Using high-throughput phenotypic profiling to screen chemicals in a rainbow trout gill cell line (RTgill-W1)

Felix R Harris[1,2], Jo Nyffeler[1,3,4], Steven Lasee[1,3], Brett R Blackwell[1], Daniel L Villeneuve[1], Joshua Harrill[1]

[1]Center for Computational Toxicology and Exposure, U.S. Environmental Protection Agency, Durham, USA; [2]Oak Ridge Associated Universities contractor at U.S. Environmental Protection Agency; [3]Oak Ridge Institute for Science and Education grantee at U.S. Environmental Protection Agency; [4]Department of Cell Toxicology, Helmholtz Centre for Environmental Research, Leipzig, Germany

5170/P271

Felix Harris | Harris.Felix@epa.gov
ORCID 0000-0002-0256-7452

United States
Environmental Protection
Agency

THE RTGILL-W1 CELL LINE

- RTgill-W1 cells have a history of use for toxicity testing.
- In 2021, the Organisation for Economic Cooperation and Development released Test Guideline 249 (TG 249), describing a plate-reader based method for assessing acute toxicity in RTgill-W1 cells in 24-well plate format .
- We have miniaturized TG 249 to run in 384-well plate format, and conducted this assay (CV-PR) in tandem with an imaging-based cell viability assay (CV-IB) and Cell Painting assay (CP)

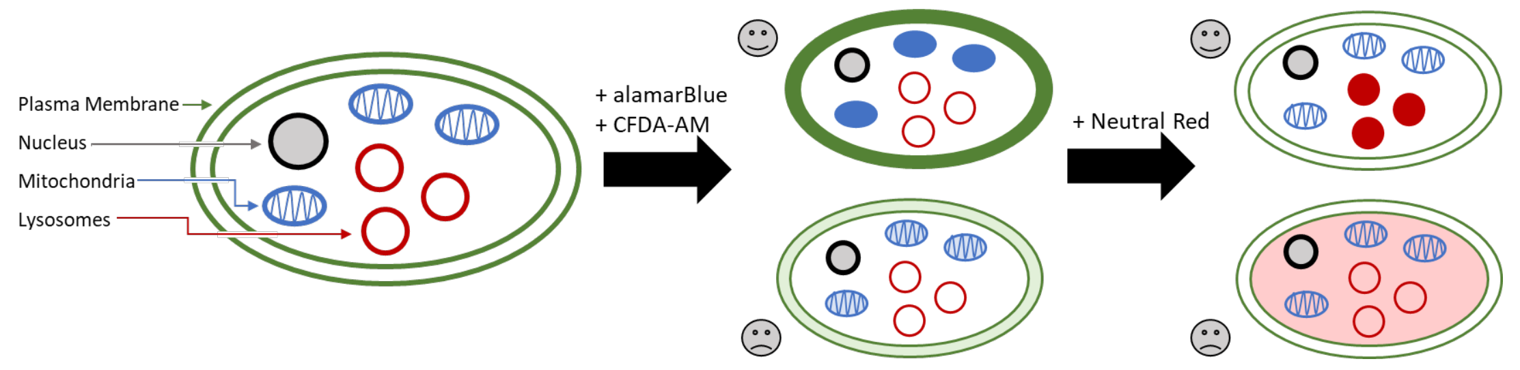


Figure 1. Graphical depiction of the three assay endpoints described in TG 249. Acute toxicity is measured by applying alamarBlue to measure metabolic activity, CFDA-AM to measure membrane integrity, and Neutral Red to measure lysosomal membrane integrity. For all three chemicals, increased fluorescence is associated with high viability (top, smiling cells), and low fluorescence is associated with cytotoxic conditions (bottom, frowning cells).

WHAT IS HIGH THROUGHPUT PHENOTYPIC PROFILING?

