AI-enabled automated compound screening for toxicity effects using healthy intestinal 3D organoids

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Introduction

The most common side effect of anti-cancer drugs is their toxicity to intestinal cells which often limits the dose that can be administered to treat patients. *In vitro* assays using organoids can evaluate toxicity effects on the intestine and provide valuable information in the process of drug development. Conventional toxicity testing *in vitro* using 2D cell lines may not accurately reflect true clinical effects. Organoids are three-dimensional (3D) multicellular tissue constructs that originate from human pluripotent stem cells (iPSCs) or adult stem cells. They can recreate the physiological structure and function of human organs through self-assembly in matrix. Studies show that patients and their derived organoids respond similarly to drugs, highlighting the potential benefits of utilizing organoids in screening to improve therapeutic outcomes. Combining assay automation with high content imaging and advanced analysis using artificial intelligence (AI) will greatly increase productivity and scale the use of these models, as well as the accuracy of assays that involve complex 3D biology.

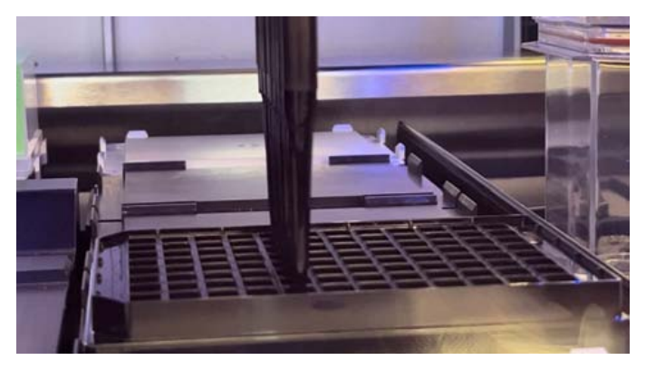
We demonstrated automation of compound testing assay for toxicity assessment using human and mouse 3D intestinal organoids. Intestinal organoids were cultured, passaged, and expanded in Matrigel domes in 24 wells using the CellXpress.ai™ Automated Cell Culture System. Media exchanges and transmitted light (TL) imaging were automatically performed every 24 hours during organoid culture. For compound screening, organoids were automatically plated into 96 multi-well plate format and treated with seven concentrations of eight compounds known to cause toxicity to intestines.

For evaluation of toxicity effects by imaging and analysis of phenotypic changes in 3D organoids, we used the ImageXpress® HSC.ai High Content Screening System (Molecular Devices). This next generation platform has confocal optics and delivers enhanced speed, robust autofocus, and an optimized optical path—enabling higher throughput and enhanced signal-to-noise ratio. After treatment with compounds, organoids were stained with a mix of dyes for nuclei, mitochondria, and cytoskeleton integrity markers. Then organoid images were taken with a confocal option using 10X magnification.

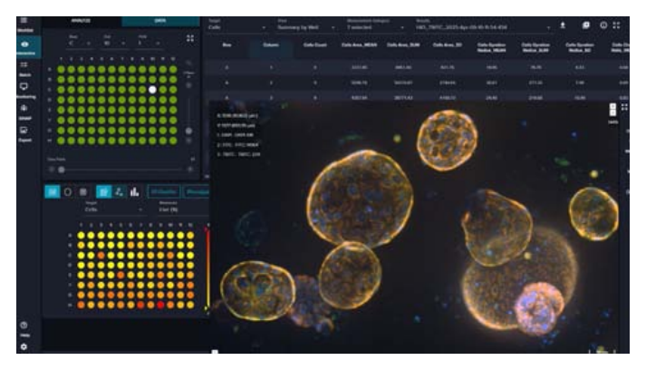
Images were analyzed using machine-learning enabled IN Carta® Image Analysis Software. Organoid objects were first identified, individual cells resolved using nuclear staining, then concentration-dependent phenotypic changes in organoids and individual cells were quantified for different markers through multi-parametric feature extraction. These features were used in a machine-learning classifier to distinguish various organoid phenotypes. First, un-supervised machine-learning identified multiple classes automatically, then supervised training was applied to distinguish between affected from unaffected organoids. This method is suitable for automating toxicity assessment studies, significantly reducing manual cell processing while enhancing productivity and assay scalability. AI-powered data analysis allows for the automation of complex analysis steps, enabling reproducible and efficient compound testing.

