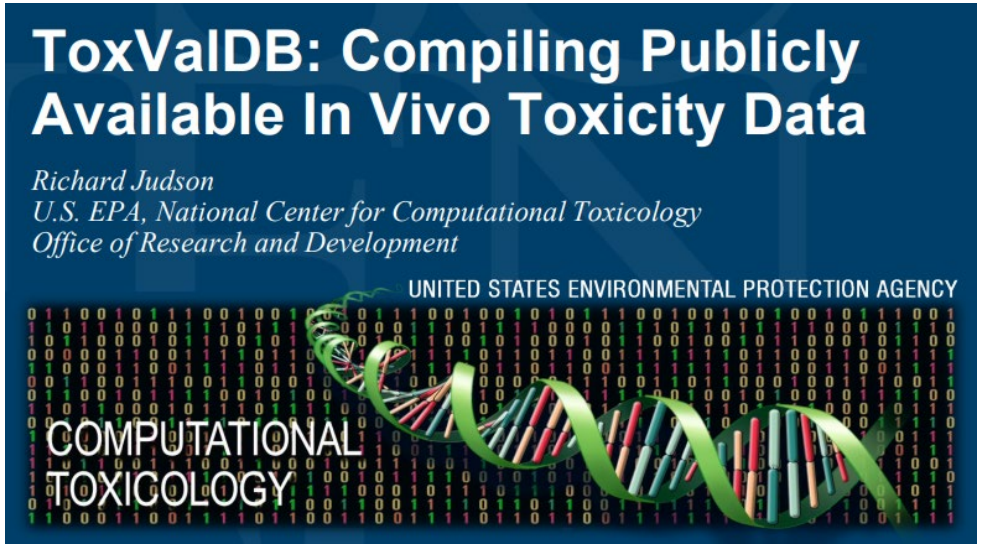
- **Bioactivity exposure ratio (BER)** analysis is a potential method for prioritization of chemicals for more in depth hazard assessment.
- **Negative BER** indicates that the predicted human exposure overlaps with the administered equivalent dose (AED) predicted from *in vitro*.
- When cell lines are considered in combination, ~10% of chemicals had negative ratios.
- Most extreme negative ratios associated with drugs and chemicals found in consumer products.
- Most extreme negative ratios associated with pesticides and herbicides.



Comparison of AEDs to "Traditional" *In Vivo* PODs



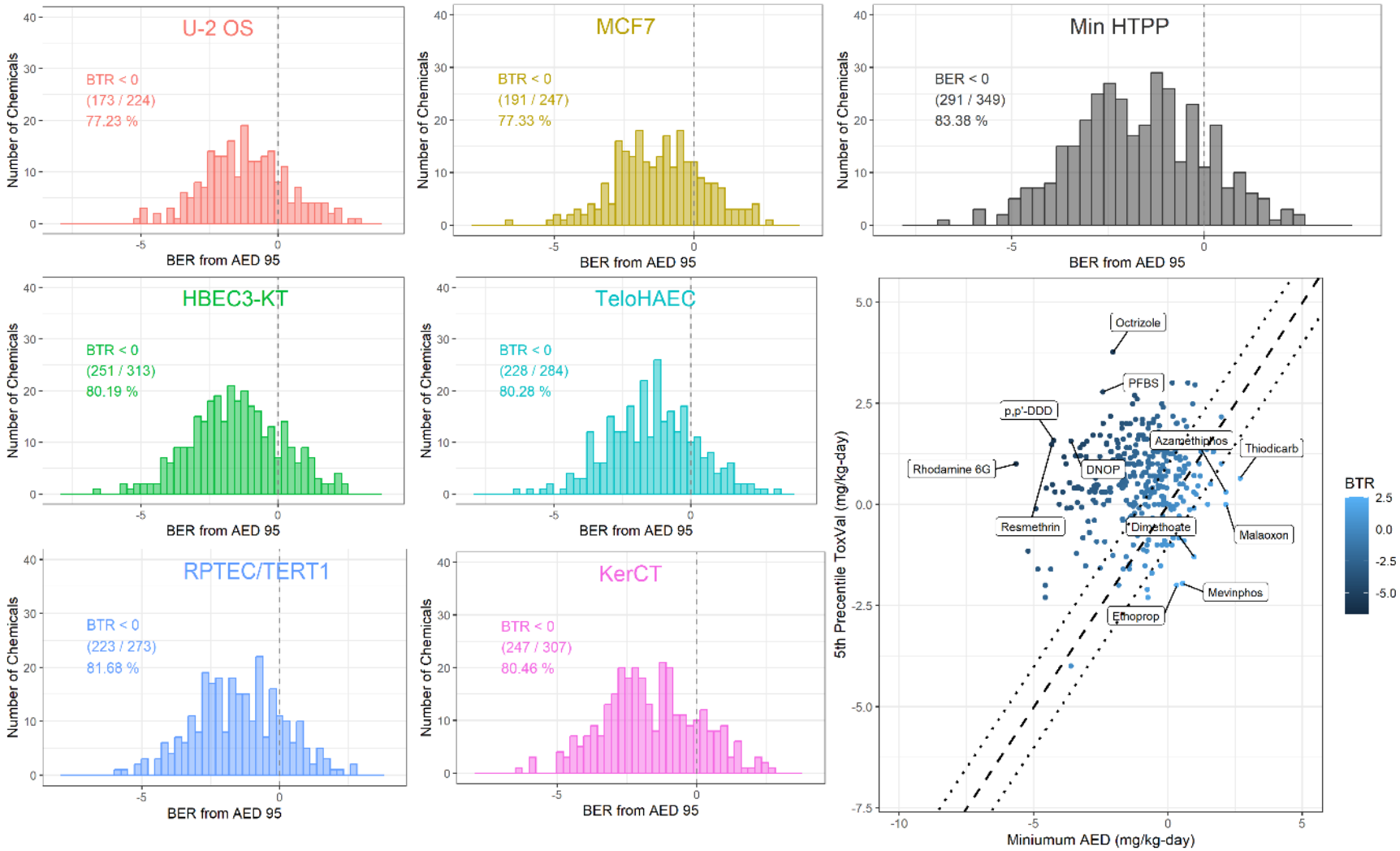
- Is the administered equivalent dose from an in vitro assay a conservative surrogate for a "traditional" *in vivo* POD?



https://www.epa.gov/sites/default/files/2018-12/documents/comptox_cop_dec_20_2018_final.pdf

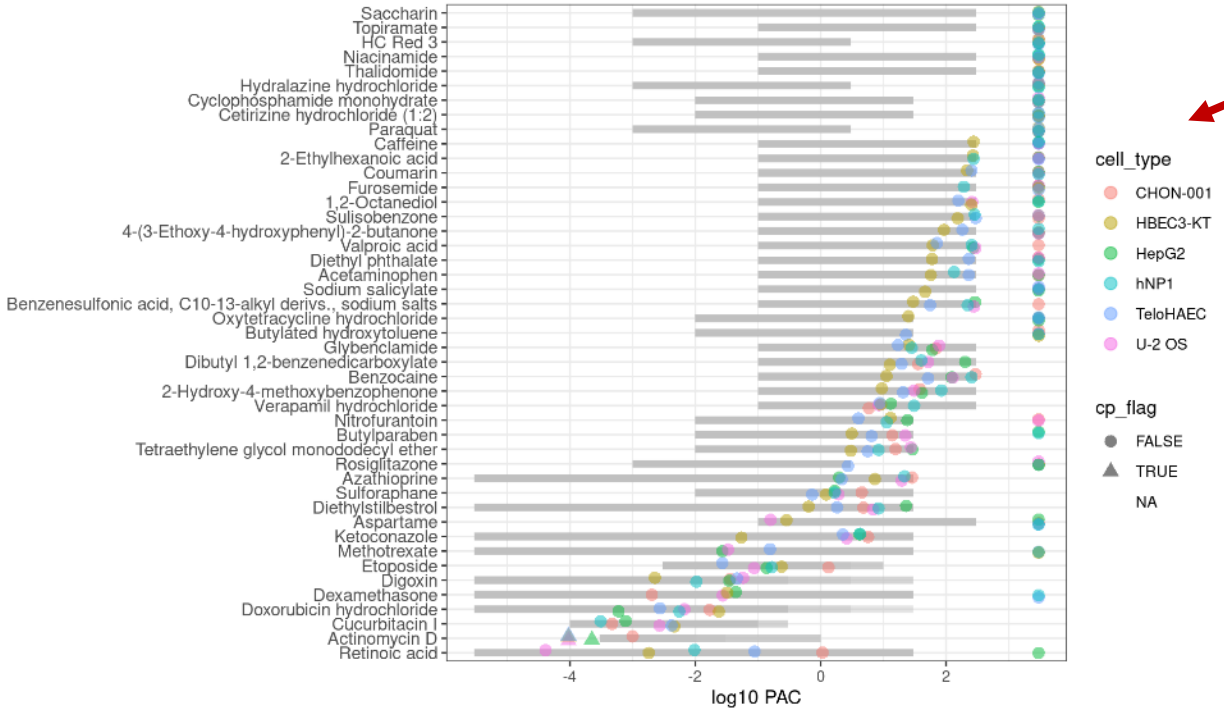
Bioactivity / *In Vivo* Effect Value Ratio Analysis

