Automated 3D Cell-Based Assays Using a Novel Flowchip System and High Content Imaging

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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. 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[1,2]. We present here results from automated organoid assays using a Pu·MA System coupled with high content imaging and fast fluorescence kinetic read-outs. The flowchip contains organoid sample wells connected to multiple reservoirs that can contain various assay reagents. Organoids are located in protective chambers that allow media exchange, sample staining, wash steps, and other processing all to be performed without disruption to, or loss of 3D sample. The bottom of the sample chamber is thin optically clear plastic compatible with high resolution fluorescence imaging[3]. We present two applications for disease modeling using the Pu·MA System.

- Assaying cytotoxicity effects of anti-cancer drugs on cancer spheroids.
- Functional evaluation of calcium oscillations in neurospheroids.

This novel assay method using microfluidics enables automation of 3D cell-based cultures that mimic *in vivo* conditions, performs multi-dosing protocols and multiple media exchanges, provides gentle and convenient handling of spheroids and organoids, and allows a wide range of assay detection modalities.

Pu·MA SYSTEM 3D CELL ASSAYS

Pu·MA System 3D Flowchips are designed with chambers and reservoirs arranged in a convenient multi-well plate format (384-well spacings) and provide up to 32 organoid samples 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 a built-in program. Assay protocols are pre-loaded into the system and accessed using an intuitive touch-screen interface. The whole Pu·MA System can be placed into an incubator to run assays at 37°C and 5% CO_2 (**Figure 1**). The system architecture and use of pneumatics to move fluids provides gas exchange to the sample chambers to maintain cell health.

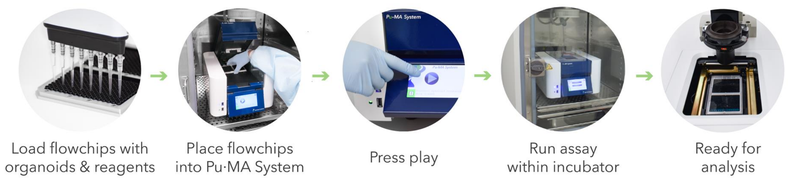

Figure 1. Schematic of the automated organoid assay workflow coupled with high content 3D imaging.

INSTRUMENTATION

The Pu·MA System and 3D Flowchip features:

- Automated media exchanges occur with cells in protected chambe
- 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 assay protocols can be edited via the Pu·MA System Software

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

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

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

References:
1. Human organoids: Tools for understanding biology and treating diseases. Schutgens, F. & Clevers H. (2020) 15(1): 211. Mech. Of Disease.
2. Three-dimensional in vitro cell culture methods in drug discovery and drug repositioning. Langhans, S.A (2018) 9:1 Frontiers in Pharma.
3. High-Content Assays for Characterizing the Viability and Morphology of 3D Cancer Spheroid Cultures. Sirenko, O. et al (2015) 13, 402, Assay and Drug Dev. Tech.

ONCOLOGY DISEASE MODELING

- HeLa and HepG2 cells were dispensed 2,000 - 4000 cells per well (384-well ULA round bottom plate, Corning) and incubated for 72 hours until they formed tight spheroids.[2]
- Formed spheroids and assay reagents were dispensed into to Pu·MA System flowchips and the processed as shown in **Figure 2**. Spheroids were treated with a serial dilution of staurosporine in triplicate. Staining was done after overnight incubation.
- Imaging was done with an ImageXpress Micro Confocal Imaging system and analysis performed using MetaXpress High-Content Image Acquisition and Analysis software[3]. Customized analysis for multiparametric outputs was done using a protocol created in the MetaXpress Custom Module Editor. Results of imaging and analysis are shown in **Figure 3**.
- Conditioned media was recovered from wells, pooled together, and assayed for VEGF using a 96-well plate ELISA (Human VEGF DuoSet ELISA, R&D Systems)

