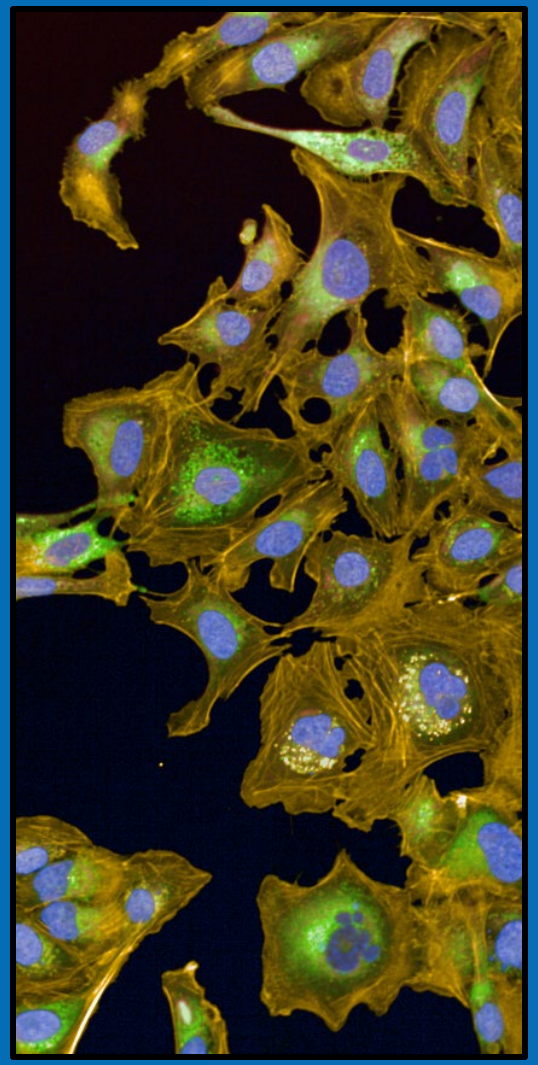
EPA United States Environmental Protection Agency



Imaging-based phenotypic profiling for high-throughput chemical screening at the U.S. Environmental Protection Agency

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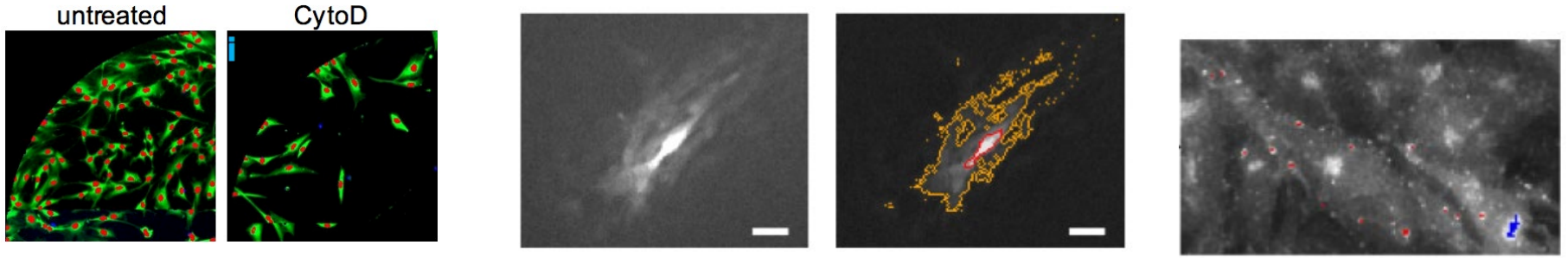
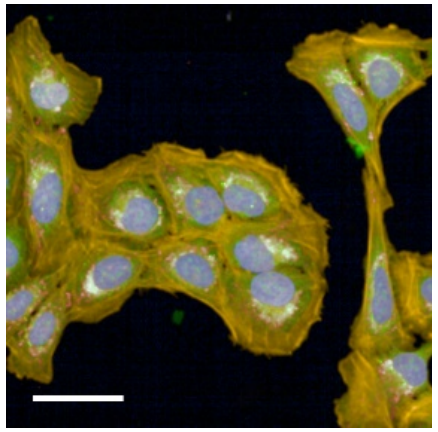
The views expressed in this presentation are those of the author and do not necessarily reflect the views or policies of the U.S. EPA.

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



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Overview

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

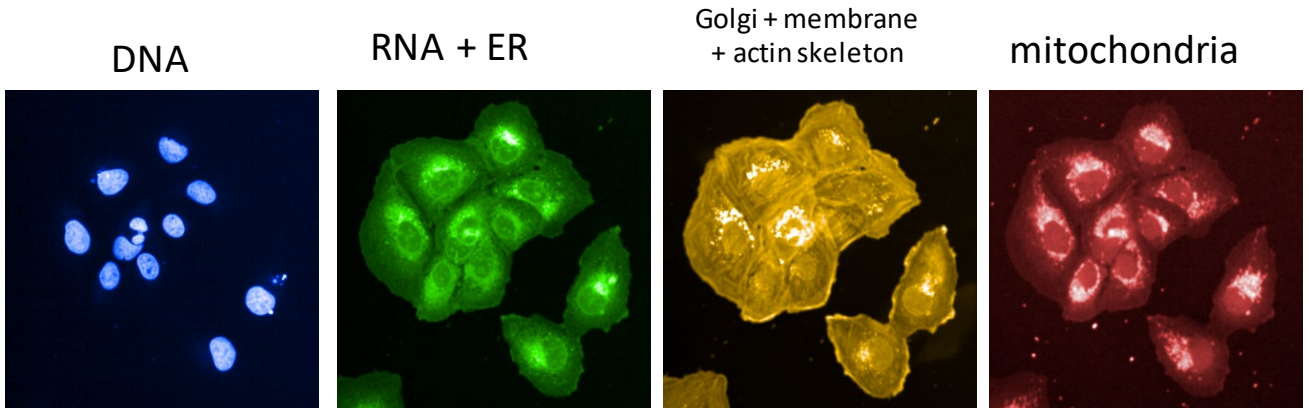
What is Imaging-Based Phenotypic Profiling?

