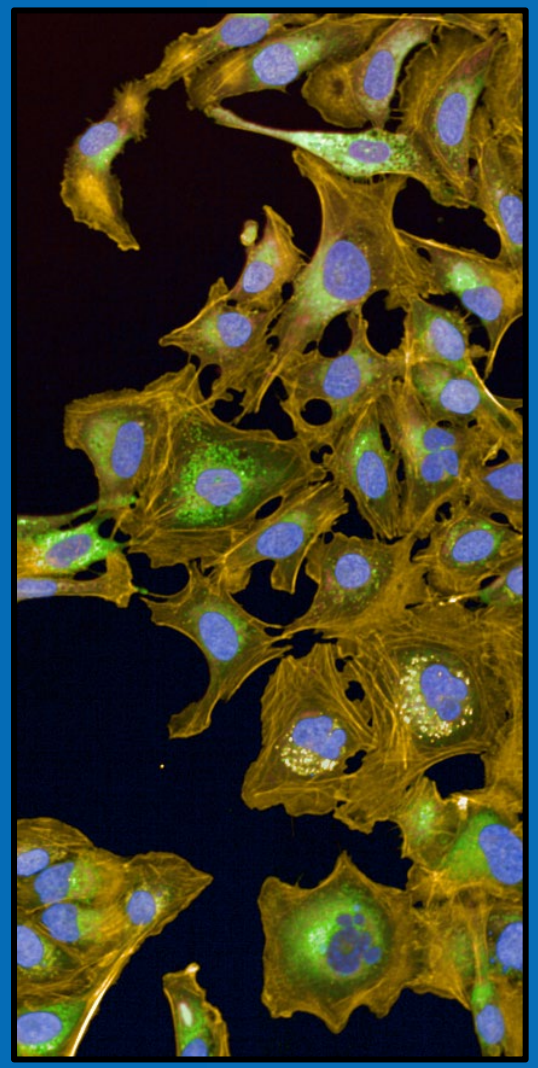
EPA
United States
Environmental Protection
Agency



Application of Cell Painting at the Center for Computation Toxicology and Exposure

Johanna Nyffeler

ORISE postdoctoral grantee at USEPA Center for Computational Toxicology and Exposure (CCTE)

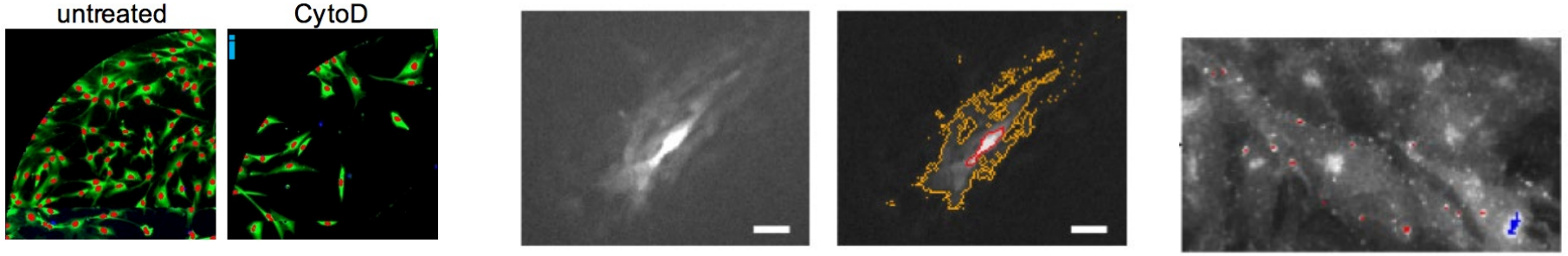
ORCiD 0000-0002-6155-9743

Nyffeler.Johanna@epa.gov

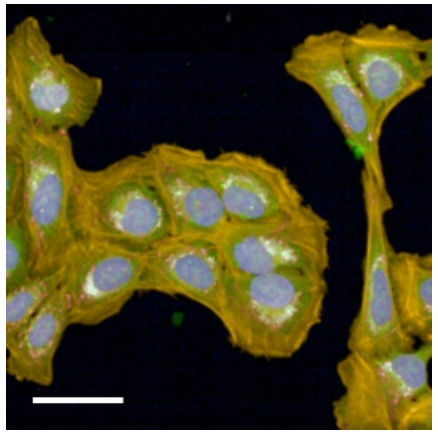
The views expressed in this presentation are those of the author and do not necessarily reflect the views or policies of the U.S. EPA.

Introduction: Dr. Johanna Nyffeler

- BSc in Biochemistry, MSc in Genetics
- **PhD at University of Konstanz, Germany**
  - group of Dr. Marcel Leist
  - development of high-content assays for *in vitro* developmental neurotoxicology



- **PostDoc at Center for Computational Toxicology & Exposure (CCTE), US EPA**
  - group of Dr. Joshua Harrill
  - high-throughput image-based profiling ('Cell Painting'), computational toxicology



Overview

1. **What is imaging-based phenotypic profiling?**
2. **Implementation at CCTE/EPA**
   - Workflow
   - Image analysis pipeline
   - QC reports
3. **Aims/Focus for CCTE/EPA**
4. **Application 1: Potency estimation**
5. **Application 2: Mechanistic prediction**

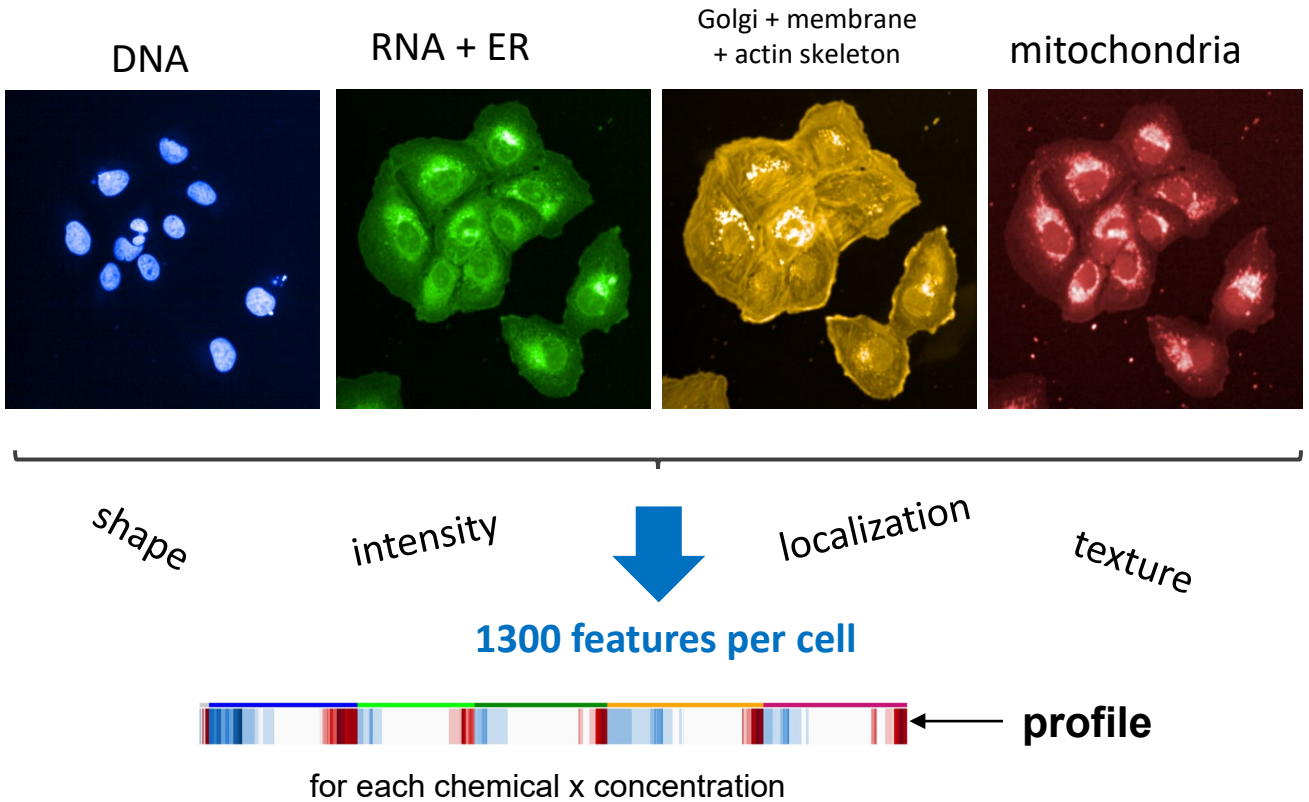
What is Imaging-Based Phenotypic Profiling?

- labeling of various cell organelles with fluorescent probes in *in vitro* cultures
- assessing a large variety of morphological features on individual cells

'Cell Painting' assay

Gustafsdottir *et al.* 2013

Bray *et al.* 2016



Flourescent labels
DNA: H-33342
RNA: SYTO14
ER: Concanavalin A-488
Actin: Phalloidin-568
Golgi + Membrane: wheat germ agglutinin (WGA) -555
Mitochondria: MitoTracker

Nyffeler *et al.* 2020

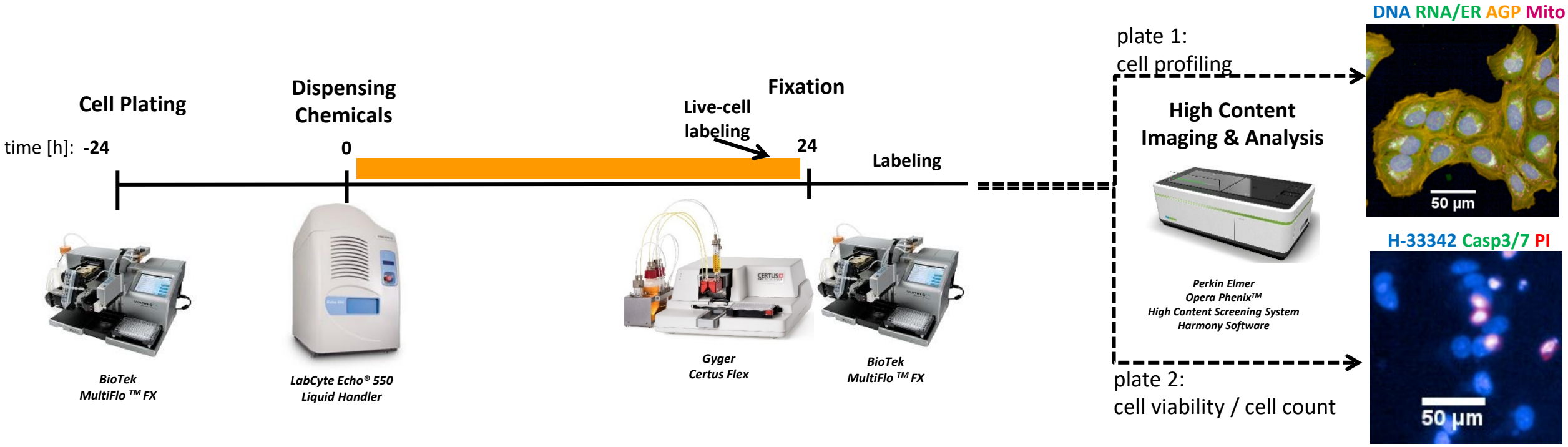
Cell Painting = Phenotypic Profiling

High-Throughput Phenotypic Profiling = HTPP

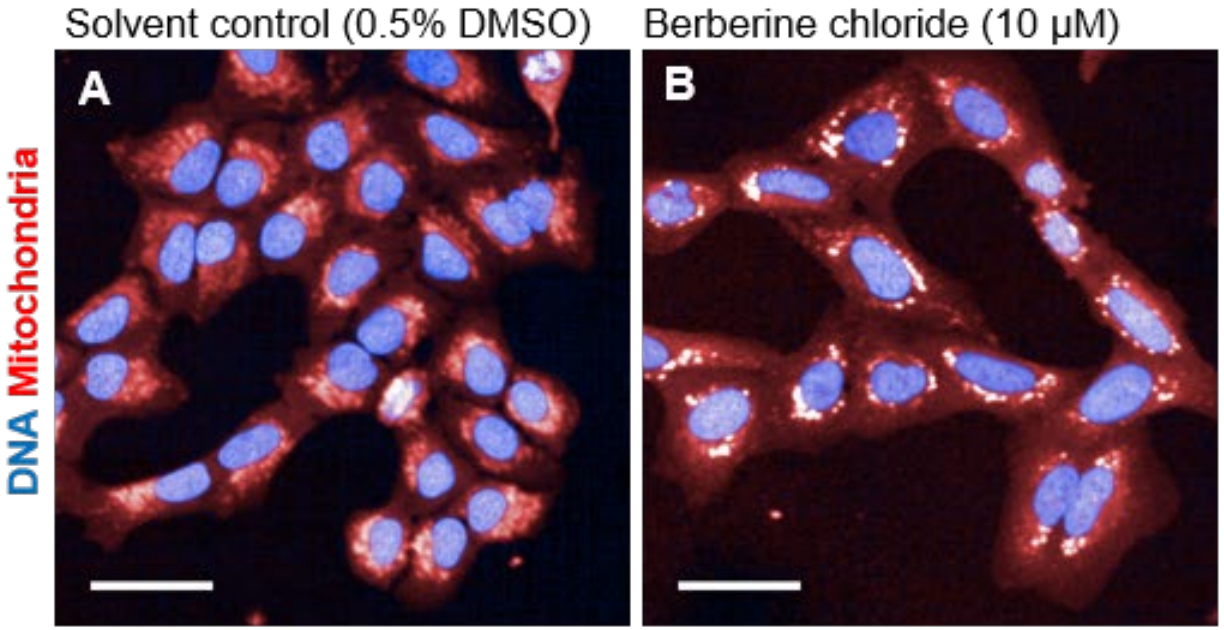
EPA United States Environmental Protection Agency

Implementation at CCTE/EPA

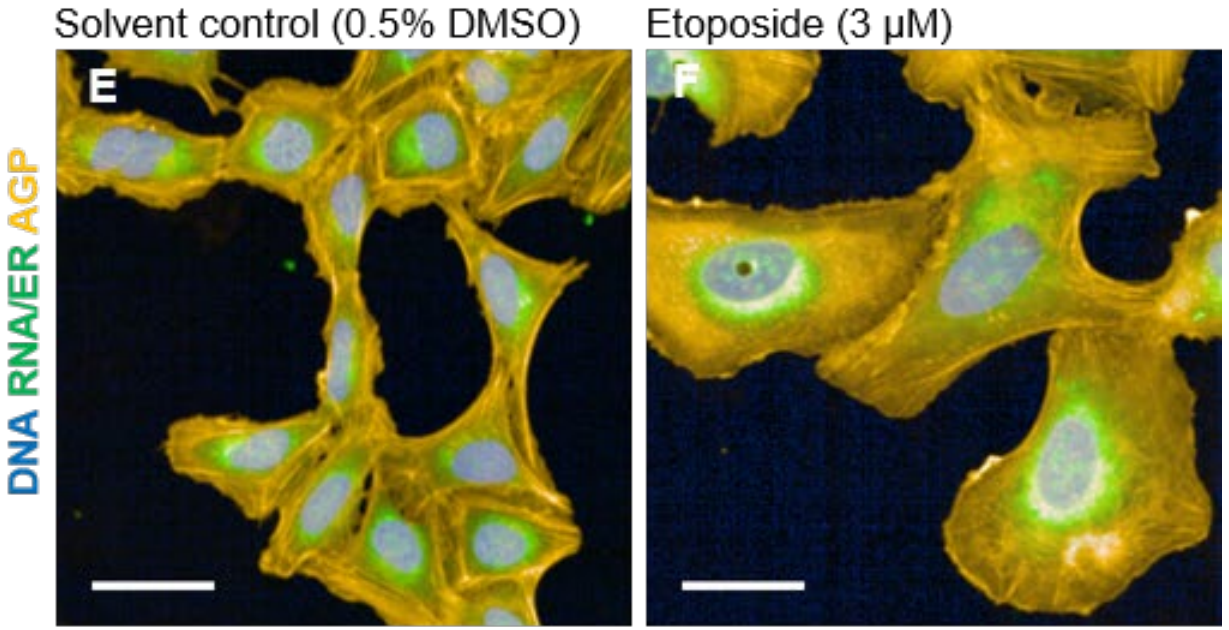
Laboratory Workflow



Example Chemicals: Qualitative Observation



→ Mitochondrial compactness/texture



→ Cells are larger

⇨ **Strong phenotypes are observable qualitatively**

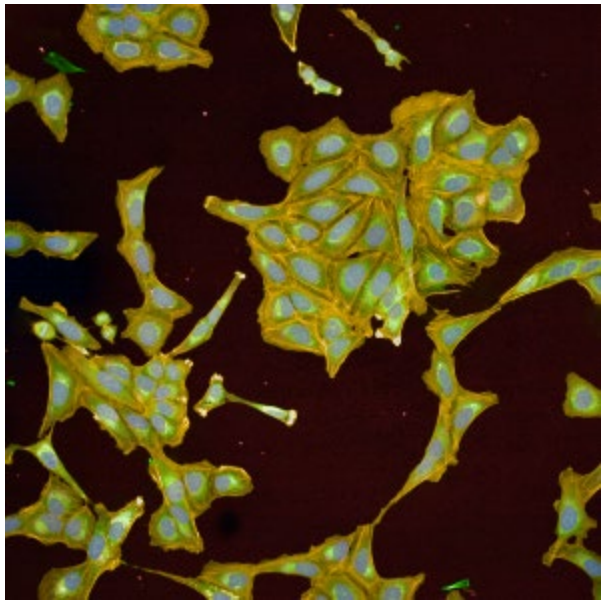
adapted from Nyffeler *et al.* 2020

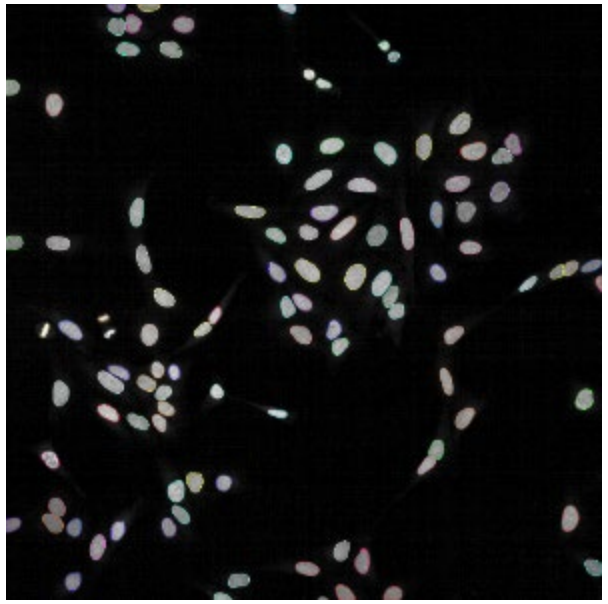
Image Analysis Workflow → Image Segmentation