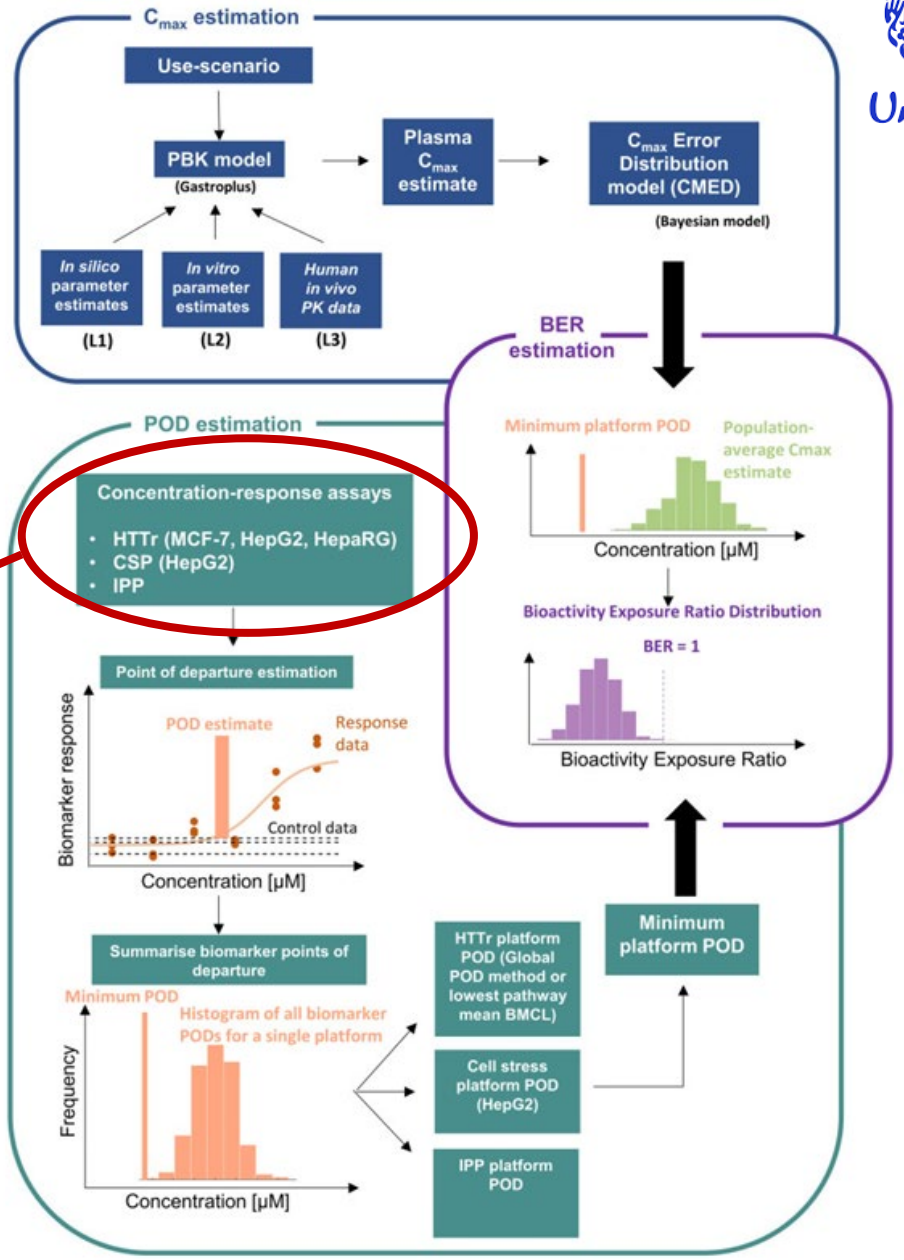
- **Negative ratios** indicate that AEDs derived from HTPP are **conservative** surrogates for traditional *in vivo* PODs.
- When cell lines are considered individually, **~77-81%** of chemicals had negative ratios.
- When considered in combination, the number and percentage of chemicals with negative ratios **increased (83.8 %)**.
- Paul-Friedman et al. (2020)[a]:
  - Using ToxCast, **89 %** of APCRA chemicals had negative ratios.
- Positive ratios observed for several organophosphate and carbamate pesticides.



CCTE / Unilever CRADA

- Jointly explore the utility of a battery of NAMs, which are non-animal based, for evaluating the safety and hazard of chemicals using an **exposure-led, hypothesis-driven approach**.
- Toolbox approach inclusive of Cell Painting and HTTr.
- Bioactivity to exposure (BER) ratio the key value of interest.





From Middleton et al., 2022. *Tox Sci* (PMID:35822611)

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Molecular Initiating Event (MIE) Prediction / Mechanistic Inference

Feature Selection & Profile Comparison for Mechanistic Inference

1300 features

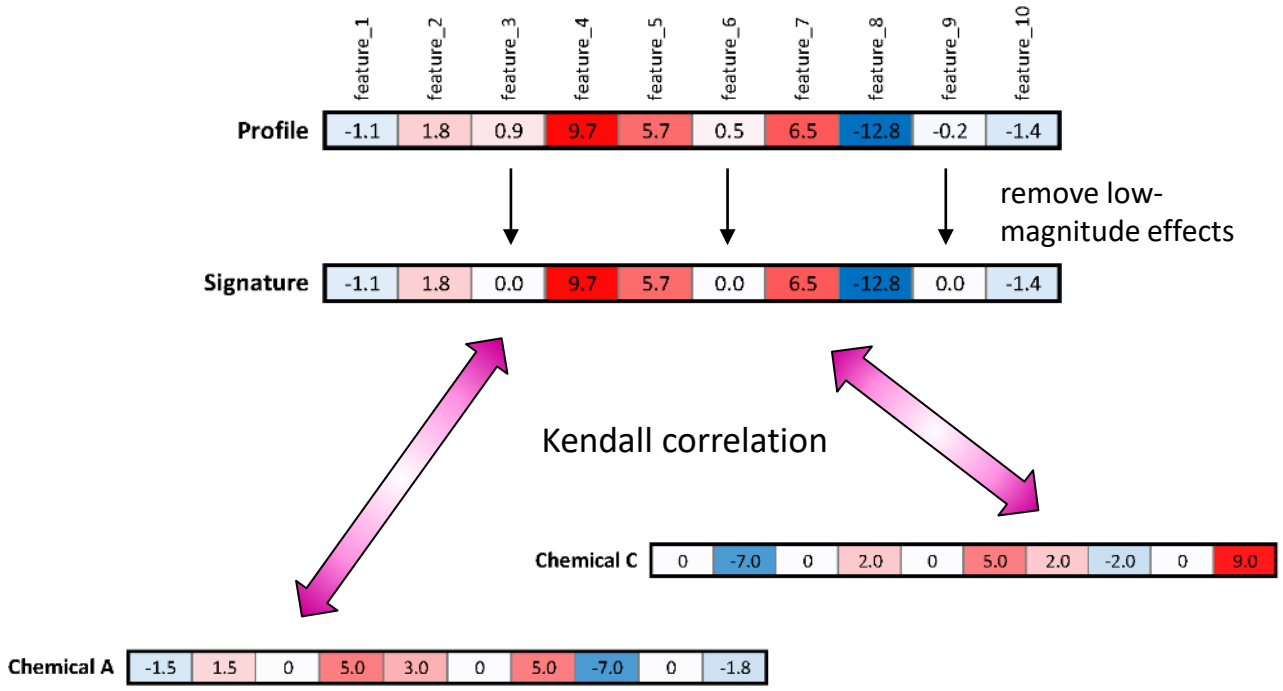
Feature Selection

1. remove features that do not provide any information (i.e. have 0 variance)

2. remove features that are not reproducible (high variation between treatments of different biological replicates)

3. remove features that are highly correlated (using recursive feature elimination)

