Design and application of an 8-plex multiplex immunofluorescence panel for deep phenotypic profiling of the tumor microenvironment using InSituPlex technology

Alexis Wong[1], Mara Koerner[1], Katir K. Patel[1], Anna Potier[1], Stephanie Hennek[1], Mustapha Abubakar[2], Mary E. Olanich[3*], Yelena G. Golubeva[3], Karun Mutreja[3], Scott Lawrence[3*]

[1]Ultivue, Inc., Cambridge, MA; [2]National Cancer Institute, Rockville, MD; [3]Frederick National Laboratory for Cancer Research, Frederick, MD

Cancer Genomics Research

Supporting the Division of Cancer Epidemiology and Genetics

Frederick National Laboratory for Cancer Research

sponsored by the National Cancer Institute

leidos Leidos Biomedical Research, Inc.

AACR Poster # 386

Abstract

The tumor microenvironment is composed of a complex network of different cell types and activation states, the exact composition of which is important for immuno-oncology research efforts to correlate treatment options and outcome. The number of biomarkers necessary to sufficiently profile the various cellular phenotypes and elucidate contextual relationships in situ is larger than can be accommodated by standard immunohistochemistry (IHC) approaches. Furthermore, conventional IHC requires more sections, potentially exhausting tissue sample availability. To overcome these limitations, Ultivue has developed InSituPlex® technology and UltiMapper™ assays that enable biomarker multiplexing in tissue samples with an automated workflow to support high sample throughput.

Introduction

In translational oncology research, understanding and evaluating the phenotypic profiles of cells in the tumor immune microenvironment can yield deeper understanding into the complex interactions within the immunological landscape of the tumor.

Although the immune microenvironment is traditionally assessed on hematoxylin and eosin (H&E) stained slides, biologic and therapeutically-relevant distinctions cannot be made between the different cell phenotypes (**Figure 1**).

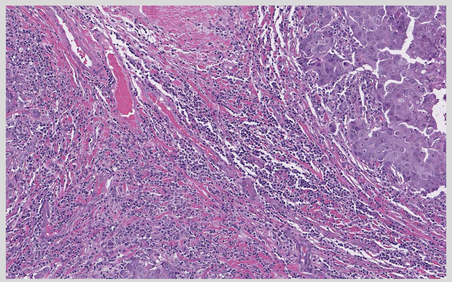


Figure 1. Representative H&E image of the tumor microenvironment in a breast cancer sample.

Multiplex immunofluorescence (mIF) can study this interplay through rapid identification, quantification, and mapping of many cell types. Having the flexibility to look for specific biological markers and their co-localization within this environment allows researchers to better understand the mechanisms of action for compounds of interest.

Here, a customized 8-plex mIF panel was used to study markers in non-small cell lung cancer (NSCLC), colorectal cancer (CRC), and ductal carcinoma in situ (DCIS) samples.

Leveraging the insights gained through staining, imaging, and analysis of these samples, we show how the InSituPlex mIF platform is a beneficial tool in understanding and characterizing the tumor microenvironment, helping to accelerate the hypothesis-to-answer workflow.

Methods and Materials

An 8-plex immune-profiling panel using the InSituPlex approach was designed and tested to detect and classify T cells, macrophages, and tumor cell populations along with PD-L1 checkpoint expression in multiple tumor types. This assay was designed, developed, and tested using UltiMapper reagents and corresponding protocols. The assay was run on multiple formalin-fixed paraffin-embedded (FFPE) tissue samples from NSCLC, CRC, and DCIS cases. Each sample was stained for the following 8 markers: CD3, CD4, CD8, CD68, FoxP3, PD-1, PD-L1, and pan-cytokeratin (CK). Staining was performed in a single step using the Leica Biosystems BOND RX autostainer. Whole slide imaging (WSI) was performed in two steps on the ZEISS Axio Scan.Z1 slide scanner, and image analysis was performed using HALO software from Indica Labs.

Overview of Customized 8-Plex InSituPlex Panel

A.

PHENOTYPE	MARKERS							
	CD3	CD4	CD8	CD68	FoxP3	PD-1	PD-L1	CK
T cell	X							
T helper cell	X	X						
Cytotoxic lymphoid cell			X					
Cytotoxic T cell	X		X					
FoxP3+ T cell	X				X			
Exhausted T cell	X					X		
T-reg	X	X			X			
CD4/CD8 T cell	X	X	X					
CD8+ T-reg	X		X		X			
Exhausted, cytotoxic T cell	X		X			X		
Macrophage				X				
Immunosuppressive Macrophage				X			X	
Tumor cell								X
Immuno-evading tumor cell							X	X

B.

