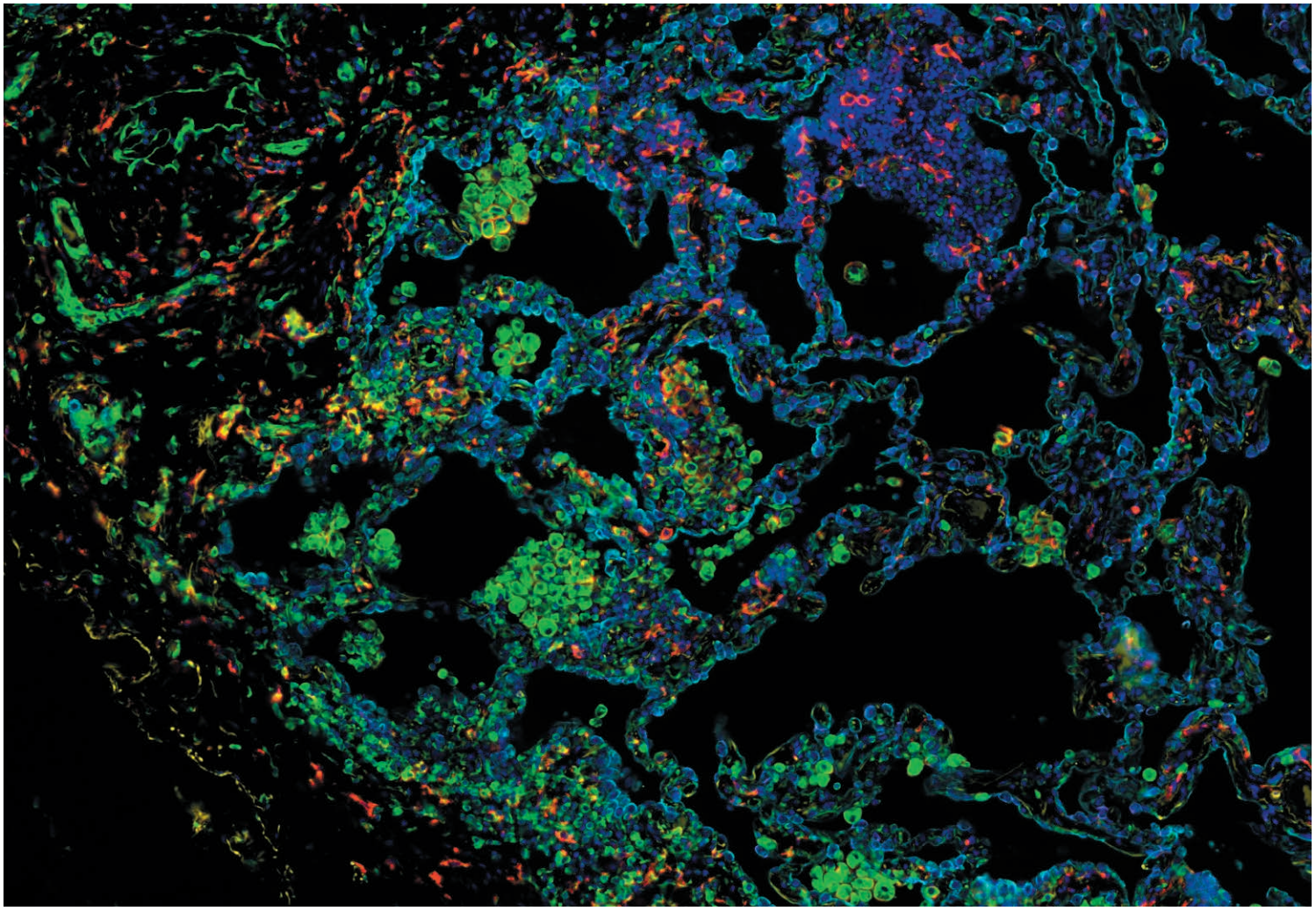

Advancing Spatial Biology through Highly Multiplexed Protein Imaging: The Orion™ Platform

By Eric Kaldjian, MD, Chief Medical Officer

Introduction

Tissue multiplexing to enable spatial biology

Tissues contain a complex micro-environment of cells and structural components that are altered in cancer by growth of the malignancy itself as well as the body's response to it. The investigation of immune cell populations in tumors has received significant recent attention as evidence accumulates that they impact cancer prognosis and response to treatment. Analysis of cell types and states present in the tumor micro-environment is increasingly recognized as an essential part of oncology research. The list of cell types is broad: cancer cells, immune cell subsets including B cells, T cells – with helper, cytotoxic, activated, and regulatory sub-types – macrophages, dendritic cells and others, and stromal cells such as vascular endothelial cells and fibroblasts. In addition, the spatial positioning of immune cells in and surrounding the tumor can reveal further insight into the tumor-host response relationship.

