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Highly Multiplexed IHC Assays to Examine Immune Checkpoints and Biomarkers for Immunotherapy

INTRODUCTION

The emergence of an increasing number of immunotherapy biomarkers and the importance of their context within the tumor microenvironment has resulted in a need for high-plex immunohistochemistry (IHC) assays. Using highly specific and validated antibodies developed for this purpose, we constructed several fluorescent multiplexed, TSA-based assays to examine the frequency, spatial localization, and proximity of immune cells within the tumor microenvironment.

Our data demonstrates the feasibility of simultaneous detection of seven fluorochromes in order to visualize immunosuppressive receptors associated with the exhausted T cell phenotype, myeloid-derived suppressor cells, and the PD-1:PD-L1 axis. Our findings demonstrate the utility of multiplex IHC to deconvolute protein expression and interactions within the complex tumor microenvironment.

METHODS

Tyramide signaling amplification (TSA) was used to serial stain tumor tissue of various types. This protocol allows for the use of multiple rabbit monoclonal antibodies in a single panel. A Mantra quantitative pathology workstation (PerkinElmer) was used to spectrally unmix the fluorescent signal in each image, and the InForm Image Analysis software (PerkinElmer) was used to provide quantitative data. Immuno-oncology-centric panels have been constructed, as well as those that focus on receptors involved in certain targeted cancer therapies.

CONCLUSIONS

- Multiplex IHC panels consisting of up to six targets plus DAPI were constructed and validated in various tumor types.
- Highly detailed images illustrating the utility of mIHC to detect:
  - Spatial localization of immune cells within the tumor micro-environment.
  - Co-localization and frequency of immune checkpoint receptors.
  - Proximity of suppressive immune cells and immune checkpoints indicative of receptor-ligand interactions.
- mIHC may provide a more in-depth understanding of the role of suppressive immune cells and their interactions with tumor cells in the process of immune evasion.
- *Any Cell Signaling Technology, Inc. IHC-validated antibody can be used to construct mIHC panels.*
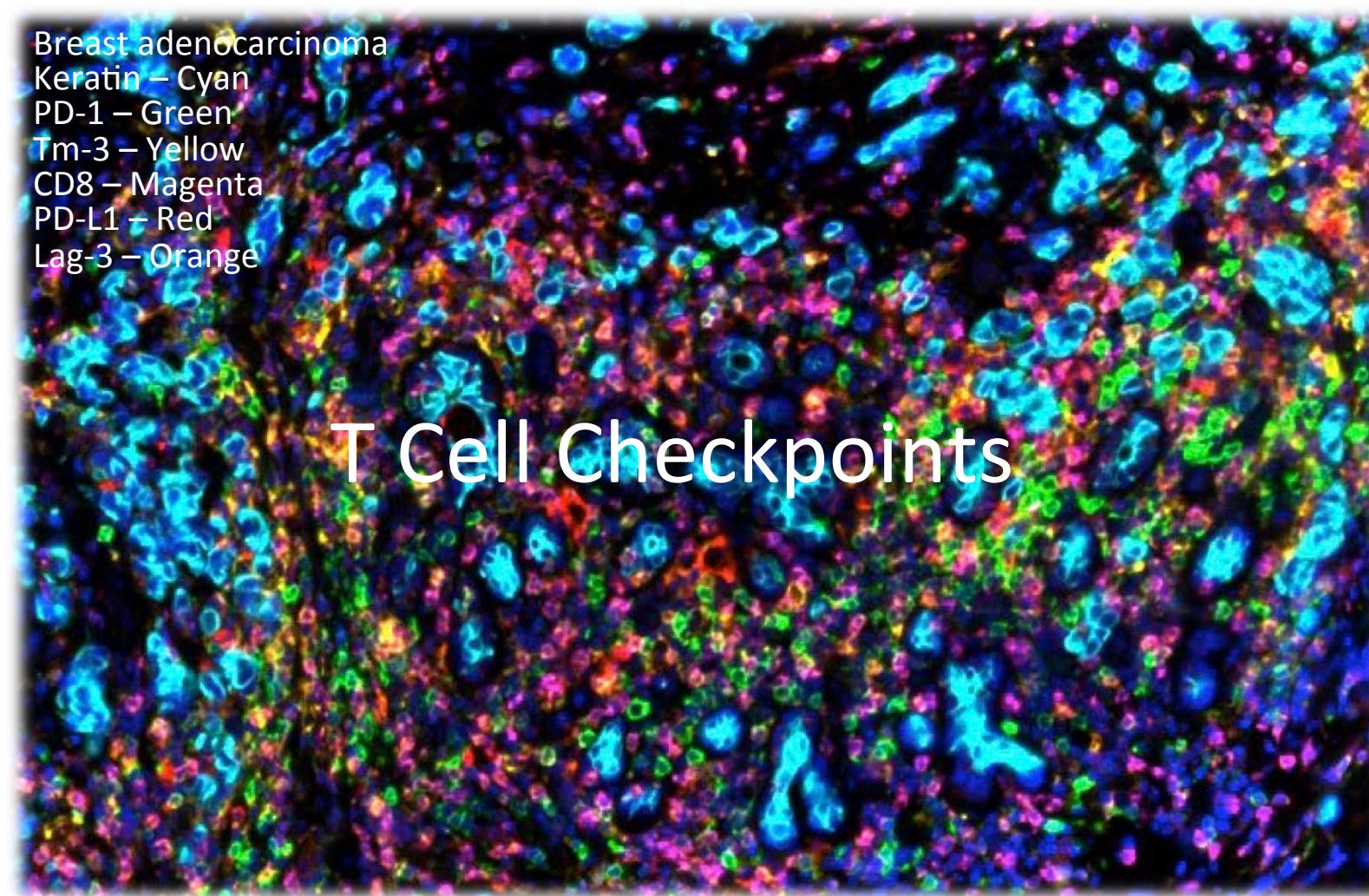
**Seven-plex IHC of key immuno-oncology phenotypic markers and therapeutic targets**

