

Introduction

- Drug-induced liver injury (DILI) remains a critical challenge in pharmaceutical development, emphasizing the need for more comprehensive and physiologically relevant evaluation methods.
- While conventional 2D primary human hepatocyte (PHH) cultures are widely used for high-throughput toxicity screening, they suffer from rapid dedifferentiation and limited physiological relevance. In contrast, 3D PHH spheroid models exhibit a higher degree of physiological relevance to the native liver, maintaining long-term phenotypic stability and enhanced metabolic function. Conventional approaches in both systems often focus on viability endpoints, offering limited insight into cellular morphological perturbations.
- This study explores how the Cell Painting assay can complement established viability assessments in both 2D and 3D PHH models by capturing rich, high-dimensional morphological information.

Methods

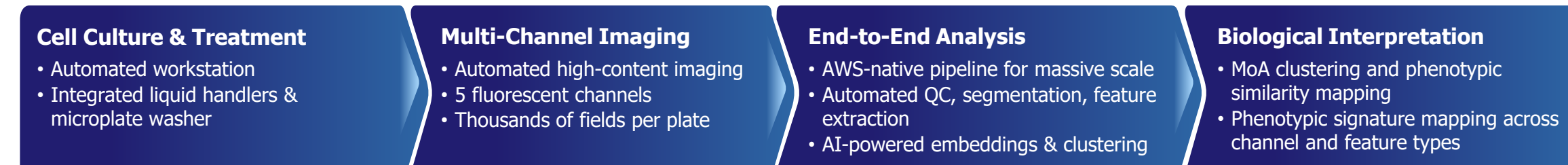
- PHHs were cultured both as conventional 2D monolayers and as self-assembled 3D spheroids. Both models were exposed to a panel of FDA-categorized DILI compounds representing different concern levels, across six concentrations spanning sub-cytotoxic to cytotoxic ranges.
- Cytotoxicity was assessed using the CellTiter-Glo (CTG) assay to determine IC50 values.
- In parallel, the Cell Painting assay was performed using a multiplexed staining panel. High-content images were acquired on an Operetta CLS system, and quantitative morphological features were extracted to construct phenotypic profiles for both culture formats.

Conclusion

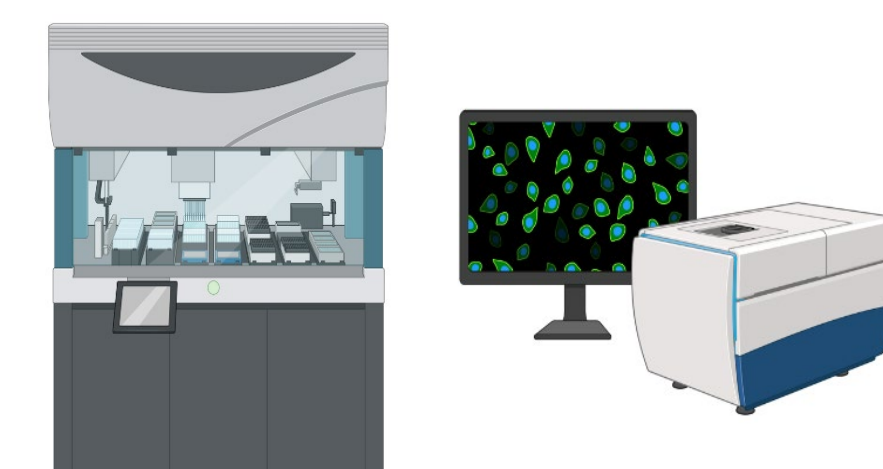
- Cell Painting detects morphological changes that may occur at or below cytotoxic concentrations, complementing viability assays by providing additional biological context and mechanistic insight (e.g., mitochondrial damage).
- Phenotypic profiling in 2D PHHs shows sensitivity for DILI comparable to or exceeding viability readouts (e.g., tolcapone, pioglitazone), supporting its value as an early and informative toxicity screen.
- Integration of Cell Painting with viability data enables a multi-dimensional assessment. The 2D vs. 3D comparison highlights the importance of physiologically relevant models for capturing complex toxicological responses.

