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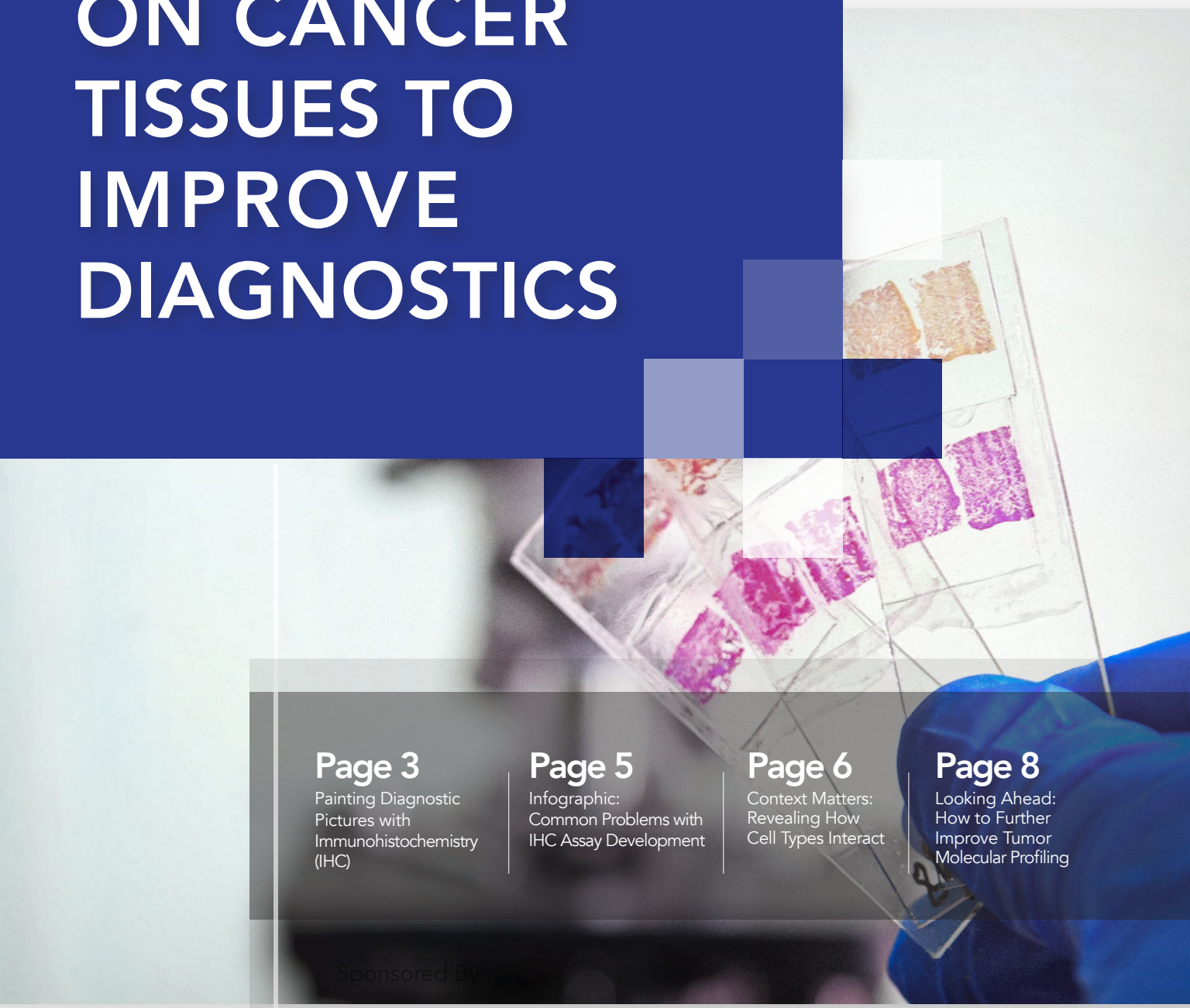