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

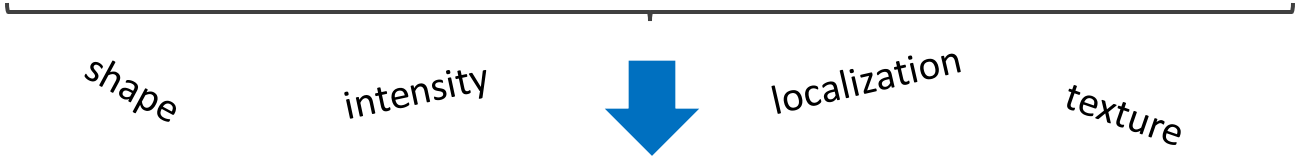
'Cell Painting' assay

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



1300 features per cell

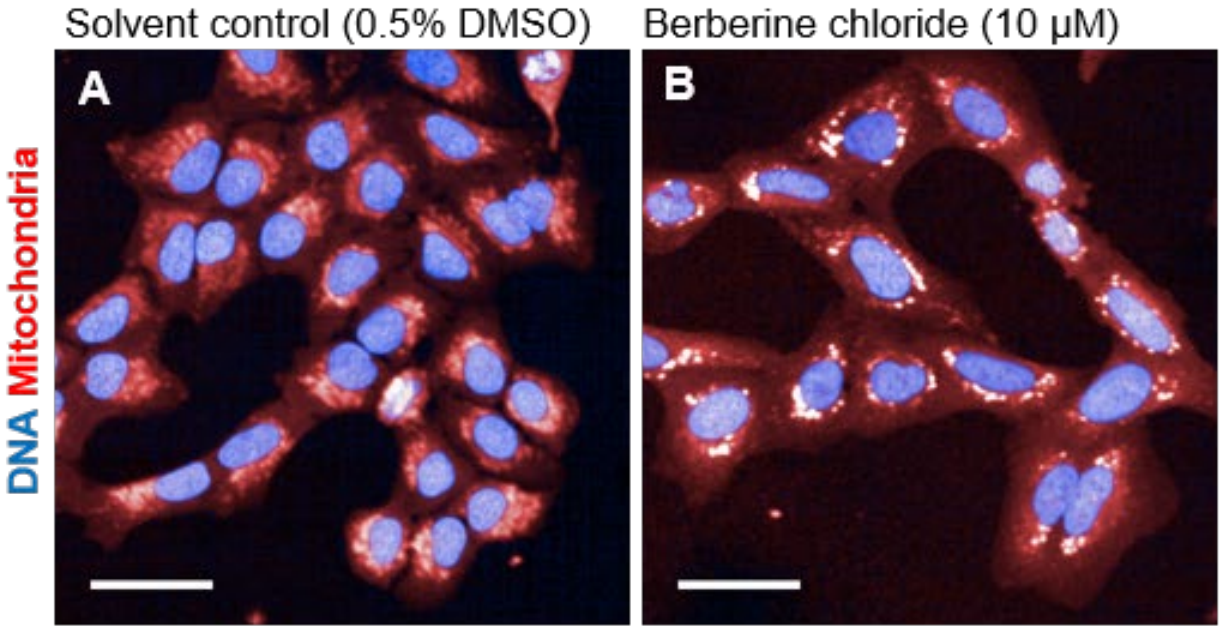
Nyffeler *et al.* 2020

for each chemical x concentration

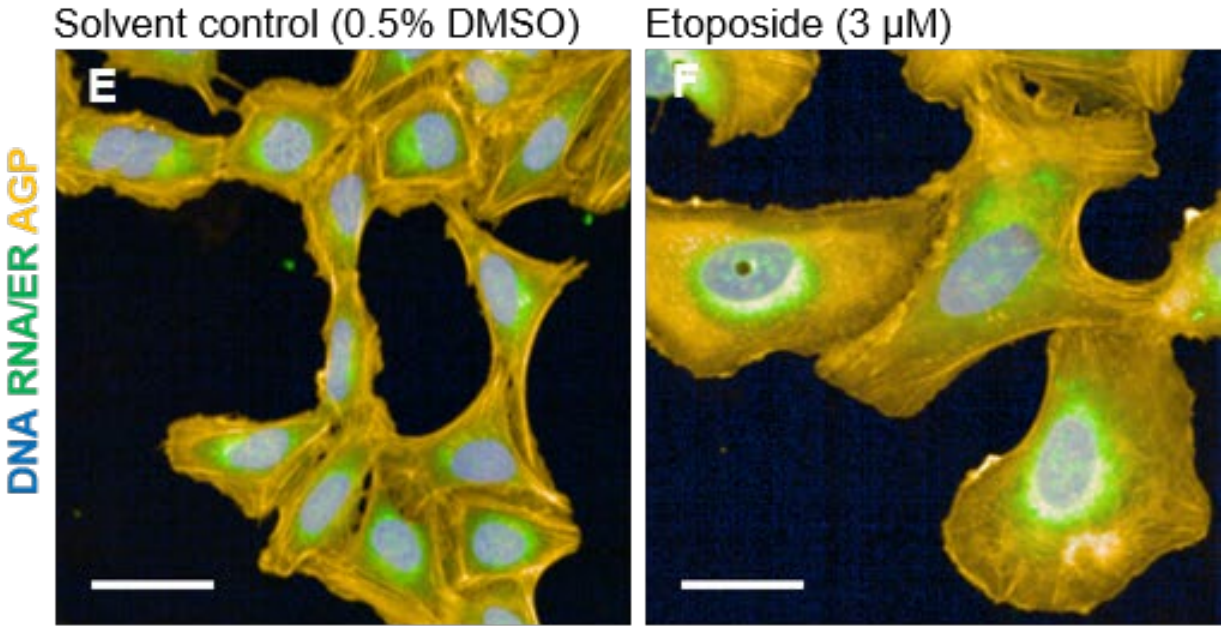
Cell Painting = Phenotypic Profiling

High-Throughput Phenotypic Profiling = HTPP

Example Chemicals: Qualitative Observation



→ Mitochondrial compactness/texture



→ Cells are larger

⇨ **Strong phenotypes are observable qualitatively**

adapted from Nyffeler *et al.* (2020) Toxicol Appl Pharmacol. PMID: 31899216

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Implementation at CCTE/EPA

Laboratory Workflow

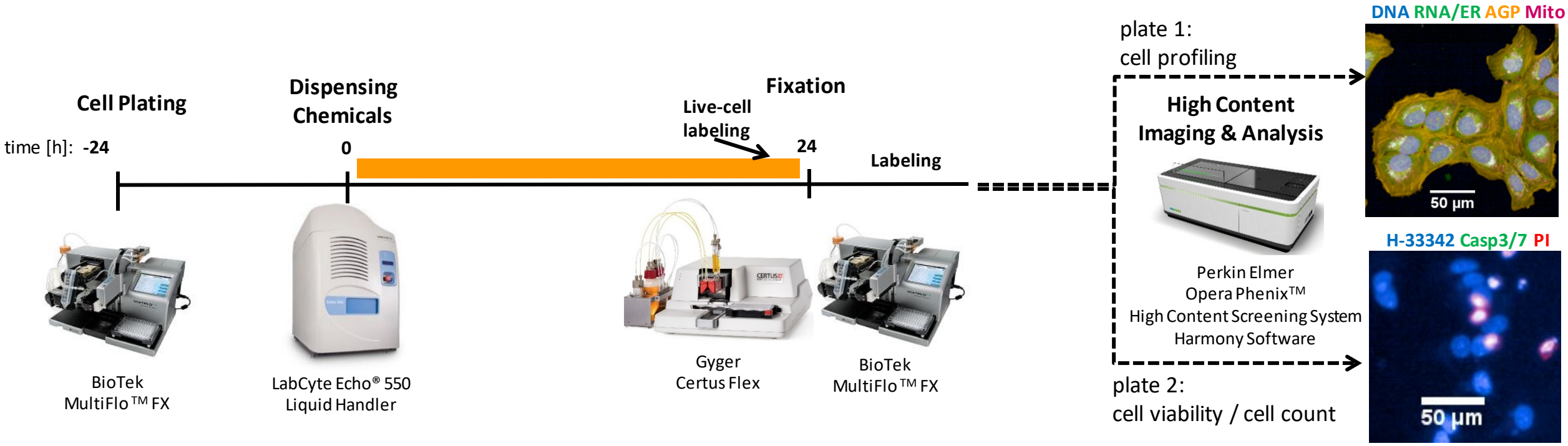
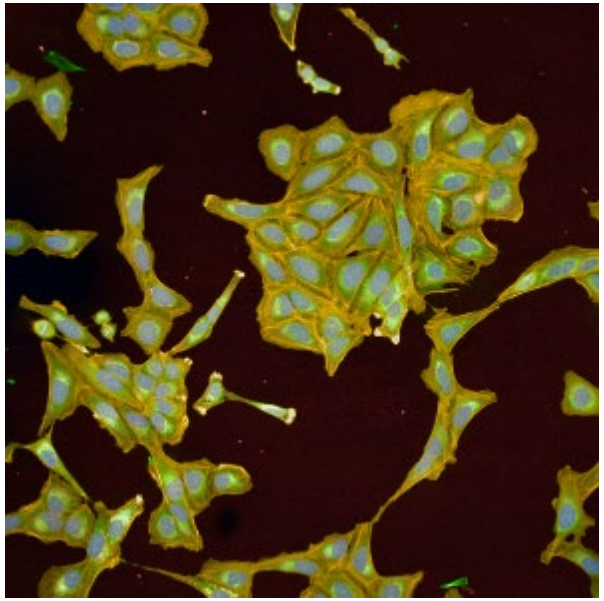


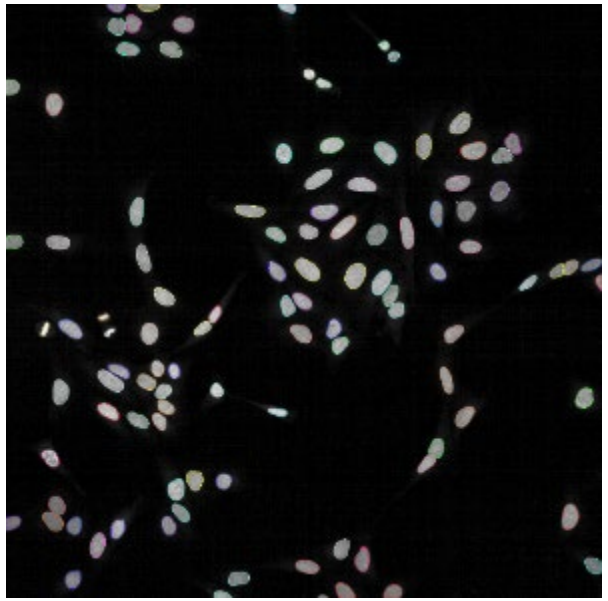
Image Analysis Workflow → Image Segmentation

