Automated Biomarker Identification in 3D Cell Models Using Microfluidic Pu·MA System and CellVoyager CQ1 Benchtop High-Content Analysis System

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INTRODUCTION

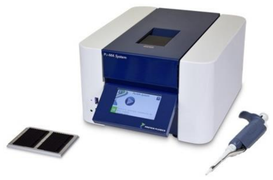
Physiologically relevant 3D cell models are essential for pre-clinical research and drug discovery because they better represent physiological processes and disease phenotypes. Biomarker studies have become a cornerstone in preventive and personalized medicine. There is an increased interest in using cellular biomarkers to complement genomic ones. Development of functional assays and identification of cellular biomarkers in 3D models is critical as they are expected to have a higher clinical importance than in 2D culture systems. 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 immunofluorescence staining in 3D cell models using our proprietary microfluidic-based Pu·MA System combined with high resolution confocal imaging technology from Yokogawa Electric Corporation.

INSTRUMENTATION

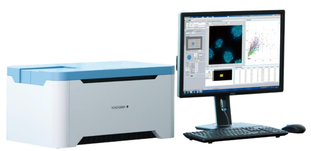
The Pu·MA System and flowchips

- 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



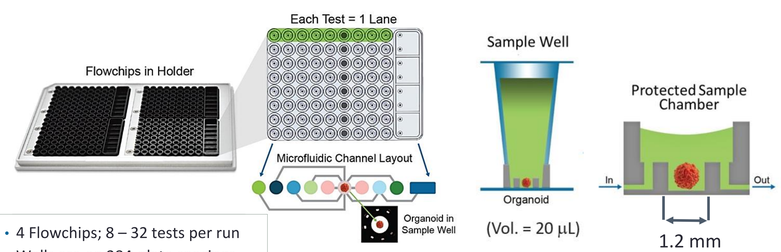
The CellVoyager CQ1 Benchtop High-Content Analysis System

- Confocal spinning disc technology
- High precision stage incubator and low phototoxicity
- Four fluorescence channels + transmitted light
- Integration with CellPathfinder high content analysis software



MICROFLUIDIC FLOWCHIP

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. Each test lane designated to one organoids/spheroid sample and consists of sample well connected to the reagent wells via microfluidic channels. Flowchip can be filled with any reagents (media, compounds, stains, etc.) depending on the assay configuration. Organoids/spheroids are loaded 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. The bottom of the flowchip is a thin cyclic olefin copolymer (COC) film which makes it compatible with high resolution imaging.



- 4 Flowchips; 8 – 32 tests per run
- Wells are on 384-plate spacings
- Thin optically clear COC bottom

Figure 1. 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.

References

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LOADING 3D SAMPLES INTO FLOWCHIP

In this study IF staining for biomarker detection was performed in two 3D cell models:

- MCF7 breast cancer spheroids
- TU-BcX-4IC (4IC) patient-derived triple negative breast cancer tumoroids

Spheroids and tumoroids were created by seeding 2500 cells/well into ultra-low attachment 96-well plates and culturing over 72 hours.

The staining was performed in two different extracellular matrices:

- Matrigel (Basement Membrane Extract-Based Matrix).
- VitroGel Hydrogel Matrix (TheWell Bioscience). It is a ready-to-use xeno-free, room temperature stable, tunable and permeable engineered hydrogel that closely mimics the natural extracellular environment.

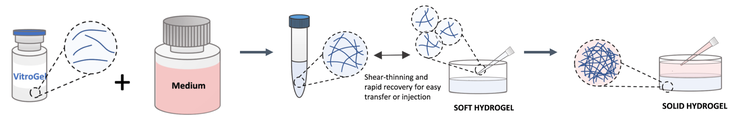


Figure 2. VitroGel gelation/formation starts by mixing the VitroGel with cell culture medium. Hydrogel molecules interact with ionic molecules such as Ca^{2+} and Na^{+} from the cell medium and form the matrix structure.

MCF7 spheroids and 4IC tumoroids were resuspended in 25% Matrigel (diluted in cell culture medium) or in VitroGel and loaded to the Sample Well of the flowchip in 2 µL of volume to create a minidome inside the Protected Sample Chamber. After that samples were fixed inside the flowchip followed by automated IF staining in Pu·MA System.

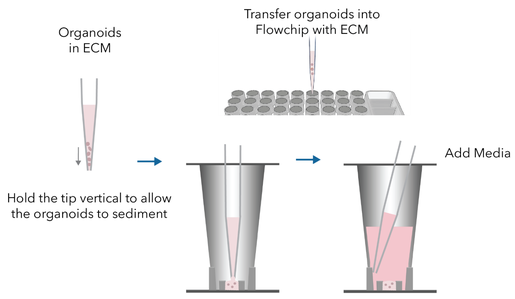


Figure 3. Schematic for loading spheroids or tumoroids into the flowchip.

AUTOMATED IF STAINING WORKFLOW

The workflow starts with loading the flowchip with reagents. Automated execution of 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. Confocal high-resolution imaging using CellVoyager CQ1 System was performed within the flowchip. 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 separated by 10 µm was acquired.

