Spatial characterization of T-cell receptors at subcellular resolution using sequencing-by synthesis & spatial protein detection on the same tissue

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Introduction

Here, we present a novel spatial multiomics method combining *in situ* sequencing-by-synthesis (SBS) with high-plex protein detection (30+ markers) to simultaneously analyze RNA and protein on a single FFPE tissue section. The technology is based on iterative interrogation of rolling circle amplified RNA detection probes and directly conjugated antibodies, expanding beyond traditional hybridization-based RNA detection to uniquely enable 4-color *in situ* SBS of RCA products.

Using this method, we studied samples from a groundbreaking case of a 17-year-old patient with a relapsed/metastatic, heavily pre-treated kidney tumor (nephroblastoma/Wilms' tumor) treated with experimental TCR-engineered T-cell therapy at the Hopp Children's Cancer Center Heidelberg (KiTZ), Germany. This enabled detailed spatial characterization of TCR clonotypes, immune cell infiltration, and key protein markers within the tumor microenvironment. Please also see the poster presentation: **Point-of-care-manufactured PRAME-specific TCR T cell therapy induces deep remission in a heavily pre-treated pediatric patient with extensive, multifocal nephroblastoma (LB326, abstract 10580)**.

Methods and Materials

FFPE tissue samples from peritoneal and lung metastases of a patient treated with transgenic TCR-T cells, generated using the **CliniMACS Prodigy® platform**, were studied. The peritoneal sample was collected nine days post-treatment and the lung sample at 100 days post-treatment. Bulk sequencing of extracted TCR α/β RNA was performed using **Miltenyi's TCR α/β RNA Profiling Kit**. **RNAsky® Detection Probes** were designed against the CDR3 regions of the ten most abundant clonotypes identified in the lung sample, as well as against the 2A peptide sequence part of the engineered TCR-T cells polyprotein single ORF. Sequential tissue sections were hybridized, ligated, and amplified and then imaged on both the **MACSima® Platform** and a new spatial multiomics application. On both cases, spatial transcriptomics was followed by proteomics using fluorescent MICS antibody-based protein detection on the same tissue section. The new spatial multiomics application additionally employed in situ sequencing via 4-color sequencing-by-synthesis (SBS), enabling detection of both known and unknown transcript sequences.

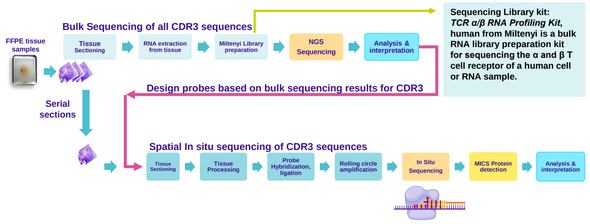


Fig 1. T cell receptor transcript detection (CDR3) using bulk sequencing TCR profiling followed by spatial in situ sequencing & protein detection on FFPE tissue

Results

1. Bulk TCR Sequencing

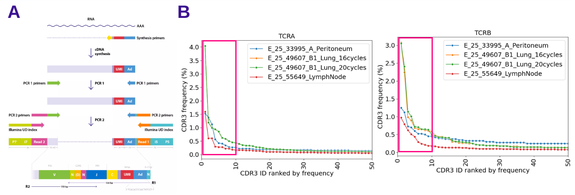
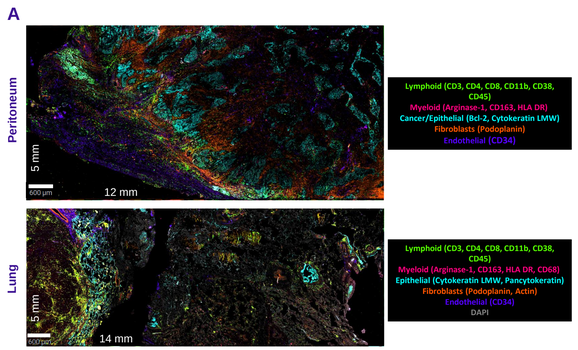


Fig 2. Bulk sequences identifies most abundant TCR α/β. A. Schematic overview of TCR library generation and composition. B. Bulk sequencing results for alpha and beta chains with clone frequency distribution from FFPE peritoneum, lung and lymph node tissue.

