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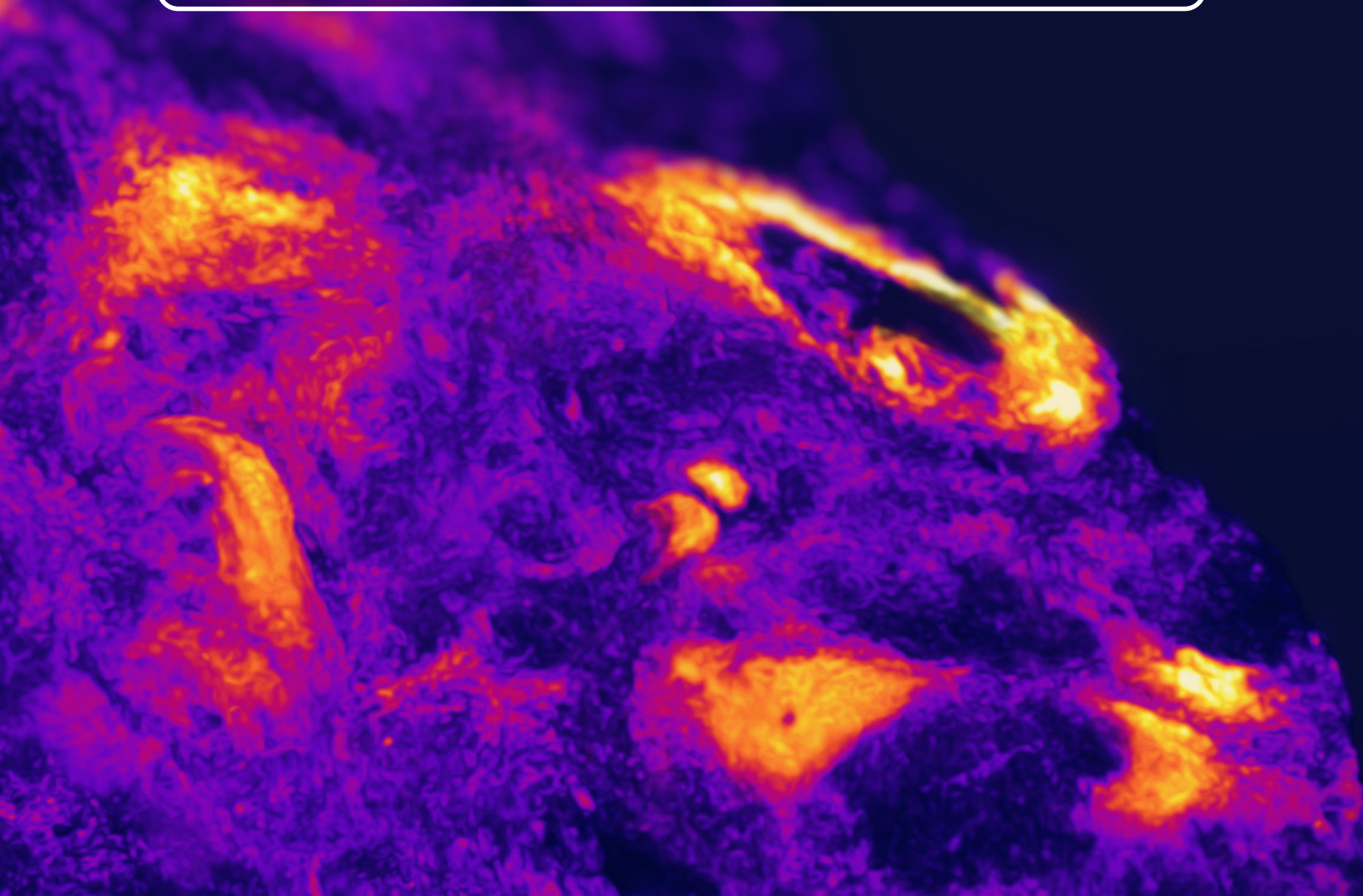
Spatial Segmentation and Analysis of Tertiary Lymphoid Structures in Non-Small Cell Lung Cancer Tissue with the Alpenglow Biosciences 3D*i* Spatial Imaging Platform and Computational Hematoxylin and Eosin Staining