- Cell Painting (CP) is an assay within high throughput phenotypic profiling where cellular organelles are labeled with fluorescent probes and imaged. Cell morphology is measured quantitatively using high-content image analysis.
- Features analyzed (~1300 total) include measurements such as intensity, texture, localization, and shape.
- CP is used to screen chemicals in concentration/response format.
- Mahalanobis distances are calculated to reduce dimensionality across all features, as well as by feature categories defined by cellular location, organelle/channel, and measurement.
- Generalized cytotoxicity is excluded from analysis using a propidium iodide cell viability assay (CV-IB)
- Phenotypic profiles are generated by comparing CP data from treated cells to controls and used to compare the bioactivity of chemicals (Nyffeler et al 2020).

Table 1. Organelles targeted by cell painting, the fluorescent probes used for each organelle, and the corresponding channel output.

Targeted Organelle	Stain	Channel
Nucleus	Hoechst 33342	DNA
Nucleoli + RNA	SYTO14	RNA/ER
Endoplasmic reticulum	Concanavalin A/Alexa Fluor 488 conjugate	RNA/ER
Actin skeleton	Alexa Fluor 568 Phalloidin	AGP
Golgi body + plasma membrane	Wheat Germ Agglutinin/Alexa Fluor 555 conjugate	AGP
Mitochondria	MitoTrackerDeepRed	Mito

SCREEN DESIGN

- 237 chemicals were selected for screening:
  - 129 with *in vivo* rainbow trout data
  - 69 with *in vitro* rainbow trout data
  - 29 detected in the water of the North American Great Lakes
  - 110 tested previously with HTPP at EPA in human U-2 OS osteosarcoma cells
- Each chemical was tested at 8 concentrations (½ $\log_{10}$ spacing)
- Four technical replicates of each assay (CP, CV-IB, and CV-PR)
- Passage 8 cells are grown on a 7-day cell culture cycle, plated at passage 10
- Plating media is changed to serum-free minimal media as recommended in TG 249 before dosing

