Development and use of a high-throughput phenotypic profiling assay for bioactivity screening of environmental chemicals

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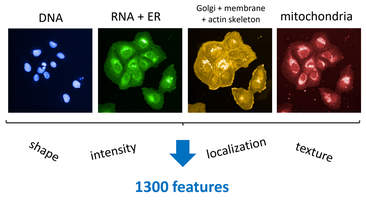
Background

- Image-based phenotypic profiling is a chemical screening method that measures a large variety of morphological features of individual cells in *in vitro* cultures.
- Successfully used for functional genomic studies and in the pharmaceutical industry for compound efficacy and toxicity screening.
- No requirement for *a priori* knowledge of molecular targets.

⇨ May be used as an efficient and cost-effective method for evaluating the bioactivity of environmental chemicals.

⇨ May be used to determine effect thresholds (i.e. *in vitro* point-of-departure, POD) for comparison to toxicity values from animal studies (i.e. *in vivo* POD)



Aims

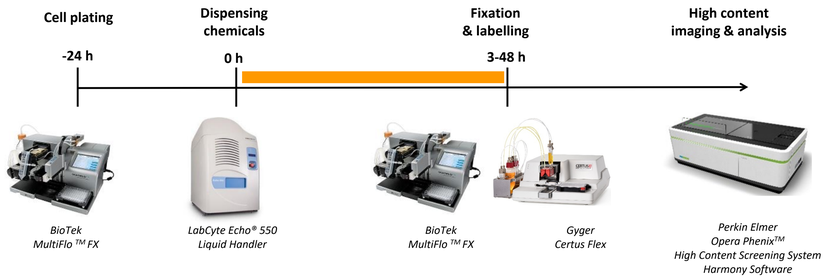
1. **Miniaturize an existing assay (Bray *et al.* 2016) and evaluate performance by replicating published results.**
2. **Investigate how *in vitro* point-of-departures (POD) change**
   a) **across biological space (i.e. cell types)**
   b) **with exposure time**
3. **Screen a set of environmental chemicals and compare the obtained *in vitro* PODs to *in vivo* toxicity data.**

Conclusions

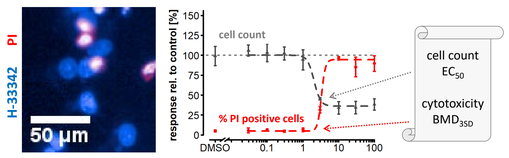
1. **Published results could be reproduced; distinct profiles were observed.**
2. **PODs varied**
   a) **less than 2 orders of magnitude across different cell lines**
   b) **less than 1 order of magnitude from 6 – 48 h of exposure**
3. ***In vitro*-to-*in vivo* extrapolation (IVIVE) demonstrated that *in vitro* PODs were as protective or more protective that *in vivo* toxicity data for 2/3 of the chemicals tested.**

Methods

1. Experimental workflow



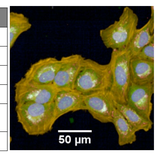
Assay 1: Cytotoxicity & cell viability (CV)



Experimental design	Reference chemicals	Test chemicals
Cell types	6	1 (U2OS)
Exposure time	3 – 48 h	24 h
# chemicals	14 positive 2 negative	160
# concentrations	7	8
Dose spacing	½ $\log_{10}$	½ $\log_{10}$
Solvent controls/plate	24	24
Replicates/plate	3	1
# independent exp	3	4

Assay 2: Cytological profiling (CP)

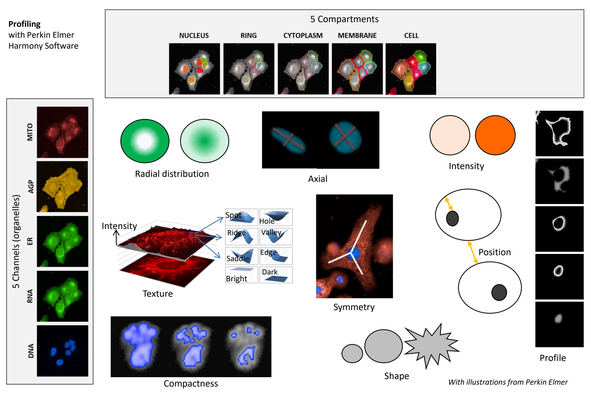
Flourescent labels
DNA: H-33342
RNA: SYTO14
ER: Concanavalin A-488
Actin: Phalloidin-568
Golgi + Membrane: wheat germ agglutinin (WGA) -555
Mitochondria: MitoTracker