Instruments


CellXpress.ai Automated Cell Culture System


Seeding organoids with the CellXpress.ai system


ImageXpress HCS.ai High Content Screening System


IN Carta Image Analysis Software

Methods

Organoid culture. Primary Mouse Intestinal organoids (StemCell Technologies), or primary Human Intestinal organoids (Molecular Devices, OES-DP41N2-CXP1) were cultured in Matrigel domes using IntestiCult media according to protocols from StemCell Technologies. During organoid culture, automated media exchanges and monitoring by transmitted light imaging were done every 24h. Organoids self-organized, grow and developed structures as expected for their phenotype. For toxicity evaluation assay organoid domes were seeded into 96-well plates (Ibidi plates or U-bottom Corning), with 50% Matrigel domes, 15 µL per dome. Each dome contained approximately 50 organoids. Organoids were plated using the CellXpress.ai system. Compounds were added to organoids after 48h in culture. Compound treatments were applied using 7-point dilution range, 5X dilutions, starting from 100 µM concentration, except staurosporine that was started from 10 µM concentration. Organoids were cultured with compounds for 3 days. After compound treatments organoids were stained with Hoechst and MitoTracker orange, then fixed with 4% formaldehyde and additionally stained with Alexa488 Phalloidin in the presence of 0.05% of TritonX. All Dyes were from Thermo Scientific.

Imaging organoids. Organoids were imaged using the ImageXpress HCS.ai System with confocal option (60 µm pinhole) in three fluorescent channels DAPI, FITC, TRITC, and 10X magnification. Then, 3x3 sites per well were taken at 10X magnification to cover the entire dome area. Additional images were taken using 4X magnification, in this case, 4 tiled images were used to cover the organoid dome area. Z-stacks of 16 images were taken at 8um interval, covering a z-range of 160 µm. Maximum projection 2D images were used for analysis.

High-content image analysis. Image analysis was completed on 2D maximum projection images using IN Carta software. Within IN Carta software, the Custom Module Editor (CME) tool was used to create a multi-step protocol that defined organoids as "blobs" using the DAPI channel (nuclear stain). Numbers of organoids, average organoid area, and average organoid fluorescent intensities for DAPI (Hoechst stain), FITC (Alexa488 Phalloidin) and TRITC (MitoTracker) were measured. First, a Gaussian filter was used to blur the Hoechst signal to facilitate segmentation of organoids. Next, nuclei were segmented and used to define cells. Cells were scored as positive or negative depending on the signal intensities for Phalloidin (actin cytoskeleton) or MitoTracker (mitochondria). Thresholds for positive and negative cell scoring were set empirically using control (untreated) samples and samples treated with toxic compound. Cells that were scored positive for actin or mitochondria were defined as having an intact cytoskeleton or intact mitochondria, respectively. Positive and negative cells per organoid were counted and average area and average intensity of positive cells was measured. After analysis, concentration dependencies for different readouts were plotted as 4-parametric curve fit (for 0.06–100 µM concentration range) to calculate EC50s for compound toxicity effects. SoftMax® Pro Software was used for the curve fit and calculations of EC50s.

Flexi protocol analysis. For analysis of human intestinal organoids, the SINAP deep-learning segmentation module was used to identify objects and extract features, and the Phenoglyphs object classifier module was used to identify different phenotypic groups. The deep-learning segmentation model was created using an integrated software tool including the use of the SAM (segment anything model) to quickly annotate new datasets. Flexi protocol was used to extract multiple measurements for transmitted light and for three fluorescent channels, extracting measurements for organoid areas, intensities, intensity distribution, texture, and many others.

