3D spatial quantification of fibrosis and inflammation in the tumor microenvironment using the novel workflow: 3D I/O Pro™

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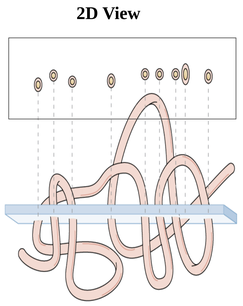
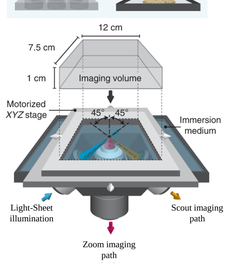
Introduction

The composition of the tumor microenvironment (TME) is a major determinant of response to therapy in many solid tumors. To-date, characterization of the TME has been based on limited analysis of thin tissue sections mounted on glass slides. Here we demonstrate the utility of an end-to-end 3D spatial biology workflow, the 3D I/O Pro™, based on imaging of whole tissue samples to identify and quantitate tumor cells, fibrosis (collagen fiber features), inflammation, and tertiary lymphoid structures in human FFPE tissue samples.



Hybrid Open-Top Light-Sheet (HOTLS) Microscopy



HOTLS features

- Whole Tissue or "3D" imaging
- Easy mounting of multiple samples
- High throughput imaging:
  - Quickly "Scout" large tissue volumes at low resolution
  - Select regions of interest for high resolution "Zoom" scans for single cell imaging
- Combine imaging with machine learning and spatial analysis

Tissue Preparation

Five de-identified human colorectal cancer (CRC) FFPE blocks were deparaffinized, 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*™ (Glaser *et al.* 2022). Smaller regions of interest (ROIs), 0.5 mm^3, were re-imaged at higher resolution, 0.33 µm/pixel. Nuclear and collagen segmentation along with 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 spatially into tumor associated stromal or parenchymal. The variation in stromal to parenchymal TILs was then quantified in 3D and compared to virtual 2D sections of the 3D images.

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

REFERENCES:
Glaser *et al.*, Nat Meth; 2022
Stoltzfus *et al.*, Cell Rep; 2020

Results

All tissue samples were imaged in 3D and computational approaches were used to segment and classify tumor cells, TILs, and collagen fibers within the high-resolution ROI. The ratio of stromal to parenchymal TILs varied from 1.4 up to 9.1 in 3D volumes and 1 to 25 in 2D images. The ratio of the perpendicular to parallel collagen fibers within 100 µm of the tumor border was 0.37 in a proof-of-concept study with a TIL infiltration ratio of 12. Qualitatively, areas with perpendicular collagen appeared to have more TIL infiltration than areas with parallel collagen orientation.

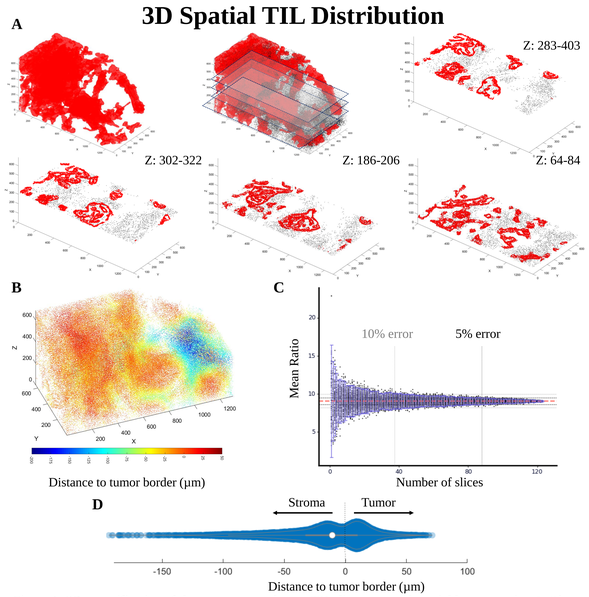
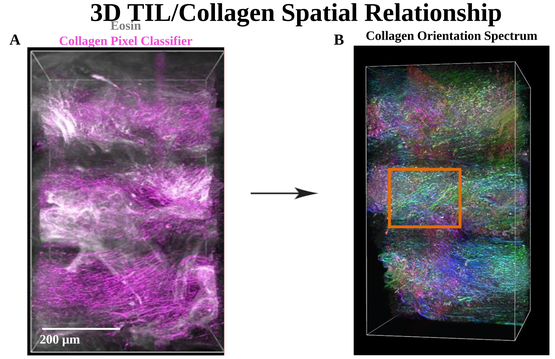
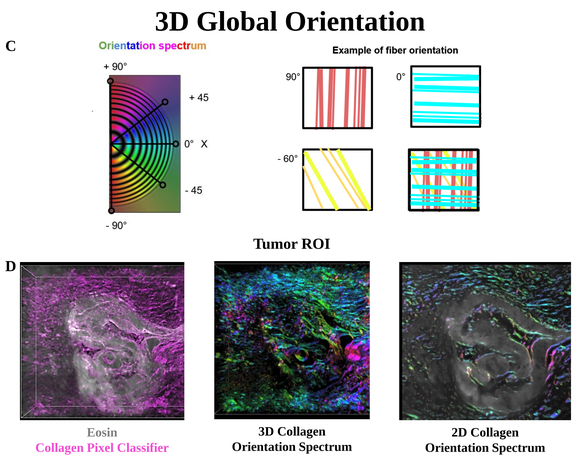


Figure 1: 3D quantification of the tumor parenchyma, TIL distances, and spatial heterogeneity. A: These positional plots show the 3D structure of the tumor region in red for a representative sample. The top middle plot shows the positions of TILs as black dots. The rightmost plot and bottom row show a thin section of the 3D data from various depths. **B:** 3D positional plot from CytoMAP showing the positions of all cells color coded by distance to the tumor parenchyma. **C:** 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. **D:** A violin plot of the distance to tumor parenchyma for all lymphocytes identified in the ROI. The thickness of the violin corresponds to the number of cells at that distance to the border.