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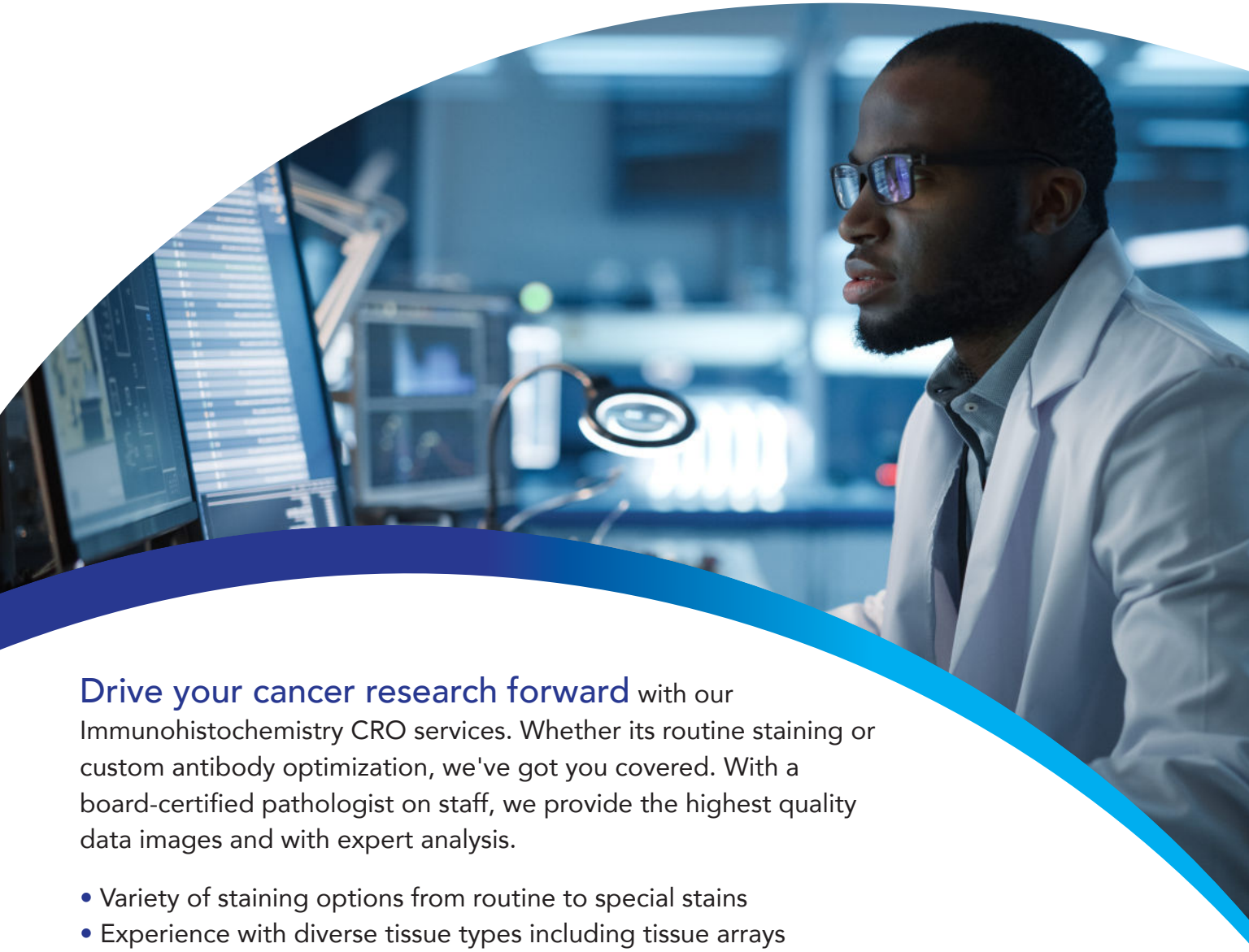

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PAINTING DIAGNOSTIC PICTURES WITH IMMUNOHISTOCHEMISTRY

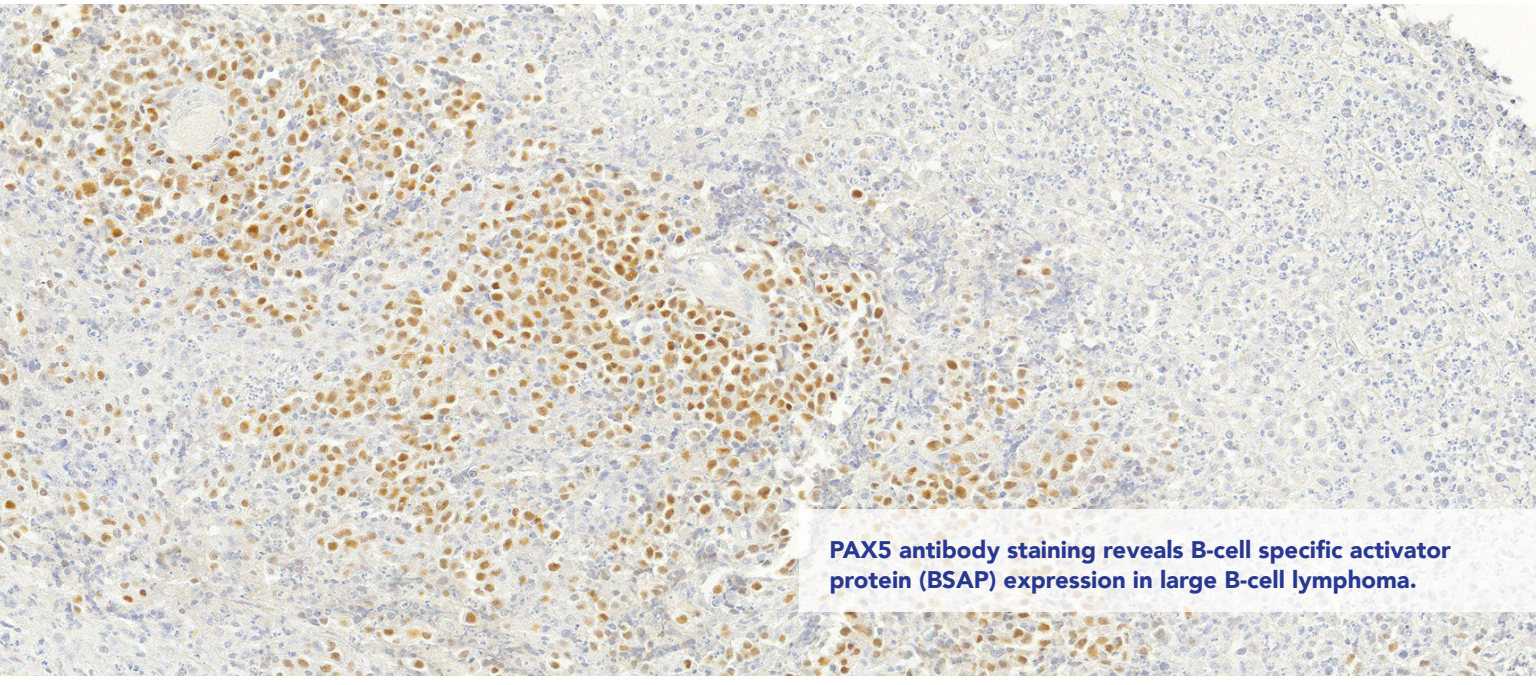
Histological analysis of biological specimens is a staple in the diagnostic toolbox of pathologists, laboratory medicine professionals, and translational scientists. Because unstained tissue samples lack contrast, researchers must use histological techniques to microscopically evaluate them. For diagnostic purposes, scientists typically use hematoxylin and eosin (H&E), two dyes that differentially stain nuclei and cytoplasm to reveal cellular morphology and tissue architecture. Researchers can also perform immunohistochemistry (IHC), which employs antibodies that mark specific proteins, to detect biomarkers and cell types within the sample. Because H&E and IHC stains are incompatible, scientists typically perform these stains on consecutive tissue sections.[1] While these technologies have existed for decades—or even centuries in the case of H&E staining—researchers continue to optimize them, developing novel applications and instruments to probe ever deeper into cellular shapes, expression patterns, and tissue functions.