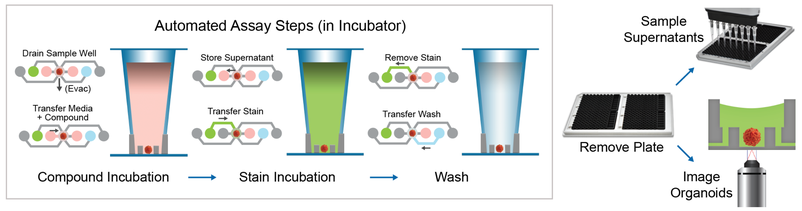

Figure 2. Protocol steps for cancer spheroid assay. Spheroids were treated overnight with staurosporine. Staining reagents (1 µM calcein AM, 3 µM EthD-1, 33 µM Hoechst 33342) were added to flowchips after incubation period due to instability at 37°C. Plates with flowchips were removed from the Pu·MA System for downstream analysis.

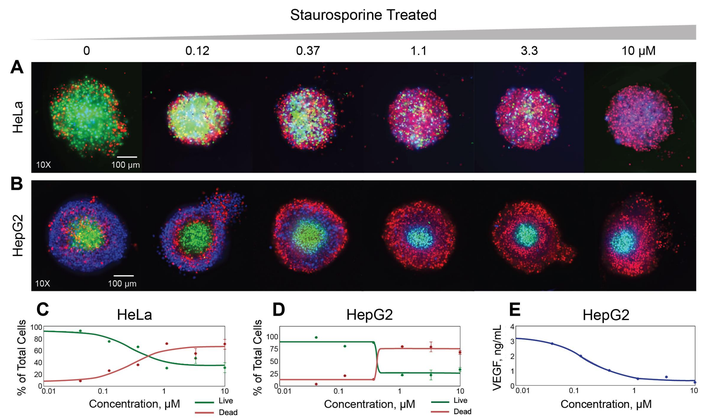

Figure 3. Quantitative assessment of the effect of staurosporine on spheroid morphology and viability by high content imaging. Maximum projection images shown for HeLa (**A**) and HepG2 (**B**) control spheroids and spheroids treated with indicated increasing concentrations of staurosporine. HeLa cells were stained with Hoechst nuclear dye (blue), calcein AM viability dye (green) and EthD-1 dead cells dye (red). HepG2 cells were stained with Hoechst nuclear stain (blue), EthD-1 dye (red), NucView dye for apoptotic cells (green). **C & D:** Concentration-dependent plots shown below for percentages of Live call and Dead cells quantitated in the images of spheroids for different concentrations of staurosporine. **E:** Concentration of VEGF measured in supernatants from HepG2 spheroids as a function of staurosporine concentration. Supernatants from two spheroids were combined and analyzed by ELISA for each measurement. No VEGF was detected in HeLa spheroid supernatants.

MICROFLUIDIC FLOWCHIP TECHNOLOGY

Each Pu·MA System Flowchip contains eight lanes of reagent wells connected by microfluidic channels. Four flowchips are placed in holder that locates all wells in a 384 multiwell plate format providing for 32 samples per assay. The Compound wells in each lane hold media, compounds, or additional assay reagents. Organoids are placed into the Sample Well and located in a protected chamber at the bottom of the well **(Figure 4).** This allows reagents to be directed in and out of the Sample well without disturbing or drying out the microtissue.

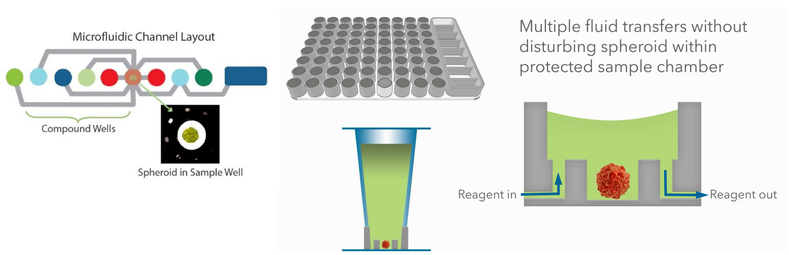

Figure 4. Schematic of Flowchips showing channel layout and sample well with proprietary protected chamber. The diameter of the sample well clear aperture is 1.2 mm.