Segmentation of cells → Profiling of cell compartments → Normalization → Dose-response modelling → ***in vitro* point-of-departure (POD)**

2. Image processing (Cell profiling)

Profiling with Perkin Elmer Harmony Software



= 1300 endpoints

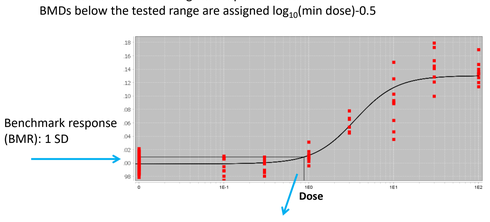
49 ontologies

Examples:
- Mito_Compactness _Cells
- AGP_Texture _Cytoplasm
- DNA_Intensity _Nuclei

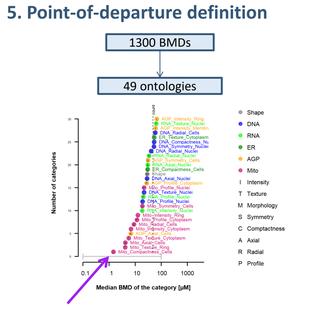
3. Data reduction & normalization

4. Dose-response modelling

- Scaled well-level data is clipped above the first cytotoxic dose (determined by the CV assay)
- Software: BMDExpress 2.2
- 4 models: Hill, Linear, Poly2, Power
- Best model selection:
  1. nested Chi2 to select the better polynomial (Linear vs Poly2)
  2. best AIC (Hill, Power, Poly)
- BMDs above the tested range are reported as NA
  BMDs below the tested range are assigned $\log_{10}$(min dose)-0.5



5. Point-of-departure definition



Point-of-departure (POD):
Median BMD of the most sensitive ontology
(where ≥ 30% ontology elements affected)

Results I: Assay setup

An existing assay (Bray *et al.* 2016) was miniaturized and adapted to a microfluidics-based laboratory workflow. To evaluate assay performance, 14 reference chemicals were tested in the same cell line (U2OS cells) at the same exposure time (48 h), aiming to reproduce published results.

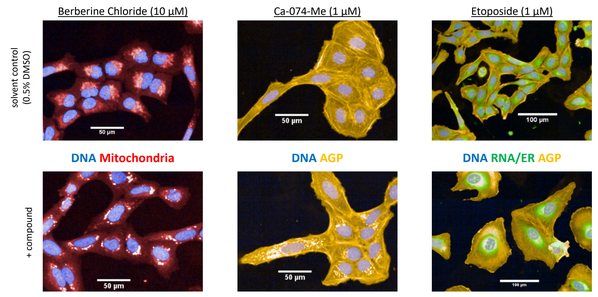


Fig 1.: Examples of chemical-specific cytological phenotypes. U2OS cells were treated for 48 h with the compounds before cells were live-labeled for mitochondria, fixed, permeabilized and remaining labels applied. Images were acquired with a 20x water immersion objective. Only selected channels are shown to highlight the phenotypes. Affected endpoints are mentioned below the images.

⇨ **Different chemicals induce different cytological phenotypes**

⇨ **Phenotypes are mostly consistent with literature (Gustafsdottir *et al.* 2013)**

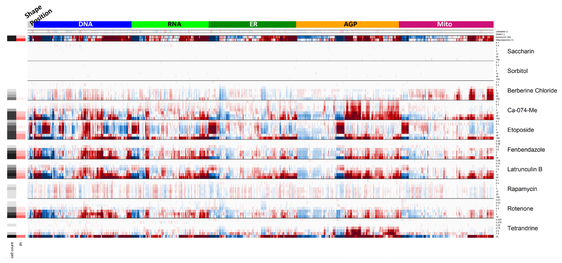


Fig 2.: Examples of chemical-specific profiles. Standardized well-level data of U2OS cells were averaged across 3 technical and 3 biological replicates. Endpoints are ordered according to the corresponding channel/organelle. The color key on the left indicates reductions in cell count and increases in cell death.