A Closer Look Into Tissue Pathology

Despite the wide variety and availability of complex staining methods, H&E remains the most used stain in biological research because of its reliable performance regardless of tissue type or fixation method.[1,2] The ancient Mayans initially used hematoxylin, extracted from logwood tree cores, to dye their fabrics.[3] In the 17th century, Spanish explorers introduced the wood to European scientists, who discovered the dye's nuclear staining capabilities and combined it with counterstains, such as eosin, for better visualization through improved contrast.[4,5] For most tissues, the H&E staining protocol colors nuclei blue and paints the cytoplasm and extracellular matrix in various shades of pink, revealing the specimen's subcellular and extracellular organization. In addition, the stain uncovers cell- and cancer-type–specific heterochromatin condensation patterns, which enables diagnostic pathologists to identify and classify disease when examining patient biopsies.

Because cancer tissues typically display a high level of cellular heterogeneity, it may be difficult to score a patient's cancer based on a stained tissue section.

Structural information alone is often insufficient for scoring biopsies, especially from cancer tissues, to determine best treatment options or eligibility for ongoing clinical trials. Therefore, pathologists increasingly perform IHC on



PAX5 antibody staining reveals B-cell specific activator protein (BSAP) expression in large B-cell lymphoma.

patient samples to detect molecular signatures that can be specifically targeted with immunotherapy. To do this, scientists incubate sections with primary antibodies that detect proteins of interest and subsequently add secondary antibodies, tagged with peroxidase or alkaline phosphatase, which will bind the primary antibodies.

They next visualize the antibody complexes by adding the enzymatic tag's substrates, which will deposit a colored product at the target protein's location. Alternatively, fluorescently-labeled antibodies allow scientists to apply differently-colored antibodies and visualize multiple markers at once.

When in Doubt, CLIA It Out

As technologies to characterize patient tissue samples become more sophisticated and permit simultaneous analysis of several cellular and molecular markers, it becomes challenging to stay up to date with the literature and select the most appropriate biomarkers for a certain cancer type. In addition, because cancer tissues typically display a high level of cellular heterogeneity, it may be difficult to score a patient's cancer based on a stained tissue section. For these reasons, scientists should consider partnering with a Clinical Laboratory Improvement Amendments (CLIA)-certified service provider when performing diagnostic IHC and histopathology on patient samples. CLIA is a set of regulations that establish quality standards for US-based facilities that test human specimens to diagnose, prevent, or treat disease.[6] While clinical trials and basic research are excluded from CLIA regulations, scientists working with patient samples should consider incorporating these standards because they ensure accurate, reproducible, and timely results.

The diagnostics team at Canopy Biosciences' Core Laboratory combines histology experts and board-certified pathologists to offer a range of CLIA-certified services that meet both clinical and biopharmaceutical needs.[7] Researchers can directly submit patient samples to the Core Laboratory, where they are processed, grossed, embedded in paraffin and subsequently sectioned. Next, Canopy's histology experts work with researchers to develop, test, and validate custom assays with established and novel stains or antibodies. Finally, board-certified pathologists score all stained specimens to analyze tissue patterns, biomarkers, and cell types to identify the underlying pathology. Apart from H&E and IHC stains, the Core Laboratory also offers CLIA-certified fluorescence in situ hybridization (FISH), Nanostring™-based gene expression analysis, and nucleic extraction services, enabling scientists to reliably profile patient samples in depth.

