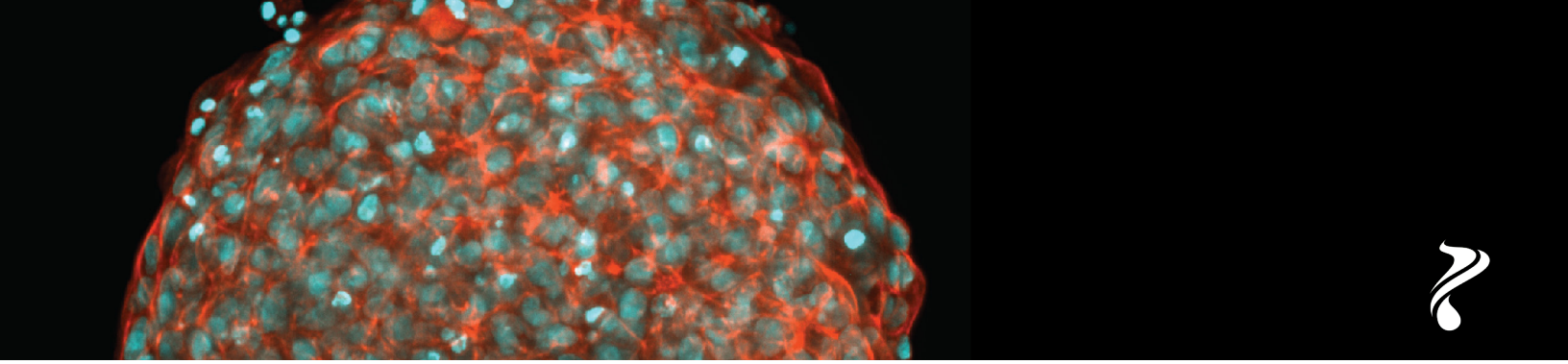

APPLICATION NOTE

Automated Biomarker Assays for 3D Cell Models on the Pu·MA System® with Xeno-free VitroGel® Hydrogel

Introduction

For decades cell biology principles, drug activities, cell responses, and tissue morphogenesis have been determined in 2D cell monolayer culture systems. But these 2D cultures lack most of the interactions occurring in 3D in native tissues. 3D in vitro cell culture systems are increasingly popular and have great potential as tools for disease modeling and drug discovery[1]. Their ability to resemble tissue-like structures, characteristics, interactions, and spatial organization can help accelerate the emergence of novel and effective therapies. For creating physiological in vitro environments, 3D cell culture systems often require either an extracellular matrix (ECM) or hydrogel, to provide structural support and stability for the cells, but also to facilitate cell signaling that influences morphology and function[2]. The Pu·MA System® is a benchtop instrument that uses unique microfluidic flowchip consumables to automate complex multi-step cell assays and data acquisition from 3D cell models[3,4]. Here, we have developed an automated workflow using the Pu·MA System while incorporating the xeno-free hydrogel VitroGel® (TheWell Biosciences)[5] with the 3D cell models.

In this study, we optimized a workflow for creating minidomes with 3D cell models in VitroGel Hydrogel Matrix within the protected sample chamber of the flowchips. The flowchips were loaded with assay reagents for automated assays using the Pu·MA System, followed by imaging of 3D cell models within the flowchip, providing high-quality imaging data for phenotypic profiling (Figure 1). We have established protocols using either complex, animal-based ECM (e.g., Matrigel®) or the easy-to-use VitroGel hydrogel. Although Matrigel is widely used, it has assay limitations like undefined composition, lot-to-lot variability, murine components, and temperature dependency. Chemically defined, xeno-free VitroGel can be modified for desired outcomes and provides reproducible results. In this application note, we demonstrate applications using ECMs and engineered hydrogels for automated image-based profiling 3D assays (Figure 1). We present the use of the Pu·MA System for two 3D cell culture applications using VitroGel:

1. Formation of 3D cell models
2. Cell biomarker detection of 3D cell models.

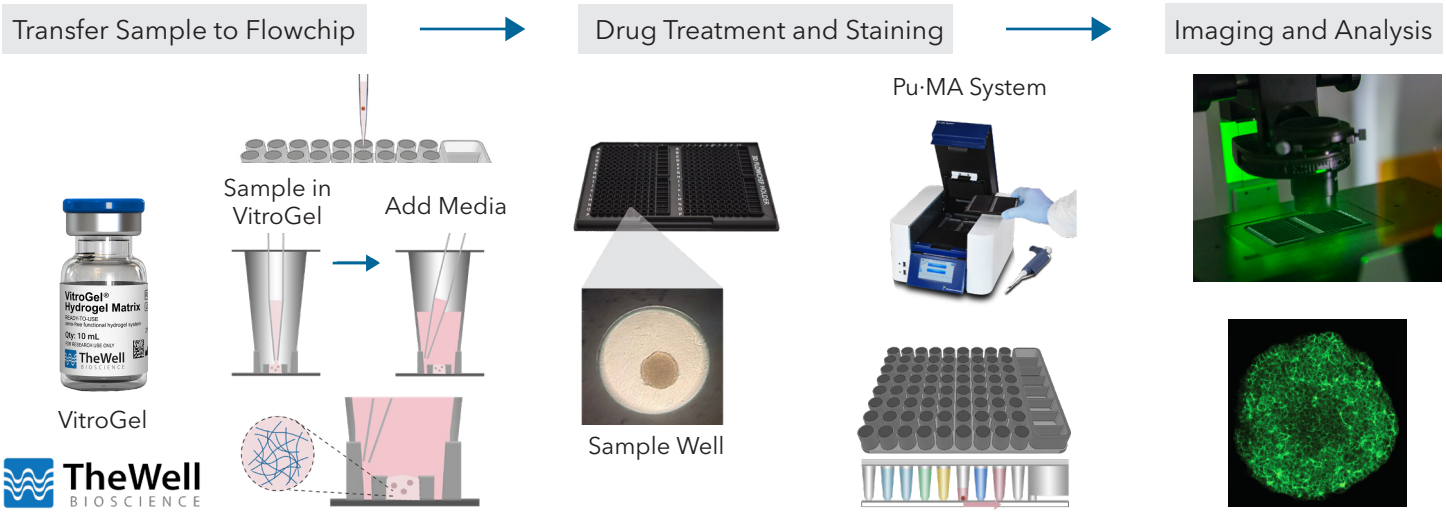

Figure 1. The workflow outlined shows creating minidomes with 3D cell model in VitroGel within the protected sample chamber of the sample well, loading flowchips with assay reagents for automated assays using Pu·MA System, followed by imaging of 3D cell models within the flowchip.

1 Formation of 3D cell models in ECM

We have developed a method of 3D cell model formation, staining and image-based profiling in the flowchip. Spheroids are formed from a cell suspension directly within the flowchip in ECM, eliminating the transfer step of pre-formed spheroids for assays. This method requires precise dispensing of cells in high concentration ECM (80%) in very small volumes directly to the protected sample chamber of the flowchip using automated pipetting with controlled dispensing speed. In this study, we monitored spheroid formation followed by viability staining and imaging in the flowchip in different ECMs.

Methods:

3D cell models: MCF7 cell suspension was prepared in 80% Matrigel and in VitroGel® Hydrogel Matrix (ready-to-use, TheWell Biosciences). VitroGel is a synthetic, animal-free, biofunctional transparent hydrogel, compatible with high resolution imaging. 2 µL of cells in ECMs were loaded to the protected chamber of the sample well in the flowchip using an automated pipette followed by 18 µL of media. Automated pipettes (Integra) allow for flow-rate control and precisely dispensing small volumes of cells + gel to create a minidome within the flowchip (Figure 2A). Automated media exchanges were executed within the Pu·MA System.

After 48 hours of spheroid formation, samples were stained with CyQuant + Ethidium Homodimer (EthD-1) viability staining cocktail using the Pu·MA System staining protocol.

Imaging: Confocal images were acquired using CellVoyager CQ1 Benchtop High-Content Analysis System (Yokogawa Electric Corporation) with a 20X long working distance dry objective using 488nm (CyQuant) and 561nm (EthD-1) channels. Z-stack images of the spheroids were acquired – total 100 µm in 10 µm steps and maximum intensity projections (MIP) were created.

Results:

MCF7 3D cell models were formed in the flowchip from single-cell suspension. This approach created multiple smaller spheroids which were located at different planes. These were visualized by Z-stack confocal images. Spheroids can be visualized by bright field microscopy (Figure 2B). Stained images showed high viability of the formed spheroids (Figure 2C, green) with only few dead cells present (red). This spheroid formation approach allows elimination of manual spheroid transfer to the flowchip, demonstrating the possibility to form cell aggregates in different ECMs. It can be used for downstream applications like therapeutic testing, cell markers expression and others.