2. *In situ* sequencing and protein detection on whole tissue



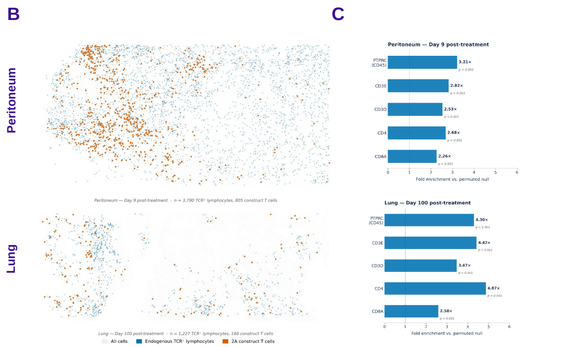


Fig 3. Miltenyi's new spatial multiomics application can image whole tissue to perform multiomic profiling of endogenous and engineered TCRs in peritoneum and lung. A. Whole tissue protein detection with 13+ antibodies in peritoneum and lung exhibit defining cells types. Scale bar = 600 µm. B. Whole tissue transcript detection shows distribution of 2A construct T cells and endogenous TCR⁺ lymphocytes. C. Cells containing 2A transcripts are highly enriched for lymphoid and T cell markers indicating these cells are in the T cell lineage at both time points (vs. 1,000-permutation null, $p < 0.001$ for all).

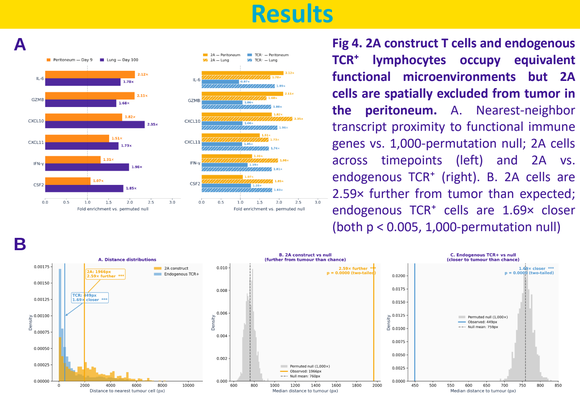


Fig 4. 2A construct T cells and endogenous TCR⁺ lymphocytes occupy equivalent functional microenvironments but 2A cells are spatially excluded from tumor in the peritoneum. A. Nearest-neighbor transcript proximity to functional immune genes vs. 1,000-permutation null; 2A cells across timepoints (left) and 2A vs. endogenous TCR⁺ (right). B. 2A cells are 2.59× further from tumor than expected; endogenous TCR⁺ cells are 1.69× closer (both $p < 0.005$, 1,000-permutation null)

3. In depth spatial TCR multiomic profiling of the peritoneum

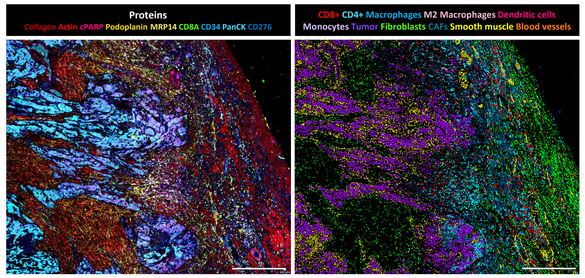


Fig 5. In depth multiomic profiling of the peritoneum tumor microenvironment with the MACSima platform. A. 9 selected markers of a 44 marker protein panel highlight the complexity of the metastasized tumor microenvironment. B. 11 main cell populations were identified by unsupervised Leiden clustering of normalized multiplexed protein expression data, followed by marker-guided compartment assignment and sub clustering. Scale bar = 500 µm.