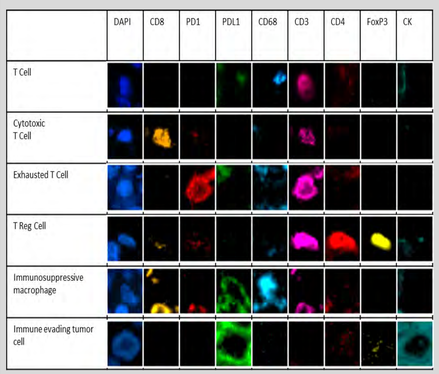
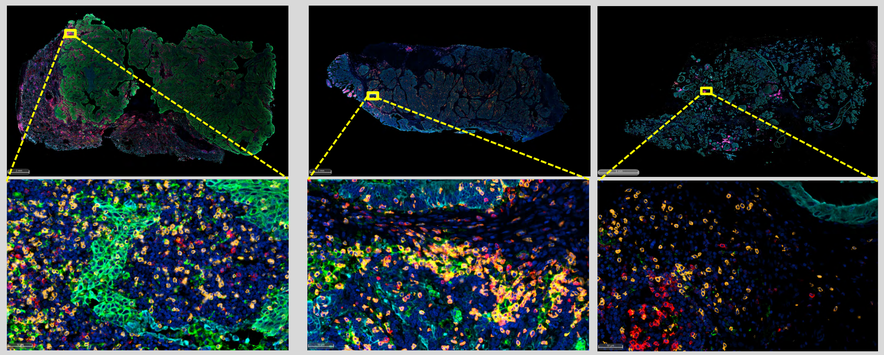


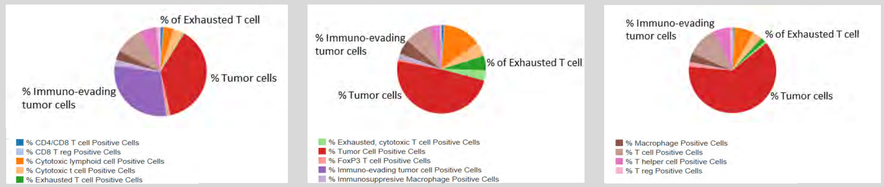
Figure 2. A) A customized panel of 8 markers, including CD3, CD4, CD8, CD68, FoxP3, PD-1, PD-L1, and pan-CK, was developed to allow phenotypic profiling of immune and tumor cell populations. **B)** Representative images of immunophenotypes identified by the 8-plex mIF assay are shown.

Immunophenotyping and Spatial Relationships in NSCLC, CRC, and DCIS

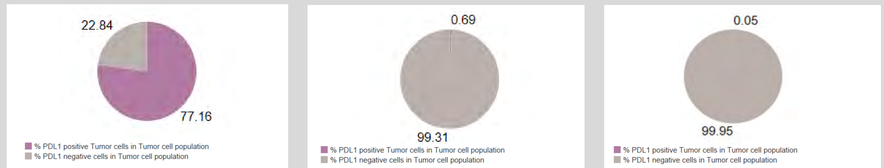
A.