1

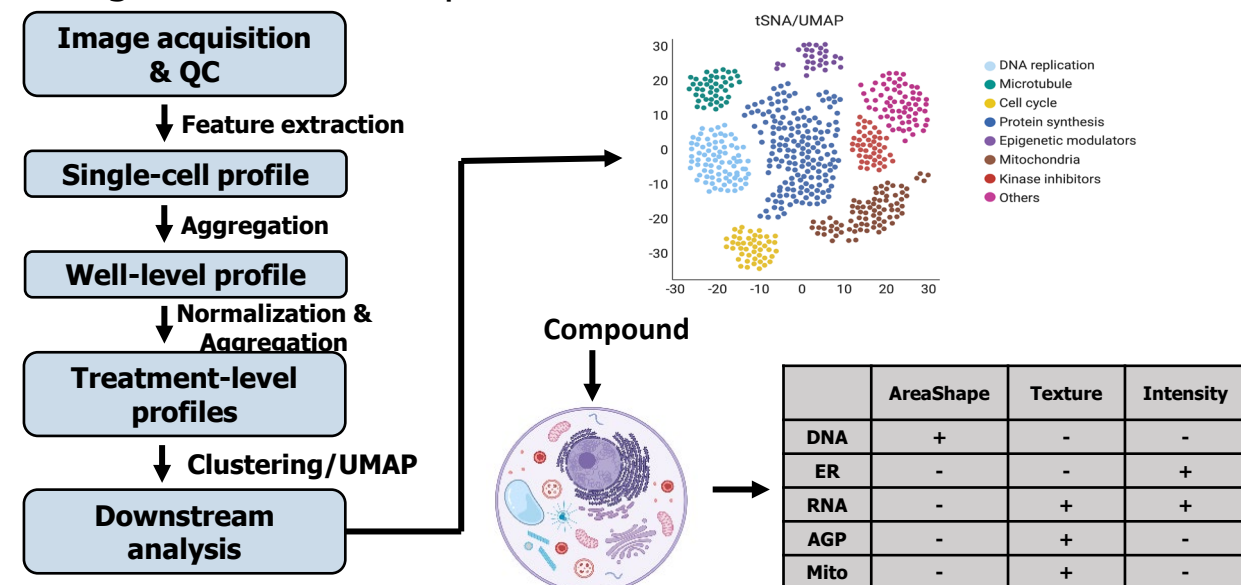
Workflow of Cell Painting Experiment



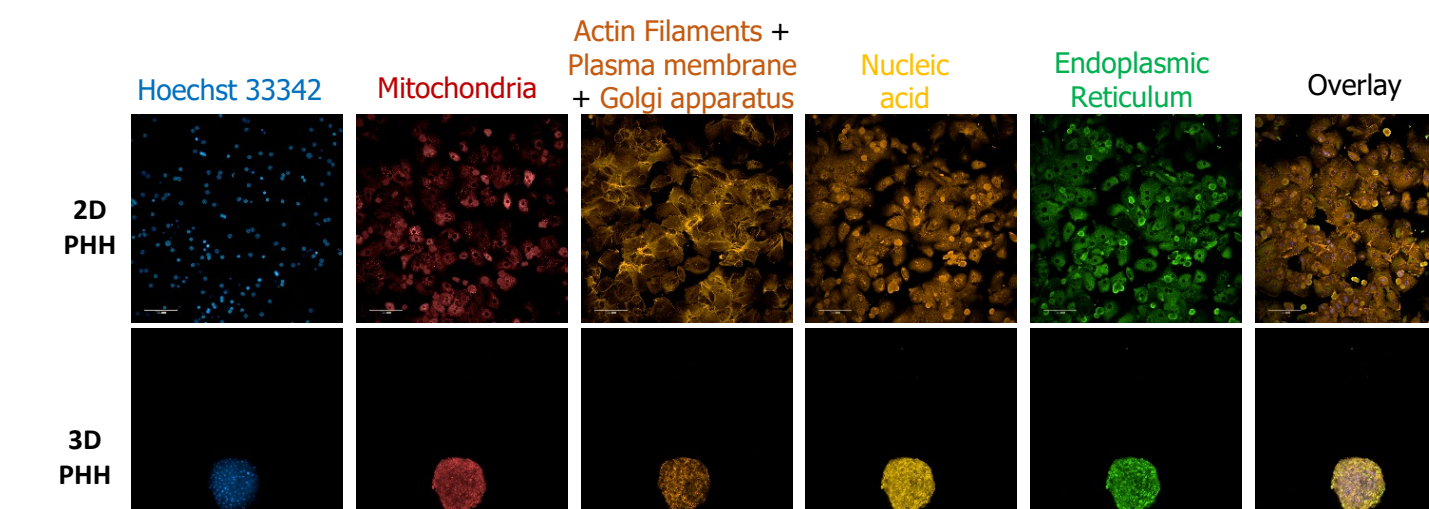
Wet-lab: Fully automated high-throughput cell preparation, staining, and imaging with reproducible hands-free operation



Data analysis: Automated high-throughput pipeline from raw images to multi-level profiles



Target	Dye
Nuclear	PhenoVue Hoechst33342
Mitochondrial	PhenoVue 641 Mitochondrial stain
Actin Filaments	PhenoVue Fluor 568 Phalloidin
Plasma membrane + Golgi apparatus	PhenoVue Fluor 555 WGA
Nucleic acid	PhenoVue 512 nucleic acid stain
Endoplasmic Reticulum	PhenoVue 488 Concanavalin A



Upper panel: General workflow for cell painting assay.

Bottom panel: Summary of the fluorescent dyes used in the cell painting assay and representative images of vehicle-treated 2D/3D PHHs. The table specifies each dye, its main cellular target, and the corresponding imaging channel used on the Operetta CLS high-content imaging system. The accompanying image panel shows the distinct subcellular structures visualized under basal conditions, reflecting the fluorescent channels employed for phenotypic profiling.

