Applications of image-based high-throughput phenotypic profiling (HTPP) for hazard evaluation of environmental chemicals

Johanna Nyffeler[1,2], Clinton Willis[1], Megan Culbreth[1], Richard E. Brockway[1,3], Logan J. Everett[1], Grace Patlewicz[1], Imran Shah[1], Daniel Chang[1], John Wambaugh[1], Katie Paul Friedman[1], Joshua Harrill[1]

1 Center for Computational Toxicology and Exposure, Office of Research and Development, US Environmental Protection Agency, Durham, NC 27711, United States.
2 Oak Ridge Institute for Science and Education (ORISE) Postdoctoral Fellow, Oak Ridge, TN, 37831, United States.
3 Oak Ridge Associated Universities (ORAU) National Student Services Contractor, Oak Ridge, TN, 37831, United States.

ORCiD 0000-0002-6155-9743 | Nyffeler.Johanna@epa.gov

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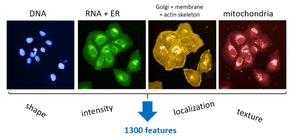
Background

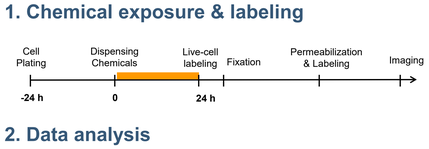
What is phenotypic profiling?

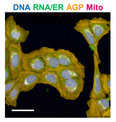


- Image-based phenotypic profiling is a chemical screening method that measures a large variety of morphological features of individual cells in *in vitro* cultures.
- No requirement for *a priori* knowledge of molecular targets.
- **May be used as an efficient and cost-effective method for evaluating chemical bioactivity.**

Method: High-throughput phenotypic profiling (HTPP)

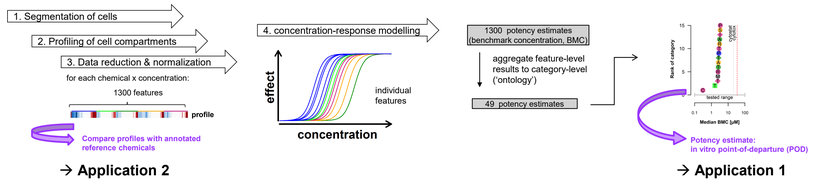
1. Chemical exposure & labeling





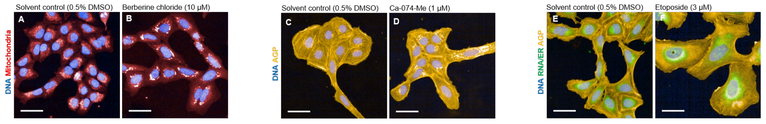
Organelle/Structure	Label
DNA	H-33342
RNA	SYTO14
Endoplasmic reticulum (ER)	Concanavalin A-488
Actin	Phalloidin-568
Golgi + Membrane	wheat germ agglutinin - 555
Mitochondria	MitoTracker

2. Data analysis



Results

Examples



⇨ compactness of mitochondria ⇨ compactness/texture of Golgi ⇨ nuclear and cell size increased

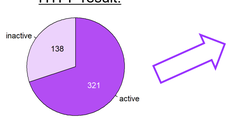
High-throughput screening

Screen 1: 462 bioactive chemicals

Chemicals from our inventory were selected that had information about *in vivo* bioactivity and for which toxicokinetic measurements and exposure estimates were available (Paul Friedman et al. 2020). A majority of chemicals are pesticides, the remaining chemicals are drugs, food additives and industrial chemicals.

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

HTPP result:



Potency estimates from active chemicals are compared to:
1) *in vivo* effect values
2) potency estimates from other NAMs (ToxCast assays) and TTC
3) exposure estimates.

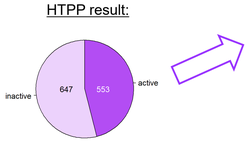
→ see **Application 1**

Screen 2: 1201 ToxCast chemicals

Chemicals from the ToxCast phase 1 and 2 libraries were selected. Of the 1201 chemicals, 179 chemicals had molecular targets annotated in the RefChemDB database (Judson et al. 2019).

