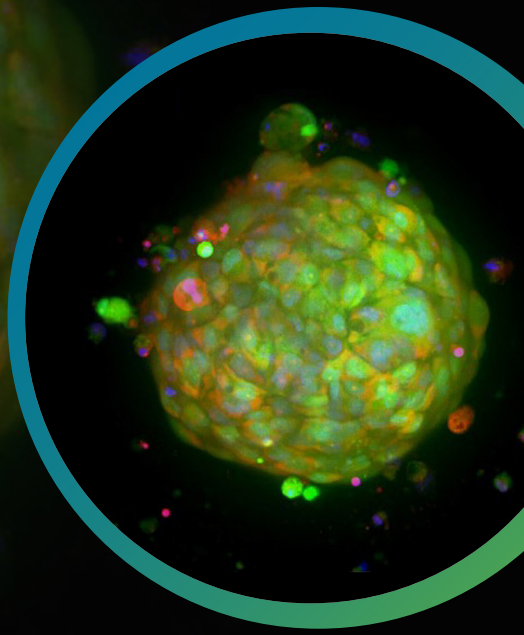
APPLICATION NOTE

In vitro 3D cancer assays using a microfluidics system, magnetic nanoparticles, and high-content imaging



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Introduction

In recent years, the need to have physiologically accurate 3D cell models for research and drug development has been steadily growing. Researchers have been perfecting the formation and maintenance of various 3D cell models for understanding both disease and normal physiology[1,2]. Some of the limiting factors have been the ability to perform complex assays easily and quickly with precious samples, especially with patient-derived material. The manual treatment, staining, and processing of spheroids and organoids is typically labor-intensive and prone to disruption or loss of samples. In addition, high-content imaging can be challenging because organoids tend to be located at the edges of wells, or may be located in different positions and heights within a well. Also, when performing drug treatments and assays in multiwell plates, one is limited by the number of readouts per sample.

New technologies are rapidly developing to streamline and facilitate the process. We used a microfluidic-based device, Pu·MA System® 3D MAG and 3D flowchips (Protein Fluidics) to perform automated assay steps with magnetically coated 3D cell models (Figure 1). 3D cell models coated with magnetic nanoparticles, NanoShuttle™,[3] were transferred and centered into flowchip wells using embedded magnets. The automated microfluidics system allowed automated media exchange, compound addition, and processing of microtissues. Then the ImageXpress® Micro Confocal High-Content Imaging System (Molecular Devices) was used to provide efficient resolution of the 3D structures along with advanced analysis to characterize and quantify spheroid and organoid morphology and compound effects.

Benefits

- Streamline complex 3D assays with automated microfluidic control
- Overcome common 3D assay challenges with magnetically coated 3D cell models and an automated assay workflow
- Measure multiple assay readouts from secreted factor analysis to high-content image analysis

We demonstrated the automated workflow for two complex 3D assays:

- Drug treatment, staining, and analysis of 3D cancer spheroids: HeLa spheroids were treated with compounds for 24–48 hrs. Automated viability staining was used to determine concentration response curves compound treatments.
- Evaluation of drug sensitivity and immunofluorescence analysis for biomarkers: Triple-negative breast cancer (TNBC) patient-derived organoids (PDOs)[4] were assayed for drug response. PDOs were stained with IF markers and imaged in the flowchips.

Pu·MA System and 3D MAG workflow

Pu·MA System and 3D flowchips have been designed for streamlined automated organoid assays. Pu·MA System 3D MAG consists of the standard Pu·MA System device with the 3D MAG modification which enables magnetically coated 3D cell models to be held within the protected sample chamber during the assay steps (Figure 1 & 2).

The NanoShuttle treatment of 3D spheroids was done according to protocols from Greiner Bio-One[3]. Here, we demonstrate a novel use of this bioprinting process coupled with our automated assay system.

The magnetized 3D cell models were transferred into the flowchips with the help of a loading tray which contains small (~1 mm dia.) magnetic rods and ensures centering and holding of the organoids coated with magnetic nanoparticles (Figure 2). The flowchips are then placed into the system within the incubator. The system architecture of the Pu·MA System and use of pneumatics to move fluids provides gas exchange to the sample chambers within the incubator environment.

