Development and Use of a High Content Imaging-Based Phenotypic Profiling Assay for Bioactivity Screening of Environmental Chemicals

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USEPA National Center for Computational Toxicology (NCCT)
Disclaimer

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Outline

• Background
  • What is NCCT?
  • What does NCCT Do?
  • USEPA Computational Toxicology Blueprint

• High-Throughput Phenotypic Profiling (HTPP)
  • Technology Overview → Cell Painting
  • Image Analysis
  • Computational Workflows

• Potential Applications for Chemical Safety Assessment
  • Bioactivity to Exposure Ratio (BER) Analysis
  • Profile Similarity for Chemical Read-Across

• Conclusions
Mission Statement:
A research organization tasked with advancing the science of toxicity testing through the development and/or application of novel experimental and computational approaches for rapidly characterizing the physiochemical properties, biological activity, exposure potential and potential human health risks associated with chemicals.
Computational Toxicology Research Areas

NCCT research programs focus on developing the **tools, approaches and data** needed to accelerate the pace of chemical risk assessment and foster incorporation of non-traditional toxicity testing data into regulatory decision-making processes.

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

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td># of assays</td>
<td># of chemicals</td>
</tr>
<tr>
<td>500</td>
<td>300</td>
</tr>
<tr>
<td>700</td>
<td>2,000</td>
</tr>
</tbody>
</table>

- Mostly targeted assays (chemical $X$ $\rightarrow$ protein $Y$)
- Incomplete coverage of biological space.

- **New Approach for Hazard Evaluation**: Employ broad-based (i.e. non-targeted) **profiling assays** that cast the broadest net possible for capturing potential hazards associated with chemical treatment and may be used to group chemicals based on similarity in bioactivity profiles.
- Cell Painting is a HCS profiling method that measures a large variety of phenotypic features in fluoroprobe labeled cells \textit{in vitro}.

- No requirement for \textit{a priori} knowledge of molecular targets.

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

- **Hypothesis:** Cell Painting may be an efficient and cost-effective method for evaluating the bioactivity of environmental chemicals.

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### Cell Painting

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cellular Component</th>
<th>Labeling Chemistry</th>
<th>Labeling Phase</th>
<th>Opera Phenix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoechst 33342</td>
<td>Nucleus</td>
<td>Bisbenzamide probe that binds to dsDNA</td>
<td></td>
<td>Fixed</td>
</tr>
<tr>
<td>Concanavalin A – AlexaFluor 488</td>
<td>Endoplasmic reticulum</td>
<td>Lectin that selectively binds to α-mannopyranosyl and α-glucopyranosyl residues enriched in rough endoplasmic reticulum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SYTO 14 nucleic acid stain</td>
<td>Nucleoli</td>
<td>Cyanine probe that binds to ssRNA</td>
<td></td>
<td>Fixed</td>
</tr>
<tr>
<td>Wheat germ agglutinin (WGA) – AlexaFluor 555</td>
<td>Golgi Apparatus and Plasma Membrane</td>
<td>Lectin that selectively binds to sialic acid and N-acetylglucosaminyl residues enriched in the trans-Golgi network and plasma membrane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phalloidin – AlexaFluor 568</td>
<td>F-actin (cytoskeleton)</td>
<td>Phalloxins (bicyclic heptapeptide) that binds filamentous actin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MitoTracker Deep Red</td>
<td>Mitochondria</td>
<td>Accumulates in active mitochondria</td>
<td>Live</td>
<td>650 760</td>
</tr>
</tbody>
</table>
Objectives

1. Adapt the Cell Painting assay (Bray et al. 2016) for use with microfluidics and imaging instruments available at NCCT.

2. Develop computational workflows for phenotypic feature extraction, data normalization, concentration-response modeling and generation of phenotypic response profiles.