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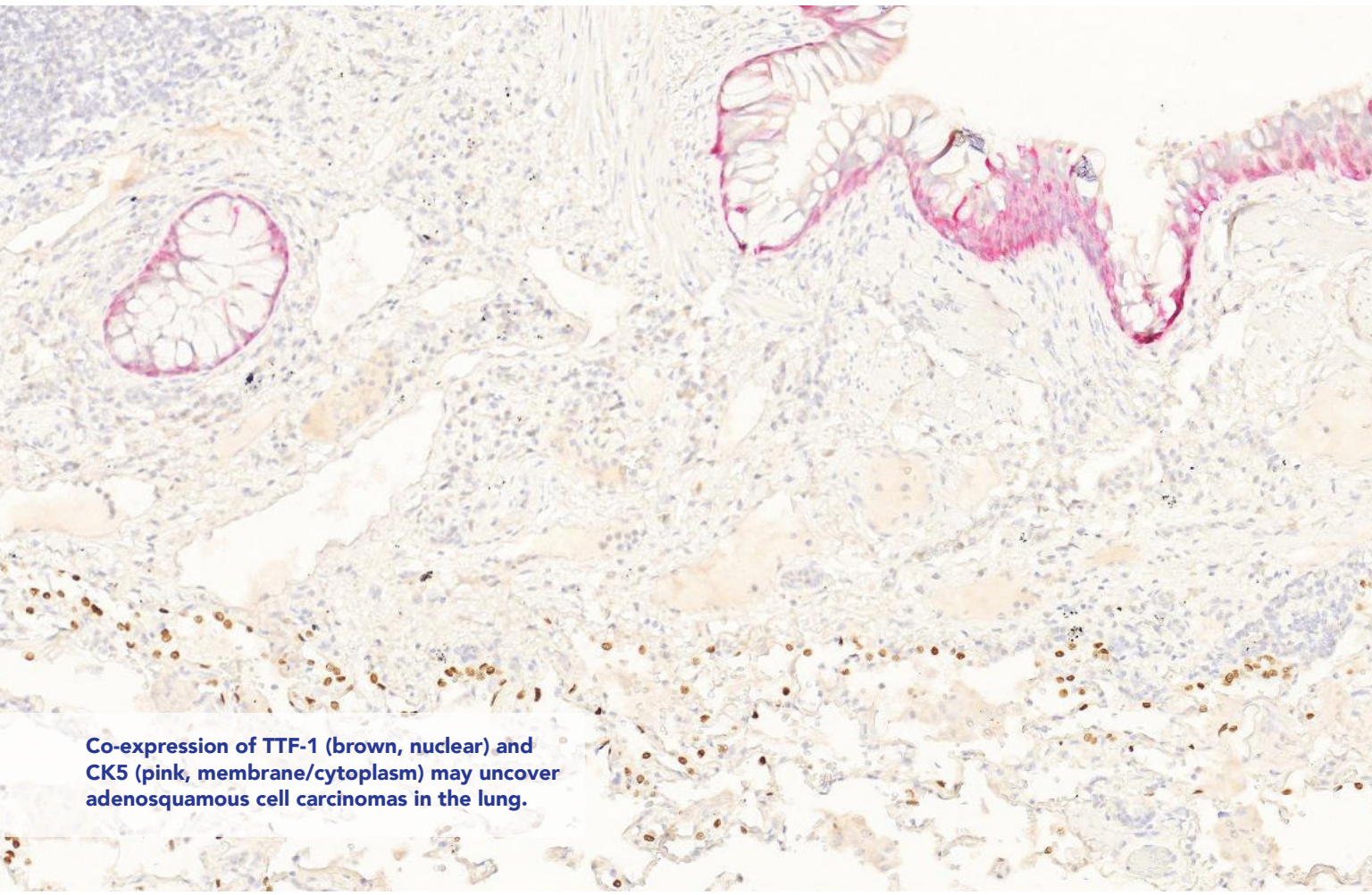


Co-expression of TTF-1 (brown, nuclear) and CK5 (pink, membrane/cytoplasm) may uncover adenosquamous cell carcinomas in the lung.

COMMON PROBLEMS WITH IHC ASSAY DEVELOPMENT

Immunohistochemistry (IHC) is an important tool for pathologists, who integrate it in routine diagnostic workflows as well as in basic and clinical research. Despite the method's widespread use, a staining protocol's success depends on many factors, and scientists must go through a rigorous optimization process whenever they introduce a new antibody to their diagnostic panel.

1. Tissue Processing and Sectioning

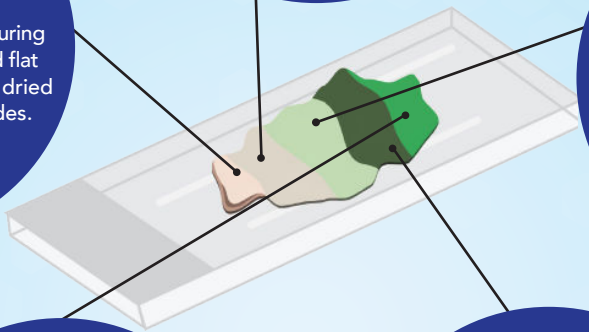
To avoid tissue detachment during the staining process, thin and flat sections should be thoroughly dried onto positively-charged slides.

2. Antigen Retrieval

For each IHC protocol, researchers must establish optimal antigen retrieval methods, which will depend on the primary antibody, tissue type, and fixation method.

3. Autofluorescence

When using fluorescent secondary antibodies, scientists should first assess the tissues' inherent fluorescence and reduce the intensity with autofluorescence quenching kits or dyes such as Sudan Black.

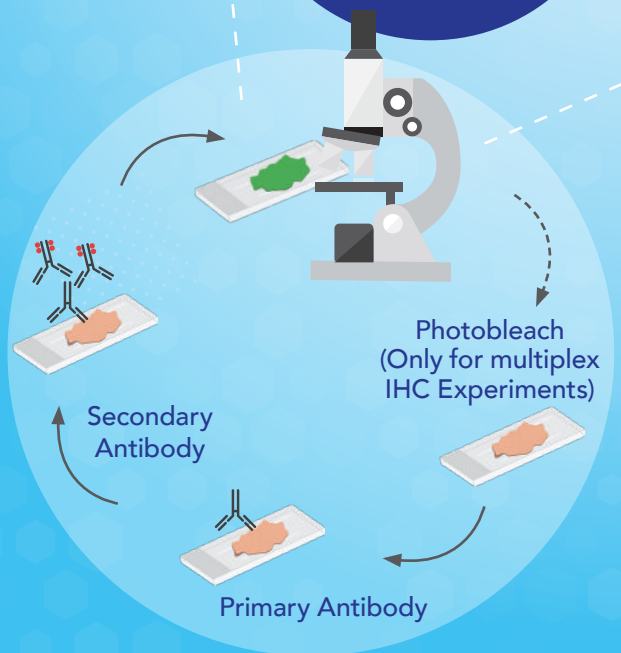


4. Weak or Saturated Target Staining

To obtain the ideal staining intensity, scientists optimize their staining protocols by varying antibody concentrations, wash step durations, detergent types, and detection systems.

5. Background Staining

Researchers should block endogenous peroxidases with hydrogen peroxide and mask molecules that might inadvertently bind the primary or secondary antibodies used for the staining with a blocking solution.



