High throughput drug response profiling of primary colorectal tumor models using a novel automation workflow and AI-assisted image analysis

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INTRODUCTION

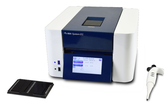
The use of human-relevant three-dimensional in vitro cell models have gained popularity as they better recapitulated key aspects of human tissues and tumor microenvironments. Methods for generating organoids, spheroids, and tumoroids have progressed to where scientists have the ability to recapitulate 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 development and the need for optimized instrumentation.[3] Here we present a novel automated workflow for phenotypic profiling of 3D tumor models that addresses those issues. We have combined the use of spheroONE® for automated sorting, isolation and dispensing of tumoroids and Pu·MA System® EC with low attachment flowchips for performing viability assays and biomarker detection.

The spheroONE, a large-particle sorter and dispenser, was optimized to dispense size-selected tumoroids into the protected sample chamber of Pu·MA System flowchips.[4,5] The Pu·MA System EC performed compound additions and staining using automated fluid transfers. Tumoroids were incubated for 48 hr in flowchips in a standard incubator to maximize efficiency of Pu·MA System. The samples were stained in the flowchips with viability dyes, imaged with ECHO microscope, and analyzed with an AI-assisted program. We used this workflow to analyze proliferation and viability of HCT116 colorectal cancer (CRC) spheroids and patient-derived CRC tumoroids. This platform is a valuable tool in a wide range of research areas including disease modeling, drug discovery and personalized medicine.

AUTOMATED REAGENT EXCHANGE

The Pu·MA System EC and 3D Flowchip features:

- Automated media exchanges occur with cells in protected chamber
- Supernatants can be collected to monitor cell secretion
- Spheroids can be stained & imaged in the flowchips
- Assay protocols can be edited via the Pu·MA System Software



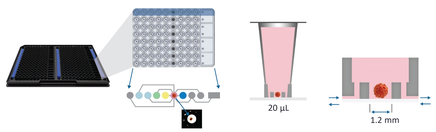
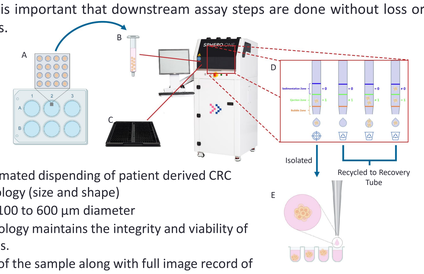


Figure 1. Pu·MA System flowchip layout and sample chamber dimensions.

SPHEROID ISOLATION AND DISPENSE

Precise isolation and dispensing of 3D cell models such as spheroids and tumoroids is critical for assay automation. It is important that downstream assay steps are done without loss or damage to the tumoroids.



spheroONE features:

- ≥95% efficiency for automated dispending of patient derived CRC tumoroids using morphology (size and shape)
- Size based sorting from 100 to 600 µm diameter
- Gentle dispensing technology maintains the integrity and viability of fragile cellular aggregates.
- Direct Visual inspection of the sample along with full image record of isolated cell aggregates.

Figure 2. SpheroONE workflow to isolate spheroids. (A) Cells are grown and placed into spheroid forming microcavity plate. (B) Spheroids are collected and transferred to a sample reservoir. (C) Sample and desired plate are loaded into the spheroONE. (D) Using Image-Based Isolation Software, the spheroONE images a dispensing capillary. The capillary is divided into three zones: The bubble Zone (orange) represents the volume displaced during dispense. The Ejection Zone (green) represents volume of media that will be dispensed in next drop. The Sedimentation Zone represents the region where spheroids settle and can potentially be dispensed. (E) The software ensures that only when a single spheroid is in the Ejection Zone and no spheroids are in the Sedimentation Zone are isolated into individual wells.

References

1. Matossian, M.D. et al (2019) BMC Cancer. 19(1): 205
2. Matossian, M.D. et al (2021) Clin. & Trans. Oncology 24: 127-144
3. Sirenko, O. et al (2015) ADDT 13, 402
4. Cromwell, E.F. et al (2021) SLAS Tech. 26(3): 237
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PATIENT DERIVED CRC TUMOROIDS WORKFLOW

Patient derived 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. Tumoroids were then stored in liquid nitrogen until use. Next, 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 - 13 day period before harvesting and loading into the spheroONE for automated dispense into Pu·MA System flowchips.

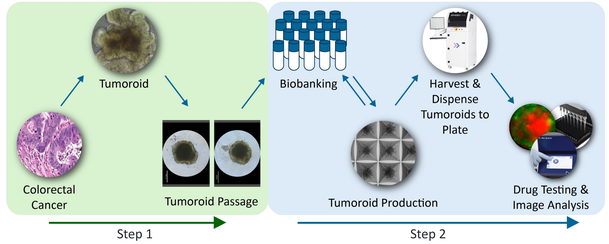


Figure 3. Workflow for formation and dispensing of patient-derived colorectal cancer spheroids.

