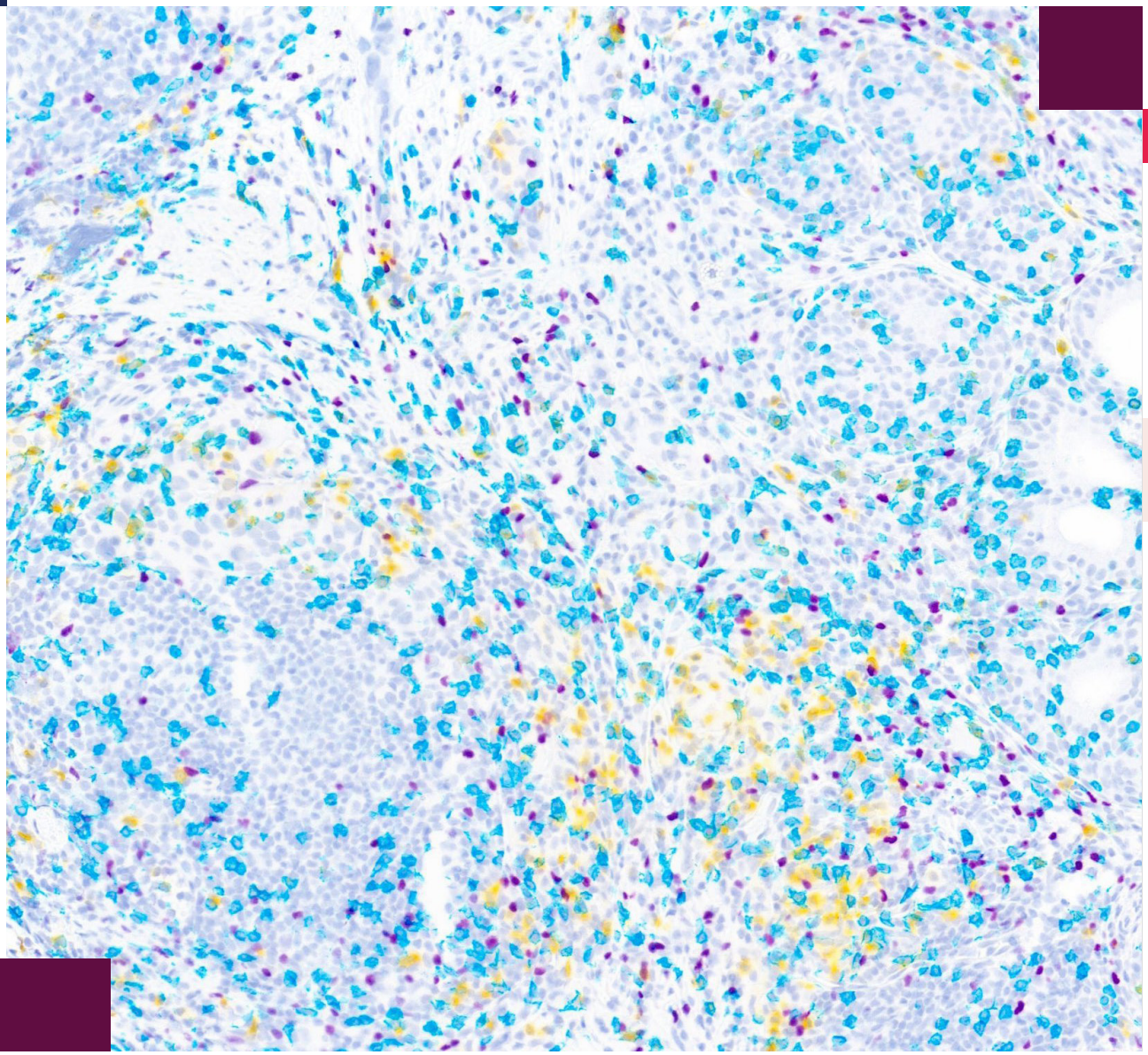
CHROMOGENIC MULTIPLEX IHC ASSAY

CellCarta

formerly
Caprion-HistoGeneX



MAPPING PRECISION MEDICINE

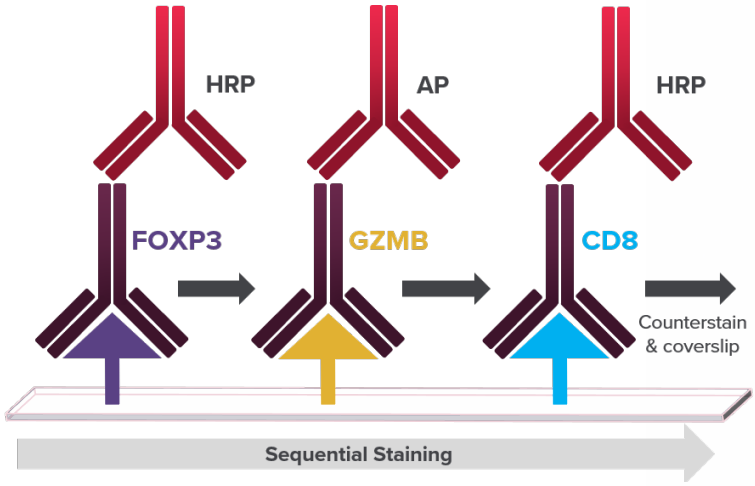
Why Chromogenic Multiplex Immunohistochemistry?

Targeted immunotherapies, both novel and effective, have become pivotal to the clinical management of patients with various types of cancer[1-8]. Multiplex immunohistochemistry (mIHC) has emerged as a powerful technology in the field of immuno-oncology and has become a standard tool for biomarker studies[1-3]. This method allows simultaneous detection of multiple protein biomarkers on the same tissue section, enabling the identification of different immune cell subsets, their activation or exhaustion state, and spatial distribution in the tumor microenvironment (TME)[1-3]. IHC has long been the gold standard method for understanding the *in situ* expression patterns of therapeutically relevant proteins that are prognostic, predictive, or selective for patient management in targeted oncology. However, the detection of one or two proteins no longer provides enough information to draw effective conclusions for patient stratification and therapy guidance.

New demands placed on clinical tissue specimens, an emphasis on teasing more information from increasingly small biopsy material, have further spurred the development of multiplex IHC strategies. Here we describe how we optimize and validate one of our brightfield chromogenic triplex IHC assays, and how this assay could be used for future clinical applications to better predict response to therapy.

Benefits of Chromogenic mIHC

- Uses a standard light microscope
- Familiar methodologies and staining characteristics
- Established technique with defined standards
- Reduced image size and associated storage costs compared to multiplex IF