NEUROSPHEROID FUNCTIONAL ASSAYS

- Intracellular Ca^{2+} oscillations can be used to observe neuronal "firing" in 3D neural cell cultures and assay effects of neuroactive compounds.
- In this assay demonstration neurospheroids (microBrain 3D, StemoniX) were pre-loaded into Pu·MA System flowchips, stained with FLIPR Calcium 6 Kit, and treated with compounds.
- Assay workflow (**Figure 5**) provides complete media exchange and addition of increasing concentrations of compounds to the same micro-tissue.
- Ca^{2+} oscillations were measured using FLIPR Tetra High-Throughput Cellular Screening System. The flowchips and holder are compatible with the FLIPR optical system allowing quick measurements of the neurospheroids and return to incubator environment **(Figure 6)**.

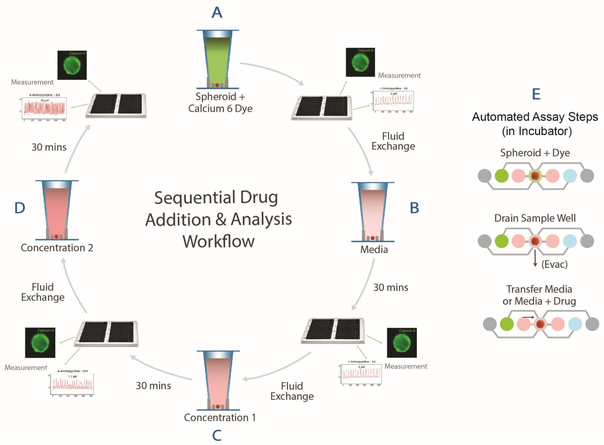

Figure 5. Assay protocol workflow for neurospheroid function assay. Compounds are automatically transferred into the sample well. Flowcihps are easily removed from the PuMA System for FLIPR measurements. Multiple compound treatments were done on the same neurospheroids.

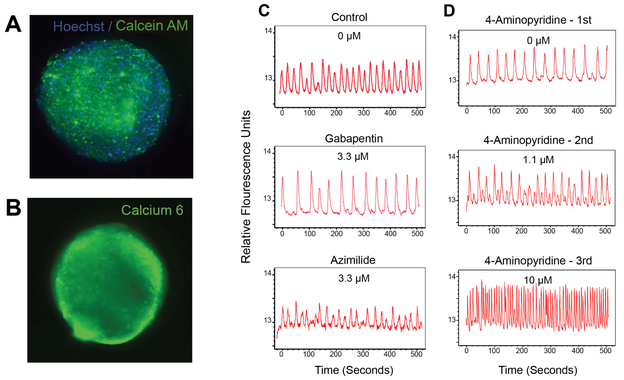

Figure 6. High content imaging and recordings of Ca^{2+} oscillations in neurospheroids. **A:** 2D Projection image of a neurospheroid stained with Hoechst (blue) and calcein AM (green). **B:** Image of neurospheroid stained with Calcium 6 dye. **C & D:** Kinetic fluorescent recording or calcium oscillations measured by FLIPR Tetra High-Throughput Cellular Screening System. Recorded patterns were analyzed, and peaks counted by PeakPro2 software. **D:** Concentration response of a single neurospheroid to 4-Aminopyridine. 1st measurement was with media only; 2nd with 1.1 µM 4-Aminopyridine after 30 min incubation; 3rd with 10 µM 4-Aminopyridin after an additional 30 min incubation.

CONCLUSIONS

- We have demonstrated capabilities of a novel automated 3D cell-based assay system that performs complex protocols with 3D cell models in an incubator environment.
- Cancer spheroid treatments were assayed using high resolution confocal imaging of spheroids incubated with various compounds and Live/Dead staining and analysis.
- Automated neurospheroid treatments were performed with the Pu·MA System. Compatibility with FLIPR allows multiple measurements of individual spheres with minimal time outside an incubator environment.
- The ability to analyze spheroids and organoids *in situ* in order to capture toxicity information and perform functional assays shows great promise for drug discovery.

MOLECULAR DEVICES

PROTEIN FLUIDICS