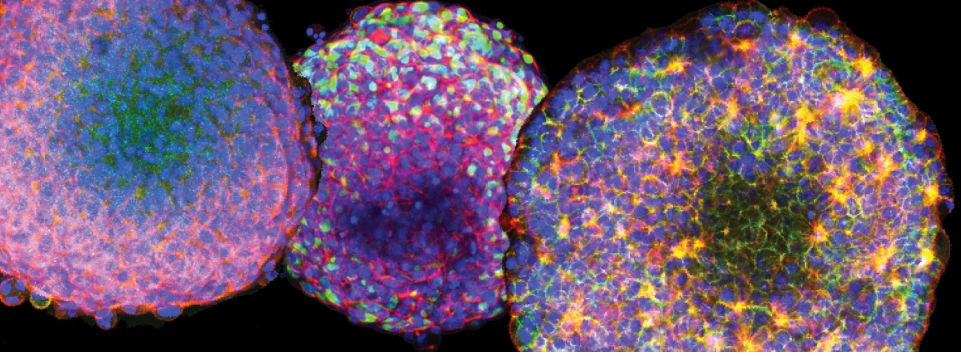

APPLICATION NOTE

Automated Biomarker Identification using IF Staining using the Pu·MA System® followed by High-Content Confocal Imaging

Introduction

Physiologically relevant 3D cell models are essential for pre-clinical research and drug discovery because they better represent physiological processes and disease phenotypes[1,2]. Biomarker studies have become a cornerstone in preventive and personalized medicine. There is an increased interest in using cellular biomarkers to complement genomic ones[3,4]. Both development of functional assays and identification of cellular biomarkers in 3D models are critical, as they are expected to have a higher clinical importance than in 2D culture systems[5]. Immunofluorescence staining (IF) is a widely used method to identify and quantify biomarker expressions and their cellular localizations. One of the challenges faced by researchers is that IF staining of delicate spheroids/organoids tend to be manual and tedious.

Culture systems like hanging drop or ultra-low attachment are difficult to handle for IF staining due to a risk of losing or damaging the spheroids. There is a further technical challenge of plate compatibility and locating spheroids for confocal microscopy, which is typically used for imaging of stained spheroids. The commonly used approach of embedding, and sectioning is cumbersome, time consuming and prone to errors. To overcome these challenges, we have developed and describe here a breakthrough advancement in biomarker staining and detection. We present the automated IF staining in 3D cell models using our proprietary microfluidic-based Pu·MA System[4,6,7] combined with high resolution confocal imaging technology from Yokogawa Electric Corporation (Figure 1).