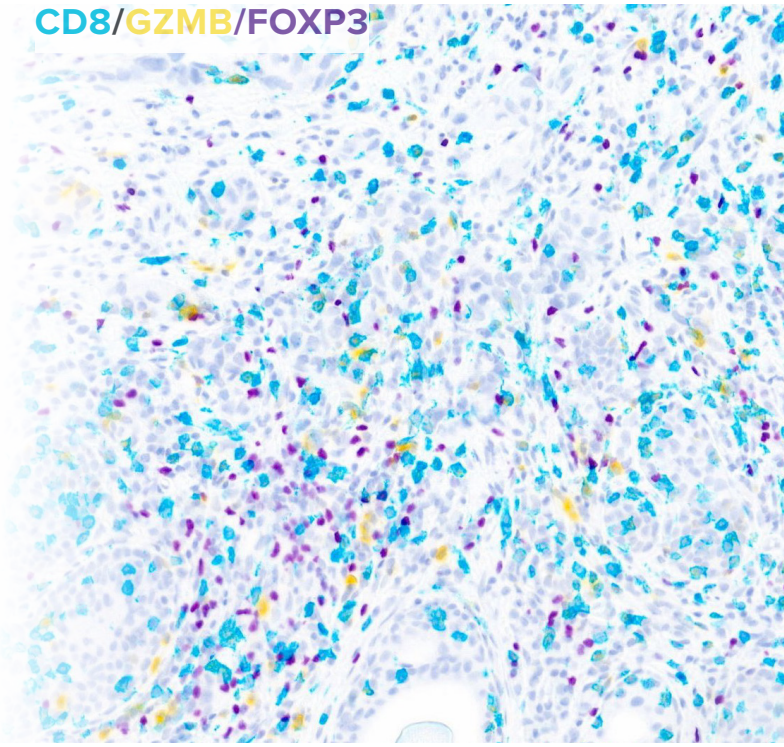


FIGURE 1.
Staining scheme for the CD8/GZMB/FOXP3 triplex IHC assay. All IHC staining performed on the Ventana DISCOVERY ULTRA autostainer (Roche).

CD8/Granzyme B (GZMB)/FOXP3 Chromogenic mIHC Assay

The CD8/GZMB/FOXP3 chromogenic mIHC assay is a fully automated assay that has been developed for use in formalin-fixed paraffin-embedded (FFPE) tissues. The assay involves sequential staining of three unmodified primary antibodies from the same or different species. Each secondary antibody is raised against the primary antibody host species and is conjugated to a reporter enzyme (horseradish peroxidase (HRP) or alkaline phosphatase (AP)). Different chromogen substrates are used for each enzymatic reaction which will yield a colored product (teal, yellow or purple) at the site of antibody/antigen binding[1,2].

Since both the FOXP3 and CD8 antibodies are raised in the same species (mouse), the triplex IHC assay protocol requires a stripping step. This step removes the FOXP3 antibody and detection system antibodies before the CD8 stain is applied.

Stages of mIHC development

OPTIMIZE

THE SINGLE IHC FOR EACH TARGET USING POSITIVE CONTROL TISSUE

Identify the clone, antigen retrieval conditions, and primary antibody incubation time for each target[1,2].

Monoclonal antibodies are ideal due to their high specificity, batch-to-batch reproducibility, and minimal background noise[2].

ASSEMBLE

THE MULTIPLEX PANEL

The order of antibody protein target and chromogen deposition is determined. The most effective utilization of chromogenic multiplexing is when the proteins of interest are expressed on exclusive cell types or unique subcellular locations.

TEST

THE OPTIMIZED CONDITIONS FOR A SINGLE IHC ASSAY

These conditions are then combined into a multiplex protocol. Calibration experiments will be applied to the assembled multiplex panel. For each antibody, samples will be stained with different dilutions. Increasing dilutions should result in gradual loss of the IHC signal. The appropriate dilutions will be selected to obtain optimal signal-to-noise ratios.