The flowchips are designed in a convenient multiwell plate format (384-well spacings SLAS/ANSI standard) which makes them amenable to multichannel or automated liquid dispense system. They have optically clear bottoms for imaging with any fluorescence or confocal imaging system (Figure 1). This, along with the ability to center and maintain the position of the magnetically coated 3D models throughout the entirety of the assay, facilitates the automation of the same acquisition field across all sample wells. Furthermore, the robust autofocus capabilities of the ImageXpress Micro Confocal system, aids in acquiring high-resolution images of the entire spheroid or organoid in each sample well.

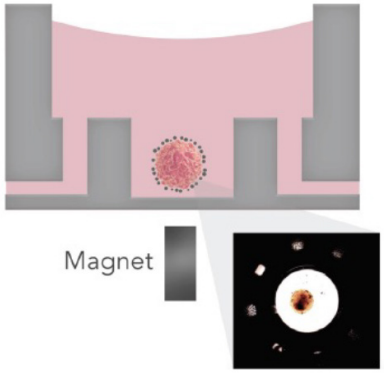

Figure 1. *Schematic of a positioned spheroid within flowchip protected sample chamber in a Pu·MA System 3D MAG. Inset shows a bright field image of Nanoshuttle coated mouse islets within the sample chamber.*

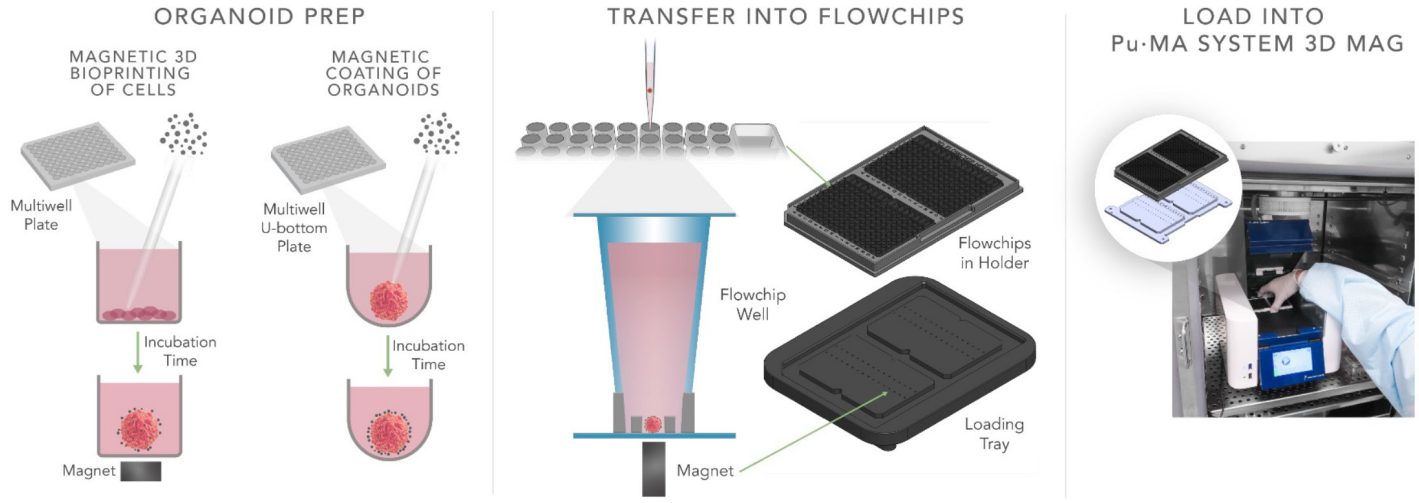

Figure 2. *Assay steps for magnetic coating, transfer into flowchips, and loading into Pu·MA System 3D MAG. The workflow used 1 µl of NanoShuttle (Greiner Bio-One) per 10,000 cells for cells that were magnetically bioprinted, while pre-formed 3D cell models were incubated with NanoShuttle at a ratio of 0.1 µl per sample (3D microtissue). After incubation, they are transferred into flowchips with the aid of the loading tray. Reagents are loaded in the wells adjacent to the sample well and the flowchip holder placed into the Pu·MA System 3D MAG within the incubator. Upon the completion of the automated assays, the flowchips were taken to the ImageXpress Micro Confocal system for subsequent high-content, 3D imaging and analysis.*

