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T-Cell Profiling Paper

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Abstract

The immune system heavily regulates disease development, progression, and recurrence, and, as such, the need for reliable predictors is high. These markers are used to assess the best course of treatment, help assess survivability, and provide insight into a possible recurrence. Interest in immune profiling and the tumor immune microenvironment (TIME) has increased, and new tools and techniques have arisen as a result. It is important in most cases to use assays appropriate to the situation and recognize that a combination is often necessary for a comprehensive analysis of the disease state. Here, we discuss the benefits of analyzing the TIME in a multiplex immunofluorescence setting using some key T-cell markers and what information researchers can glean utilizing this technique.

Overview of T-cells

As central regulators of our immune systems, T-cells are crucial to overall body functions and act as a primary defense system against foreign bodies and the development of harmful diseases. Following the presence of an antigen-presenting cell (APC), a signal is sent to the naïve T-cell population. These naïve cells then mature and differentiate according to the signal received, resulting in three types of effector cells: cytotoxic, helper T, or regulatory (Suárez-Fueyo et al. 2018).

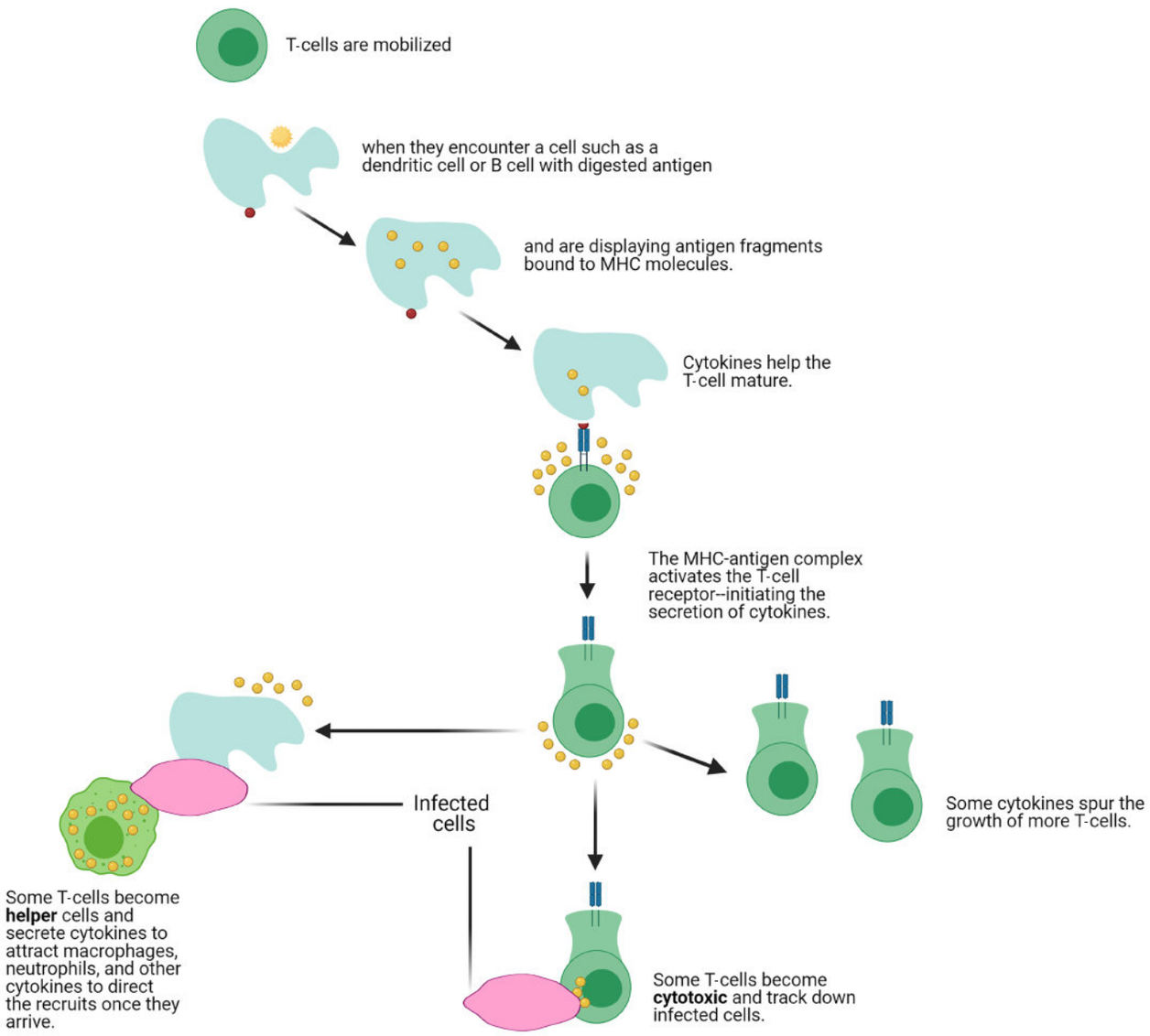

Figure 1: T-lymphocyte differentiation pathway (adapted from *T-cells 2021*, made with BioRender.com)

T-cells have very individualistic properties that, when quantified, can provide significant diagnostic insight. For example, naïve cell count provides insight on the adaptability of the current immune system, effector cells are highly useful in assessing the method of treatment available to the affected patient, and memory cell presence is crucial to drug discovery and potential development of vaccines.

Importance of Immune Profiling

Of the many disease states that threaten our body, cancer is more pervasive and least discriminatory. The CDC ranked cancer as the second leading cause of death in the United States—claiming more than a half million lives each year (CDC 2021). Cancer accounted for nearly 10 million deaths last year globally, per the WHO's most current report. Prevention and early detection are critical components to curbing incidence—factors that physicians and researchers alike have been pouring extensive time and effort into improving.