COMPARE

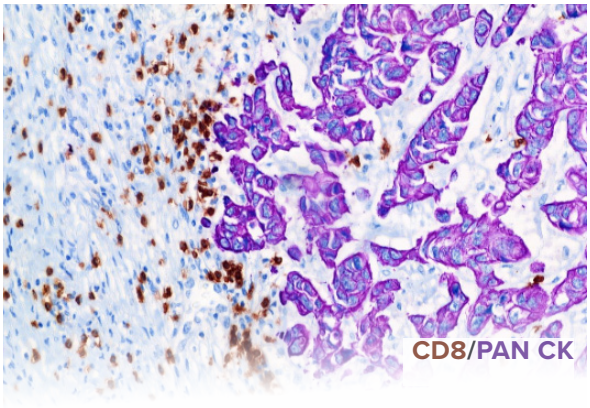
mIHC ASSAY TO ITS RESPECTIVE VALIDATED SINGLEPLEX IHC COUNTERPARTS

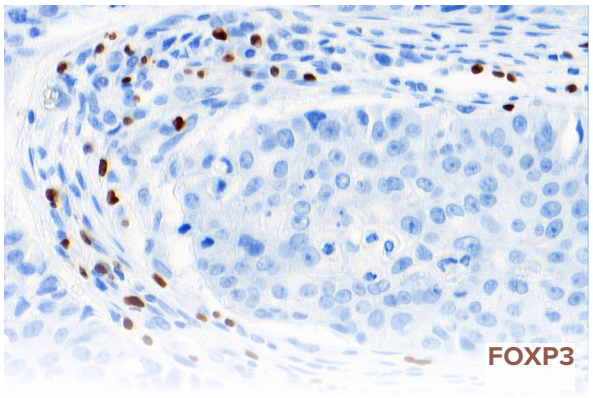
Serially stained slides are evaluated qualitatively by a pathologist (Fig. 2), and assessed semi-quantitatively via image analysis software (Fig. 3).

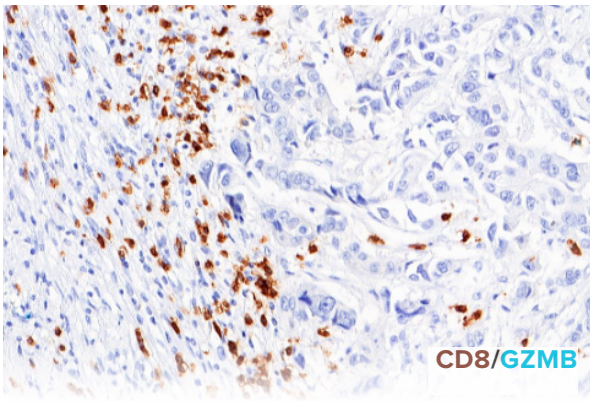
PRECISION TEST

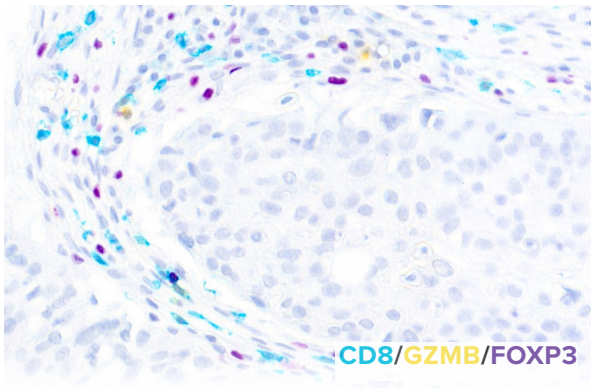
THE FINAL STAGE OF MULTIPLEX DEVELOPMENT

Precision testing using serial slides that are stained with the antibodies or IgG isotype matched control (negative control).