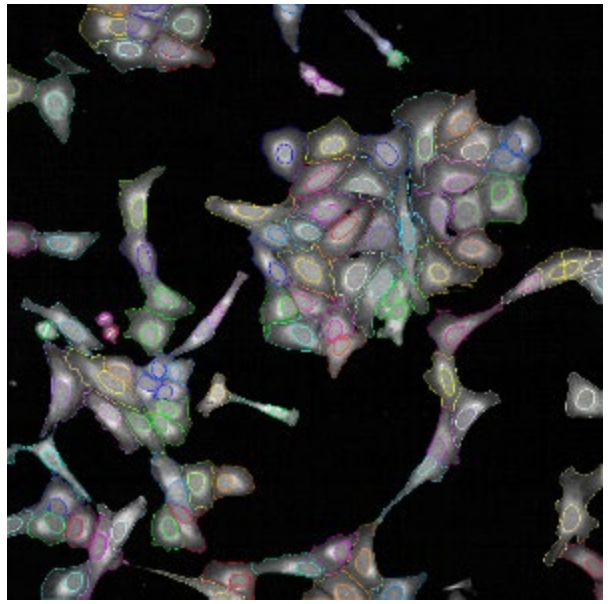
1. find nuclei

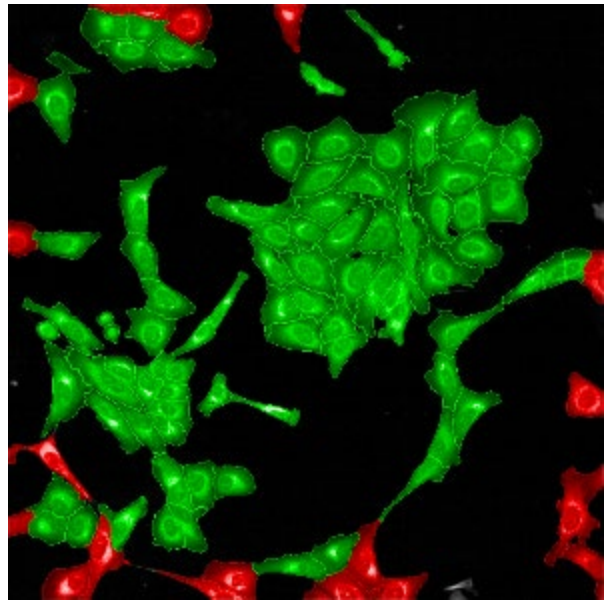
2. find cell outline

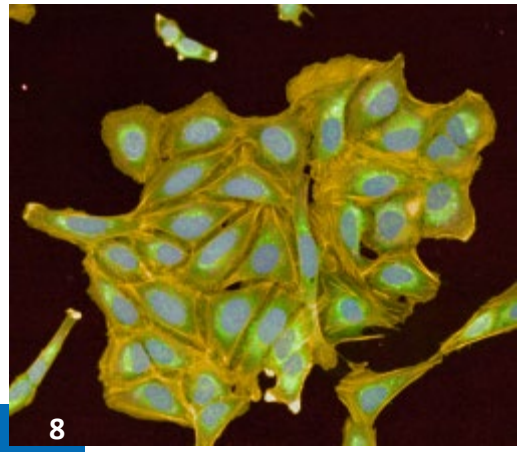
3. reject border objects

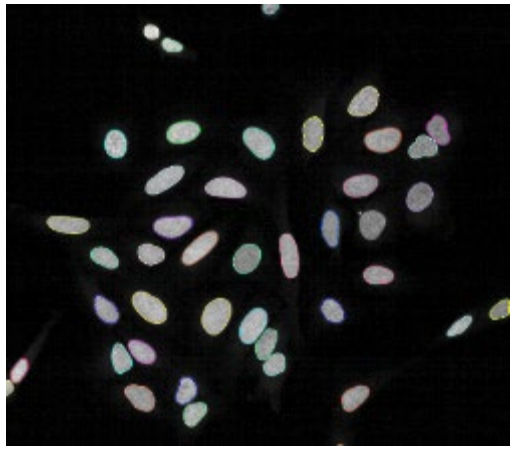


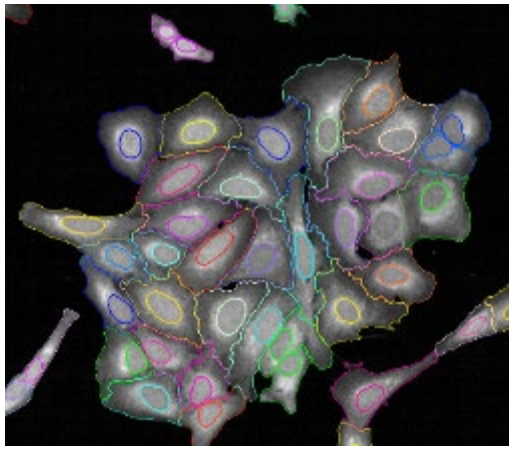






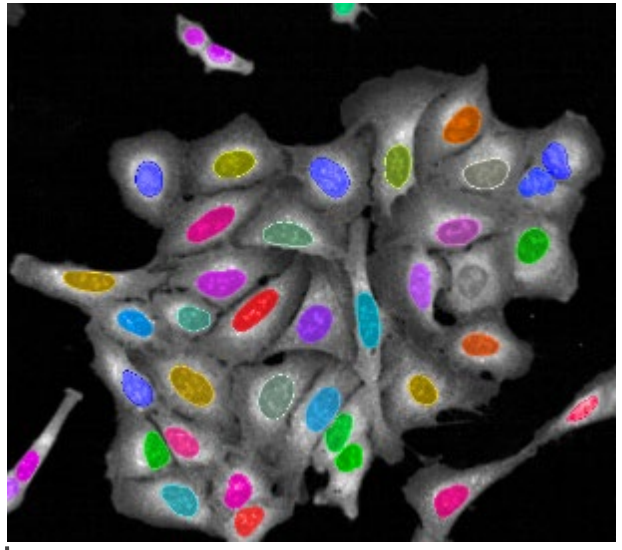




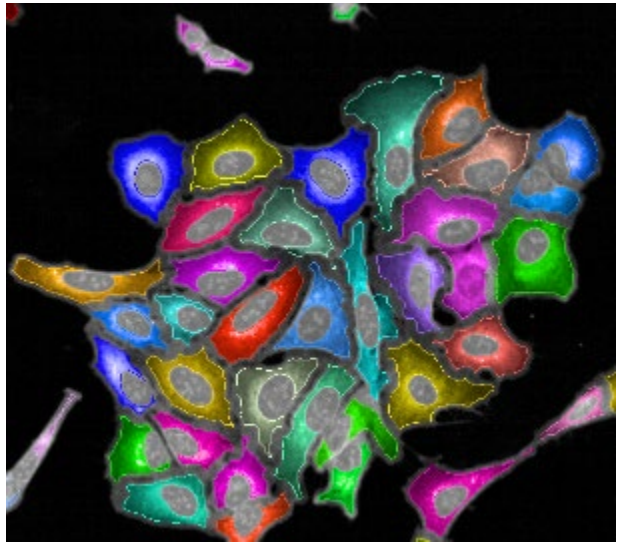


Define Cellular Compartments

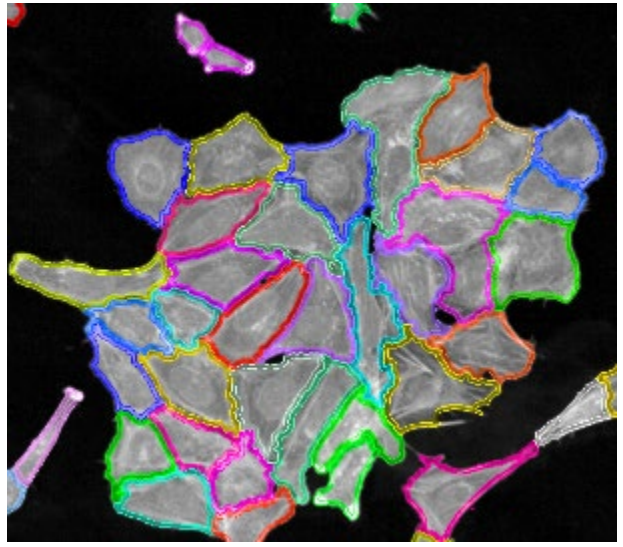
nuclei



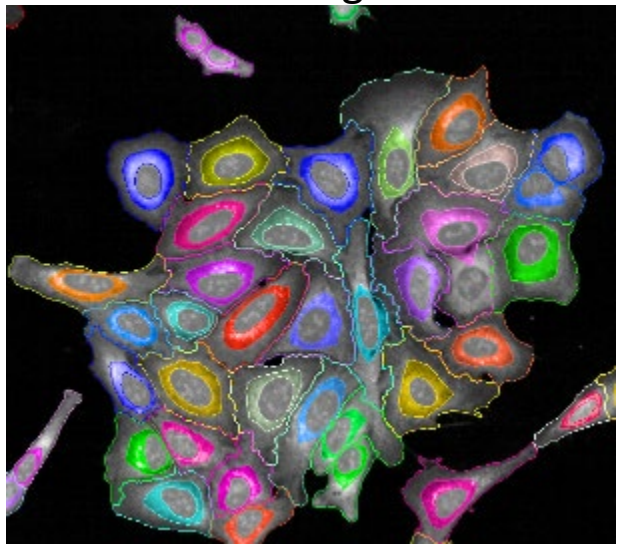
cytoplasm



membrane



ring



cell

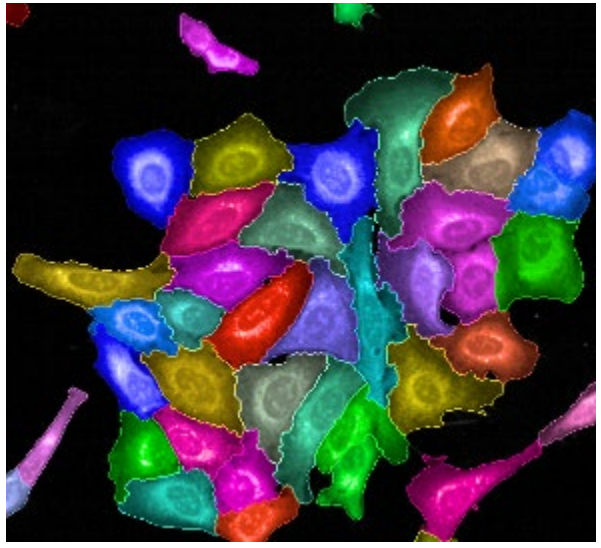
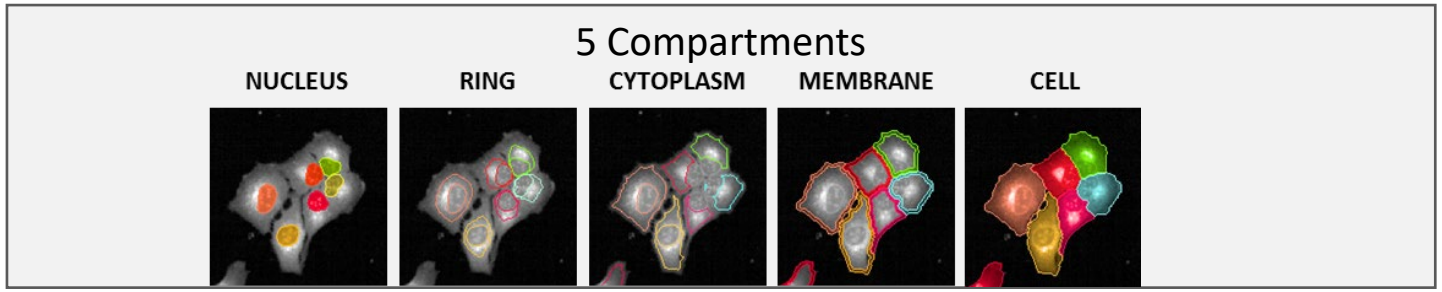
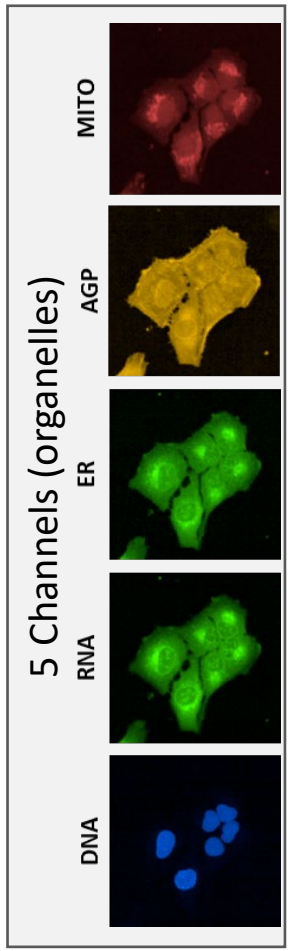
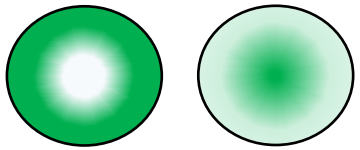


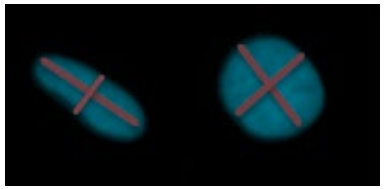
Image Processing

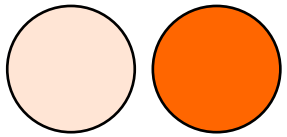
Profiling
with PerkinElmer
Harmony Software