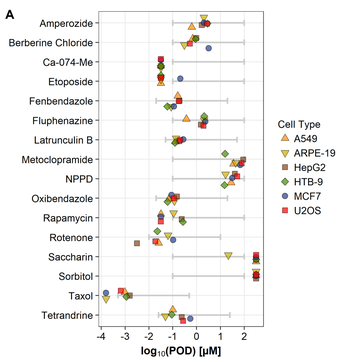
⇨ **Effects observed at non-cytotoxic concentrations**

⇨ **Qualitative observations of cell phenotypes correspond to quantitative changes in endpoint profiles as a function of dose.**

Results II: Time & biological space

Using the same 16 chemicals from above, variation of the *in vitro* point-of-departure (POD) was measured across different cell lines (i.e. biological space) and different exposure durations. To assess biological space, chemicals were tested on 6 cell lines at 48 h of exposure. Variation of PODs with time was assessed in U2OS cells for exposure times of 3 – 48 h.

1. Variation of PODs across biological space



B

Cell line	Description
A549	Human lung adenocarcinoma epithelial cells
ARPE-19	Human retinal pigment epithelial cell line
HepG2	Human liver carcinoma cells
HTB-9	Human urinary bladder epithelial cells
MCF7	Human breast adenocarcinoma cell line
U2OS	Human bone osteosarcoma epithelial cells

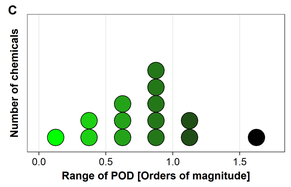


Fig 3.: The 16 reference chemicals were tested in 6 cell types. PODs were defined as the median BMD of the most sensitive ontology with enough coverage. For negative chemicals, the POD was defined as ½ order of magnitude above the highest tested dose. (A) PODs by individual chemicals. (B) Tissue origin for the tested cell lines. (C) For the 14 positive chemicals, the range of PODs was calculated as $\log_{10}$(max POD) – $\log_{10}$(min POD).

⇨ **PODs vary less than 2 order of magnitude across cell types**

2. Variation of PODs with exposure time

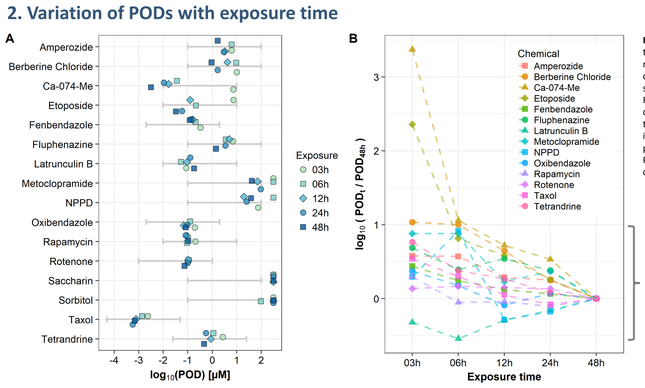


Fig 4.: The 16 reference chemicals were tested in U2OS cells with exposure times ranging from 3 h to 48 h. PODs were defined as the median BMD of the most sensitive ontology with enough coverage. For negative chemicals, the POD was defined as ½ order of magnitude above the highest tested dose. (A) PODs by individual chemicals. (B) PODs for each positive chemical were normalized to its POD at 48 h to illustrate the temporal change.

⇨ **PODs vary less than 1 order of magnitude within 6 – 48 h of exposure**

Results III: Screening

A set of 160 known bioactive chemicals was screened in U2OS cells following 24 h of exposure. The obtained *in vitro* PODs were transformed to administered equivalent doses (AEDs, mg/kg bw/day) using the httk R-package to compare to traditional *in vivo* toxicity data.

1. Hit rate in both assays

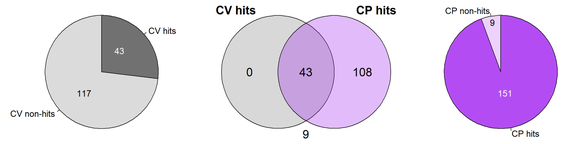


Fig 5.: Overview of hit rates for cytotoxicity & cell viability (CV) as well as cytological profiling (CP) assays. Compounds were defined as a CV hit, if their cytostatic EC_{50} or cytotoxic BMD_{3SD} was below the highest tested dose. Compounds were defined as a CP hit, if they had a POD below the highest tested dose, i.e. if for at least one ontology ≥ 30% of endpoints had a BMD below the highest tested dose.