The clinical use of immune checkpoint blockade (ICB) agents designed to target CTLA-4 and PD-1/PD-L1 has significantly changed how many cancers are treated (Pilla and Maccalli 2018). Moreover, industry focus on the tumor immune microenvironment has helped predict patient survivability (Galon et al., 2006) and tumor recurrence (Mlecnick et al. 2016). By taking a retrospective look at patient population data in which clinical blockade agents were ineffective, it has been noted that, if taken into consideration initially, the TIME of these patients would have revealed tumors not prone to this avenue of treatment (Binnewies 2018). The recent emphasis on evaluating a patient's unique immune environment has revolutionized not only the prognosis but the treatment options available and the probability of these treatments working (Mlecnick et al. 2018).

Immune profiling can provide unique insights into how well the patient's adaptive immune response functions, the current disease state/progression, and/or the patient's overall health (McAllister 2021). High-quality, specific, and consistently performing antibodies are essential to aid in early detection. In addition, knowing the best biomarkers to screen for is not enough—these markers must also be available on the market. While immune cell profiling is no novel concept, the methods available vary significantly—flow cytometry, qPCR, and immunohistochemical methods are just a handful of tools currently being utilized.

Application of T-cell Profiling Using Multiplex Immunohistochemistry

T-cell profiling can be a potent tool in a multiplex immunofluorescence setting. This method involves taking a cancerous/diseased tissue, applying antibodies against the expected biomarkers, and profiling the various T-cells present. Immune profiling via multiplex immunofluorescence can help guide pathologists in determining how to proceed with the patient's case. Interestingly, in some instances, multiplex immunohistochemistry immune profiling was more successful at detecting Treg numbers than flow cytometry analyses (Halse et al., 2018).

Furthermore, in the field of IHC, technological advancements have significantly increased the number of biomarkers screened at once with improved clarity. Fluidigm's Hyperion System and corresponding CyTOF® software leverage imaging mass cytometry to analyze 80+ markers using a metal-tagging system. PerkinElmer's Vectra Polaris™ System builds upon traditional fluorescent tagging by utilizing their sensitive Opal™ reagents combined with whole slide scanning capabilities—lending the system to enhanced multi-spectral imaging and minimized photobleaching.