6. THE BENEFITS OF PARTNERING WITH EXPERIENCED SERVICE PROVIDERS

The combination of IHC-related variability with the inherent heterogeneity of patient samples makes it difficult to confidently identify disease. When researchers partner with CLIA-certified service providers for their histological needs, researchers can rest assured that all analyses are performed to a consistent, high standard.

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CONTEXT MATTERS: REVEALING HOW CELL TYPES INTERACT

Conventional immunohistochemistry (IHC) has long been the gold standard for tissue pathology, but the technology's limited ability to stain for multiple markers at once curbs its diagnostic and prognostic potential. IHC's lowplex nature means pathologists must process multiple tissue sections to run through complete diagnostic antibody panels and gather all the data required to provide accurate diagnoses. The emergence of multiplex immunohistochemistry (mIHC) methods, which permit simultaneous analysis of multiple markers within a tissue section, hold the potential for pathologists to accelerate multiparametric analyses of whole tissue sections and improve the diagnostic process.[1,2]

Strength in Numbers

Typical diagnostic workflows consist of standard stains, such as hematoxylin and eosin (H&E), followed by a variety of IHC experiements to stain for a set of diagnostic antibodies that will provide sufficient information to properly categorize a disease. This is especially true for complex specimens, such as cancer biopsies, where cell subpopulations and the tumor microenvironment provide insights into the disease's biology and clinical progression. In addition, scientists must also phenotype tumor-infiltrating immune cells to determine patient responses to targeted therapies. Given the wide array of markers needed to functionally profile immune cells, assessing therapeutic success further increases the number of experiments needed for accurate cancer diagnostics and prognostics.[3]

Because researchers can perform mIHC with the same commercially-available antibodies they have used and vetted for decades, ChipCytometry allows reliable biomarker and cell type identification within the tumor microenvironment

While traditional IHC protocols only stain for one to two proteins at a time, scientists can repeatedly probe the same section for numerous markers with mIHC.[1,2] To do this, researchers perform iterative staining cycles with fluorescent antibodies that are photobleached after image acquisition.[1,3] This manual method allows scientists to increase the amount of information they obtain from a single tissue section but is quite laborious and time-consuming. To accelerate and automate mIHC experiments, scientists at Canopy Bioscience developed CellScape™, a ChipCytometry™-based imaging platform.[4] This instrument combines microfluidic, advanced imaging, and walk-away automation technologies to repeatedly stain, image, and photobleach specimens. For every staining, CellScape acquires stacks of images at different brightnesses to compile a high dynamic range image that allows scientists to simultaneously

The new CellScape instrument from Canopy Biosciences is a fully automated system for high-plex spatial -omics that uses open source antibodies.

quantify rare and abundant proteins.[5] In addition, the instrument records a sample's background fluorescence between photobleaching steps to increase signal-to-noise ratios. Finally, the instrument comes with innovative software that analyses images and converts data into flow-like dot plots to ease data interpretation.[4,5] This technology has great potential to accelerate cancer research, including translational and biomarker studies, and tumor diagnostics because it enables scientists to identify and phenotype every cell in the tumor microenvironment while preserving structural context.

The Multiplexed Data Is In

Since its development, scientists have optimized ChipCytometry-based mIHC workflows for cell suspensions, fresh-frozen tissues, and formalin-fixed, paraffin-embedded tissues, enabling liquid and solid tumor analyses regardless of sample collection or processing methods.[1] Because researchers can perform mIHC with the same commercially-available antibodies they have used and vetted for decades, ChipCytometry allows reliable biomarker and cell type identification within the tumor microenvironment.

For example, Andrea Tüttenberg's research team at the University Medical Center of the Johannes Gutenberg University Mainz compared ChipCytometry's ability to characterize immune cell infiltration with flow cytometry and traditional IHC.[6] Initially, flow cytometry analysis revealed elevated CD8+/CD4+ ratios in the tumor sample compared to ChipCytometry, suggesting flow cytometry might be better suited for tumor infiltration analysis. However, a closer look at the stained tissue section revealed that effector T cells failed to migrate into the tumor and instead remained in the periphery. Flow cytometry cannot make this distinction because it analyzes the entire tumor tissue. This revealed the importance of spatial context for immune cell infiltration analysis.[6]

Similarly, Louisa von Baumgarten's laboratory at the Ludwig Maximilian University of Munich used ChipCytometry to study the characteristics of chimeric antigen receptor (CAR) T cells that successfully infiltrated primary central nervous system lymphoma to mount an immune response.[7] The researchers analyzed 16 markers and found two that correlate with enhanced infiltration, suggesting these could be possible biomarkers to assess treatment response in patients. Together, these studies show that ChipCytometry is an ideal platform for mIHC-based diagnostic and research workflows.

