ADVANCES IN MULTIPLEXED ION BEAM IMAGING (MIBI) FOR IMMUNE PROFILING OF THE TUMOR MICROENVIRONMENT

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Abstract 48

Background
Multiplexed ion beam imaging (MIBI) combines time-of-flight secondary ion mass spectrometry (ToF-SIMS) with metal labeled antibodies to image 40+ proteins in a single scan at subcellular spatial resolution. Here, we show that the recently released MIBIscope provides improved sensitivity for detecting immune checkpoint markers and offers greater throughput at higher resolution than the alpha instrument.

Methods
Serial sections from three FFPE NSCLC samples, in addition to a control slide consisting of various unremarkable tissues, were stained with a panel of 25 metal labeled antibodies. The tissue was imaged at subcellular resolution using the MIBIscope and the alpha instrument. Masses of detected species were assigned to target biomolecules given the unique label of each antibody and multi-step processing was used to create images. Cell classification was performed using two complementary methods that differed in the need for cell segmentation to phenotypically characterize the tissue environment and quantify marker expression.

Results
Replicate regions of interest (ROIs) were collected on both instruments with similarly sized ROIs acquired in 17 minutes with the MIBIscope compared to 280 minutes with the alpha instrument. Fourier Ring Correlation (FRC) showed the resolution to be greater on the MIBIscope as compared to the alpha instrument with FRC also demonstrating uniform resolution across an ROI 2.5X greater in size. Even with the 16X greater speed of the MIBIscope, the signal of the 25 markers across replicate ROIs was increased (y=x^1.07) and showed similar expression patterns to those observed on the alpha instrument (figure 1). This resulted in greater sensitivity to markers with low expression, such as checkpoint markers. Eleven cell populations were classified across the ROIs utilizing two methods, with both methods showing a similar frequency of tumor cells and B, T, and myeloid cell subsets between instruments. Segmentation enabled the number of cells within a population to be calculated but defining boundaries is laborious and signal from neighboring cells can result in misclassification. Performing classification at the pixel level, without segmentation, enabled the fraction of the tissue that is tumor or any other cell type to be rapidly determined.

Conclusions
The MIBIscope enables the phenotypic characterization of tumor and non-tumor microenvironments. Co-expression of markers can be used to classify tumor and immune populations and to quantify the expression of markers associated with immune suppression. The increased sensitivity and throughput of the MIBIscope, in combination with the 40-parameter capability and subcellular resolution, provides a platform uniquely suited to understanding the complex tumor immune landscape.

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IN SITU PHENOTYPIC ANALYSIS OF T CELLS IN THE TUMOR MICROENVIRONMENT OF A PRE-CLINICAL MODEL OF NON-SMALL CELL LUNG CANCER (NSCLC) BY TISSUE SECTIONING AND WHOLE-MOUNT IMMUNOFLORESCEENCE IMAGING

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Background
Understanding the interactions between tumor and immune cells is critical for improving current immunotherapies. Pre-clinical and clinical evidence has shown that failed T cell infiltration into lung cancer lesions might be associated with low responsiveness towards checkpoint blockade.1 For this reason, it is necessary to characterize not only the phenotype of T cells in tumor-bearing lungs but also their spatial location in the tumor microenvironment (TME). Multiplex immunofluorescence staining allows the simultaneous use of several cell markers to study the state and the spatial location of cell populations in the tissue of interest. Although this technique is usually applied to thin tissue sections (5 to 12 μm), the analysis of large tissue volumes may provide a better understanding of the spatial distribution of cells in relation to the TME. Here, we analyzed the number and spatial distribution of cytotoxic T cells and other immune cells in the TME of tumor-bearing lungs, using both 12 μm sections and whole-mount preparations imaged by confocal microscopy.

Methods
Lung tumors were induced in C57BL/6 mice by tail vein injection of a cancer cell line derived from KrasG12D/+ and Tp53-/- mice. Lung tissue with a diverse degree of T cell infiltration was collected after 21 days post tumor induction. Tissue was fixed in 4% PFA, followed by snap-frozen for sectioning. Whole-mount preparations were processed according to Weizhe Li et al. (2019)2 for tissue clearing and multiplex volume imaging. T cells were labeled with CD8 and FOXP3...
antibodies to identify cytotoxic or regulatory T cells, respectively. Tumor cells were labeled with a pan-Keratin antibody. Images were acquired using a Leica SP8 confocal microscope. FJI3 and IMARIS were used for image processing.

**Results** We identified both cytotoxic and regulatory T cell populations in the TME using thin sections and whole-mount. However, using whole-mount after tissue clearing allowed us to better evaluate the spatial distribution of the T cell populations in relation to the tumor structure. Furthermore, tissue clearance facilitates the imaging of larger volumes using multiplex immunofluorescence.