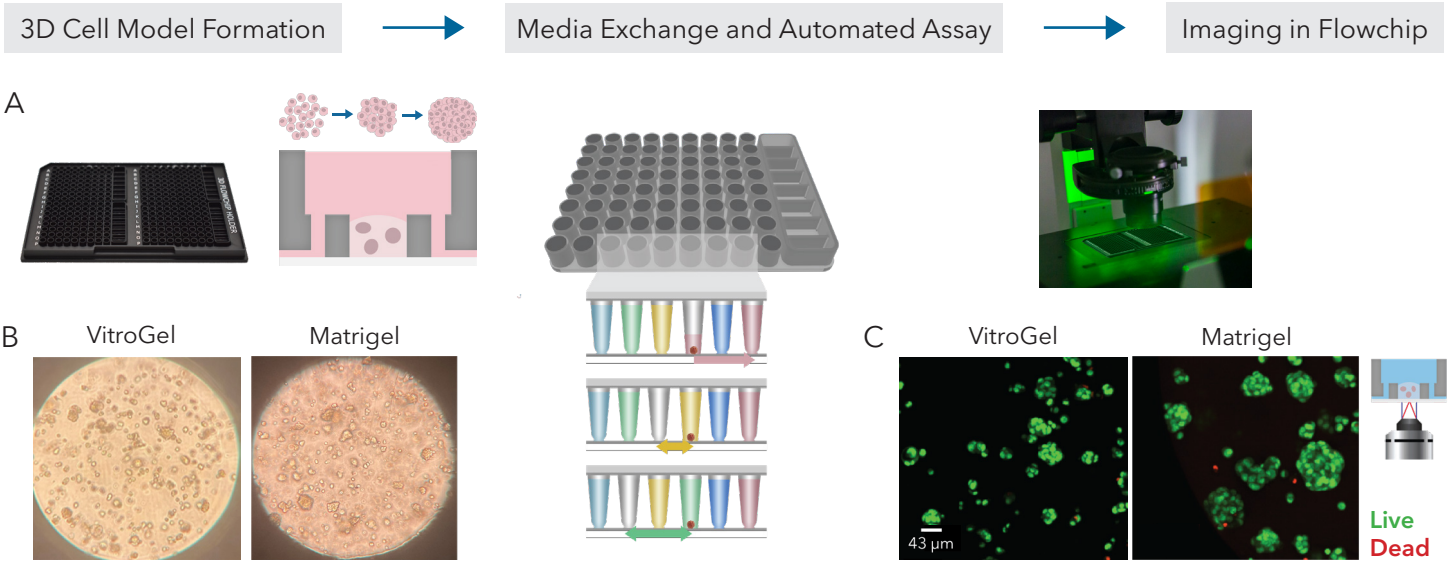

Figure 2. (A) Workflow for 3D cell model formation from single cell suspension. (B) Brightfield images of MCF7 spheroids formed within the flowchip using different ECM material (C) Viability staining of MCF7 spheroids using CyQuant (live, green) and Eth-D1 (dead, red) and imaged CellVoyager CQ1 confocal microscope.

2 Cell marker detection of 3D cell models in VitroGel

E-cadherin (an epithelial cell-cell adhesion protein) and Vimentin (intermediate filament protein in mesenchymal cells) are regarded as major markers for epithelial to mesenchymal transition[6,7,8]. Loss of E-cadherin is associated with cancer progression and unfavorable prognosis in breast cancer[9,10]. In this study, we have compared the expression of E-cadherin, Vimentin and F-actin (cytoskeletal protein) in two different breast cancer 3D models within VitroGel:

1. Cell line-based spheroids (MCF7): ER positive, non-aggressive, non-invasive, low metastatic
2. Patient-Derived Tumoroids (TU-BcX-4IC, designated as 4IC): cells derived from a primary triple-negative breast cancer patient. This tumor was classified as a metaplastic subtype with rapid growth, highly metastatic, aggressive, and resistant to therapy[11]

Methods:

3D Cell Models: For this study, spheroids were formed in an ultra-low attachment well plate and loaded into the flowchip in VitroGel RGD, a tunable hydrogel. VitroGel was diluted in VitroGel Dilution Solution (1:3 ratio). VitroGel RGD concentration was optimized to support spheroids in the flowchip without affecting the performance of microfluidic channels or fluid transfers. Samples were fixed using 4% Paraformaldehyde within the flowchip and followed by automated immunofluorescence (IF) staining in the Pu·MA System.

Imaging: High-resolution confocal imaging was done using CellVoyager CQ1 System within the flowchip. Confocal images were acquired at 10X and 40X using 405 nm (Nuclei Hoechst), 488 nm (E-Cadherin or Vimentin) and 561 nm (Phalloidin) channels. Z-stack images were acquired – total 120 µm z-stack in 10 µm steps and MIP created.

Results:

The epithelial-like and low malignant MCF7 spheroids showed intense peripheral E-cadherin staining prevalent at cell-cell contact areas, and no detectable expression of Vimentin (Figure 3A). 4IC tumoroids showed loss of E-cadherin expression while being positive for Vimentin. Loss of E-cadherin is consistent with high aggressiveness of the patient's native tumor (Figure 3B). F-actin distribution and organization were detected by Phalloidin staining. The acquired images were of high quality without autofluorescence. The data showed firstly that 3D cell models can be used for studying the interplay between cell-adhesion machinery, Vimentin, and F-actin, their role in epithelial-to-mesenchymal transition and metastasis. Secondly, VitroGel RGD, an easy-to-use, xeno-free, tunable, and reproducible matrix, can be considered as a Matrigel alternative for 3D cell-based assays with no imaging issues. Thirdly, the Pu·MA System can automate assays with 3D cell models that require ECM.

