Quantitative assessment of the tumor microenvironment using the Aurora™ 3D Spatial Biology Platform

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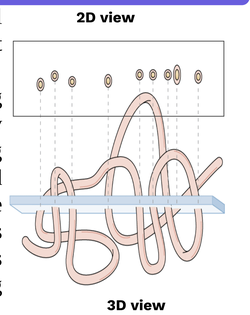
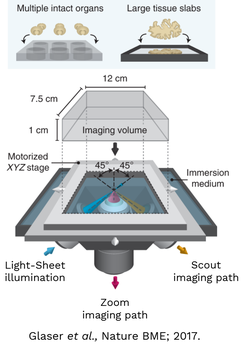
Introduction

Analysis of the three-dimensional (3D) tumor microenvironment (TME) landscape has the potential to transform diagnosis and drug development. Current 2D histology techniques introduce sampling error, interobserver variability, and fail to capture the full tissue biology. Our technology breaks down the practical barriers hampering widespread 3D imaging and quantification adoption. The Alpenglow Biosciences Aurora™ 3D Spatial Biology platform stains, chemically clarifies, images, and analyzes entire, intact tissue samples. Here we report an analysis of CRC samples using the Aurora platform to characterize tumor, immune cell, and collagen features in the TME and to quantify and compare lymphocyte exclusion in the tumor microenvironment in 3D and 2D.



Hybrid Open-Top Light-Sheet (HOTLS) Microscopy



Glaser *et al.*, Nature BME; 2017.

HOTLS features

- Easy mounting of multiple samples
- High throughput imaging
- Quickly "Scout" large volumes at low resolution
- Select regions of interest for high resolution "Zoom" scans for machine learning and spatial analysis

Tissue Processing, Imaging, and Analysis

Five de-identified human colorectal cancer (CRC) FFPE blocks were deparaffinized and stained with nuclear (TO-PRO-3) and general protein (eosin) fluorescent dyes, and optically cleared using a modified iDISCO protocol. Samples with a volume of 200-500 mm^3 were imaged at 2 µm/pixel resolution with the HOTLS 3D*i*™ (Glasser, *et al.*, 2017). Smaller regions of interest (ROIs), 0.5 mm^3, were re-imaged at higher resolution, 0.33 µm/pixel. Nuclear segmentation and 3D spatial analysis were performed using our 3D*ai*™ tools consisting of a U-Net, CytoMAP (Stoltzfus *et al.*, 2020), and custom python scripts capable of scaling to the 10–20-billion-pixel image datasets the 3D*i*™ can produce. Tumor infiltrating lymphocytes (TILs) were identified and classified according to spatial location in the tumor stroma or tumor parenchyma within high-resolution ROIs. The variation in stromal to parenchymal TILs was then quantified in 3D and compared to virtual 2D sections of the 3D images. Using the OrientationJ plugin, collagen fibers were then color coded in high-resolution ROIs based on the orientation of fibers.

REFERENCES:
Glaser *et al.*, Nature BME; 2017
Stoltzfus *et al.*, Cell Rep; 2020

ABBREVIATIONS: HOTLS – Hybrid Open-Top Light-Sheet; TME – Tumor Microenvironment; TIL – Tumor Infiltrating Lymphocyte; TLS – Tertiary Lymphoid Structure; ROI – Region Of Interest; CRC- Colorectal Cancer

Results

For the 5 tissues analyzed, 1 high resolution ROI was selected from 4 of the tissues and 2 ROIs were selected from 1 of the tissues. Within the 6 ROIs selected, a total of 641,800 TILs were identified and spatially profiled. A marked variation in the ratio of stromal to parenchymal TILs was observed across the 3D volumes. Interestingly, due to this variation in the TME in 3D space, for an accurate representation (within 10% error) of the spatial organization of the TILs, between 9 and 39 traditional 4 micrometer slices would be needed in the five samples analyzed. This finding underscores both the heterogeneity of the TME and the sampling power that 3D imaging offers over conventional techniques. The detailed visualizations below, including the 3D structure of the tumor region, positional plots of TILs, and analyses of virtual 2D sections at various depths, highlight the rich information gleaned from the data and subsequent quantification.