T-cell markers FOXP3, CD3e, CD8a, and T-bet were examined on an FFPE colon carcinoma tissue sample in the example below. The tissue was then digitally scanned, T-cell populations were analyzed using inForm®, and phenotype plots were constructed with R.

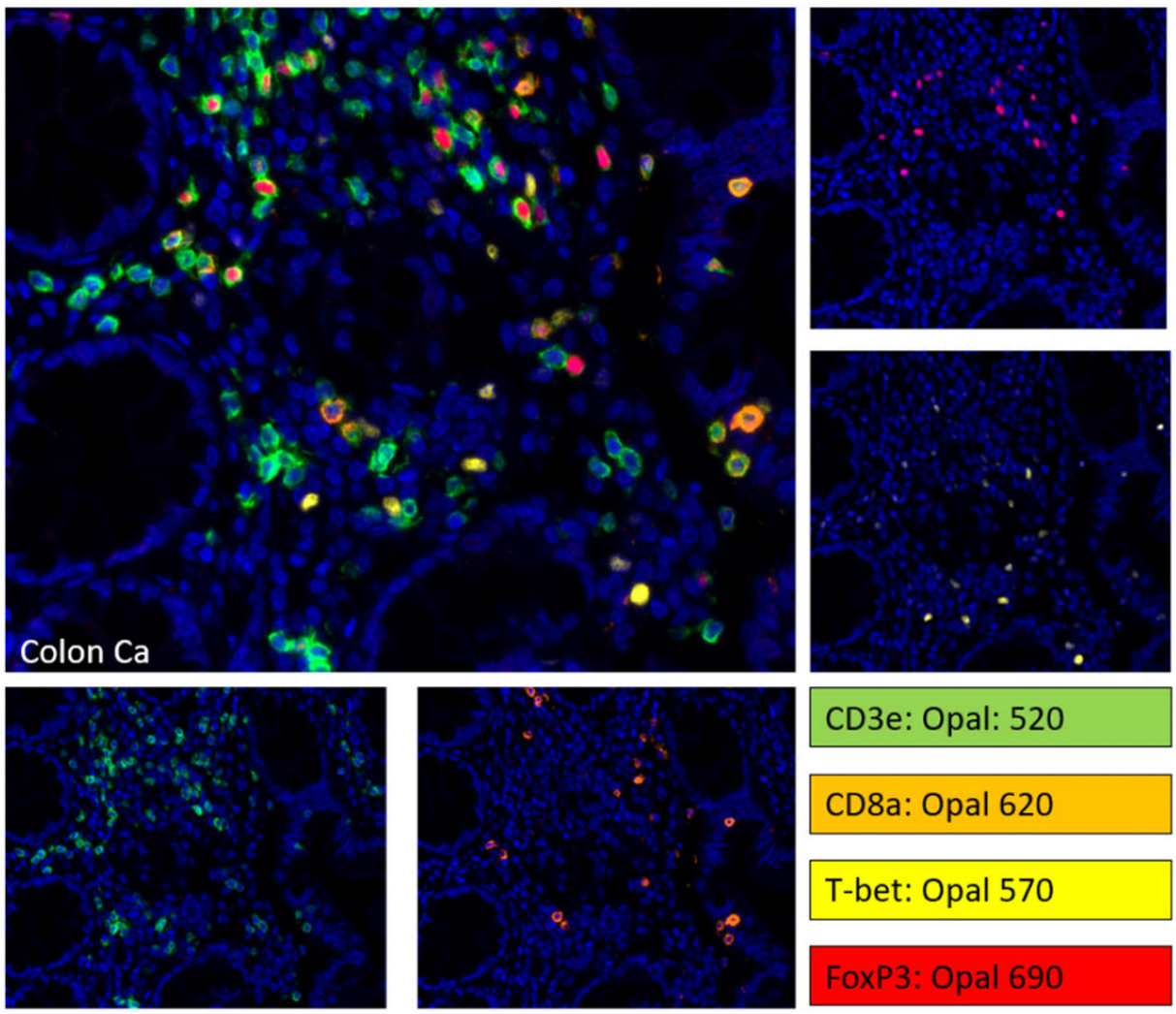

Figure 2: **Detection of human CD3e (green), CD8 (orange), T-bet (yellow), and FOXP3 (red) in FFPE colon carcinoma by mIF.** Rabbit anti-CD3e recombinant monoclonal [BL-298-5D12] (A700-016), rabbit anti-CD8 alpha recombinant monoclonal [BLR044F] (A700-044), rabbit anti-T-bet/TBX21 monoclonal [BLR110H] (A700-110), and rabbit anti-FOXP3 recombinant monoclonal [BLR034F] (A700-034). Secondary: HRP-conjugated goat anti-rabbit IgG (A120-501P). Substrate: Opal™ 520, 570, 620, and 690. Counterstain: DAPI (blue).