The traditional way to spatially assess the cellular composition of a tumor is to perform multiple immunohistochemistry (IHC) stains on serial tissue sections taken from the same tissue block, staining each microscope slide with an antibody directed against a target cell marker. This allows assessment of that single marker within the architectural context of the tumor on

that slide. With this approach, however, the spatial relationships between multiple cell types (defined by different markers) cannot be precisely discerned since each section reveals a new collection of cells deeper in the tissue block. Additionally, cells with complex phenotypes – requiring more than one marker for identification – cannot be identified.

With multiplexed staining, many cell types – including ones with complex phenotype – can be identified in the same tissue section, allowing the spatial relationships between them to be understood directly in the same micro-environment. **Figure 1** shows an example of how multiplexed staining can resolve the heterogeneity of a densely cellular lymphoid tissue into cells of different lineages. By increasing plex depth further, physiological states such as proliferation and activation can also be assessed. In short, multiplexing allows a more complete picture of the expression of cell proteins, the functional end products of transcribed genes, throughout a tissue. Hence, it is increasingly being employed as a biomedical research tool, as evidenced by the rapidly rising number of publications over the past two decades (see **Inset 1**).

The potential impact of multiplexed imaging extends beyond research into translational science and diagnostics. One area of intense focus is the development of predictive tests for response to immune checkpoint inhibitors (ICIs), including therapies directed against the checkpoint proteins PD-1 and PD-L1. Despite the profound and durable success of these drugs, it has been reported that more than two-thirds of patients given ICIs do not respond to the therapy.[1] Effective biomarkers to predict benefit from ICIs are important to minimize the toxicity risk for patients who are unlikely to benefit and for earlier stage use in the curative setting. Companion diagnostic tests for PD-L1 by IHC and molecular tests associated with increased mutation rates have been shown to enrich populations for responders, but there remains a need for better predictive tests, since a positive biomarker test does not guarantee response to ICIs, and a negative test does not preclude one.[2] A meta-analysis of biomarkers used to predict response to ICIs concluded that multiplexed imaging had higher predictive value than these tests.[3] In another recent report, a 7-plex panel of markers was used to investigate association between multiplex phenotype and therapeutic outcome in patients with melanoma treated with an ICI; a signature of phenotypes was identified that showed predictive value for both response and non-response.[4] Such results suggest that diagnostic multiplexed imaging may soon have an impact on clinical management by directing effective ICI therapies to potential responders and using other therapies for non-responders.

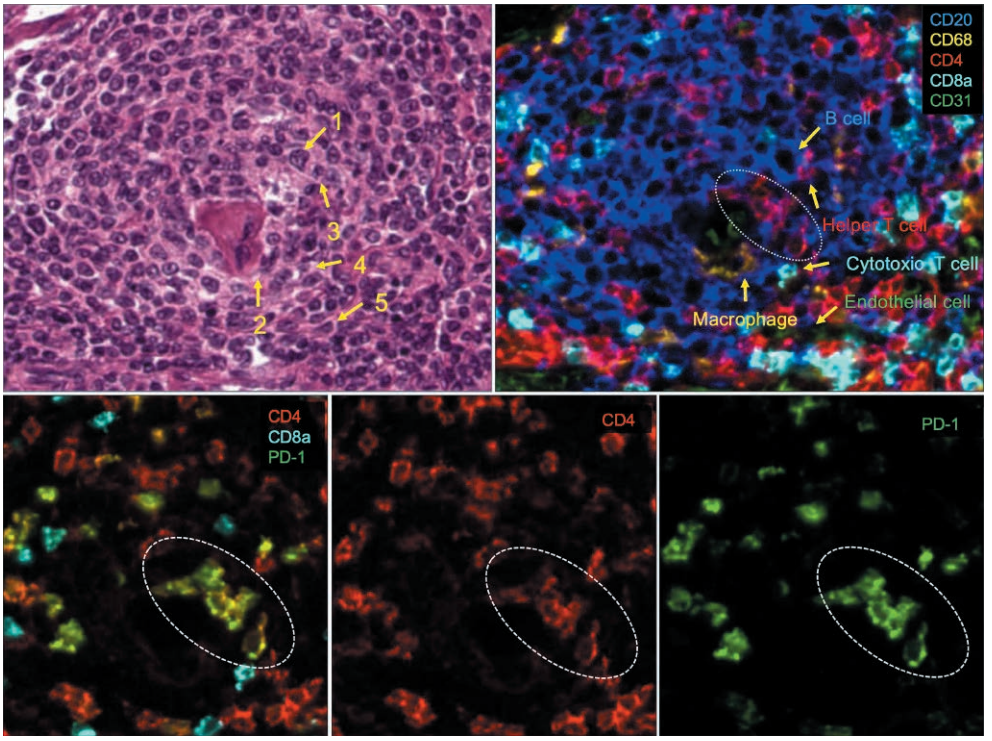

Figure 1: Multiplexed imaging identifies various cell types. Within this lymph node follicle there are cells of different sizes and shapes; most of them are similar in appearance in the H & E-stained section (upper left panel). The five cells pointed to by arrows cannot be differentiated. Markers for B cells (CD20), helper T cells (CD4), cytotoxic T cells (CD8a), macrophages (CD68) and endothelial cells (CD31) reveal the "invisible" identities of the cells (upper right panel). Multiplex imaging on the Orion Instrument using a 17-plex StrataPlex™ Panel was performed on the on the same section as the H & E. The PD-1 checkpoint molecule is present in helper T cells, but not in cytotoxic T cells (lower panels).