2

3D PHHs Outperform 2D PHHs in DILI Sensitivity Using CellTiter-Glo Assay

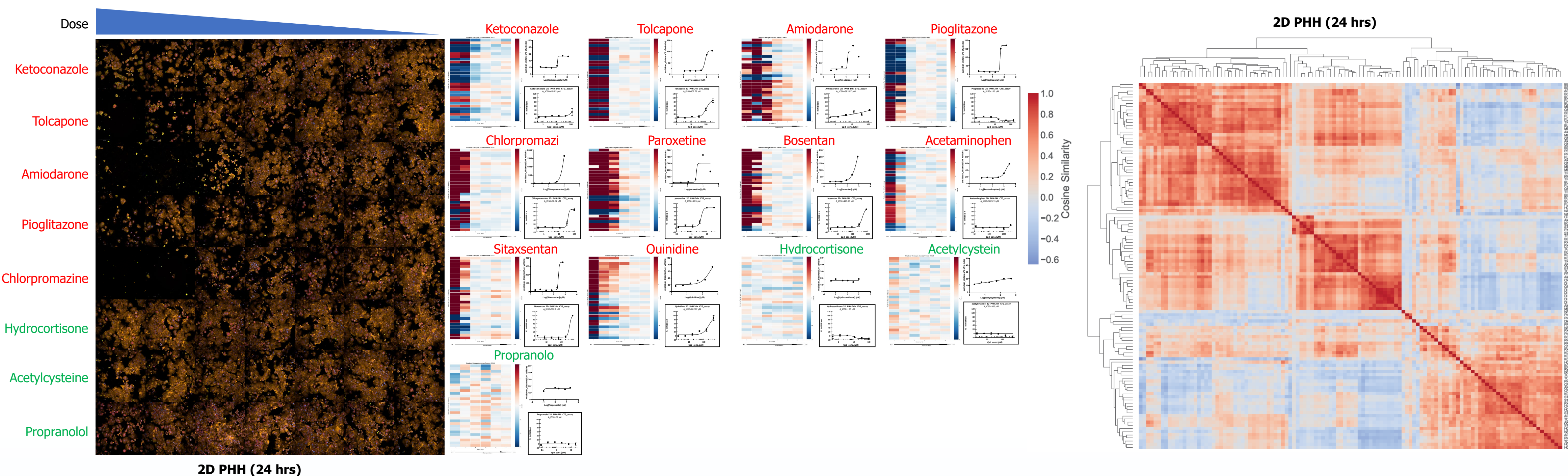
No.	Compound ID	Cmax (μM)	DILI Positive/ Negative ¹	vDILIConcern ²	Severity Class ²	2D PHH (24 hrs)			Prediction Outcome (MOS=50*) ²	3D PHH (7 days)			Prediction Outcome (MOS=50*) ²	Sensitivity in 3D vs 2D
						A_IC50(μM)	Max % inh	IC50/Cmax		A_IC50(μM)	Max % inh	IC50/Cmax		
1	Amlodaron	5	POS	vMost-DILI-Concern	8	382.97	40.9	76.59	FN	8.51	92.16	1.70	TP	3D>2D
2	Ketoconazole	11.29	POS	vMost-DILI-Concern	8	195.2	29.96	17.29	TP	8.88	100.02	0.79	TP	3D>2D
3	Tolcapone	47.58	POS	vMost-DILI-Concern	8	107.79	86.23	2.27	TP	31.78	100.77	0.67	TP	3D>2D
4	Sitaxsentan	30	POS	vMost-DILI-Concern	8	372.7	99.55	12.42	TP	91.6	100	3.05	TP	3D>2D
5	Flutamide	5.43	POS	vMost-DILI-Concern	8	>200	13.04	>36.83	FN	54.16	100.02	9.97	TP	3D>2D
6	Troglitazone	6.4	POS	vMost-DILI-Concern	8	33.88	100.19	5.29	TP	38.06	98.24	5.95	TP	3D=2D
7	Paroxetine	0.2	POS	vLess-DILI-Concern	8	3.99	99.82	19.95	TP	2.26	100.07	11.30	TP	3D=2D
8	Bosentan	7.43	POS	vMost-DILI-Concern	7	423.15	92.22	56.95	FN	40.88	100.09	5.50	TP	3D>2D