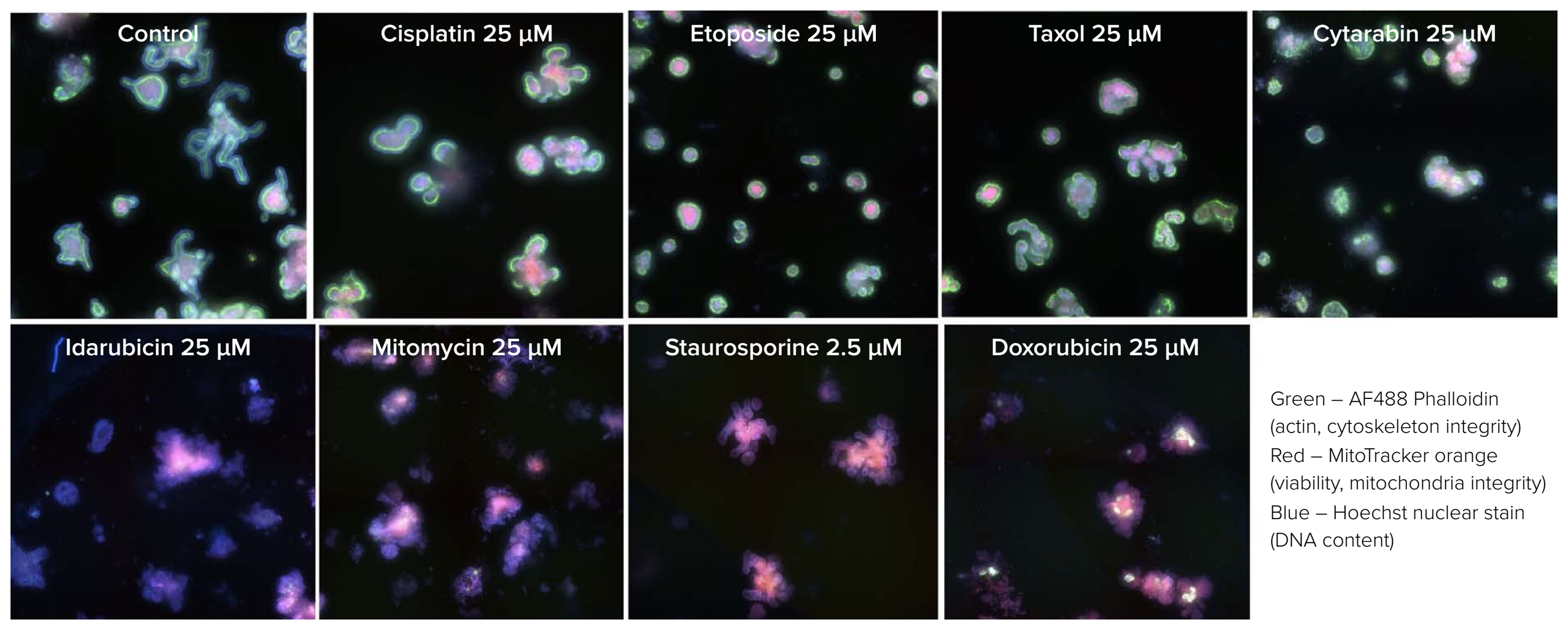
Using phenoglyphs for organoid classification. After analysis and feature extraction was performed by either CME or by Flexi protocols, measurements were further processed either for evaluation of individual readouts, or for classification via Phenoglyphs. Phenoglyphs processing involved un-supervised classification of organoids selecting automatically up to 20 classed of organoid phenotypes. Then a supervised learning step which manually defines the selected phenotypes of the organoids. After manual re-assigning of classes, training processes were applied with several iterations. Selection of Live/Dead organoid classes, or Live, Cytostatic, Cytotoxic classes were successfully used for classification of all organoids into those few classes. Classification analysis data (e.g. % of Live organoids) were used to define IC50s for compound effects.

Results

Toxicity assessment using 3D mouse intestinal organoids

Intestinal organoids were expanded in 24-well plates then seeded into 96-well Ibidi or 96 U-bottom Corning plates on the CellXpress.ai system. Each Matrigel dome contained approximately 50 organoids. Organoids were monitored by TL imaging every 12 hours. Organoids self-organized and developed crypts as expected organoid phenotypes. After 48h in culture, organoids were treated with selected anti-cancer compounds that were also added automatically.