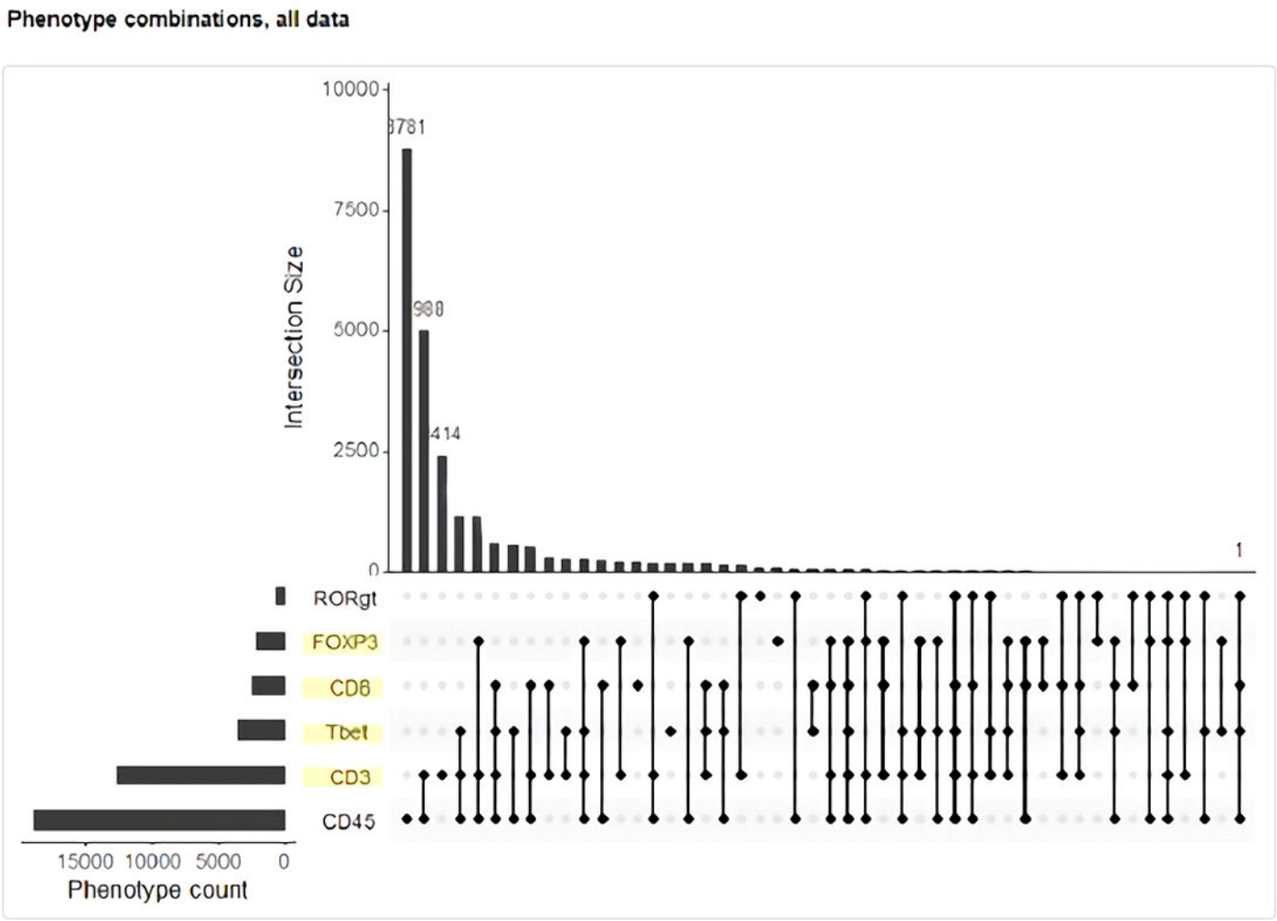

Figure 3. Breakdown of cell count by phenotype. Each phenotype was examined on a series of 14 fields of view. T-cell biomarkers of interest are highlighted in yellow. In this sample, it should be noted that, as expected, CD3e+ cells represent a significant portion of the tissue while individual T-bet, CD8a+, and FOXP3+ represent smaller subsections.

Results

In the above example, populations of single FOXP3+(red), T-bet+(yellow), and CD8+(orange) cells were low as compared to CD3e (green) cells. Overall, the observed colon carcinoma tissue had high T-cell counts, indicating a favorable prognosis (Dahlin et. al., 2011). When looking at individual T-cell markers, the moderate presence of CD8+ T-cells is notable as this marker is now known as a predictor for patient survival. T-bet+ cell counts were higher overall than CD8+ and FOXP3+ cells--further contributing to the likelihood of a favorable prognosis. Higher relative T-bet+ infiltration combined with lower FOXP3+ expression generally yields a more positive outlook (Ling et al., 2015). The relatively low FOXP3+ cell count contributes to the likelihood of a good prognosis—high expression is typically linked to poor prognosis in certain cancers--colorectal, non-small cell lung cancer, melanoma, pancreatic, and several others (Szylberg et. al. 2016). Finally, co-expression of CD3e and CD45+ cells was high—as CD45 is ubiquitously expressed in hematopoietic cells, overlap with CD3e T-cells would be expected. As with all assays, it is helpful to have a foundation/control like this that can indicate proper staining technique, proper localization, and other metrics.

In summary, these results suggest that a patient expressing these conditions will likely have a good prognosis and high survivability dependent upon the treatment prescribed. Spatial analysis, among other tools, can also be instrumental in further monitoring and exploring the TIME.

Notable T-cell Biomarkers

FOXP3

FoxP3+ cells are regulatory T-cells, playing an integral role in maintaining immune tolerance and regulating homeostasis within the body, and are recognized as a key marker for CD4+ Tregs (Kim 2009). Research on FoxP3+ Treg cells is relatively extensive, albeit conflicting. On the one hand, it is suggested that FoxP3+ Treg cells "*may lack stability*" and, under some immunological instances, "*may be easily converted into Th-like Treg cells instead,*" suggesting some significant plasticity (Li et al. 2015). Conversely, some suspect that these cells lack this plasticity. Instead, it is believed that they may actually "*display distinct sequences and are derived from separate clones with little to no possibility of inter-conversion*" (Li et al. 2015).

FOXP3 memory T-cells are highly efficient at suppressing effector T-cell function, while naïve FOXP3 T-cells are skilled at suppressing the earliest immune responses in lymphoid tissues (Chang 2009). Thus, these cells play a large part in helping prevent autoimmune disease, graft rejection, and an extensive suite of inflammatory diseases.