9	Acetaminophen	136	POS	vMost-DILI-Concern	5	3629.13	16.25	26.68	TP	1221.99	60.77	8.99	TP	3D>2D
10	Mexiletine	35	POS	vMost-DILI-Concern	3	176.48	93.24	5.04	TP	187.08	97.29	5.35	TP	3D>2D
11	Bepidil	2	POS	Not listed	/	45.77	46.89	22.89	TP	16.19	99.97	8.09	TP	3D>2D
12	Pioglitazone	3.3	POS	vLess-DILI-Concern	3	>150	7.57	>45	FN	>150	31.24	>45	FN	3D=2D
13	Quinidine	8.66	POS	vLess-DILI-Concern	3	202.87	88.52	23.43	TP	79.23	100.02	9.15	TP	3D>2D
14	Chlorpromazine	0.94	POS	vLess-DILI-Concern	2	38.52	92.24	40.98	TP	27.2	100	28.94	TP	3D>2D
15	Bumetanide	0.08	NEG	Ambiguous DILI-concern	3	>20	5.57	>250	TN	>20	24.7	>250	TN	3D=2D
16	Propranolol	0.2	NEG	Ambiguous DILI-concern	3	>20	8.57	>100	TN	>20	26.56	>100	TN	3D=2D
17	Hydrocortisone	0.84	NEG	vNo-DILI-Concern	0	>100	3	>119	TN	>100	-1.47	>119	TN	3D=2D
18	Acetylcysteine	2.45	NEG	vNo-DILI-Concern	0	>500	0.45	>204	TN	>500	-21.61	>204	TN	3D=2D
19	Digoxin	0.005	NEG	vNo-DILI-Concern	0	0.12	90.1	24.00	FP	0.15	99.92	30.00	FP	3D=2D
						Sensitivity			71.4%				92.9%	
						Specificity			80.0%				80.0%	

