Tissue and Liquid Biopsy Profiling Using Multiplexed Immunofluorescence

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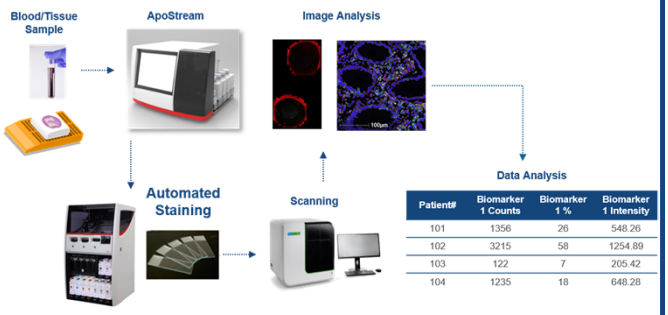
INTRODUCTION

Background:
Precision medicine relies on a good diagnosis and screening methods for cancer tumors. The primary challenges to precision medicine are the difficulties involved in sampling a very heterogeneous microenvironment in its entirety, and being able to perform a good quality profiling. Liquid Biopsies help analyze the tumor using Circulating Tumor Cells (CTCs) released into the blood by the primary tumor, providing valuable genetic and proteomic information. On the other hand, using a tissue biopsy can generate a more in-depth tissue profile. Multiplexing technology can accurately detect up to 8 markers to be able to phenotype and find the most important cell populations. By using software able to track each cell and its data, it is possible to explore the architectural context of the tumor microenvironment.

Methods:
FPE samples of primary tumors were immunostained using Opal™ reagents manually or on a Leica BOND RX™. In brief, tissue samples were de-waxed, rehydrated, and subject to heat-induced antigen retrieval. Using a serial immunostaining protocol the samples were incubated in primary antibody for 30', washed, then incubated with a secondary rabbit/mouse HRP polymer for 10'. After washing, the samples were incubated with the appropriate Opal fluorophore for 10' , washed, and subjected to another antigen retrieval. This process was repeated for each marker. Finally, samples were rinsed, and incubated with DAPI for 5' , prior to coverslipping. For CTCs, blood samples are Ficolled, and then run through ApoStream for CTC enrichment. The post-apostream sample is permeabilized with TritonX100, rinsed, blocked with a serum mix, incubated overnight with cocktail of directly labeled antibodies, counterstained with DAPI and coverslipped.

WORKFLOW

Workflow for CTC and Tissue Multiplexed Profiling



Patient#	Biomarker 1 Counts	Biomarker 1 %	Biomarker 1 Intensity
101	1356	26	548.26
102	3215	58	1254.89
103	122	7	205.42
104	1235	18	648.28

PANEL DESIGN

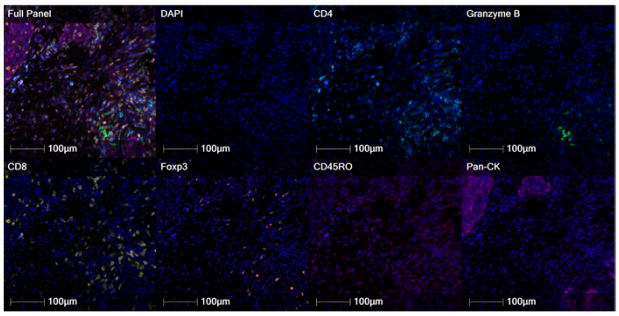
Overview of the Multiplexed Panel Design

Round	1	2	3	4	5	6
Primary Antibody	CD4	CD8	Granzyme B	FoxP3	CD45RO	Pan-CK
Opal Dye	Opal 480	Opal 570	Opal 520	Opal 620	Opal 690	TSA-DIG + Opal 780

This Immuno-oncology panel was chosen to provide insight into the immune profile of the tumor and its microenvironment. Then panel was limited to 6 markers to be able to use the Whole Slide Multi-spectral Imaging mode from our Vectra Polaris system.

3 commercially available antibodies were titrated in triplicate for each marker. The best one was chosen based on specificity, intensity, and variability. Opal fluorophores were titrated to optimize the fluorophore concentration. Tonsil was used as an intra-tissue positive and negative control.

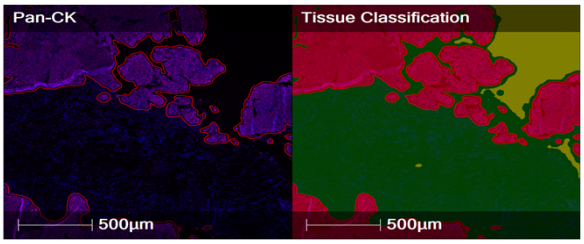
REPRESENTATIVE IMAGES



Representative images showing the fully multiplexed panel and its individual components in an ovarian cancer FFPE tumor biopsy.

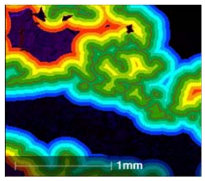
SPATIAL ANALYSIS - INFILTRATION

A



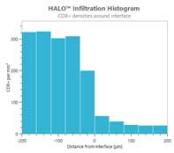
Pan-CK IF and tumor vs non-tumor classifier as defined by the HALO image analysis platform. An AI classifier is trained based on CK expression; this system can also be correlated with a pathologist's annotations on a traditional H&E slide.

B



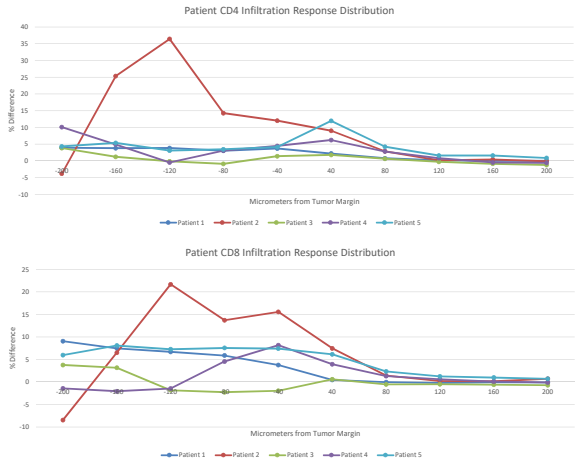
After defining the tumor region, the Infiltration Analysis tool is used to define concentric regions inside and outside the margin to quantify infiltration of immune cells.

C



Graphical representation of CD8+ cell densities within the regions defined by the Infiltration Analysis tool.

INFILTRATION ANALYSIS DATA



The above plots show the changes in immune cell infiltration (monitoring cell densities) for the 5 patients evaluated in this pilot study. The X axis represents the distance in micrometers from the tumor margin (negative values are inside the tumor margin); the y-axis represents the % change of immune cell density (cells/mm^2) in each concentric region in the post-treatment sample versus the pre-treatment sample.

CONCLUSIONS

The Precision for Medicine profiling pipeline is a great tool that allows you to describe CTCs, and tissue samples. Our study shows the ability of this pipeline to successfully combine cutting edge technology in order to automate processes, reduce costs, variation and allow greater scalability. These tools provide our team of experts the means to run the assays faster and more efficiently. Our pathology solutions empower our pathologists by the use of AI. Classifiers can be trained to automatically annotate whole slide scans in either brightfield or fluorescence samples; these annotations can be used for further spatial analysis. We show here the ability to detect patients that might show better response based on tumor infiltration by CD4+ and CD8+ cells. Other potential analysis include nearest-neighbor and proximity analysis.