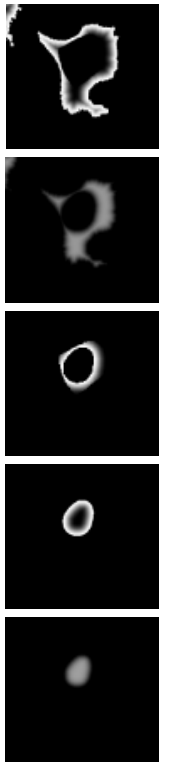


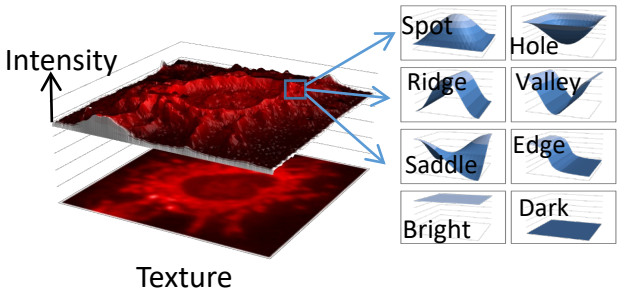



Radial distribution

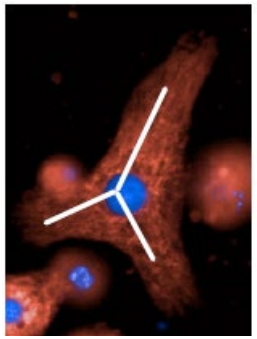

Axial

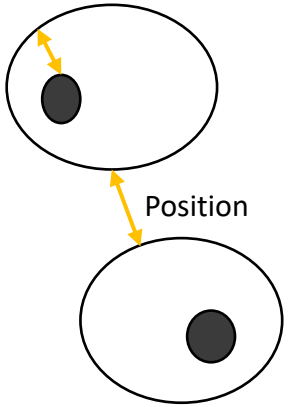

Intensity

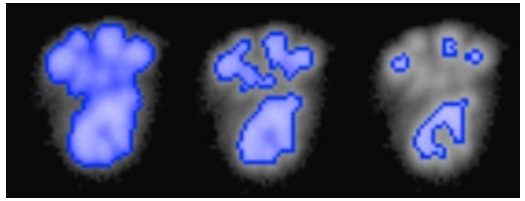

Profile

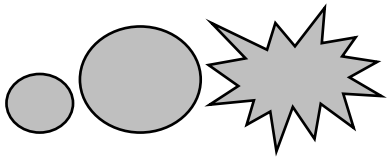


Texture


Symmetry



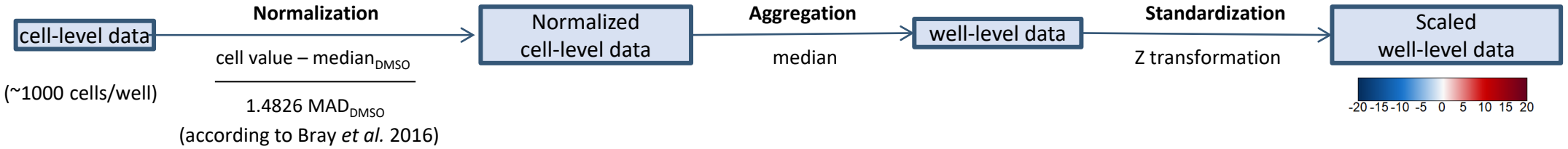

Compactness

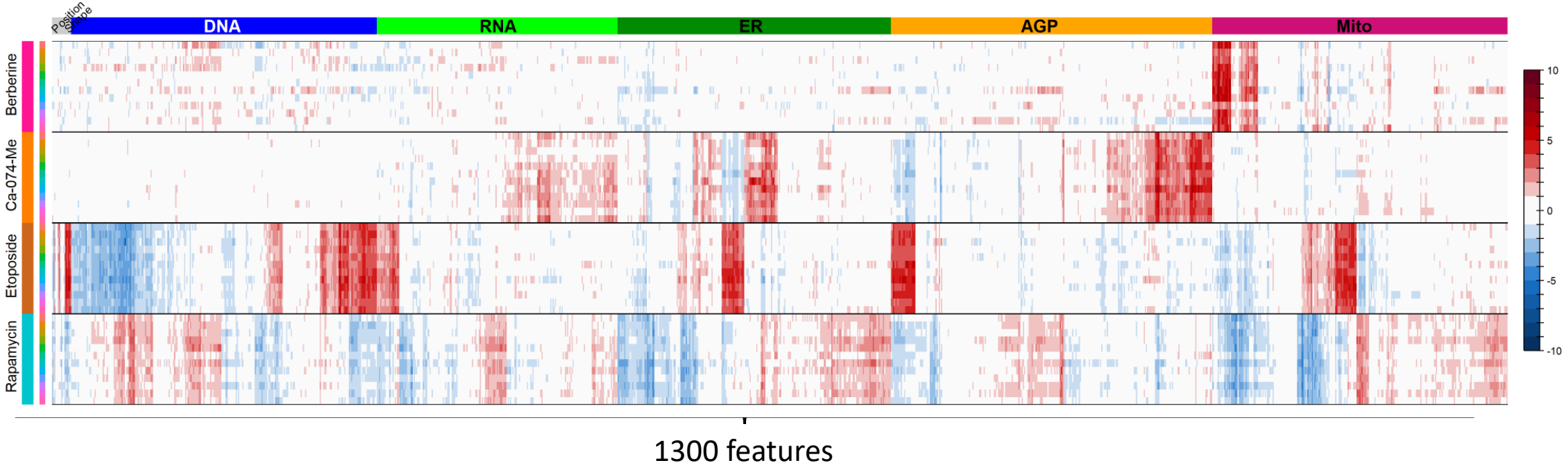

Shape

= 1300 features

With illustrations from Perkin Elmer

Example Chemicals: Quantitative Observation

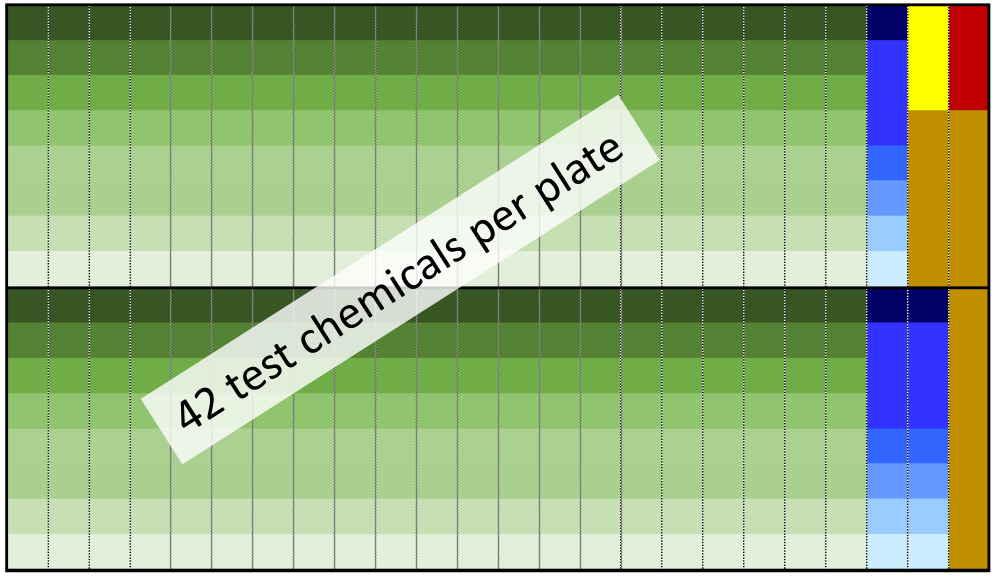


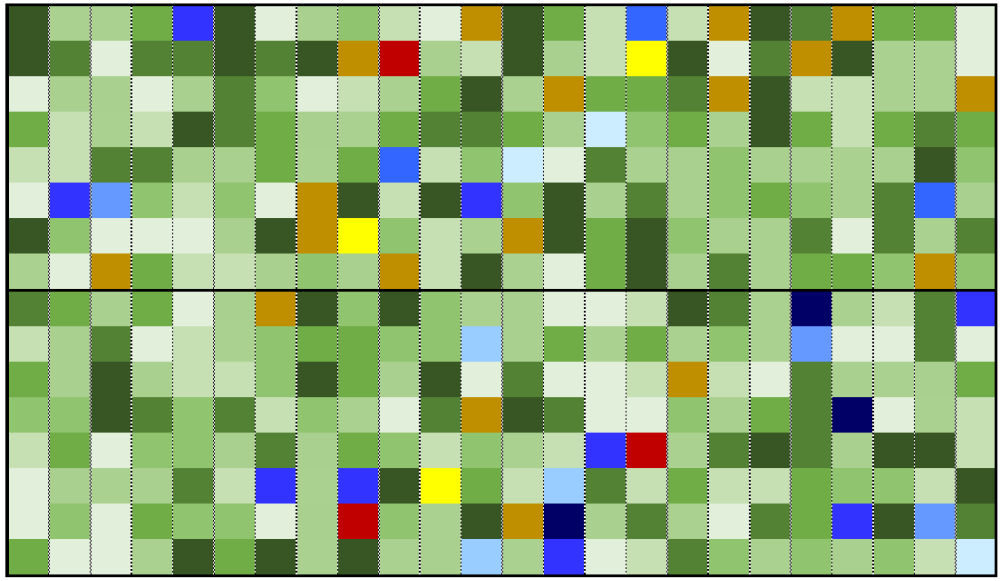


⇨ **Qualitative observations can be quantified**

adapted from Nyffeler *et al.* 2020

Example for Dose Plate Design



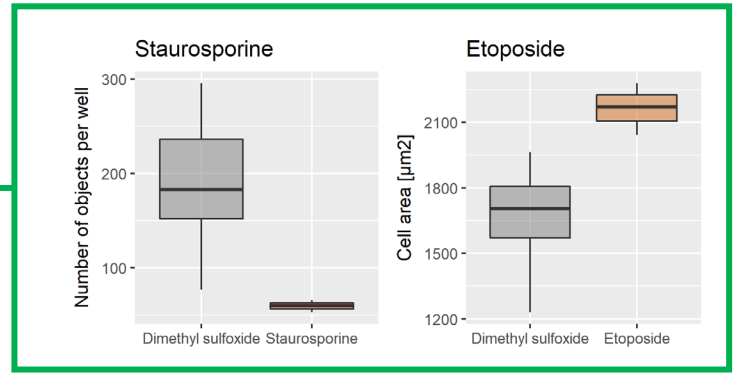


each test plate is uniquely randomized
→ no systematic edge effects

Color	Reference Chemicals:
	Phenotypic reference chemicals (concentration-response)
	Transcriptomics reference chemical (single concentration)
	Viability positive control Staurosporine
	Vehicle control (0.5% DMSO)

Quality Control Reports (1)

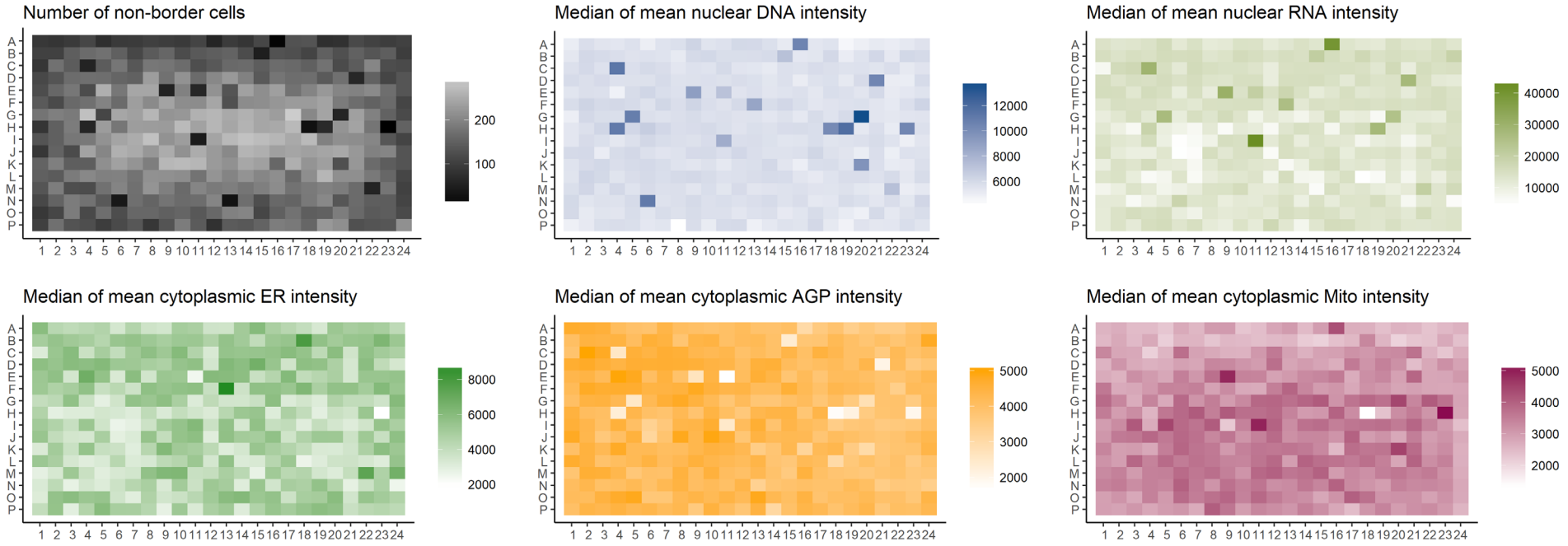
plate matched sample key



TC00000193

	Median
Number_of_Objects	180
DNA_Nuclei_Intensity_Mean	5600
RNA_Nuclei_Intensity_Mean	13000
ER_Cytoplasm_Intensity_Mean	4200
AGP_Cytoplasm_Intensity_Mean	4000
Mito_Cytoplasm_Intensity_Mean	3500

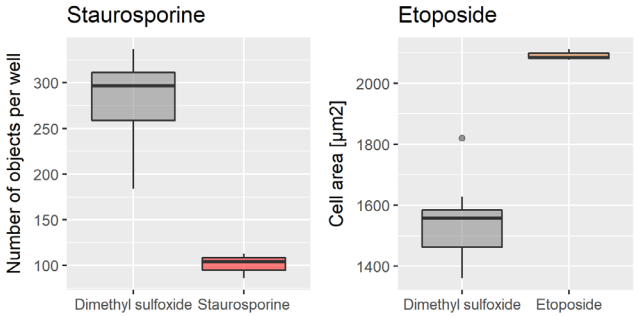
uniform intensity of labels across the plate



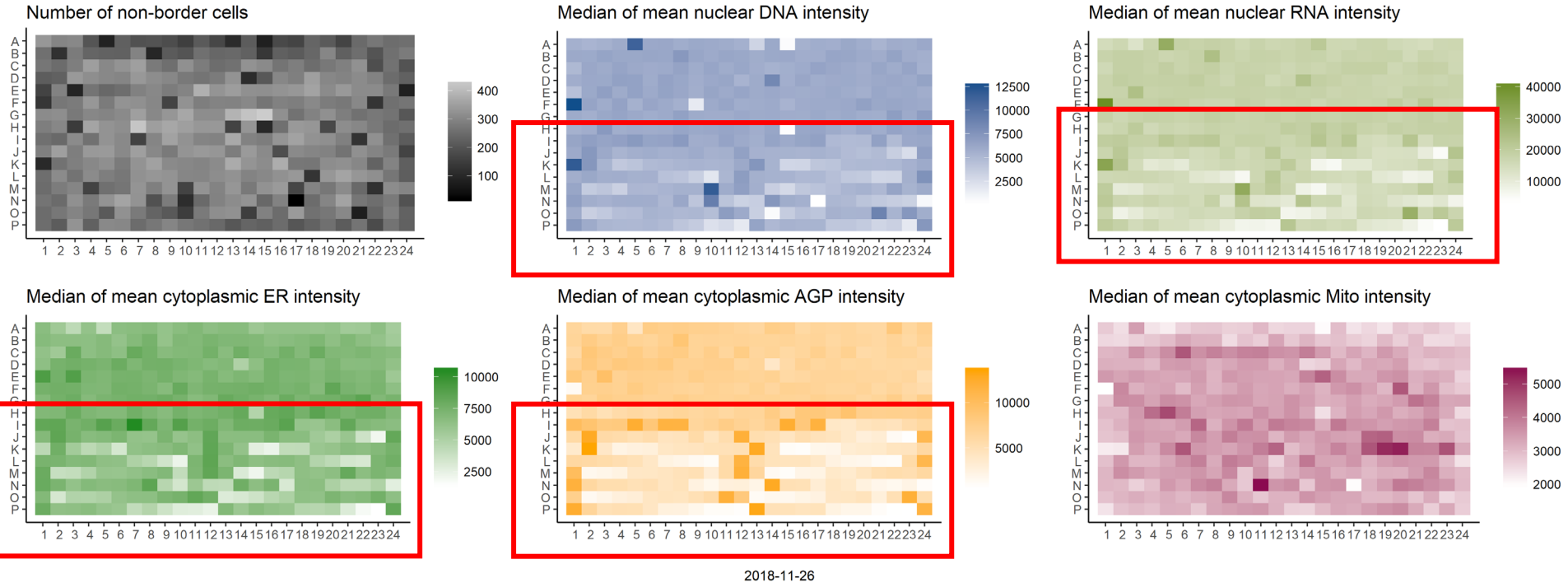
2018-11-26

Quality Control Reports (2)

TC00000269

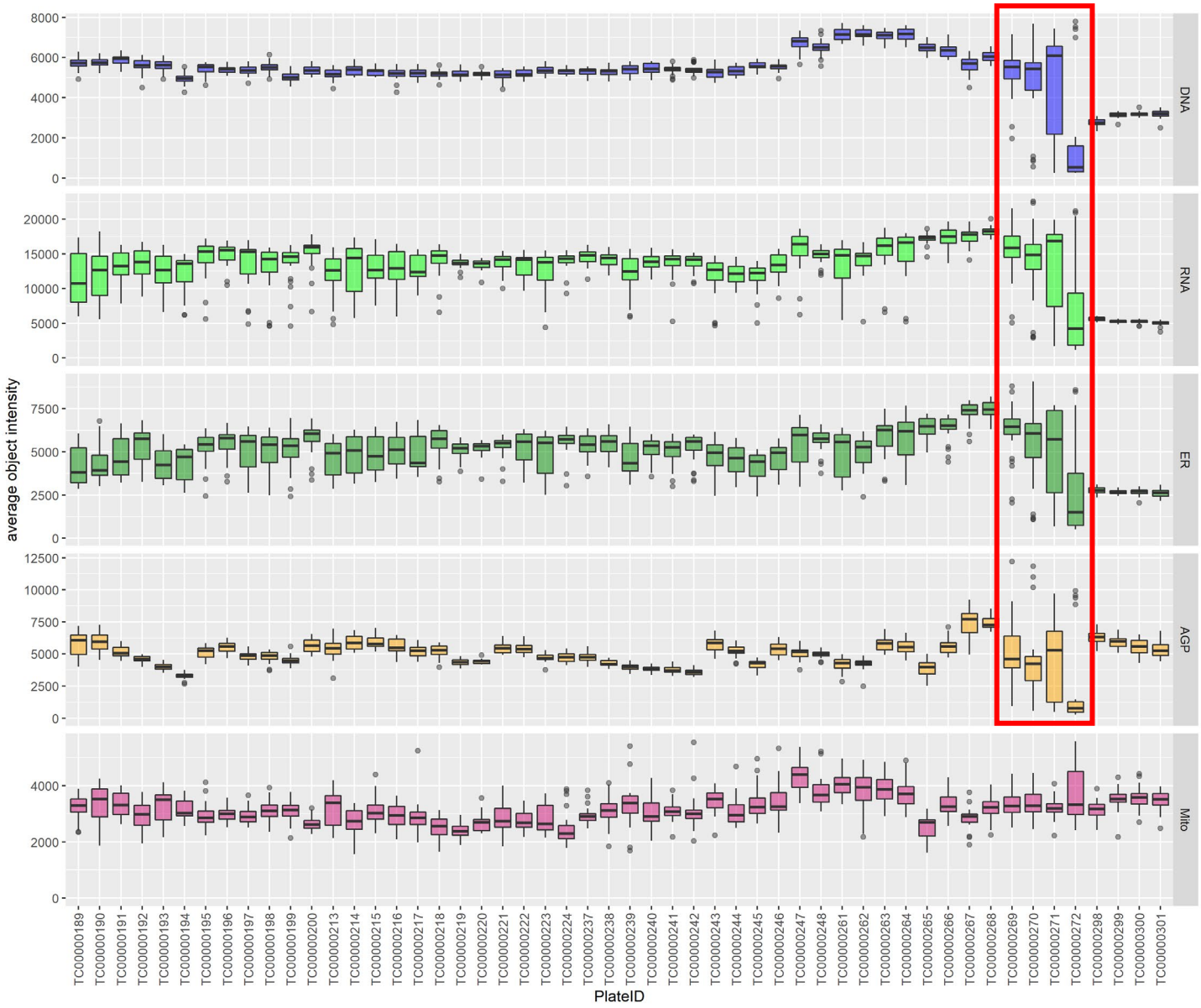


	Median
Number_of_Objects	300
DNA_Nuclei_Intensity_Mean	5500
RNA_Nuclei_Intensity_Mean	16000
ER_Cytoplasm_Intensity_Mean	6400
AGP_Cytoplasm_Intensity_Mean	4600
Mito_Cytoplasm_Intensity_Mean	3300



failure during label dispensing

Quality Control Reports (3)



Aim for CCTE/EPA

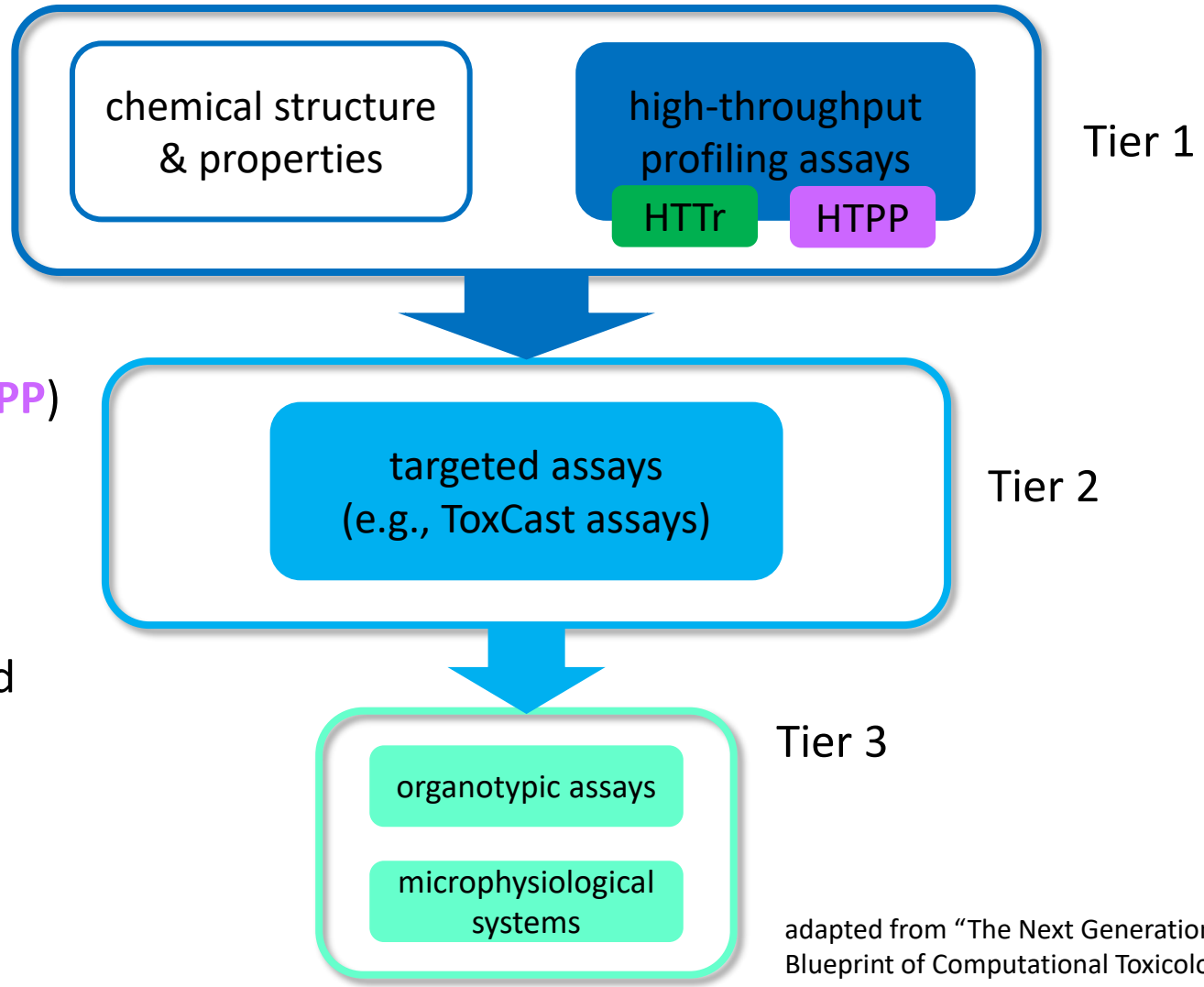
Tiered Hazard Evaluation Strategy based on New Approach Methods (NAMs)

Profiling Assays

- untargeted
- measure large number of endpoints (e.g., transcripts, phenotypic features)
- high-throughput transcriptomics (**HTTr**) (Harrill et al. 2021, PMID: 33538836)
- high-throughput phenotypic profiling (**HTPP**) (Nyffeler et al. 2020, PMID: 31899216)

Focus

- Prioritization: False positives are preferred over false negatives



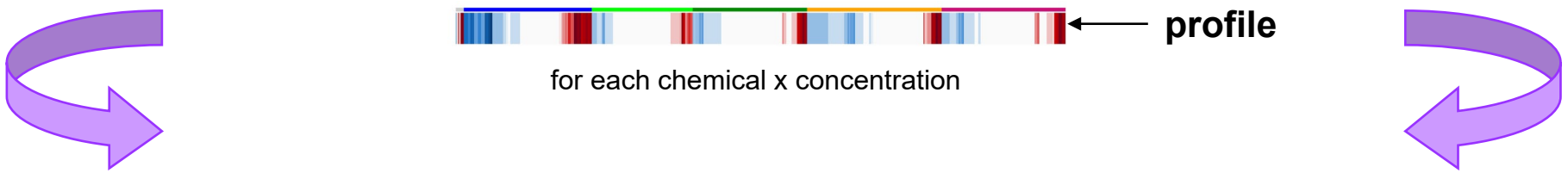
adapted from "The Next Generation Blueprint of Computational Toxicology at the U.S. EPA", Tox. Sci. 2019; 169(2):317-322. PMID: 30835285

Challenges of Environmental Chemicals

- Often low expected bioactivity
- Often lack a specific molecular target in human-based cell models
- ‘poly-pharmacology’
- Responses can be associated with general cell stress

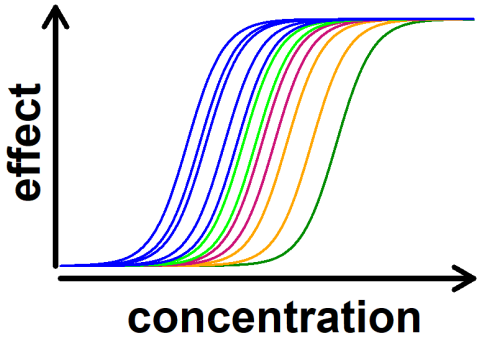
⇨ **more challenging for hit identification than drug-like chemicals**