3. Evaluate assay performance by replicating published results (Gustafsdottir et al. 2013).

4. Perform concentration-response screening of a set of environmental chemicals selected from USEPA ToxCast collection.

5. Demonstrate data usability in down-stream applications of potential interest to chemical safety assessment practitioners:
   1. *In vitro-to-in vivo* (IVIVE) and bioactivity-exposure ratio (BER) analysis
   2. Chemical read across using phenotypic profile similarity
Image Analysis Workflow → Image Segmentation

1. find nuclei
2. find cell outline
3. reject border objects
Define Cellular Compartments

- **nuclei**
- **cytoplasm**
- **membrane**

**cell**

**ring**
**Phenotypic Feature Extraction**

5 Compartments: NUCLEUS, CYTOPLASM, MEMBRANE, CELL

- **Radial distribution**
- **Axial**
- **Intensity**
- **Symmetry**
- **Compactness**
- **Profile**
- **Position**
- **Shape**

1300 features / cell

---

### Module

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Nuclei</td>
<td>Nuclei</td>
<td>Nuclei</td>
<td>Nuclei Cell</td>
<td>Nuclei</td>
</tr>
<tr>
<td>RNA</td>
<td>Nuclei</td>
<td>Nuclei</td>
<td>Nuclei</td>
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<tr>
<td>ER</td>
<td>Cell</td>
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<tr>
<td>AGP</td>
<td>Cell</td>
<td>Cell</td>
<td>Cell</td>
<td>Cell</td>
<td>Nuclei</td>
</tr>
<tr>
<td>Mito</td>
<td>Cell</td>
<td>Cell</td>
<td>Cell</td>
<td>Cell</td>
<td>Nuclei</td>
</tr>
</tbody>
</table>

Not associated with a channel: Nuclei, Cell
Analysis of phenotypic features

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 $\chi^2$ to select the better polynomial (Linear vs Poly2)
  2. best AIC (Hill, Power, Poly)
- BMCs above the tested range are reported as NA
- BMCs below the tested range are assigned $\log_{10}(\text{min dose})-0.5$

Phenotypic Category Analysis

- 1300 BMDs
- 49 ontologies

Benchmark response (BMR): 1 SD

Bioactivity Altering Concentration (BAC): Median BMC of the most sensitive category (where ≥30% ontology elements affected)
HTPP Assay Overview

**A**

Cell Plating → Dispensing Chemicals → Live-cell labeling → Fixation

-24 h → 0 → 3-48 h

**B**

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

**C**

plate 1: cell viability (CV)

Segmentation of nuclei → Quantification of intensities → Data reduction → Concentration-response modelling

H-33342 PI normalized cell count

% PI positive cells

Cytoxicity BMC → Cytoxicity BMC

**D**

plate 2: cell profiling (CP)

Segmentation of cells → Profiling of cell compartments → Data reduction & normalization → Concentration-response modelling

1300 BMCs → 49 categories

in vitro point-of-departure HTPP POD

CP BAC
## Pilot Study Design

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Multiplier</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Type(s)</td>
<td>1</td>
<td>U-2 OS</td>
</tr>
<tr>
<td>Culture Condition</td>
<td>1</td>
<td>DMEM + 10% HI-FBS (^a)</td>
</tr>
<tr>
<td>Chemicals</td>
<td>16</td>
<td>14 phenotypic reference chemicals, 2 negative controls</td>
</tr>
<tr>
<td>Time Points:</td>
<td>1</td>
<td>48 hours</td>
</tr>
<tr>
<td>Assay Formats:</td>
<td>2</td>
<td>Cell Painting</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HCI Cell Viability &amp; Apoptosis</td>
</tr>
<tr>
<td>Concentrations:</td>
<td>8</td>
<td>3.5 log(<em>{10}) units; semi log(</em>{10}) spacing</td>
</tr>
<tr>
<td>Biological Replicates:</td>
<td>3</td>
<td>Independent cultures</td>
</tr>
</tbody>
</table>
### Reference Chemical Set

