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Drug response profiling of individual primary colorectal cancer tumoroids using a novel automation workflow and AI-assisted image analysis

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INTRODUCTION

The use of three-dimensional in vitro cell models have gained popularity as they better recapitulate key aspects of tissues and tumor microenvironments. Generating organoids, spheroids, and tumoroids have progressed to where scientists can emulate most human organs and cancer types *ex vivo* [1,2]. These models are being used in many aspects of drug discovery and development, but adoption has been limited due in part to challenges related to sample handling, assay standardization and the need for optimized instrumentation [3]. **Here we present a novel workflow that addresses those issues and analyzed the proliferation and viability of established cell line-derived spheroids (HTC116) and patient-derived CRC (P-D CRC) tumoroids after drug treatment.**

We combined:
- Pu·MA System® EC and protected sample chamber flowchips [4,5] (PFI) for automatic fluid transfer during drug treatment and viability assays;
- Automated sorting and isolation of size-selected tumoroids using spheroONE® (SCIENION), directly into the flowchips;
- Precise reagent loading and dispensing using I.DOT LT (Dispendix), for addition/dilution of drugs, wash and staining reagents into flowchips;
- High content imaging with a Revolution microscope (Discover Echo);
- AI-assisted image analysis.

This workflow provides automation and standardization of 3D model-based drug development, while offering complete flexibility in terms of 3D model types, drugs, volumes and readouts, and is applicable to a wide range of research areas, incl. disease modeling, drug discovery and personalized medicine.

PRECISION LIQUID DISPENSING

- **Accuracy Built-In**: Real-time droplet detection verifies volume and alerts when source liquid runs out.
- **Prevents Cross-Contamination:** Non-contact, pressure-based dispensing eliminates carryover and tip usage.
- **Reagent-Saving Miniaturization**: Dispenses as low as 17.3 nL with <1 µL dead volume to cut reagent costs.
- **Flexible & Compatible**: Supports 17.3 nL–30 µL, handles viscous liquids (up to 43% glycerol), and fits all SBS-format plates, including up to 1536-well plates.
- **Simplified Setup**: Assay Studio software enables protocol creation within minutes - no programming needed

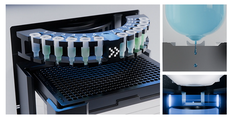


Figure 1. *The I.DOT LT utilizes a* ***positive pressure dispensing system*** *formed between the dispense head and* ***12 disposable source wells****, each holding up to* ***490 µL*** *of reagent. It precisely releases* ***17.3–50 nL droplets*** *through a* ***100-µm orifice*** *at a rate of* ***100 droplets/s****. The built-in* ***DropDetection*** *system counts every droplet in real time and alerts users when a source well runs dry. After each run, a* ***color-coded dispense report*** *provides a clear summary of all liquid transfers, ensuring accuracy, traceability, and confidence in every experiment.*

AUTOMATED REAGENT EXCHANGE

- **Automated media exchanges** occur with cells in protected chamber: approximately 95% of fluid is exchanged allowing for efficient washing and minimalize compound carryover.
- **Supernatants can be collected** to monitor cell secretion.
- **Spheroids can be stained and imaged** in the flowchips.
- **Assay protocols can be edited** via the Pu-MA System Software.

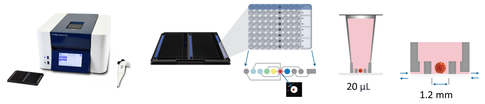


Figure 2. *Pu·MA System® EC workflow allowing media exchange, drug treatment, and immunostaining of individual spheroids. The Pu·MA System EC system workflow allows for the processing of 32 individual spheroids inside an environmentally controlled tabletop device. Precise microfluidic channels allow for the* ***addition, removal, and recovery of up to seven different reagents/samples****. Spheroids are contained within a microcavity to prevent sheer damage during fluid exchange. High clarity bottom allows for* ***excellent imaging*** *with an inverted scope.*

SINGLE SPHEROID ISOLATION AND DISPENSING

- **Standardization of 3D model** size and morphology.
- **≥95% accuracy** for automated image-based dispending of patient-derived CRC.
- **Gentle dispensing technology** maintains the integrity and viability of fragile cellular aggregates, from 100 to 600 µm diameter.
- **Direct visual inspection** of the sample along with full image record.