HCT116 SPHEROID FORMATION AND LOADING

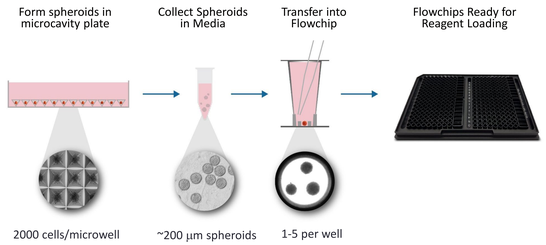
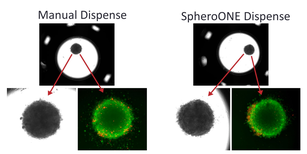
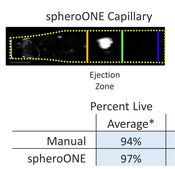


Figure 4. Spheroids are formed in microcavity plates (Aggrewell 800, Stemcell Technologies) over a 2 - 3 day period. Spheroids are harvested according to the manufacturer's protocol and are collected into a 1.5 ml tube. Spheroids are picked up in 3 µl of media and dispensed directly into the bottom of a primed sample well. After dispense the well is filled with 18 µl of media or media + compound.

spheroONE AUTOMATED FLOWCHIP LOADING

An important design criteria of the spheroONE is to not adversely affect spheroid viability. To characterize this sister spheroids were dispensed into flowchips using manual pipetting and spheroONE automated dispense. The sample wells were filled with 20 µL of media (McCoy's Complete) and incubated overnight. Media was replaced with viability staining solution using Pu·MA System and then spheroids were imaged for Live and Dead cells. Equivalent phenotypes and viability were observed between two methods with % Live cells > 90%.

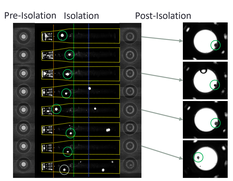




Percent Live

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

*n=5



- Pre-dispense imaging allows for post-dispense validation of spotting
- Imaged based isolation ensures only spheroids are dispensed (All images are saved for records)
- Post-dispense image shows that our spheroid was dropped into the correct location
- Visualization under microscope confirms isolated of spheroid into center microcavity

Figure 5. Top: Comparison of spheroid viability using manual and spheroONE dispense in Pu·MA System flowchips. Bottom: Images captured by spheroONE showing isolation of tumoroids and position in flowchips.

VIABILITY ASSAY SETUP & WORKFLOW

Assay reagents are loaded into flowchip wells and then fluid exchanges occur with the sample chamber according to pre-determined assay protocols. Tumoroids were protected during these operations and approximately 95% of fluid is exchanged. This makes for very efficient wash and minimizes compound carry-over. The assay protocol here included compound incubation, viability staining, and wash steps. Tumoroids were characterized by automated high resolution transmitted light and fluorescence imaging.

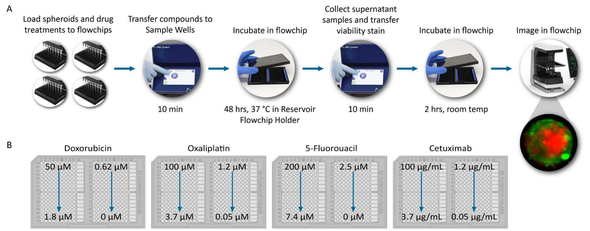
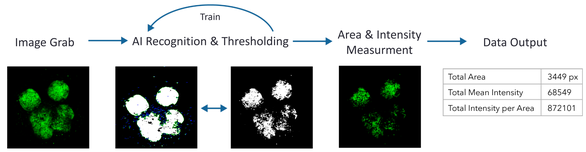


Figure 6. Workflow for viability assays using P-D CRC tumoroids. Multiple plates were loaded with tumoroids and compounds were dispensed in serial dilutions (e.g., 32 samples per plate 3-fold serial dilution, 8 conc., n = 4). Flowchips were placed in incubators using holders with water reservoirs to limit evaporation. After 48 hr incubation, staining solution (CyQuant Green + EthD-1 or PI, Invitrogen) was loaded and transferred into the sample wells using Pu·MA System. Imaging was done with either ECHO Revolution (10X, widefield, ECHO) or CQ1 (10X, confocal, Yokogawa).

AI-ASSISTED VIABILITY ANALYSIS



Total Area	3449 px
Total Mean Intensity	68549
Total Intensity per Area	872101

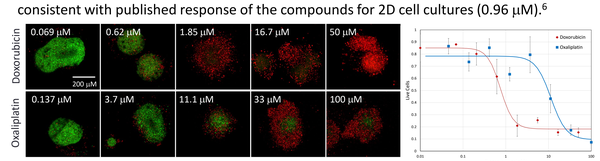
Figure 7. Workflow of AI-assisted analysis of fluorescently stained spheroids for viability assessment.