Phenotypic changes in organoids



Green – AF488 Phalloidin (actin, cytoskeleton integrity)
Red – MitoTracker orange (viability, mitochondria integrity)
Blue – Hoechst nuclear stain (DNA content)

Figure 2. Intestinal organoids were treated with cytotoxic compounds for 72h, with 5X dilutions, 0–100 µM concentration range, in triplicates. Then stained with Hoechst, MitoTracker and AF488 Phalloidin. Changes in morphology and viability were measured using high-content imaging. Organoids were imaged using confocal option (60" disk) on the HSC.ai instrument with 10X magnification. Z-stack of 21 confocal images were taken 8 µm apart, covering approximately 160 µm range, then maximum projection images were used for analysis.

Machine learning-based classification of compound toxicity effects

High content analysis and feature extraction with Custom Module Editor: High-content imaging and analysis allows quantification of the number of organoids in the dome and measures the organoid size (area) and fluorescent intensities with different markers. In addition, it allowed to identify individual cells score cells as positive, if those had a high signal for actin or MitoTracker and count intact or damaged cells in organoids.

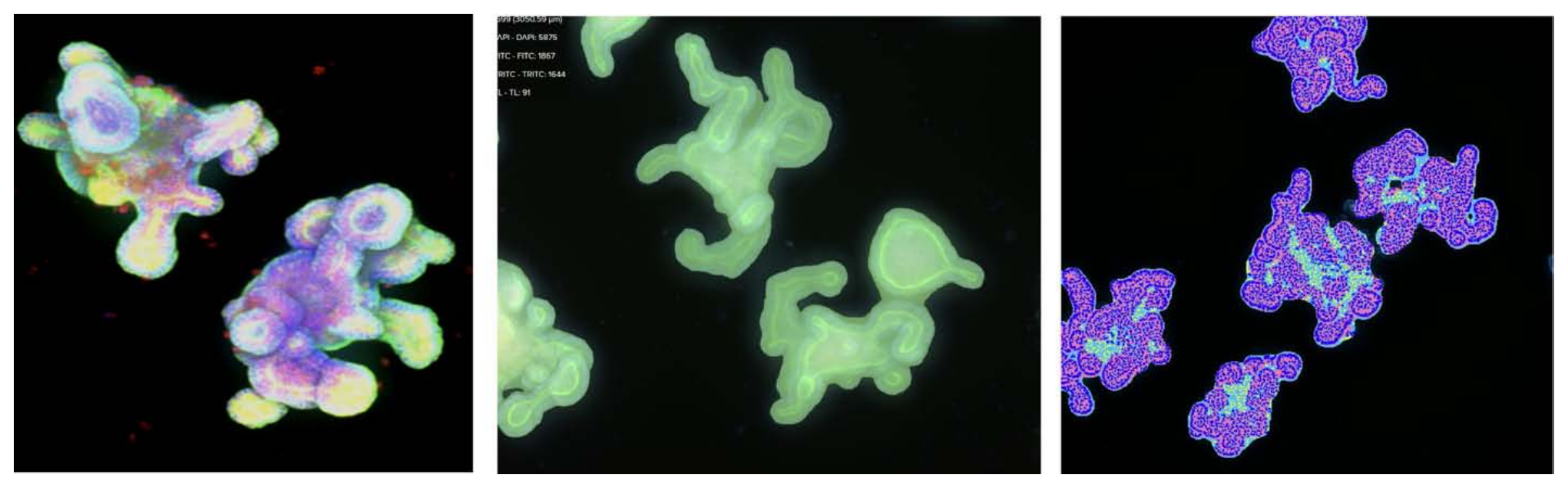


Figure 3. Organoids stained with Hoechst, MitoTracker and AF-488 Phalloidin. Organoids segmented and analyzed in different fluorescent channels, then individual cells identified within organoids, analyzed, and scored as positive or negative for different markers.

Machine-learning classification by Phenoglyphs: Machine learning and AI reduces time-consuming and labor-intensive tasks and provides automated decision-making—an unbiased approach to image analysis and data consistency. Image analysis was completed using IN Carta software. First, the Custom Module Editor software module was used to find organoid objects and characterize those objects using images captured in three fluorescent channels. Nuclei and individual cells were found inside organoids and were then characterized using various measurements featuring intensities, areas, and linear measurements. More than sixty measurements in total were extracted from each image.

