Multiparameter tissue section imaging and retrieval of image-defined micro-regions for RNA sequencing using the RareCyte platform

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Background

Tumor tissue imaging allows for a contextual understanding of tumor cells in relation to the immune microenvironment. The ability to interrogate tissues for multiple proteins that define microscopic regions of interest (ROI) and investigate gene expression of cells in those regions is needed for advancing immuno-oncology therapeutic and biomarker discovery. Here we introduce Pick-Seq™, a novel method enabled by the RareCyte CyteFinder® Imaging System with integrated multi-parameter imaging and micro-region retrieval capabilities for sequencing and transcript-level analysis.

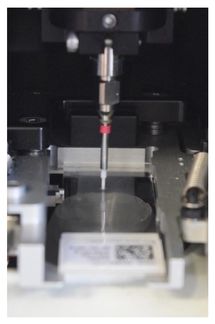
Methods

Formalin-fixed, paraffin-embedded (FFPE) sections of tonsil and lung carcinoma sections were stained with a panel of antibodies to CD3, CD4, CD8a, CD20, and cytokeratin. Frozen sections were stained with antibodies to T cell (CD3, CD4, and CD8a) and B cell markers (IgG/IgM). All sections were also stained with SYTOX™ Orange or DAPI (nuclear dyes). Whole-slide six-color scanning and ROI identification was performed with the CyteFinder Imaging System. For frozen sections, 40 µm diameter micro-regions were retrieved directly from the antibody-stained section using the CytePicker® Retrieval Module. For FFPE sections, ROI were identified on an antibody-stained section, and micro-regions were retrieved from the same location on an adjacent section stained with DRAQ5™ only. RNA from retrieved micro-regions was amplified with SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara) followed by Nextera XT library prep (Illumina). Sequencing was performed on an Illumina MiSeq followed by differential expression analysis (DESeq2). Cell compositions of each micro-region were deconvolved with CIBERSORT using the LM22 signature.

RareCyte Technology

A

B



C

Prep	Tissue Section	Antigen Retrieval	Staining Panel	Purpose
Frozen	1	No	Original	Identify and retrieve micro-regions
	2	No	Informed	Validate Pick-Seq results
FFPE	1	Yes	Original	Identify micro-regions
	2	No	DRAQ5	Retrieve micro-regions
	3	Yes	Informed	Validate RNA-seq results

Figure 1. RareCyte technology and Pick-Seq experimental workflow overview. Stained tissue sections were scanned and analyzed with the CyteFinder system (A), followed by retrieval of micro-regions of interest with the CytePicker Retrieval Module (B). Experimental workflow for tissue Pick-Seq (C). For frozen tissue, the first section is stained without antigen retrieval (AR) with the original panel, followed by micro-region retrieval from the same slide. For FFPE tissue, one section is antigen-retrieved and stained with the original panel to define regions of interest, followed by retrieval of those regions on a serial section stained with DRAQ5 without AR. This strategy was employed to prevent RNA degradation that occurs during AR process. Resulting RNA-seq data informs a second staining panel that is applied to another serial section.