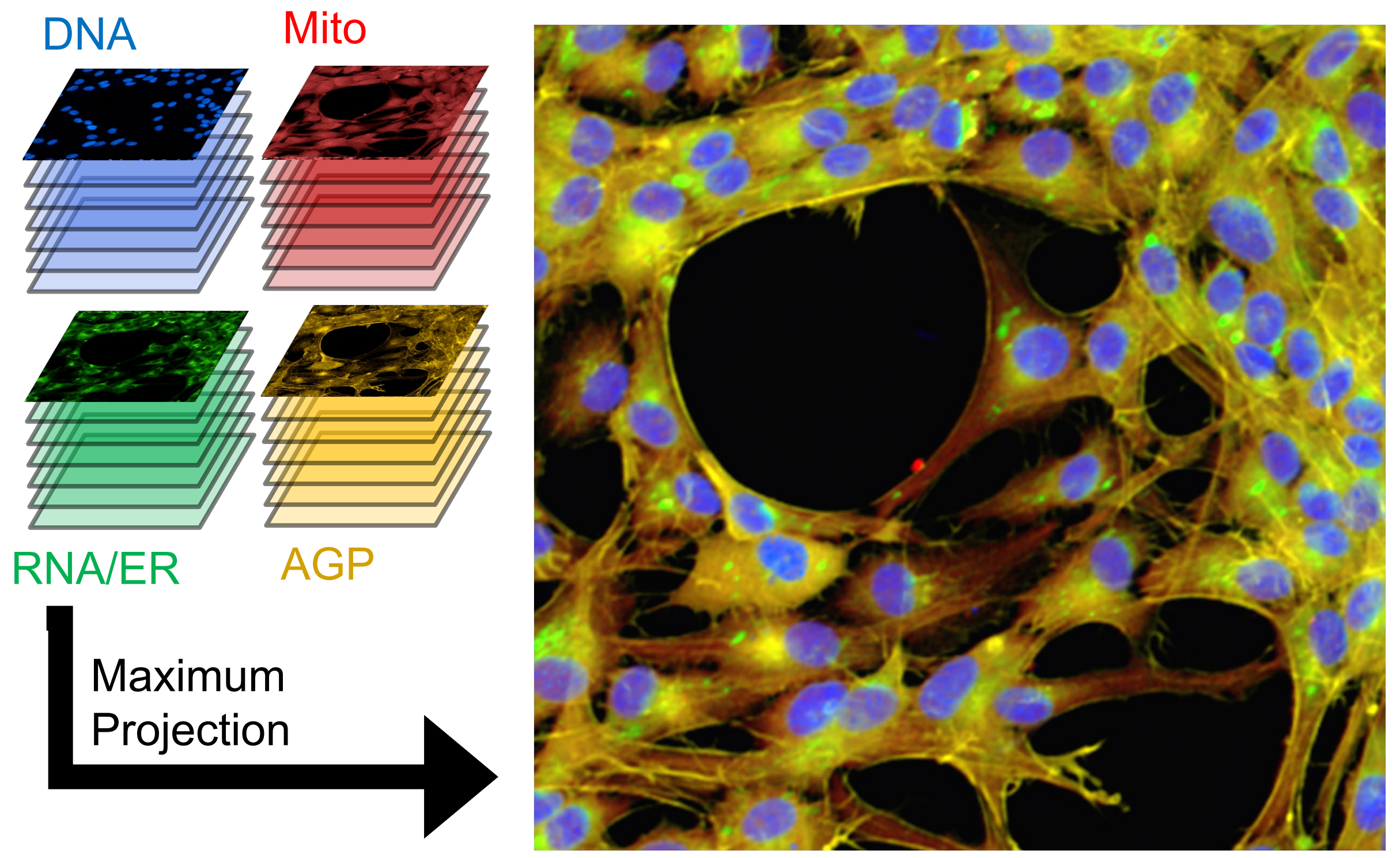


Figure 2. RTgill-W1 cells are imaged across 7 optical planes in each well, which is then flattened across each channel forming a maximum projection for analysis. The right is a composite image is created in R by converting fluorescence intensity in each channel to different colors and combining each channel.