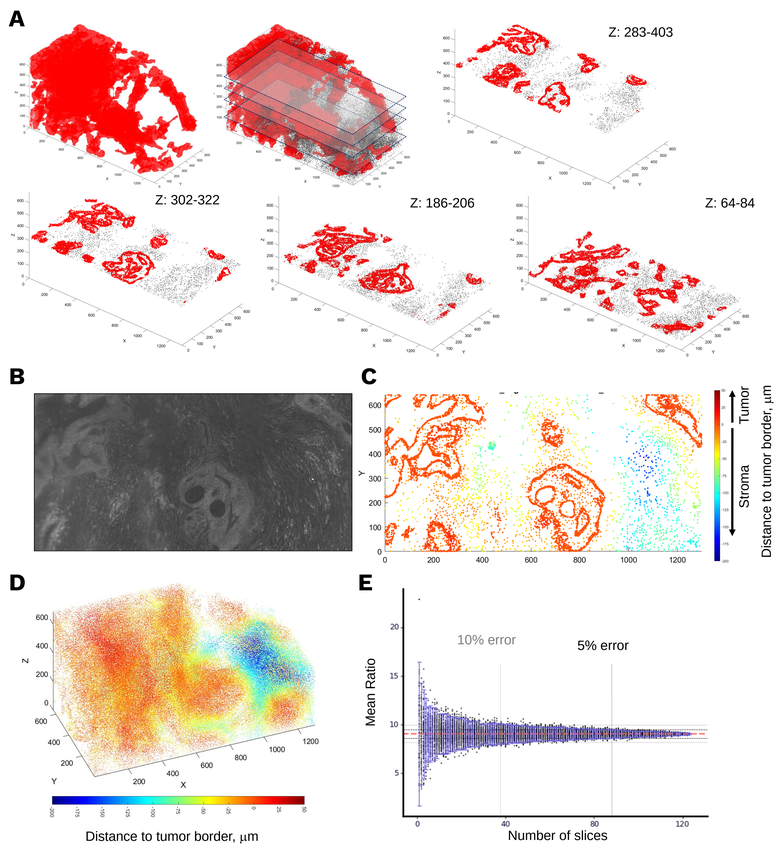


Figure 1: 3D quantification of the tumor parenchyma, TIL distances, and spatial heterogeneity. A: These positional plots show the 3D structure of the tumor region in red for a representative sample. The top middle plot shows the positions of TILs as black dots. The rightmost plot and bottom row show a thin section of the 3D data from various depths. **B:** Single 2D slice from the same ROI showing the nuclear channel. **C:** A positional plot from CytoMAP of the same section shown in B. Each dot corresponds to a cell color-coded by 3D distance to the tumor parenchyma border. This border is highlighted in orange. Distances are split into the tumor parenchyma (distances > 0) and the tumor stroma (distances < 0). **D:** 3D positional plot from CytoMAP showing the positions of all cells color coded by distance to the tumor parenchyma. **E:** Plot of the variation in the stroma to parenchymal TIL distribution as a function of the number of slices sampled from the full 3D dataset. Each dot represents a simulated experiment with N randomly selected slices of the 3D dataset.

Figure 2: A single 2D image plane from the 3D dataset showing a cross-section of a TLS.

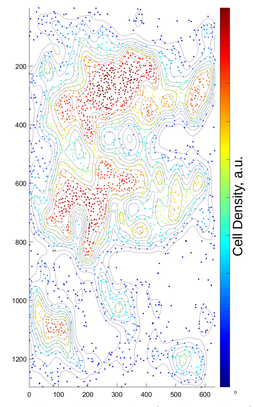


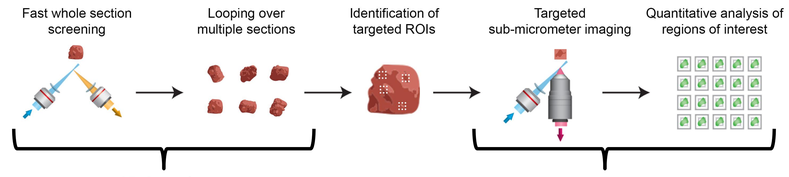
Figure 3: A Contour density positional plot in CytoMAP showing the positions of the TILs in the same 2D plane shown in Figure 2, color coded by the density of cells.