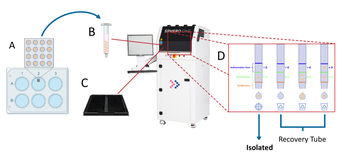


Figure 3. *spheroONE® was used to standardize isolate 3D cellular models. For spheroid experiments: (A) Cells are grown and placed into spheroid forming microcavity ULA plate. (B) Spheroids are collected and transferred to a sample reservoir. (C) Sample and target plate are loaded into the spheroONE®. (D) The* ***image-based isolation*** *software analyses the trajectory of particles in the dispensing capillary and defines the Ejection Zone, i.e., the area of the capillary image that represents the volume that will be dispensed in next drop. When isolation criteria are met (i.e., when* ***one 3D model of the right size and morphology*** *is in the ejection zone), the capillary is driven onto the target and the drop containing the spheroid is dispensed.*

GENERATION OF PATIENT-DERIVED CRC TUMOROIDS

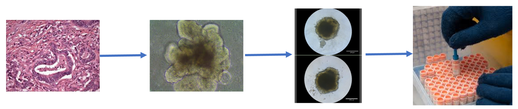


Figure 4. Generation of patient-derived CRC tumoroids (P-D CRC). *Primary tumors were obtained from Next Oncology (San Antonio, TX) as primary or secondary passages. Further passaging and expansion of the tumoroids were performed at MatTek.*

- **Cancer epithelial cells were expanded from frozen stock** and then seeded into multicavity plates (Heidolph Sphericalplate 5D 24-well plate) with a seeding density of approximately 600 cells per microcavity.
- **Tumoroids were allowed to form over a 10- to 13-day period** before harvesting and loading into the spheroONE for automated dispense into Pu·MA System flowchips.

VIABILITY ASSAY DEVELOPMENT

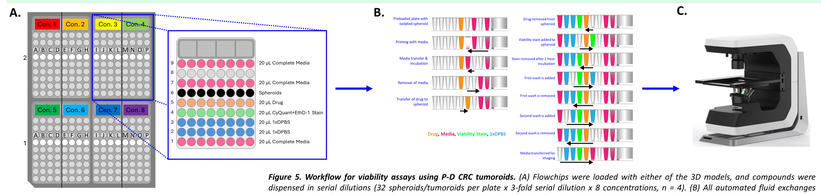


Figure 5. Workflow for viability assays using P-D CRC tumoroids. *(A) Flowchips were loaded with either of the 3D models, and compounds were dispensed in serial dilutions (32 spheroids/tumoroids per plate x 3-fold serial dilution x 8 concentrations, n = 4). (B) All automated fluid exchanges occurred within the sample chamber, and tumoroids were protected by the flowchip design. Flowchips were incubated at 37°C for 48 hours, with water reservoirs to limit evaporation. After incubation, staining solution (CyQuant Green + EthD-1 or PI, Invitrogen) was added into flowchips using Pu·MA System. (C) Imaging was performed with either Revolution (10X, widefield, ECHO) or CQ1 (10X, confocal, Yokogawa).*

AI-ASSISTED VIABILITY ANALYSIS

- Images undergo **preprocessing** operations to ensure compatibility with the VGG16 model (standardization of image size and intensity normalization).
- **Pre-trained VGG16 regional convolutional neural network** (R-CNN) is employed to extract features from the images.
- **Custom-built fully connected layers** learn discriminative features through iterative training specific to spheroid detection.
- The model, facilitated by the **Adam optimizer and binary cross-entropy loss function**, measures spheroid parameters and produces data outputs.