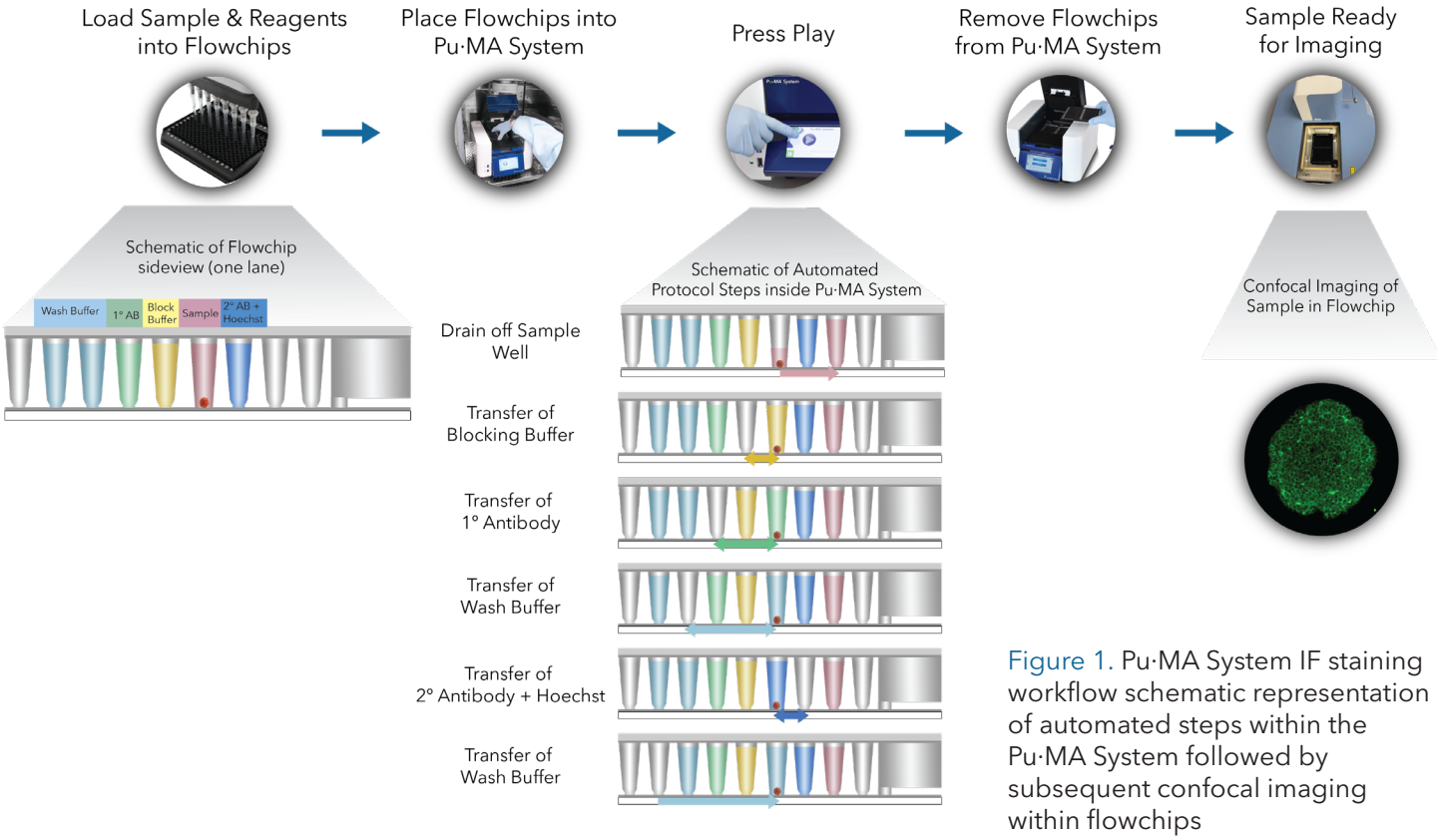

Figure 1. Pu·MA System IF staining workflow schematic representation of automated steps within the Pu·MA System followed by subsequent confocal imaging within flowchips

Samples and ECMs

In this study, automated IF staining for biomarker detection was performed using two 3D cell models:
MCF7 breast cancer spheroids
TU-BcX-4IC patient-derived triple negative breast cancer tumoroids (4IC Tumoroids) Tulane Univ[4,7.]

Spheroids and tumoroids were created by seeding 2500 cells/well in ultra-low attachment 96-well plates and cultured for 72 hours. Two different matrices: Matrigel (Extracted from Engelbreth–Holm–Swarm mouse sarcoma), complex, temperature dependent VitroGel® Hydrogel Matrix (TheWell Bioscience) Ready-to-use, Xeno-free, room temperature stable, tunable and engineered hydrogel, closely mimics natural ECM.

We demonstrate the compatibility of Pu·MA System with engineered ECMs like VitroGel. VitroGel is transparent and compatible with high resolution imaging. Both MCF7 spheroids and 4IC tumoroids show similar E-cadherin expression patterns as in Matrigel (Figure 2).

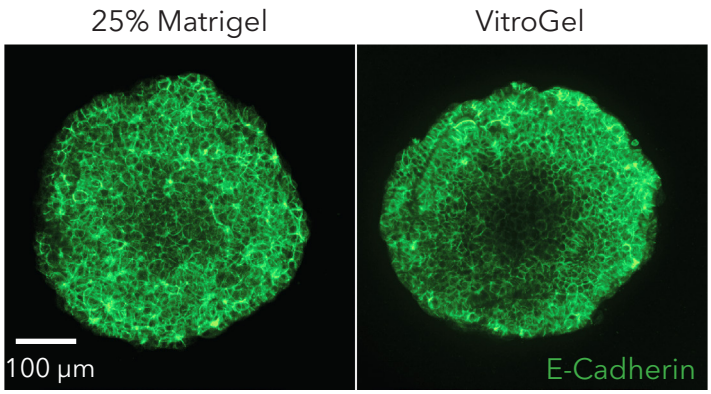

Figure 2. MCF7 spheroids were loaded into Pu·MA System flowchips within either 25% Matrigel or VitroGel. Spheroids were stained using identical conditions and subsequently imaged using the Yokogawa CQ1 confocal system. Maximum intensity projections are shown.