High-plex spatial biology solution: The Orion Platform

The higher the plex depth of protein imaging, the greater the capacity to assess tissue microenvironments. However, technologies that have achieved high plex depth have had trade-off costs in time, workflow complexity, optical resolution, and amount of tissue that is analyzed. There is a need for a technology solution that

INSET 1

Rapidly increasing research utilizing multiplexed imaging

A PubMed search on "multiplexed imaging" shows that the number of scientific publications in the last two decades has grown from less than 40 in 2000, to about 200 in 2010, to over 700 in 2020.

https://pubmed.ncbi.nlm.nih.gov/?term=multiplexed+imaging&filter=years.2000-2020&timeline=expanded

Figure 2: Spectral resolution of overlapping fluorescence signals. Lung section with emission signals from CD68, cytokeratin, CD4, FOX3, and CD8 antibodies conjugated to fluorescent dyes that overlap within the orange region of the spectrum (imaged by traditional fluorescence). Orion extracts the overlapping fluor signals into separate channels for visualization of the individual biomarkers.

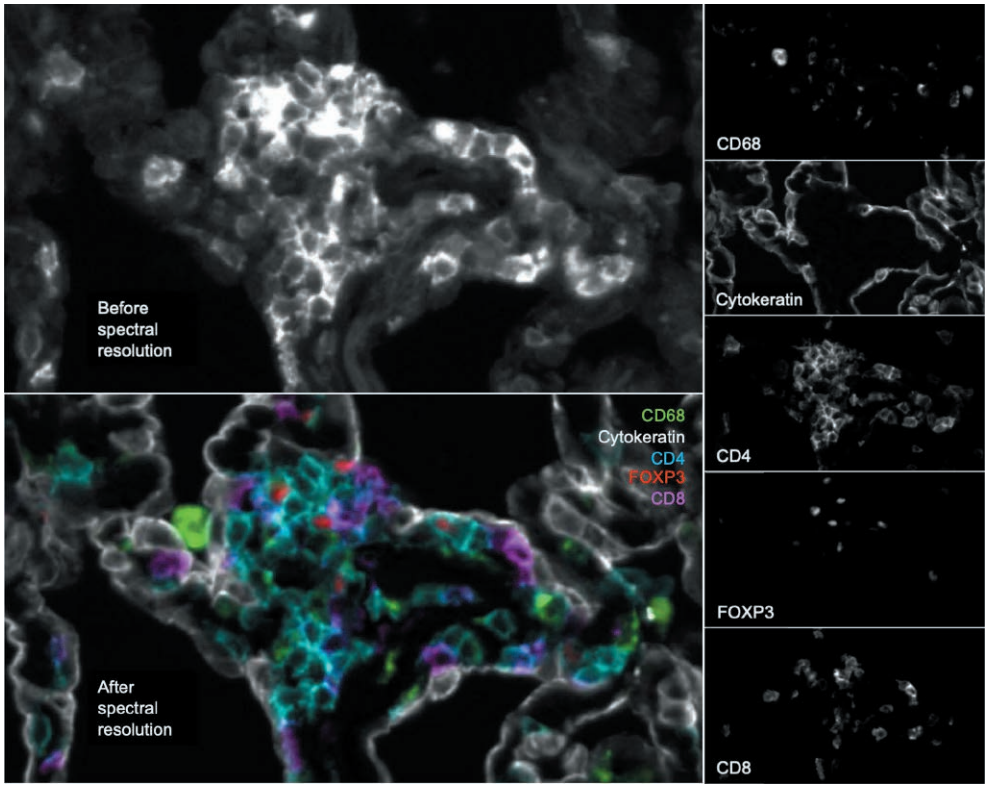

Figure 3: Orion multiplexed imaging workflow. (1) Tissue sections are prepared on standard microscope slides. (2) Slides are stained in a single staining process using routine immunofluorescence protocols. (3) One scan is performed for the complete staining panel on the Orion Instrument. (4) Images are processed and visualized using Artemis; files are exported in OME-TIFF format compatible with third-party image analysis software systems.

balances depth of multiplexing with time and resolution for practical research and eventual clinical applications. A versatile multiplexed imaging platform would effectively address the following:

- **Plex depth.** 10 to 15-plex imaging would provide adequate depth to enable extensive investigation of immune response in disease.
- **Resolution.** Sub-cellular resolution is necessary to localize biomarker signals within cellular compartments (membrane, cytoplasm, nucleus); this yields important information on cell state, as well as confirms specificity of staining.
- **Image area.** Whole-slide tissue imaging is required to obtain complete tissue section information for analysis free of regional sampling bias. Tissue microarrays containing tens to hundreds of tissue cores are an important sample type for translational clinical studies; these can occupy most of the microscope slide surface area.
- **Staining.** The staining process must be able to preserve protein epitopes that are the binding sites of labelled antibody reagents to generate reliable data. Staining panels should be flexible to enable incorporation of custom targets. Staining should be performed in a single round to increase efficiency and eliminate possibility of co-imaging residual stains from previous staining rounds.
- **Scanning.** The image acquisition should be automated and rapid to enable practical analysis of large sample sets. Output file format should allow flexible analysis by software tools.
- **Sample types.** The platform should be agnostic to the type of specimen to support broad research and diagnostic applications.
- **Morphology.** Light microscopic characteristics of the tissue, including cells and non-cellular components, should be part of the available imaging data set.
- **Turn-around time.** The complete workflow from sample preparation to image result must allow sufficiently high throughput for multi-sample research studies, and rapid turn-around time for diagnostic clinical applications.

RareCyte has directly addressed each of these requirements with its recently commercialized Orion platform, which includes: (1) the Orion Instrument for high-plex imaging; (2) StrataPlex Reagents utilizing ArgoFluors™ that are optimized for use with the Orion Instrument; and (3) Artemis™ Software which drives the instrument and image processing, enables visualization of high-plex datasets, exports images for analysis, and provides a clinically compatible environment for data management. StrataPlex Modular panels of validated sets of biomarkers will be available initially for oncology/immunology applications. These can be customized by substituting or adding other validated biomarkers. Tailored antibodies can be developed for specific biomarker targets. Further investigational flexibility is provided with kits using Orion-compatible ArgoFluors that allow scientists to incorporate custom biomarker antibodies into panels in their own laboratory.

The Orion platform overcomes a major obstacle encountered by traditional fluorescence microscopy – overlapping fluorescent dye spectra – that limits imaging to one marker per spectral region. Orion technology utilizes tunable, narrow band emission to exploit the full range of available fluorescent dyes by extracting specific signals away from spectrally overlapping signal to increase the number of markers per spectral region. Laser illumination is used to overcome the intensity loss due to the narrow band emission. The system sensitivity is sufficient to allow direct antibody-fluor conjugates to be used for labeling most targets. High numerical aperture objective lenses generate subcellular resolution images. **Figure 2** demonstrates how signals from closely overlapping fluors that cannot be discriminated by traditional methods are extracted by Orion into separate channels for visualization of individual biomarkers.

In the Orion workflow, shown in **Figure 3**, the entire tissue section is stained in one round, and imaging is performed in a single scan. A 20x-objective scan of a 1 cm x 1 cm tissue section in 15-channels takes less than 1.5 hours. Turn-around-time from unstained slide to multiplex image is less than 2 days. Any specimen that can be placed on a microscope slide with cover slip may be analyzed: formalin-fixed paraffin-embedded (FFPE) or frozen sections, tissue or slide smear samples. Hematoxylin and eosin (H & E) staining can be performed after fluorescence imaging is completed on the same section, integrating morphological assessment with the phenotypic analysis. The importance of evaluating entire sections cannot be overstated, as it is increasingly recognized that tissues contain a variety of micro-environments that provide information that may be missed if the multiplexing analysis is limited to a few microscopic fields of view. This intra-tissue heterogeneity is illustrated in the lung section shown in **Figure 4**. In summary, the Orion platform is a rapid, high-resolution, high-plex spatial biology solution developed for research and eventual clinical applications.