Two Applications



Application 1

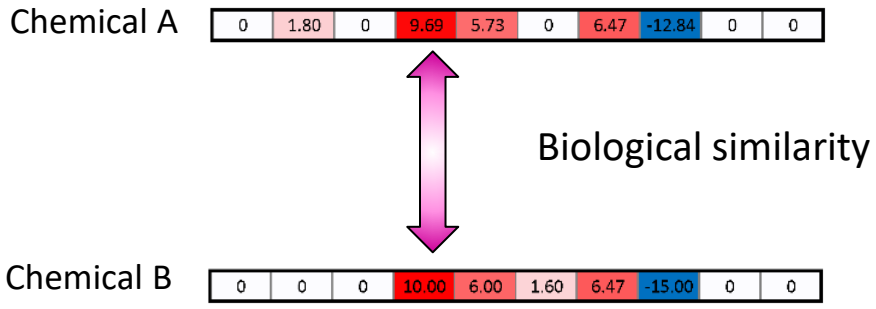
concentration-response modelling



Potency estimation:
***in vitro* point-of-departure (POD)**

- Nyffeler *et al.* (2020) Toxicol Appl Pharmacol. PMID: 31899216
- Willis *et al.* (2020). SLAS Discov. PMID: 32546035
- Nyffeler *et al.* (2021). SLAS Discov. PMID: 32862757

Application 2



Compare profiles with annotated reference chemicals
→ putative mechanisms

work in progress

EPA United States Environmental Protection Agency

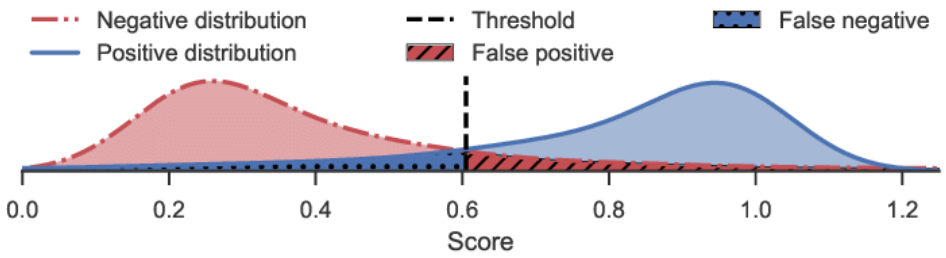
Application 1: Potency Estimation

Challenges in Analysis of Profiling Data

Targeted Assays

- Response is predictable
- Often have a positive control
- Often have known negative controls

➔ Use of positive and negative controls to set a threshold for hit calls



https://www.researchgate.net/profile/Denis_Reis/publication/327847657/figure/fig1/AS:674446763380738@1537812047280/Threshold-and-score-distribution-for-a-binary-classification-process.png

Profiling Assays

- Measure 100s – 1000s of features
  → not feasible to define a threshold for each feature in an analogous manner to targeted assays.
- Multiple diverse phenotypes can be observed
  → no single 'positive control'
- Multiple testing problem can lead to identification of false actives

➔ How should thresholds be chosen to ensure reliable hit calls?

⇨ **no widely accepted standard practices for hit identification from phenotypic profiling data ➔ potential barrier for regulatory applications**

Screen of Environmental Chemicals

- 462 test chemicals
  - pesticides (~ 75%), drug-like chemicals, food additives, industrial chemicals
  - 448 chemical from the 'APCRA' list
    - available in vivo effect values
    - available toxicokinetic parameters for in vitro to in vivo extrapolation (IVIVE)

APCRA
ACCELERATING THE PACE OF CHEMICAL RISK ASSESSMENT

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

Experimental design	
Cell type	U-2 OS
Exposure time	24 h
Cell seeding density per well	400
# unique chemicals	**462**
# concentrations	8
Concentration spacing	½ $\log_{10}$
# solvent controls/plate	24
# replicates/plate	1
# independent experiments	4

Reference chemicals run on each plate

	Specific	**Broad**
Subtle	Berberine chloride	Rapamycin
Strong	Ca-074-Me	Etoposide

Procedure

- **Data from the APCRA set**
  - Well-level data for 478 chemicals
  - 8 concentrations
  - 4 biological replicates

100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	A	B	C	DMSO
30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	A	B	C	DMSO
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	A	B	C	DMSO
3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	A	B	C	DMSO
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	A	B	C	DMSO
0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	A	B	C	DMSO
0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	A	B	C	DMSO
0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	A	B	C	DMSO
100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	D	Stauro	DMSO	DMSO
30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	D	Stauro	DMSO	DMSO
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	D	Stauro	DMSO	DMSO
3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	D	Stauro	DMSO	DMSO
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	D	Stauro	DMSO	DMSO
0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	D	Stauro	DMSO	DMSO
0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	D	Stauro	DMSO	DMSO
0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	D	Stauro	DMSO	DMSO

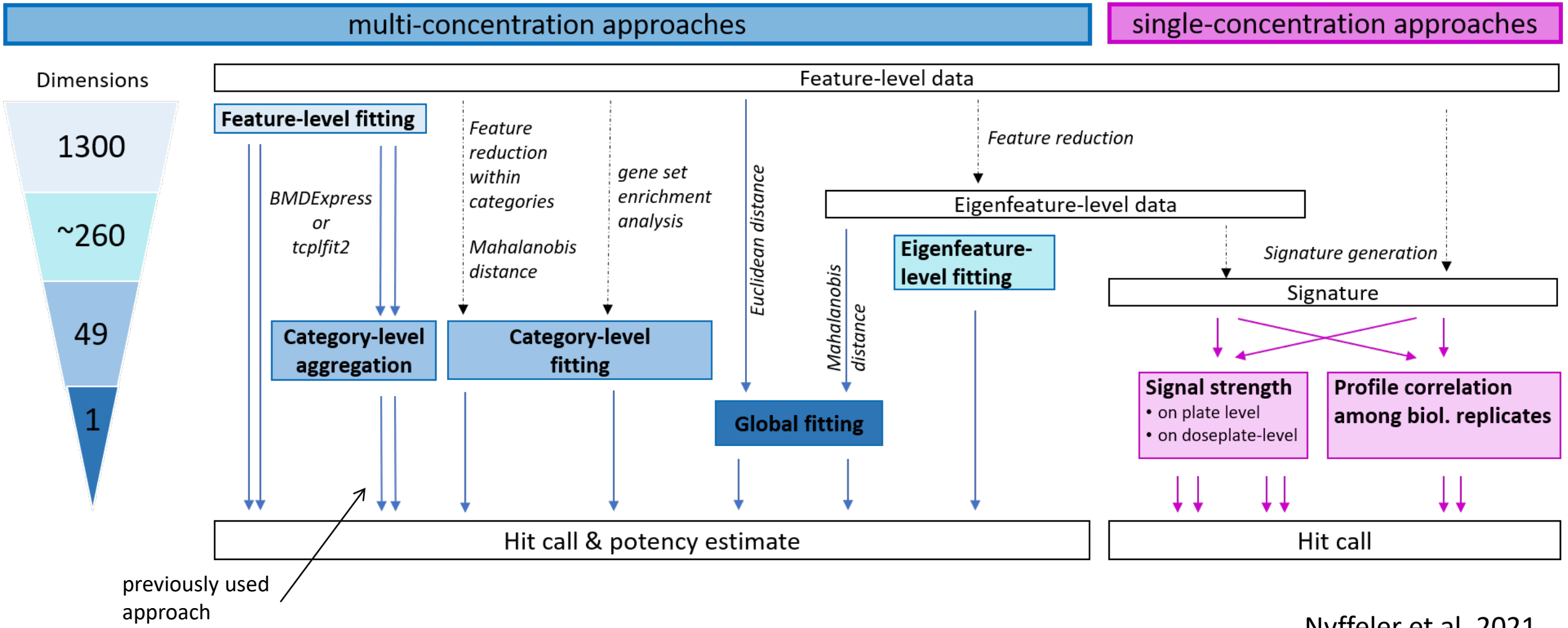
- **Constructed a null data set**
  - Sampling of well-level data from the lowest two tested concentrations of test chemicals
  - 108 ‘null chemicals’ were generated, with 8 concentrations and 4 biological replicates

  → False positive rate
- **Reference chemical berberine chloride**
  - 12 independent replicates

  → True positive rate
- **Test chemicals run in duplicates**
  - 16 test chemicals were screened twice

  →Concordance
- **15 different approaches were compared at a fixed false positive rate of ~10%**

Different Approaches to Identify Hits



Nyffeler et al. 2021

potency estimate = phenotype altering concentration = PAC

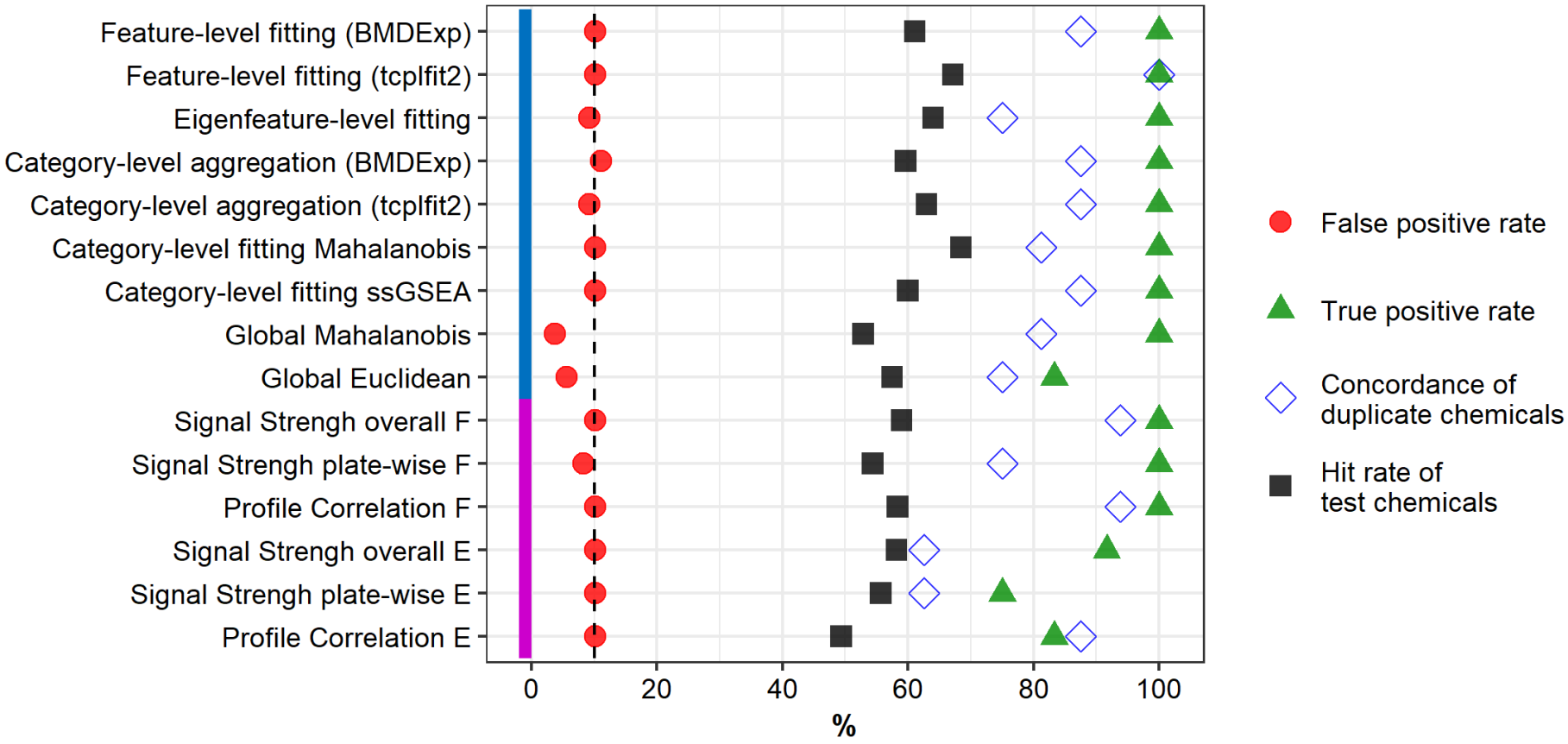
Metrics

- False positive rate (FPR) = % of null chemicals that are positive
  - Null sets are constructed from the lowest 2 concentration of all test chemicals
- True positive rate (TPR) = % of APCRA Berberine that are positive
  - Berberine chloride: weak chemical with specific effects in only 100-200 features → most closely resembles expected behavior from positive test chemicals
- Hit rate = % of test chemicals that are active
- Concordance:
  - % of test chemicals with concordant hit calls (all inactive or all active)
  - Number = # chemicals that are active

Thresholds for each approach were individually optimized for

1. False positive rate of ~ 10%
2. Highest true positive rate (100%)
3. Best possible concordance & high hit rate

Optimizing Approaches to Achieve Equivalent False Discovery Rate

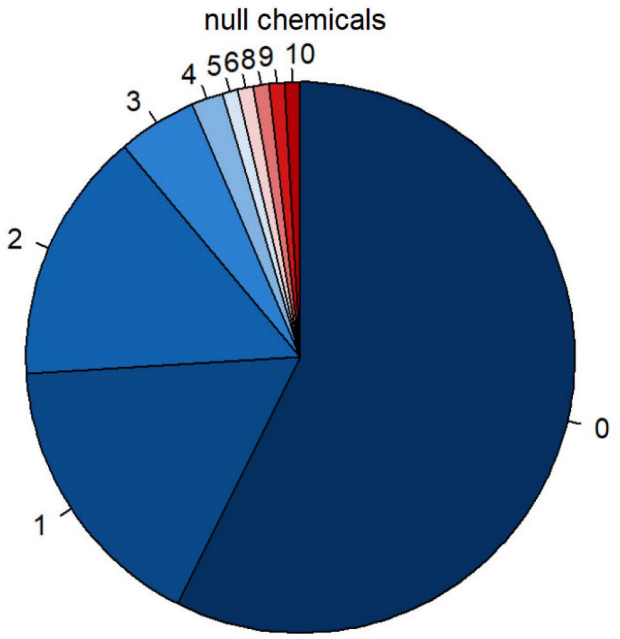


Nyffeler et al. 2021

- ⇨ **11/15 approaches identified 100% of true positives**
- ⇨ **Hit rate is overall between 50-70%**

Concordance of Hit Calls Across Approaches

B



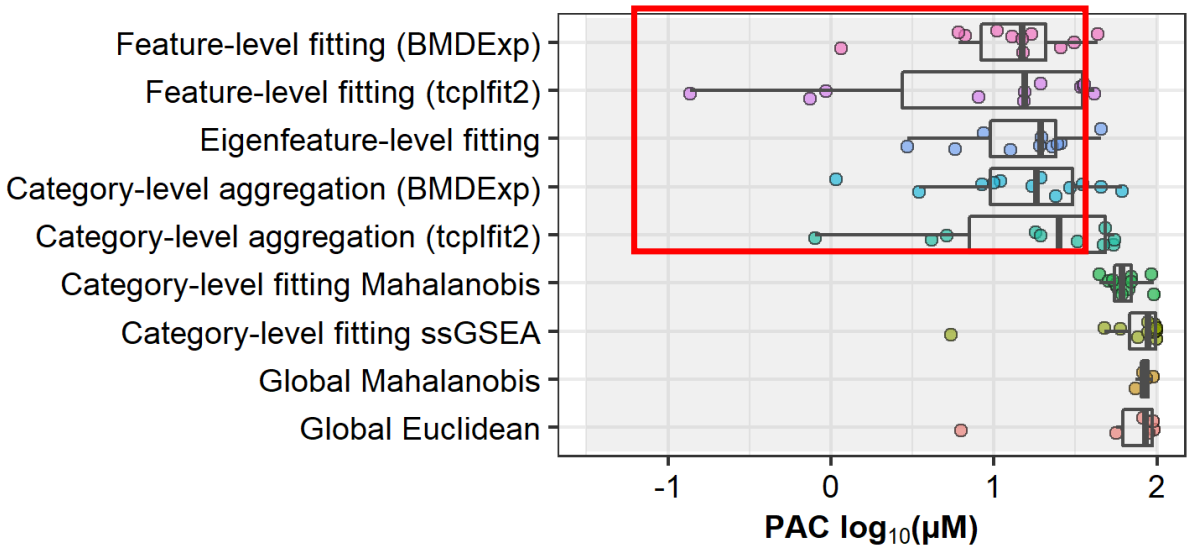
⇨ **87% of null chemicals were inactive in 9 or more approaches**

Nyffeler et al. 2021

Concordance of Potency Estimates

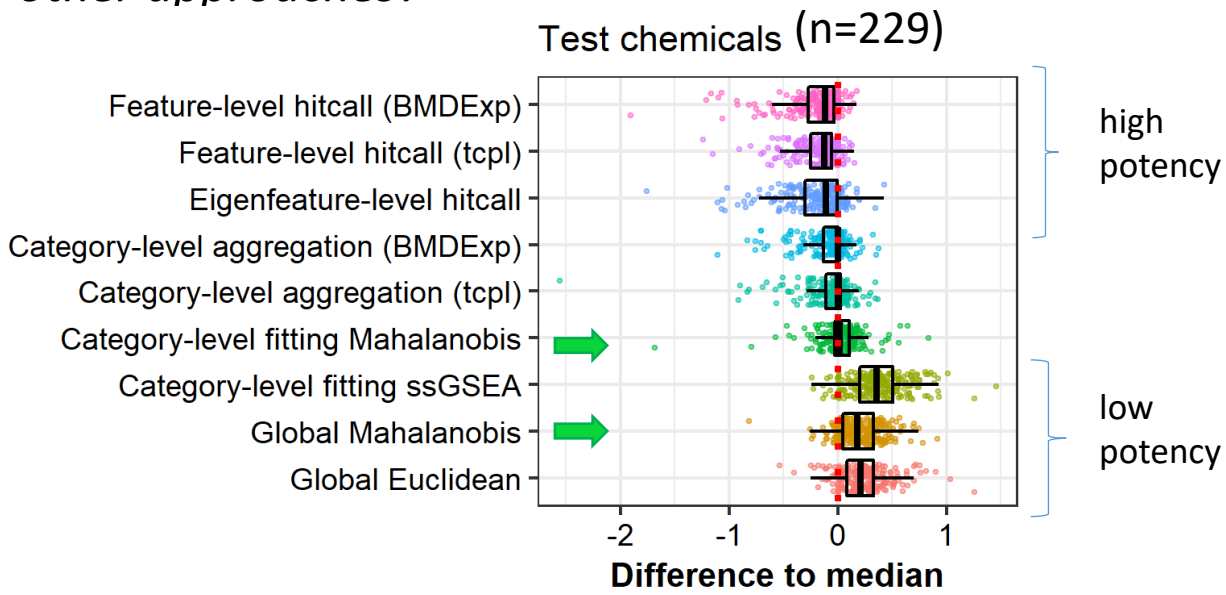
Null chemicals

Does the approach produce many high-potency false positives?



Test chemicals

How sensitive is the approach relative to the other approaches?