Drug treatment, staining, and imaging of 3D cancer spheroids

The application of the workflow using microfluidics sample processing and magnetic beads was demonstrated with HeLa spheroids for cell viability and cell death in response to the effects of selected cytotoxic drug treatments[5]. HeLa cells were magnetically bioprinted into a cell-repellent surface 384-well microplate at 2,000 cells per well following protocols from Greiner Bio-One[3]. HeLa cells, coated with NanoShuttle, were incubated at 37°C, 5% CO_2 for one hour on top of the magnetic holding drive. The plate was then left in the incubator for a day prior to transferring the formed spheroids into the flowchip wells and processed using Pu·MA System 3D MAG.

In the example below, HeLa 3D spheroids were automatically treated with different compound concentrations (staurosporine, 0 to 10 µM) for 22 hours, then media was exchanged to staining solution and incubated for two hours (Calcein AM, Hoechst and Ethidium Homodimer 1), then samples were washed with PBS.

After processing steps, flowchips were taken out of the device and imaged within the flowchips using ImageXpress Micro Confocal Imaging system. Images were acquired with 10X or 20X objectives with a 3D Z-stack of 12-20 images 510 µm apart, and the 2D projection images were analyzed with a simple custom module in MetaXpress® High-Content Image Acquisition and Analysis Software to rapidly quantify cell viability (Figure 3). Numbers of total live or dead cells upon treatment with staurosporine were quantitated using MetaXpress software. Live dead analysis application module was used for analysis of either 2D projection images (maximum projection) or 3D analysis with Custom Module Editor.