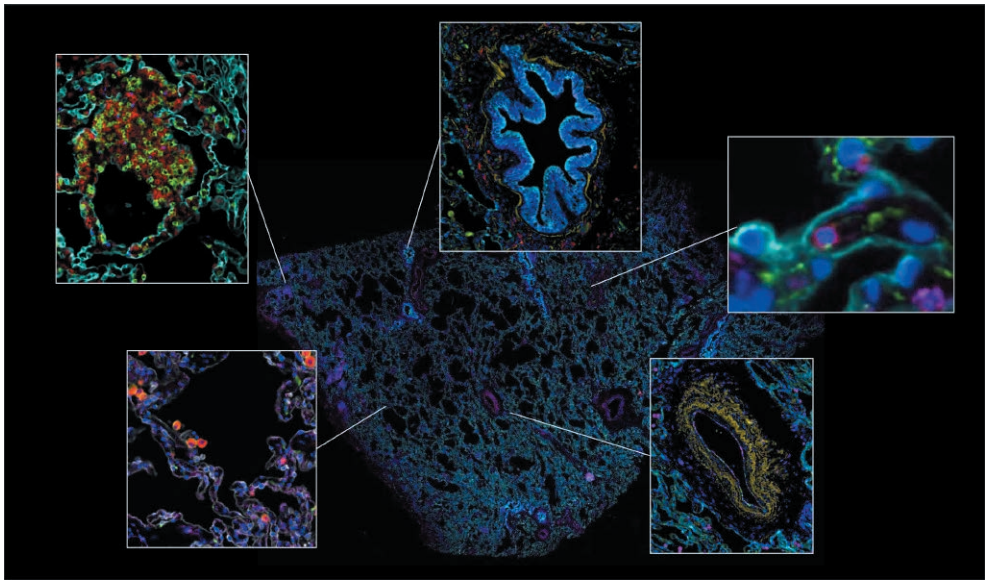

Figure 4: Value of whole-slide multiplexed imaging. The background image in this figure is of a non-neoplastic lung section stained with a 17-plex panel and imaged on the Orion platform. Insets show the diversity of cells present in the tissue. Counter-clockwise from the upper center: (1) Bronchus with epithelial lining stained with cytokeratin and E-cadherin. (2) Lymphoid aggregate showing T cell subsets CD4 (red) and CD8 (green) in interstitium. (3) Lung alveolar space defined by cytokeratin lining (white) containing macrophages expressing cytoplasmic CD68 (red) and surface membrane CD163 (green); capillaries in the alveolar septum are stained by CD31 (magenta). (4) Artery with endothelial lining stained with CD31 (green) and vimentin (magenta); The Orion Platform can use inherent tissue fluorescence as an additional marker – here the elastic lamina of the artery is visualized in yellow, effectively making the panel an 18-plex stain. (5) High-magnification detail of a CD4 T cell (red) squeezing through a capillary (CD31, green) in the alveolar septum; this was a regulatory T cell as determined by nuclear staining with FOXP3 (not shown).

Quantitative image analysis

Data-rich sample sets

Since whole-slide high-plex imaging is information rich, and since human visualization can typically comprehend no more than 6 or 7 displayed colors, analysis strategies that use computer algorithms are typically employed to analyze the images. By using the H & E image of the same tissue section, a trained observer (such as a pathologist) can define regions by architectural and cytologic features for a directed analysis using computational methods. **Figure 5** describes the use of a model "pathologist-driven workflow" for research analysis of a colorectal cancer sample stained with a 19-plex immuno-oncology panel5. An algorithm was used to "segment" each cell in the tissue from neighboring cells. Cell types defined by single marker phenotype were compared between pathologist-defined regions of carcinoma and normal colonic mucosa and quantitative differences determined to understand immune cell composition changes in the cancer. The analysis demonstrated that the normal mucosa region has a higher proportion of CD8+ T cells and CD31+ endothelial cells, while the tumor region has higher proportion of cytokeratin (pan-CK) expressing epithelial cells and proliferating (Ki-67+) cells; regulatory FOXP3+ T cells were similar in each region (**Figure 5B**). By using a computational tool that reduces high dimensionality, groups of cells that are phenotypically related across the high-plex marker set can be clustered together in two dimensional plots that can be comprehended by inspection. The groups of cells can then be queried for various biomarkers within them and differences between spatial tissue regions can be understood. **Figure 5C** highlights different cell populations defined by phenotypic markers E-cadherin for epithelial/tumor cells and CD31 for stromal/endothelial cells.

Orion has been used to investigate differences between primary and metastatic cancers,[5] as shown in Figure 6. Paired lung adenocarcinoma and brain metastasis samples from the same patient were stained with an 18-plex panel. 37 different cell types were identified by using phenotypic combinations of relevant markers after cell segmentation and bioinformatics analysis. Spatial location of the cell types was then mapped to the tissue section as an overlay. When the spatial distribution of cell subpopulations was compared in paired sets of primary and brain metastasis samples, statistically higher proportions of T and B lymphocytes (CD8a+, FOXP3+, CD20+ and CD3d+) were found in primary tumors, while proliferative cells (Ki67+) were higher in the brain metastases confirming cellular differences in compositions between primary and metastatic sites (data not shown).