Background

Tertiary lymphoid structures (TLSs) are aggregates of immune cells that form in tissues that are non-lymphoid in nature and share many features, including an anatomical resemblance and similar immune cell composition with secondary lymphoid organs (SLO), such as lymph nodes. TLSs are three dimensional structures which span hundreds of microns in multidimensional space and are believed to replicate SLO function, where adaptive immune responses can be generated or enhanced within these structures [1]. While TLSs can form in a large variety of pathophysiological conditions, their presence in cancer has been well documented in many different indications, with TLS formation found to be present in the stroma, invasive margin, and the core of tumors [2]. In some indications, such as colorectal cancer (CRC) and non-small cell lung cancer (NSCLC) the presence of TLSs, the lymphocyte density, organization of cells within, and the presence of B cells are a few factors that contribute to a positive prognosis [3,4].

One significant benefit of 3-dimensional (3D) imaging is the capability to observe the entire TLS, rather than a single, thin cross-section. The ability to view the whole structure removes potential sampling error where a specific section may not represent the entire structure in terms of overall size and shape, cellular density, immune cell composition, or even simple presence or absence. Factors such as this impact how TLS are characterized in terms of their enumeration, maturity and organization. Additionally, their proximity to structures such as vasculature or even tumor cells could be misstated based on the small sample size incurred with a 4-micron tissue section. Traditional imaging approaches like slide scanners or electron microscopy are incapable of capturing the vast size and adjacent spatial information that is crucial in further understanding TLSs in their biological context.

Alpenglow Biosciences has been able to visualize tertiary lymphoid structures within human clinical NSCLC tissues. Using computational analysis, TLSs were identified via tissue morphology segmentation. Image analyses were performed to gain a deeper understanding of the spatial relationships to surrounding structures and to achieve greater biological insights.

Methods

The human NSCLC lung biopsy featured here was taken from a 20-sample cohort from the Mayo Clinic's thoracic specimen registry and the sample dimensions of approximately 6 mm x 6 mm x 2 mm (~72 mm3). The sample was stored frozen in OCT, and once received at Alpenglow, it was post-fixed in 4% paraformaldehyde. The sample was stained with a computational hematoxylin and eosin (H&E) stain comprised of a nuclear marker (TO-PRO-3), and a general protein fluorescent dye (Eosin). The sample was optically cleared post-staining using a modified iDISCO+ protocol [5] with ethyl cinnamate used as the refractive index matching solution.

The entire tissue sample was then imaged non-destructively and slide-free on Alpenglow's open-top 3D lightsheet microscope (**3D*i***) using a resolution of 2 µm/pixel (Scout mode) to capture a multi-channel, 3D image of the entire tissue. The imaging volume captured is equivalent to imaging 400-500 serial glass slide sections and was completed in a few hours for each sample. A region that contained a TLS was then imaged at a resolution of 0.167 µm/pixel (Zoom modality) to reveal subnuclear features and perform cell segmentation. Multichannel images were rendered into a 3D image with Aivia software (Leica Microsystems).

Segmentation analysis was performed on the Zoom images using the pixel classification feature in Aivia. Once segmentation was complete, object meshes were generated with **3D*i*** and then classified using the cell morphology (size and shape) to distribute the cell by regions and identify TLSs. The volume (μm^3) and surface area (μm^2) of each TLS structure was then calculated.