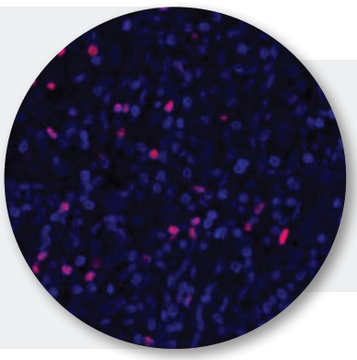

Figure 4. Detection of human FOXP3 (red) by IHC

Sample: FFPE section of human breast carcinoma. *Antibody:* Rabbit anti-FOXP3 recombinant monoclonal antibody [BLR034F] (A700-034 lot 1) used at 1:250. *Secondary:* HRP-conjugated goat anti-rabbit IgG (A120-501P). *Substrate:* Opal™. *Counterstain:* DAPI (blue).

T-bet/TBX21

Originally, T-bet was considered as a critical transcription factor for Th1 cells though it is now clear that it aids in the generation of multiple different lymphocyte lineages. Its presence is restricted almost solely to the immune system (Kallies et al. 2017). T-bet expression is essential for the clearance of pathogens and maintenance of immunity. This is due to its role in mediating the differentiation, migration, and overall survival of effector and memory T-cell subsets in the diseased tissue. In addition to its role in the T-cell pathway, research suggests that T-bet plays a vital role in B cell transcription and cell-fate decisions (Stone et al. 2019).

T-bet, a subset of CD4+ T cell (Th1), has also been shown to play a crucial role in metabolic regulation. It was determined that t-bet deficiency contributes to larger depositions of visceral adipose tissue—making this target of particular importance in obesity studies (Stolarczyk et al. 2014).

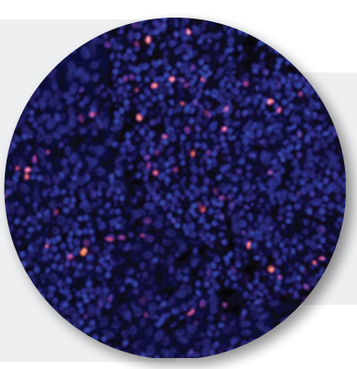

Figure 5. Detection of human T-bet/TBX21 (orange) by IHC

Sample: FFPE section of human tonsil. *Antibody:* Rabbit anti-T-bet/TBX21 recombinant monoclonal antibody [BLR110H] (A700-110 lot 1) used at 1:250. *Secondary:* HRP-conjugated goat anti-rabbit IgG (A120-501P). *Substrate:* Opal™. *Counterstain:* DAPI (blue).

CD3e

As CD3e antibodies generally correlate with the absolute value of total T-cells, an increased value often indicates an inflammatory response due to fighting an infection or cancerous disease. Moreover, its appearance at all stages of T-cell development and its high specificity makes it an excellent marker for the detection of both normal T-cells and lymphocytic tumors. High total T-cell counts are often correlated with lower stage/grade of disease in a clinical setting. Similarly, some studies have shown high total T-cell counts correlated to more prolonged cancer-specific survival (Dahlin et al. 2011).

CD3e plays a ubiquitous role in TCR activation and a crucial role in differentiation (Uniprot 2021). Its involvement in autoimmune disease and the associated harmful inflammation resulting from its expression make it a crucial target.

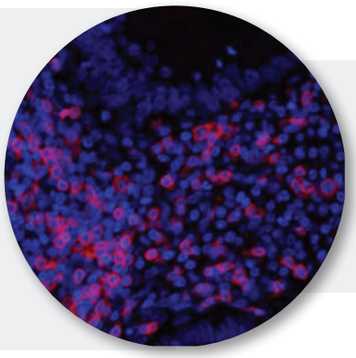

Figure 6. Detection of human CD3E by IHC.

Sample: FFPE section of appendix. *Antibody:* Rabbit anti-CD3E recombinant monoclonal antibody [BL-298-5D12] (A700-016 lot 1) used at 1:250. *Secondary:* DyLight® 594-conjugated goat anti-rabbit IgG (A120-101D4).