317 features



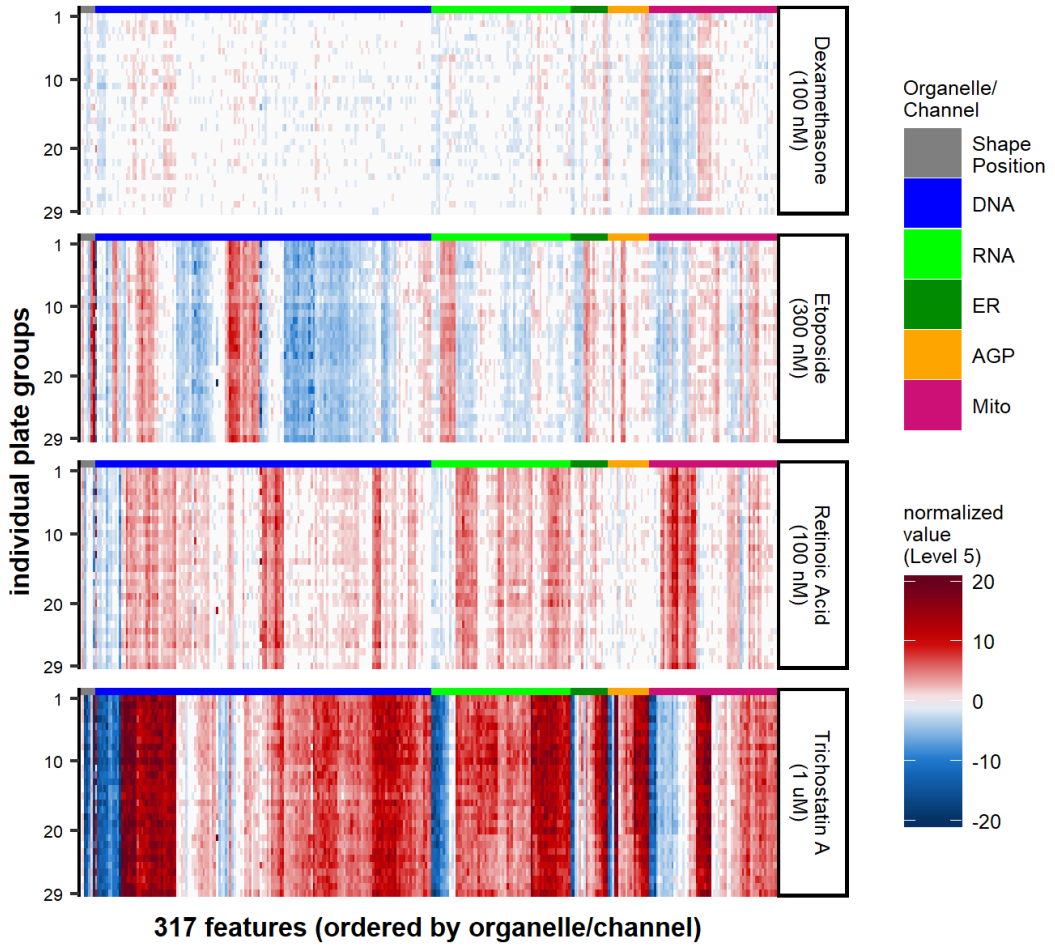
Hypothesis: *Chemicals acting through similar mechanisms will display similar profiles.*

Slide courtesy of Jo Nyffeler

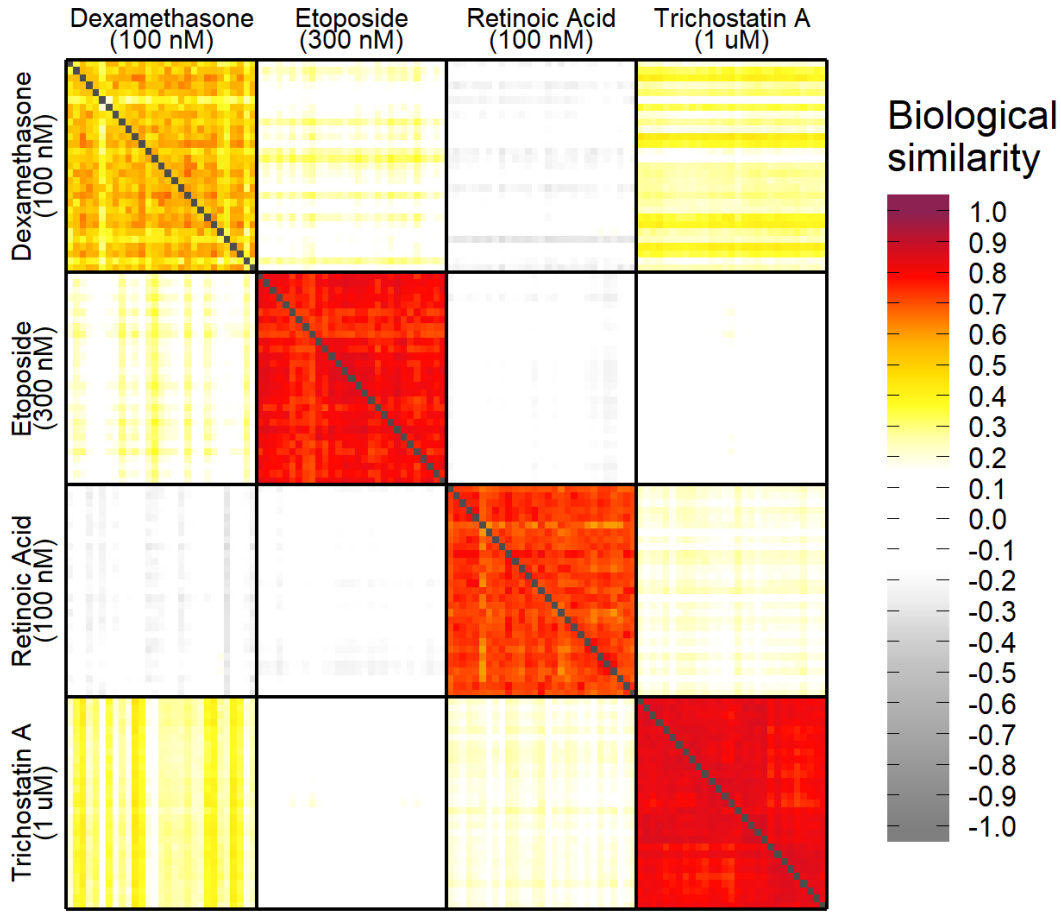
Reproducibility of Phenotypic Profiles

Jo Nyffeler

Profile Visualization



Profile Correlation

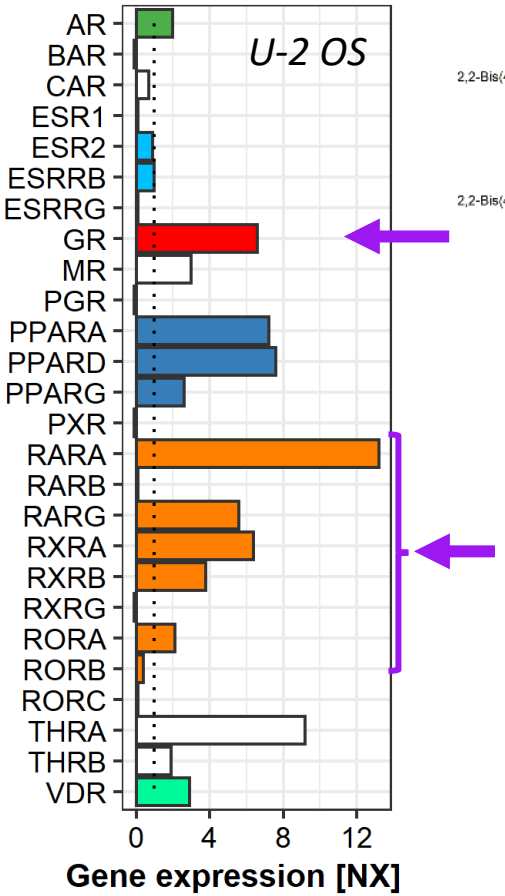


- Phenotypic profiles are highly reproducible across many plate groups (weeks / months)