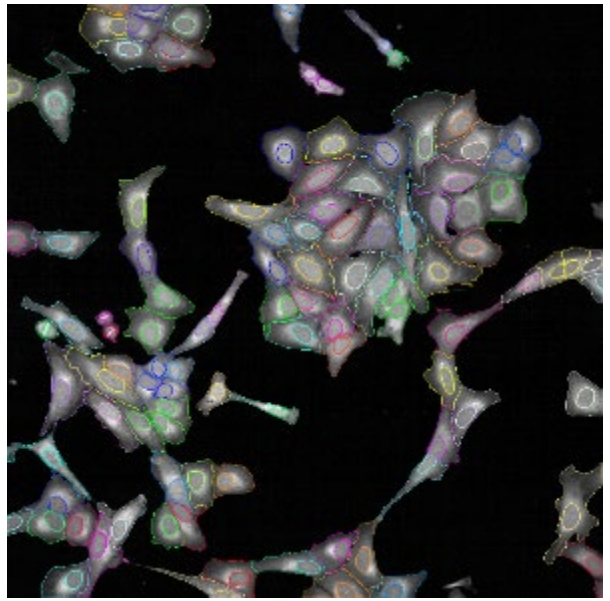
1. find nuclei

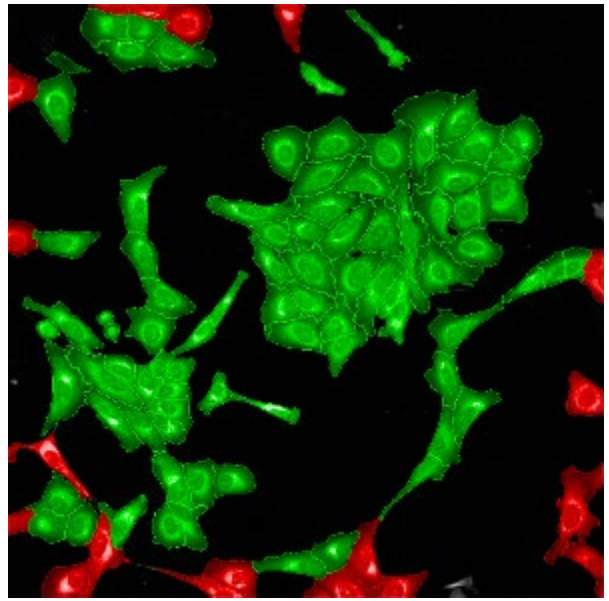
2. find cell outline

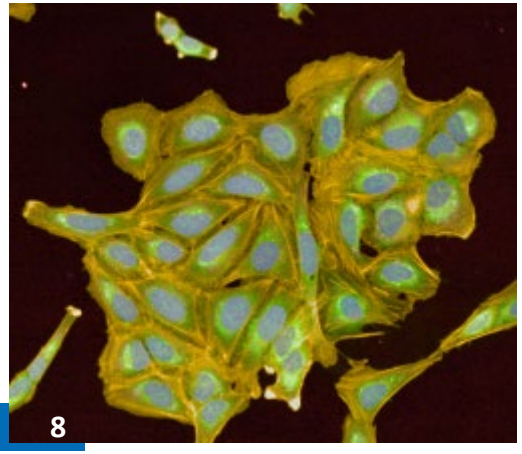
3. reject border objects

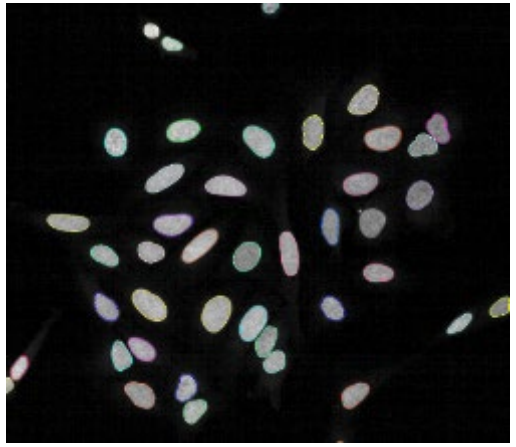


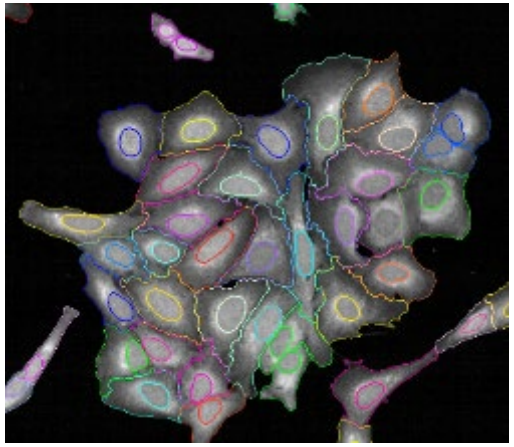






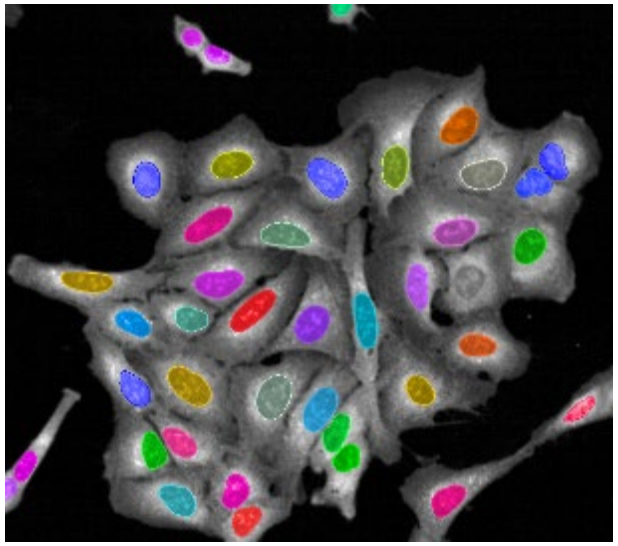




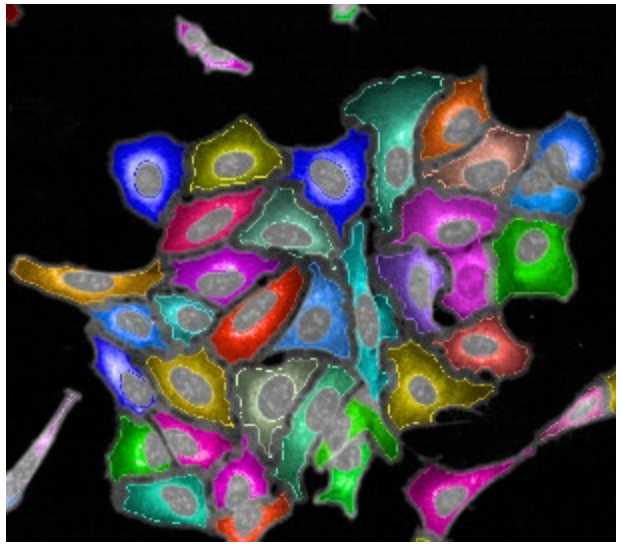


Define Cellular Compartments

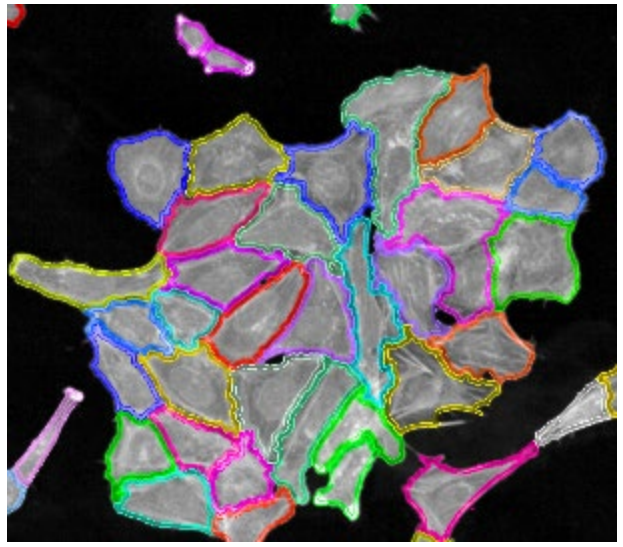
nuclei



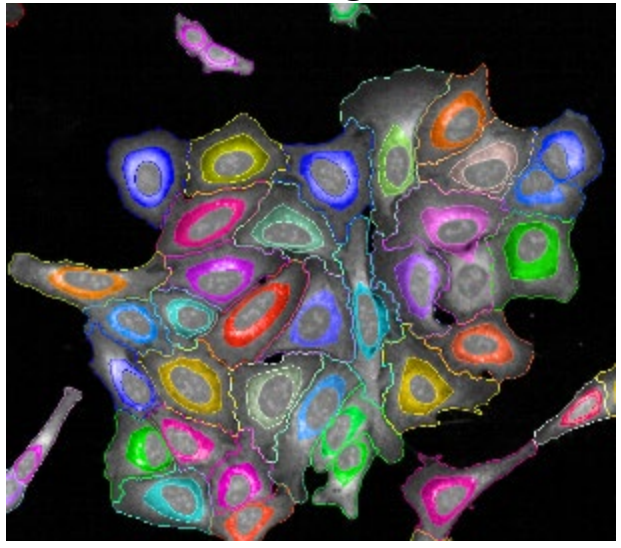
cytoplasm



membrane



ring



cell

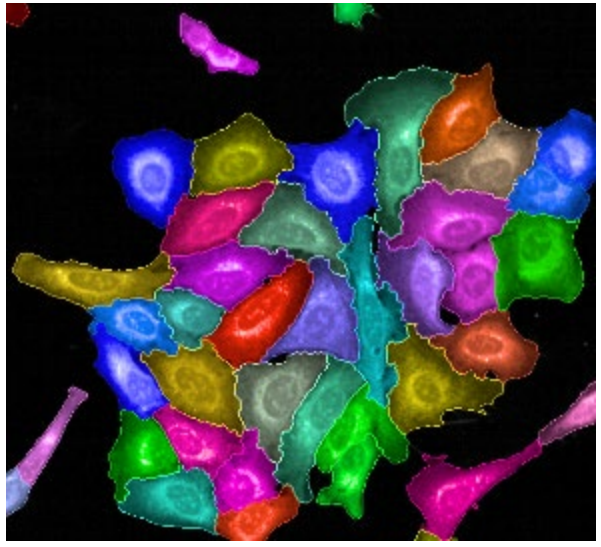
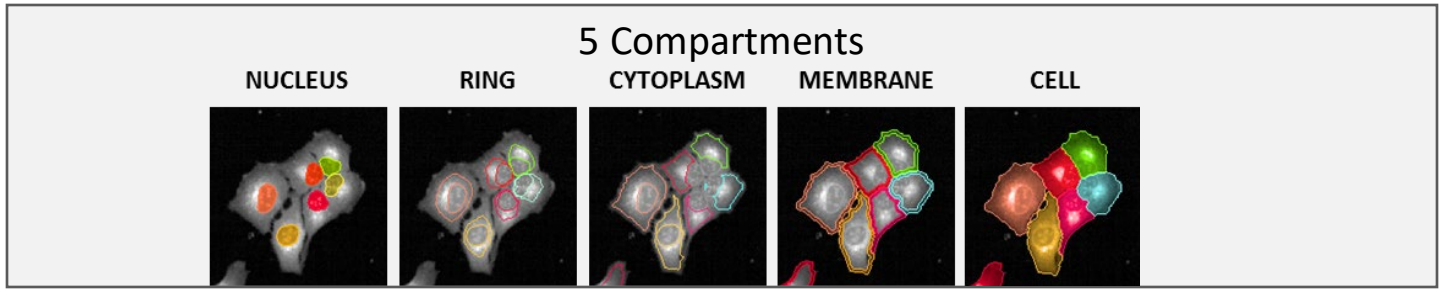
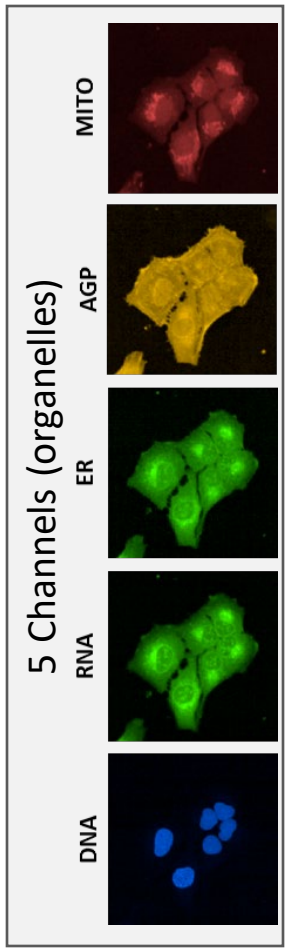
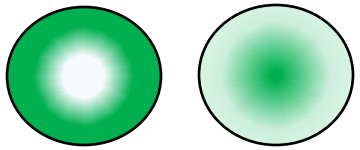


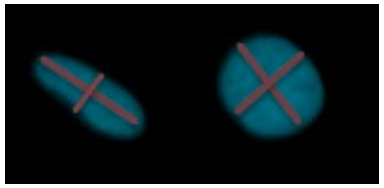
Image Processing

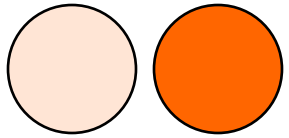
Profiling
with PerkinElmer
Harmony Software

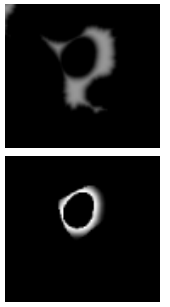


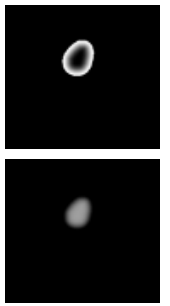



Radial distribution

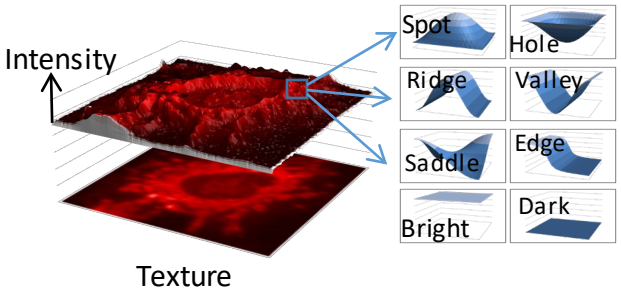

Axial


Intensity

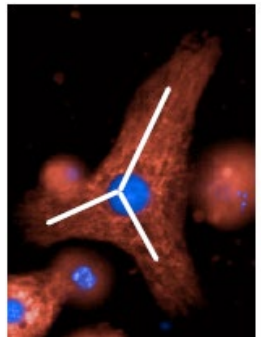


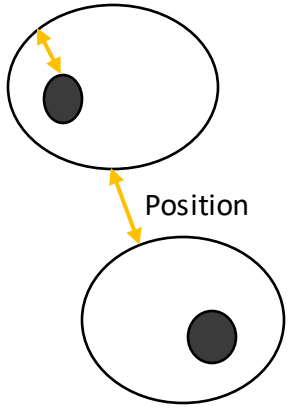


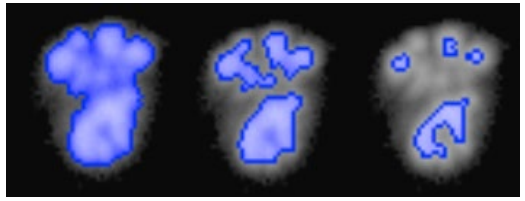
Profile

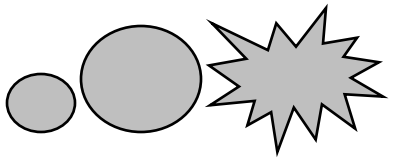


Texture


Symmetry



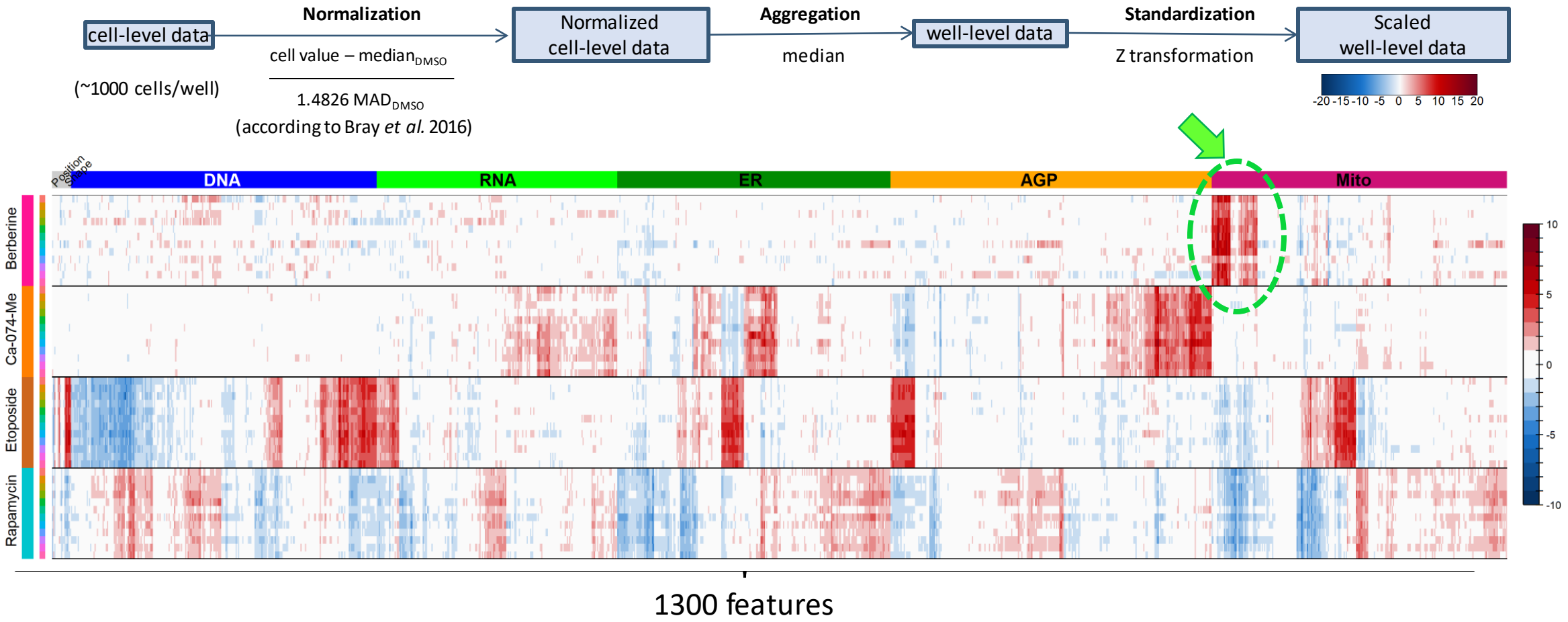

Compactness


Shape

= 1300 features per cell

With illustrations from Perkin Elmer

Example Chemicals: Quantitative Observation



⇨ **Qualitative observations can be quantified**

adapted from Nyffeler *et al.* (2020)
Toxicol Appl Pharmacol.
PMID: 31899216

EPA United States Environmental Protection Agency

Aim for CCTE/EPA

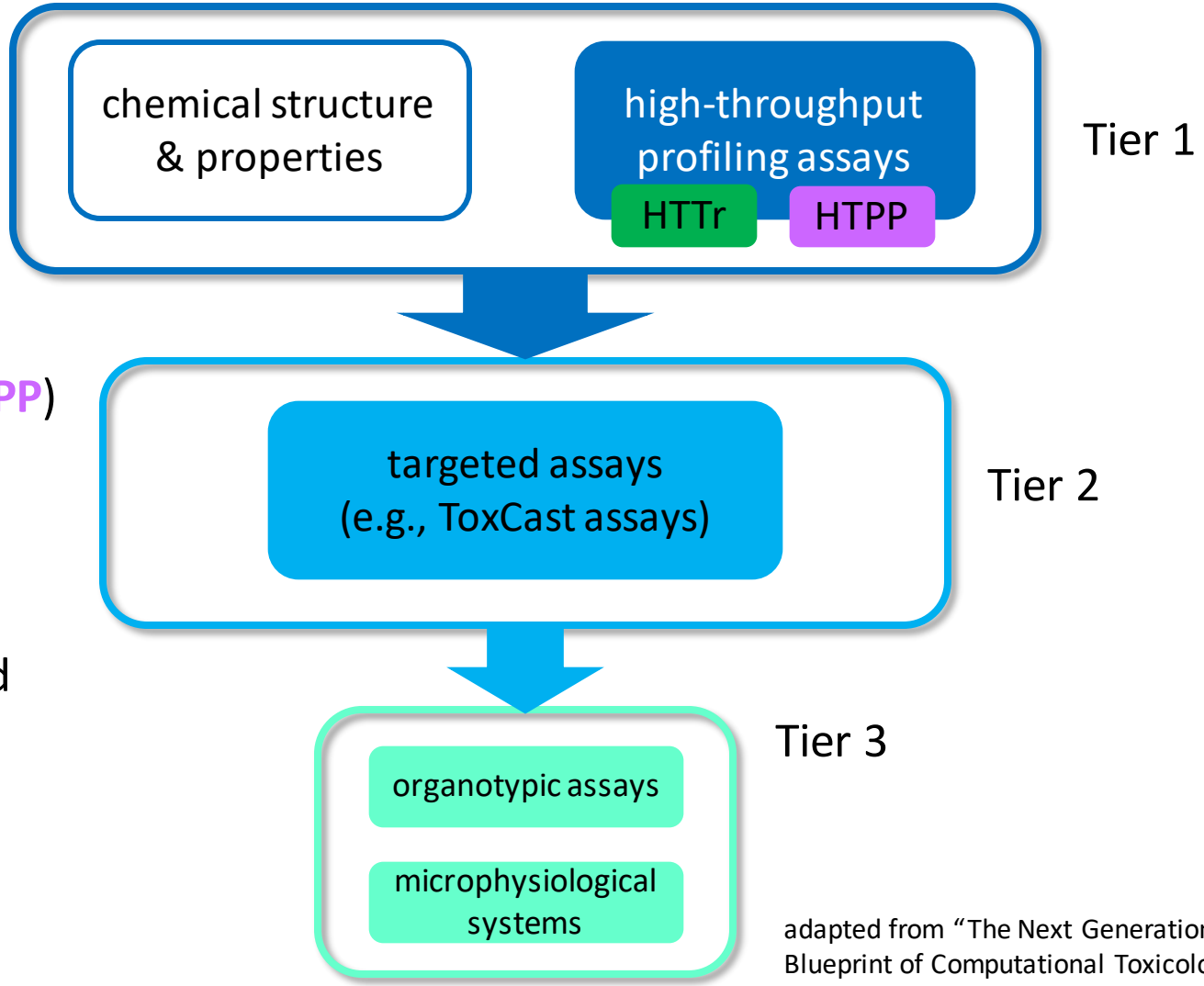
Tiered Hazard Evaluation Strategy based on New Approach Methods (NAMs)