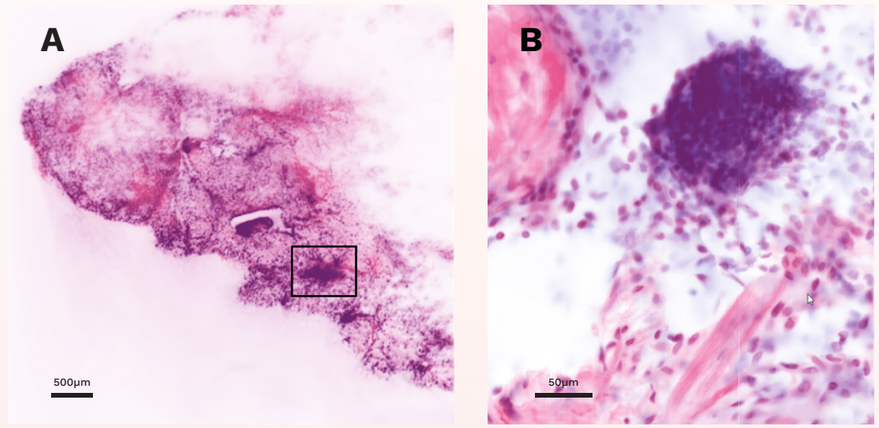


Figure 1. Images of the NSCLC tissue biopsy stained with the computational H&E and acquired on the **3D*i*** microscope using the Scout function. **Figure 1A** shows a region of densely clustered nuclei were identified as a TLS, the rectangle in the image was selected as an ROI to be visualized at a higher resolution with the Zoom function. **Figure 1B** shows a higher resolution image of the rectangle from **Figure 1A**.

Results

In **Figure 1**, a NSCLC tissue sample obtained from a cohort of twenty samples provided by the Mayo Clinic's thoracic specimen registry was visualized. The sample had been fixed and stained with Alpenglow's computational H&E before being cleared and imaged with the **3D*i*** open-top-light-sheet microscope. **Figure 1A** was acquired by whole tissue, low resolution Scout scan. Here, an area of dense nuclei, likely lymphocytic cells, was initially observed (marked by a black rectangle). The Scout feature enables efficient scanning of large, whole tissue sections for notable pathology features and landmarks while reducing the amount of generated data and scan time required; at this image resolution, vasculature, tumor nests, and immune cell infiltration is visible. **Figure 1B** is an ROI selected after Scout imaging of the whole tissue specimen, which includes the area of densely packed nuclei. This area was scanned using the **3D*i***'s high resolution Zoom function with single cell resolution. Cell nuclei of densely packed lymphocytes are easily observable enabling confirmation of structures such as a TLS.

When this image was visualized in 3D (**Figure 2**) and this ROI was observed from a slightly different angle, it was noted that this large group of densely packed nuclei was not one TLS, but rather two TLSs that were in close proximity to one another. The ability to view these structures has enabled us to see their spatial complexity; had this cluster of dense nuclei been visualized in only 2D, the presence of two distinct TLSs would have been completely overlooked. These two TLS structures were located at the center of the tissue at approximately z scan 560 in a tissue of over 960 total z scans. Had this block been sectioned for 2D imaging, this structure was much deeper than several tissue sections from the surface and would not have been captured.

Next, to be able to perform analyses on these structures, the 3D images must be translated into mesh objects. This process begins with rendering a 3D image (**Figure 3A**), followed by pixel classification, and finally a mesh is created (**Figure 3B**). Mesh objects are used to view the morphology and classify the different objects, including the two TLS structures. In addition to being able to identify structures within the tissue based on cell morphology, this workflow can also be used for single cell analysis to quantify cell populations and density and measure the infiltration of cells into tumor tissue, to name a few.

After segmentation into different structures, we then applied quantitative spatial statistics to enumerate several characteristics of the TLS. Using the nuclear stain, we were able to quantify the entire surface area as well as volume of TLS 1 and TLS 2. (**Figure 4**). It is crucial to note here that while these calculations are ostensibly simple in nature, a significant amount of

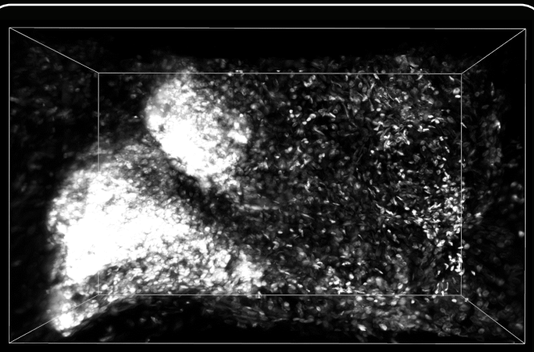


Figure 2. A 3D image of the Zoom acquired ROI shown in **Figure 1B** in greyscale where the nuclei labelled by TO-PRO-3 is in white. Upon rotation of the image, this TLS structure was in fact, 2.