Reference chemicals (n=14) with narrative descriptions of observed phenotypes were identified from Gustafsdottir et al. 2013. Candidate negative control chemicals (n=2) with no anticipated affect on cell phenotype were included in the reference set.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Chemical Use</th>
<th>Expected Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amperozide</td>
<td>Atypical antipsychotic</td>
<td>Toroid nuclei</td>
</tr>
<tr>
<td>Berberine Chloride</td>
<td>Mitochondria complex I inhibitor</td>
<td>Redistribution of mitochondria</td>
</tr>
<tr>
<td>Ca-074-Me</td>
<td>Cathepsin B inhibitor</td>
<td>Bright, abundant golgi staining</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Chemotherapeutic</td>
<td>Large, flat nucleoli</td>
</tr>
<tr>
<td>Fenbendazole</td>
<td>Anthelmintic</td>
<td>Giant, multi-nucleated cells</td>
</tr>
<tr>
<td>Fluphenazine</td>
<td>Typical antipsychotic</td>
<td>Enhanced golgi staining and some cells with fused nucleoli</td>
</tr>
<tr>
<td>Latrunculin B</td>
<td>Actin cytoskeleton disruptor</td>
<td>Actin breaks</td>
</tr>
<tr>
<td>Metoclopramide</td>
<td>D₂ dopamine receptor antagonist</td>
<td>Enhanced golgi staining and some cells with fused nucleoli</td>
</tr>
<tr>
<td>NPPD</td>
<td>Chloride channel blocker</td>
<td>Redistribution of ER to one side of the nucleus</td>
</tr>
<tr>
<td>Oxibendazole</td>
<td>Anthelmintic</td>
<td>Large, multi-nucleated cells with fused nucleoli</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Macrolide antibiotic / antifungal</td>
<td>Reduced nucleolar size</td>
</tr>
<tr>
<td>β-dihydrorotenone</td>
<td>Mitochondria complex I inhibitor</td>
<td>Mitochondrial stressor</td>
</tr>
<tr>
<td>Saccharin</td>
<td>Artificial Sweetener</td>
<td>Negative Control</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>Artificial Sweetener</td>
<td>Negative Control</td>
</tr>
<tr>
<td>Taxol</td>
<td>Microtubule Stabilizer</td>
<td>Large, multi-nucleated cells with fused nucleoli</td>
</tr>
<tr>
<td>Tetrandrine</td>
<td>Calcium channel blocker</td>
<td>Abundant ER</td>
</tr>
</tbody>
</table>

\*β-dihydrorotenone not commercially available. Tested rotenone instead
Reference Chemical Phenotypes (1)

A

- **DNA Mitochondria**
  - Solvent control (0.5% DMSO)
  - Berberine chloride (10 μM)

- **DNA AGP**
  - Solvent control (0.5% DMSO)
  - Ca-074-Me (1 μM)

- **DNA RNA/AGP**
  - Solvent control (0.5% DMSO)
  - Etoposide (3 μM)

- **RNA/ER**
  - Solvent control (0.5% DMSO)
  - Rapamycin (100 μM)
Reference Chemical Phenotypes (2)
Reference Chemical Phenotypes (3)
## U-2 OS APCRA Screen Experimental Design

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Multiplier</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Type(s)</td>
<td>1</td>
<td>U-2 OS</td>
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<tr>
<td>Culture Condition</td>
<td>1</td>
<td>DMEM + 10% HI-FBS</td>
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<tr>
<td>Chemicals</td>
<td>462</td>
<td>APCRA Case Study Chemicals + Duplicates Unilever CRADA Consensus Chemicals HTTr Pilot Chemicals</td>
</tr>
<tr>
<td>Time Points:</td>
<td>1</td>
<td>24 hours</td>
</tr>
</tbody>
</table>
| Assay Formats:       | 2          | Cell Painting
                      Cell Viability                                                   |
| Concentrations:      | 8          | 3.5 log\(_{10}\) units; semi log\(_{10}\) spacing                   |
| Biological Replicates: | 4     | Independent cultures                                                 |

- International collaboration of regulatory scientists focused on developing case studies for evaluating the use of New Approach Methodologies (NAMs) in chemical risk assessment.

- ECHA Workshop (2017) case study focuses on **deriving quantitative estimates of risk based on NAM-derived potency information and computational exposure estimates**
<table>
<thead>
<tr>
<th>Label Reference Chemicals:</th>
<th>Phenotypic Observations</th>
<th>Test Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Berberine Chloride</td>
<td>Specific mitochondrial effects</td>
<td>0.03 – 10 uM</td>
</tr>
<tr>
<td>B Etoposide</td>
<td>Cell hypertrophy control that produces effects in every channel / organelle</td>
<td>0.03 - 10 uM</td>
</tr>
<tr>
<td>C Ca-074-Me</td>
<td>Effects on AGP channel at sub-cytotoxic doses</td>
<td>0.03 - 10 uM</td>
</tr>
<tr>
<td>D Rapamycin</td>
<td>Effects on RNA and DNA channels</td>
<td>0.03 - 10 uM</td>
</tr>
<tr>
<td>STS Staurosporine</td>
<td>Cytotoxicity Control</td>
<td>0.01 - 3 uM</td>
</tr>
</tbody>
</table>