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



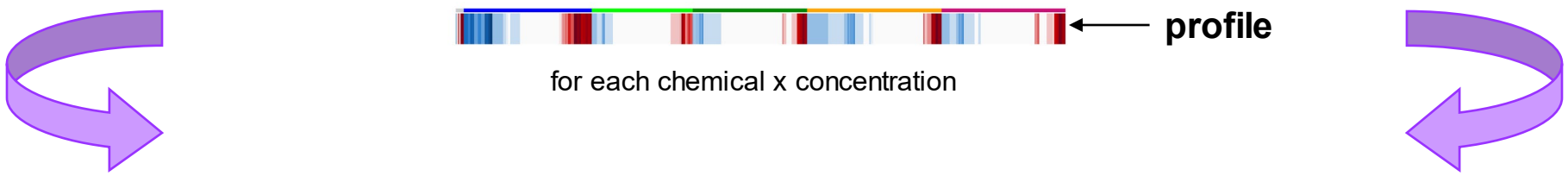
adapted from "The Next Generation Blueprint of Computational Toxicology at the U.S. EPA", Tox. Sci. 2019; 169(2):317-322. PMID: 30835285

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

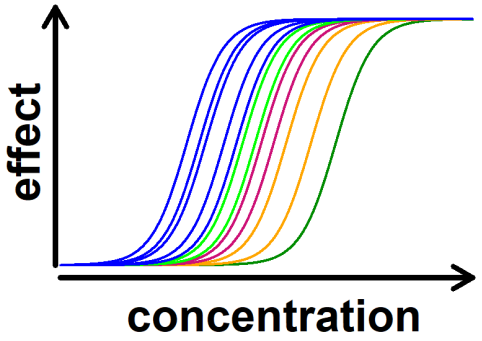
⇨ **more challenging for hit identification than drug-like chemicals**

Two Applications



Application 1

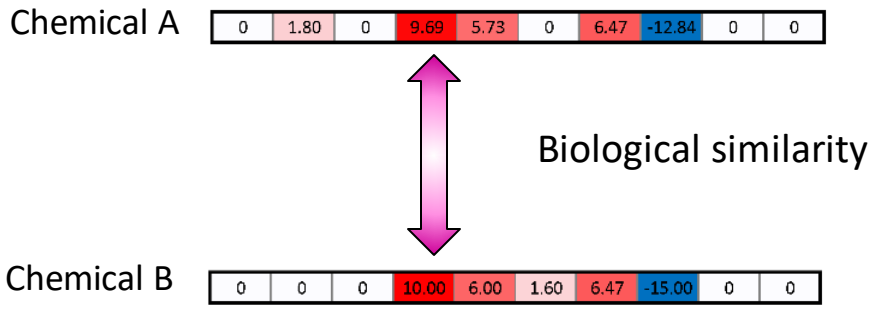
concentration-response modelling



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

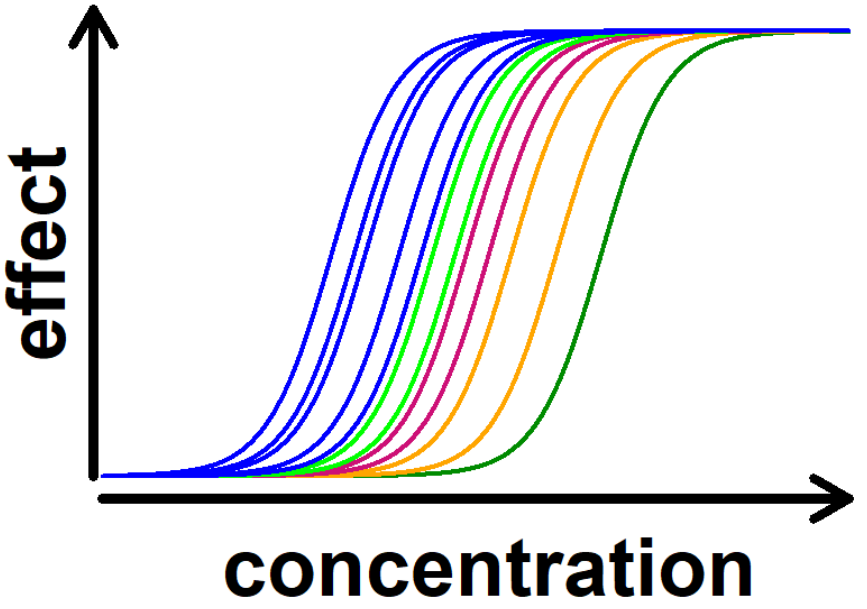
Application 2



Compare profiles with annotated reference chemicals
→ putative mechanisms

work in progress

Application 1: Potency Estimation



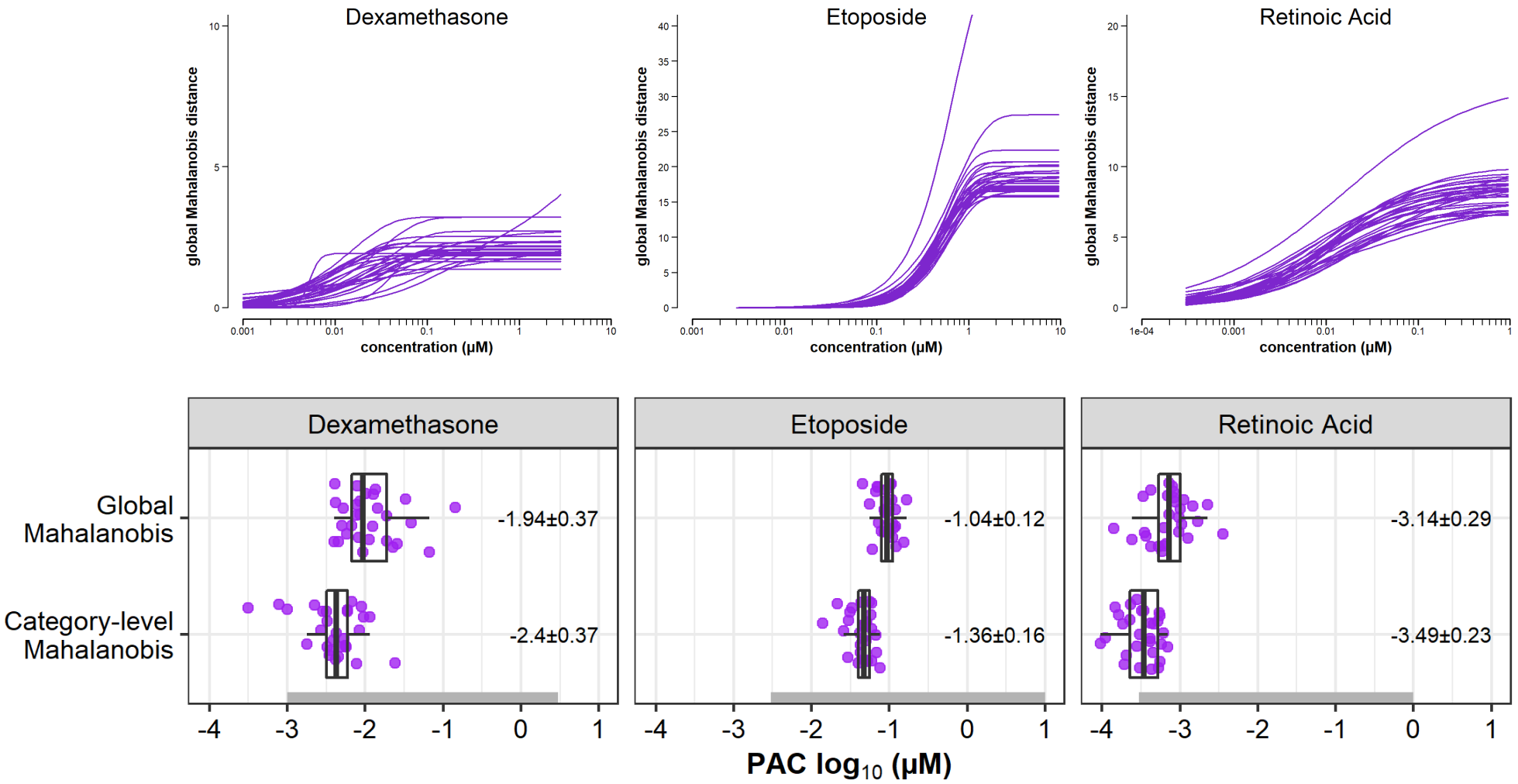
U-2 OS ToxCast Screen Experimental Design

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

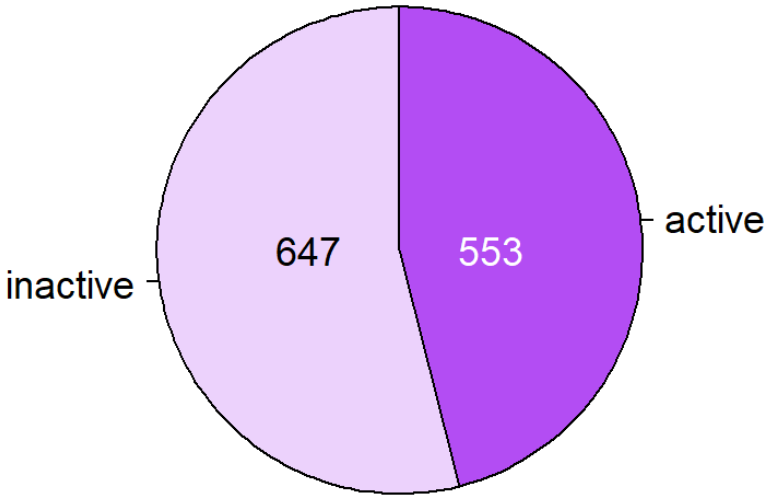
Reproducibility: Potencies

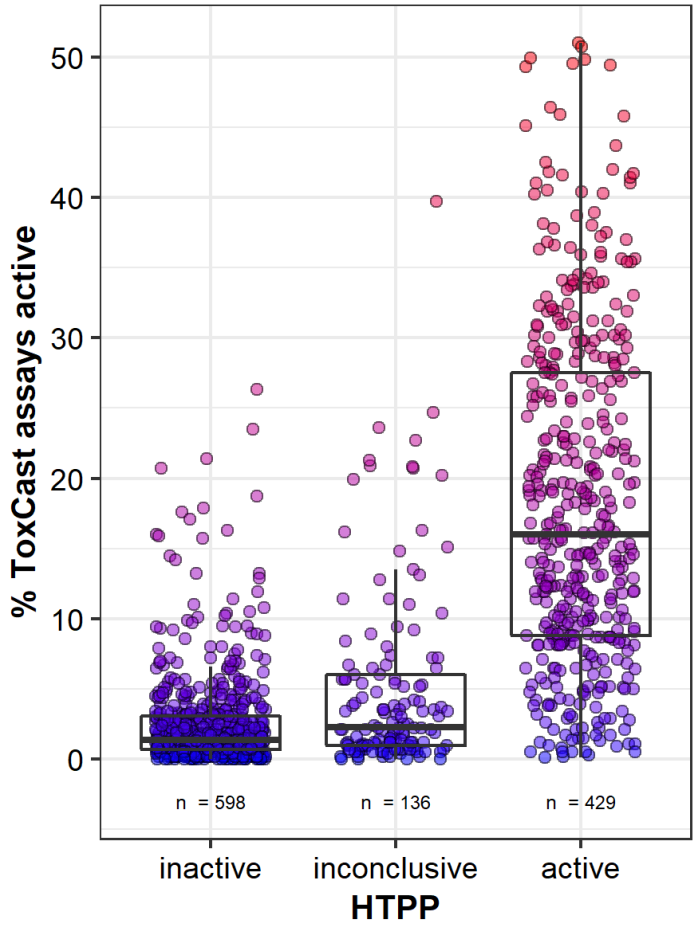


⇨ **Potency estimates vary less than ½ an order of magnitude**

HTPP Screening Results (1)

Active chemicals:

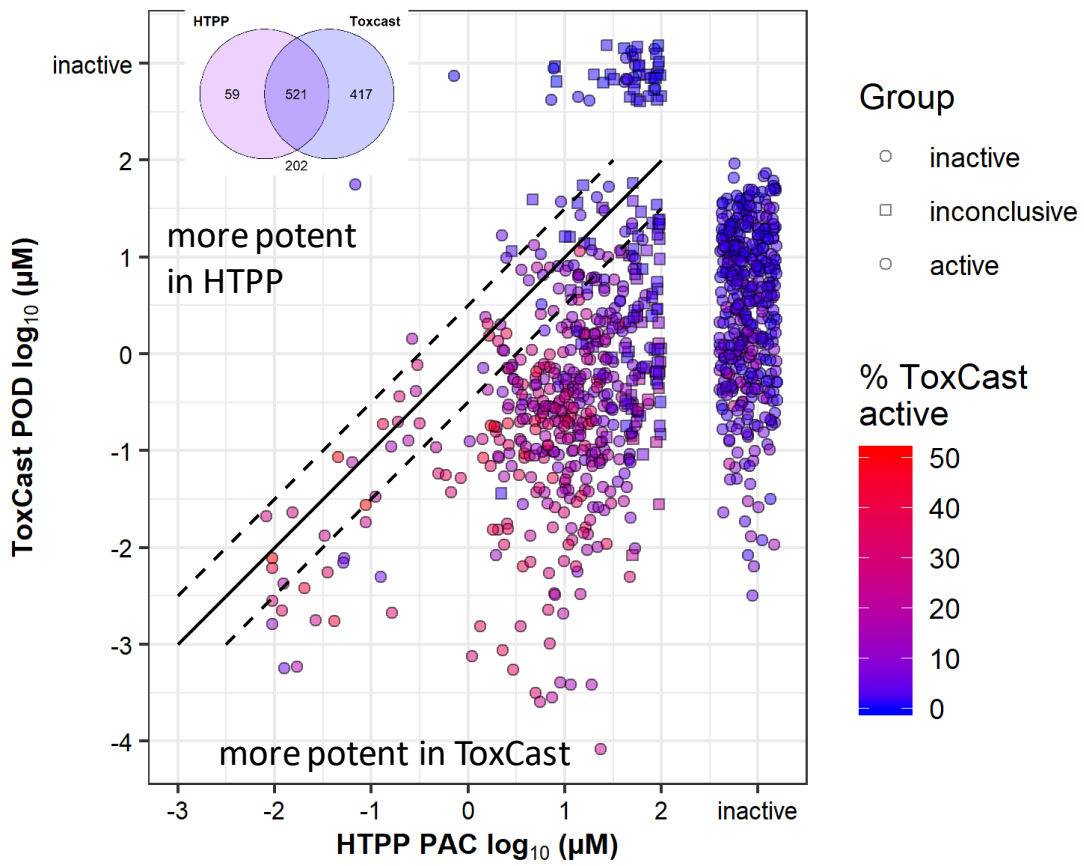


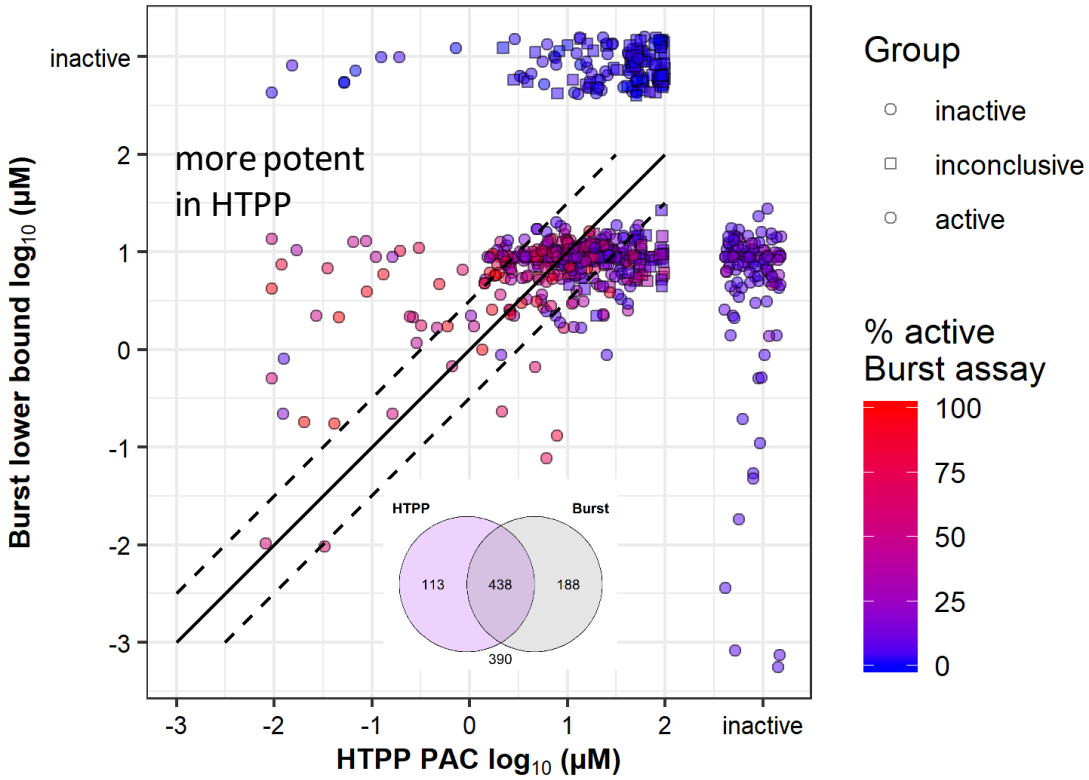


- ~ 40% of chemicals were active
- Most activity is > 10 µM
- Chemicals active in HTPP are more often active in many ToxCast assays

HTPP Screening Results (2)

Comparison with ToxCast screening results:



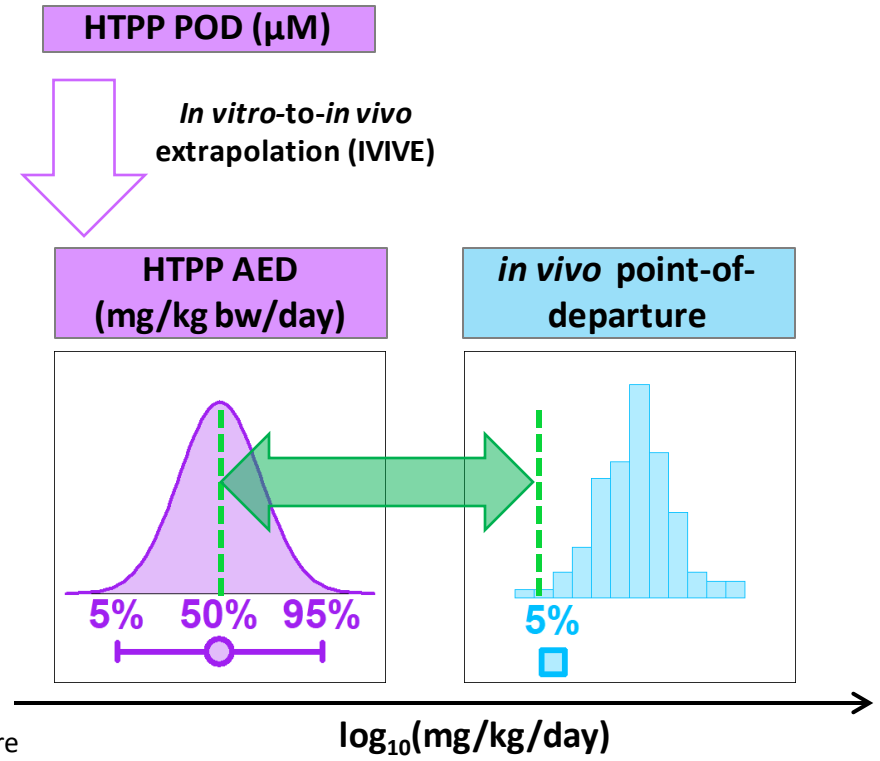


⇨ **HTPP with a single cell line less sensitive than all ToxCast assays combined….**

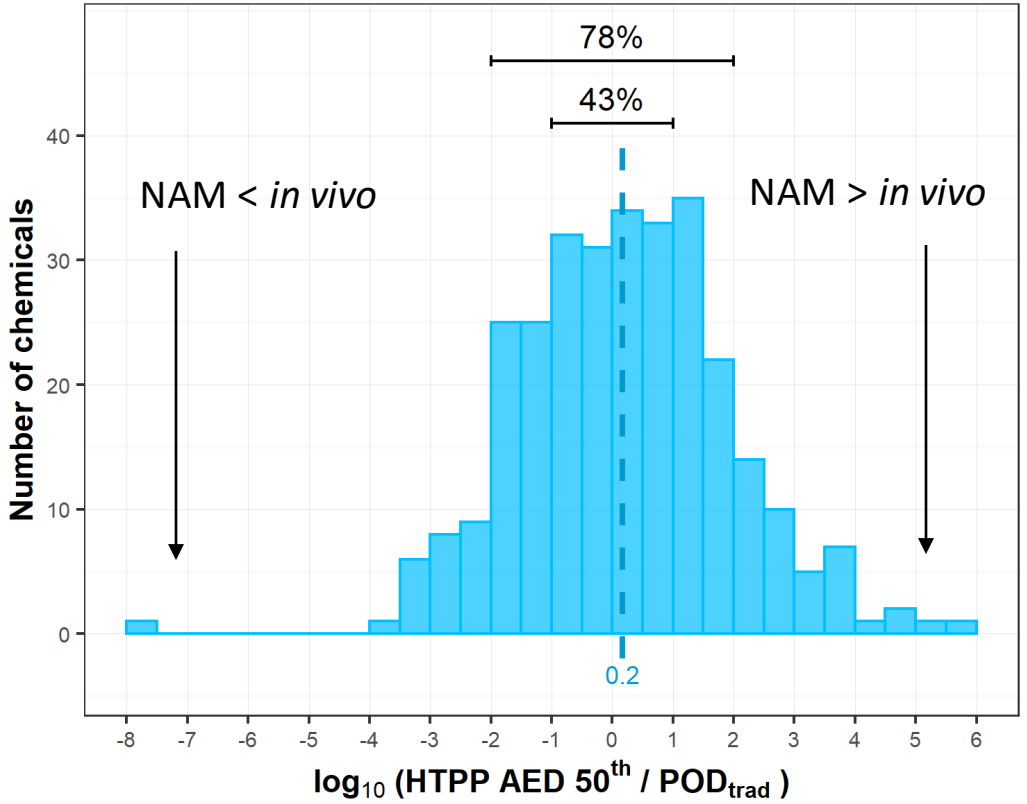
⇨ **… but more sensitive than the ToxCast cytotoxicity burst estimate**

Comparison to *in vivo* Effect Values

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



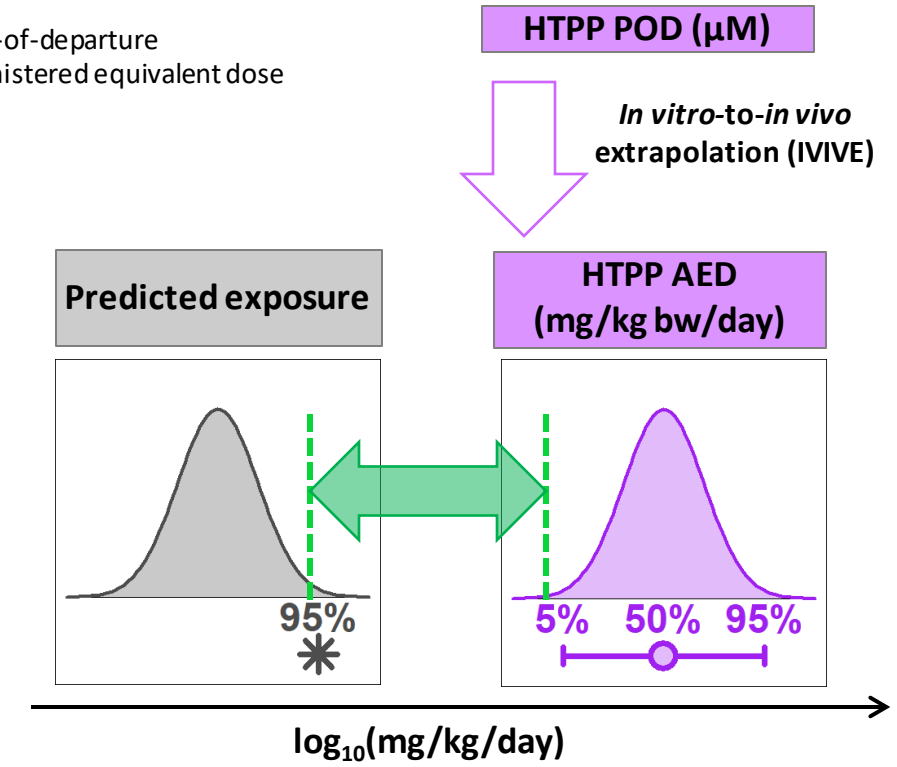
POD: point-of-departure
AED: administered equivalent dose