Phenoglyph analysis generated un-supervised clustering into 10 classes, we then consolidated these into just two classes—Live (intact) organoids, and Dead (damaged) organoids—then training was initiated. After the training run was complete, we reviewed the classifications and corrected classes by manual re-assignment of images into either the Live or Dead category. After some corrections, we repeated the training and, after several cycles, shifted the classification score close to 1. As a result, we obtained accurate classification of objects in the wells obtained classification as % of Live or Dead organoids. The % Live organoids numbers were decreasing with increasing concentrations of compounds and reflected the trends expected for effects of tested compounds.

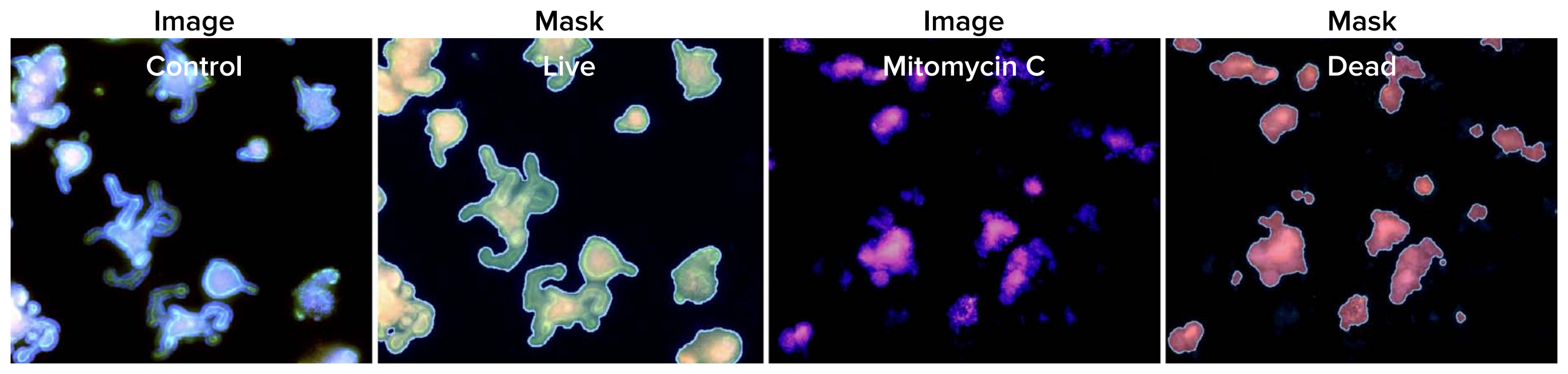
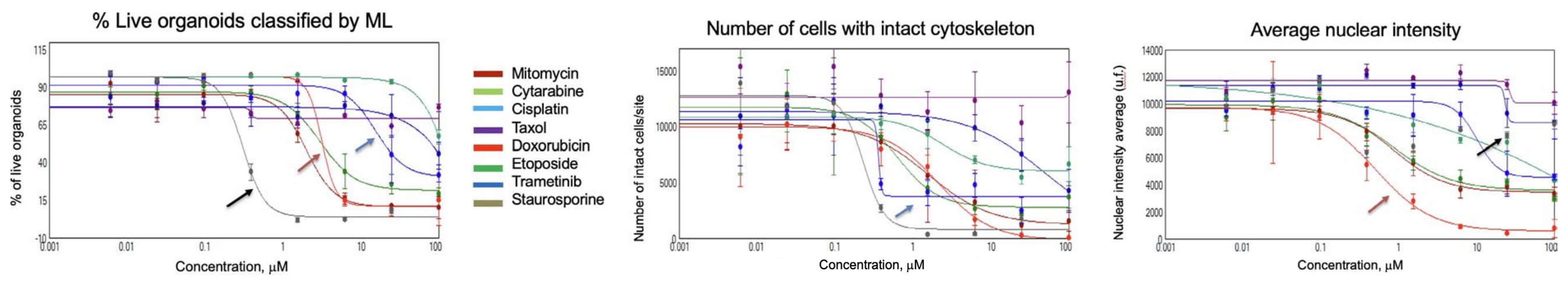


Figure 4. Intestinal organoids stained with AF-488 – conjugated Phalloidin, Hoechst nuclear dye and MitoTracker orange. Live (green) and Dead (deteriorated) organoids after treatment with Mitomycin C indicated by software and marked with green and red masks respectively.