SCREEN RESULTS

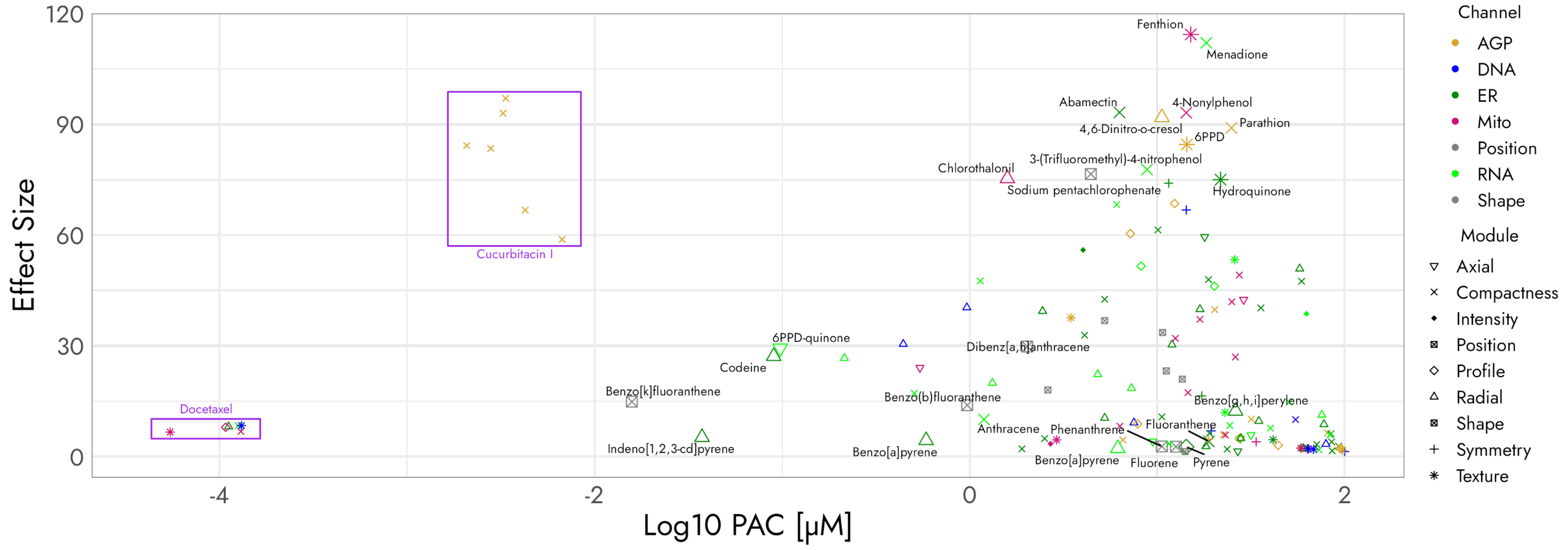
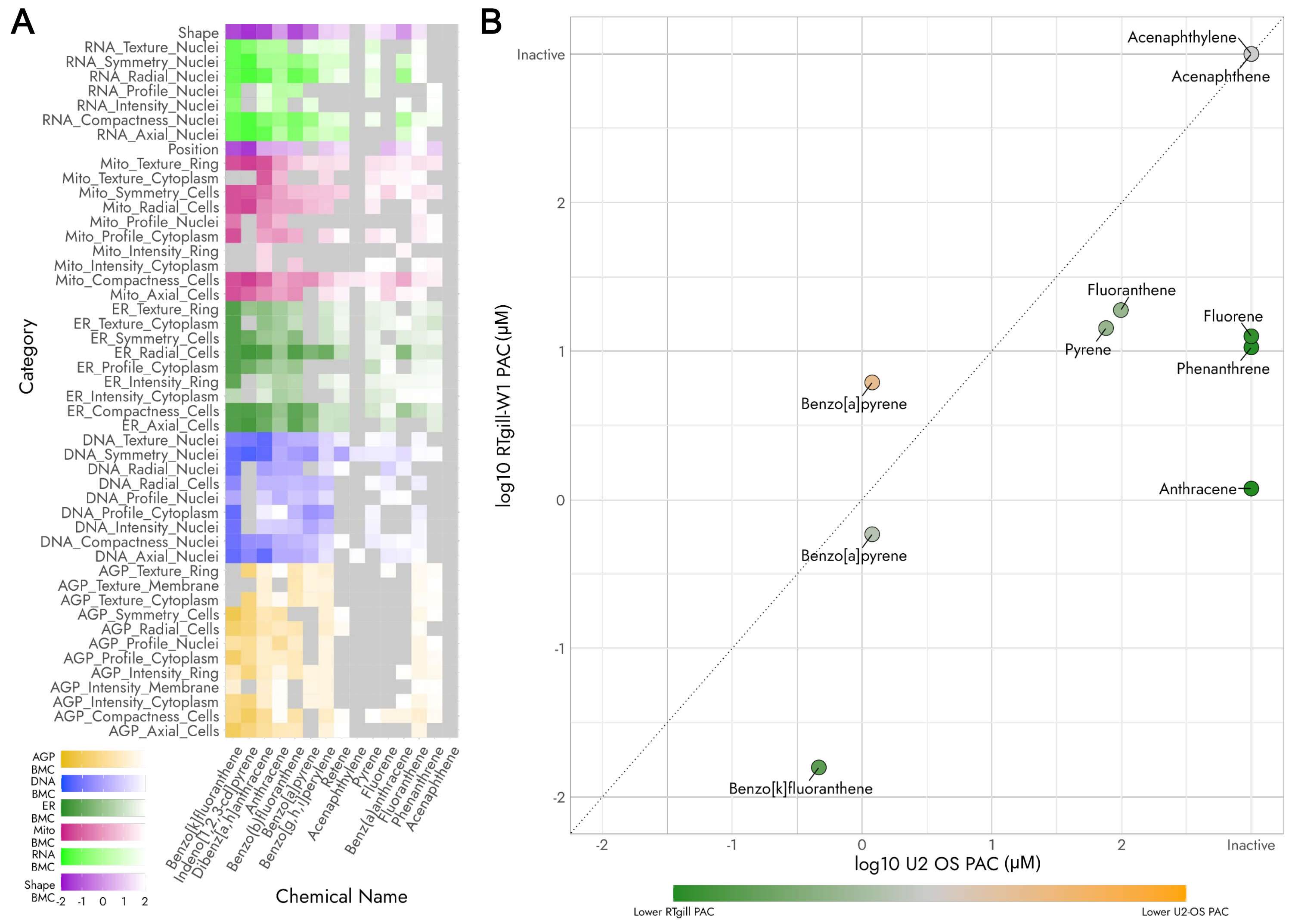