Results

Multi-parameter immunofluorescence tissue imaging

A B

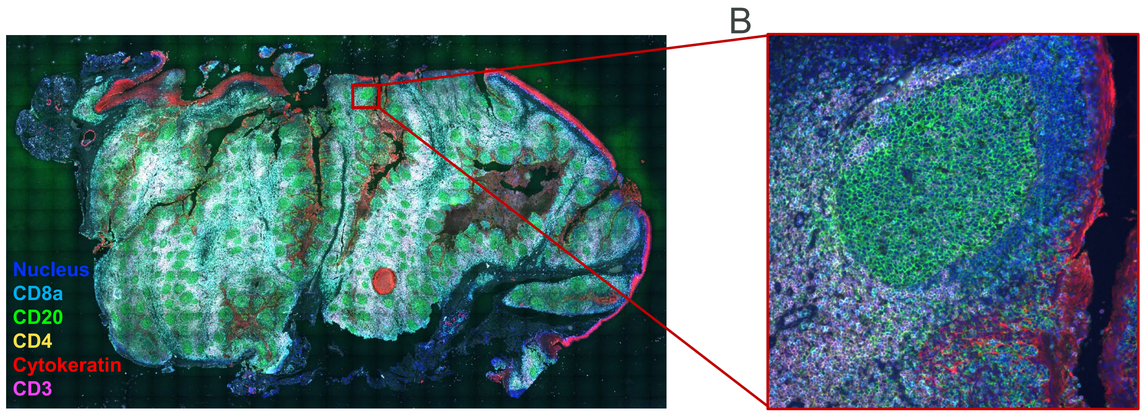


Figure 2. 6-color immunofluorescence imaging of FFPE tonsil tissue. (A) Whole section composite multi-parameter image and (B) germinal center shown at 20X magnification. Tonsil staining distinctly identified crypt lining (cytokeratin), T cell (CD3, CD4, CD8) and B cell (CD20) compartments.

A B

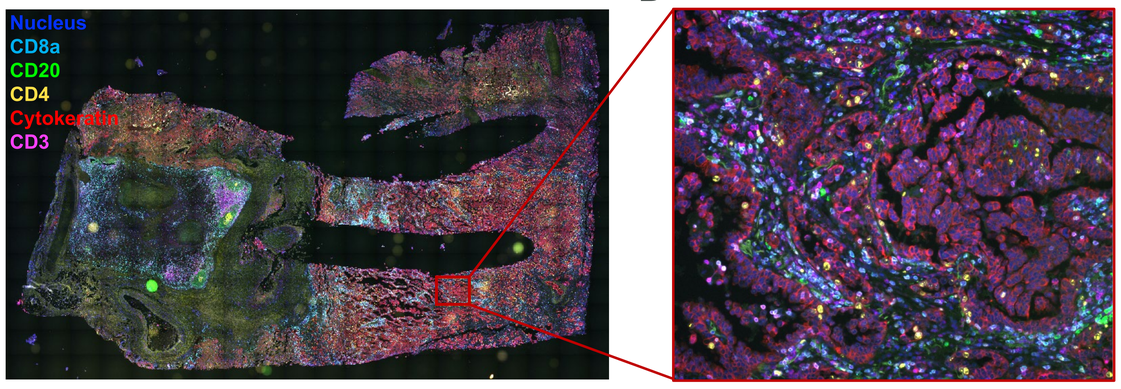


Figure 3. 6-color immunofluorescence imaging of FFPE lung cancer tissue. (A) Whole section composite multi-parameter image. Cytokeratin-positive carcinoma is prominent on the right side of the panel. Bronchus-associated lymphoid tissue is seen on the left. (B) Representative region of cytokeratin-positive (red) tumor cells with surrounding infiltrating CD8-positive (blue) T cells (20X objective magnification).