⇨ **Profiling identifies more compounds as bioactive than cytotoxicity measurement alone.**

⇨ **Over 90% of bioactive compounds were identified with the profiling assay.**

2. Comparison to *in vivo* data

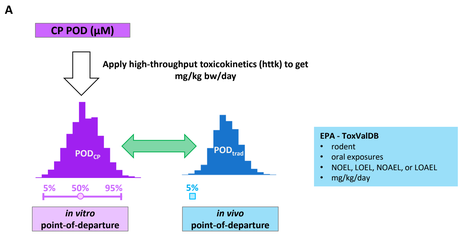
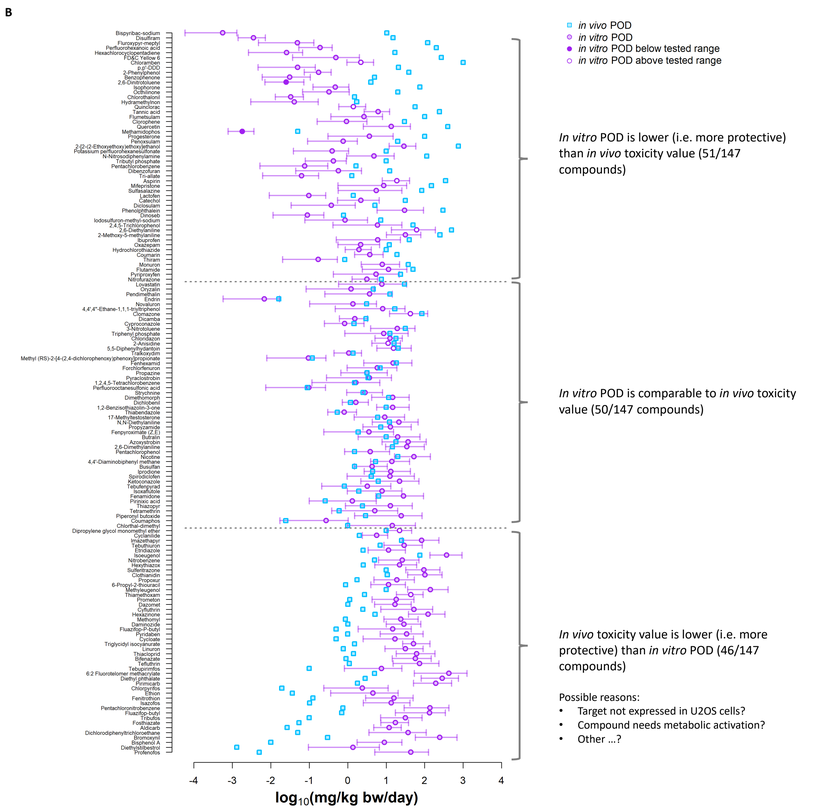


Fig 6.: Comparison of *in vitro* POD to *in vivo* bioactivity data. (A) Procedure to compare *in vitro* PODs (in µM) to *in vivo* data (in mg/kg bw/day). AEDs that would give a plasma concentration corresponding to the *in vitro* POD were estimated using *in vitro*-to-*in vivo* extrapolation, using high-throughput toxicokinetic information and models in the httk R package (v1.8). The displayed interval indicates inter-individual variability in toxicokinetics (5-95%). The AED is then compared to *in vivo* data from the ToxValDB database. All rodent data from oral exposures was considered. In this study, the *in vivo* POD was defined as the 5th percentile of the distribution of available NOEL, LOEL and similar. (B) Comparison of calculated AEDs from the profiling POD and the 5th percentile of *in vivo* data. Only chemicals are displayed that have both *in vivo* data and toxicokinetic information. This was the case for 147/160 chemicals. Chemicals that were inactive in the *in vitro* assay are displayed as transparent circles while chemicals where the *in vitro* POD was below the lowest tested dose are denoted with closed circles.



⇨ **Phenotypic profiling-derived *in vitro* PODs, when used to estimate AEDs in mg/kg bw/day units, were as protective or more protective than *in vivo* toxicity values for 2/3 of the chemicals.**

⇨ **Future efforts will aim to refine *in vivo*-to-*in vitro* comparisons through testing of multiple cell lines and incorporation of metabolic activation of chemicals.**

Future directions

- Increase the number and diversity of chemicals by screening the USEPA ToxCast library in multiple cell types.
- Compare how profiling PODs compare to other *in vitro* assays (ToxCast assays, high-throughput transcriptional profiling) and to predicted exposure levels.
- Investigate whether mechanistic information can be obtained from the profiles.

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