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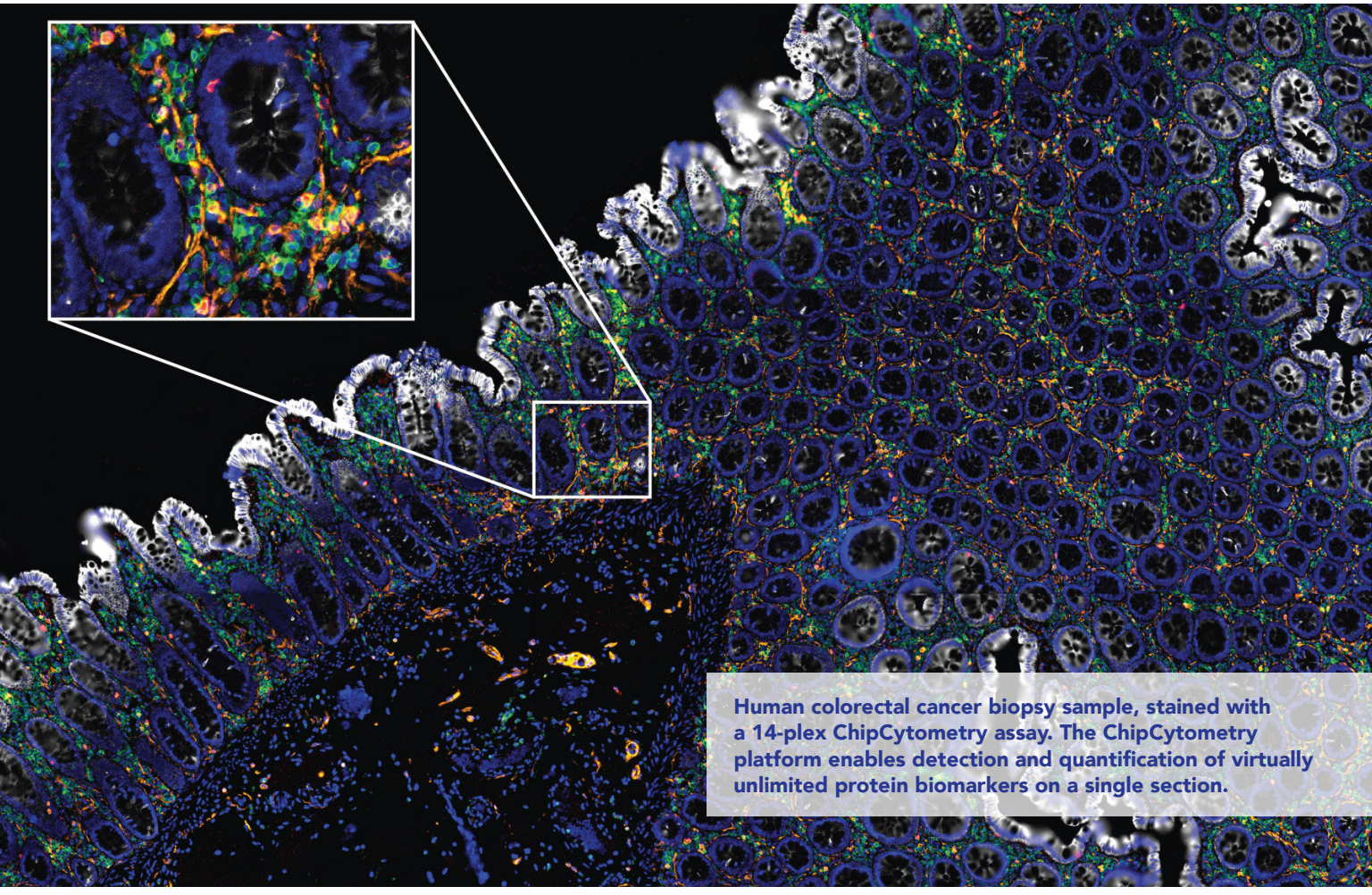


Human colorectal cancer biopsy sample, stained with a 14-plex ChipCytometry assay. The ChipCytometry platform enables detection and quantification of virtually unlimited protein biomarkers on a single section.