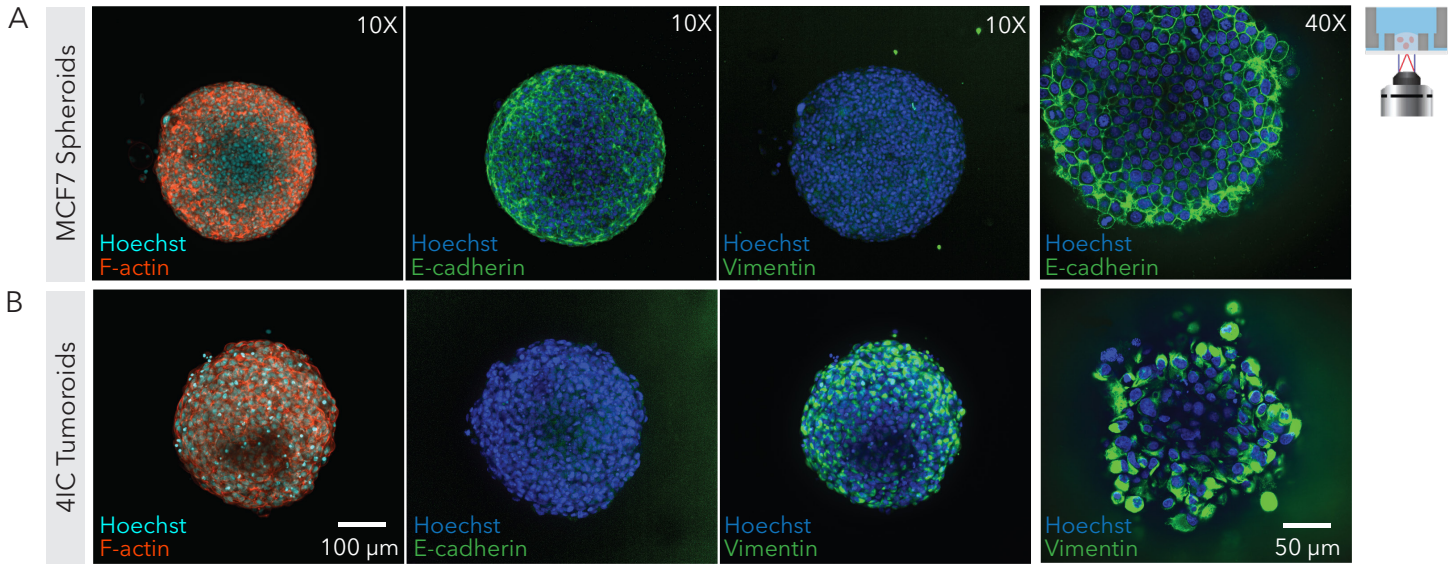

Figure 3. IF Staining of MCF7 spheroids (A) and 4IC tumoroids (B) in VitroGel. Cell marker detection and expression of E-cadherin, Vimentin and F-actin in these cell models. Confocal images acquired on the CQ1 confocal imaging system. 120 µm Z-stack images of the samples were acquired at 10 µm increments.

Summary

In this application note, we have demonstrated that other types of matrices, such as VitroGel hydrogels, can be integrated with the Pu·MA System automation to create robust assay workflows for matrix-grown 3D cell models. These workflows streamline the process of 3D cell model formation, interrogation, and measurement. Through automation of complex multi-step protocols and high-content imaging within the flowchip, the Pu·MA System enables examining a large set of parameters including biomarkers, cell morphological changes, proliferation, toxicity, and secreted factors in the physiological-like environment. The approaches demonstrated in this note here are applicable to other 3D cell models and other types of matrices.

We found that VitroGel as a 3D hydrogel matrix is very effective for 3D cell models (spheroids and tumoroids) without any external carcinogenic factors which other animal-derived matrices can contain. The tunable nature of this product helped us choose the right mechanical strength for the assays without affecting microfluidic performance of our system. Also, VitroGel had no autofluorescence which affected imaging.

The increase in imaging throughput, analytical frameworks and high-performance computational resources open avenues of data-rich phenotypic profiling for disease modeling and drug discovery. Imaging data and computational image analysis tools are extremely valuable for disease modeling, understanding disease progression and therapeutic responses in the context of the right microenvironment.

Acknowledgments

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- Mahomi Suzuki, Arvonn Tully & Kevin Jan (Yokogawa Electric Corporation)

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Instruments

- Pu·MA System and 3D Flowchips (Protein Fluidics, Inc.)
- CellVoyager CQ1 Benchtop High-Content Analysis System (Yokogawa Electric Corporation)

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