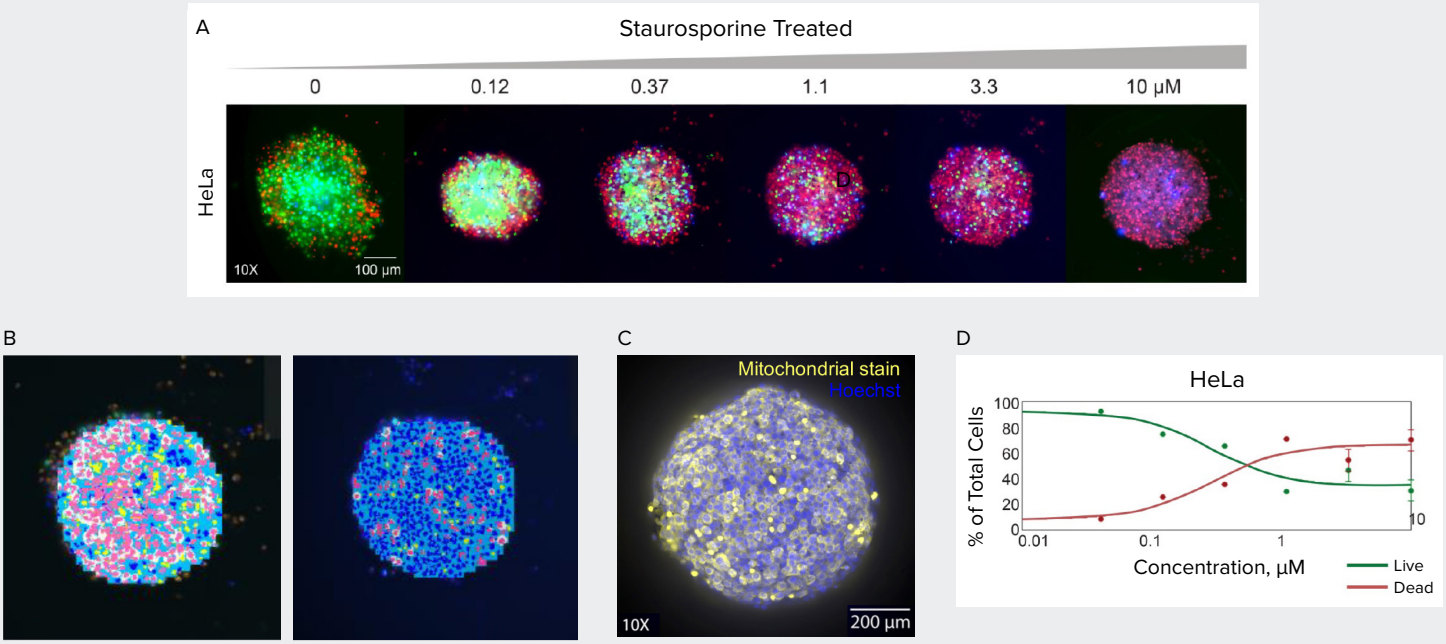

Figure 3. *Drug treatment and staining. A) Confocal imaging of HeLA spheroids treated with increasing concentrations of staurosporine (0 to 10 µM) and stained with nuclear stain (Hoechst, Blue), cell viability dye (calcein AM, Green), cell death stain (EthD-1, Red). Images were taken using a 10X Plan Fluor objective. B) Image analysis masks showing treated and untreated samples indicating spheroid area (light blue), live cells (pink), and dead cells (dark blue). C) Untreated HeLa spheroid stained and imaged within flowchips (Hoechst and Mitochondrial marker for cell viability (Mitotracker™ Orange). D) Graph indicating percentage cell number live versus dead cells upon treatment with different staurosporine concentrations (0.01–10 µM).*

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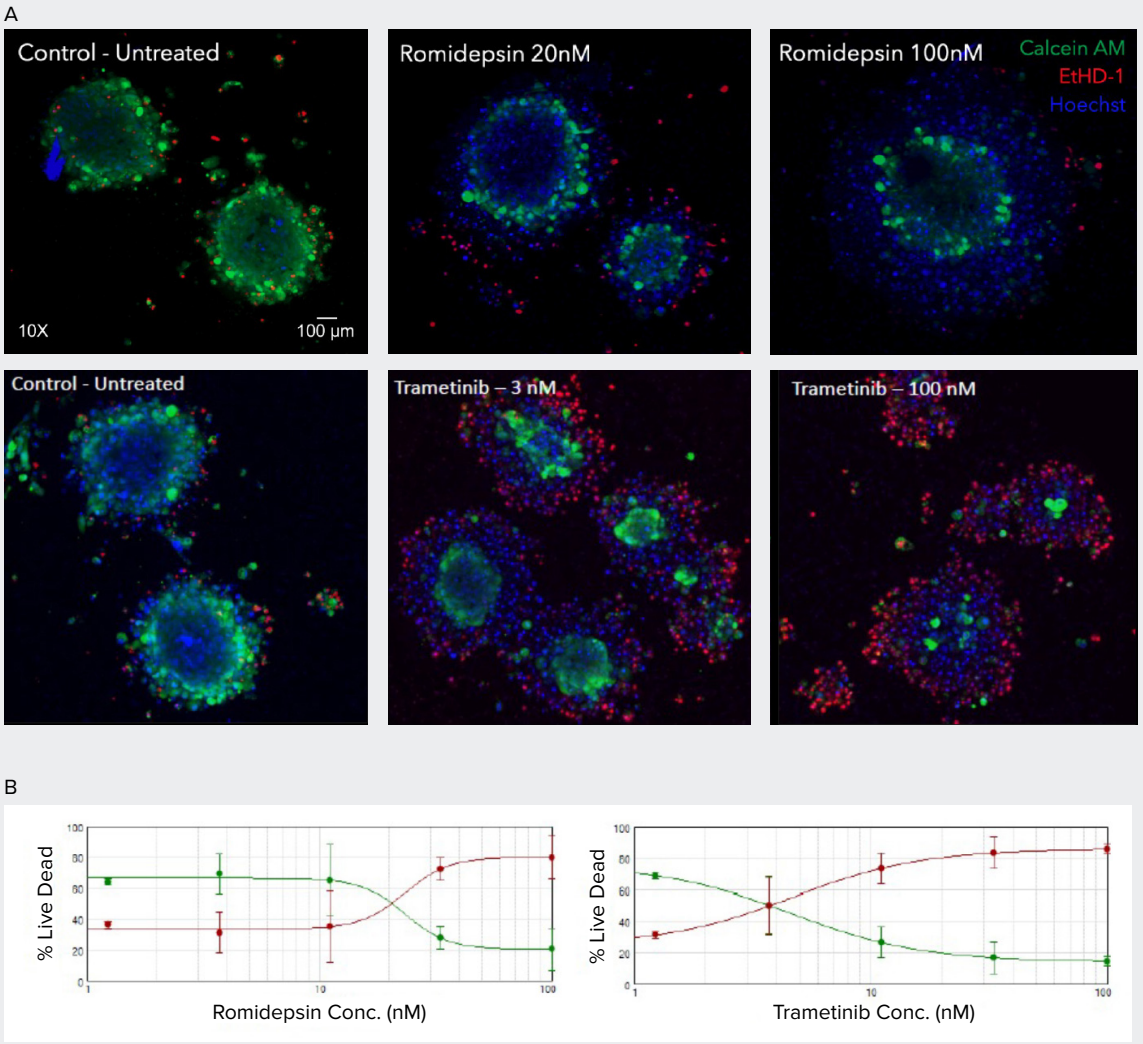