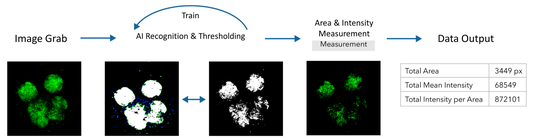
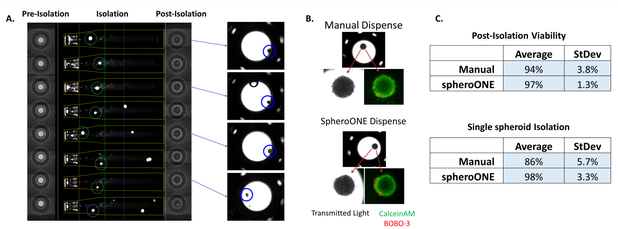


Figure 6. Workflow of AI-assisted analysis of fluorescently stained spheroids for viability assessment*. We have developed a comprehensive method for automated spheroid detection and analysis of fluorescent images, employing state-of-the-art deep learning techniques.*

COMPARISON OF MANUAL VS. SPHEROONE SPHEROID ISOLATION

- Spheroids were dispensed into flowchips either via manual pipetting or automated spheroONE isolation.
- Post-dispense microscopy showed **correct spatial positioning** of 3D models into the center of flowchip wells.
- Equivalent phenotypes and viability were observed between two methods with **>90% viable cells**.
- spheroONE dispensing was in average **4 times faster** and achieved **greater single spheroid isolation accuracy**, with an average **98%** of the wells containing one spheroid.



Post-Isolation Viability

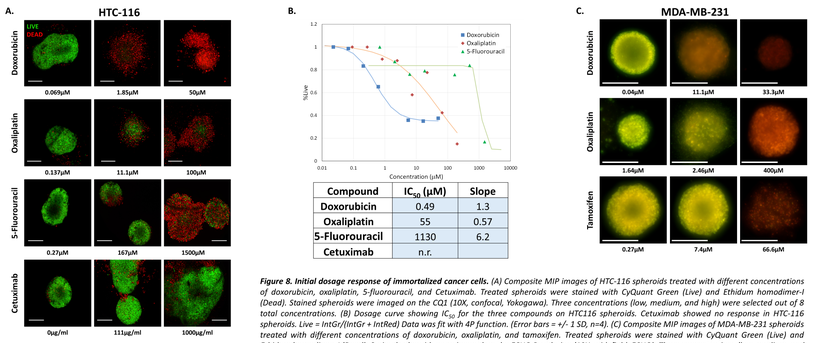
	Average	StDev
Manual	94%	3.8%
spheroONE	97%	1.3%

Single spheroid Isolation

	Average	StDev
Manual	86%	5.7%
spheroONE	98%	3.3%

Figure 7. *(A) Images captured by spheroONE showing isolation of tumoroids and position in flowchips. (B) Comparison of spheroid viability using manual or spheroONE dispensing. Sample wells were filled with 20 µL of media and incubated overnight. Media was replaced with viability staining solution using Pu·MA System and subsequently imaged. (C) Comparison of post-isolation viability and single spheroid isolation accuracy.*

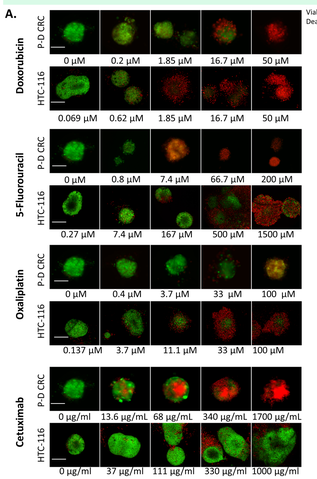
DOSAGE RESPONSE CURVES FOR IMMORTALIZED CELL LINES



Compound	IC_{50} (µM)	Slope
Doxorubicin	0.49	1.3
Oxaliplatin	55	0.57
5-Fluorouracil	1130	6.2
Cetuximab	n.r.	