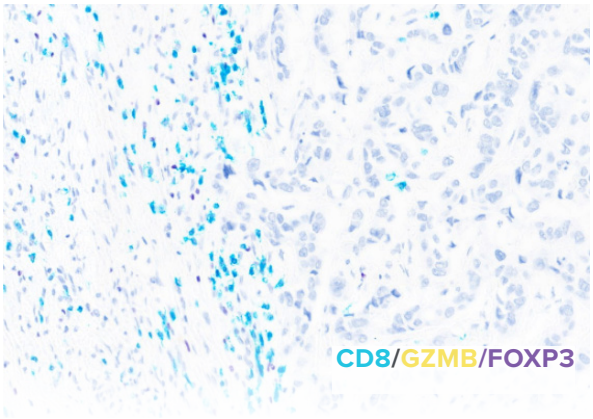
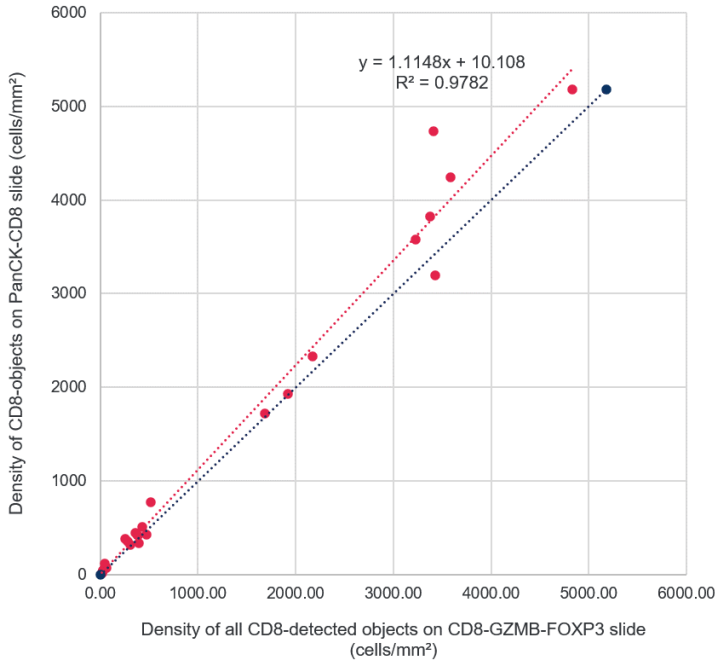
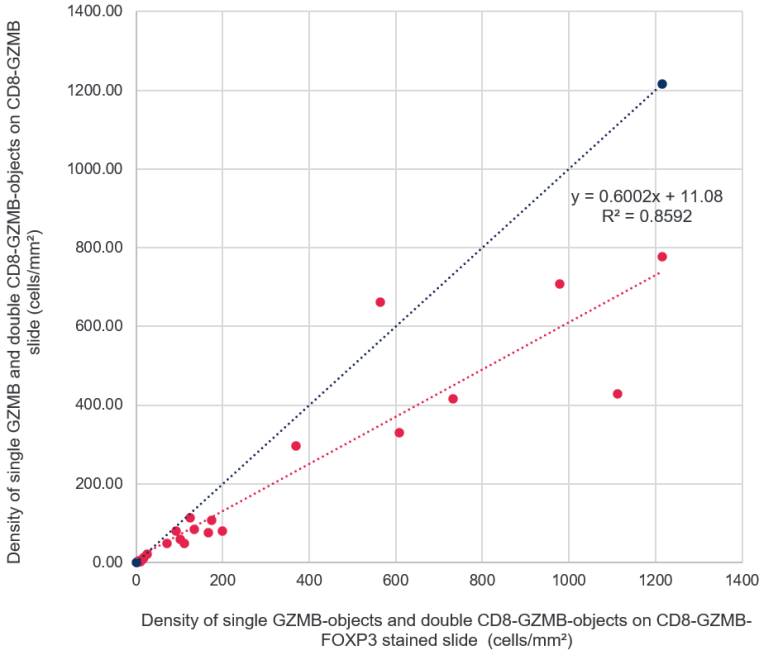


FIGURE 2.
The optimized mIHC assay is compared against its counterpart single-color or other validated brightfield assays using serial slides. *Left column*: CD8 staining across 3 serial sections, *right column:* FOXP3 staining across 2 serial sections.