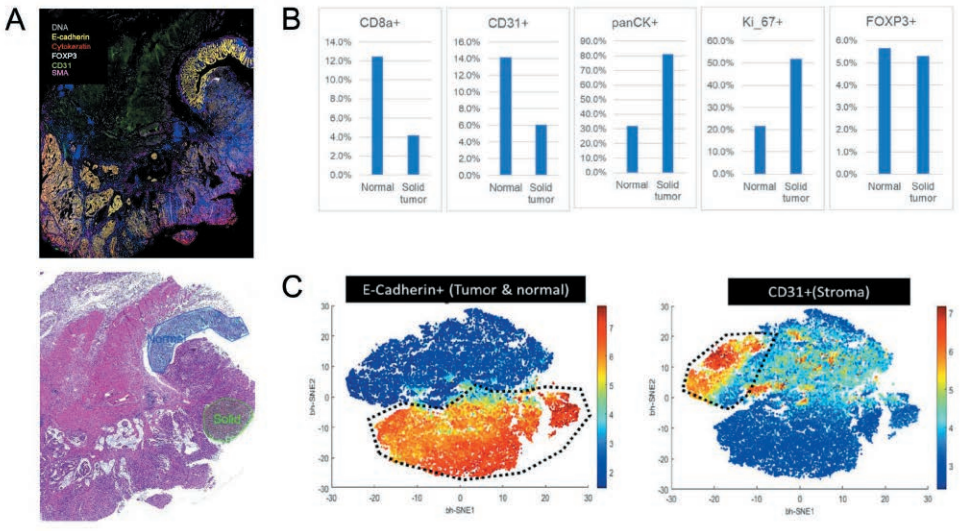

Figure 5: Pathologist-driven workflow: colorectal cancer example. The Orion platform allows acquisition of an H&E brightfield image after the multiplexed IF image on the same slide section. (A) Using a single colorectal cancer section, we acquired a 19-plex Orion image (upper panel), then stained with H&E and acquired a brightfield image (lower panel). A pathologist identified two regions of normal mucosa and solid adenocarcinoma in the H&E image for cell type analysis. (B) Single-cell phenotypes were derived from the fluorescence image after cell segmentation and data quantification. Markers were identified that were differentially expressed in the normal mucosa and solid adenocarcinoma regions. (C) Multi-dimensional-reduction plots (t-SNE) were created to visualize populations of cells into clusters based on expression of all markers in the panel.

Using artificial intelligence to analyze whole slide multiplexed tissue images

Artificial intelligence (AI)-powered image analysis is increasingly being used in pathology to assist pathologists in diagnosis using routine light microscopy as well as IHC stains. AI analysis can also be employed in the analysis of highly multiplexed whole-slide tissue staining to mine patterns that are associated with disease conditions.

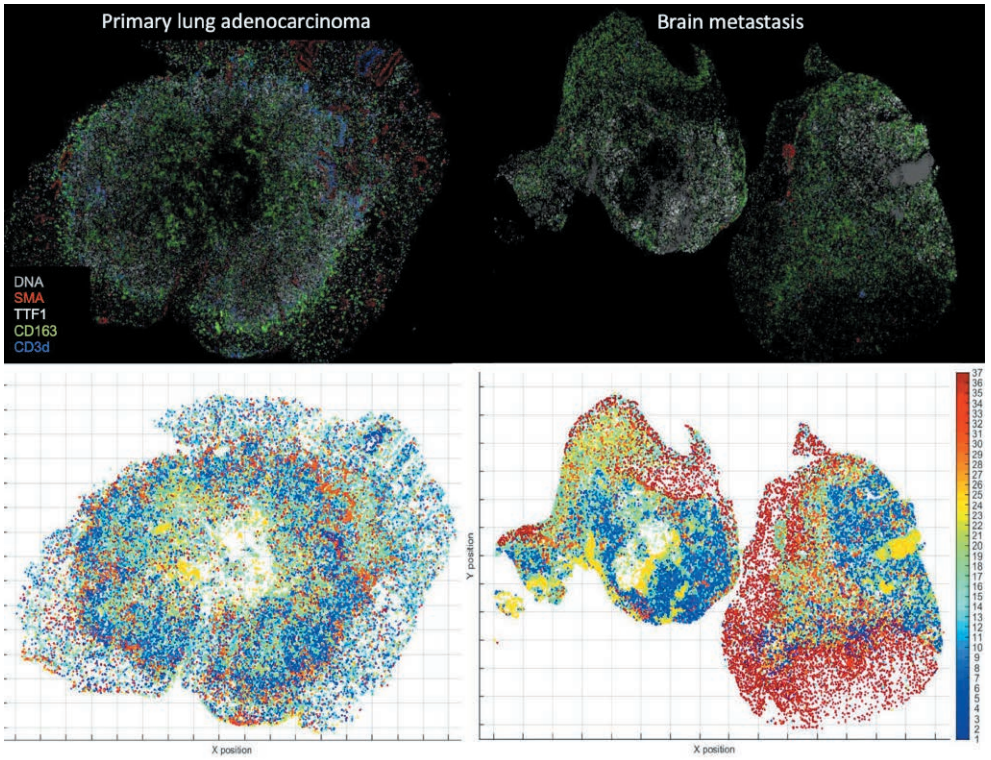

Figure 6: Cell type immune profile maps comparing primary lung cancer and brain metastasis from the same patient. The upper panel shows images from a multiplexed panel of a primary lung adenocarcinoma (left) and a metastatic nodule from the same lung cancer that was present in the brain (right). 37 different cell types were identified. The cell types were then mapped to the tissue sections (lower panel); cells with the same phenotype are given the same color.