Figure 4. *PDOs drug treatment assay. A) Confocal images of PDOs treated with romidepsin (top) and trametinib (bottom) and stained with nuclear stain (Hoechst, Blue), cell viability dye (calcein AM, Green), cell death stain (EthD-1, Red). Images are maximum projections of confocal stacks acquired with a 10X objective. B) Concentration response curves for PDOs treated with romidepsin and trametinib. EC_{50} values for romidepsin and trametinib were found to be consistent with previously obtained results using 2D cell models. C) 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. Images are maximum projections of confocal stacks acquired with a 20X objective.*

In vitro drug sensitivity assay and biomarkers detection in primary tumor 3D models

Patient-derived organoids (PDOs) were formed from TU-BcX-4IC (cells derived from a primary triple negative breast tumor) grown into tight spheres as described previously[4]. Here, we further optimized the method by using micro-patterned plates for formation of organoids. Using a single well of a 24-well AggreWell™ 400 microwell plate allowed us to generate approximately 1200 consistently sized PDOs. To create organoids, the TU-BcX-4IC cells were plated at a density of 2,000 cells per microwell, and the plate was incubated for two days to allow for formation of compact 3D organoids. 100 µl of NanoShuttle was added to the wells, and the organoids were incubated with the NanoShuttle for three hours before harvesting and transferring the PDOs into the flowchips. The drugs used in this study were romidepsin and trametinib. 3D organoids were treated at different concentrations of drugs for 48 hours. Then automated drug treatments and immunofluorescence staining were performed within Pu·MA System 3D MAG. Live samples were stained with viability stains (Calcein AM, Hoechst and Ethidium Homodimer 1) as described above, then samples were imaged with ImageXpress Micro Confocal system and analyzed using Live-Dead analysis module of MetaXpress software (Figure 5A and 5B). Images were captured as a confocal Z-stack, and the stack of images were compressed into 2D projection images for analysis of cell viability. Multiparametric readouts were generated and concentration response curves were analyzed (Figure 4A, 4B).

Percent of live and dead cells were determined by analysis and used to derive effective concentrations for compounds. Then samples were fixed and restained for specific markers E-cadherin and CD44.

Immunofluorescence staining of PDOs for specific markers was performed within the Pu·MA System following romidepsin treatment. E-cadherin and CD44 markers were detected in the PDOs after treatment as shown in Figure 5C. Confocal images were acquired with the ImageXpress Micro Confocal Imaging system. The results showed disruption of the PDOs upon drug treatments as well as decreased expression of E-cadherin.

Conclusion

We have demonstrated improved assay workflow using magnetic coated 3D cell microtissues on the Pu·MA System 3D MAG followed by high-content imaging and analysis with ImageXpress Micro Confocal system. The combined workflow enhances the ability to perform: automated organoid/spheroid processing, in situ media sampling for secreted factors, drug treatment and multiparametric analysis, and immunofluorescence staining for biomarkers.

Using streamlined workflows and Pu·MA System protocols, we showed staining and imaging using different 3D cell models in response to different drugs and concentrations. We demonstrated automated drug treatment, staining, and processing of samples, as well as analysis of viability, marker expression, and phenotypic changes by imaging that can be used for rapid evaluation of drug sensitivity in 3D tumor samples.

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