Concentration dependencies of organoid phenotypes based on AI-classification or cellular measurements



Left: Live (intact) vs Dead (deteriorated) organoids were classified by software. Plots are showing concentration-dependent decrease in % of live organoids with increasing concentrations of indicated drugs. Concentration-dependencies can be different between classification and individual read-outs, depending on compound mechanism of action (pointed with arrows). For example, DNA-intercalating agents showing marked decrease in nuclear intensity by Hoechst stain, but not immediate deteriorated phenotype. **Center and right:** Plots are showing concentration-dependent decrease in the number of cells with intact cytoskeleton (positive for Phalloidin); or decrease in the nuclear intensity (Hoechst stain) with increasing concentrations of indicated drugs.

IC50, µM	Live Class by %	Actin Positive Cells	Mitochondria Positive Cells	Nuclear Intensity Average	Mechanism of Action
Staurosporine	0.3	0.23	0.3	100*	protein kinases inhibitor
Mitomycin	2	1.5	1.02	0.87	DNA intercalator, blocks DNA, RNA synthesis
Cytarabin	2.97	0.6	1.07	0.87	DNA cross-linking
Doxorubicin	3.2	2.1	n/d	0.51	DNA intercalation
Trametinib	15.6	0.36	0.33	10.2	MEK 1/2 inhibition
Cisplatin	49	49.5	23.1	23.7	DNA cross-linking
Etoposide	100*	2.4	2.1	100*	topoisomerase II inhibitor
Taxol	100*	118	82	100*	stabilize and prevent microtubules depolymerization

100* means effect was observed only at highest concentration

IC50s were obtained from concentration dependencies from Live/Dead phenotypic classification of organoids, also from concentration – dependencies for other read-out obtained from High Content Imaging. Notably, IC50s for various read-outs are typically different, depending on compound mechanism of action.

IC50s determined from phenotypic classification by Machine Learning helps to determine IC50s from phenotypic changes that represent phenotypes of interest (intact or deteriorated phenotypes). IC50s from phenotypic classification may or may not correlate with selected single read-outs, depending on mechanism of action of compound, or phenotype selected for analysis.

Figure 5. Concentration dependencies and effective concentrations for selected readouts that reflect corresponding phenotypic changes caused by toxic compounds.

Results

Toxicity assessment using 3D healthy human intestinal organoids

Human intestinal organoids were seeded into 96 U-bottom Corning plates using the CellXpress.ai system, monitored by TL imaging every 12 hours, then treated with selected anti-cancer compounds.

