Automated Long-term 3D Cell-Based Toxicity Studies Using a Flowchip System

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INTRODUCTION

There is an increasing interest in using three-dimensional (3D) cell structures for modeling tumors, organs, and tissue to accelerate translation research.[1] Significant progress has been made in formation of such structures to recapitulate the *in vivo* environment but performing complex assays with them can be challenging. For example, manual treatment, staining, and processing of spheroids and organoids is typically labor-intensive and prone to disruption or loss of samples. Here we report on use of a novel microfluidic-based system (Pu·MA System®) to perform automated assays with spheroids, organoids, and microtissues. It is ideal for applications for long-term toxicity, oncology therapeutics, single organoid secretion, and metabolite sampling.

Pu·MA System flowchip: This flowchip has sample chambers connected to multiple reservoirs to enable media and buffer exchange, supernatant sampling, and *in situ* lysing. A key aspect to the device is a protective chamber that holds 3D cell models and allows reagent exchanges without disrupting the structures.

Spheroid Toxicity & Imaging: Capabilities of the system were demonstrated by assaying spheroid toxicity over a 48-hour period with HCT116 spheroids (colon cancer cell line). Spheroids were incubated in Pu·MA System flowchips along with media or "media + compound". After incubation, the spheroids were analyzed by ImageXpress™ Micro Confocal Imaging System for Live/Dead cells and by CellTiter-Glo luminescence using a SpectraMax® iD5 Plate Reader for ATP activity.

Single Spheroid Metabolomics: Ability to perform single spheroid metabolomics was also demonstrated with HCT116 spheroids. Spheroids were incubated 2 hours with compounds and then lysed *in situ*. Spheroid lysate was analyzed for a panel of metabolites using mass spectrometry (Children's Medical Center Research Institute Metabolomics Facility at UT Southwestern) showing significant differences between Treated and Untreated spheroids.

MICROFLUIDIC ASSAY BACKGROUND

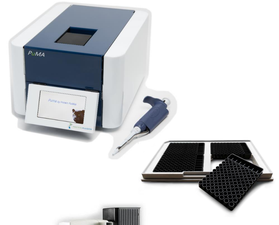
Pu·MA System Flowchips are designed with chambers and reservoirs arranged in a convenient multi-well plate format (384-well spacings) and provide up to 32 tests per plate. Once spheroids and reagents are loaded into the flowchips, the plate is placed into the Pu·MA System and reagent exchanges are done automatically through microfluidic channels connected to the protected sample chamber.

Multiple reagent exchanges are performed with assay protocols using built-in program. Assay protocols are pre-loaded into the system and run using an intuitive touch-screen interface. The whole Pu·MA System can be placed in an incubator to run assays at 37°C and 5% CO_2. The system architecture and use of pneumatics to move fluids provides gas exchange to the sample chambers.

INSTRUMENTATION

The Pu·MA System and flowchips includes:

- Automated media exchanges occur with cells in protected chamber
- Supernatants can be collected to monitor cell secretion
- Cells can be lysed in situ for sensitive metabolomic profiling
- Spheroids can be imaged in the flowchip, or samples removed for immunoassay or metabolomics analysis



The ImageXpress™ Micro Confocal Automated Imaging System (IXM-C) includes:

- Four colors + transmitted light
- Environmental control
- Automated data analysis

The system is controlled by MetaXpress™ Automated Imaging Acquisition and Analysis Software

References:
[1]Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning, Langhans, S.A. Frontiers in Pharma. 2018, 9, 1.
[2]High-Content Assays for Characterizing the Viability and Morphology of 3D Cancer Spheroid Cultures, Sirenko, O. et al. Assay and Drug Dev Tech. 2015, 13, 402.
[3]Metabolomics and Metabolic Diseases: Where DoWe Stand? Newgard, C.B. Cell Metabolism 2017, 25, 4380.
[4]Cancer cell specific inhibition of Wnt/β-catenin signaling by forced intracellular acidification, Melnki, S. et al., Cell Discovery, 2018, 4, 37

SPHEROID TOXICITY & IMAGING

- HCT116 cells were dispensed 2,000 cells per well (384-well ULA round bottom plate, Corning) and incubated for 48 hours until they formed tight spheroids.[2]
- The spheroids were transferred to a Pu·MA System flowchip in either media (Control) or media + compound (Treated).
- Flowchips were placed into a Pu·MA System in an incubator (37°C & 5% CO_2 and then automatically processed. At the end of the compound incubation, spheroids were stained and washed prior to imaging.
- Imaging was done with an ImageXpress Micro Confocal Imaging system and analysis performed using the Custom Module Editor with MetaXpress imaging and analysis software.
- Incubations can be performed for 1 to 5 days with up to 3 media exchanges with the current format.