Cell viability was measured by CellTiter-Glo assay in 2D PHHs (24 h) and 3D PHHs (7 days). True positives (TP) and false negatives (FN) are DILI-positive drugs correctly or incorrectly predicted as hepatotoxic. True negatives (TN) and false positives (FP) are DILI-negative drugs correctly or incorrectly predicted as nonhepatotoxic. *MOS: Margin of safety, IC50/Cmax ratio=50

- The definition of DILI positive or negative is based on the classification in the FDA's Drug-Induced Liver Injury Severity and Toxicity (DILIST) Dataset (<https://www.fda.gov/science-research/liver-toxicity-knowledge-base-ltkb/drug-induced-liver-injury-severity-and-toxicity-dilist-dataset>)
- The level of DILI concern is defined based on the classification in the FDA's Drug Induced Liver Injury Rank (DILIRank 2.0) Dataset (<https://www.fda.gov/science-research/liver-toxicity-knowledge-base-ltkb/drug-induced-liver-injury-rank-dilirank-20-dataset>)

3

Dose-Dependent Morphological Changes and Phenotypic Profile Similarity of DILI-Positive and DILI-Negative Compounds in 2D PHHs



Phenotypic profiling matches or exceeds viability assay in detecting DILI.

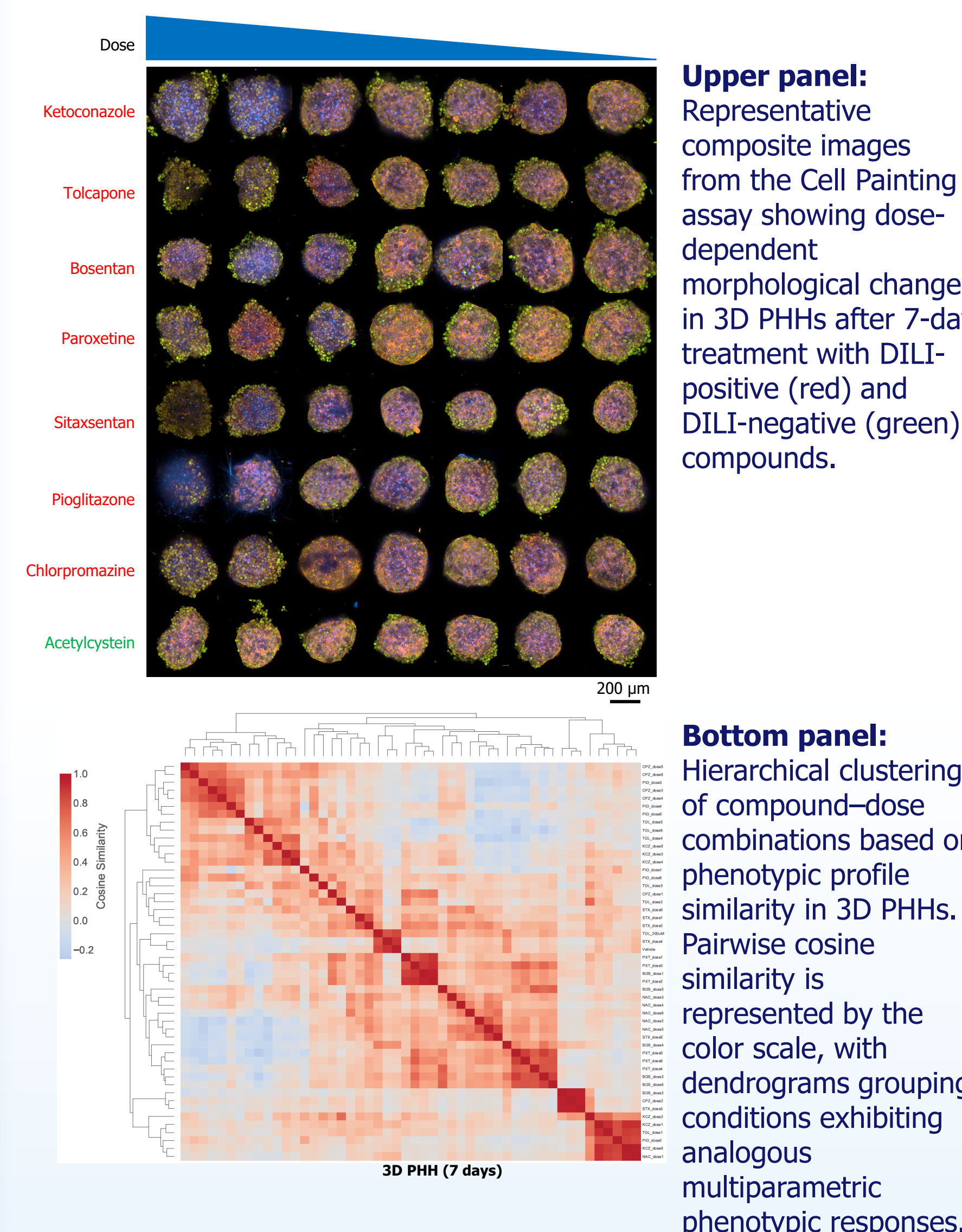
Left panel: Representative composite images from the Cell Painting assay showing dose-dependent morphological changes in 2D PHHs after 24-hour treatment with DILI-positive (red) and DILI-negative (green) compounds.