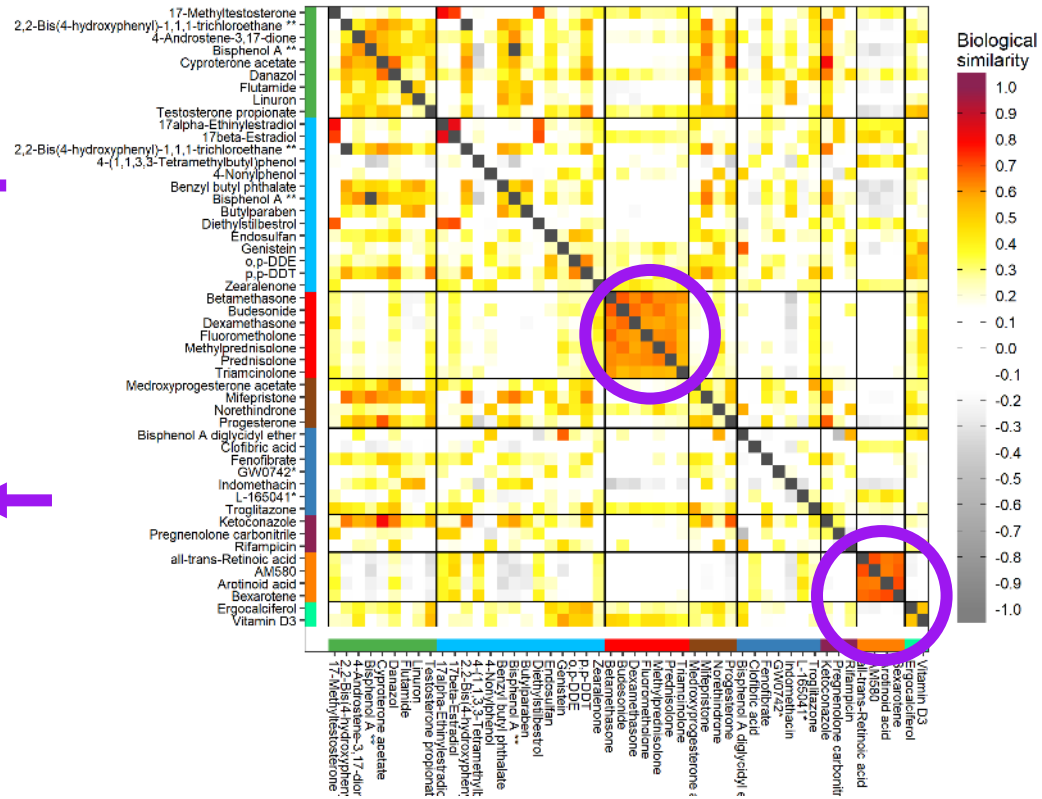
Profile Comparison: Nuclear Receptor Modulators

Jo Nyffeler

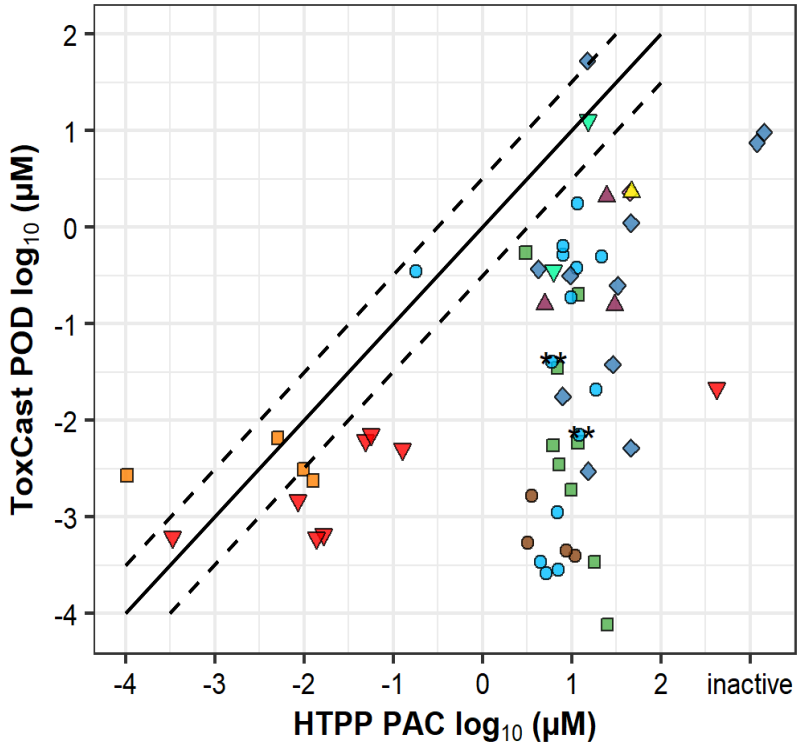
Target expression



Profile similarity in Cell Painting



Potency Versus ToxCast



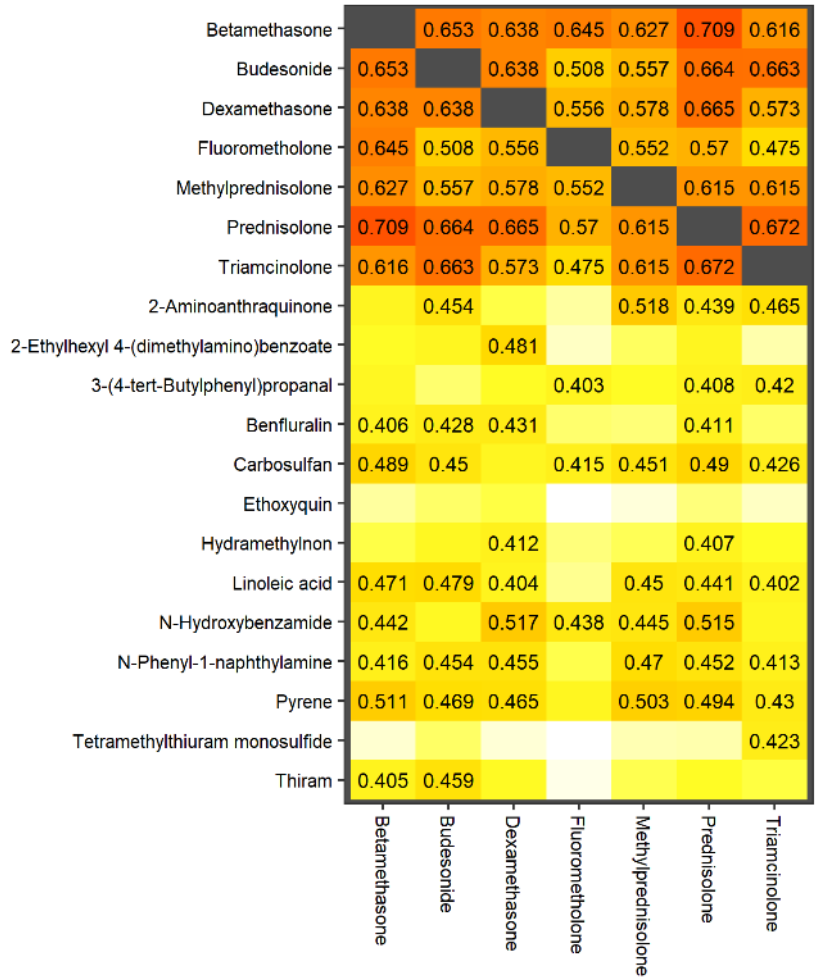
- Agonists of glucocorticoid receptor (GR) and retinoic acid receptor (RAR) yield characteristic profiles.
- Expression of a target does not guarantee a characteristic profile will be observed (e.g., PPAR).

Preliminary results from Nyffeler et al. (submitted). Do not cite or quote.

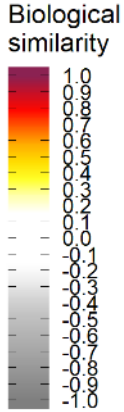
Profile Similarity & Targeted Follow-Up Reveal Novel Bioactivity