A) Correlation of all CD8 detected objects



B) Correlation of single GZMB and double CD8-GZMB detected object



C) Correlation of single FOXP3 detected objects

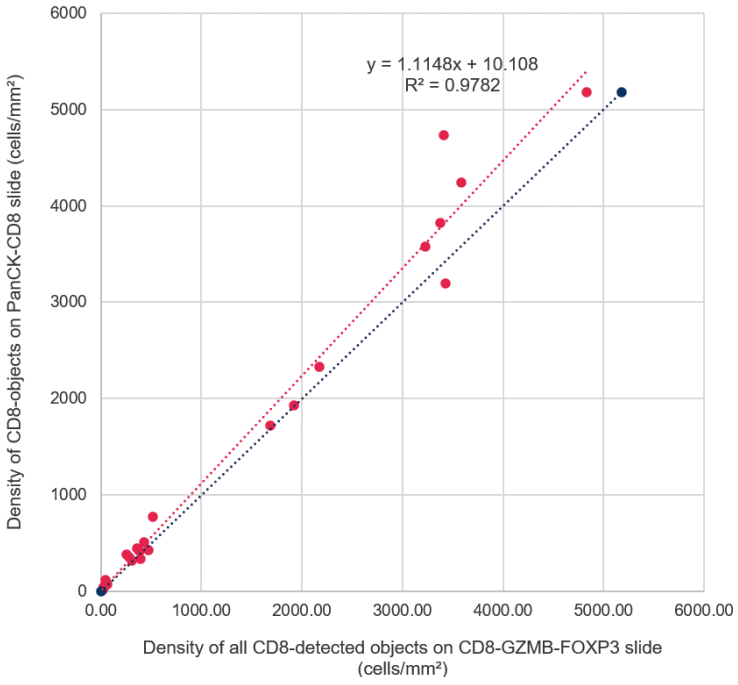


FIGURE 3.
Comparison of total CD8, GZMB and FOXP3 density (positive cells/mm^2) in serial slides stained for:

A) PanCK/CD8 or CD8/GZMB/FOXP3 (R^2 = 0.9782)

B) CD8/GZMB or CD8/GZMB/FOXP3 (R^2 = 0.8592)

C) FOXP3 or CD8/GZMB/FOXP3 (R^2 = 0.9782)

Interestingly, beyond quantitative image analysis, this chromogenic multiplex assay lends itself well to interpretation by a pathologist. Our pathology team has developed a robust and reproducible scoring algorithm which can be applied to the CD8/GZMB/FOXP3 assay.

This allows us to quickly assess the immune phenotype of a tumor biopsy (desert, excluded, inflamed) as well as characterize the level of CD8 activation as 'low' or 'high', making it an ideal partner to your immune therapy biomarker development strategy.

Clinical Applications

Immunotherapy has revolutionized cancer treatment[1-17]. Despite recent advances, there are subsets of patients who do not respond to treatment[4-7]. TME is emerging as a critical factor in understanding the relationship between a patient's immune system and their cancer[7]. Numerous studies have provided strong evidence that immune cells contribute to determining prognosis[3-13].

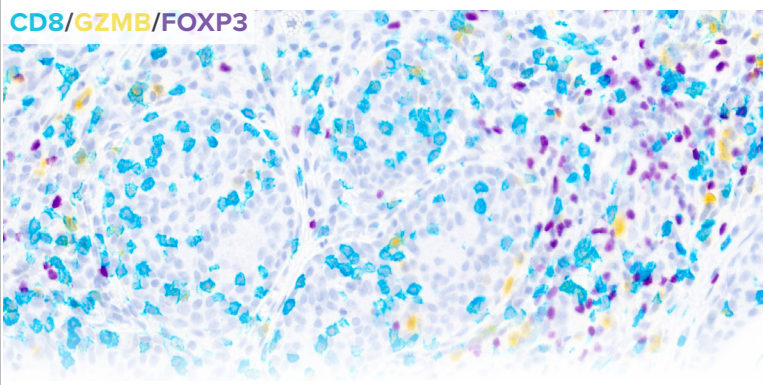
Chromogenic multiplexing assays with specific biomarkers such as our CD8/GZMB/FOXP3 triplex can provide valuable prognostic information.

GZMB is an essential mediator in lymphocyte activity and is used as a surrogate marker of activated CD8+ cells. Studies have shown that the presence of T cells with dual CD8 and GZMB expression correlated with a better clinical outcome, supporting the fact that these immune cells could represent a predictive biomarker of response to immunotherapy[5,8,9] (Fig. 4).

We recently published a paper in *Nature Medicine* in collaboration with researchers from the Barts Cancer Institute. The publication describes a Phase 2 study looking at neoadjuvent atezolizumab as a monotherapy before cystectomy in patients with bladder cancer[8]. The study analyzed biomarker expression (IHC and mIHC) of over 60 paired (pre-treatment vs. post-treatment) patient samples and concluded that pre-existing T-cell immunity correlated with response[8].