Figure 3, left. Screening results for all active chemicals. Color and shape are coded to represent the most sensitive category for each chemical. Technical replicates for reference chemicals Docetaxel and Cucurbitacin I are bounded within purple boxes. Labeled points fall into one or more of the following categories: the top 15 lowest BMC values, the top 15 highest responses, polycyclic aromatic hydrocarbons (PAHs), and 6PPD along with 6PPD-quinone. For clarity, the size of labeled points has been increased.



- Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous organic compounds found in fuel which are released into the environment upon combustion.
- Many of the lowest BMC values produced in this study belonged to PAHs, such as Benzo[k]fluoranthene and Indeno[1,2,3-cd]pyrene.
- 9 of the 15 PAHs screened in this study have previously been screened using Cell Painting in human U-2 OS osteosarcoma cells
- 3 of the 5 PAHs which were in active in U-2 OS cells were active in RTgill-W1 cells.
- Except for one technical replicate of Benzo[a]pyrene, RTgill-W1 cells were more or just as sensitive to PAHs as U-2 OS cells.

Figure 4, left. A: Category-level results for all polycyclic aromatic hydrocarbons (PAHs) tested in this screen, colored by channel. Vibrancy of the colors correspond to lower benchmark concentration (BMC) values, NA values are represented in gray. B: Comparison of phenotype altering concentrations (PACs) for all PAHs tested in both RTgill-W1 and U-2 OS cells with Cell Painting.

SCREEN RESULTS CONT.