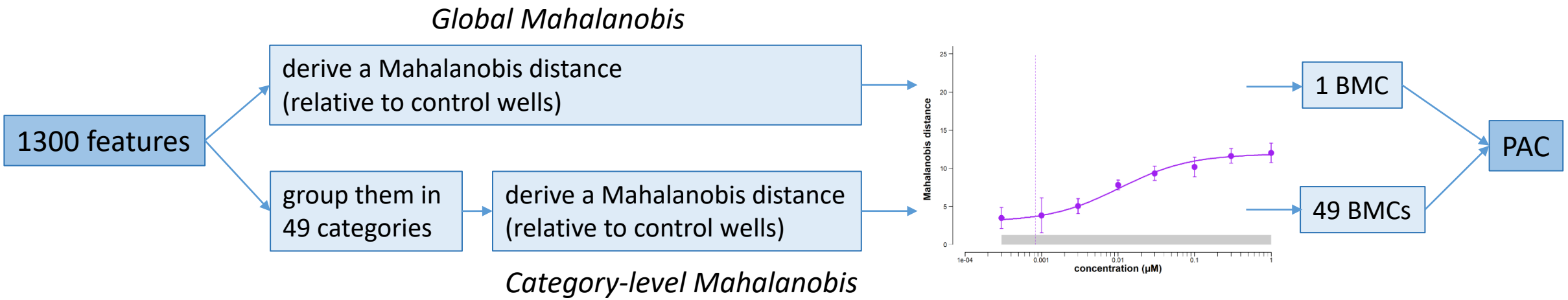


⇨ **Feature-based approaches (including category-level aggregation) have a higher risk of false positive, highly potent results**

Nyffeler et al. 2021

Final Choice of Analysis Approach

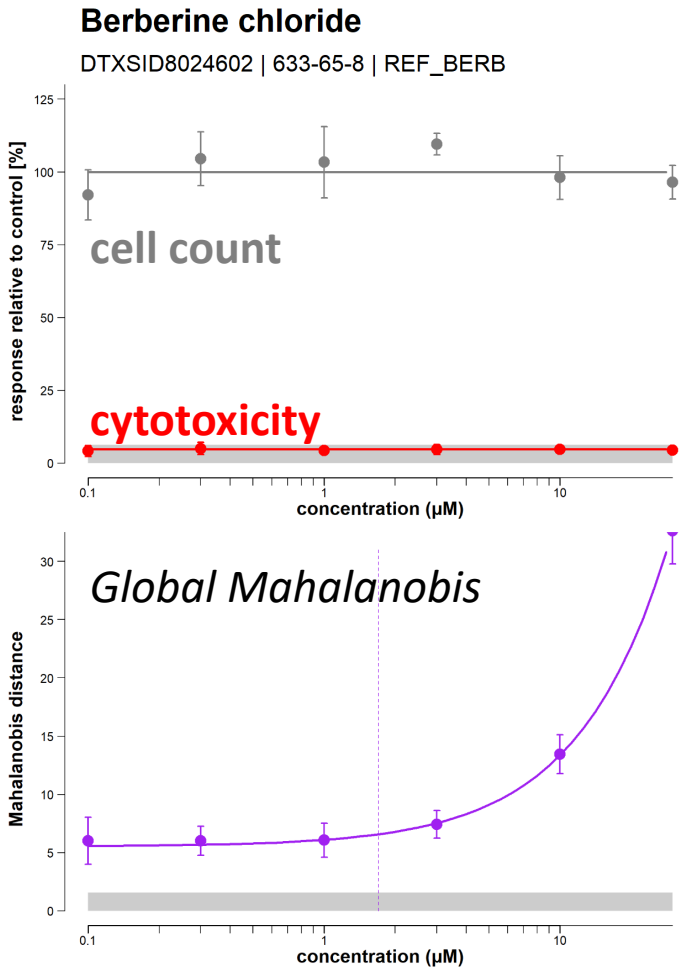
- **Mahalanobis Distance (D_M):** A multivariate distance metric that measures the distance between a point (vector) and a distribution.

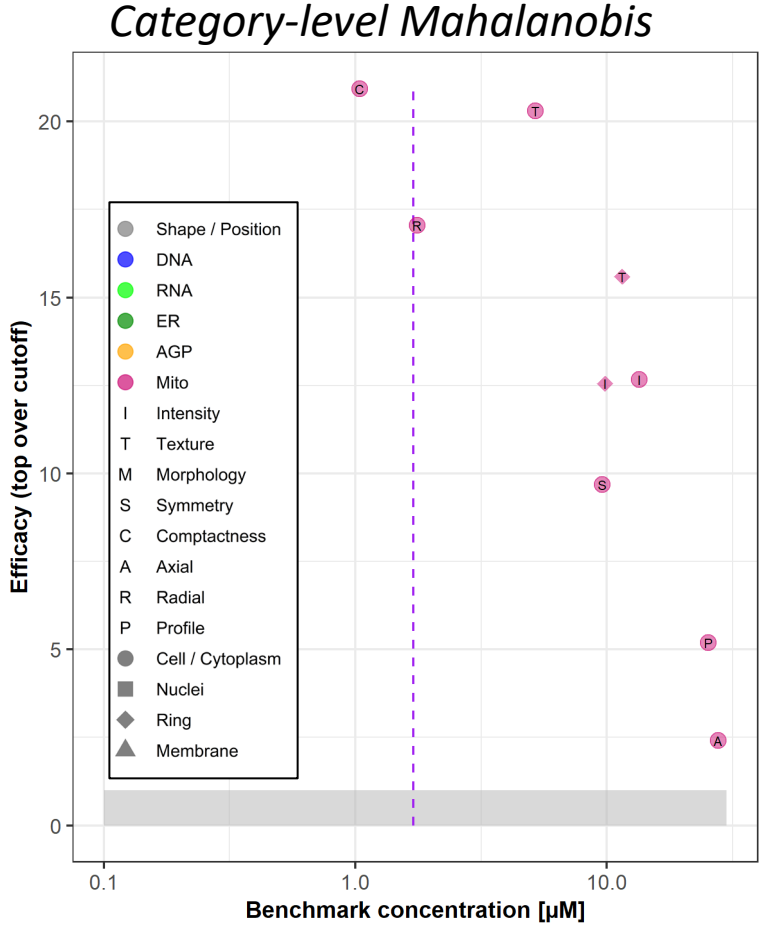


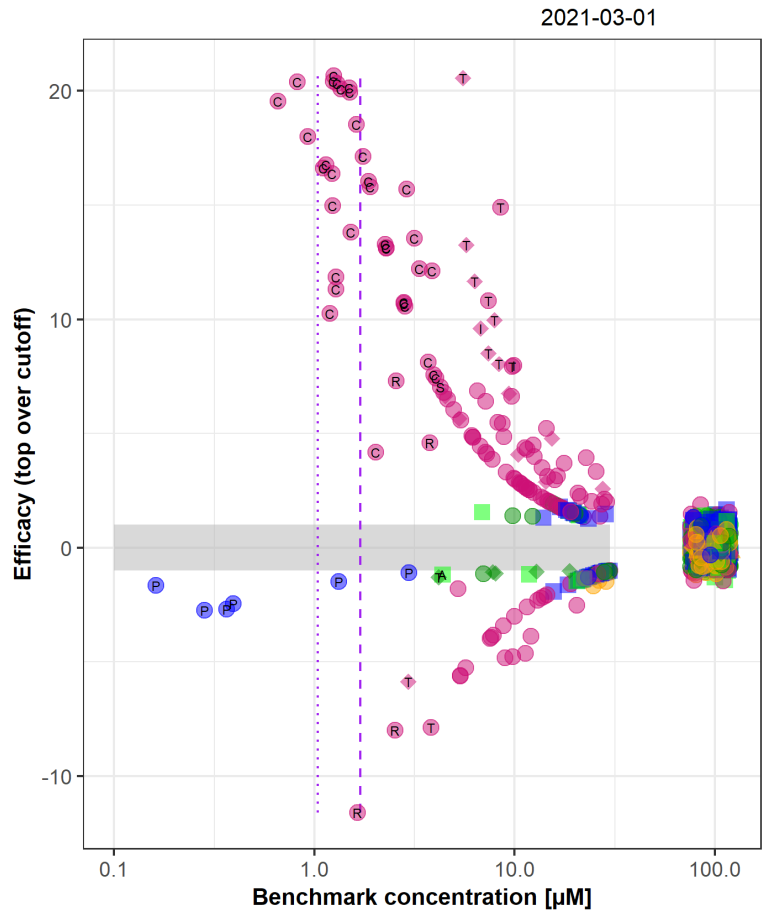
- Chemicals where a BMC can be determined using either the global or category D_M approach are considered active.

- The minimum of the global or most sensitive category BMC is the **Phenotype Altering Concentration** (PAC)

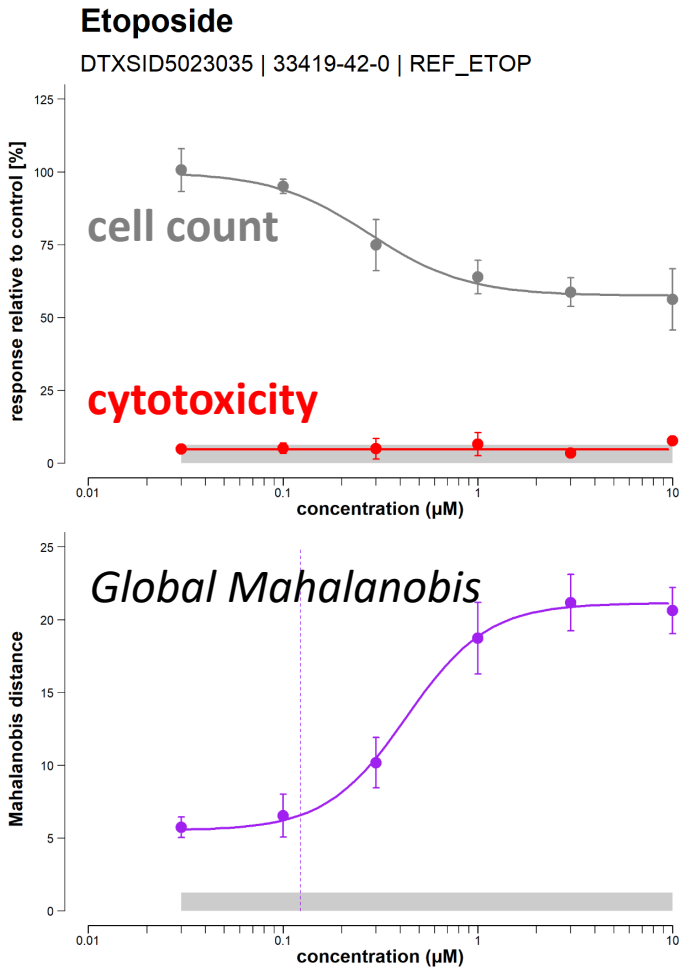
Visualization of High-Dimensional Data (1)

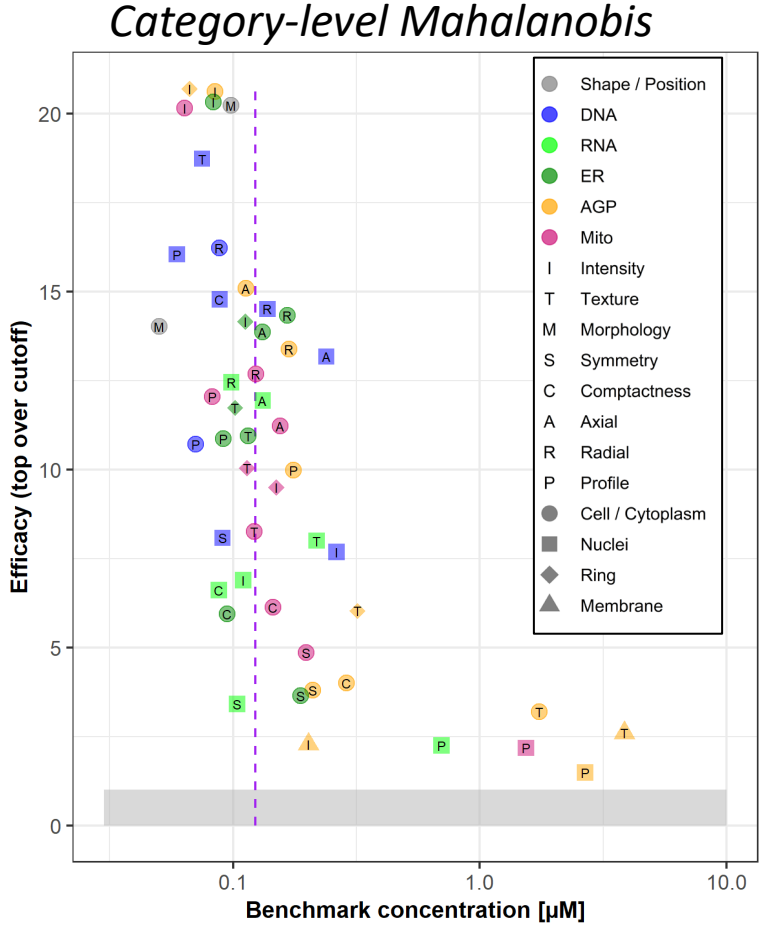


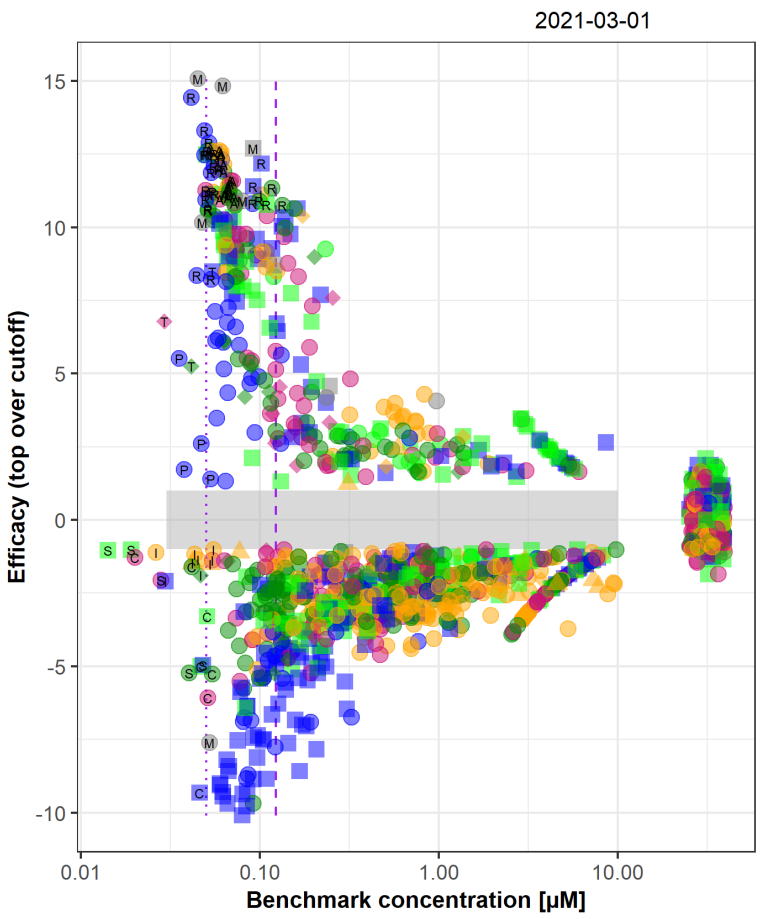




Visualization of High-Dimensional Data (2)







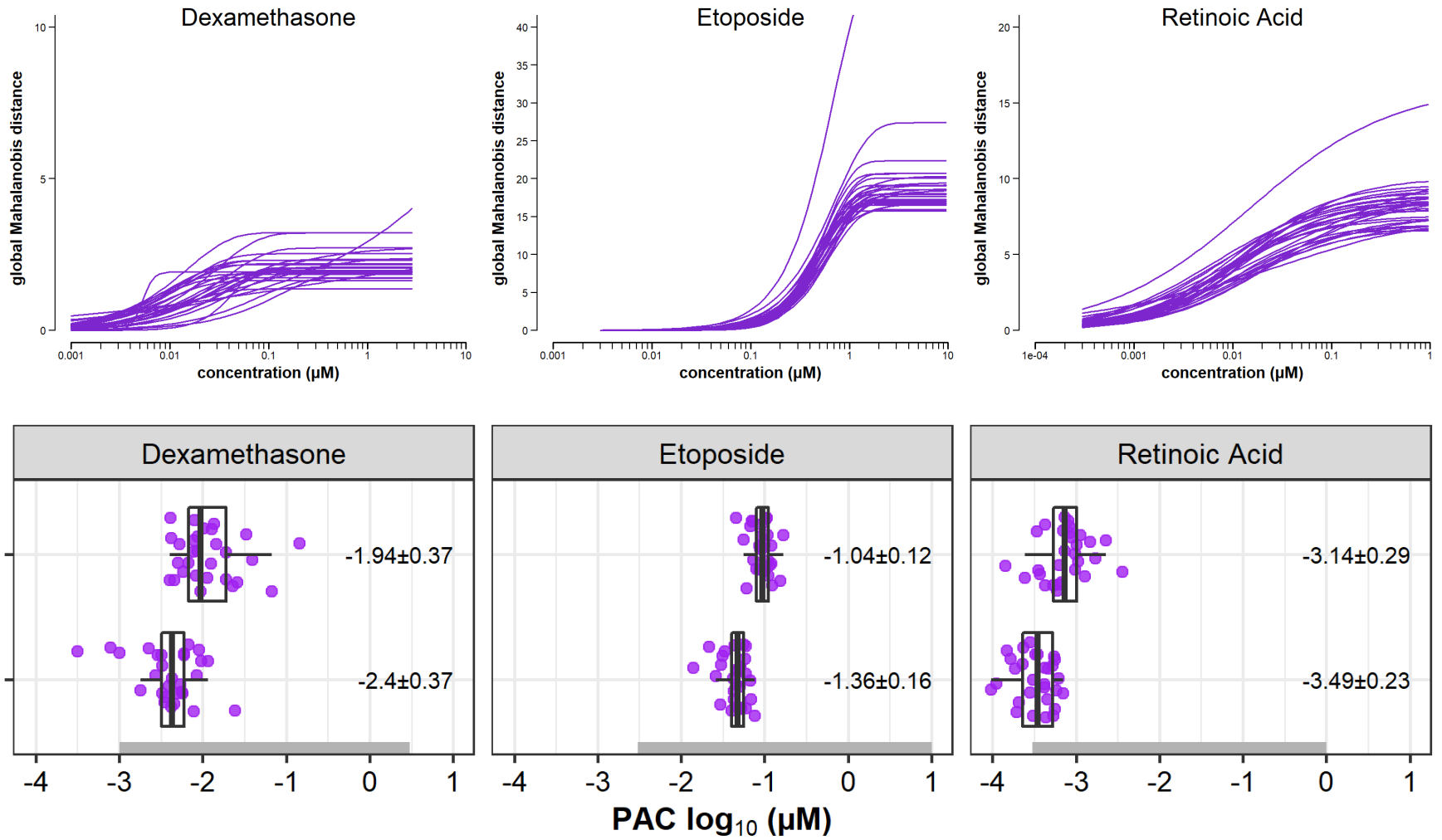
U-2 OS ToxCast Screen Experimental Design

Parameter	Multiplier	Notes
Cell Type(s)	1	U-2 OS
Time Points:	1	24 hours
Chemicals	1,202	TSCA Chemicals of interest to US EPA • Includes 462 APCRA case study chemicals • Includes 179 chemicals with annotated molecular targets
Concentrations:	8	3.5 $\log_{10}$ units; ~half-$\log_{10}$ spacing
Biological Replicates:	4	--

Reference chemicals run on each plate

	Chemical	Molecular Target	Tested Range
Weak	Dexamethasone	Glucocorticoid receptor agonist	0.001 – 3 µM
Medium	all-trans-Retinoic Acid	Retinoic acid receptor agonist	0.0003 – 1 µM
Strong	Etoposide	DNA topoisomerase inhibitor	0.03 - 10 µM
Extra strong	Trichostatin A	Histone deacetylase inhibitor	1 µM

