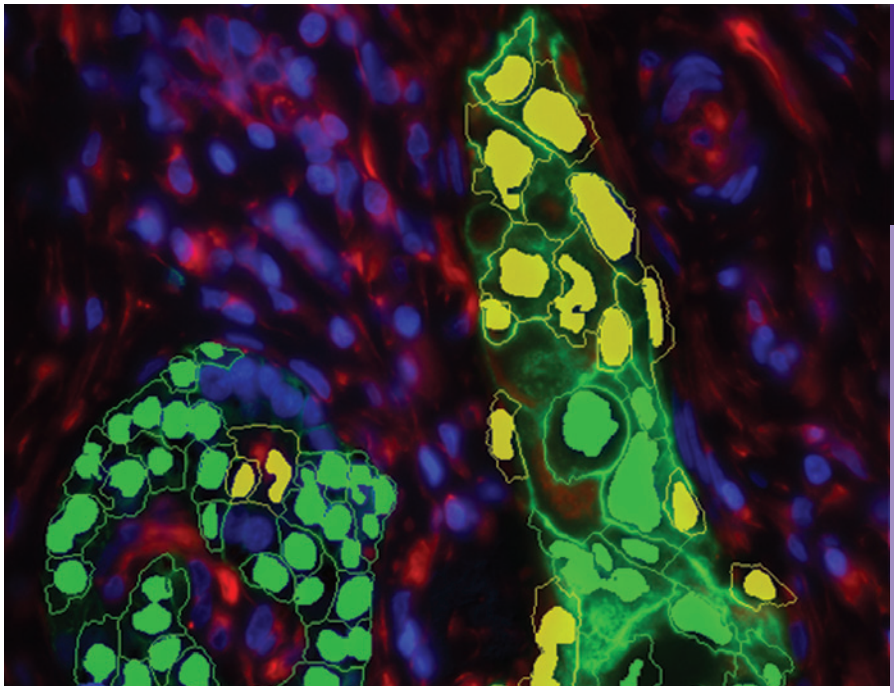


Image with cell segmentation mask for tumor cells and their membrane. Cells positive for only E-cadherin are displayed in green, cells double positive for E-cadherin and vimentin are displayed in yellow, and cells negative for both markers are shown in blue.

APPLICATION NOTE

inForm Advanced Image Analysis Software

Highlights

- Quantitating per-cell co-expression of EMT markers *in situ* in tumors
- Understanding cancer invasion and interactions with the tumor microenvironment
- Unmixing of fluorescence markers using multispectral imaging systems
- Identifying tumor regions automatically with trainable pattern-recognition algorithms using inForm Tissue Finder software

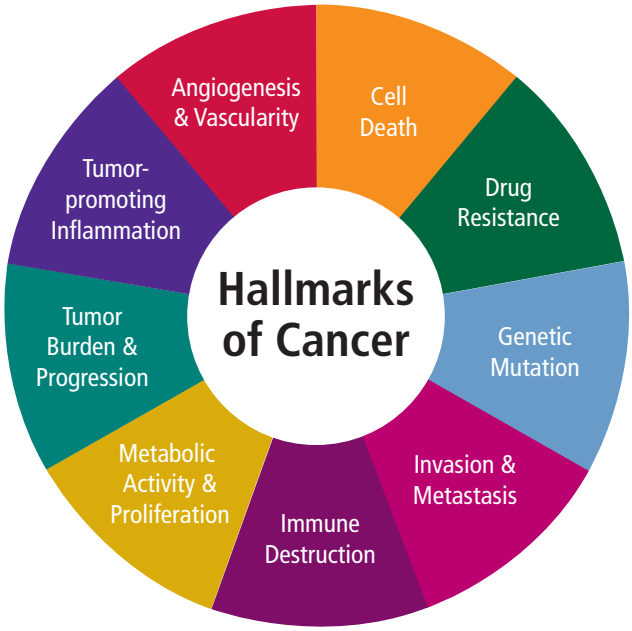
Evaluating EMT in FFPE Tissue Sections Using the inForm Data Analysis Software

Introduction

Epithelial-mesenchymal transition (EMT) is a process whereby epithelial cells acquire mesenchymal, fibroblast-like properties and display reduced intracellular adhesion and increased motility. This is a tightly regulated process utilized by malignant epithelial cells to spread beyond their origin, and is associated with a number of cellular and molecular events. Recent studies indicate that cancer cells have up-regulated N-cadherin in addition to loss of E-cadherin. This change in cadherin expression is called the "cadherin switch" and down regulation of E-cadherin is one of the hallmarks of EMT. Vimentin is an intermediate filament of mesenchymal origin and is present at early developmental stages. Vimentin's dynamic spatial re-organization during EMT is another hallmark.