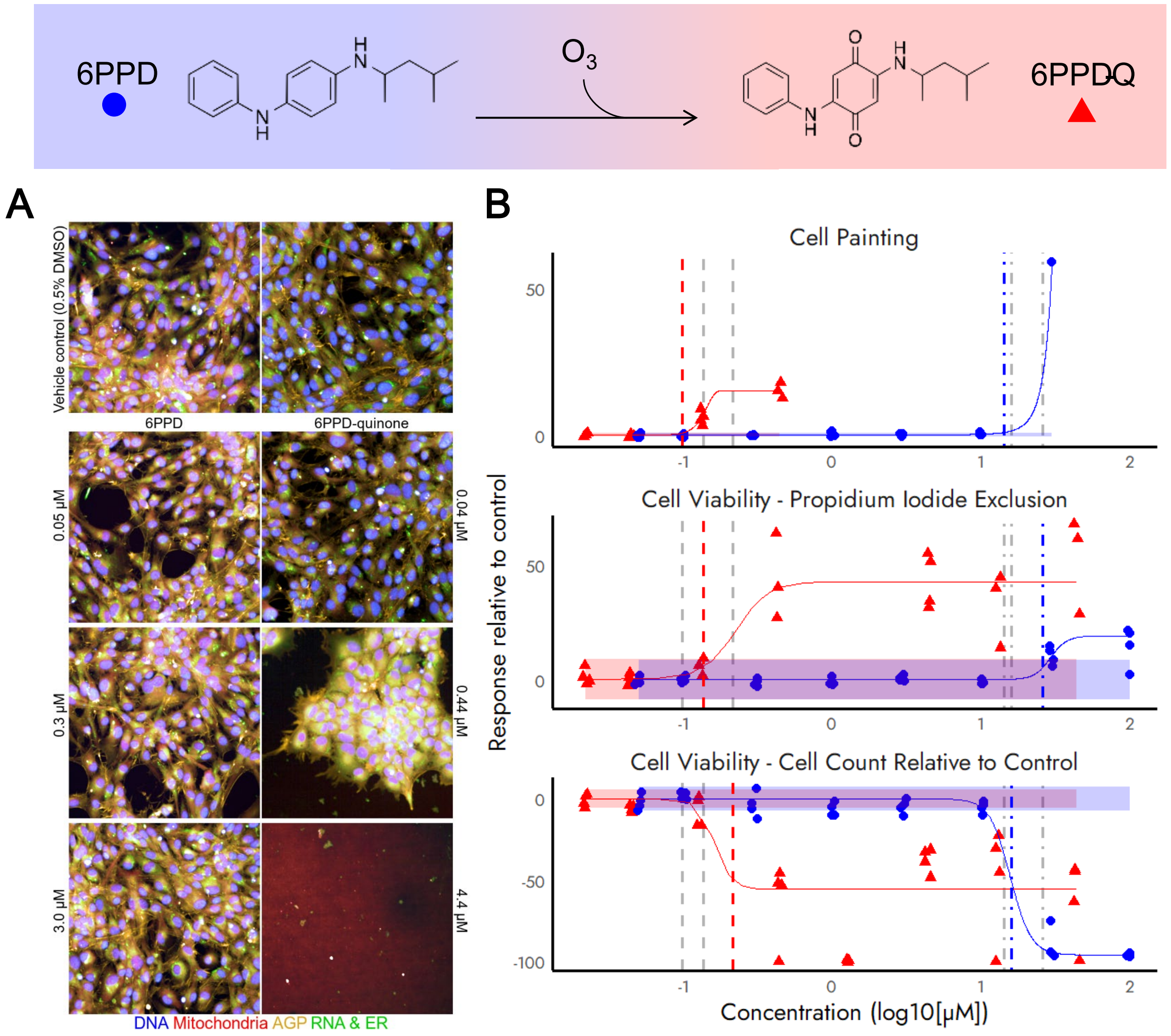


Figure 5. Screening results for 6PPD and 6PPD-quinone (6PPD-Q). A: Representative composite images at comparable concentrations of 6PPD (left) and 6PPD-Q (right), compared to vehicle control (top). B: Concentration/response curves for global Cell Painting and CV-IB data. The mechanism of oxidation from 6PPD to 6PPD-Q is displayed in the graph key for clarity (top). Vertical lines represent benchmark concentration (BMC) values for each endpoint: 6PPD has dot-dashed lines and 6PPD-Q has dashed lines. All three endpoints are shown in each facet for comparison, but the BMC value corresponding to each endpoint is highlighted in red or blue on the respective graphs.

- Out of all chemicals tested in this screen, 6PPD-quinone has one of the lowest BMC values.
- 6PPD is an antiozonant added to rubber in tires which is oxidized to 6PPD-quinone
- 6PPD-quinone in motorway runoff has been implicated as a causative agent for coho salmon die-offs in the urban streams and presumed to impact populations in Puget Sound (Tian et al 2022).
- In this screen, 6PPD-quinone was approximately 2 orders of magnitude more sensitive than 6PPD in both the Cell Painting and CV assays.

REFERENCES

Nyffeler J, Willis C, Lougee R, Richard A, Paul-Friedman K, Harrill JA, 2020. Bioactivity screening of environmental chemicals using imaging-based high-throughput phenotypic profiling. Toxicol Appl Pharm. 389:114876. doi:10.1016/j.taap.2019.114876

OECD, 2021. Test No. 249: Fish Cell Line Acute Toxicity - The RTgill-W1 cell line assay, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris. doi:10.1787/c66d5190-en.

Tian Z, Gonzalez M, Rideout CA, Zhao HN, Hu X, Wetzel J, Mudrock E, James CA, McIntyre JK, Koloziej EP, 2022. 6PPD-Quinone: Revised Toxicity Assessment and Quantification with a Commercial Standard. Environ Sci Technol Lett. 9(2):140-146. doi:10.1021.acs.estlett.1c00910

Changes in RTgill-W1 cell morphology (phenotype) in response to chemicals can be quantified with the Cell Painting assay. Potency ranking demonstrates PAHs and 6PPD-quinone as the most toxic chemicals that were screened.

U.S. Environmental Protection Agency
Office of Research and Development