Middle panel: Heatmap (right) displaying the top 30 most variable phenotypic features across compound concentrations (high to low, left to right), with row-normalized values to visualize relative changes. Corresponding dose-response curves show aggregate phenotypic change (Euclidean distance; upper right) and cell viability (CTG assay; bottom right). In this case, phenotypic changes demonstrated sensitivity for DILI detection comparable to, or exceeding that of, the cell viability assay in 2D PHHs (e.g., tolcapone and pioglitazone).

Right panel: Hierarchical clustering of compound-dose combinations based on similarities in their phenotypic profile. Pairwise cosine similarity is shown by the color scale, with dendrograms grouping conditions with comparable multiparametric responses. DMSO and most non-hepatotoxic drugs (HC, PRO, NAC) clustered together across doses, although a few at the high-dose points grouped with hepatotoxicants, suggesting non-specific effects. Known hepatotoxicants at higher doses separated from controls into a distinct toxic cluster, with lower doses shifting toward the non-toxic cluster, demonstrating dose-dependent responses. Subclustering within the toxic cluster suggests underlying mechanism-based differences.

4

Clustering of Compounds by Phenotypic Profile Similarity in 3D PHHs



Upper panel: Representative composite images from the Cell Painting assay showing dose-dependent morphological changes in 3D PHHs after 7-day treatment with DILI-positive (red) and DILI-negative (green) compounds.

Bottom panel: Hierarchical clustering of compound-dose combinations based on phenotypic profile similarity in 3D PHHs. Pairwise cosine similarity is represented by the color scale, with dendrograms grouping conditions exhibiting analogous multiparametric phenotypic responses.