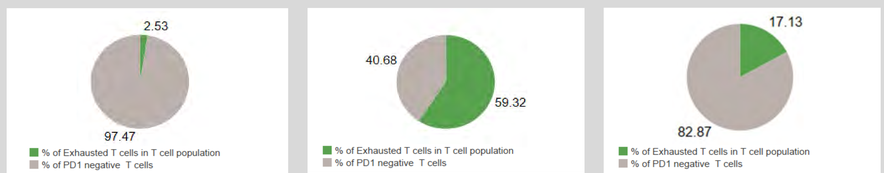
B. Immune Signature



C. Comparison of Immuno-Evading T Cell Population



D. Comparison of Exhausted T Cells



E. Spatial Analysis

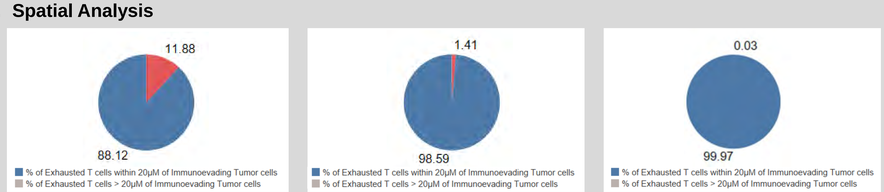


Figure 3. A) ***Top:*** Whole slide images of NSCLC, CRC, and DCIS FFPE tissues stained with the customized 8-plex mIF panel. ***Bottom:*** Representative high-resolution images illustrate regions of interest selected from whole slide images. Image analyses of whole slide images enables **B)** profiling of immunophenotypes; characterization and comparison of particular cell populations of interest, such as **C)** immuno-evading T cells and **D)** exhausted T cells; and **E)** interrogation of spatial relationships within the tumor immune environment.

Conclusions

The 8-plex immune-profiling panel demonstrated specific biomarker staining and was verified for staining reproducibility. The 8-plex workflow was compatible across multiple tumor tissue types. The samples were characterized by cell analysis and phenotyped based on their expression of specific biomarkers. The following cell phenotypes were detected: T cells (CD3+), cytotoxic T cells (CD3+/CD8+), regulatory T cells (CD4+/FOXP3+), exhausted T cells (CD3+/PD-1+), macrophages (CD68+), and tumor cells (CK+) with a dynamic range of PD-L1 expression levels.

Discussion

The 8-plex panel revealed important immunophenotypes across multiple sample types. The assay was automated and optimized for a high-throughput workflow. By testing across multiple tissue types, this assay showcases the potential for broad application across additional cancer types. The ability to interrogate an 8-plex I/O panel on a single slide with WSI presents an attractive approach for the next generation of IHC, enabling profiling within the tumor microenvironment and sensitive detection of PD-L1 expression in cancer tissues.

References

Barua, S., Fang, P., Sharma, A., Fujimoto, J., Wistuba, I., Rao, A. U. K., & Lin, S. H. (2018). Spatial interaction of tumor cells and regulatory T cells correlates with survival in non-small cell lung cancer. Lung Cancer (Amsterdam, Netherlands), 117, 73–79. https://doi.org/10.1016/j.lungcan.2018.01.022

Giraldo, N. A., Nguyen, P., Engle, E. L., Kaunitz, G. J., Cottrell, T. R., Berry, S., … Taube, J. M. (2018). Multidimensional, quantitative assessment of PD-1/PD-L1 expression in patients with Merkel cell carcinoma and association with response to pembrolizumab. Journal for ImmunoTherapy of Cancer, 6(1), 99. https://doi.org/10.1186/s40425-018-0404-0

Lazarus, J., Maj, T., Smith, J. J., Perusina Lanfranca, M., Rao, A., D'Angelica, M. I., … Frankel, T. L. (2018). Spatial and phenotypic immune profiling of metastatic colon cancer. JCI Insight, 3(22). https://doi.org/10.1172/jci.insight.121932

Ma, J., Zheng, B., Goswami, S., Meng, L., Zhang, D., Cao, C., … Zhang, X. (2019). PD1Hi CD8+ T cells correlate with exhausted signature and poor clinical outcome in hepatocellular carcinoma. Journal for ImmunoTherapy of Cancer, 7(1), 331. https://doi.org/10.1186/s40425-019-0814-7

Tsakiroglou, A. M., Fergie, M., Oguejiofor, K., Linton, K., Thomson, D., Stern, P. L., … West, C. M. L. (2020). Spatial proximity between T and PD-L1 expressing cells as a prognostic biomarker for oropharyngeal squamous cell carcinoma. British Journal of Cancer, 122(4), 539–544. https://doi.org/10.1038/s41416-019-0634-z

Contact Information

*Mary E. Olanich: mary.olanich@nih.gov
*Scott Lawrence: lawrencesm@mail.nih.gov

This project has been funded in whole or in part with Federal funds from the National Cancer Institute, National Institutes of Health, under NCI Contract No. 75N91DD00024. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

DEPARTMENT OF HEALTH AND HUMAN SERVICES • National Institutes of Health

The Frederick National Laboratory for Cancer Research is a Federally Funded Research and Development Center operated by Leidos Biomedical Research, Inc. for the National Cancer Institute

Contract No. 75N910D00024 - Funded by the National Cancer Institute