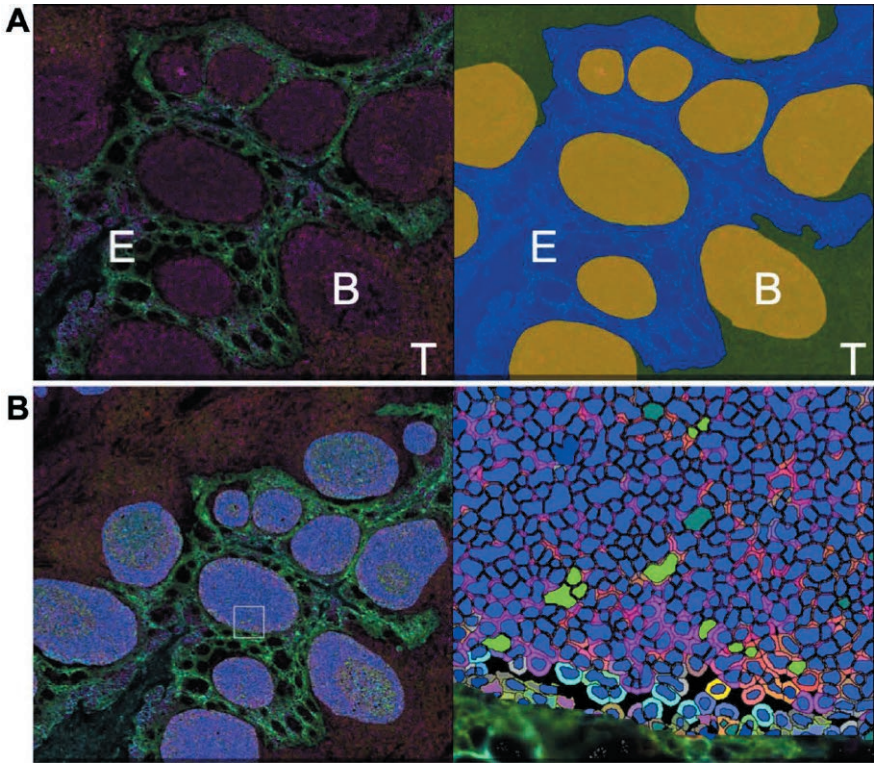

Figure 7: Use of artificial intelligence for image analysis on tonsil section stained with 17-plex marker panel. (A) A tissue classifier algorithm (HALO AI®) was trained to recognize different regions, including B cell, T cell and epithelial regions. Left panel is the Orion image; right panel shows the results of tissue classification by region. (B) Segmentation of individual cells was performed followed by separation of cells into phenotypic classes based on the expression of the markers in the panel. Left image shows the application of the phenotyping to the B cell regions (circular/oval follicles); right panel shows high magnification view of individual cells by phenotypic class within the square area in the left panel.

By training AI algorithms to identify areas of interest, similar regions across the entire tissue sample can be efficiently grouped together for subsequent phenotypic analysis[6] (**Figure 7**). By providing the Orion platform and related products (such as biomarker panel kits), RareCyte will enable the creation of AI-based protocols within the workflow of highly multiplexed imaging analysis that identify complex phenotypic signatures and lead to high-value diagnostic tests.

Potential high-plex applications for Orion beyond immuno-oncology

The current interest in multiplexed imaging in oncology has been intensely focused on the investigation of cellular immune profiles to guide the use of immunotherapies targeting solid cancers. There are many other anticipated applications of the Orion platform.

Hematologic malignancies

Many of the markers in immuno-oncology panels are used to define immune cell subsets; these and similar markers may be used in the diagnostic classification of lymphomas, cancers of the immune system. Lymphomas are typically characterized by flow cytometry, which tests a cell suspension made from fresh tissue biopsies to establish a multi-dimensional phenotype for classification. High-plex tissue multiplexing could similarly assess lymphoma phenotype but on a tissue section. Because it simultaneously can provide pathologists with the context of cell morphology and tissue architecture, this approach could be especially useful in diagnostically challenging cases. Furthermore, if a paraffin tissue block is available, multiplexing has the potential to eliminate the need to procure fresh tissue by additional biopsy.

Infectious diseases and auto-immunity

Immune cell markers can be assessed to provide deep information on immune response regardless of the stimulus. Multiplexed imaging can be used in infectious diseases to investigate immune response to pathogens, as well as in the development of vaccines. Auto-immune disease is caused by aberrant immune response directed against host tissues. Multiplexed imaging is anticipated to be a powerful tool to decipher the details of that immune response and to develop therapies that block it.

Neuroscience

Neuroscience research is an area that will benefit from imaging multiplexed markers by elucidating the complex spatial interactions between cells of varying types within the context of highly ordered nervous system anatomy. There are many

functionally and phenotypically distinct cells within neural tissues, necessitating many of markers to map them, and there is a large body of mRNA expression data being developed that requires verification at the protein level.