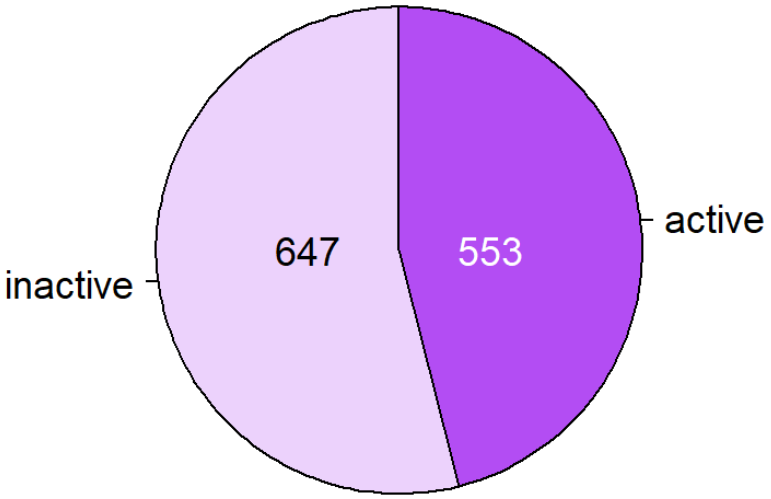
Reproducibility: Potencies

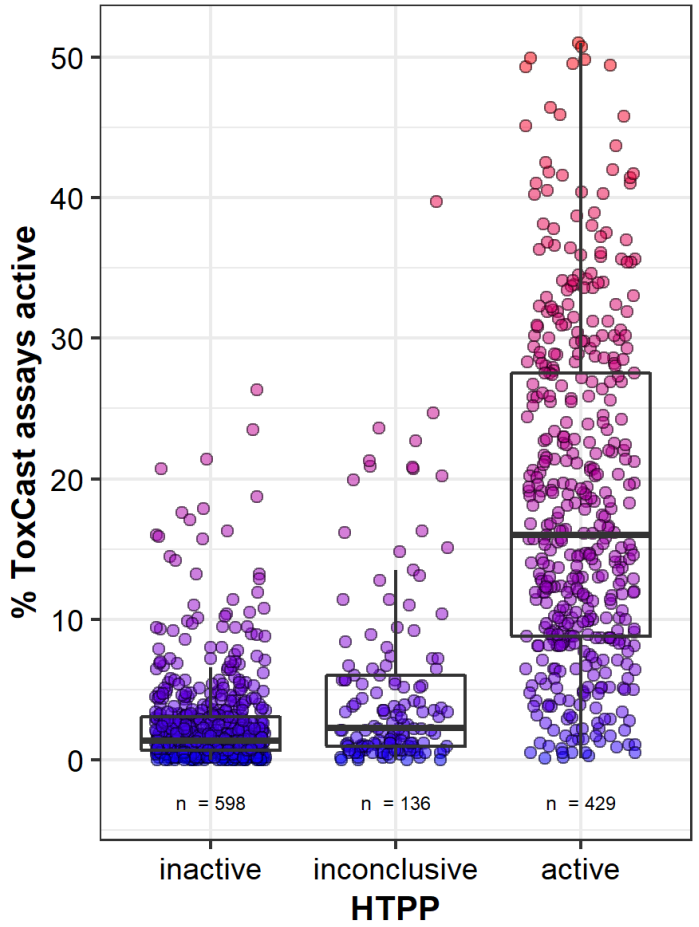


⇨ **Potency estimates vary less than ½ an order of magnitude**

HTPP Screening Results (1)

Active chemicals:

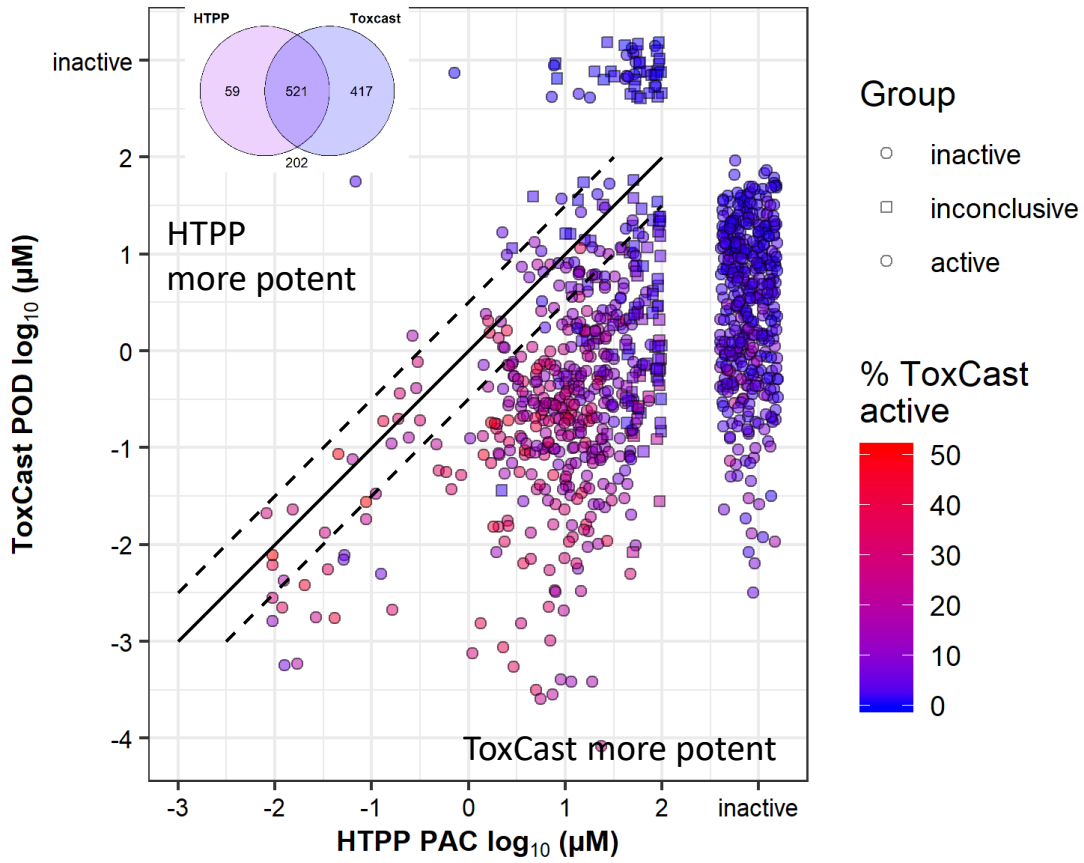




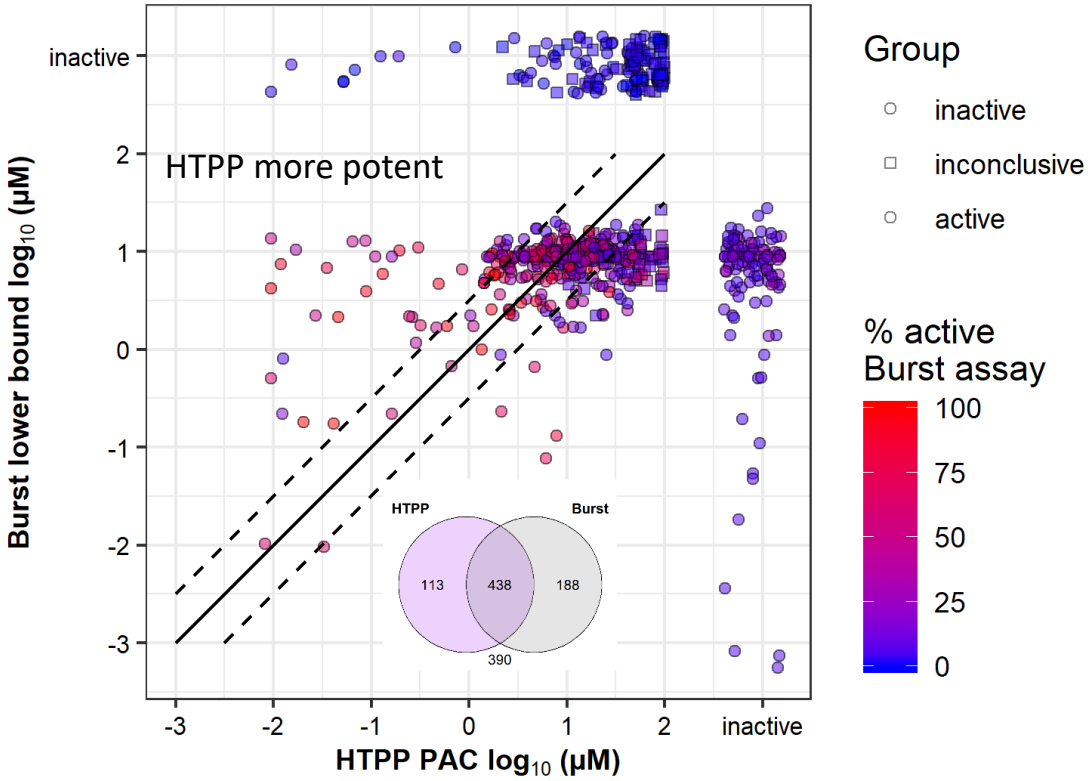
- ~ 40% of chemicals were active
- Most activity is > 10 µM
- Chemicals active in HTPP are more often 'promiscuous' in ToxCast

HTPP Screening Results (2)

Comparison with ToxCast screening results:

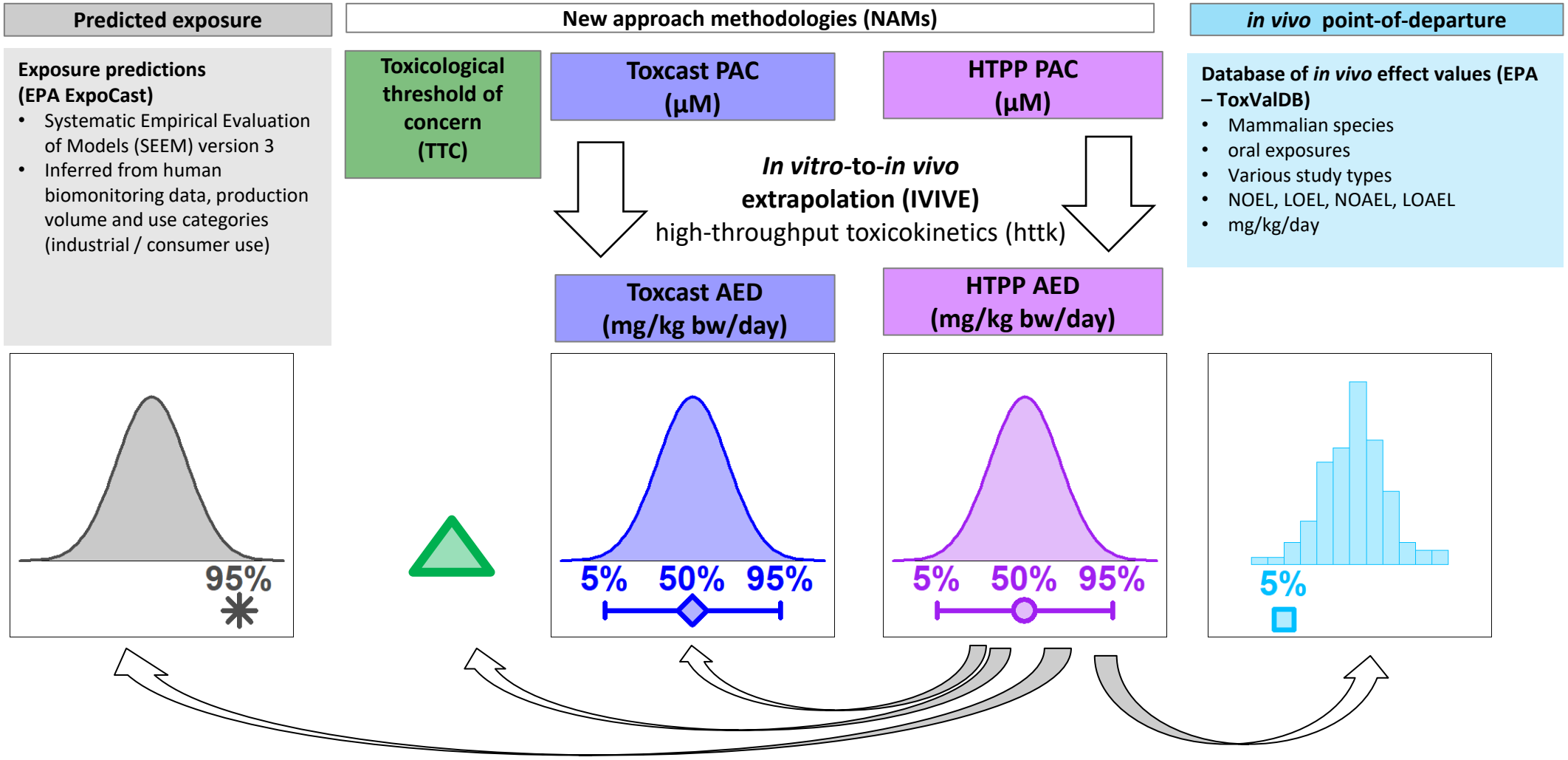


⇨ **Less potent than ToxCast POD**



⇨ **More potent than the ToxCast cytotoxicity burst estimate**

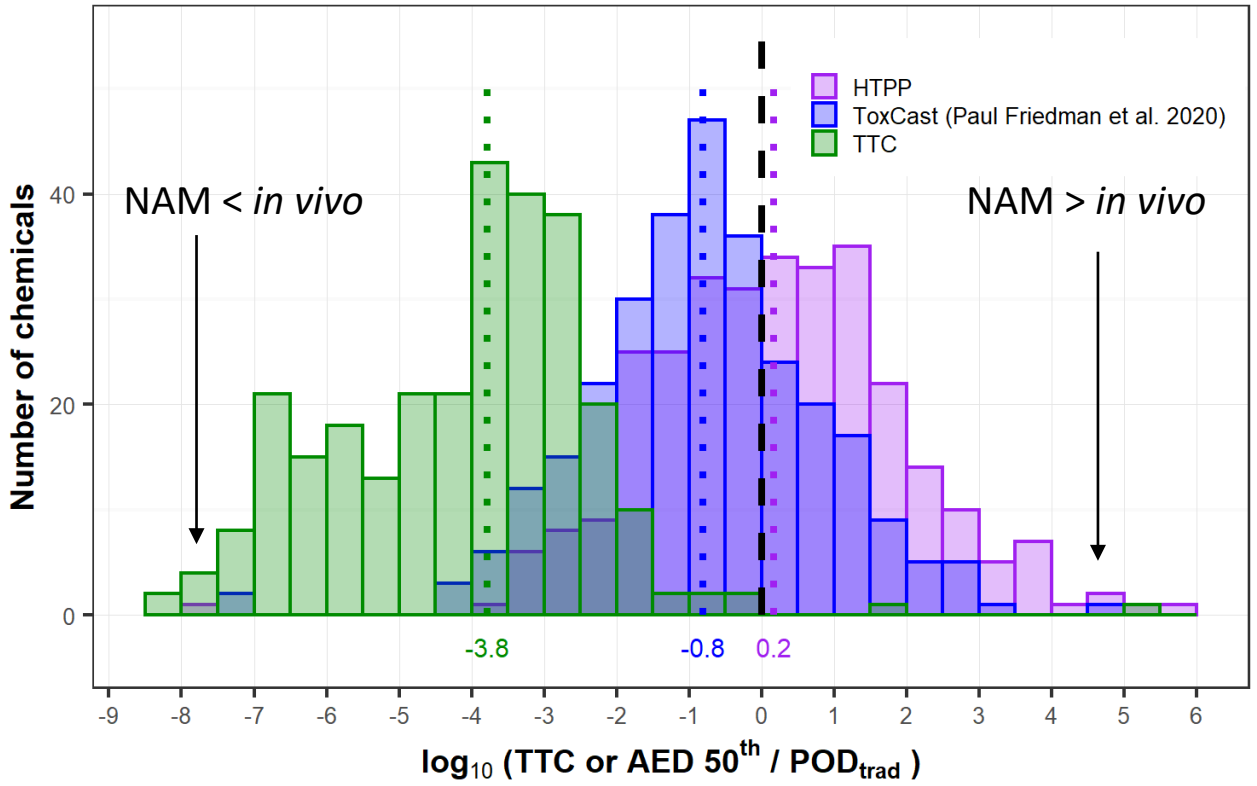
Comparison to *in vivo* data and exposure

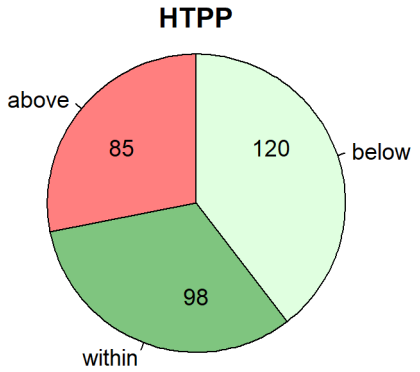


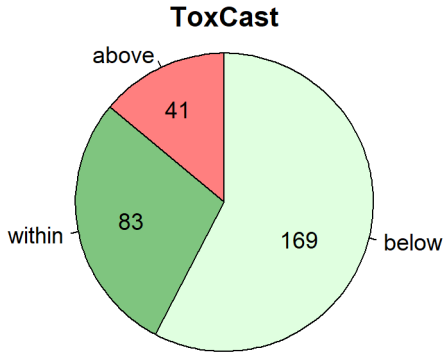
PAC: phenotype altering concentration
AED: administered equivalent dose

Comparison to *in vivo* Effect Values & other NAMs

- 303 chemicals were active and had pharmacokinetic (PK) information



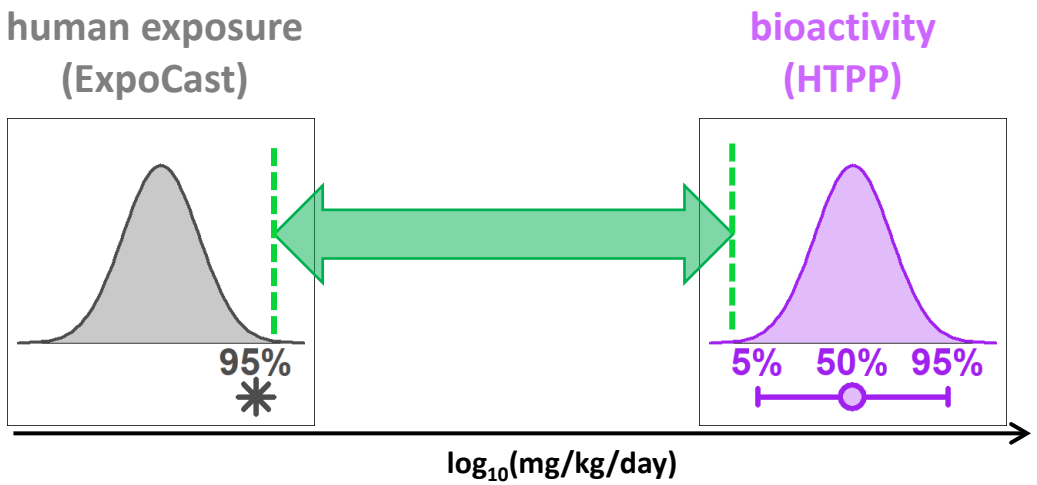


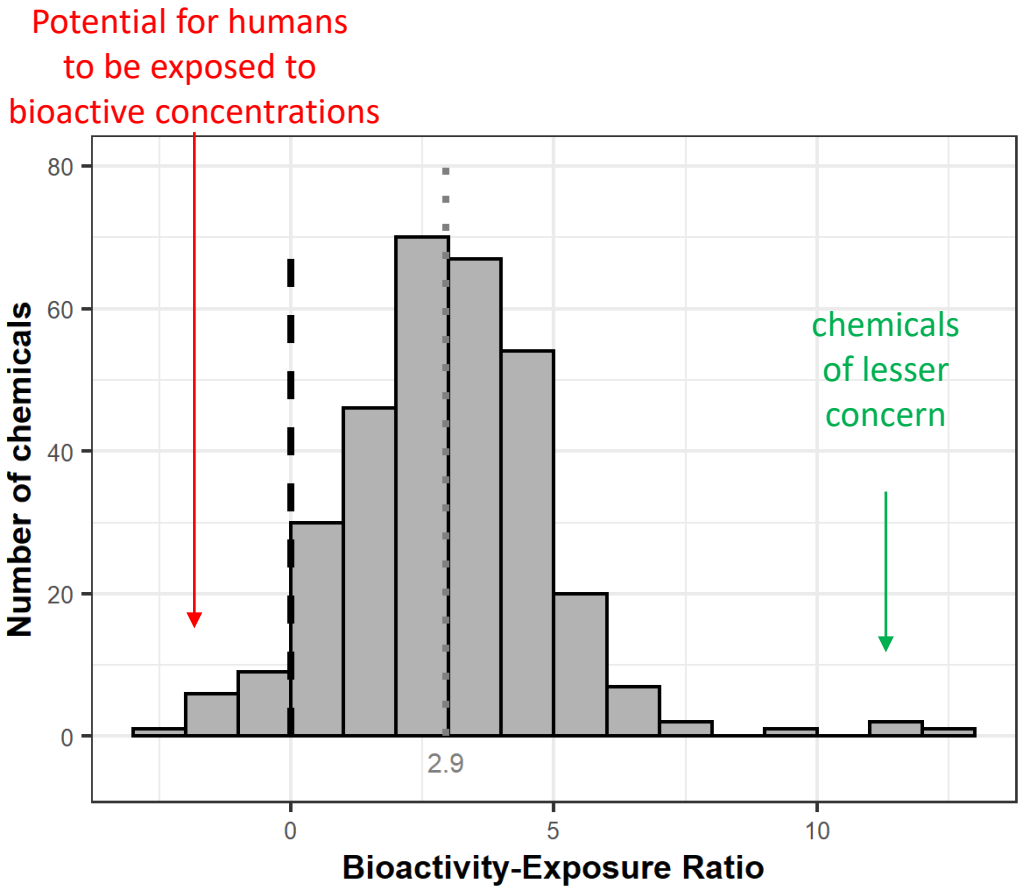


⇨ **HTPP AEDs are higher than ToxCast-derived AEDs and TTC values**

⇨ **78% of HTPP AED are within 2 orders of magnitude of the *in vivo* POD**

Comparison to Exposure Estimates





- ⇨ **for 49% of chemicals, predicted exposure is > 1000x lower than estimated bioactivity**
- ⇨ **for a small set of chemicals, the BER was negative, indicating a potential for humans to be exposed to bioactive concentrations of these chemicals**