Phenotypic changes in organoids after compound treatment

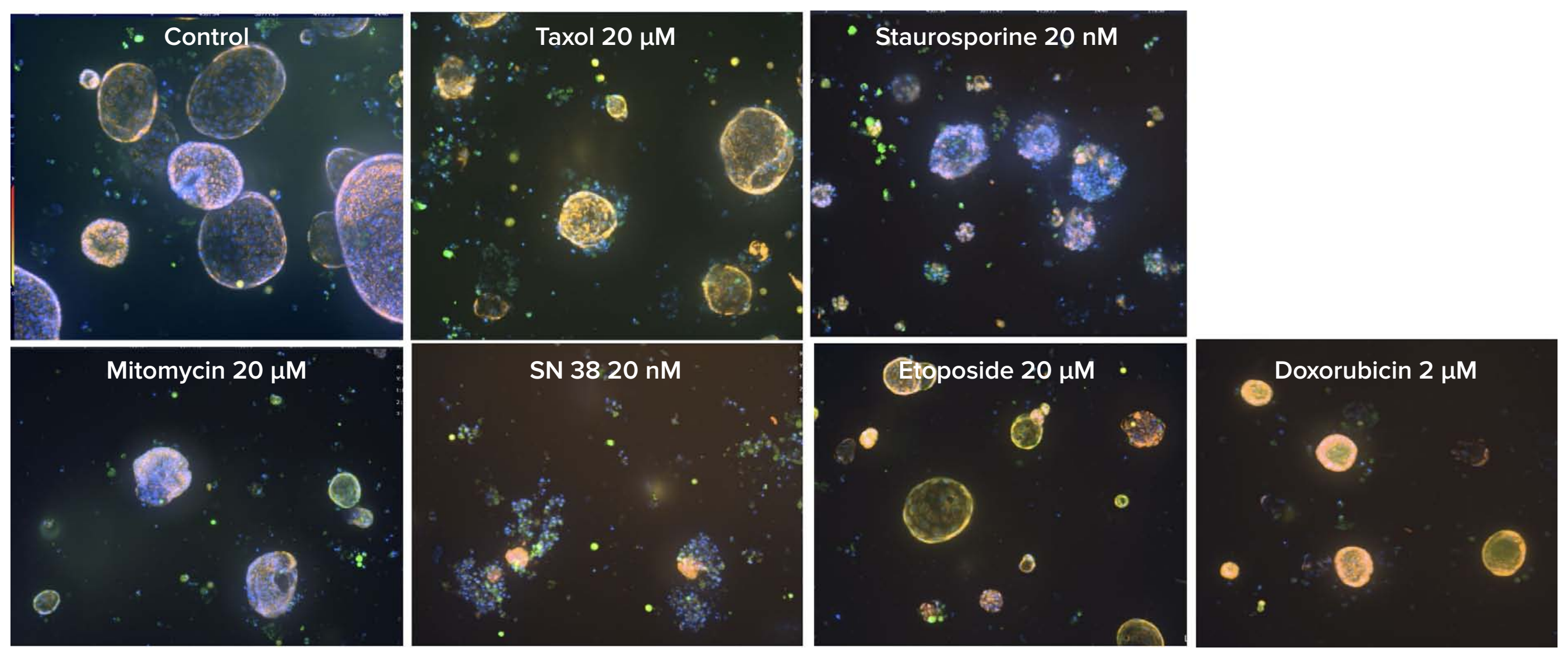


Figure 6. Intestinal organoids were treated with cytotoxic compounds for 72h, with 5X dilutions, 0–100 µM concentration range, in triplicates. Then stained with Hoechst, MitoTracker and CalceinAM. Organoids were imaged live using confocal option (60" disk) of ImageXpress HSC.ai instrument with 10X magnification. Z-stack of 17 confocal images were taken 8 µm apart, covering approximately 136 µm range, then maximum projection images were used for analysis.

IN Carta software was used for analysis. Organoids were segmented by Segmentation Is Not A Problem (SINAP) then feature extraction was completed by Flexi protocol that identified 107 measurements in the images, including intensities, areas, textures, distributions, etc. SINAP is a deep learning-based tool for image segmentation. Pre-trained models (such as for nuclei, cells) can be customized by users to fit their analysis goals. The SAM (Segment Anything Model) allows users to more easily annotate images.

Organoid segmentation (recognition) by SINAP

Figure 7. Image showing steps for segmentation: First find organoids using SINAP deep learning tool for segmentation; with the SAM (segment anything) tool. Then train the model selecting 20–50 images first, then correct and re-train as needed.