Experimental design	
Cell type	U-2 OS
Exposure time	24 h
Cell seeding density per well	3000
# unique chemicals	**1201**
# concentrations	8
Concentration spacing	½ $\log_{10}$
# solvent controls/plate	18
# replicates/plate	1
# independent experiments	4

HTPP result:



Compare phenotypic profiles of active chemicals with profiles from annotated (reference) chemicals.

→ see **Application 2**

Application 1: Estimation of potency thresholds for chemical bioactivity

In vitro-to-*in vivo* extrapolation (IVIVE) was performed using reverse dosimetry to extrapolate the HTPP POD to an administered equivalent dose (AED) to compare it with *in vivo* effect values, other alternative methods and to exposure predictions:

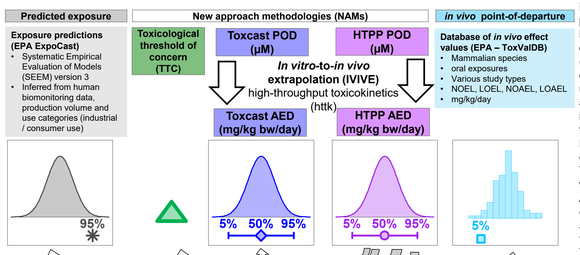


Fig. 1: Procedure to compare *in vitro* POD (in µM) to *in vivo* bioactivity data (in mg/kg bw/day), other alternative approaches and exposure. Administered equivalent doses (AEDs) that would give a plasma concentration corresponding to the *in vitro* POD were estimated using high-throughput toxicokinetic information and models in the *httk* R package. The displayed interval indicates inter-individual variability in toxicokinetics (5-95%). In this study, the *in vivo* POD was defined as the 5th percentile of the distribution of available effect values in the ToxValDB database. Additionally, the HTPP AED was compared to two other alternative methods: A POD from a collection of ToxCast assays was calculated in a previous study (Paul-Friedman et al. 2020). The same study also reported toxicological thresholds of concern (TTC) values. The upper confidence bound of exposure was predicted using SEEM3 model (Wambaugh, et al. 2014).

Comparison to *in vivo* effect values & other NAMs

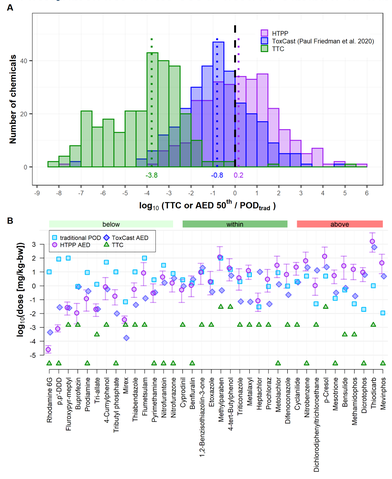
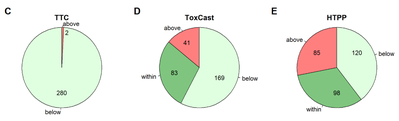


Fig. 2: Comparison of HTPP assay results to *in vivo* toxicity values and published NAM results for chemicals that were active in the HTPP assay. (A) Comparison of different NAMs to the *in vivo* POD (POD_{trad}). Vertical dotted lines and numbers below indicate the median of the distribution for each NAM. The vertical dashed line indicates the unity line. The histogram comprises only chemicals that had available *httk* and *in vivo* data (ToxCast n=293, TTC n=282, HTPP n=303). (B) IVIVE results for representative chemicals. Chemicals were sorted according to the difference between the HTPP AED distribution and the *in vivo* POD.(C) Chemicals in the "above" group had AEDs that overpredicted the *in vivo* effect dose. Chemicals in the two latter groups provided either a comparable (POD_{trad} lies within the AED range) or conservative surrogate for the *in vivo* effect dose, respectively. As for the TTC approach no dose range exists, the chemicals were grouped into "above" and "below" only.