Application 2: Mechanistic Prediction

Feature Selection & Profile Comparison

Feature Selection

1300 features

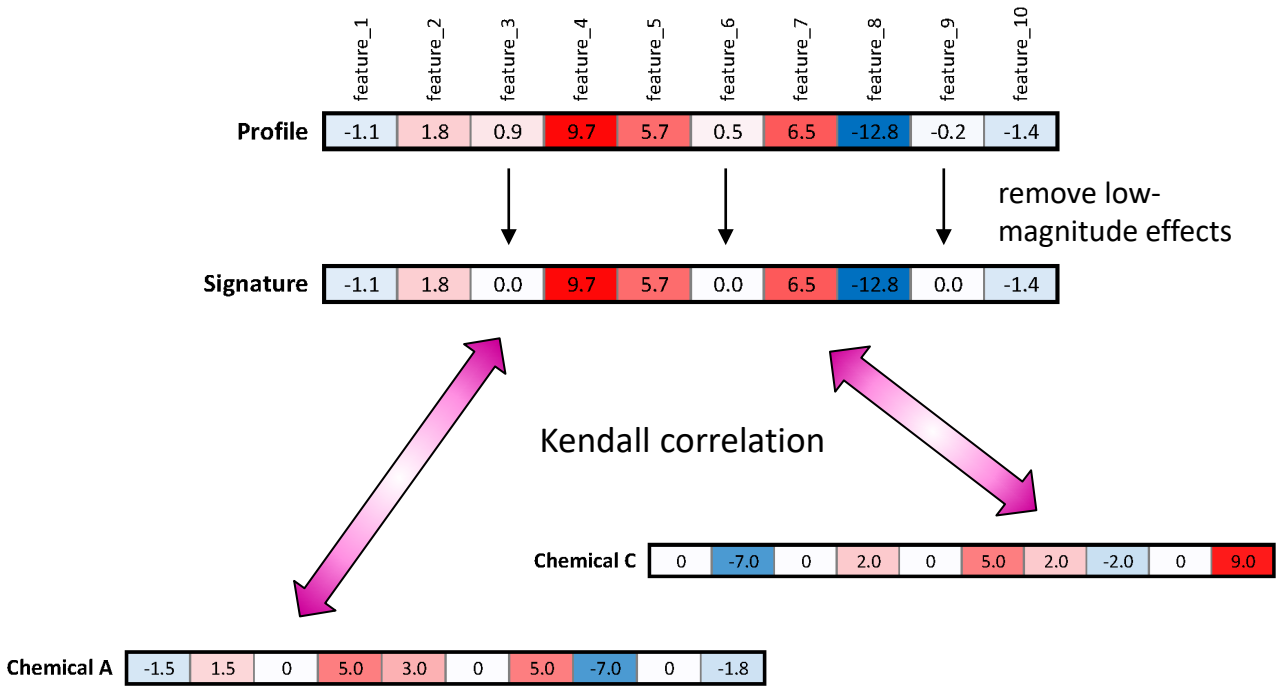
1. remove features that do not provide any information
(i.e. have 0 variance)

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

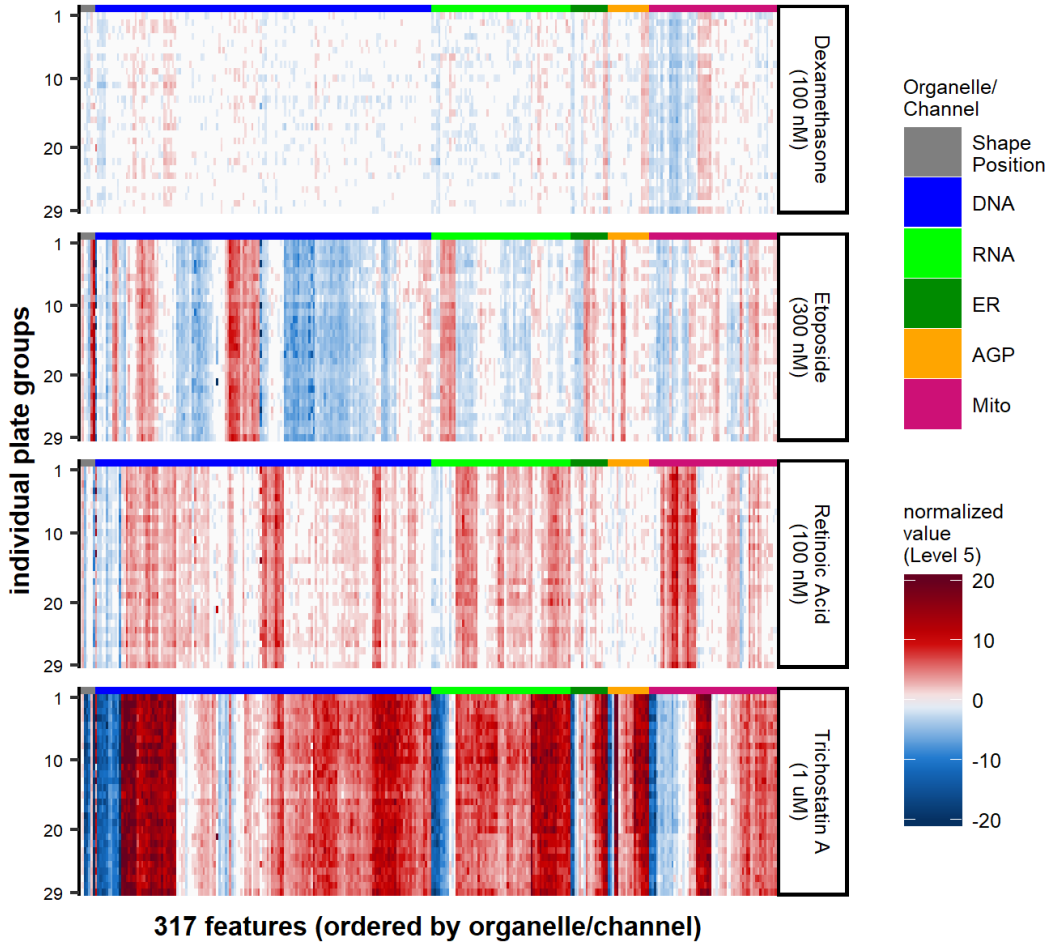
3. remove features that are highly correlated
(using recursive feature elimination)

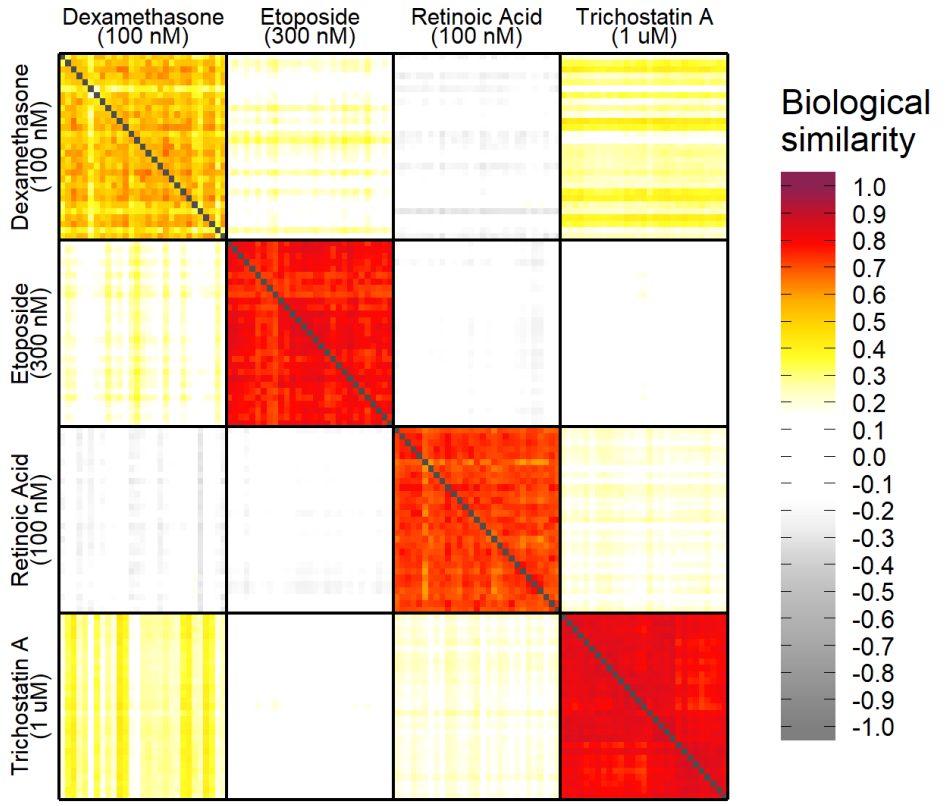
317 features

Profile Comparison



Reproducibility: Phenotypic Profiles





⇨ **Phenotypic profiles are highly reproducible across different plates**

Hypothesis: Chemicals with similar mechanisms will display similar profiles.

Example: Nuclear Receptor Modulators (I)

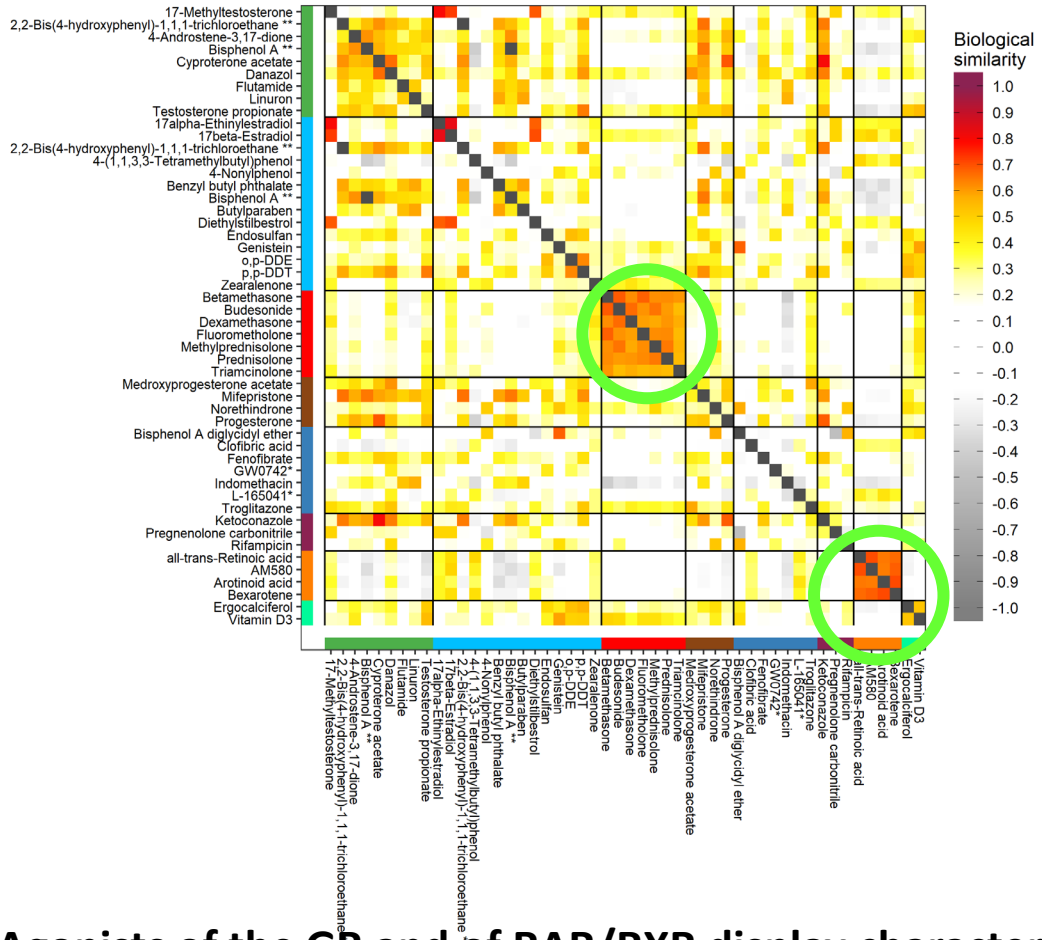
- 52 chemicals were annotated as targeting a nuclear receptor

Preliminary results. Do not cite or quote.

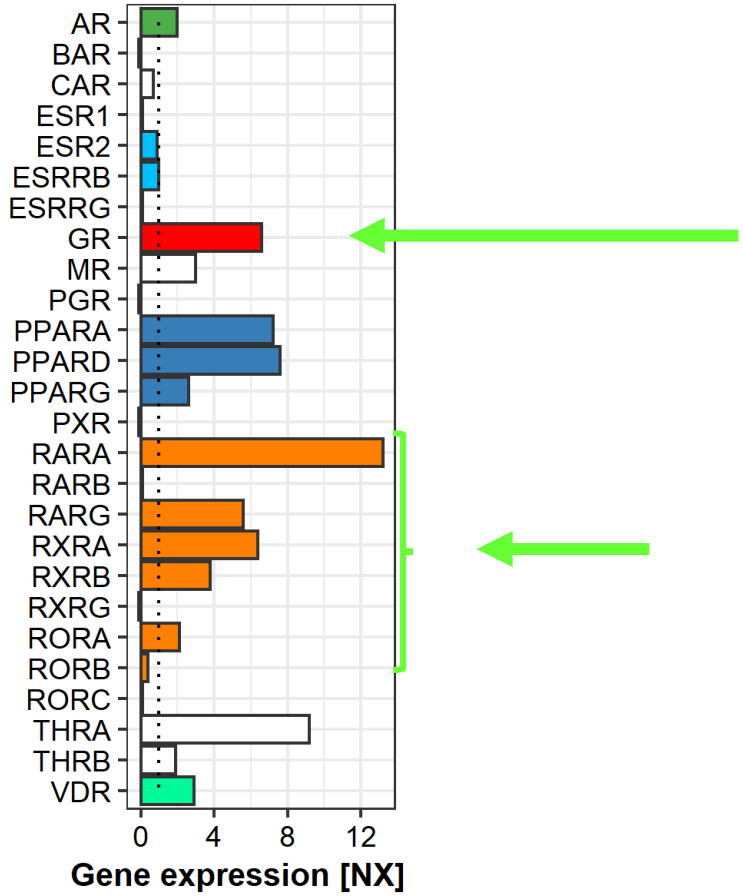
target

- AR
- ESR
- GR
- PGR
- PPAR
- PXR
- RAR
- VDR

Biological similarity in HTPP



Gene expression in U-2 OS



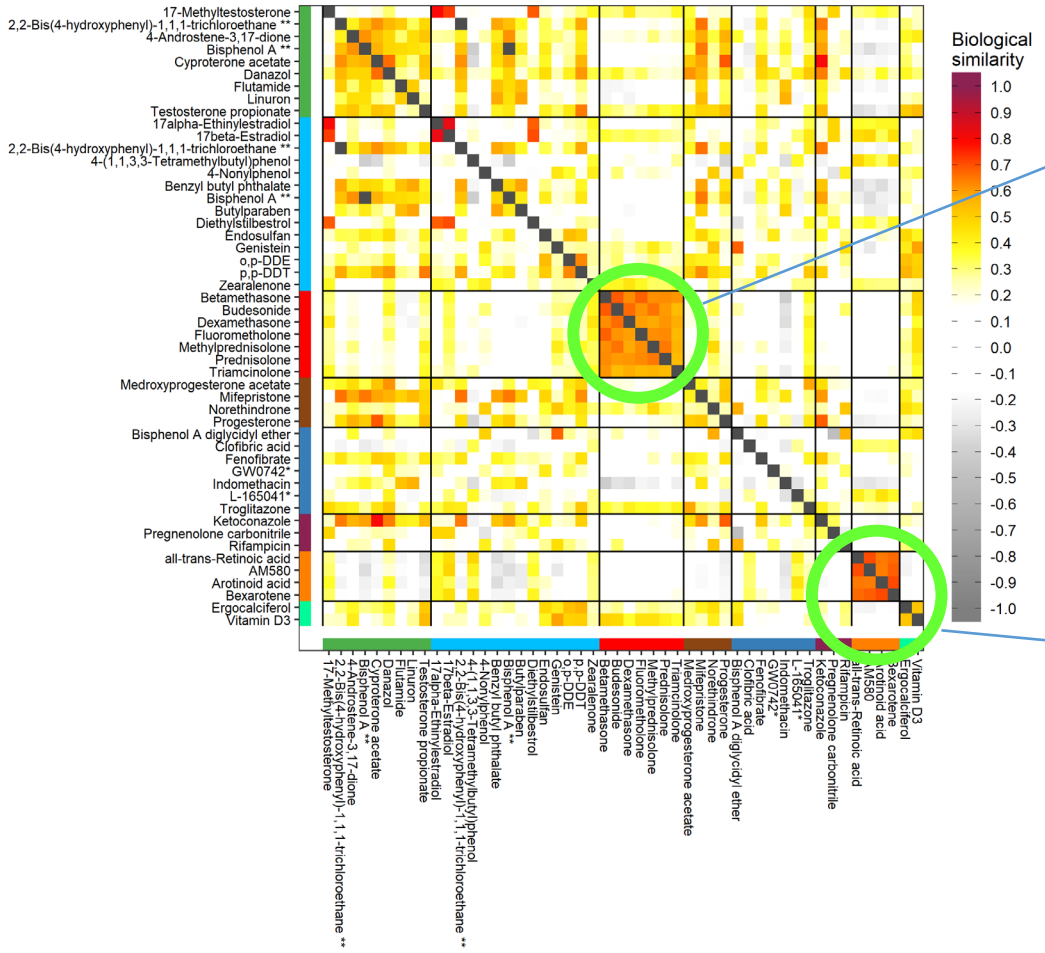
⇨ **Agonists of the GR and of RAR/RXR display characteristic profiles**

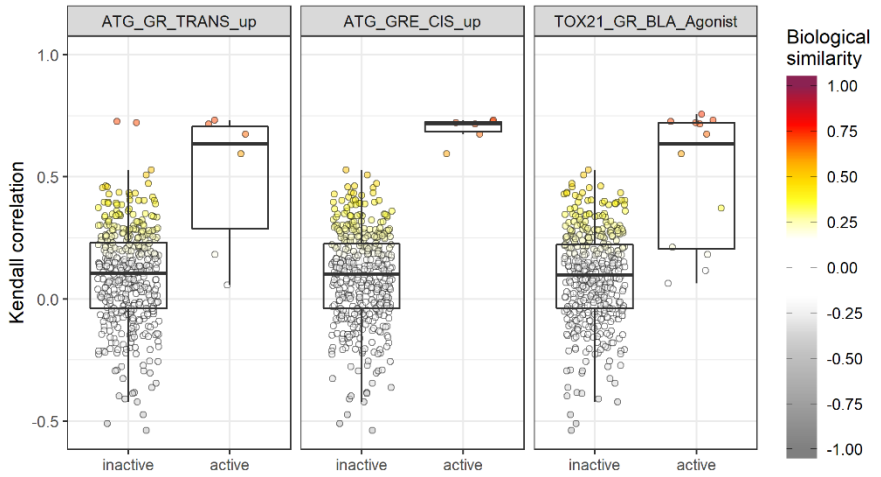
⇨ **Expression of a target does not guarantee that characteristic profiles are observed (e.g., PPAR)**

Preliminary results. Do not cite or quote.