We have developed a comprehensive method for automated spheroid detection and analysis of fluorescent images, employing state-of-the-art deep learning techniques. The approach (see **Fig 7**) combines the power of transfer learning using a pre-trained VGG16 regional convolutional neural network (R-CNN) with custom-built fully connected layers for precise spheroid detection.

- Images undergo preprocessing operations to ensure compatibility with the VGG16 model (standardization of image size and intensity normalization).
- Pre-trained VGG16 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.

HCT116 COMPOUND RESPONSE

HCT116 spheroids were formed in a microwell cavity plates over 72 hr and collected into media. Spheroids were dispensed into flowchips sample wells using manual pipetting. Spheroids were treated for 48 hours with compounds doxorubicin then stained with viability dyes using Pu·MA System. The spheroids were imaged in the flowchips and analyzed for number of Live and Dead cells (see **Fig 8**). Spheroid viability results were found to be consistent with published response of the compounds for 2D cell cultures (0.96 µM).[6]



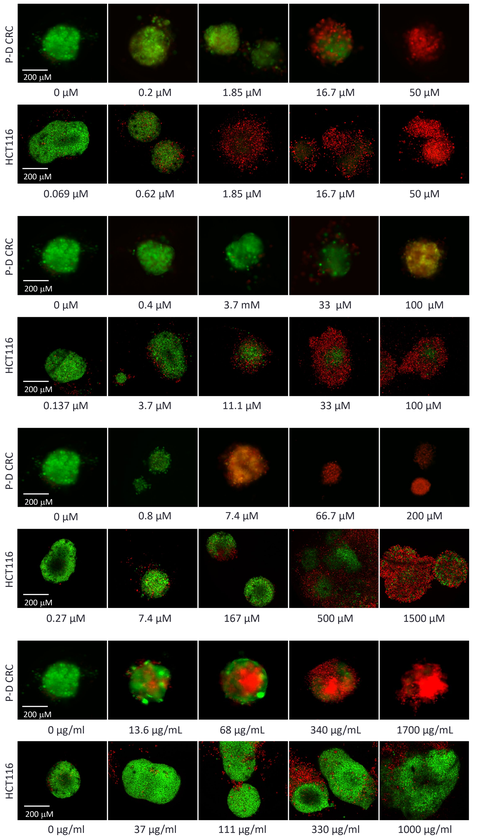
	Doxorubicin	Oxaliplatin
IC_{50}	0.75	11.8
Slope	2.9	2.44

Figure 8. Above: Composite MIP images of HCT116 spheroids treated with different concentrations of doxorubicin and oxaliplatin. **Right:** Viability-response curve for incubation with compound. Image analysis was done with AI-assisted process. Live (Green) and Dead (Red) channels were analyzed independently. Live Cells value was determined from normalized integrated intensities: Live = IntGr/(IntGr + IntRed) Data was fit with 4P function. (Error bars = +/- 1 SD, n=4)

- Doxorubicin[6]: 0.96 µM
- Oxaliplatin[7]: ~5 µM

6. Khaleel, S.A. et al (2016) Sci Reports 6, 36855
7. Narvi, E. et al, (2018) Sci Reports 8, 16579
8. Richard, S.M. et al, (2015) 11, 336

HCT116 VS PATIENT-DERIVED CRC TUMOROIDS



Doxorubicin

- Anthracycline, blocks enzyme topo isomerase 2. Widely used chemo agent susceptible to drug resistance.[6]
- P-D CRC and HCT116 show similar sensitivity

Oxaliplatin

- Ligand-Pt complex causes DNA damage, induces apoptosis.[7,8]
- P-D CRC show less sensitivity than HCT116

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 HCT116

Cetuximab

- EGFR mAb for RAS wild-type metastatic CRC.[7]
- P-D CRC sensitivity seen at 68 ug/mL.
- HCT116 not sensitive

Figure 9. Representative images of P-D CRC tumoroids and HCT116 spheroids after incubation with compounds for 48 hrs. Spheroids were stained with CyQuant Green (Live) and EthD-1 or PI (Dead). P-D CRC images were taken with ECHO widefield; HCT116 images are MIP's taken with CQ1 confocal.

Next Steps:

- Immunofluorescence staining of tumoroids for biomarker expression (e.g., EGFR, HER2)
- Analysis of conditioned media for cytokines, chemokines, and other secreted factors
- Comparison of different patient-derived samples for correlation to clinical data

CONCLUSIONS

- We have demonstrated capabilities of a novel automated spheroid and tumoroid assay system that performs complex protocols with 3D cell models .
- Tumoroids are automatically sorted, isolated and dispensed into flowchips for downstream assays providing precise control over size and number.
- Assays and fluid exchanges are performed in a novel microfluidic device that protects the cell models and enhances assay precision and control.
- The ability to analyze spheroids and tumoroids *in situ* in order to capture toxicity information and perform functional assays shows great promise for disease modeling and drug discovery.

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