Jo **Nyffeler**

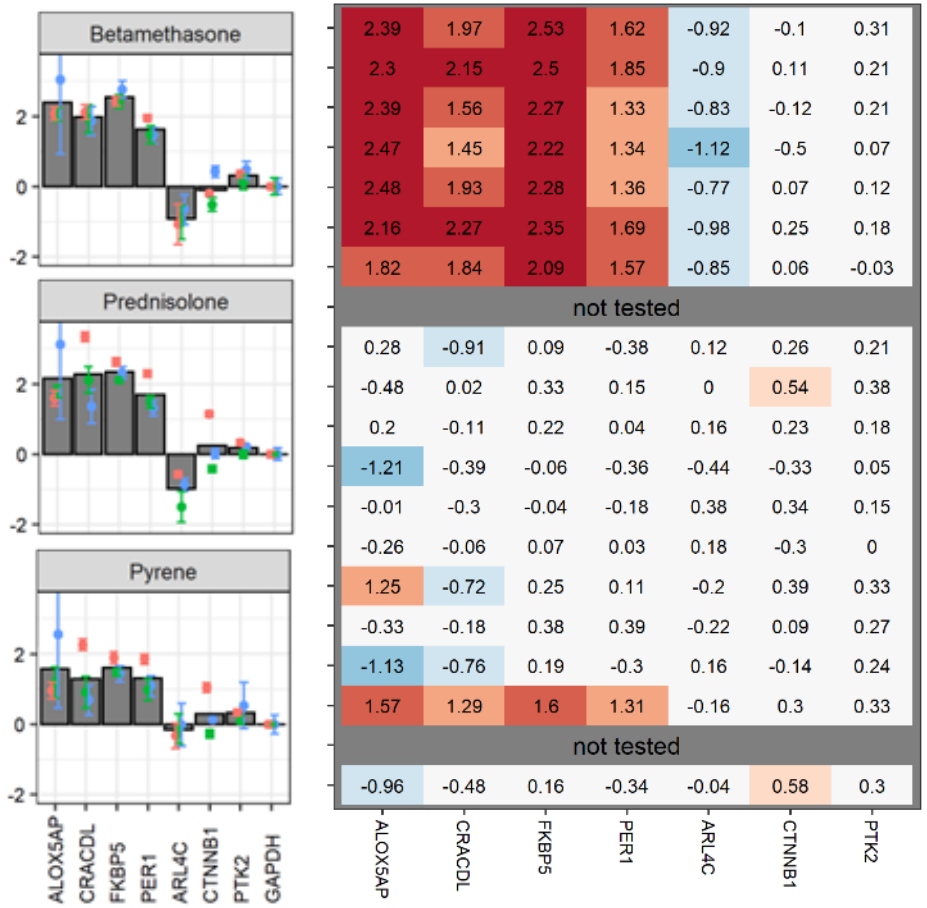
Primary screen



Secondary screen



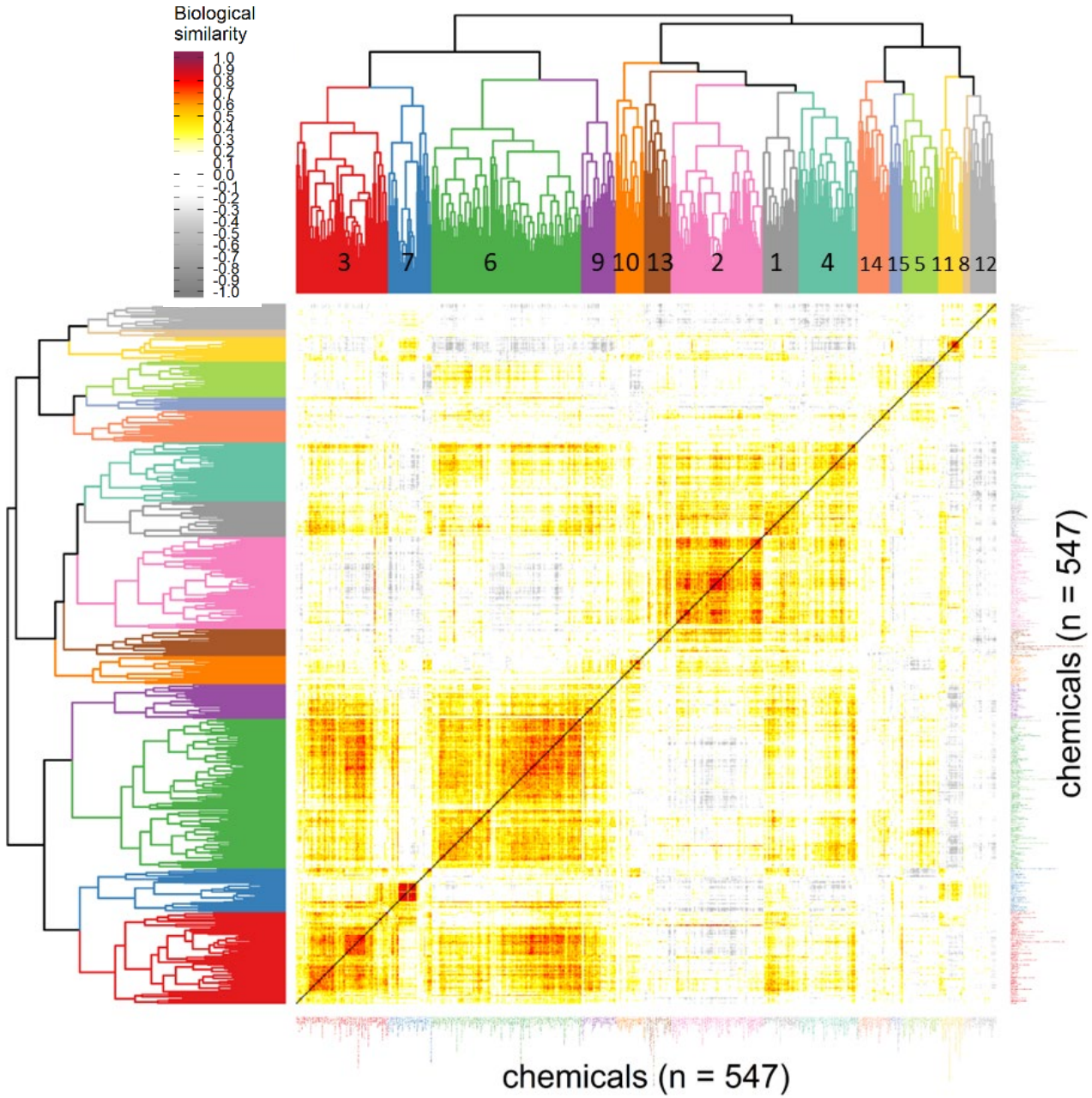
Orthogonal qRT-PCR Assay



- **GR** identified as a novel molecular target for **pyrene**, a polycyclic aromatic hydrocarbon (PAH)

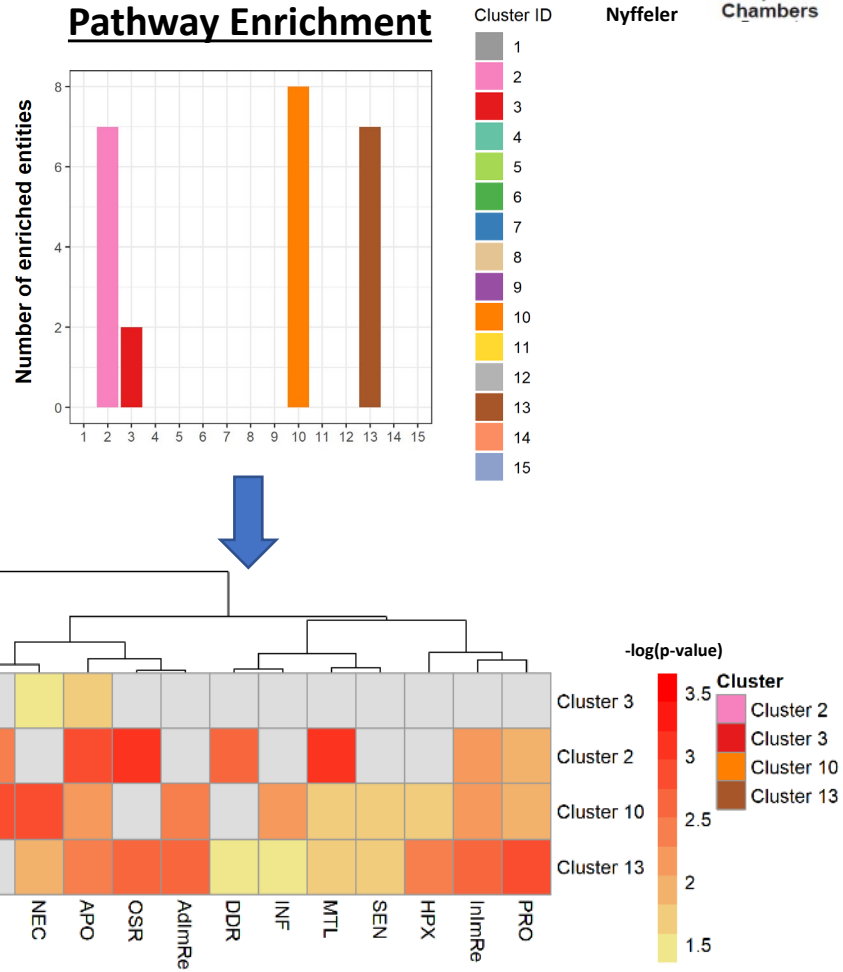
Preliminary results from Nyffeler et al. (submitted). Do not cite or quote.