3D TIL/Collagen Spatial Relationship





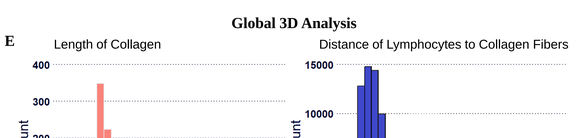


Figure 2: 3D spatial analysis of collagen and TILs. A: 3D pixel classification of collagen fibers shown in 3D. **B:** A visual representation of the 3D global collagen analysis by implementation of the OrientationJ plugin (ImageJ) on the same tissue shown in **Figure 2A** (images to the left). **C:** A color key of the 3D Global Orientation where the hue of collagen represents the angle or direction of the fiber relative to the selected imaging plane. **D:** A visual representation of collagen fibers throughout a selected tumor ROI (orange box in **Figure 2B)**. A 2D representative slice is shown in the rightmost image. **E:** (Left) Distribution of collagen length (µm) throughout the 3D image, and (right) the distribution of TILs and their distance to the nearest collagen fiber.

2D TIL/Collagen Spatial Relationship

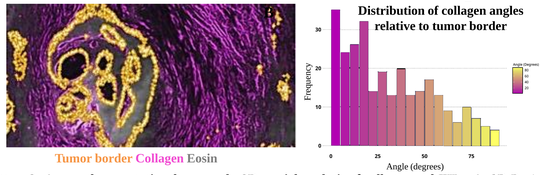
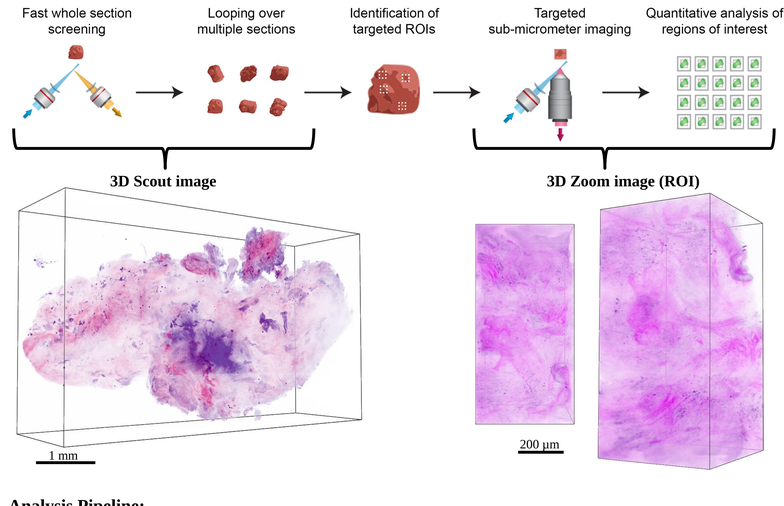


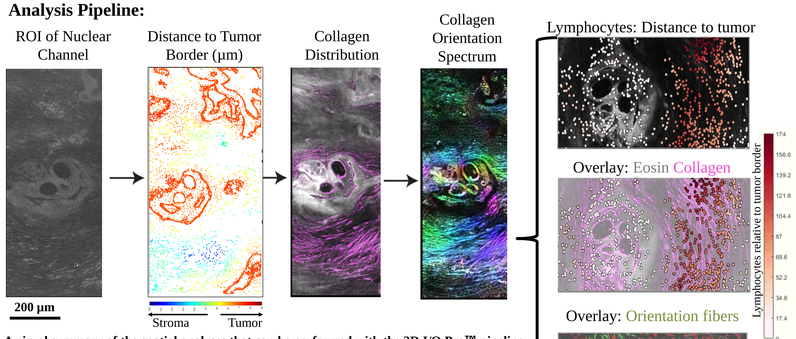
Figure 3: A second computational approach; 2D spatial analysis of collagen and TILs. A: 2D Regional orientation analysis of collagen at the tumor border using CurveAlign & CTFire. The tumor border is defined, as shown in **Figure 1.** The distance to TILs and the local collagen orientation with respect to this border is calculated. **B:** Plot of the distribution of local collagen fibers within 100 µm of the tumor border.

3D I/O Pro™ Workflow

Imaging Pipeline:



Analysis Pipeline:



A visual summary of the spatial analyses that can be performed with the 3D I/O Pro™ pipeline: Beginning with a 2D slice ROI showing the nuclear channel (left), a positional plot from CytoMAP of the same section shown (middle, left) where each dot corresponds to a cell color-coded by 3D distance to the tumor parenchyma border. This border is highlighted in orange. This same ROI is overlaid with a pixel classifier trained to identify collagen fibers (middle, right). These collagen fibers are further colored based upon their angle of orientation (right). Using the 2D computational approach (right column): TILs (dots) are overlaid onto the eosin channel where the color gradient depicts relative distance to the tumor border (top), TILs are overlaid onto the eosin and collagen pixel classifier (middle), and the bottom image shows the local orientation of collagen within 100 µm of the tumor border and their direction (shown in green).

Conclusions

We demonstrated that the 3D I/O Pro™ workflow can be used analyze inflammation and fibrosis within the TME. This pipeline allows us to classify tumors based on a large number of complex spatial relationships and could have broad applicability in research and development of novel cancer therapies as well as profound impact on clinical diagnostics. In the future, we plan to correlate features from the 3D TME with response to immunotherapy and use these features to refine histologic definitions of immune phenotypes.