Example: Nuclear Receptor Modulators (II)

Biological similarity in HTPP





Chemicals with similar profiles to dexamethasone tend to be active in **ToxCast GR assays**

Chemicals with similar profiles to all-trans retinoic acid tend to be active in **ToxCast RAR / RXR assays**

⇨ **Certain molecular mechanisms result in characteristic phenotypic profiles**

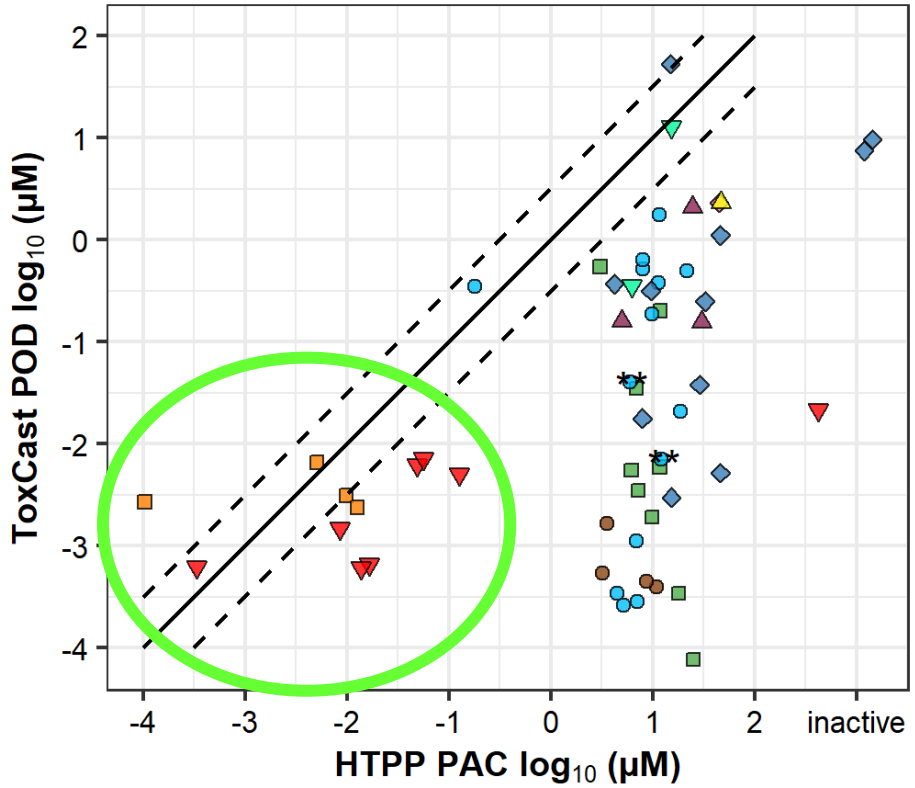
Example: Nuclear Receptor Modulators (III)

Preliminary results. Do not cite or quote.

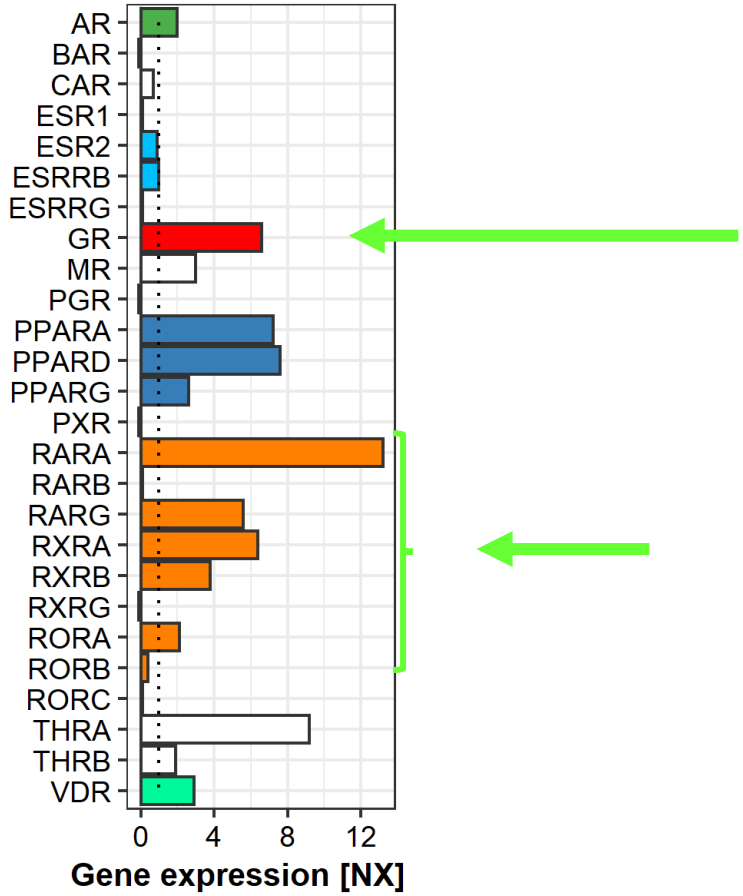
target

- AR
- ESR
- GR
- PGR
- PPAR
- PXR
- RAR
- VDR

Comparison to ToxCast potencies



Gene expression in U-2 OS

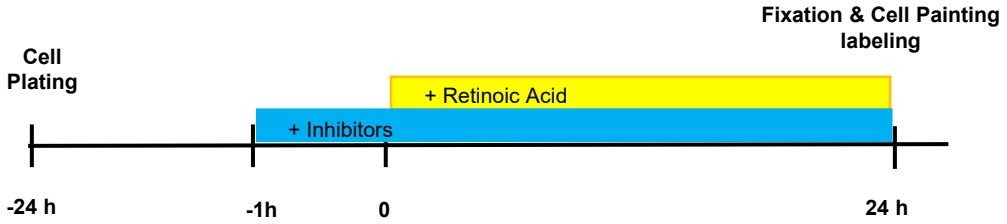


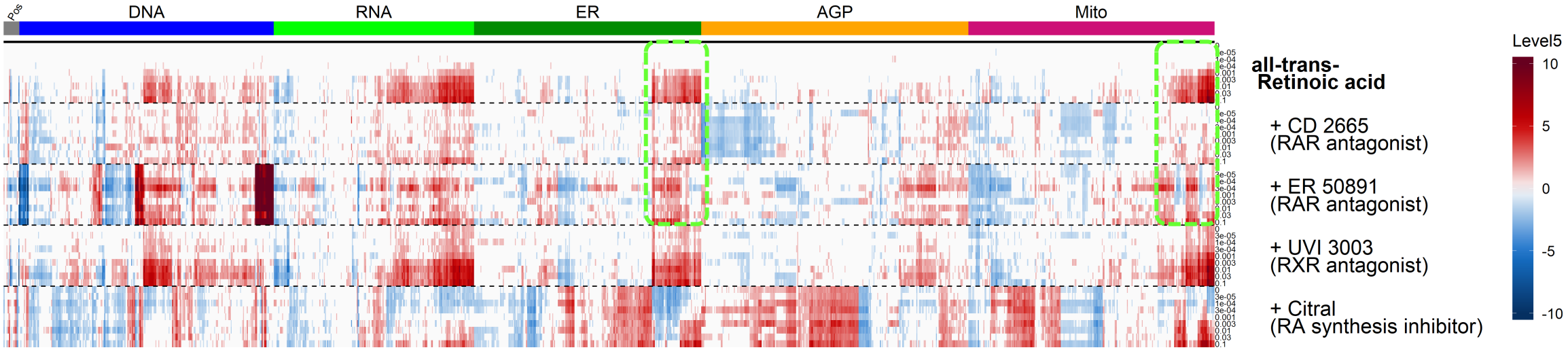
⇨ **For two receptor systems that are expressed (GR, RAR/RXR) potencies were comparable with ToxCast**

⇨ **For all other receptors, we are much less sensitive than ToxCast (off-target effects?)**

Preliminary results. Do not cite or quote.

Pharmacological Blockade of Phenotypic Effects

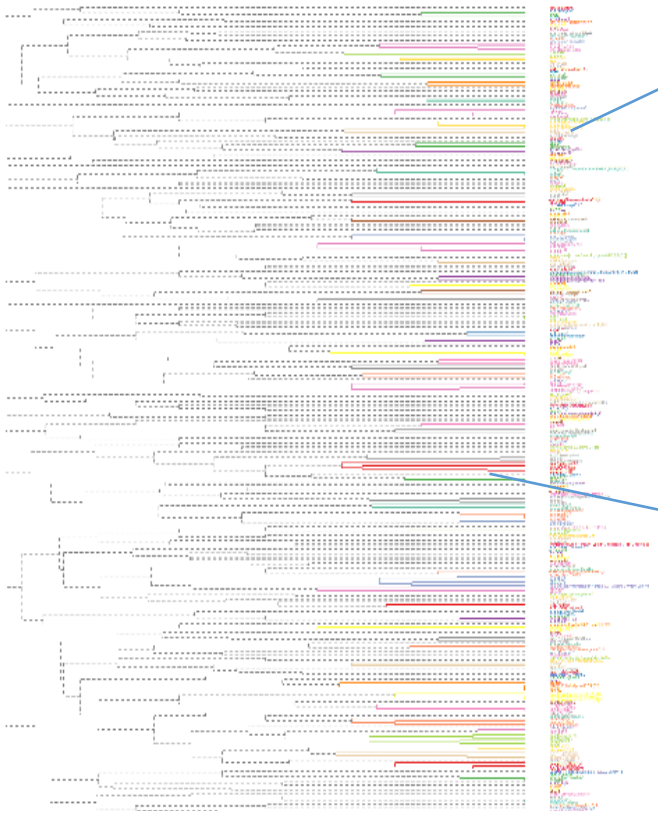


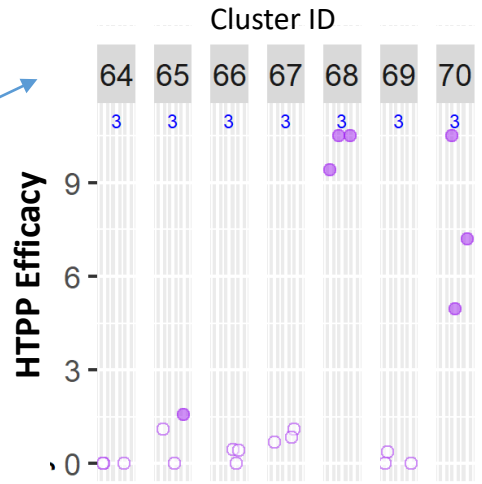


⇨ **RAR but not RXR antagonists block the retinoid phenotype**

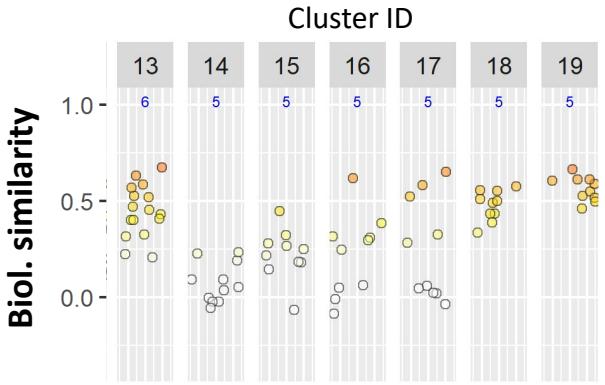
Structural Similarity Translates to Biological Similarity

Dendrogram using structural information (ToxPrints) → 680 clusters





⇨ **Structurally similar chemicals tend to display similar efficacies**



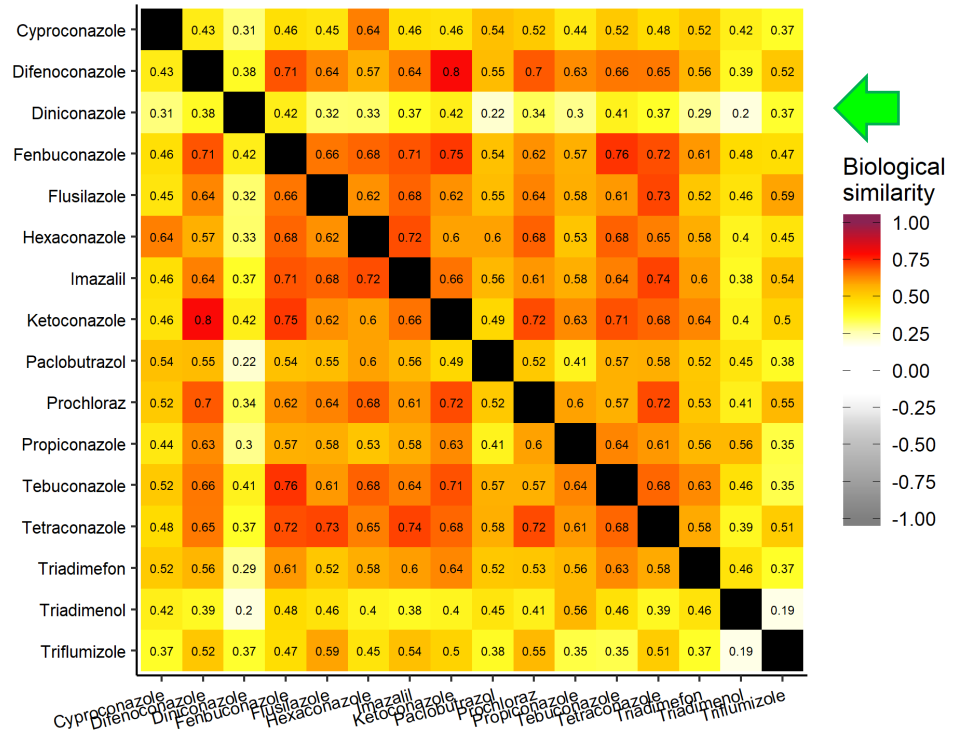
⇨ **Structurally similar chemicals tend to display similar phenotypes**

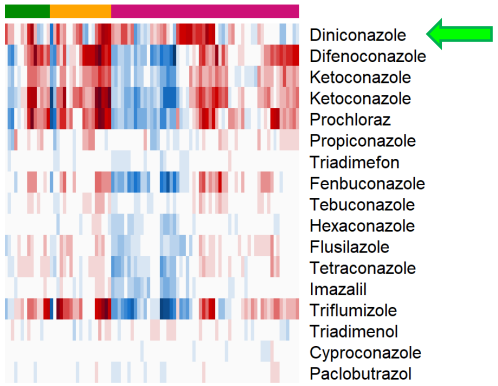
⇨ **Structurally similar chemicals tend to be biologically similar**

Application to Environmental Chemicals:
Example: Conazoles

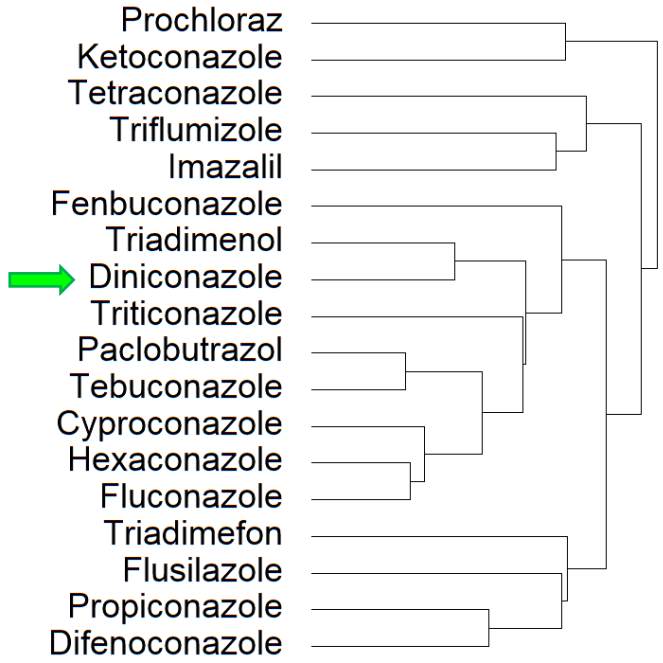
- group of fungicides
- disturb ergosterol synthesis via CYP51 and CYP61 (target absent in mammals)

biological similarity





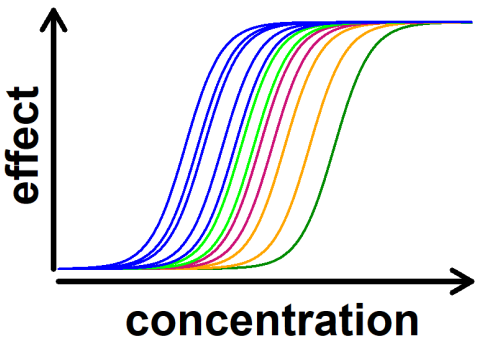
structural similarity
(based on ToxPrints)



⇨ **most conazoles are phenotypically similar**

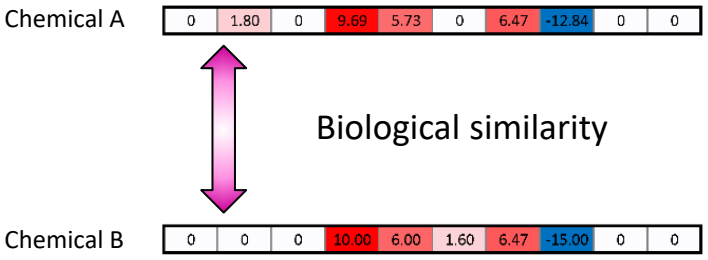
⇨ **Diniconazole is phenotypically different from the other active conazoles**

Conclusions



Application 1: Potency estimation

- HTPP can be used to derive *in vitro* potency estimates
- These *in vitro* potency estimates are often comparable or more conservative than *in vivo* PODs



Application 2: Mechanistic prediction

- Structural similarity → biological similarity
- Similar mechanisms → biological similarity

Outlook

- **Combine HTPP with HTTr**
  - compare results, both in terms of potencies and mechanisms
  - increased potential to discern molecular mechanisms

- **Expand Coverage of Biological Space**
  - deploy assay across diverse cell lines that express different receptors/pathways
  - proof-of-concept (Gustafsdottir *et al.* 2013, Willis *et al.* 2020)
  - expansion to other species

Acknowledgements

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

Harrill Lab team

- **Joshua Harrill**
- **Clinton Willis**
- Rick Brockway
- Megan Culbreth
- Felix Harris
- Dan Hallinger
- Terri Fairley

Data analysis

- Daniel Chang
- Kathy Coutros
- Logan Everett
- Derik Haggard
- Richard Judson
- Ryan Lougee
- Grace Patlewicz
- Katie Paul Friedman
- Ann Richard
- Woody Setzer
- Imran Shah
- John Wambaugh

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

- Scott Auerbach