Tools to quantify EMT in intact FFPE tissue sections would facilitate significantly the understanding of how cancer behaves in the in-situ context of tumor microenvironment. However, since individual cells rarely bridge across FFPE serial sections, measuring co-expression of EMT markers in individual cells requires analysis of multiple markers in the same section, which requires multi-color labeling protocols and image analysis tools that can identify and analyze individual cells of interest in complicated morphologies. Furthermore, understanding EMT depends on determining the location of cells going through EMT relative to the invading edge of a tumor. While flow cytometry is capable of determining the per-cell, multi-marker phenotype of samples that have been disaggregated, this loses key spatial distribution information.

AKOYA BIOSCIENCES



To obtain quantitative multi-marker per-cell data, there are several challenges that must be overcome: a) multiplexed immunofluorescence labeling protocols that yield robust, balanced, independent and specific signals; b) image acquisition system capable of capturing multiplexed signals and isolating them from one another and background fluorescence; and c) image analysis software capable of identifying tumor areas and individual cells within those areas, and collecting multi-analyte signals from them.

Akoya's multispectral slide imaging systems collect accurate and sensitive signals from multicolor FFPE tissue samples. inForm® image analysis software from Akoya is then used to perform multispectral unmixing to isolate label signals from one another and from background signals, and, using a trainable pattern-recognition-based algorithm (inForm Tissue Finder™), identifies tumor areas and individual tumor cells within those areas and extracts data from those cells. A key capability in the latest release of inForm (2.0) is an interactive tool for setting thresholds to determine double-positivity of tumor cells, to reveal their location relative to the edge of a tumor. To provide an effective solution for EMT analysis in FFPE, Akoya has collaborated with Cell Signaling Technology® to provide reagent kits (EMT kit # 7771), that are easy-to-use, optimized for FFPE.

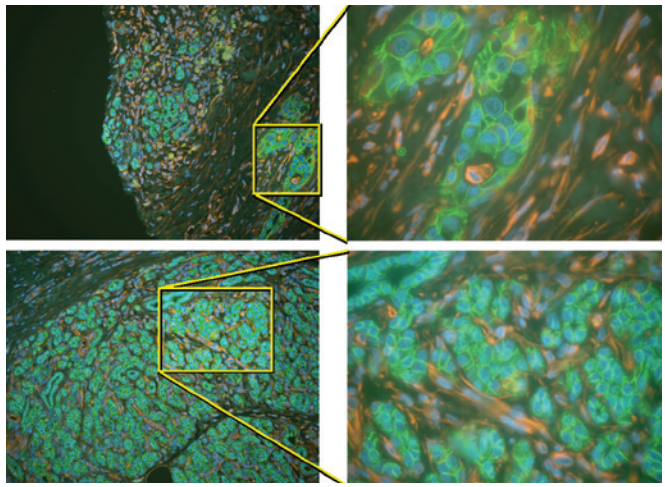


Figure 1. Multispectral 20x (left) and 60x (right) images of two pancreatic cancer tissue sections labeled with an EMT kit from Cell Signaling Technology. From this imagery alone it is difficult to visually determine differences in these morphologies.

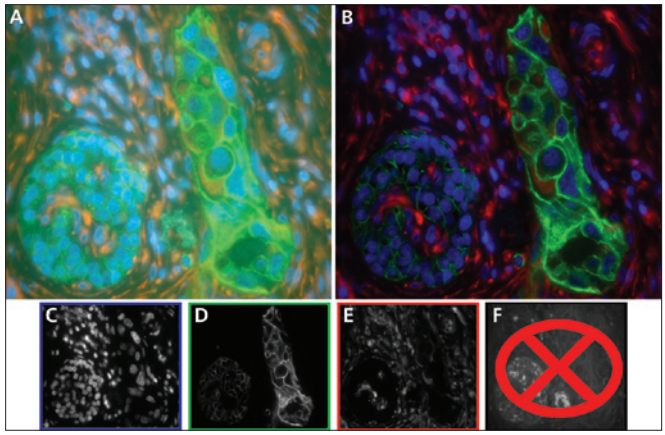


Figure 2. (A) RGB representation of a multispectral image from a pancreatic cancer tissue section. (B) Pseudo-colored composite image of spectrally unmixed components with the removal of autofluorescence. Shown underneath are the individual unmixed component images of (C) DAPI, (D) E-cadherin, (E) vimentin, and (F) autofluorescence, which is removed from imagery.