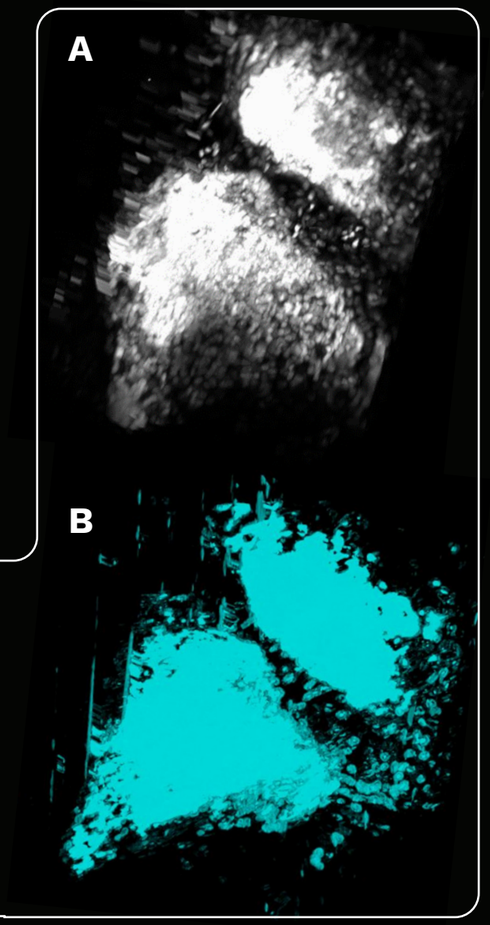


Figure 3. The current pipeline to move from a 3D computational H&E image to TLS identification through tissue segmentation. A) This process begins with a 3D image labelled with TO-PRO-3 (white), B) next an artificial image is generated with pixel classification and finally that classification is used to make mesh objects; these meshes can then be classified into objects based on tissue morphology.

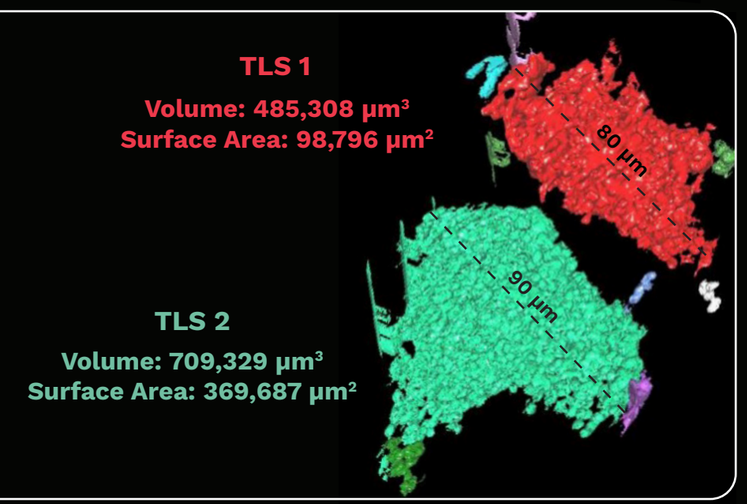


Figure 4. Using the 3D tissue segmentation function, two distinct TLS structures (TLS 1 shown in red and TLS 2 in light green) were identified. These two TLS structures were further quantified to find the total volume and surface area of each of the structures.

computational power and an eloquent workflow were required to obtain these values. Additionally, being able to characterize the overall shape, volume, or surface area of any structure in a 2-dimensional (2D) field of view would prove to be an impossible task.

3D imaging, image analysis and quantitative spatial statistics opens a realm of new possibilities in the quantification and categorization of TLSs- including enumeration of TLSs in whole biopsies or resected tissues. Moreover, the overall 3D shape and size of these structures can be completely visualized using simple small molecule fluorescence dyes to simulate a standard H&E; the presence of nearby vasculature and cell populations within the TLS can be quantified not only by cell density, but also by cellular phenotype through fluorescent labeling of these populations. Additionally, the spatial relationship of the TLS to surrounding features like blood vessels, fibrosis, or even tumor cells and nests can also be spatially quantified.