CD8a

A class of cytotoxic T-cells generated by the thymus is important to immune defense against intracellular pathogens (i.e., viruses, bacteria, etc.) and overall tumor surveillance. These cells can be implicated in the rejection or failure of transplants. An overabundance or dysregulation of CD8+ cells in the body can also lead to immunopathological effects resulting from an excessive immune response (Wissinger n.d.).

The three possible mechanisms employed by this cytotoxic T-cell are as follows:

1. Secretion of cytokines with anti-viral, anti-tumor, and anti-microbial effects
2. Production and secretion of cytotoxic granules (also found in NK cells) that work in conjunction with one another to form an entrance into the foreign body and initiating apoptosis of the invading cells (nicknamed “serial killers”)
3. Destruction of any infected cells by using Fas/FasL interactions (these interactions present amongst the CD8 t-cells also allow for fratricide, necessary for the end of an immune response).

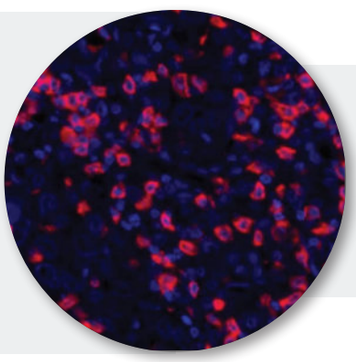

Figure 7. Detection of human CD8 alpha (red) by IHC

Sample: FFPE section of human breast carcinoma. *Antibody:* Rabbit anti-CD8 alpha recombinant monoclonal antibody [BLR044F] (A700-044 lot 1) used at 1:250. *Secondary:* HRP-conjugated goat anti-rabbit IgG (A120-501P). *Substrate:* Opal™. *Counterstain:* DAPI (blue).

Conclusion

Multiplex immunofluorescence is a valuable assay for immune profiling and provides better insight into the tumor immune microenvironment. Analysis of a patients' TIME within a sample demonstrates a tremendous impact this application has on the course of treatment available, the patients' initial immune response to the disease, and how it serves as a predictor for overall survivability. A deeper understanding of the disease state is revealed in analyzing fluorescent tissue staining of several key T-cell markers. Although this assay can be somewhat time-consuming upfront (optimizing antibody concentration and staining order can be lengthy), the information obtained is extensive. Furthermore, initial optimization can be significantly reduced by consistent, specific antibodies.

Fortis Life Sciences recognizes the need for high-quality, consistently performing antibodies for applications such as these, and the urgency to produce targets not currently on the market. Our catalog offers a suite of products validated for multiplex immunofluorescence, western blot, flow cytometry, and other applications.

Table 1. Suggested monoclonal T-cell markers from Fortis Life Sciences

Product	Catalog No	T-cell	Clone	Application	Host
FoxP3	A700-034	Regulatory	BLR034F	WB, IP, IHC, ICC, IHC-IF, mIF	Rabbit
T-bet/TBX21	A700-110	TH1 cells	BLR110H	WB, IP, IHC, ICC, mIF	Rabbit
CD3e	A700-016	Total T-cell	BL-298-5D12	WB, IP, IHC, ICC, IHC-IF, mIF	Rabbit
CD8a	A700-044	Cytotoxic	BLR044F	WB, IHC, IHC-IF, mIF	Rabbit
CD8a	A500-021A	Cytotoxic	C8/144B	IHC, IHC-IF	Mouse
EOMES	A700-104	Tcm, Tem, Teff	BLR104H	WB, IP, IHC, ICC	Rabbit
Granzyme B	A700-022	Cytotoxic	BLR022E	WB, IHC, ICC, IHC-IF, mIF	Rabbit
CD4	A700-015	Helper T-cell	BL-155-1C11	WB, IP, IHC, mIF	Rabbit
GATA3	A700-121	TH2 cells	BLR121H	WB, IP, IHC, ICC	Rabbit
STAT5a	A700-124	Regulatory	BLR124H	WB, IP, IHC, ICC	Rabbit
HLA-DR	A500-022A	Activated T-cells, Tem,	LN3	WB, IHC, ICC, IHC-IF	Mouse

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