



Overview



The importance of 3-dimensional extracellular matrix on cellular function and behavior is clear from studies in differentiation, tissue engineering and more recently, molecular pharmacology. If given the proper 3D scaffold, mammary epithelial cells will undergo polarization and differentiation resembling in vivo structures¹. Important differences exist between migration of fibroblasts and other cell types on flat plastic compared to 3D environments². Certain therapeutic agents show differential responses between 2D substrates and biological 3-D matrix^{3,4}. Yet due to technical challenges, cellular assays are still predominantly performed in 2D. Leveraging our previous work in high content 3D tumor cell migration using an array of microchannels⁵, we present here a highly miniaturized and automated approach for high content immunocytochemistry in 3D matrices. The validation was performed on a pancreatic tumor cell model using cells embedded in 3D fibrillar collagen compared to collagen coated substrates. A series of protocol enhancements will be described, along with representative experimental results from cell cycle analysis. This approach yields precise and reproducible inhibitor potencies, and produces screenable quality assay windows. The miniaturization inherent in the platform should prove enabling for studies using primary cells and customized extracellular matrices.

Morphological differences between cells in 2D vs. 3D

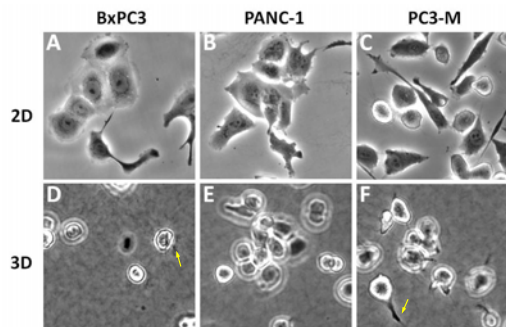


Figure 1. Cells were seeded in microchannels either on a 2D surface coated with 50µg / mL type I collagen (a-c), or in 3D by embedding in 1 mg/ml type I collagen (d-f). Clear morphological differences are apparent when cells are viewed via phase contrast microscopy. Cells in 2D spread flat on the bottom surface of the channel. In 3D, cells remain rounded and interact with collagen fibers around them (yellow arrows.)

Cells embedded throughout full height of microchannel

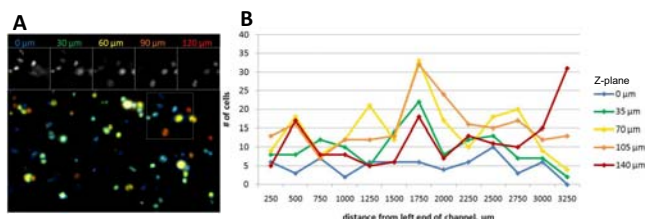


Figure 2. Cells were embedded in 3D type I collagen by flowing an ice-cold neutralized collagen-cell suspension into a channel. The plate is then warmed to 37°C while continuously rotating. As the collagen warms, the suspension gels and traps the cells in 3D. Here, M4A4 cells (a) or BxPC3 cells (b) embedded in 3D type I collagen were stained with Hoechst 33342, and imaged at a range of Z-heights with a 20x objective. These images were colored and overlaid to construct a 3D-view of the embedded cells (a), or manually scored to determine the distribution of the cells throughout the height of the channel (b). Cells are distributed throughout the 140-µm height of the channel.

INSERT A SCALE BAR ON FIGURE A

Cells were evenly stained across the length of a channel

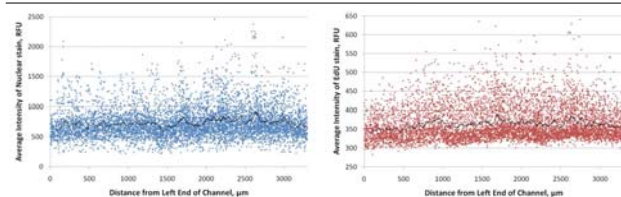


Figure 3. 3D type I collagen is fibrillar and porous, and therefore permits flow of reagents through the matrix from one end of the channel to the other. BxPC3 cells were seeded in 3D collagen and incubated overnight. Cells were assayed for S-phase using the Invitrogen Click-IT™ EdU kit. The channels were imaged with a 4x objective with sufficient depth of focus to capture the 140 µm height of the channel. Both the nuclear dye, Hoechst and the EdU label was able to evenly stain cells across the full length of the channel, as shown by the moving average (black line, period= 100 points).

Optimization of identification of nuclei in an image

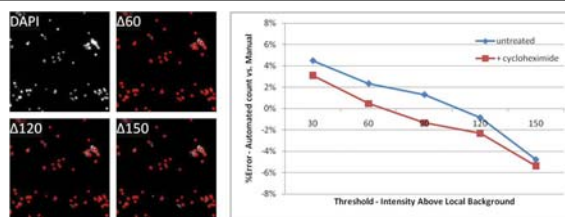


Figure 4. Metamorph® software was used for all automated image analysis. Metamorph®'s applications use a threshold setting for intensity above local background in order to identify the border of objects. This parameter was optimized for accurate identification of nuclei in images of BxPC3 cells culture in 3D, treated with 10 µM cycloheximide or left untreated, and stained with Hoechst 33342 dye. The red overlay shows the resulting segmentation of nuclear objects by this application with the indicated threshold setting. Segmenting in 3D is a challenge due to overlapping nuclei. At lower thresholds, objects are not adequately segmented but rather clumped together into single objects, and some fragmented nuclei are counted as multiple objects. At higher thresholds, the application excludes apoptotic fragmented nuclei altogether. Accurate counts are obtained at intermediate threshold settings between Δ90 and Δ120. The two conditions were analyzed separately to determine if one treatment condition was being preferentially skewed.