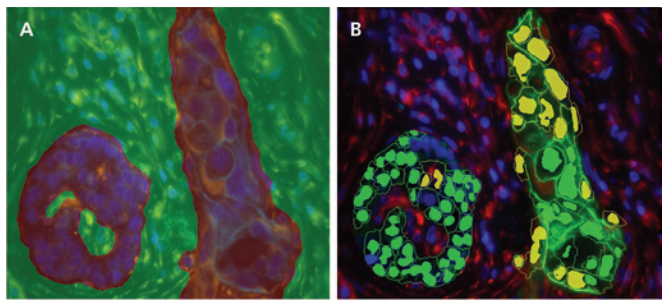


Figure 3. (A) Image with tumor segmentation mask, showing tumor in red and stroma in green. (B) Image with cell segmentation mask for tumor cells and their and membrane. Cells positive for only E-cadherin are displayed in green, cells double positive for E-cadherin and vimentin are displayed in yellow, and cells negative for both markers are shown in blue.

Methods

- De-identified tissue sections were stained using Epithelial-Mesenchymal Transition (EMT) Antibody Sampler kit (#9782) from Cell Signaling Technology, which labels:
  - E-cadherin with AlexaFluor 488,
  - vimentin with AlexaFluor 594,
  - and includes a DAPI counterstain.
- The samples were then scanned with Vectra multispectral slide analysis system from Akoya.
- The multispectral images were analyzed using the new inForm Tissue Finder image analysis software from Akoya to:
  - spectrally unmix the DAPI, E-cadherin, and vimentin signals and remove tissue autofluorescence;
  - train for automatic segmentation of the images into compartments of tumor, stroma, and blank background;
  - segment tumor cells and their membranes;
  - score tumor cells based on co-expression of vimentin and E-cadherin.

Results

Specific, independent, and strong signals were obtained through multispectral imaging that could be readily unmixed from each other and autofluorescence. Trainable, pattern-recognition-based image analysis (inForm Tissue Finder) reliably identified areas of tumor and phenotyped tumor cells according to co-expression of vimentin and E-cadherin. This co-expression was determined based on visually-defined single-positivity thresholds for E-cadherin expression within the membrane and vimentin expression within the cytoplasm. For the image shown in Figure 3, 44% of tumor cells were found to be double positive for both markers. The signal counts for these cells can be seen in the scatter plot in Figure 4.

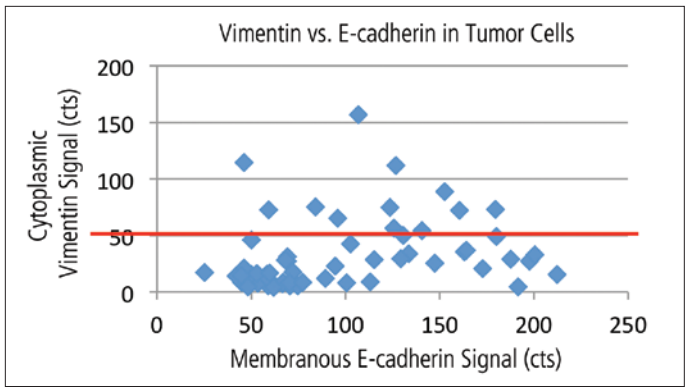


Figure 4. Scatter plot displaying the average membranous E-cadherin signal versus the average cytoplasmic vimentin signal on a per-cell basis. The red line shows the threshold for vimentin positivity. Cells below this threshold are phenotyped as epithelial while those above are phenotyped as epithelial and mesenchymal.

Conclusion

Characterizing the progression of individual cells undergoing an epithelial-to-mesenchymal transition (EMT) and their distribution in a tumor is feasible using a combination of multiplexed labeling kits, multispectral microscopy, and trainable pattern-recognition-based image analysis.

Performance of the platform for automated multiplexed tissue cytometry analyses supports its application to routine clinical studies and works for both IHC and IF staining methodologies.

Authors

C. Hoyt

Akoya Biosciences, Inc.
Hopkinton, MA, USA

C. Gagen

R. Wetzel

Cell Signaling Technology
Danvers, MA, USA

Akoya Biosciences, Inc.
68 Elm Street
Hopkinton, MA 01748 USA
P: (+1) 855-896-8401
www.akoyabio.com

AKOYA BIOSCIENCES