**U-2 OS APCRA Screen: Dose Plate Design**

<table>
<thead>
<tr>
<th>Dose Plate ID</th>
<th>c2018-10-14</th>
<th>c2018-10-15</th>
<th>c2018-10-16</th>
<th>c2018-10-17</th>
<th>c2018-12-11</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CP</td>
<td>CV</td>
<td>CP</td>
<td>CV</td>
<td>CP</td>
</tr>
<tr>
<td>LAB-000005</td>
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<td>TC00000201</td>
<td>TC00000213</td>
<td>TC00000225</td>
<td>TC00000237</td>
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<td>TC00000214</td>
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<td>TC00000238</td>
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<td>LAB-000007</td>
<td>TC00000191</td>
<td>TC00000203</td>
<td>TC00000215</td>
<td>TC00000227</td>
<td>TC00000239</td>
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<tr>
<td>LAB-000008</td>
<td>TC00000192</td>
<td>TC00000204</td>
<td>TC00000216</td>
<td>TC00000228</td>
<td>TC00000240</td>
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<tr>
<td>LAB-000009</td>
<td>TC00000193</td>
<td>TC00000205</td>
<td>TC00000217</td>
<td>TC00000229</td>
<td>TC00000241</td>
</tr>
<tr>
<td>LAB-000010</td>
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<td>TC00000206</td>
<td>TC00000218</td>
<td>TC00000230</td>
<td>TC00000242</td>
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<tr>
<td>LAB-000011</td>
<td>TC00000195</td>
<td>TC00000207</td>
<td>TC00000219</td>
<td>TC00000231</td>
<td>TC00000243</td>
</tr>
<tr>
<td>LAB-000012</td>
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<td>TC00000208</td>
<td>TC00000220</td>
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<td>TC00000221</td>
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<td>TC00000245</td>
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<tr>
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<td>TC00000222</td>
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<td>TC00000212</td>
<td>TC00000224</td>
<td>TC00000236</td>
<td>TC00000248</td>
</tr>
</tbody>
</table>

- Plates in gray failed QC and had to be re-run

- Plates in gray failed QC and had to be re-run
U-2 OS APCRA Screen: Assay Reproducibility
U-2 OS APCRA Screen: Results
In Vitro-in-In Vivo Extrapolation (IVIVE) & Bioactivity Exposure Ratios (BER)

High-throughput toxicokinetic (httk) modeling: Conversion of *in vitro* bioactivity to *in vivo* steady state concentration ($C_{ss}$)

**Reverse dosimetry:** Conversion of predicted $C_{ss}$ to an administered equivalent dose (AED)

**EPA - ExpoCast**

- **HTPP POD** ($\mu$M)
  - Apply high-throughput toxicokinetics (httk) to get mg/kg/day

- **httk v1.8**
- **Single compartment**
- **Steady-state**
- **100% bioavailability**
- **Restrictive clearance**

**Figure courtesy of Katie Paul-Friedman & John Wambaugh**
Ber results show a trend that chemicals with small BER ratio would be of higher priority than chemicals with a large BER ratio.
# Read Across Pilot, Experimental Design

<table>
<thead>
<tr>
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<td>U-2 OS</td>
</tr>
<tr>
<td>Culture Condition</td>
<td>1</td>
<td>DMEM + 10% HI-FBS</td>
</tr>
<tr>
<td>Chemicals</td>
<td>120</td>
<td>Pharmacological Tool Compounds Model Toxicants Structure Series</td>
</tr>
<tr>
<td>Time Points:</td>
<td>1</td>
<td>24 hours</td>
</tr>
<tr>
<td>Assay Formats:</td>
<td>2</td>
<td>Cell Painting Cell Viability</td>
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<td>8</td>
<td>3.5 log(<em>{10}) units; semi log(</em>{10}) spacing</td>
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<td>Biological Replicates:</td>
<td>4</td>
<td>Independent cultures</td>
</tr>
</tbody>
</table>

- **Actin cytoskeleton modulators**
  - DNA toxicants: alkylators
  - DNA toxicants: topoisomerase
  - DNA toxicants: antimetabolites
  - DNA toxicants: genotoxic
  - Oxidative stress
  - Proteosome inhibitors
  - Oxidative phosphorylation uncoupler

- **Actin stabilizers**
  - DNA toxicants: topoisomerase
  - DNA toxicants: antimetabolites