Automated IF Staining Workflow

The workflow starts with loading the flowchip with reagents and samples and placing the flowchip holder into the Pu·MA System[6,7]. When press PLAY, automated execution of all the IF staining steps takes place within the Pu·MA System. The system can operate between 4°C and 37°C. In this study IF staining was done at room temperature for the detection of various biomarkers in MCF7 spheroids and 4IC tumoroids. Schematic of the workflow is shown in Figure 1.

Confocal Imaging

Confocal high-resolution imaging of the 3D cell models were performed within the flowchips using CellVoyager CQ1 System (Yokogawa Electric Corporation). Confocal images were acquired with a 20X long working distance dry objective using 405nm (Nuclei Hoechst), 488nm (E-Cadherin or Vimentin) and 561nm (Phalloidin) channels. A 120 µm z-stack of images at 10 µm intervals were acquired (Figure 2 and 3).

Biomarker Analyses

We investigated the expression of E-cadherin (an epithelial cell-cell adhesion protein), Vimentin (intermediate filament protein in mesenchymal cells) and F-actin (cytoskeletal protein) show in Figure 3. These experiments were done with samples in 25% Matrigel.

The epithelial-like and low malignant MCF-7 spheroids show intense peripheral E-cadherin staining prevalent at cell-cell contact areas, and no detectable expression of Vimentin (Figure 3A). The highly aggressive 4IC tumoroids showed loss of E-cadherin expression while being positive for Vimentin. F-actin distribution and organization was detected by Phalloidin staining (Figure 3B).

Confocal Image Analyses

Collaborating with scientists at Yokogawa, we show advanced image analysis approach for 3D cell models using CellPathfinder. In Figure 3C we show schematic of the segmentation: the cell borders and cell segmentation was performed using Phalloidin image input. Cells were partitioned into “Inner Region” and “Outer Region” corresponding to the cytoplasmic and membrane regions. Nuclei were identified using Hoechst stain.

Accurate segmentation is challenging for 3D images. We are working on optimizing data acquisition and analysis parameters to characterize and quantify the localization and the expression level of specific biomarker in cytoplasm versus membrane region in 3D setup.

In Figure 3D, we show one of the ways to visualize the results from the image analysis approach described above. This advanced image analysis approach would allow to quantify the biomarker membrane/cytoplasm intensity within cells under various interrogation conditions. Specific cell populations can be extracted and classified by gating the feature value data of identified objects (cells).

