Novel assay methods for cancer patient derived organoids

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Introduction

In recent years, researchers have transitioned from traditional 2D assays to more complex 3D cell models, as they are shown to recapitulate the *in vivo* environment and serve as a more predictive tool for drug discovery. One area gaining attention is the use of patient-derived organoids (PDO) for oncology research. PDOs are generated from tumor biopsies and can serve as models to understand tumor growth and resistance to therapies. With this transition, there has been an increased need to automate the processing and analysis of 3D cell culture assays for a more simplified, "hands-off" workflow that yields better results. We present here results from semi-automated PDO assays using a Pu·MA System coupled with high-content imaging. The flowchip contains PDO samples located in protective chambers connected to multiple reservoirs that contain various assay reagents. The system allows media exchange, sample staining, wash steps, and other processing all to be performed without disruption to, or loss of the samples. In the application presented here, PDOs from a triple negative breast cancer (TNBC) patient were grown and transferred into flowchips. PDOs were incubated in the presence of anti-cancer compounds for 24–48 hours and then stained for either viability markers or E-cadherin/CD44 markers. High resolution images of PDOs were obtained using ImageXpress® Micro Confocal High-Content Imaging System and analyzed with MetaXpress® High-Content Image Acquisition and Analysis Software. Resistance of PDOs to chemotherapeutic agents was observed consistent with tumor response in the patient. 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 assays

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 (Figure 1).

Multiple reagent exchanges are performed with assay protocols using built-in program. Assay protocols are preloaded into the system and run using an intuitive touchscreen 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 provide gas exchange to the sample chambers.

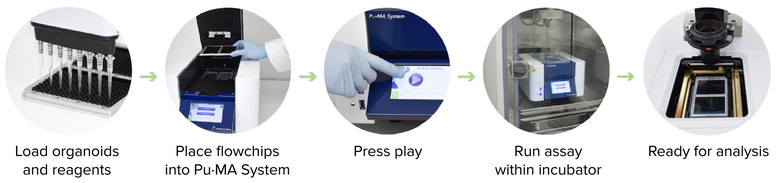
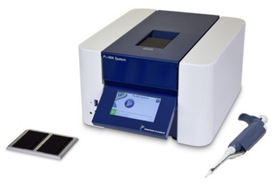


Figure 1. Schematic of the automated organoid assay workflow coupled with high-content 3D imaging.

Instrumentation

Pu·MA System and flowchips include:

- 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



ImageXpress Micro Confocal High-Content Imaging System includes:

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

The system is controlled by MetaXpress High-Content Image Acquisition and Analysis Software

PDO formation & staining

- Spheroids were formed from TU-BcX-4IC cells derived from a primary tumor. The tumor exhibited rapid pre-operative growth despite combination neoadjuvant therapy with adriamycin, cyclophosphamide, and paclitaxel. TU-BcX-4IC was classified as Metaplastic breast cancer (MBC) with a TNBC subtype.[1]
- TU-BcX-4IC 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]
- The spheroids were coated with magnetic nanoparticles (NanoShuttle (NS), Greiner BioOne) to aid in the dispense and placement of the spheres in Pu·MA System flowchips (see Figure 2).
- Spheroids were treated and stained semi-automatically using the Pu·MA System as shown in Figure 3. The system can be used for both viability and immunofluorescence staining.
- Imaging was done with an ImageXpress Micro Confocal system and analysis performed using MetaXpress software. Customized analysis for multiparametric outputs was done using a protocol created in the MetaXpress Custom Module Editor. Images of spheres stained with E-cadherin and CD44 markers are shown in Figure 4.

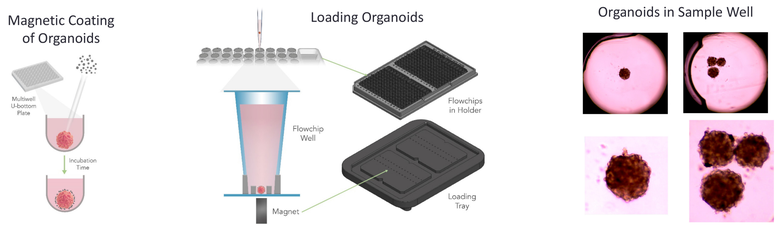


Figure 2. Schematic of the protocol for magnetic labeling and positioning of PDOs. Transmitted light images of PDOs are shown on the Right. PDOs are centered in the wells with no optical artifacts from NS particles.