- **ER modulator**
  - DNA toxicants: topoisomerase
  - DNA toxicants: antimetabolites

- **Golgi modulator**
  - DNA toxicants: topoisomerase
  - DNA toxicants: antimetabolites

- **Mitochondrial fission**
  - Oxidative stress
  - Proteosome inhibitors

- **Microtubule modulator**
  - Oxidative phosphorylation uncoupler

- **Microtubule stabilizer**
  - Oxidative phosphorylation uncoupler
  - Ca-074-Me analogues

- **DNA toxicants: alkylators**
  - Mito. Respiratory complex inhibitor
  - Autophagy inhibitor

- **DNA toxicants: topoisomerase**
  - Mito. Respiratory complex inhibitor
  - Autophagy inhibitor

- **DNA toxicants: antimetabolites**
  - RNA polymerase inhibitor
  - Benzimidazole structure series

- **DNA toxicants: genotoxic**
  - RNA polymerase inhibitor
  - Benzimidazole structure series

- **Oxidative stress**
  - RNA polymerase inhibitor
  - Benzimidazole structure series

- **Proteosome inhibitors**
  - RNA polymerase inhibitor
  - Benzimidazole structure series

- **Oxidative phosphorylation uncoupler**
  - RNA polymerase inhibitor
  - Benzimidazole structure series

- **Mito. Respiratory complex inhibitor**
  - RNA polymerase inhibitor
  - Benzimidazole structure series

- **Autophagy inhibitor**
  - RNA polymerase inhibitor
  - Benzimidazole structure series

- **Autophagy activator**
  - RNA polymerase inhibitor
  - Benzimidazole structure series

- **RNA polymerase inhibitor**
  - Benzimidazole structure series

- **Benzimidazole structure series**
  - Benzimidazole structure series

- **Rapamycin analogues**
  - Benzimidazole structure series

- **Ca-074-Me analogues**
  - Benzimidazole structure series
Profile: Feature level response magnitudes
Signature: Response threshold @ 1.5
Similarity Metric: Pearson Correlation
Comparisons: All test chemicals against each other
“Hit” Criteria: Correlation > 0.5

Etoposide | DTXSID5023035
Teniposide | DTXSID8023638

Read Across Pilot, Results (2)
Read Across Example (1)

Query:

Matches:

Endosulfan 115-29-7 | DTXSID1020560
Endrin 72-20-8 | DTXSID6020561
Aldrin 309-00-2 | DTXSID8020040
Heptachlor 76-44-8 | DTXSID3020679
Fluthiacet-methyl 117337-19-6 | DTXSID2032556
Dieldrin 60-57-1 | DTXSID9020453

0.71
0.70
0.59
0.61
0.59

0.70
0.59
Organochlorine Pesticides

- Changes in nuclear texture manifest as “holes”.

DMSO

Aldrin (30 µM)

Dieldrin (30 µM)

Endosulfan (30 µM)

Heptachlor (30 µM)
Query:

Matches:

- Benfluralin 1861-40-1 | DTXSID3023899 (0.75)
- Pendimethalin 40487-42-1 | DTXSID7024245 (0.75)
- Butralin 33629-47-9 | DTXSID3032337 (0.68)
- Tefluthrin 79538-32-2 | DTXSID5032577 (0.70)
- Pyridaben 96489-71-3 | DTXSID5032573 (0.69)
- Fenvalerate 51630-58-1 | DTXSID101017940 (0.68)
Summary

- **Workflow:** We have established the Cell Painting assay and developed computational workflows for phenotypic feature extraction, data normalization, concentration-response modeling and generation of phenotypic response profiles.

- **Reproducibility:** Demonstrated reproducibility of potency estimates and phenotypic profiles in the Cell Painting assay using phenotypic reference chemicals.

- **Chemical Screening:** Performed concentration-response screening of a set of environmental chemicals (APCRA) and a set of pharmacological chemicals and model toxicants (RefChem120).

- **Bioactivity Exposure Ratio (BER):** HTPP data may be used in combination with IVIVE and ExpoCast estimates to identify chemicals with bioactivity thresholds in human relevant exposure ranges.

- **Chemical Read Across:** Vector-based similarity approaches were able to identify structurally-related chemicals with similar response profiles.
Acknowledgments

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Derik Haggard
Katie Paul-Friedman
Rusty Thomas

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Scott Auerbach