Profile Comparison & Stress Response



Jo Nyffeler Bryant Chambers

Stress Response Pathway Enrichment

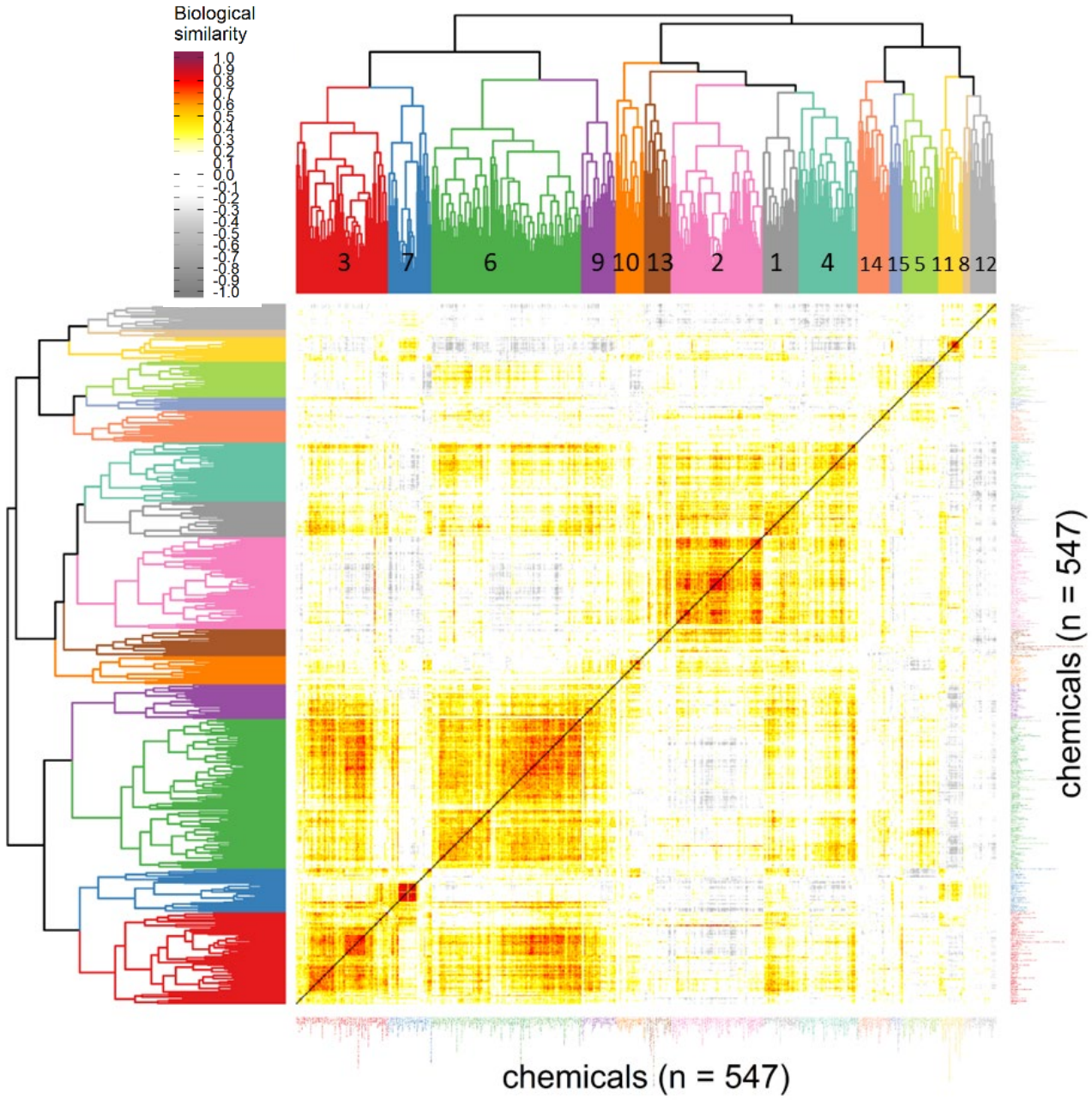


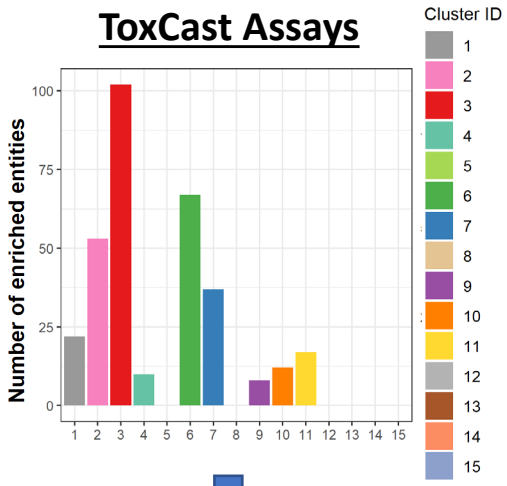
Phenotypic profile clustering associated with different patterns of cellular stress responses.

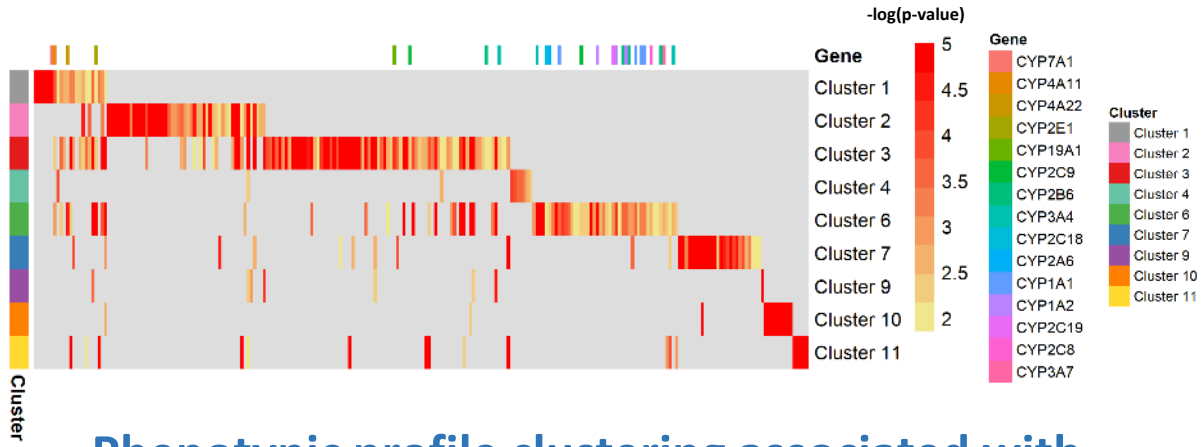
Preliminary results from Nyffeler et al. (submitted). Do not cite or quote.

Profile Comparison & ToxCast Assays

Jo Nyffeler







Phenotypic profile clustering associated with different patterns of enriched ToxCast assays.

Preliminary results from Nyffeler et al. (submitted). Do not cite or quote.

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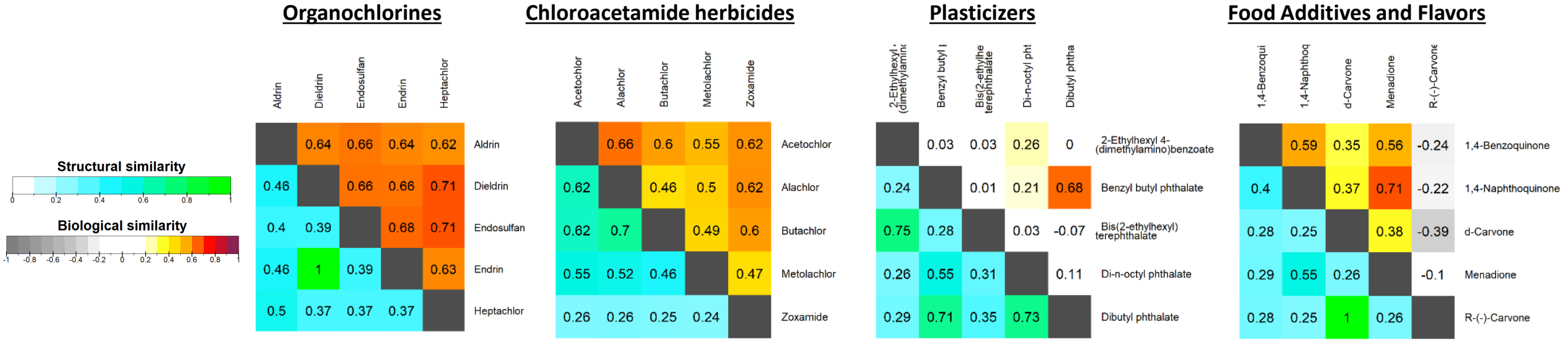
Chemical Grouping for Risk Assessment Applications

Chemical Grouping & CBRA with Cell Painting

Jo
Nyffeler

- In Chemical-Biological Read-Across (CBRA) toxicity is inferred from both chemical structural similarity and comparisons of biological responses to chemicals measured in multiple short term assays ("biological similarity) (Low et al. (2013) Chem. Res. Toxicol. (PMID: 23848138))