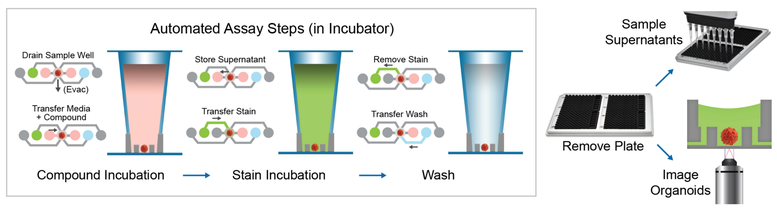


Figure 3. Protocol steps for PDO assays. Organoids were treated overnight with compounds and then stained with viability dyes (1 µM calcein AM, 3 µM EthD-1, 33 µM Hoechst 33342) or FL-labeled primary antibodies to E-Cadherin and CD44 (BD Biosciences). Conditioned media can be removed for additional analyses.

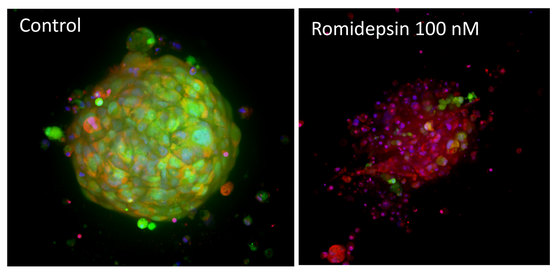


Figure 4. PDOs labeled for E-Cadherin (Green) and CD44 (Red). Images are maximum projection of confocal stacks taken with 20X water immersion (WI) objective. Treatment with romidepsin results in loss of E-Cadherin and disruption of organoids.

Microfluidic flowchip technology

Each Pu·MA System flowchip contains eight lanes of reagent wells connected by microfluidic channels. Four flowchips are placed in a 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. This allows reagents to be directed in and out of the sample well without disturbing or drying out the microtissue (Figure 5).

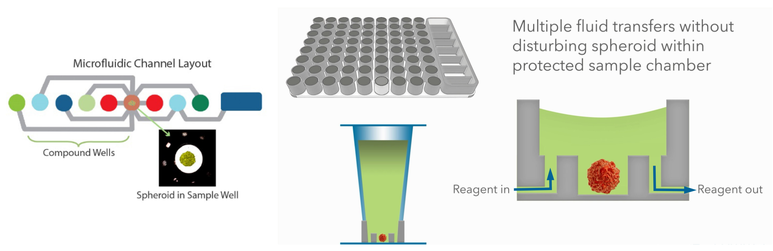
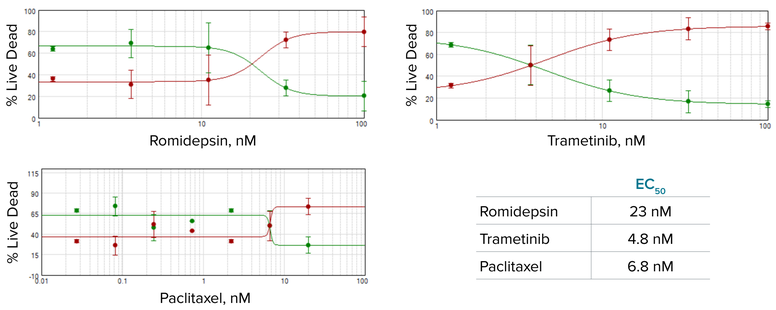


Figure 5. Schematic of flowchips showing channel layout and sample well with proprietary protected chamber. The diameter of the sample well clear aperture is 1.2 mm.

Cancer disease modeling

- TU-BcX-4IC spheres were treated for 48 hours with three compounds from the NCI 60 panel of cancer therapeutics:
  - Romidepsin – A histone deacetylase (HDAC) inhibitor
  - Trametinib – Selective reversible allosteric inhibitor of MEK1 and MEK2 activity
  - Paclitaxel – A mitotic inhibitor that interferes with microtubule growth
- Spheres were stained in a Pu·MA System with cell viability markers and imaged on ImageXpress Micro Confocal system (Figure 6). Image stacks were analyzed for percent Live (calcein AM positive) and Dead (EthD-1 positive) cells (Figure 7).
- An insensitivity of the PDOs to paclitaxel was measured consistent with the clinical presentation of the patient tumor.

Figure 6. Images of PDOs treated with romidepsin (Top) and trametinib (Bottom). Images are maximum projections of confocal stacks acquired with a 10X objective.



	EC_{50}
Romidepsin	23 nM
Trametinib	4.8 nM
Paclitaxel	6.8 nM

Figure 7. Concentration response curves for PDOs treated with romidepsin, trametinib, and paclitaxel. EC_{50} values for romidepsin and trametinib were found to be within normal ranges while paclitaxel was significantly higher. This is consistent with the response of the patient's tumor to treatment.

Conclusions

- We demonstrated capabilities of a novel automated organoid assay system that performs complex protocols with 3D cell models in an incubator environment.
- Patient-derived organoids derived from primary tumors were assayed for compound response using high resolution confocal imaging of cell surface markers and viability stains for Live/Dead.
- The ability to analyze spheroids and organoids *in situ* in order to capture toxicity information and perform functional assays shows great promise for disease modeling.

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