**Conclusions** Analysis of large lung tissue volumes provides a better understanding of the location of immune cell populations in relation to the TME and allows us to study heterogeneous immune infiltration on a per-lesion base. This valuable information will improve the characterization of the TME and the definition of cancer-immune phenotypes in NSCLC.

**REFERENCES**


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**50 SPATIALLY RESOLVED MOLECULAR INVESTIGATION OF TRIPLE NEGATIVE BREAST CANCER AND ITS IMMUNE MICROENVIRONMENT**

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**Background** Triple negative breast cancer (TNBC) accounts for 10–20% of all diagnosed breast cancer cases in the US and is characterized by loss of HER2, estrogen receptors, and progesterone receptors. TNBC is an aggressive, complex disease with a poor prognosis due to resistance to traditional therapies. Understanding the underlying biology and tumor microenvironment is critical to the development of diagnostic biomarkers and to guide the search for effective therapies. Here, we demonstrated the ability of the 10x Genomics Visium Spatial Gene Expression Solution to elucidate the immunological profile and microenvironment of TNBC samples in conjunction with standard pathological techniques.

**Methods** Spatial transcriptomics technology complement pathological examination by combining the benefits of histological stains with the throughput and deep biological insight of RNA-seq. We investigated serial sections of TNBC by using the 10x Genomics Visium Spatial Gene Expression Solution to spatially resolve the samples’ cellular composition and expressed microenvironment. Visium incorporates ~5000 molecularly barcoded, spatially encoded capture probes in spots over which a tissue section is placed and imaged. The samples are permeabilized and native mRNA is captured. Imaging and RNA sequencing data are processed together, resulting in whole transcriptome gene expression mapped to the tissue image.

**Results** We captured spatial patterns of gene expression and mapped the information back to H&E-stained images with regional annotations. Serial sections were then subject to fluorescence immunohistochemical staining for immune infiltrate paired with spatial gene expression capture. Subsequently, we combined these data with 3’ single-nuclei RNA-seq from the same tumor, generating expression profiles that were used to automatically annotate cell-types across the sections. This allowed for an understanding of the tumor microenvironment that could not be captured by image-based techniques alone. We resolved subgroups of spatially and biologically distinct immune, stem, and cancer progenitor cells. Finally, we digitally annotated tumor and normal tissue regions using expressed genetic mutations alone. Annotated tumor regions expressed more deleterious mutations than normal regions and we were able to automatically cluster regions of tumor vs. normal cells without any prior histopathological information. We also found intratumor gradients of mutational burden in oncogenes as well as non-cancer associated loci.

**Conclusions** Taken together, we demonstrated that Visium can provide a powerful complement to traditional histopathology, enabling both targeted panels and whole-transcriptome discovery of gene expression. This spatially resolved molecular information provides an unprecedented view into the tumor microenvironment and a powerful new tool for discovery of new biomarkers and therapeutic targets.

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**51 SMALL CELL/LYMPHOHISTIOCYTIC MORPHOLOGY IS ASSOCIATED WITH CD8 Positivity, Retained T CELL MARKERS, A TREND OF DECREASED PD-L1 EXPRESSION, BUT NOT OUTCOME IN ADULTS WITH ALK + ALCL**

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**Background** Several morphologic patterns of ALK+ anaplastic large cell lymphoma (ALCL) are recognized: common, small cell, lymphohistiocytic, Hodgkin-like, and composite patterns. Small cell (SC) and lymphohistiocytic (LH) patterns are thought to be closely associated with poorer outcome in children with ALK+ ALCL. However, the clinicopathologic and prognostic features of SC/LH patterns of ALK+ ALCL are not yet reported in adults. Recently, we found PD-L1 expression in a large subset of ALK+ ALCL cases, however, PD-L1 expression in SC/LH versus non-SC/LH ALCL has not been reported.

**Methods** Among 102 adult patients with ALK+ ALCL seen at our institution from January 1, 2007 through August 30, 2018, 18 (18%) cases had a SC and/or LH pattern. The clinical, pathologic, and outcome data were compared between SC/LH and non-SC/LH ALK+ ALCL cases using Fisher’s exact test. Overall survival (OS) was analyzed using the Kaplan-Meier method and compared using the log-rank test.

**Results** There were no significant differences in clinical features including age, gender, nodal versus extranodal involvement, B symptoms, stage, leukocytosis/lymphocytosis, and serum LDH levels between patients with SC/LH versus non-SC/LH ALK+ ALCL. Compared to non-SC/LH cases, SC/LH ALCL was more often positive for CD2 (92% vs. 36%, p = 0.0007), CD3 (81% vs. 15%, p = 0.0001), CD7 (80% vs. 37%, p = 0.03), and CD8 (54% vs. 7%, p = 0.0006). SC/LH ALCL showed a trend of decreased PD-L1 expression than non-SC/LH cases (24% vs. 46%, p = 0.11). There were no significant differences in the expression of CD4, CD5, CD25, CD43, CD45, CD56,