Optimization of threshold for EdU score

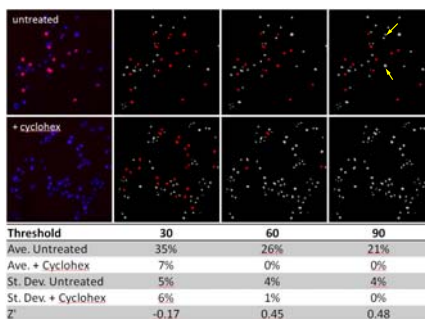


Figure 5. EdU incorporation was determined similarly as in Figure 4. Intensity above background was thresholded at a range of values. At low thresholds, background intensities are scored as "positive" for EdU incorporation. At excessively high thresholds, truly "positive" cells are not scored as such. A balance must be struck between the two. Both user expertise and Z' values must be used to select an appropriate threshold level. While a threshold of 90 provides the highest Z' value, truly positive objects, albeit of lower intensity, are being excluded as indicated by yellow arrows.

1,280 compound high content screen in 3D collagen

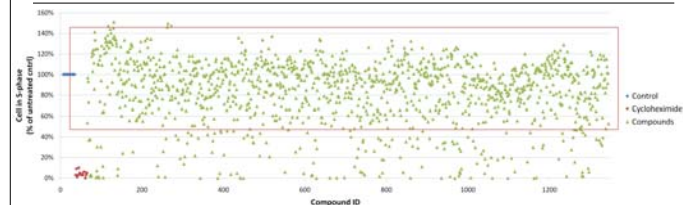
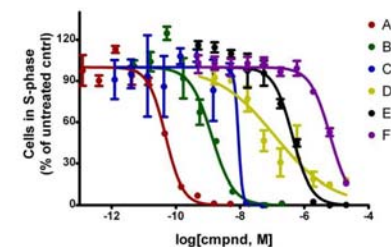


Figure 6. The LOPAC1280TM library (Sigma), a collection of 1,280 pharmacologically active compounds, was screened for inhibition of proliferation, as indicated by S-phase incorporation of EdU using Invitrogen's Click-IT™ EdU kit. Cells were seeded in 3D type I collagen (1 mg/ml) in microchannels, and treated with compounds overnight at a 10µM dose, n=2. All liquid handling was done using a CyBio CyBio®-Well 96-channel Simultaneous Pipettor. Imaging was done with an automated Nikon TE2000 Inverted Microscope. Analysis was automated using Metamorph software. The % of S-phase cells was normalized to the untreated control for each plate. The red box indicates 3 standard deviations around the average untreated control. There were several active compounds identified for cell cycle inhibition, by loss of S-phase population. Average plate Z' was 0.4.

Determining rank order of potency in 3D collagen



Compound:	A	B	C	D	E	F
pIC ₅₀ ± 95% confidence interval	10.3 ± 0.1	8.9 ± 0.2	8.1 ± 0.8	6.9 ± 0.3	6.4 ± 0.1	5.2 ± 0.1

Figure 7. Cell-cycle inhibitory compounds identified in the screen were assayed for potency in a dose response format. The assay was performed as described in figure 6, with 10 concentrations tested and a dilution factor of 3.25. The rank order potency of the compounds is shown in the table along with corresponding 95% confidence interval.

Conclusions

- Single-plane imaging can be successfully employed to accurately analyze high content assays in microchannels filled with 3D collagen.
- The assay is fully automatable using liquid handling robotics and automated microscopy.
- Compound screening and profiling using this approach are both demonstrated.

References

1. Hebner, C., V.M. Weaver, and J. Debnath, Modeling morphogenesis and oncogenesis in three-dimensional breast epithelial cultures. *Annu Rev Pathol*, 2008. 3: p. 313-39.
2. Hakkinen, K.M., et al., Direct Comparisons of the Morphology, Migration, Cell Adhesions, and Actin Cytoskeleton of Fibroblasts in Four Different Three-Dimensional Extracellular Matrices. *Tissue Eng Part A*, 2010.
3. Weigelt, B., et al., HER2 signaling pathway activation and response of breast cancer cells to HER2-targeting agents is dependent strongly on the 3D microenvironment. *Breast Cancer Res Treat*, 2010. 122(1): p. 35-43.
4. Serebriskii, I., et al., Fibroblast-derived 3D matrix differentially regulates the growth and drug-responsiveness of human cancer cells. *Matrix Biol*, 2008. 27(6): p. 573-85.
5. Echeverria, V., et al., An automated high-content assay for tumor cell migration through 3-dimensional matrices. *J Biomol Screen*, 2010. 15(9): p. 1144-51.