Tissue image atlases: knowledge bases and education

Rapid high-resolution imaging of multiple markers on whole tissue sections has the potential to allow investigation of micro-anatomy at a scale that previously has not been possible. The same can be said for virtually any tissue – one can easily imagine the use of multiplexed tissue image atlases as powerful knowledge bases for the 21st century. Two-dimensional spatial relationships between cells can be immediately understood by visualizing cell phenotypes with a mouse click; incorporating Z-dimension imaging would make three-dimensional atlases possible, further enhancing the understanding of normal and diseased tissue architecture.

Rescuing the QNS clinical sample

There are frequently specimens submitted to the pathology laboratory that have extremely sparse amount of tissue available for testing, known as "Quantity Not Sufficient" or QNS samples. Such samples – which include many minimally invasive fine needle aspirate biopsies – may have only a few slides (or perhaps even a few cells) available for analysis. In such cases, a standard complete diagnostic evaluation using a panel of single IHC stains is impossible to perform. However, the sparse specimen could provide the needed diagnostic information if the IHC panel were incorporated into a multiplexed IF panel, since it needs only a single slide. In such cases, the specimen could be "rescued" by Orion – the pathologist would not be forced to reduce the breadth of tissue analysis, and even more important, the patient may not require an additional biopsy. This represents an important potential application of multiplexed imaging in clinical medicine.

Current and Future Plans for Orion

The Orion Platform is currently available for purchase. RareCyte also offers assay development and multiplexed imaging to support translational studies and clinical trials in its BioPharma services laboratory. As an example, in a recent study an immuno-oncology assay was designed that included a drug target in the high-plex panel; the cells expressing the target in various cancer types were definitively identified by co-expression of phenotypic markers. Future considerations for the platform include the Artemis software expanded to provide users with a rapid analytic investigation interface and the StrataPlex Reagents for additional applications, such as neuroscience and murine will be explored.

Conclusion

The recent advances in multiplex imaging technology will enable the investigation of protein expression within the context of tissue architecture at a depth that has not previously been possible. Increased throughput and whole-slide tissue coverage will further accelerate understanding of the composition, distribution, activity, and interaction of cells *in situ* to inform normal function as well as disease processes. The breadth of applications of spatial biology in research and diagnostics is vast. As with the introduction of other breakthrough technologies, such as microarrays and next generation sequencing, there is growing recognition that the technological approach is powerful and has significant biomedical impact potential. RareCyte offers a practical and versatile system to realize that potential with the Orion platform. JoPM

Summary of Points

- The arrangement of cells in tumor tissues – their spatial biology – is increasingly understood to be essential to understanding disease processes and response to immunotherapies.
- Highly multiplexed imaging is a powerful spatial biology approach for identification of proteins that define individual cell phenotypes and for assessment of complex phenotypes and states within intact tissues.
- The RareCyte Orion platform provides rapid, whole-slide high-plex imaging at high resolution for spatial biology research and clinical applications.
- Images generated by the Orion system are compatible with quantitative analysis by algorithmic and artificial intelligence tools.
- The Orion platform has significant biomedical impact potential in oncology research and ultimately diagnostics, and also in neuroscience, autoimmune and infectious diseases, and others.

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Eric earned his MD and trained in pathology at the University of Michigan. He was a research fellow at the National Cancer Institute and is certified by the American Board of Pathology. Before RareCyte he was the Medical Director of Companion Diagnostics at Ventana Medical Systems/Roche Tissue Diagnostics. Eric's pharmaceutical experience has included discovery research, toxicology, and exploratory and late phase clinical development at Hoffmann-La Roche and Parke-Davis / Pfizer. He was a director of clinical genomics at Gene Logic and Chief Scientific Officer at Transgenomic. He has degree in chemistry from Harvard and studied music at Trinity College, Cambridge, England.

About RareCyte®

RareCyte, Inc. is a leading life science company headquartered in Seattle, WA that has developed innovative highly multiplexed imaging technologies for liquid biopsy and spatial biology. RareCyte's liquid biopsy platform identifies rare circulating tumor cells for characterization of drug targets and other biomarkers and has been integrated into numerous clinical trials in oncology drug development. RareCyte's Orion spatial biology platform enables rapid, whole slide, high resolution spatial biology for research and clinical applications. Both the liquid biopsy and spatial biology platforms encompass end-to-end workflows including consumables, instrumentation, analysis software, and cell retrieval capabilities that enable advances in oncology, immuno-oncology, and maternal-fetal health. The RareCyte interdisciplinary team of scientists and engineers are dedicated to designing products that advance biomedical research and contribute to companion diagnostic development. RareCyte's accredited laboratory provides a full range of services to BioPharma, including custom assay development, clinical trial testing and companion diagnostic programs.

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