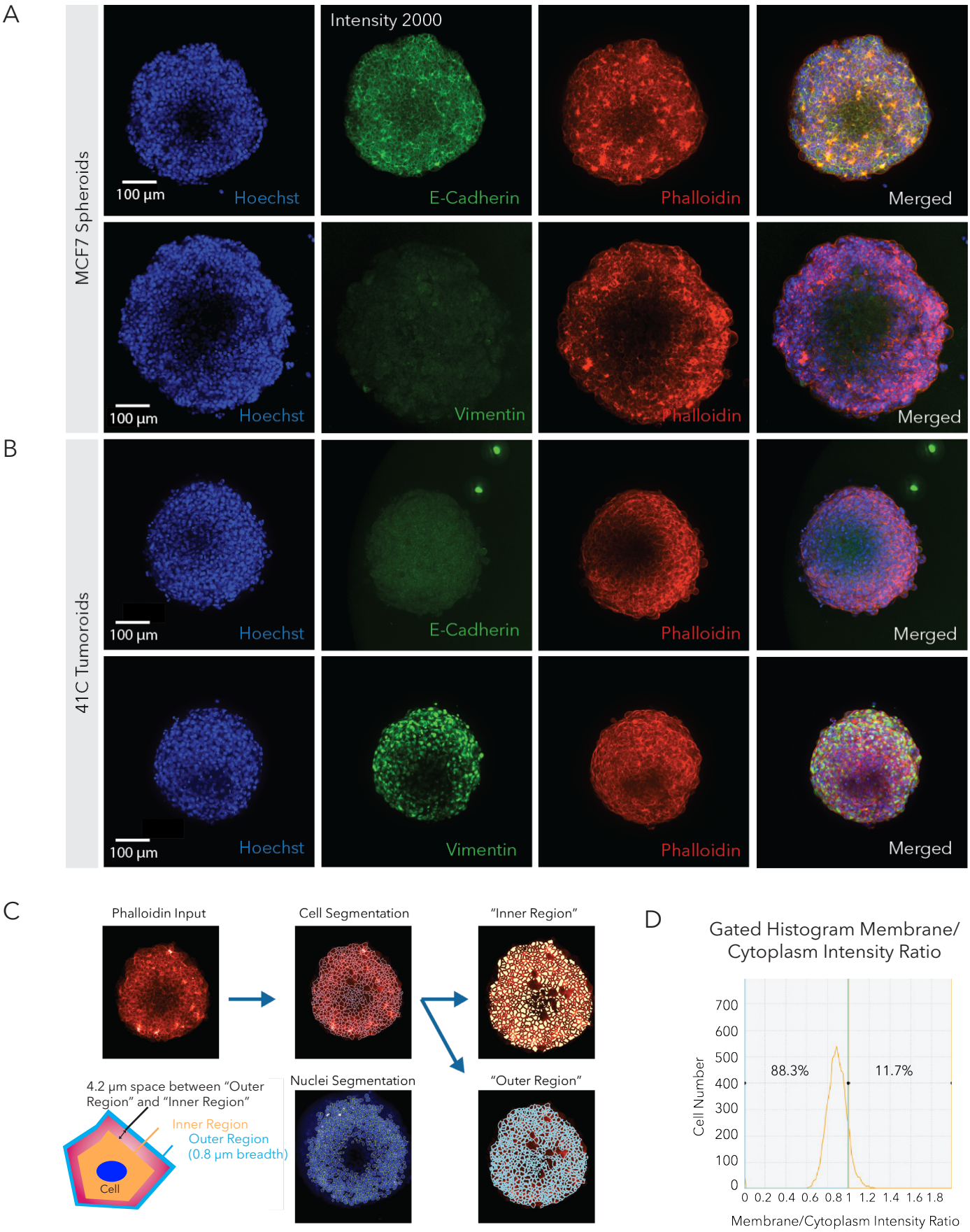

Figure 3. Confocal imaging of IF stained MCF7 spheroids (A) and 4IC tumoroids (B) for the biomarkers described. Image analysis using CellPathfinder with detailed cell segmentation (C). Example of data visualization of the acquired image analysis (D).

Summary

In this study we demonstrated the capabilities of Pu·MA System to performs complex multi-step protocols like IF staining with 3D models.
The workflow presented here has high utility and value for studying the interplay between cadherin cell adhesion machinery, Vimentin and associated partners like F-actin, its role in epithelial-to-mesenchymal transition and metastasis, as well as modulation dynamics by pharmacological agents. We also demonstrated the compatibility of the Pu·MA System flowchips for use with 3D cell models in different types of ECM. The major benefits of using the Pu·MA System are cost, time, resource saving, and minimizing human error while aiding in acquiring high quality data using high-content imaging without perturbing the samples. In combination with other automated 3D assays, the Pu·MA System enables examining a large set of parameters including biomarkers, signaling molecules, cell morphological changes, proliferation indices, and toxicity.

Acknowledgements

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- Dr. Matthew Burow's Group (Tulane University),
- Mahomi Suzuki, Arvonn Tully & Kevin Jan (Yokogawa Electric Corporation)
- Dr. John Huang & Christopher Oak (TheWell Bioscience).

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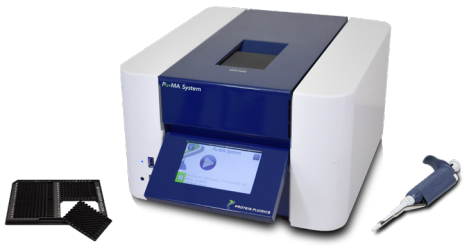
Instrumentation

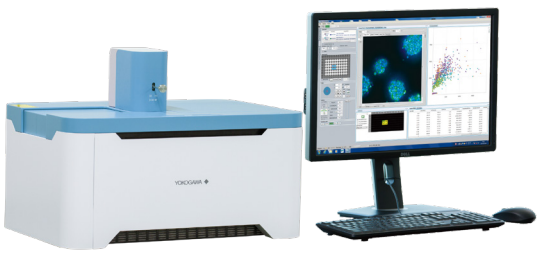
Pu·MA System and flowchips

- Automated media exchanges within protected sample chamber in the flowchips
- Spheroids are imaged in the flowchip, or supernatant samples collected for immunoassay or metabolomics analysis
- Easy touch-screen operation and protocol edits using PuMA Software



CellVoyager CQ1 Benchtop High-Content Analysis System (Yokogawa Electric Corporation)

- Confocal in the microlens dual spinning disc technology with high precision stage incubator and low phototoxicity
- Four excitation lasers + transmitted light
- Easy to use CellPathfinder high content analysis software



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