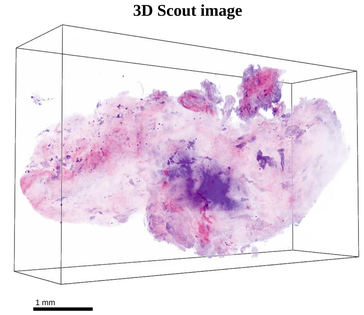
Conclusions

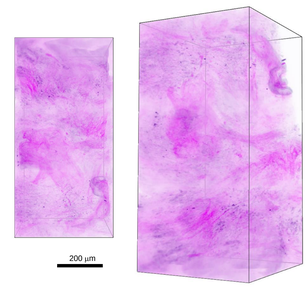
- 3D imaging techniques enable visualization of complex TME structures and nuanced features like TLS **(Figures 2 and 3),** and collagen fibers around tumor cells**.**
- High-resolution zoom scans are pivotal for precise segmentation, facilitating the quantification of lymphocytes and their 3D location, including distance to the nearest parenchyma-stroma boundary, within the TME **(Figure 1B-D)**.
- 3D imaging of tumor parenchyma reveals dense interconnected networks, presenting a marked contrast to their isolated appearance in 2D imagery **(Figure 1A)**.
- Lymphocyte ratios in stromal versus parenchymal regions exhibit heterogeneity across samples and within individual samples, requiring either 3D imaging or very large numbers of 4 µm slices for reliable quantification **(Figure 1E).**
- The pronounced heterogeneity observed in all features throughout the 3D volumes accentuates the importance of 3D imaging for an accurate assessment of tumor landscapes.

Workflow

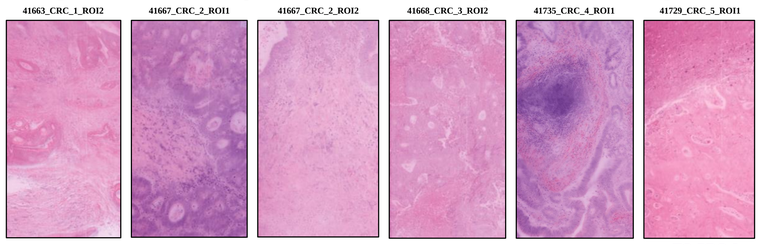
Imaging pipeline:





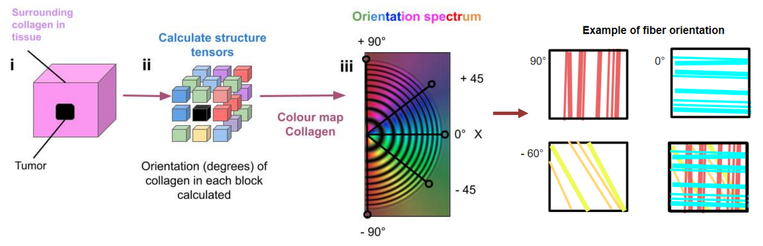


Computational H&E High-Resolution Zoom Images from Each Sample



Exploratory Collagen Analysis

Schematic representation of fiber orientation characterization using the OrientationJ plugin (ImageJ, NIH).



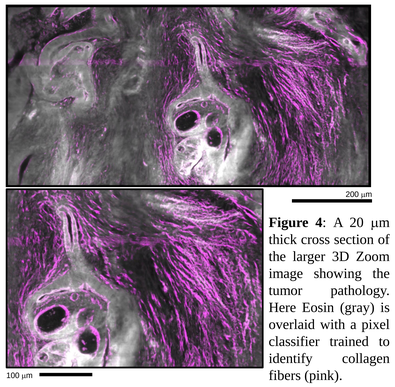


Figure 4: A 20 µm thick cross section of the larger 3D Zoom image showing the tumor pathology. Here Eosin (gray) is overlaid with a pixel classifier trained to identify collagen fibers (pink).

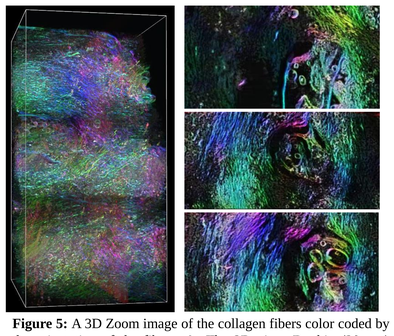


Figure 5: A 3D Zoom image of the collagen fibers color coded by the orientation of the fibers. **A:** The 3D view. **B:** thin (20 µm) sections of the image showing the orientation of the collagen fibers in various planes of the 3D dataset.