The potential benefits to using 3D imaging rather than the traditional 2D approach in visualizing and quantifying tertiary lymphoid structures in oncology samples are vast- being able to observe the entire structure rather than a single cross-section holds enormous potential. The ability to see the whole tissue, identify and enumerate complex biological structures including TLS, tumor, stroma compartments, and other areas, and then dive deeper into the tissue to visualize the complete structure, all sub-structures, and even the total population of cells within allows for a much more complete understanding of the TLS and the role it plays in the surrounding environment. This example motivates further investigation of TLSs by incorporating fluorescent immunohistochemistry to quantify the cellular populations within and around TLSs, to visualize the TLS's relationship to surrounding structures, including tumor cells and blood vessels to gain a much greater understanding of these structures and their significance to prognosis and treatment opportunities within the oncology field.

Conclusion

In this article, we have demonstrated how a 3D imaging platform, combined with image analysis and quantitative spatial statistics can be applied to capture and quantify TLSs present within human patient tumor samples. We have described the image acquisition process to obtain multi-channel 3D images and the generation of mesh structures that can be identified and classified into TLS (or other pertinent structures). TLS surface area and volume were then quantified. To our knowledge this is the first ever known 3D quantification of tertiary lymphoid structures based on non-destructive 3D imaging.

A significant amount of research has taken place in the identification of TLS using 2D spatial biology to measure, quantify and phenotype cellular components. However, it is important to note that a major limitation of 2D spatial biology methods is the inherent sampling bias, which occurs when utilizing a single 5µm cross-section of a structure that has the potential to be hundreds of microns in thickness [6]. Perhaps a single cross section is not an accurate representation of the entire TLS as a whole, thus, skewing the size, overall cellular composition, or misrepresenting its classification of maturity. Using this case as an example, these two TLS structures would have likely been unaccounted for since they were located midway through the tissue. Additionally, an entire dimension of information is lost when sampling in a single plane.

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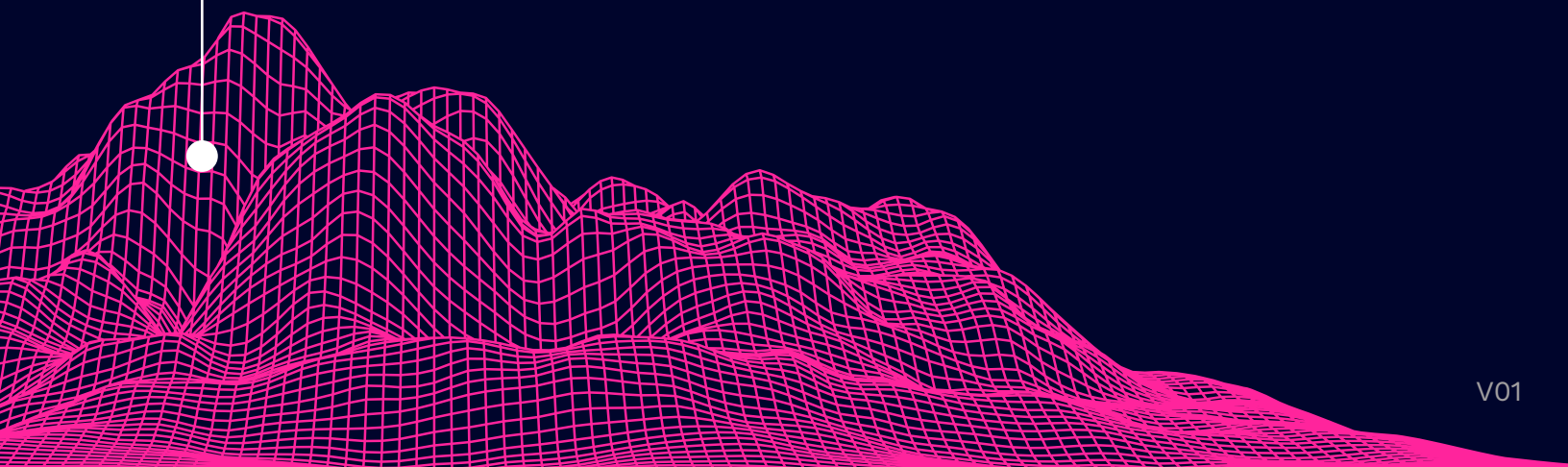

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