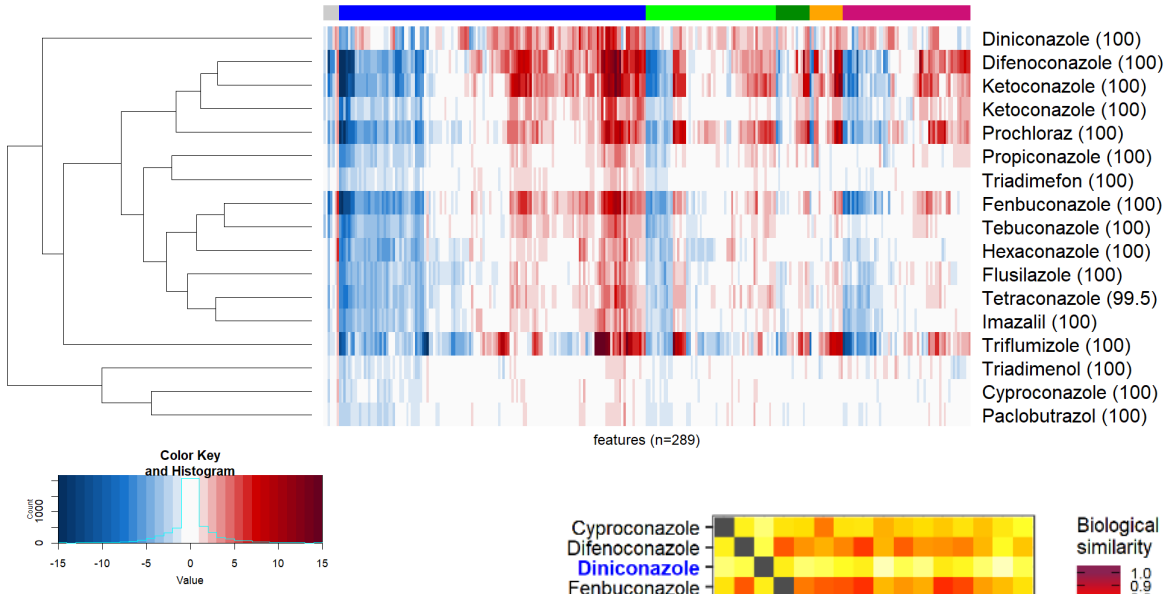
- We propose that Cell Painting may also be useful for CBRA, but need to dive deeper.

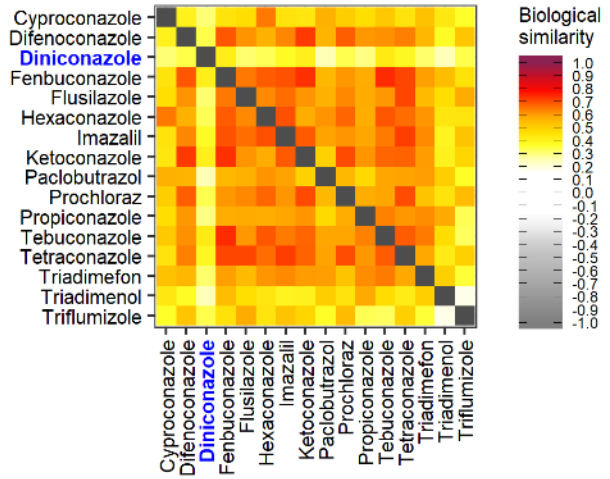
Preliminary results from Nyffeler et al. (submitted). Do not cite or quote.

Chemical Grouping & CBRA with Cell Painting

Jo Nyffeler

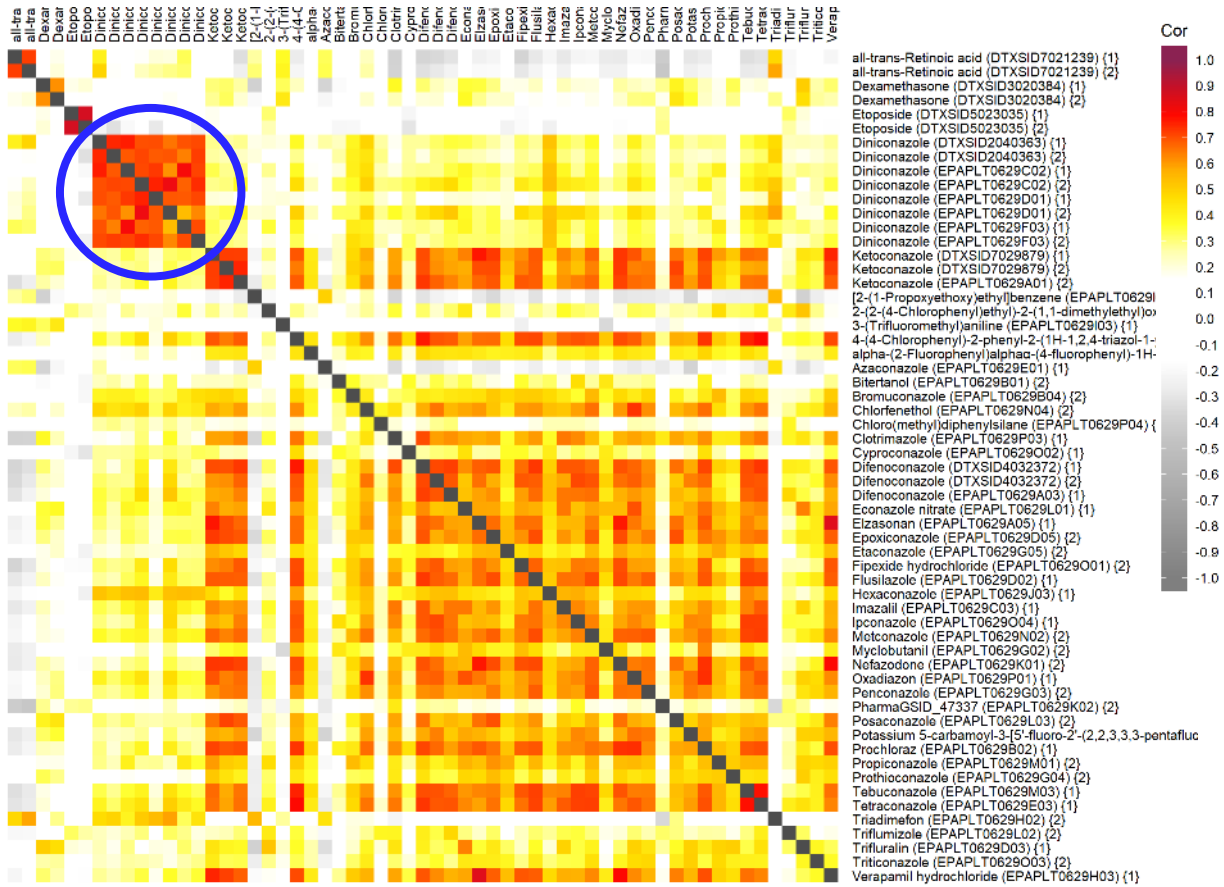
Primary Cell Painting Screen





Diniconazole identified as dissimilar biological activity compared to other conazoles.

Secondary Cell Painting Screen



Tested several independent samples of diniconazole. Profiles all similar to one another & dissimilar from other conazoles.

Preliminary results from Nyffeler et al. (submitted). Do not cite or quote.

New Chemicals Collaborative Research Program (NCCRP)

Katie Paul-Friedman

& Many Others!

Joint project within US EPA:

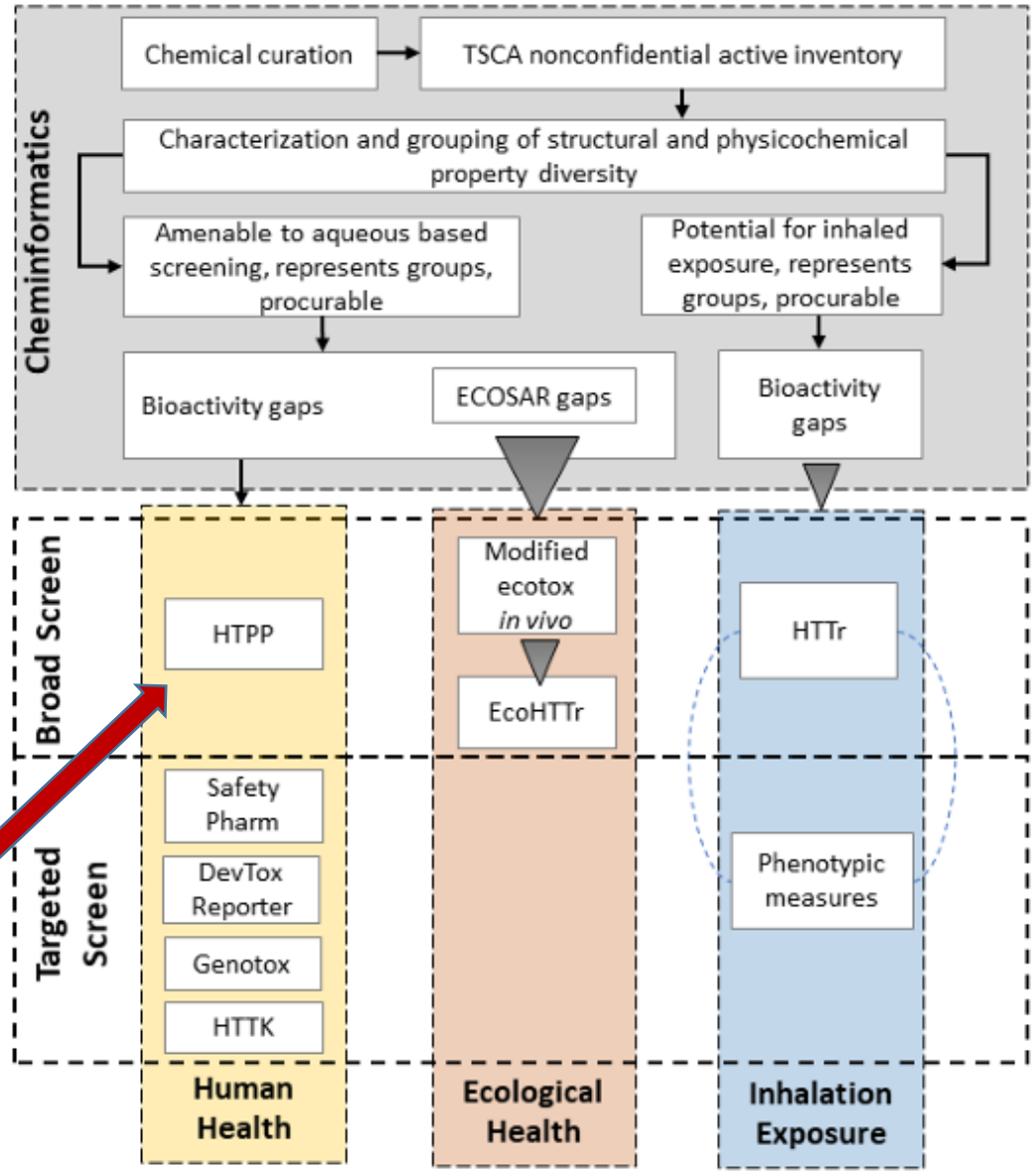
- Office of Research and Development (ORD)
- Office of Chemical Safety and Pollution Prevention (OCSPP)