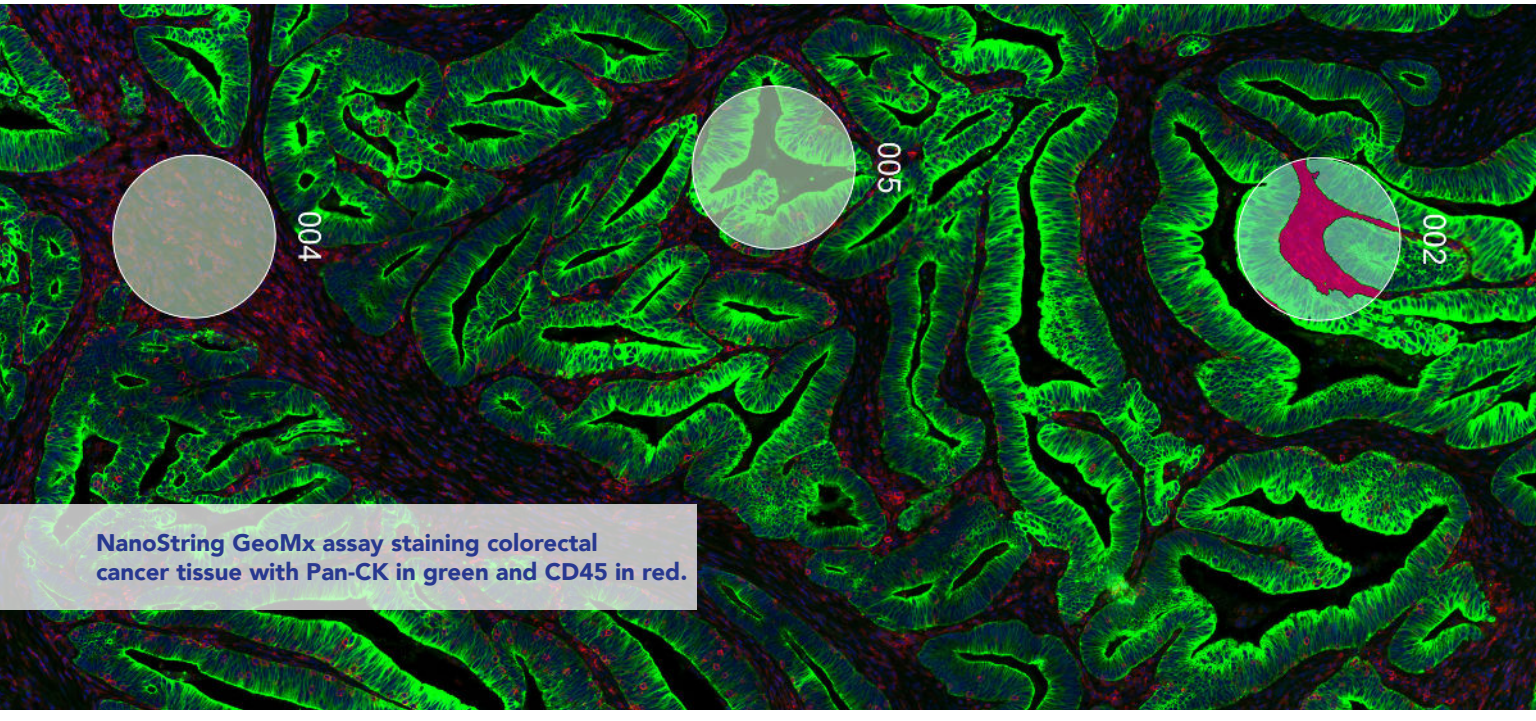
LOOKING AHEAD: HOW TO FURTHER IMPROVE TUMOR MOLECULAR PROFILING

As soon as pathologists inspected cancer tissues, it became clear that cancer heterogeneity was an important factor to consider when studying and treating the disease.[1] Indeed, biopsies from patients with the same cancer type have distinct structural features when analyzed under a microscope, a concept termed intertumor heterogeneity.[2] Tumors themselves may also not be homogenous, as individual cells or regions can contribute to intratumor heterogeneity by accumulating biological and genomic variations.[2,3] Local differences in the tumor microenvironment (TME), such as the extent of immune cell infiltration, interactions with the extracellular matrix, and proximity to vasculature, further amplify intratumor heterogeneity. Because targeted cancer treatments, such as immunotherapy, rely on effective immune cell infiltration and because cancer subclones can acquire mutations that confer resistance to such treatments, intratumor heterogeneity is a key determinant of therapeutic success.[2]

Standardizing Data Interpretation

Tumor heterogeneity makes it difficult to confidently classify a patient's cancer type upon visual inspection. In addition, multiple diagnostic immunohistochemistry (IHC) assays exist to assess biomarker levels, such as PD-L1, and determine whether a patient would benefit from immunotherapy.[4] Because these assays use distinct detection systems, scoring algorithms, and cutoffs, comparing results across assays is challenging. To reduce IHC's inter-user variability and provide an unbiased method to score patient samples, diagnostic laboratories increasingly incorporate digital pathology (DP) in their workflows.[4,5] DP combines whole slide imaging (WSI) with artificial intelligence or machine learning methods for integrated analysis. With WSI scans, pathologists can analyze both the tumor and neighboring tissues to fully assess TME heterogeneity. In addition, diagnostic labs store digital images on cloud-based servers and

The GeoMx Digital Spatial Profiler initially generates a high-resolution image of the stained tissue so that scientists can identify regions of interest for further analysis.



NanoString GeoMx assay staining colorectal cancer tissue with Pan-CK in green and CD45 in red.

share them with clinicians and pathologists for second opinions, while simultaneously building biobanks for future meta-analyses. As computational biologists improve automated analysis and quantification methods, DP promises to revolutionize molecular pathology, biobanking, and molecular tissue profiling.

Resolving Tumor Heterogeneity

Researchers have struggled to study all aspects of tumor heterogeneity. While technologies such as flow cytometry and single cell sequencing molecularly profile a tissue's various cell types, these technologies lack the spatial resolution to show how they interact with each other. In contrast, multiplex IHC (mIHC) retains spatial information so that researchers can study cellular dynamics during disease progression or in response to treatment.[6] While mIHC has led to major breakthroughs in cancer biologists' understanding of tumor heterogeneity and immune cell infiltration, the technology does not permit in-depth molecular analysis of the identified cell types.

Canopy Biosciences' service offerings on the NanoString® GeoMx® Digital Spatial Profiler platform combines the best of both worlds and allows scientists to molecularly profile a sample while retaining spatial resolution.[7-9] This technology combines two distinct tissue profiling panels so that scientists can concurrently evaluate a specimen's morphology and perform proteomic or transcriptomic analysis on outlined regions of interest.[7-9] Researchers incubate their sample with fluorescently-labeled antibodies that reveal the sample's morphological features and markers of interest. At the same time, they add a panel of barcoded antibodies or in situ hybridization (ISH) probes that will respectively bind target proteins and transcripts.[7,9] Ultraviolet (UV)-cleavable covalent bonds link the target-specific oligonucleotide barcodes to the antibodies and probes. The GeoMx Digital Spatial Profiler initially generates a high-resolution image of the stained tissue so that scientists can identify regions of interest for further analysis. The instrument next shines UV light on the outlined areas to cleave the barcodes from the antibodies or ISH probes and subsequently transfers the oligonucleotides from each region of interest to a distinct well of a 96-well-plate for downstream identification and quantification. The instrument contains a digital micromirror chip that shapes ultraviolet light to match any outlined tissue area, enabling researchers to separately collect barcodes from tumor and immune cells within a single section.[9] GeoMx's ability to spatially analyze protein and RNA expression in tumors and surrounding tissues from patients or preclinical models holds great promise to provide new insights into the complex biological and cellular processes that cause tumor heterogeneity and immune cell infiltration, and will be enable novel biomarker and precision cancer therapy development.[10]