RNA-seq of CytePicker-retrieved micro-regions

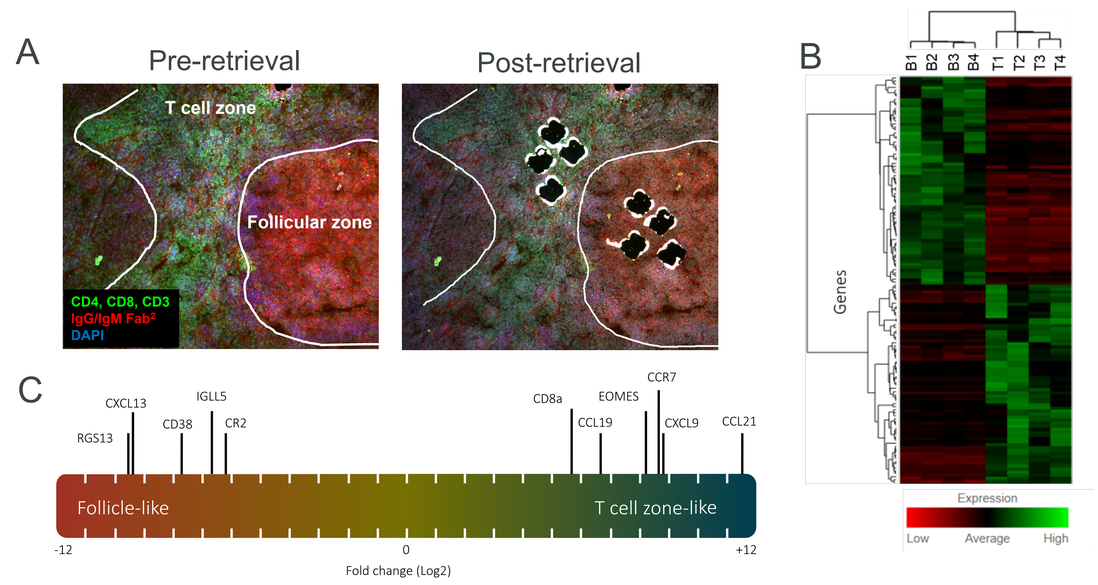
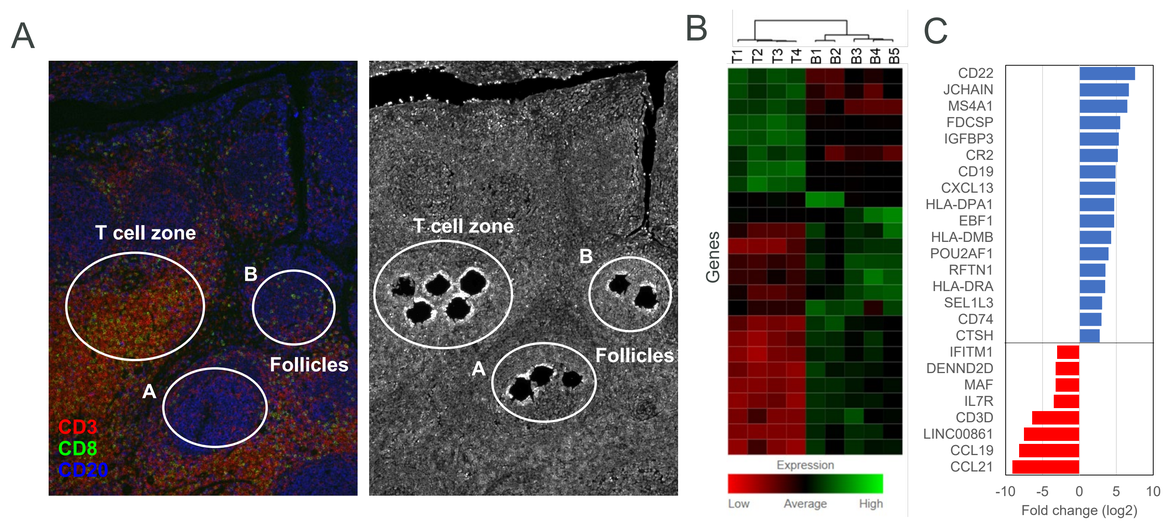


Figure 4. Micro-region retrieval and RNA-seq in frozen tonsil tissue. Micro-regions were isolated from T cell-rich and follicular zones in stained OCT tonsil tissue using CytePicker (A). RNA-seq was performed, followed by differential expression analysis. Comparative RNA analysis (B) of four frozen tonsil T cell zone micro-regions against four B cell zone micro-regions identified genes with expected differential gene expression pattern, including T cell zone upregulation of CD8a, CCL19, and CCL21 and downregulation of CD38, CR2, and CXCL13 (C).