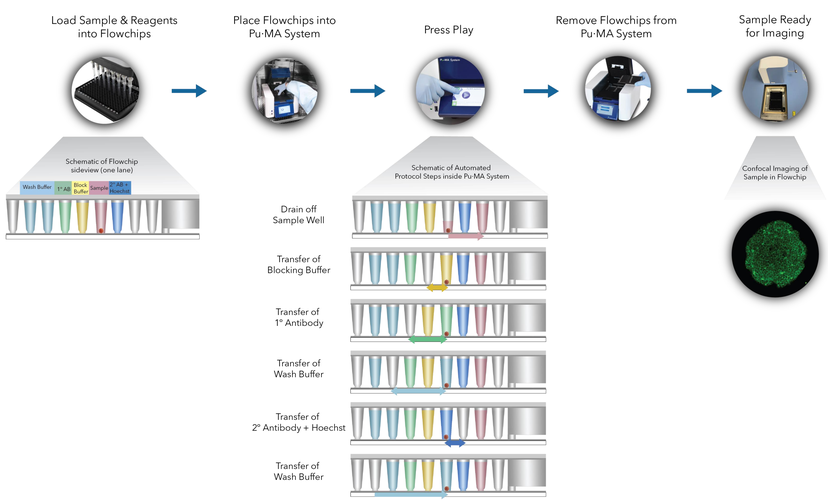
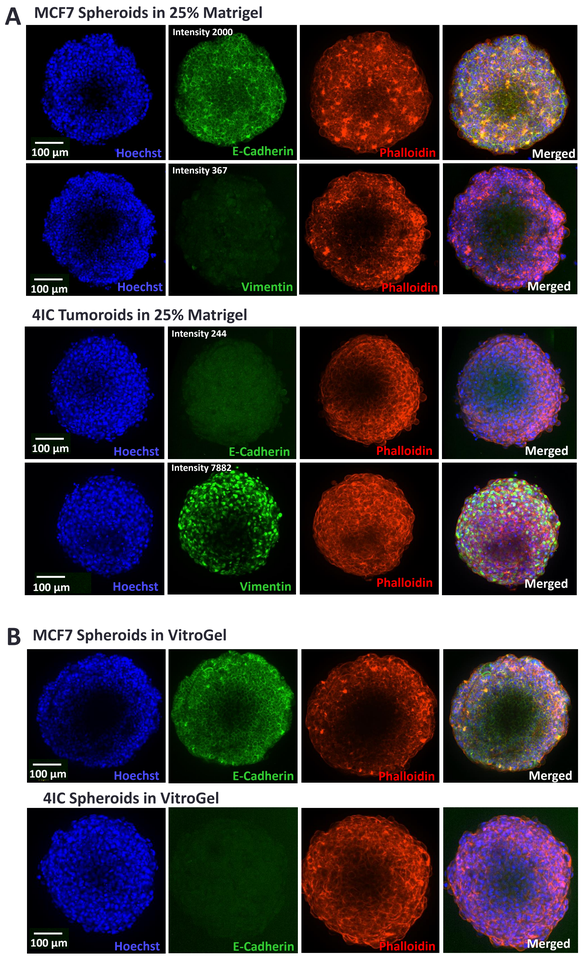


Figure 4. Schematic of the automated IF staining workflow combined with high resolution 3D imaging using CellVoyager CQ1.

CONFOCAL IMAGING AND ANALYSIS



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 5 Panels A.** The epithelial-like and low malignant MCF-7 cells shows intense peripheral E-cadherin staining prevalent at cell-cell contact areas, and no detectable expression of Vimentin. 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.

In **Panel B**, we demonstrate the compatibility of Pu·MA System with other engineered ECMs like VitroGel. VitroGel is transparent and compatible with high resolution imaging. Both MCF7 and 4IC spheroids show similar E-cadherin expression patterns as in Matrigel.

In **Panel C** we show our advanced image analysis approach for 3D cell models using CellPathfinder. 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.

Panel D shows one of the ways to visualize the results. 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).

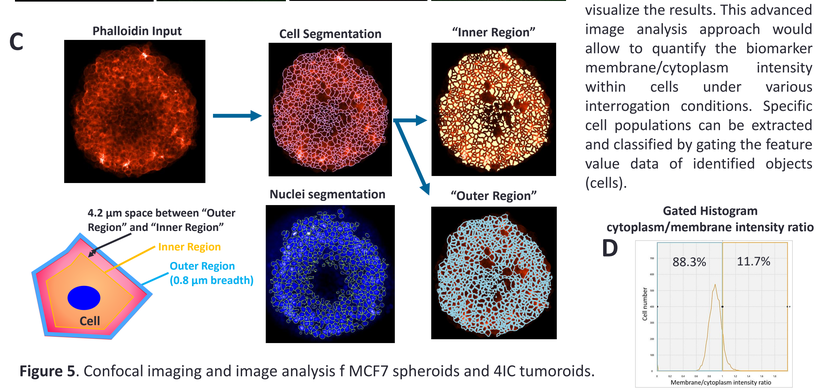


Figure 5. Confocal imaging and image analysis f MCF7 spheroids and 4IC tumoroids.

CONCLUSIONS

- In this study we demonstrated the capabilities of Pu·MA System to performs complex multi-step protocols like IF staining with 3D cell-based 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.
- The major benefits are cost, time, resource saving, and minimizing human error while aiding in acquiring high quality data.
- 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.

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