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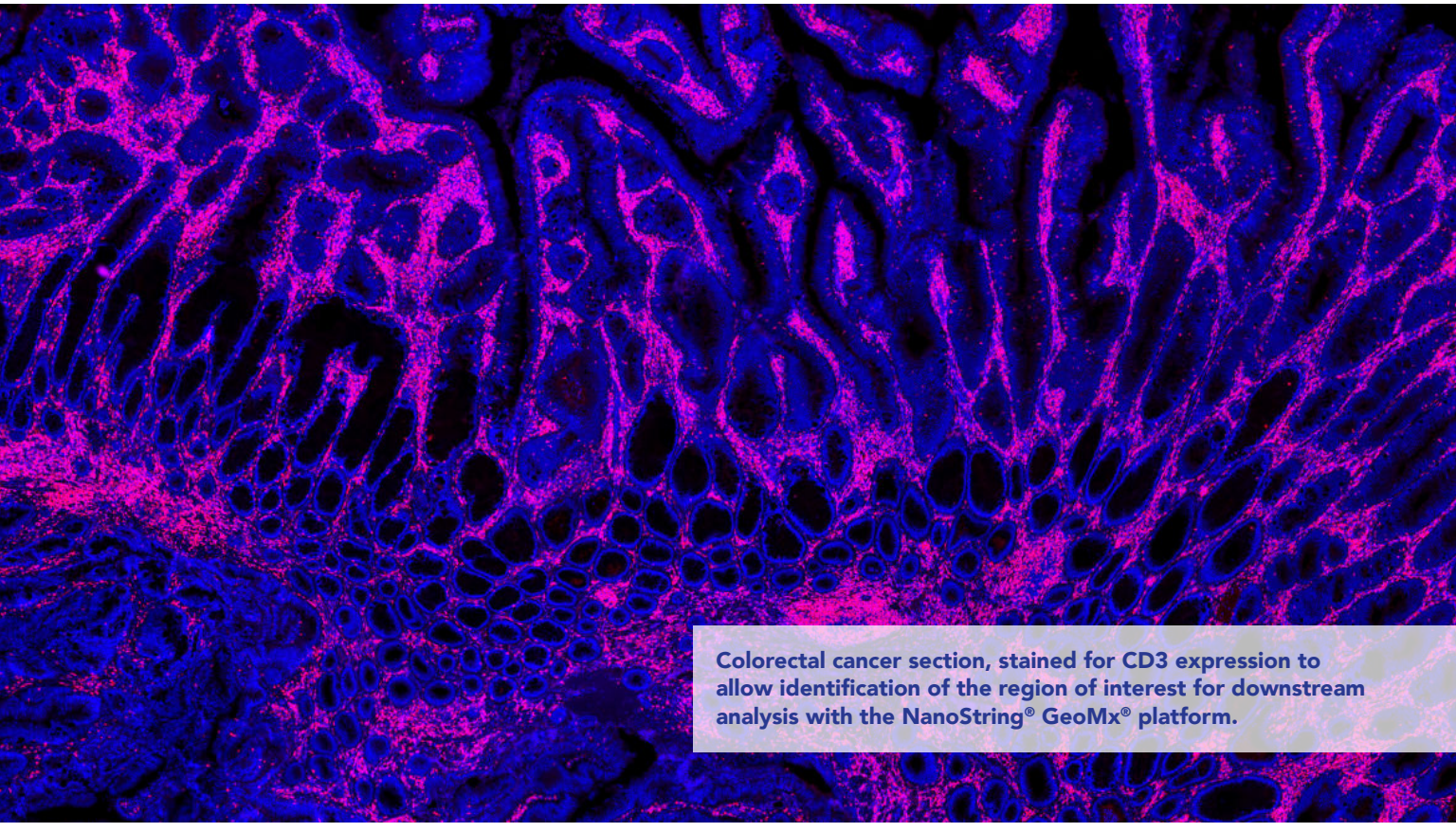

Colorectal cancer section, stained for CD3 expression to allow identification of the region of interest for downstream analysis with the NanoString® GeoMx® platform.

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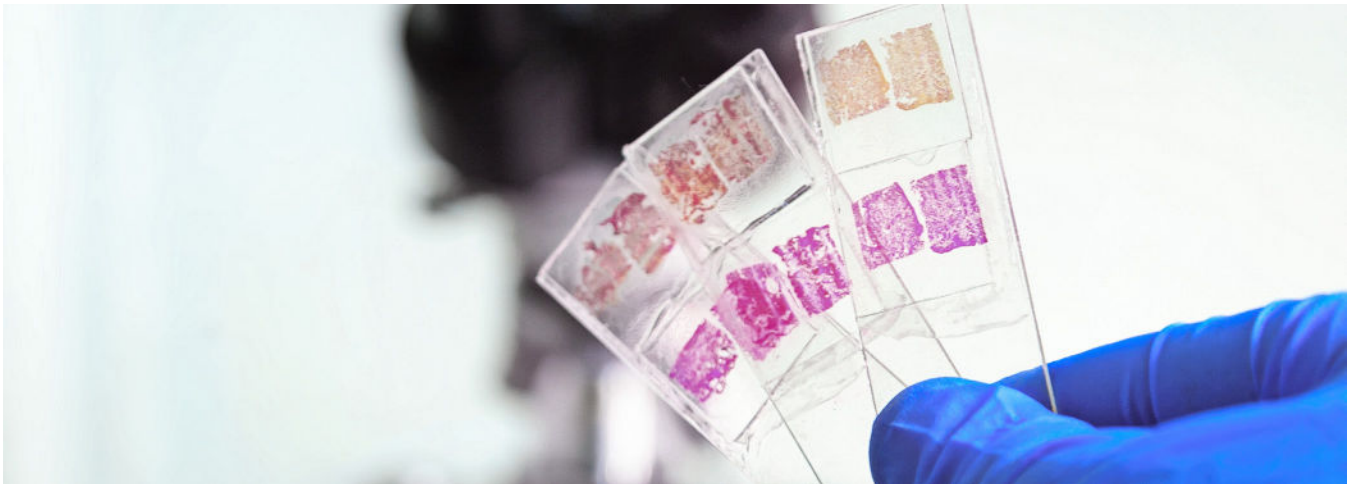

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