Figure 8. Initial dosage response of immortalized cancer cells. *(A) Composite MIP images of HTC-116 spheroids treated with different concentrations of doxorubicin, oxaliplatin, 5-Fluorouracil, and Cetuximab. Treated spheroids were stained with CyQuant Green (Live) and Ethidum homodimer-I (Dead). Stained spheroids were imaged on the CQ1 (10X, confocal, Yokogawa). Three concentrations (low, medium, and high) were selected out of 8 total concentrations. (B) Dosage curve showing IC_{50} for the three compounds on HTC116 spheroids. Cetuximab showed no response in HTC-116 spheroids. Live = IntGr/(IntGr + IntRed) Data was fit with 4P function. (Error bars = +/- 1 SD, n=4). (C) Composite MIP images of MDA-MB-231 spheroids treated with different concentrations of doxorubicin, oxaliplatin, and tamoxifen. Treated spheroids were stained with CyQuant Green (Live) and Ethidum homodimer-I (Dead). Stained spheroids were imaged on the ECHO Revolution (10X, widefield, ECHO). Three concentrations (low, medium, and high) were selected out of 8 total concentrations. Scale bars represent 200 µm in all images.*

HTC-116 SPHEROIDS *vs.* PATIENT-DERIVED CRC TUMOROIDS



B. Chemotherapeutics & observed effects

- **Doxorubicin**
  - Anthracycline, blocks enzyme topo isomerase 2. Widely used chemo agent susceptible to drug resistance [6].
  - **P-D CRC and HTC-116 show similar sensitivity.**
- **Oxaliplatin**
  - Ligand-Pt complex causes DNA damage, induces apoptosis [7,8].
  - **P-D CRC show less sensitivity than HTC-116.**
- **5-Fluorouracil**
  - Precursor of dTTP and UTP. Interferes with both DNA and RNA metabolism affecting DNA repair and DNA or RNA synthesis [8].
  - **P-D CRC ~50x more sensitive than HTC-116.**
- **Cetuximab**
  - EGFR mAb for RAS wild-type metastatic CRC [7].
  - **P-D CRC sensitivity seen at 68 ug/mL, while HTC-116 not sensitive at tested doses.**

Figure 9. *(A) Representative images of P-D CRC tumoroids and HTC-116 spheroids after incubation with compounds for 48 hrs. Spheroids were stained with CyQuant Green (Live) and EthD-1 or PI (Dead). Imaging was done with either ECHO Revolution (10X, widefield, ECHO) or CQ1 (10X, confocal, Yokogawa). (B) List of chemotherapeutic drugs used for initial trials and summary of observed effects. (C) Dosage curve showing IC_{50} for the four compounds on P-D CRC tumoroids spheroids. Scale bars represent 200 µm*

EGFR LOCALIZATION

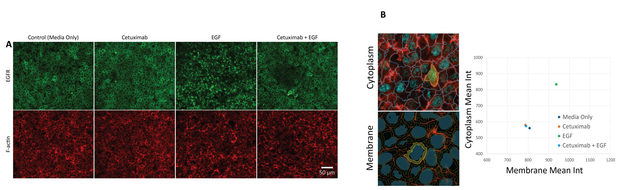


Figure 10. *HTC-116 cells treated with human recombinant EGF and/or Cetuximab. (A) Cells were incubated with Media or Cetuximab (250 ug/mL) overnight then treated with EGF (10 nM) for 20 min. Cells were fixed and stained for EGFR and F-actin. Images were acquired using a CQ1 confocal imaging system (40X Obj). (B) EGFR internalization was analyzed using CellPathfinder (Yokogawa)*

- EGFR internalization showed a **shift from membrane localization** (Fig. 10B, bottom mask) **to cytoplasmic** localization (Fig 10B, top mask) of EGFR upon EGF activation.
- Cetuximab was observed to interfere with this internalization.

CONCLUSIONS

- We have demonstrated capabilities of a novel automated 3D cellular model assay system that performs **standardized drug testing**.
- **3D models are automatically standardized and isolated** into flowchips for downstream assays, providing control over size and number.
- Fluid exchanges are performed in a novel microfluidic device that protects the cell models and enhances **assay precision and control**.
- **AI-driven analysis is currently being improved** to recognize and quantify changes in spheroid morphology to allow for further analysis of chemotherapeutic effects on spheroid behavior.
- The ability to analyze spheroids and tumoroids to capture toxicity information and perform functional assays shows great promise for **disease modeling and drug discovery**.

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