Machine-learning classification by Phenoglyphs

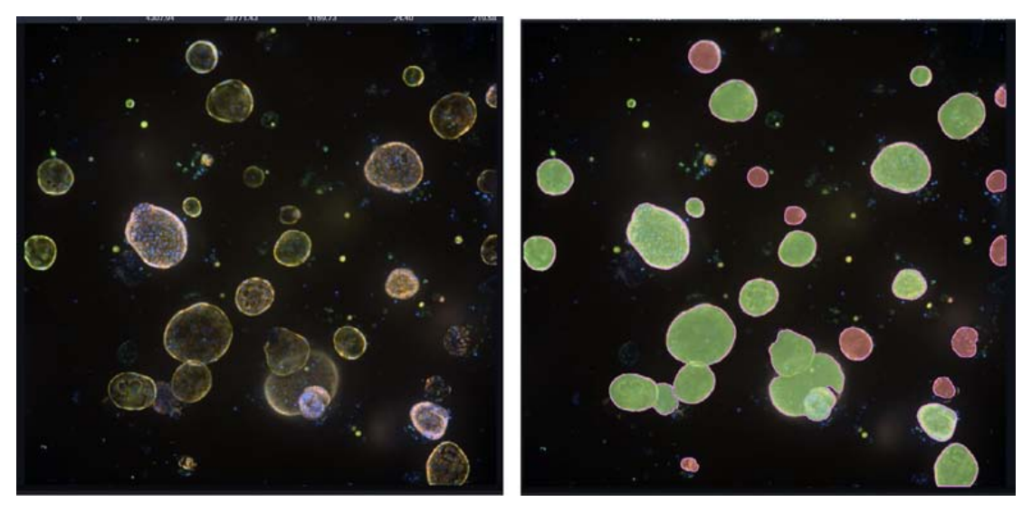
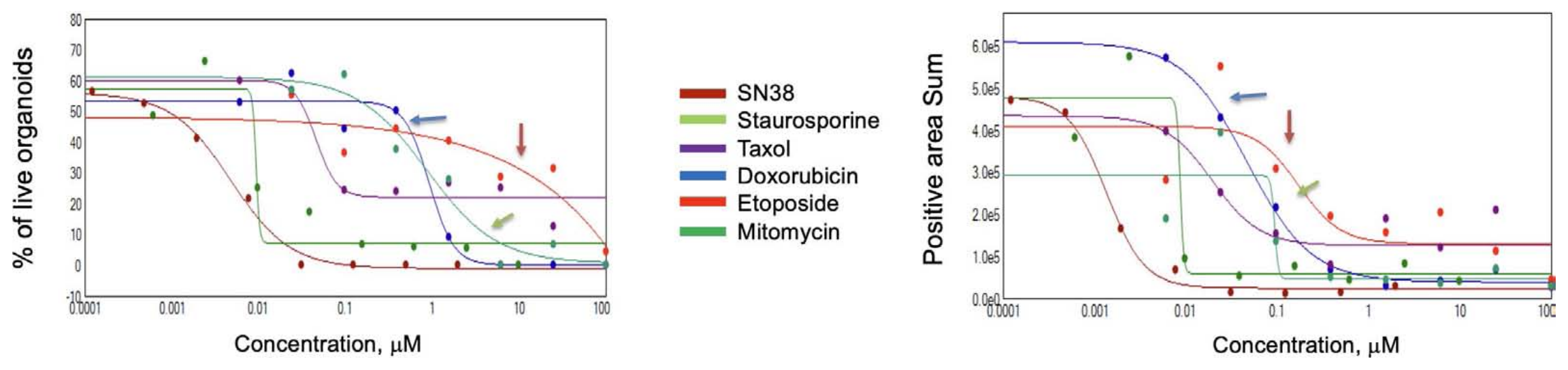


Figure 8. After feature-extraction machine-learning analysis provided unsupervised classification of organoids for different phenotypes, the user selects classes according to the experimental purpose. For example, intact organoids (green masks) vs deteriorated organoids (red masks). Then the machine-learning process is trained to recognize the phenotypes selected by scientist.

Concentration-related phenotypic changes



IC50, µM	% Live, Classification	Positive Area Sum
SN38	0.005	0.002
Staurosporine	0.01	0.01
Doxorubicin	0.936	0.048
Taxol	0.046	0.02
Etoposide	14	0.17
Mitomycin C	0.88	0.1

SN38 – topoisomerase I inhibitor

IC50s determined from phenotypic classification by machine-learning helps to determine IC50s from phenotypic changes that represent phenotypes of interest (intact or deteriorated phenotypes). IC50s from phenotypic classification may or may not correlate with selected single readouts, depending on mechanism of action of compound, or phenotype selected by scientist.

Figure 9. Concentration-dependencies and effective concentrations for selected readouts that reflect corresponding phenotypic changes caused by toxic compounds.

Summary

- 3D intestinal organoid models provide a valuable tool for studying drug effects and to evaluate potential toxicity *in vitro*, since their response to compounds provides a better prediction to clinical effects of drugs
- High-quality confocal imaging and detail resolution of 3D organoids and machine learning-powered image analysis are essential to extract essential information about biological effects
- We have demonstrated methods and results to evaluate the toxic effects of drugs using AI-based phenotypic analysis of organoids

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