Aim is to bring **innovative approaches** to address the requirements of the **Toxic Substances Control Act (TSCA)** for the review of new chemicals.

Modernizing the new chemicals review process will help overcome information gaps and help the Agency meet its statutory requirements in a timely, effective and efficient manner.

Five components:

1. Refinement of chemical category approaches
2. Expansion of databases containing TSCA chemical info
3. Develop and refine predictive models for phys-chem, environmental fate/transport, **hazard**, exposure and toxicokinetics.
4. Integration and application of *in vitro* NAMs.
5. Decision support tool.



https://www.epa.gov/reviewing-new-chemicals-under-toxic-substances-control-act-tsca/new-chemicals-collaborative

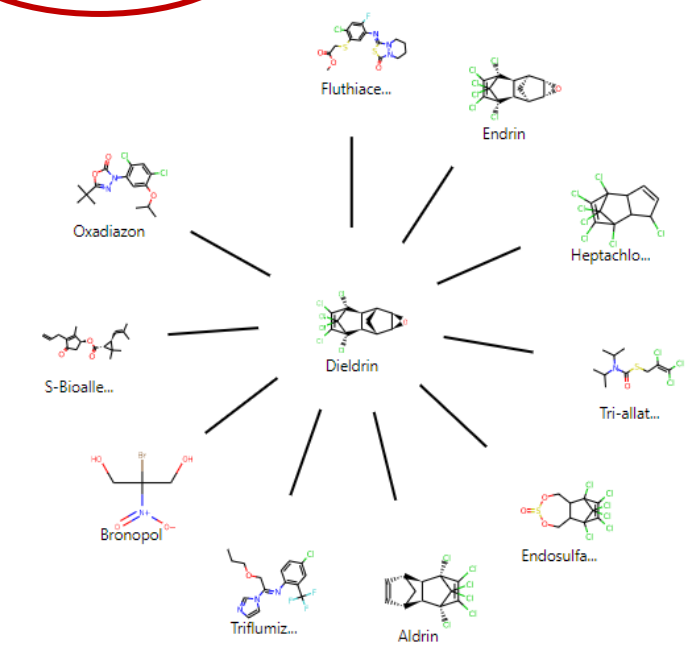
CBRA with Cell Painting Data

- Chemical biological read-across (CBRA) using Cell Painting data as input (prototype)

EPA United States Environmental Protection Agency | Generalized Read-Across (GenRA) | More Info | Ketcher | Dieldrin *DTXSID9020453*

Step Three: Data Gap Analysis & Generate Data Matrix

Neighbors by: Biology: HTPP_U2OS | **Filter by:** ToxRef data



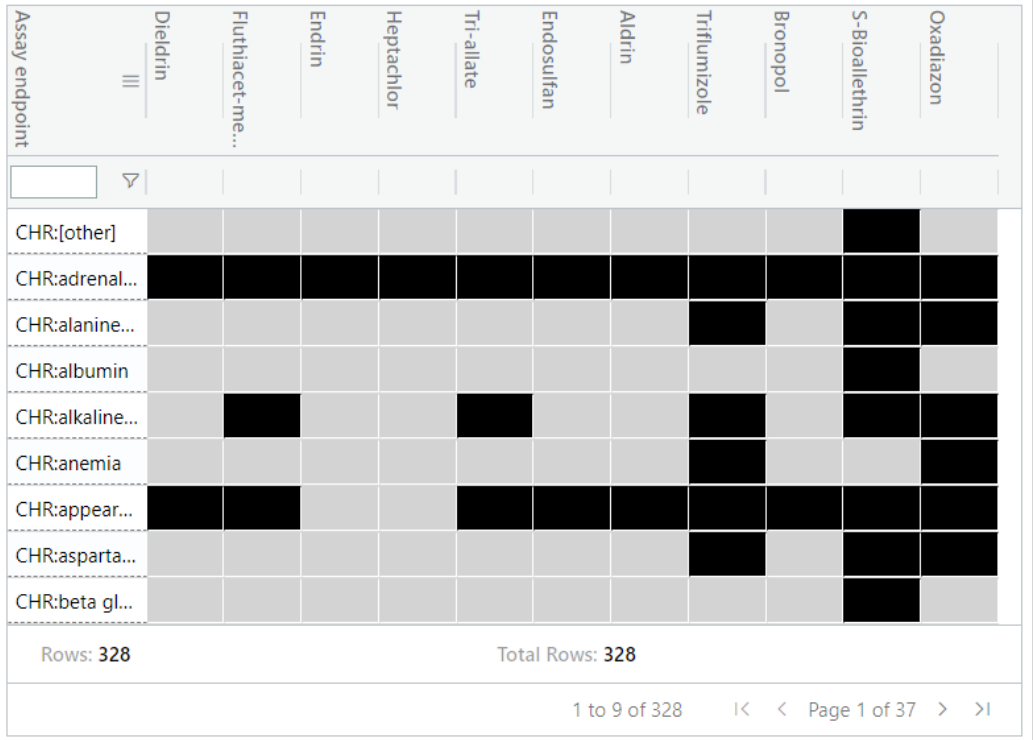
10 | Physchem Data | Neighborhood Exploration

Summary Data Gap Analysis

Chemical	bio_txct	chm_ct	chm_httr	chm_mrgn	tox_txrf
Dieldrin	183	27	62	32	120
Fluthiacet-methyl	90	22	48	69	166
Endrin	139	27	62	32	114
Heptachlor	223	19	52	33	120
Tri-allate	169	15	19	30	125
Endosulfan	247	18	57	32	136
Aldrin	201	17	60	29	120
Triflumizole	348	18	36	64	229
Bronopol	171	9	12	17	185
S-Bioallethrin	211	15	44	57	161
Oxadiazon	250	16	38	51	154

Rows: 11 Total Rows: 11

Group: ToxRef | **By:** Tox Fingerprint | **Hide Pagination** | Generate Data Matrix



Rows: 328 Total Rows: 328

1 to 9 of 328 Page 1 of 37

Summary and Future Directions

- **Cell Painting is a robust, reproducible and cost-effective method for bioactivity screening of environmental chemicals across diverse human-derived cell lines.**

- **Data can be leveraged to inform NGRA:**
  - Prioritization with BER
  - MIE Prediction / mechanistic inference
  - Chemical grouping for chemical biological read-across.

- **Future directions include:**
  - Screening chemical sets of interest to EPA across additional, diverse cell lines.
  - Methods development for screening of defined and uncharacterized chemical mixtures.
  - Implementing CellProfiler at scale to promote interoperability with other Cell Painting datasets.
  - Surfacing of additional Cell Painting data on the CompTox Chemicals Dashboard.

Acknowledgements

Office of Research and Development (ORD)
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- Joseph Bundy
- Bryant Chambers
- Joshua Witten

PerkinElmer

- Joe Trask
- Dana Hanes
- Colin Wakeham
- Jim Hostetter

Thank You & Questions ?