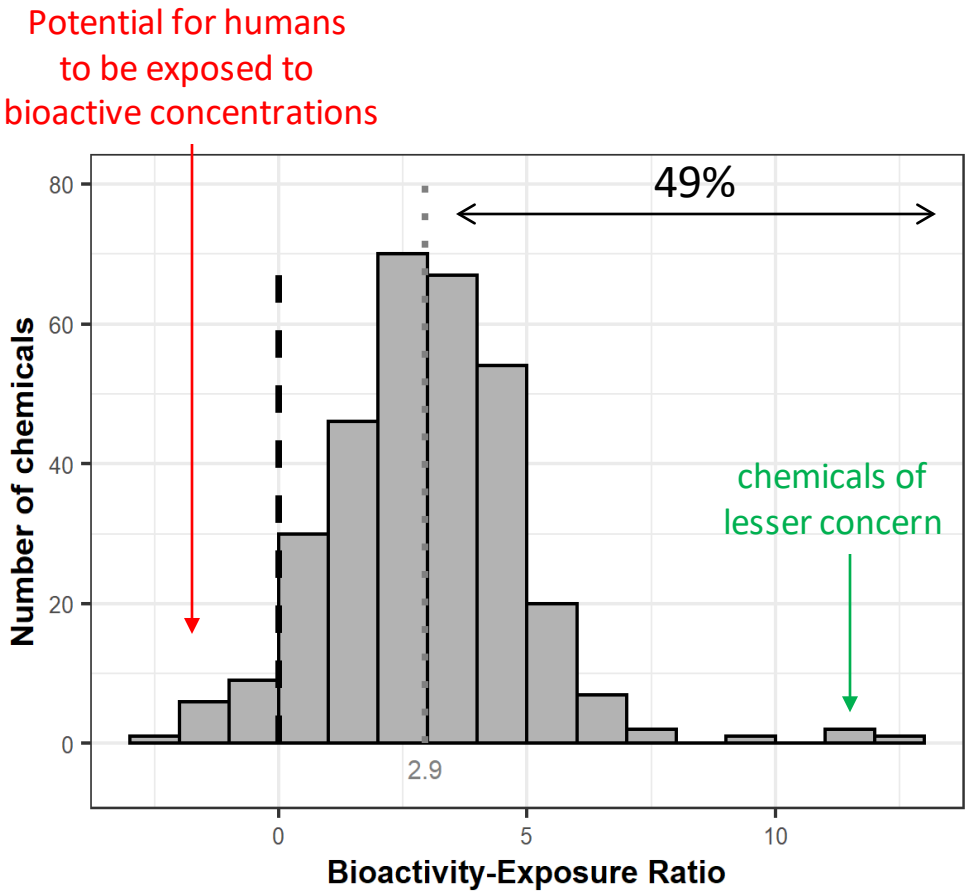


⇨ **78% of HTPP AED are within 2 orders of magnitude of the *in vivo* POD**

Comparison to Exposure Estimates

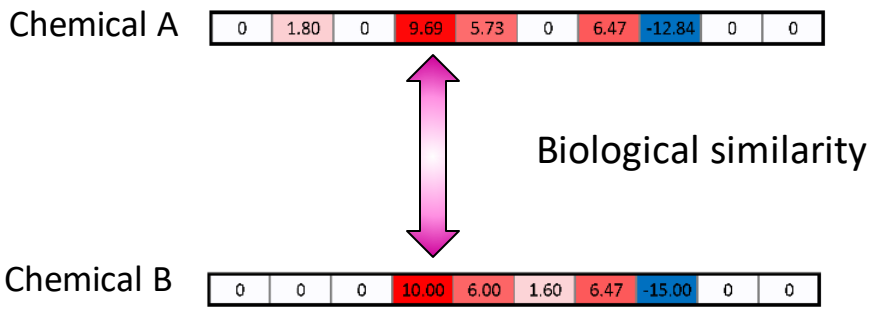
POD: point-of-departure
AED: administered equivalent dose





- ⇨ **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

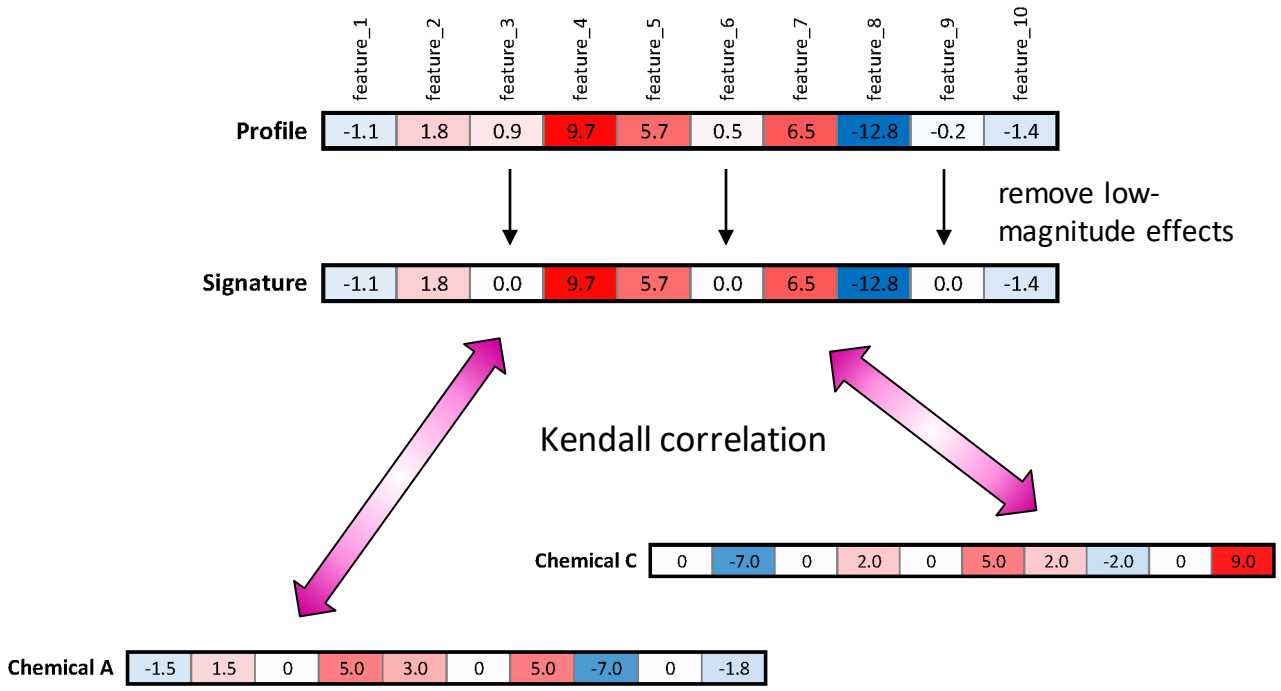
1. remove features that do not provide any information (i.e. have 0 variance)

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

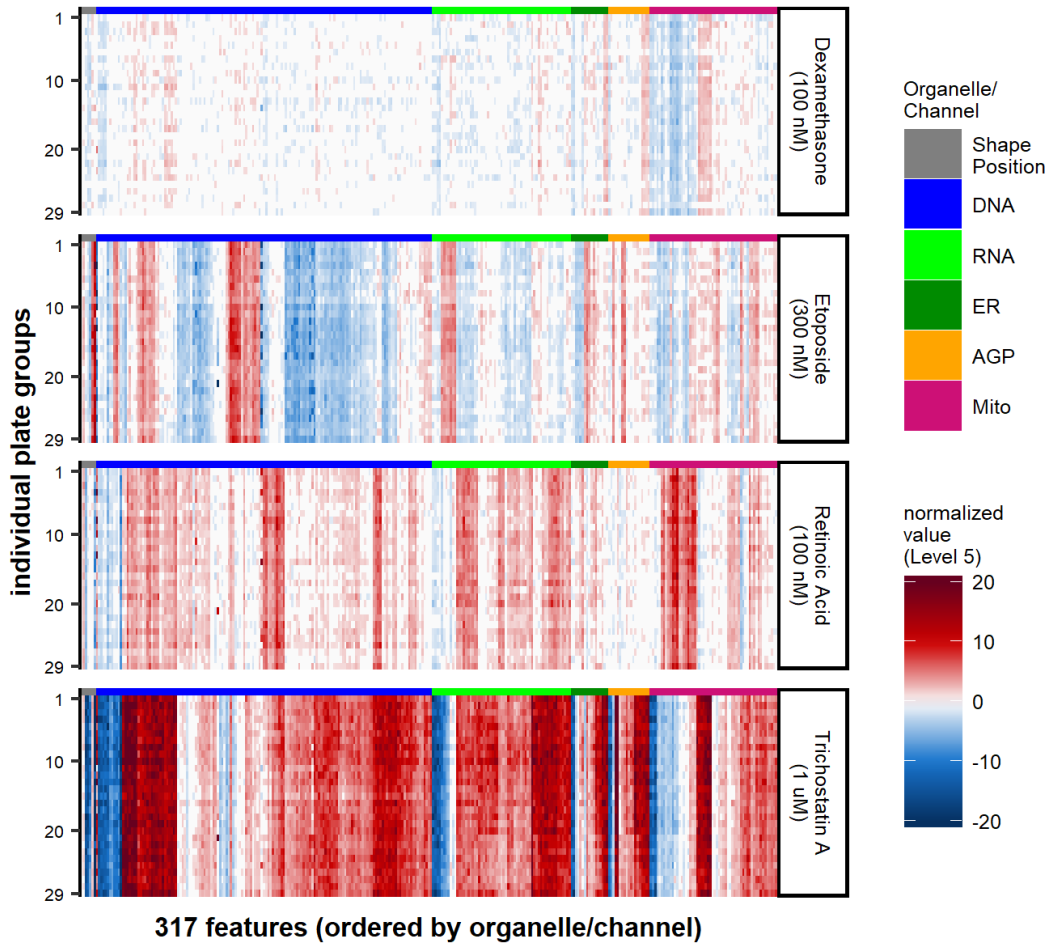
3. remove features that are highly correlated (using recursive feature elimination)

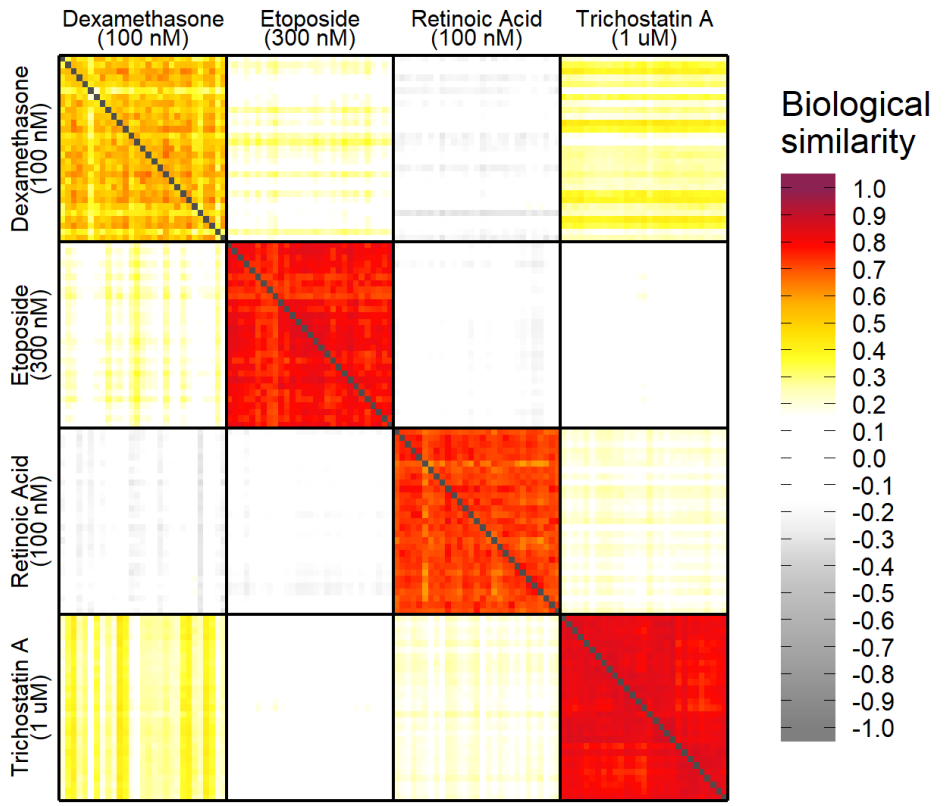
317 features

Profile Comparison



Reproducibility: Phenotypic Profiles





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

Hypothesis: Chemicals with similar mechanisms will display similar profiles.

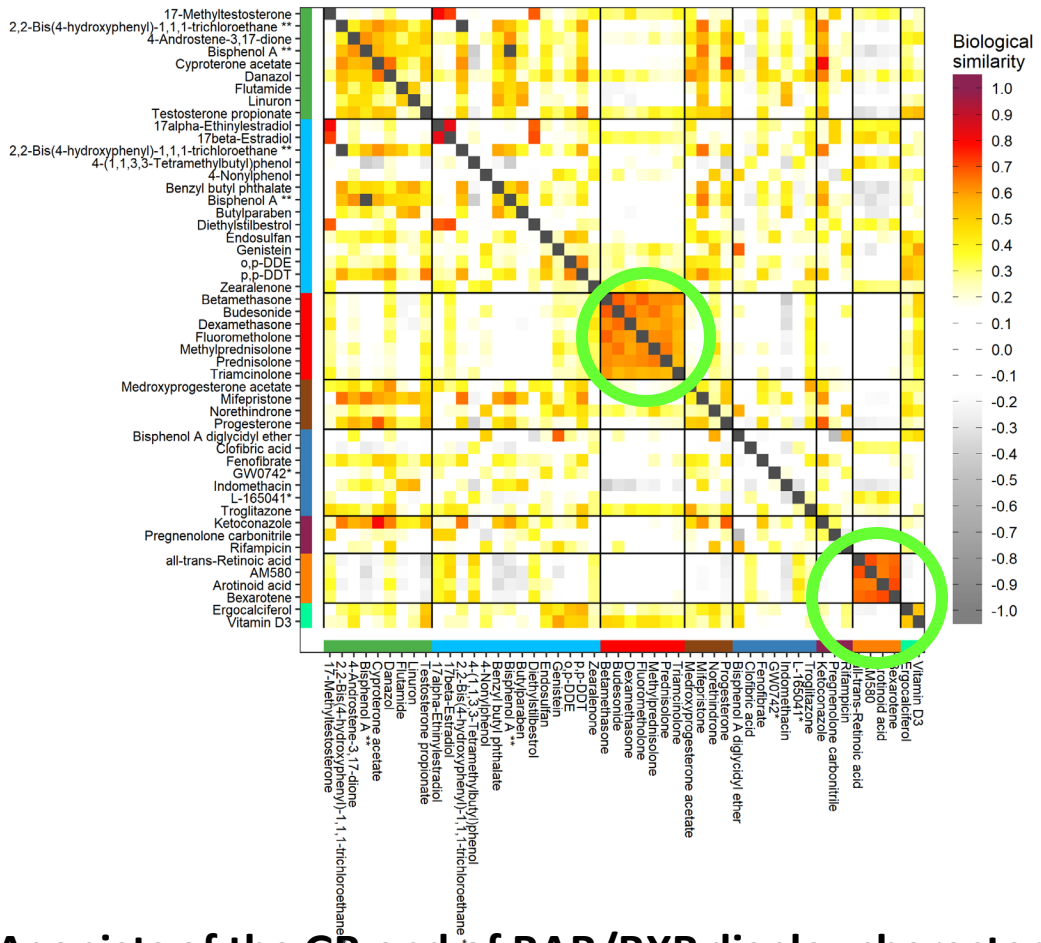
Example: Nuclear Receptor Modulators (I)