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

⇨ **78% (237/303) of HTPP AED are within 2 orders of magnitude of the *in vivo* POD**

Comparison to exposure estimates

HTPP AEDs were compared to exposure predictions and the bioactivity exposure ratio was calculated as follows:

$$\text{BER} = \frac{\text{lower bound of HTPP bioactivity}}{\text{upper bound of exposure estimate}} = \log_{10}\left(\frac{\text{HTPP AED } 5^{th}}{\text{SEEM3 } 95^{th}}\right)$$

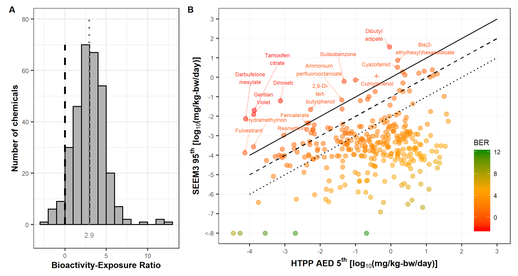


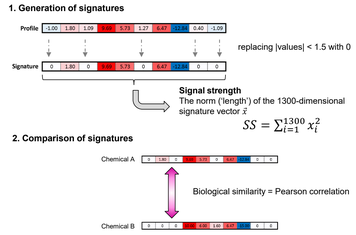
Fig. 3: Comparison of HTPP assay results to exposure predictions. (A) The bioactivity exposure ratio (BER) was defined as the ratio of the lower bound of the HTPP AED confidence interval and the upper bound of the exposure prediction from the SEEM3 framework. The gray dotted line indicates the median of the distribution. For chemicals to the left of the unity line (vertical dashed line), the bioactivity and exposure estimates overlap, indicating a potential for humans to be exposed to bioactive concentrations of these chemicals. The histogram comprises only active chemicals that had available *httk* and exposure data (n=316). (B) The 16 chemicals with a negative BER are labeled.

⇨ **for 49% of chemicals, predicted exposure is > 1000x lower than estimated bioactivity**

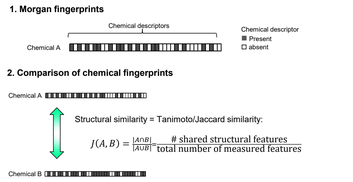
⇨ **for 5.1% (16/316) of chemicals, the BER was negative, indicating a potential for humans to be exposed to bioactive concentrations of these chemicals**

Application 2: Use of phenotypic profiles to discern putative mode-of-action (MOA)

Calculation of biological similarity



Calculation of structural similarity

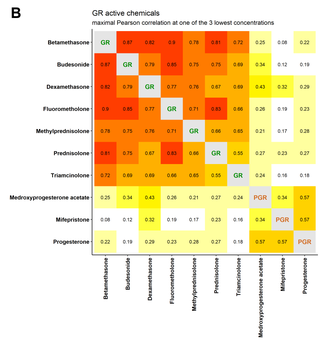


Example: Drug-like chemicals (glucocorticoids)

11 chemicals were annotated in RefChemDB with the glucocorticoid receptor (GR, NR3C1):

A

Chemical	HTPP	1° target	Pharmacology
Betamethasone	active	GR agonist	
Budesonide	active	GR agonist	
Dexamethasone	active	GR agonist	
Fluorometholone	active	GR agonist	
Methylprednisolone	active	GR agonist	prednisolone derivative
Prednisolone	active	GR agonist	
Prednisone	inactive	GR agonist	*converted to prednisolone in the liver*
Triamcinolone	active	GR agonist	
Medroxyprogesterone acetate	active	PGR agonist	progesterone derivative
Mifepristone	active	PGR antagonist	PR antagonist, GR antagonist
Progesterone	active	PGR agonist	Progesterone agonist, GR partial agonist,