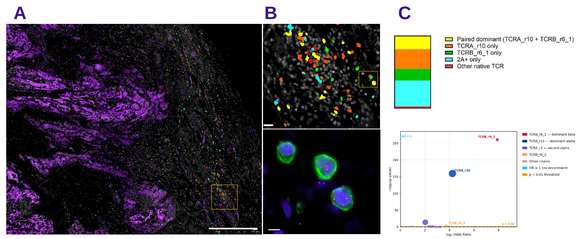


Fig 6. 2A construct-bearing cells are strongly enriched for the dominant α and β TCR chains, with 64.7% carrying TCRA_r10 or TCRB_r6_1. A. Of 488 2A+ cells, 45.3% carried the dominant α, 33.4% the dominant β, and 18.2% both chains paired. Scale bar = 500 µm. B. Zoom showing CD8+ T cells (green) with dominant α (pink) β (yellow) and 2A construct (open red) Scale bar = 25 µm (top) and 5 µm (bottom). C. TCRB_r6_1 and TCRA_r10 are strongly enriched in 2A construct-bearing cells (OR = 240.6 and 18.9; Fisher's exact test, $p<0.001$), confirming the dominant clonotype as the primary construct-associated T cell lineage.

4. Spatially resolved sequencing of unknown transcript regions

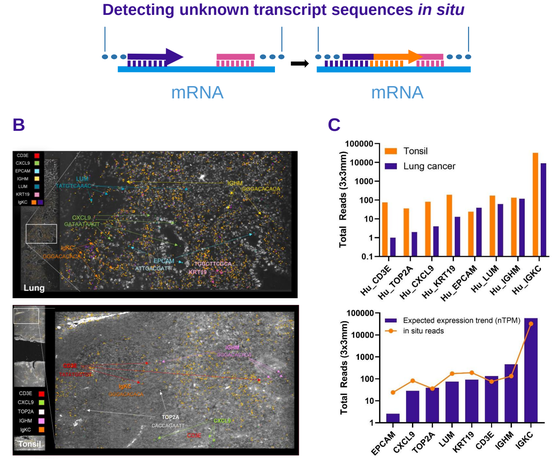


Fig 7. Miltenyi's new spatial multiomics application can identify unknown transcript sequences *in situ*. A. Schematic of the approach where a probe with 5′ and 3′ arms hybridizes to known flanking sequences of a target mRNA such that undefined (unknown) nucleotides lies between the arms. A DNA polymerase fills the gap which is ligated, incorporating the unknown sequence information. B. Fluorescent imaging of rolony signal within cells, indicating specific target transcript localization and sequence information detected in an FFPE lung cancer and tonsil tissue section (5x11 mm) using *in situ* SBS of unknown regions. C. Total reads distribution between lung and tonsil tissue sample (~22 mm²) and expression level compared to bulk sequencing transcript per million (nTPM).

Conclusions and Outlook

Comprehensive multiomic spatial profiling utilizing RNAsky® detection probes, *in situ* SBS, and direct protein detection enables simultaneous characterization of cell identity, clonal architecture, and functional state within intact tissue. Across peritoneal and lung metastases from a patient undergoing adoptive T cell therapy, this approach spatially resolved 12 cell populations and directly mapped TCR clonotypes to individual cells. Construct-bearing T cells were strongly enriched for the dominant endogenous clonotype (64.7% of 2A+ cells; OR = 240.6, $p<0.001$) yet remained spatially excluded from the tumor nest despite displaying an activated effector phenotype 9 days post treatment, implicating the microenvironment as the primary barrier to infiltration. The new multiomics application can detect unknown transcript sequences *in situ*, enabling applications such as SNP detection and immune repertoire profiling. These results demonstrate that *in situ* sequencing combined with spatial proteomics provides a uniquely powerful and non-destructive framework for dissecting immune-tumor interactions at single-cell resolution, with the capacity to interrogate both known and unknown transcript sequences within intact tissue.

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