- 52 chemicals were annotated as targeting a nuclear receptor

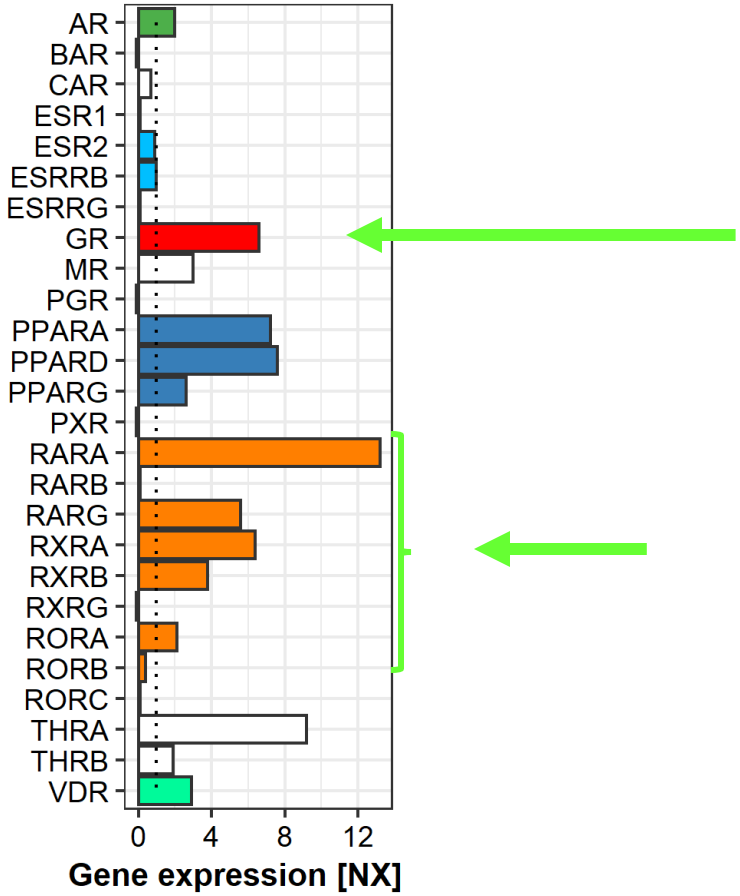
target

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

Biological similarity in HTPP



Gene expression in U-2 OS



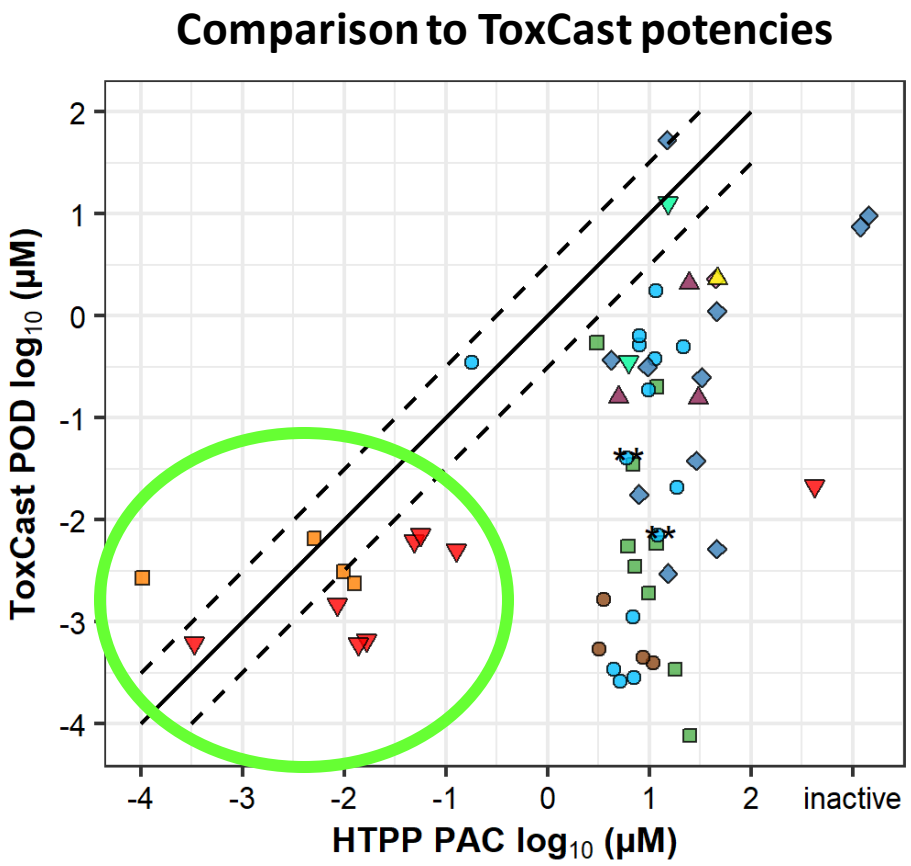
⇨ **Agonists of the GR and of RAR/RXR display characteristic profiles**

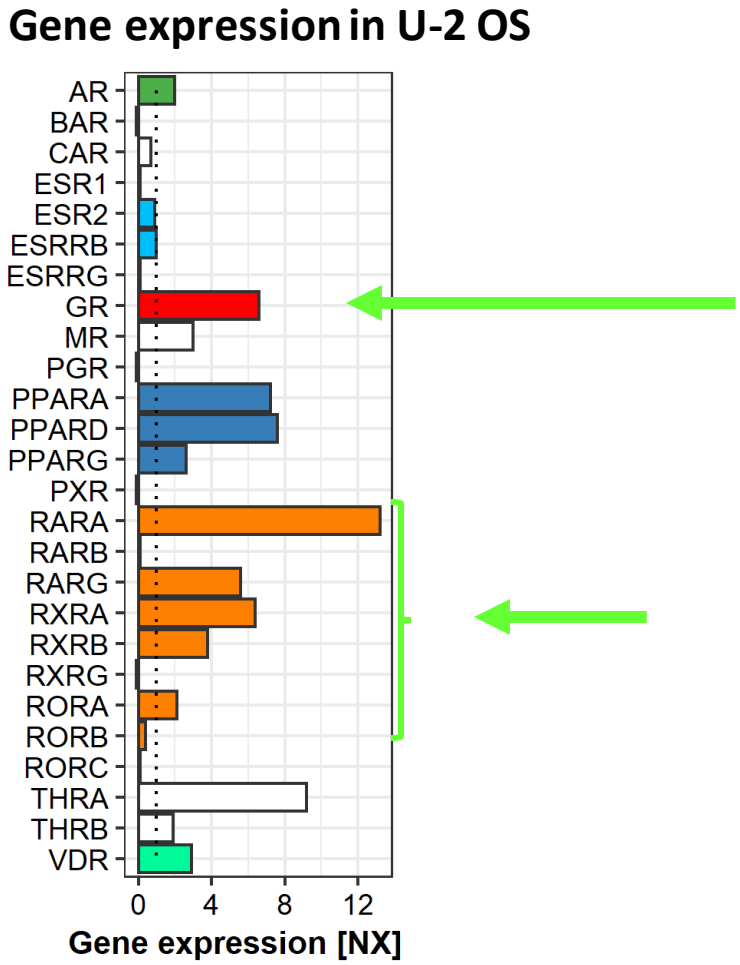
⇨ **Expression of a target does not guarantee that characteristic profiles are observed (e.g., PPAR)**

Example: Nuclear Receptor Modulators (II)

target

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

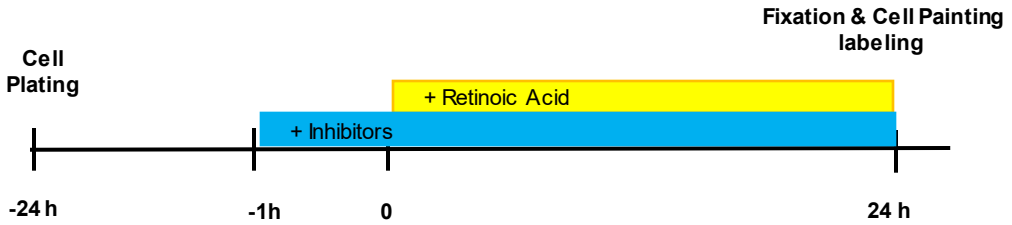


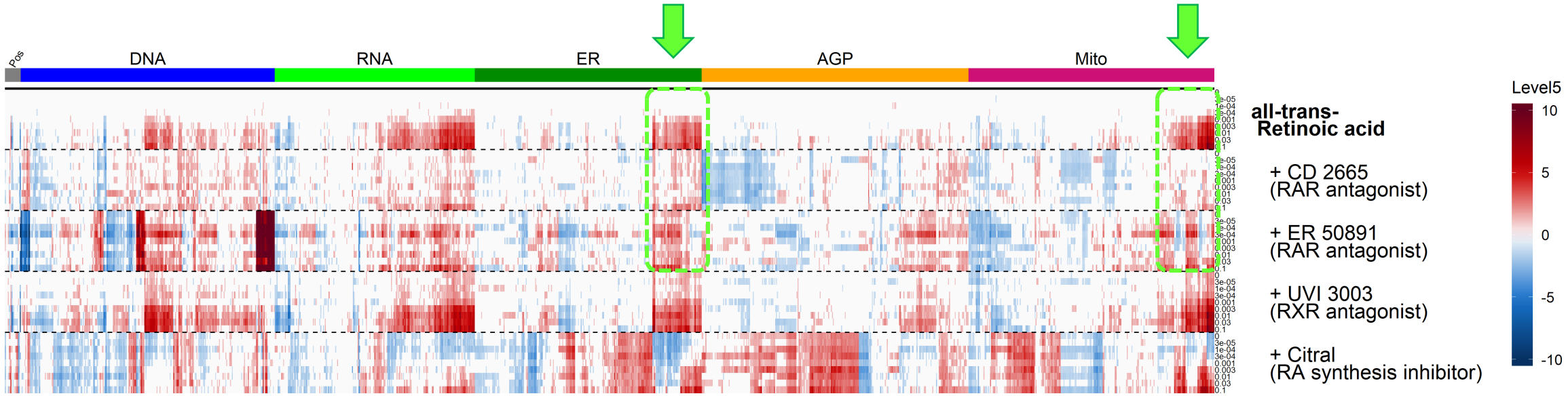


⇨ For two receptor systems that are expressed (GR, RAR/RXR) potencies were comparable with ToxCast

⇨ For receptors with no/low expression in this cell line, HTPP was less sensitive than ToxCast

Pharmacological Blockade of Phenotypic Effects





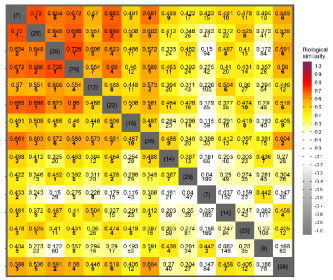
- ⇨ **RAR but not RXR antagonists block the retinoid phenotype**
- ⇨ **Phenotypic profile is related to RAR activation**

adapted from Nyffeler *et al.* (in submission)

Application: Find Retinoid-like Chemicals

RAR/RXR assays
other assays
HTPP potency

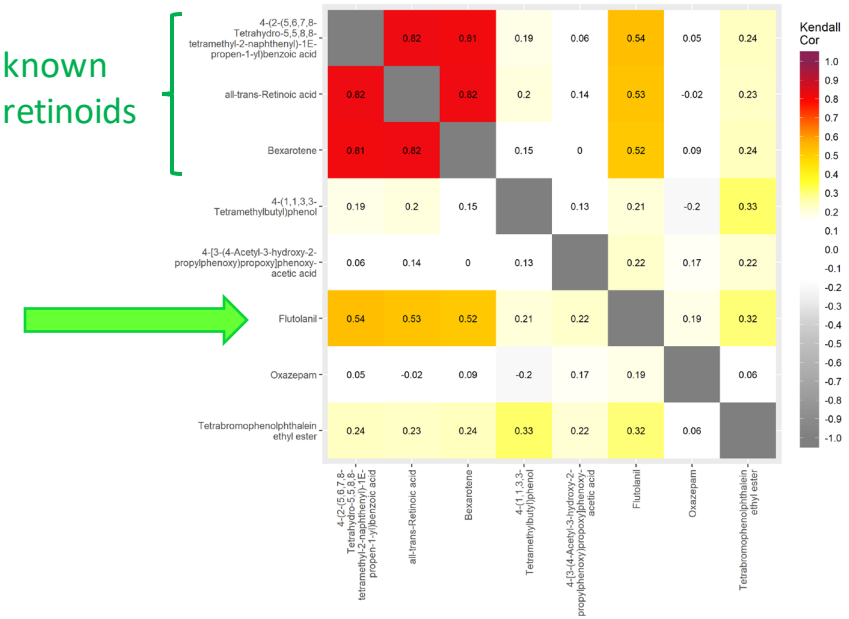
1. Compare profiles to 5 known retinoids:



→ 10 candidate chemicals

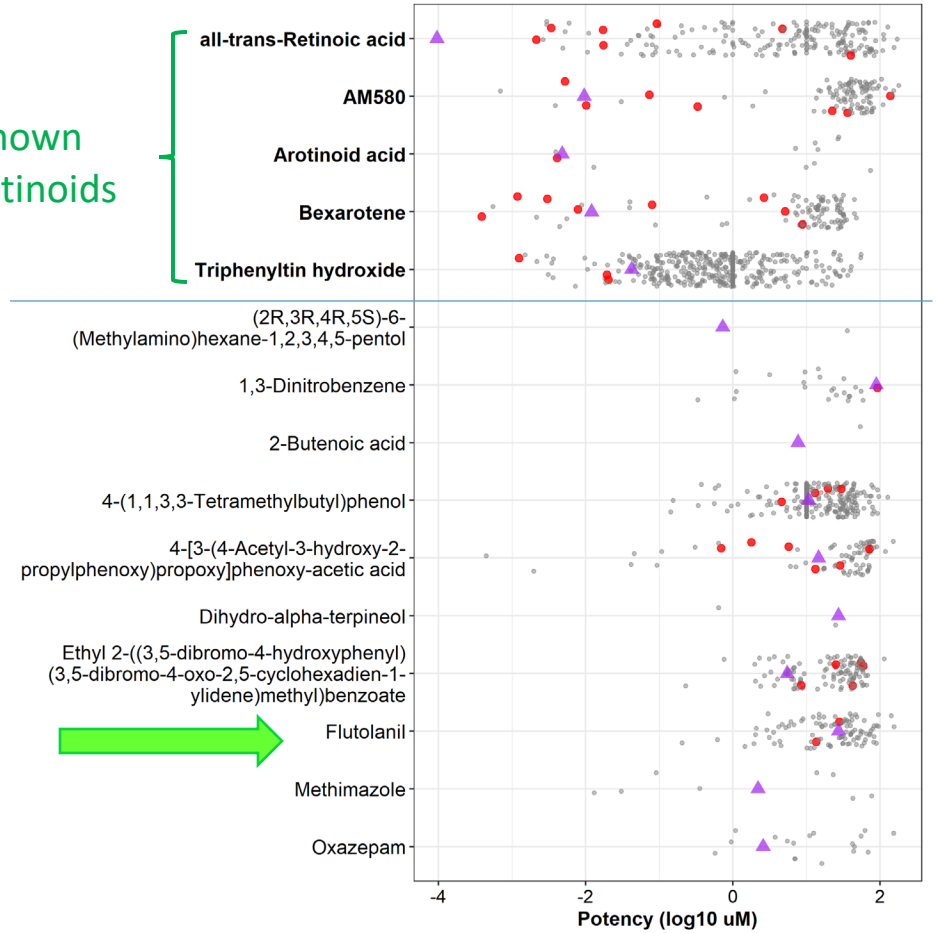
2. Repeat HTPP experiments:

known retinoids



→ Flutolanil induced a phenotype similar to retinoids

3. Compare to ToxCast results:

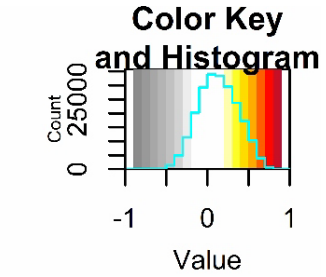


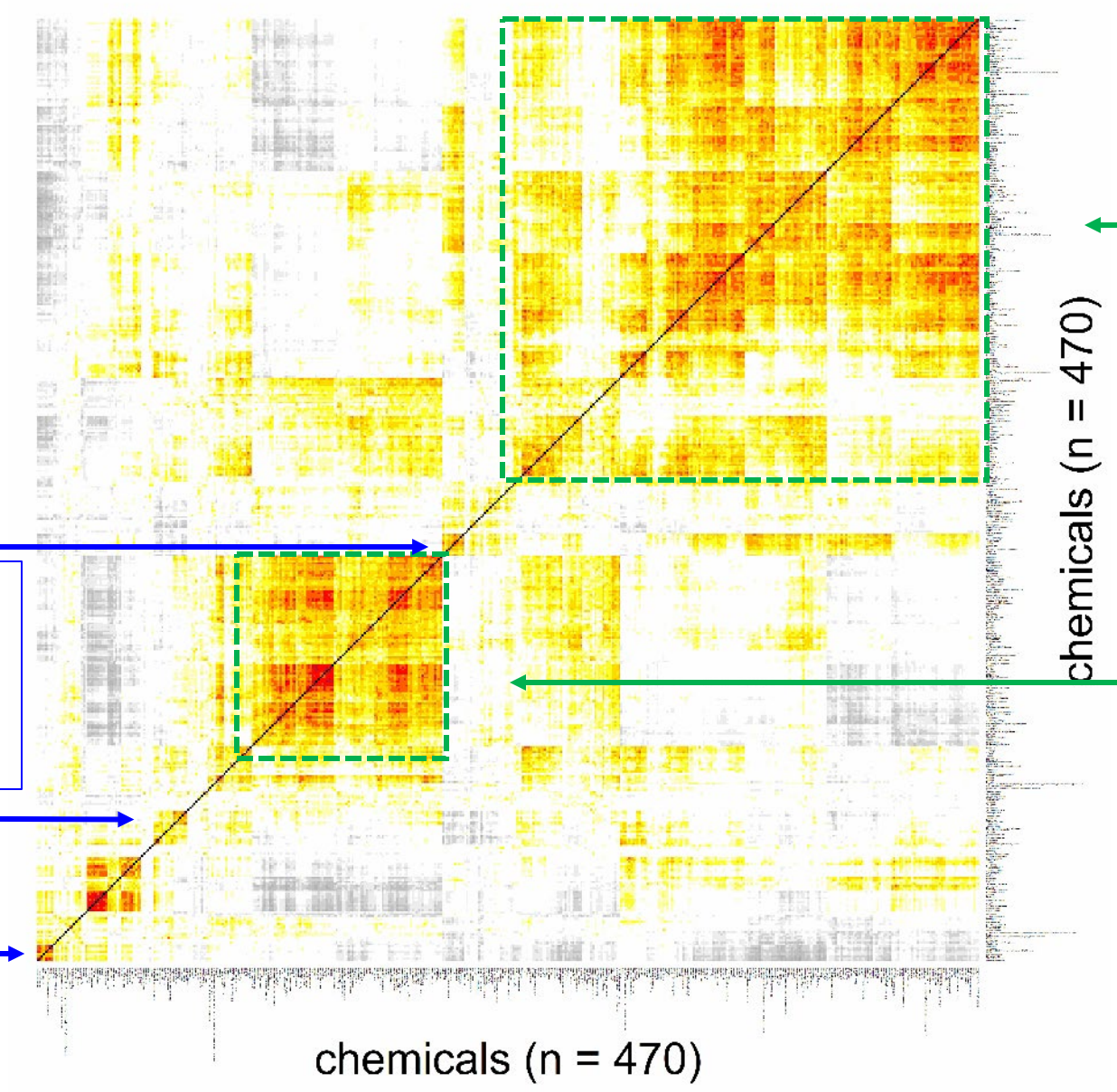
→ Flutolanil had activity in ToxCast assays targeting RAR/RXR

⇨ HTPP has the potential to identify environmental chemicals with specific activities

Preliminary results. Do not cite or quote.

Specific vs Non-specific Phenotypes





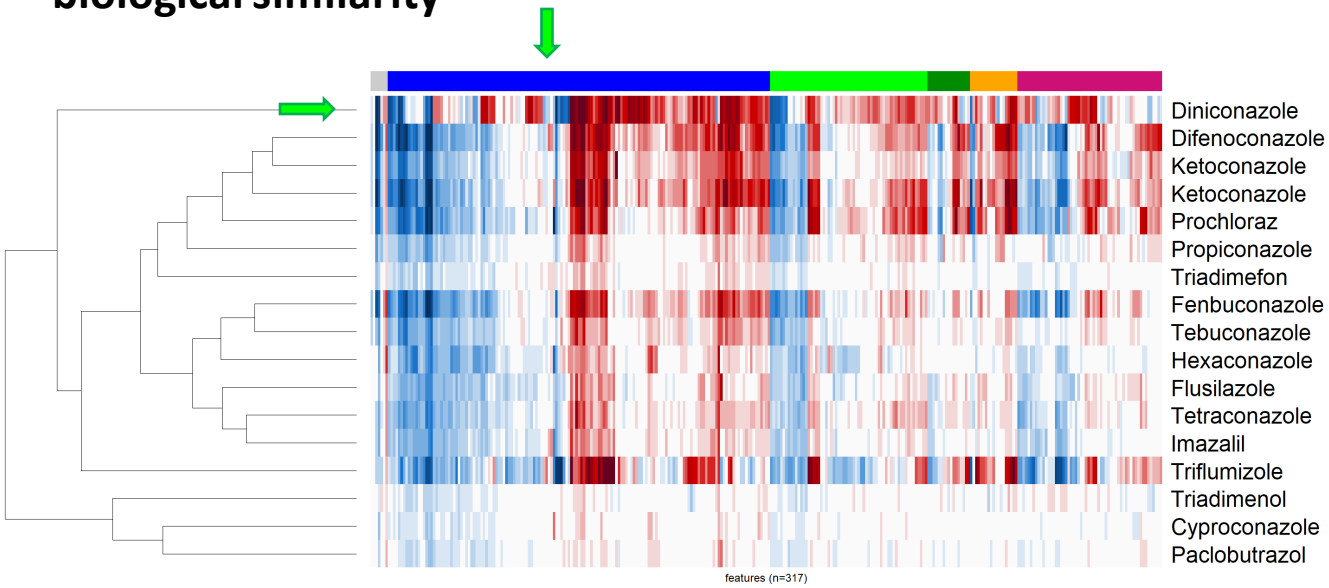
small groups of chemicals with a very **specific** phenotype

large groups of chemicals with similar phenotype → **non-specific** toxicity mechanisms?

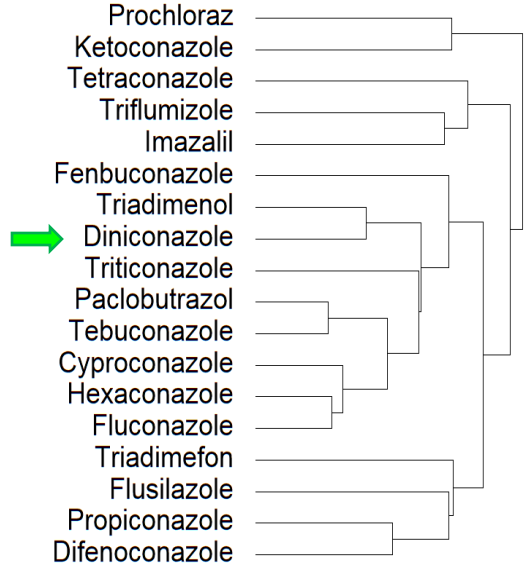
Application: Grouping of Conazoles

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

biological similarity



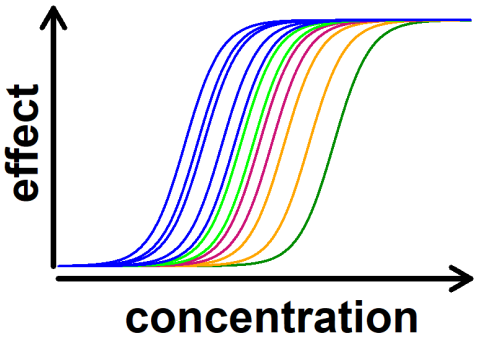
structural similarity
(based on ToxPrints)



⇨ **most conazoles are phenotypically similar**

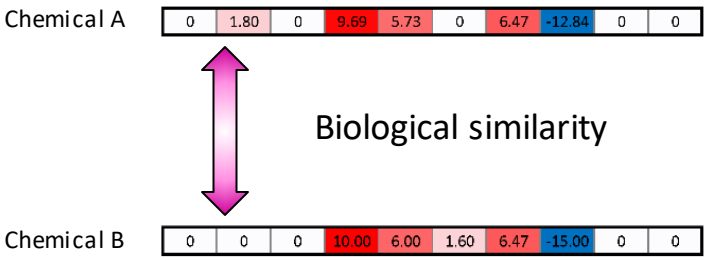
⇨ **Diniconazole is phenotypically different from the other active conazoles**

Conclusions



Application 1: Potency estimation

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



Application 2: Mechanistic prediction

- Similar mechanisms → similar phenotypes

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

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