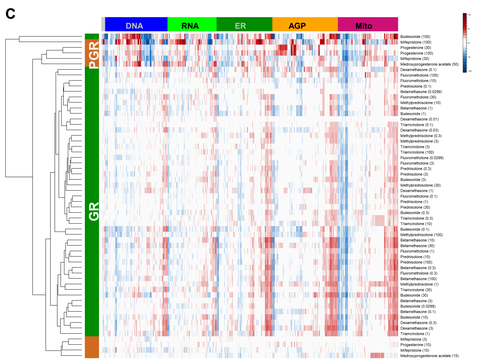


Fig. 4: Chemicals targeting glucocorticoid receptor and their profiles in HTPP assay. (A) List of the 11 chemicals annotate with glucocorticoid receptor activity in RefChemDB. (B) Biological similarity of phenotypic profiles of the 10 active chemicals. (C) Signature of all active, non-cytotoxic concentrations. Row side colors indicate the primary biological target (green: GR; brown: PGR). Abbreviations: GR; glucocorticoid receptor; PGR: progesterone receptor.

⇨ **Chemicals with the same mode-of-action display similar biological profiles.**

⇨ **Chemicals with different primary mode-of-action (i.e. GR vs PGR) can be distinguished.**

Example: Environmental chemicals (Dieldrin)

Dieldrin was used as a "seed" to retrieve chemicals with similar phenotypic profiles.

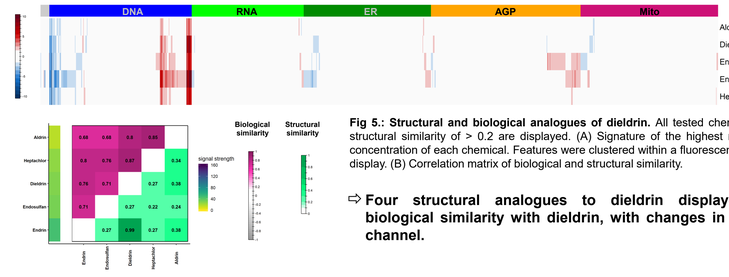


Fig 5.: Structural and biological analogues of dieldrin. All tested chemicals with a structural similarity of > 0.2 are displayed. (A) Signature of the highest non-cytotoxic concentration of each chemical. Features were clustered within a fluorescent channel for display. (B) Correlation matrix of biological and structural similarity.

⇨ **Four structural analogues to dieldrin displayed high biological similarity with dieldrin, with changes in the DNA channel.**

⇨ **HTPP *in vitro* potencies can be used for bioactivity exposure ratio analysis and prioritizing of chemicals based on inferred bioactivity in relation to predicted human exposure**

Next steps:

- **Test chemicals in multiple cell types to increase biological coverage**

⇨ **Preliminary results indicate that HTPP is able to discern putative mode-of actions of drug-like and environmental chemicals**

Next steps:

- **Look at different molecular targets, in particular targets that are not covered by the ToxCast assay battery**

References

- Bray, et al. 2016. *Cell painting, a high-content image-based assay for morphological profiling using multiplexed fluorescent dyes*. Nat. Protoc. 11 (9), 1757–1774.
- Gustafsdottir, et al. 2013. *Multiplex cytological profiling assay to measure diverse cellular states*. PLoS One 8 (12), e80999
- Judson, et al.2019. *Workflow for defining reference chemicals for assessing performance of in vitro assays*. ALTEX. 2019;36(2):261-276.
- Nyffeler et al. 2020. *Bioactivity screening of environmental chemicals using imaging-based high-throughput phenotypic profiling*. Toxicol Appl Pharmacol. 389, 114876
- Paul Friedman, et al. 2020. *Examining the utility of in vitro bioactivity as a conservative point of departure: a case study*. Toxicol. Sci. 173 (1), 202–225.

Abbreviations

AED	Administered equivalent dose
BER	Bioactivity exposure ratio
BMC	Benchmark concentration
GR	Glucocorticoid receptor
HTPP	High-throughput phenotypic profiling
IVIVE	In vitro to in vivo extrapolation
MOA	Mode-of-action
NAM	New approach methodology
PGR	Progesterone receptor
POD	Point-of-departure
TTC	Threshold of toxicological concern

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