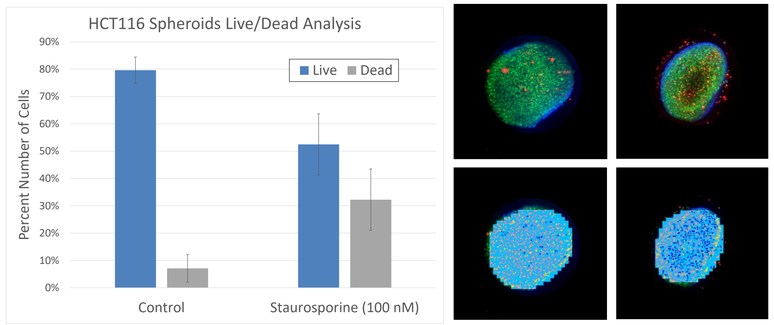


Figure 1. Live Dead Analysis of HCT 116 Spheroids incubated for 24 hours with and without 100 nM Staurosporine. Cells were stained with a mixture of DAPI, Calcein AM, and EthD.

AUTOMATED REAGENT EXCHANGE

The Pu·MA System can automatically perform complex assay steps using proprietary microfluidics. All reagents are loaded into flowchips and then incubation, media exchanges, cell secretion sampling and other steps are executed by the system program.

- 3D Cell models remain in the protected in bottom chamber
- Can exchange up to 95% of media without drying cells
- No direct fluid flow over cells to disrupt cell structures

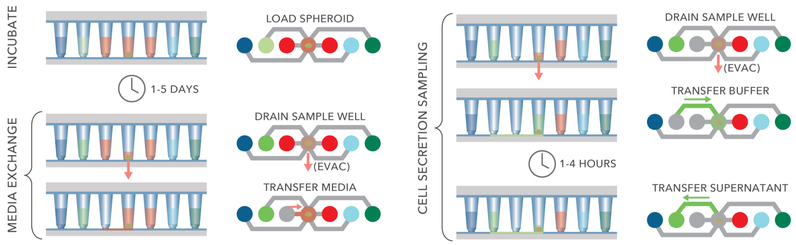


Figure 2. Schematic showing typical protocol steps available with the Pu·MA System. All steps are performed automatically inside an incubator.

Fluid transfer repeatability was demonstrated by exchange of multiple buffers back and forth between Pu·MA System flowchip wells.

- 20 µl fluid transfers in 8 lanes for 3 different flowchips (Source Well to Sample Well)
- Fluid was transferred to a Sample Well, incubated for 30 min, and then transferred back to the Source well.
- Finale volume in the Source well was measured gravimetrically
- Final Volume CV = 3%

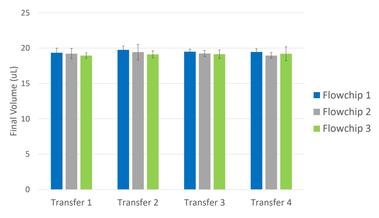


Figure 3. Fluid transfer demonstration results. Error bars represent +/- 1 SD (n=8)

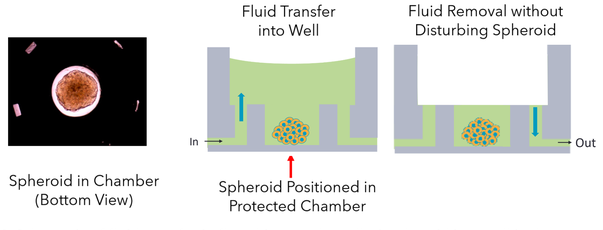
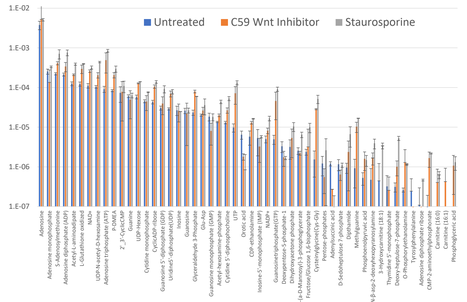


Figure 4. Schematic of fluid transfer in flowchip sample well. A spheroid in a well is shown on Left.