There was a significant correlation between presence of CD8+GZMB+ double positive cells and response in inflamed tumors; unlike relapsing tumors, which showed low levels of CD8+GZMB+ cells[8].

Early signs of metastasis in colorectal cancer (CRC) have been associated with low expression of GZMB and elevated expression was associated with improved survival[9].



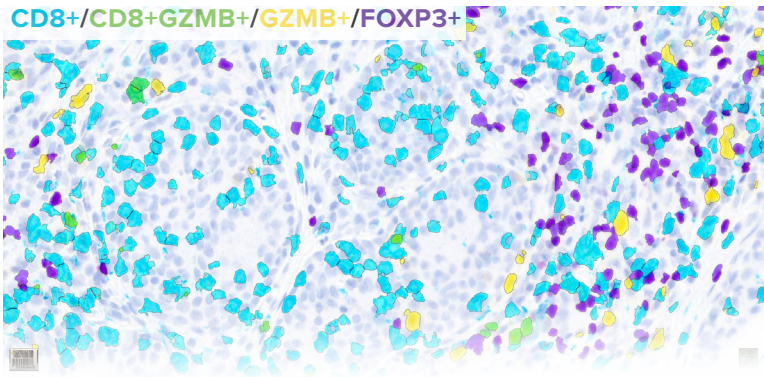


FIGURE 4.
Representative images of CD8/GZMB/FOXP3 triplex assay stain with chromogens (teal, yellow, and purple) and a hematoxylin counterstain. By combining CD8 and GZMB (double positive), the density and portion of activated cytotoxic lymphocytes (CTL) can be assessed. Activated CTLs (CD8+GZMB+ double positive cells) are prognostic factors in various types of cancers [5,8,9,16].

Clinical Applications (con't)

In addition to lymphocyte activation, we can use our chromogenic triplex assay to look at suppression of immune activity. FOXP3 is a canonical transcription factor necessary for the development and function of regulatory T cells (Tregs), and their abundance in tumor infiltrates is associated with unfavorable clinical prognosis in certain cancers[10-13]. The quantification of FOXP3 Tregs in breast tumors was shown to be valuable for assessing disease[10]. Significantly higher numbers of FOXP3 Tregs were observed in tissues of patients with *in situ* and invasive breast carcinomas as compared to healthy breast tissues[10]. Patients with invasive tumors that had high numbers of FOXP3 T cells had both shorter relapse-free and overall survival times[10]. FOXP3 increased expression in pancreatic cancers was also associated with a worse overall survival probability[12,13].

Challenges and Future Developments

Chromogenic mIHC is recommended when antigens are confined to different subcellular compartments (e.g. plasma membrane, within the cytoplasm or nucleus)[1,2]. One of the most significant challenges to chromogenic multiplexing is the spectral absorption characteristics of the dyes: if more than two colors are used, even if just two, co-localization at the pixel-level can confound even the most sophisticated downstream digital imaging and image-analysis-based quantitative tools. Mixing of light-absorbing chromogens essentially results in such dark signals that reliable spectral unmixing or quantitation is impossible. This represents an advantage of fluorescence-based methods, in which overlapping signals get brighter, not darker.

There are currently several fluorescence-based multiplex staining methodologies (e.g. tyramide signal amplification (Akoya Biosciences), modified hapten-based technology (Cell IDx), and DNA barcode labeling technology (Ultivue)) that have recently emerged to overcome some of the limitations of chromogenic mIHC and are providing new means to increase our knowledge to better understand the TME[15].

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With support from the Histopathology, Imaging, and Quantification (HIQu) team and pathologists, IHC technicians and assay development team.

At CellCarta, we have experience developing a number of multiplex IHC and IF assays for a variety of customers and programs. Our multiplex IHC and IF capabilities support our partners in drug discovery, development, and clinical sample analysis.

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