D

		CD4	CD19	CD20	CD21
T cell zone		100	13	34	41
		92	0	0	0
		64	0	0	0
		44	39	0	0
B-cell follicles	A	0	239	581	1501
		0	283	1018	380
		11	199	811	699
	B	0	566	1552	120
		0	950	1563	59

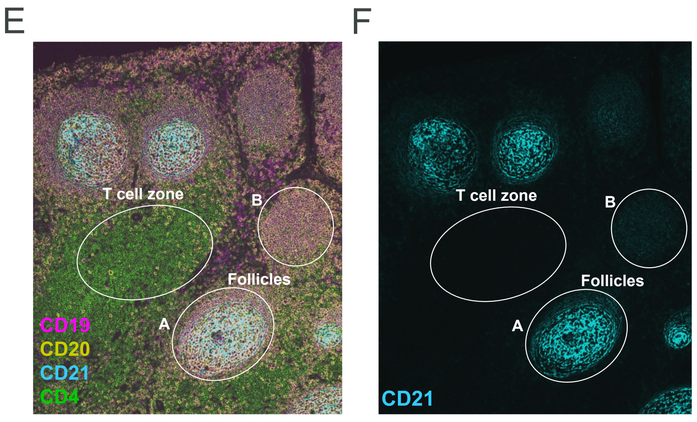
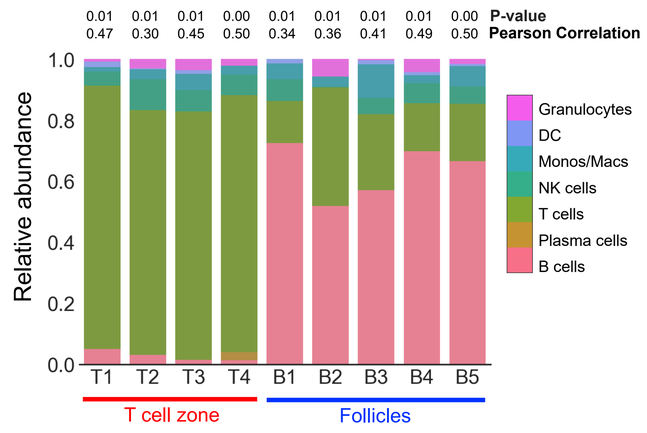


Figure 5. Micro-region retrieval and RNA-seq in FFPE tonsil tissue. Following identification of T cell-rich and follicular zones on stained FFPE tonsil tissue, micro-regions were isolated via CytePicker from a serial section with a nuclear stain (A). RNA-seq was performed, followed by differential expression analysis. Hierarchical clustering (B) and analysis of differentially expressed genes (C) showed increased expression of hallmark B cell markers in follicles (blue) and T cell markers in picks from outside the follicle. FPKM (fragments per kilobase million) analysis (D) revealed differing expression of markers in adjacent follicles, including CD19, CD20 and CD21. To interrogate and verify these results, a serial section was stained with a targeted panel containing CD21 (E), confirming the CD21 gene expression results by immunofluorescence (F), and indicating that follicle A contains a germinal center within the plane of section.

Figure 6. Immune cell populations in tissue micro-regions. CIBERSORT analysis of mRNA expression profiles (FPKM) in micro-regions. Using the LM22 matrix as signature, the CIBERSORT deconvolution showed two distinct cell compositions between T cell zones and the B cell follicles.



Conclusions

This study introduces Pick-Seq, a method of image-guided RNA discovery uniquely enabled by the CyteFinder system. Here we demonstrate the use of high resolution imaging of FFPE tonsil to guide retrieval of micro-regions from a serial section for transcriptomic analysis, which in turn revealed architecture-dependent differential gene expression. Subsequent staining of another serial section with an RNA-informed panel confirmed the RNA-seq results by immunofluorescence. These data suggest that this method can be applied to RNA-driven biomarker discovery in tissue.