SINGLE SPHEROID METABOLOMICS

Use of Pu·MA System for *in situ* lysing of spheroids for metabolomic analysis was successfully demonstrated. The ability to lyse organoids *in situ* in order to capture metabolomic profiles with minimal perturbation shows great promise for oncology research.[3]

Colon cancer spheroids (2K cells) were incubated at 37°C with compounds (C59 Wnt inhibitor[4] and Staurosporine kinase inhibitor) for 2 hours and then automatically lysed by the Pu·MA System with 80% acetonitrile solution. Over 50 metabolites were measured in the lysates with excellent signal/noise. Significant differences in metabolomic profiles were observed between untreated (Control) and treated spheroids.



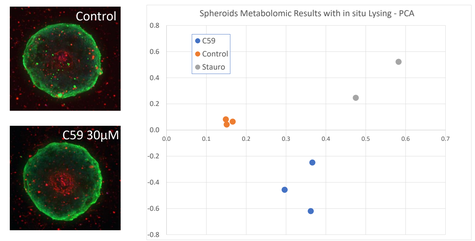
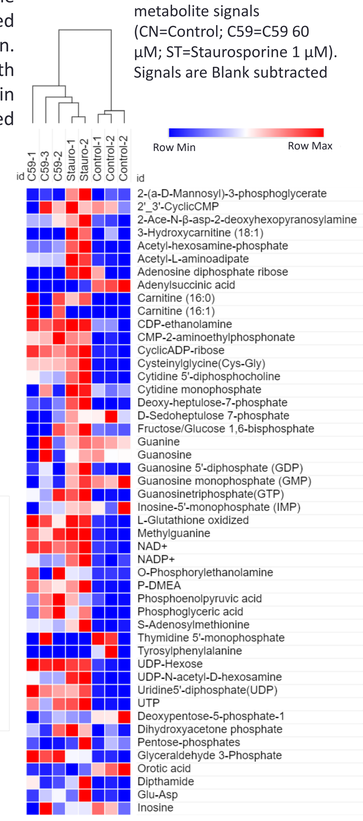


Figure 6. Top: Metabolite signals from HCT116 spheroids. Error bars represent +/- 1 SD (n=3). **Bottom:** Confocal images of representative spheroids and Principle Component Analysis of results.

Figure 5. Cluster analysis of metabolite signals (CN=Control; C59=C59 60 µM; ST=Staurosporine 1 µM). Signals are Blank subtracted



CELL VIABILITY ASSAY

Cell viability was measured for HCT116 spheroids incubated with compounds for 48 hours in Pu·MA System flowchips. (Compounds: C59 30 µM; Mitomycin 30 µM; Etoposide 100 µM).

Amount of ATP present in each spheroid sample was measured using the CellTiter-Glo assay (Promega). 10 µl of CellTiter-Glo reagent was automatically transferred into the sample wells and incubated for 10 min. Luminescence signal was measured using a SpectraMax iD5 Plate Reader (Molecular Devices).

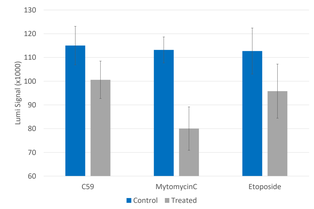


Figure 7. Relative amount of ATP in Control and Treated HCT116 spheroids as determined by luminescence signal. Error bars = +/- 1 StDev (n=3)

CONCLUSIONS

- We have demonstrated capabilities of a novel automated 3D Cell-based assay system that performs complex protocols with spheroids in an incubator environment.
- Long term toxicity was assayed using high resolution confocal imaging of spheroids incubated with various compounds and Live/Dead staining and analysis.
- The Pu·MA System has also been shown to perform *in situ* lysing of spheroids to provide highly sensitive metabolomic profiling with minimal perturbation.
- The ability to analyze spheroids and organoids *in situ* in order to capture toxicity information and metabolomic profiles shows great promise for oncology research.

MOLECULAR DEVICES

PROTEIN FLUIDICS