Figure 1A

Figure 1B

Table 1A
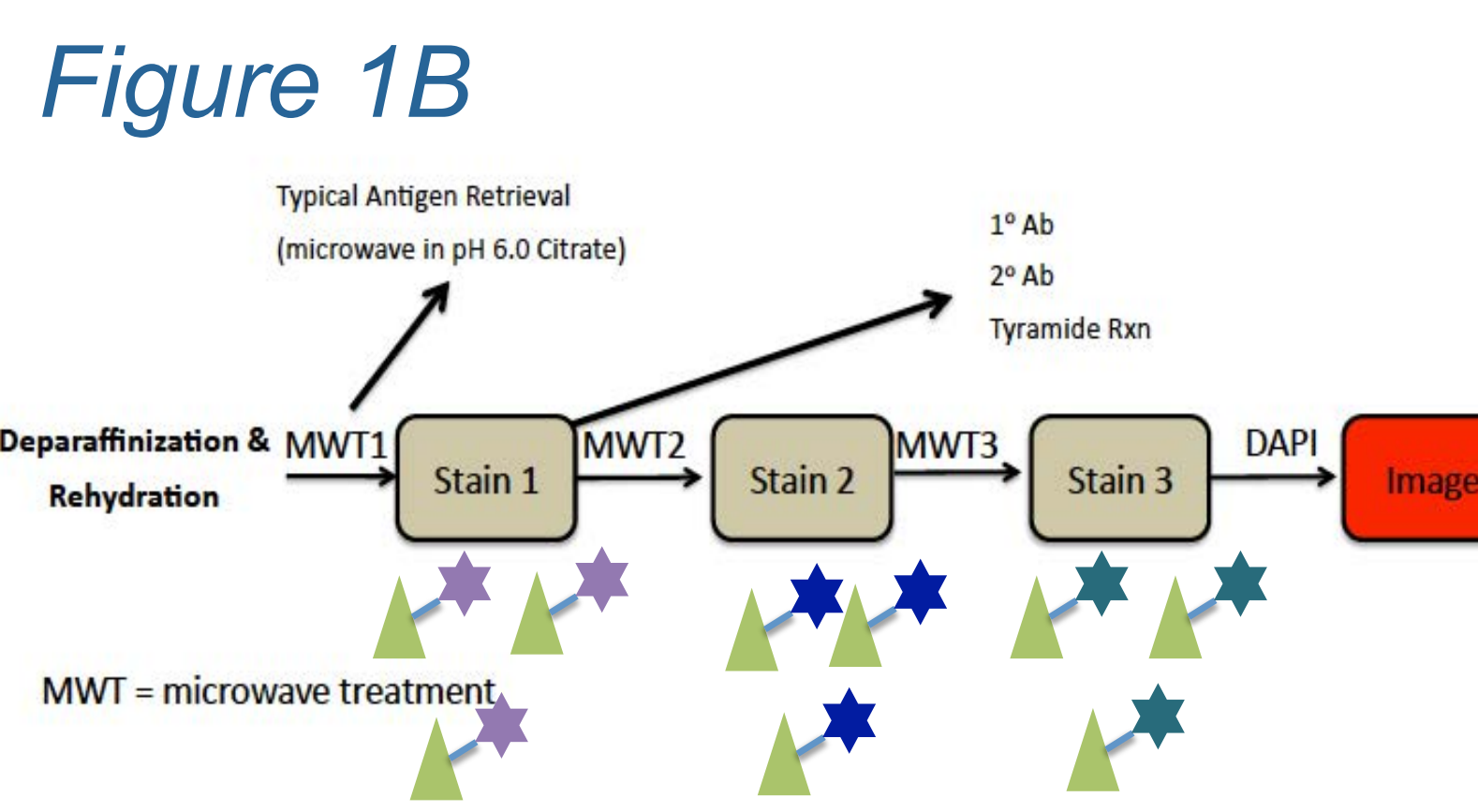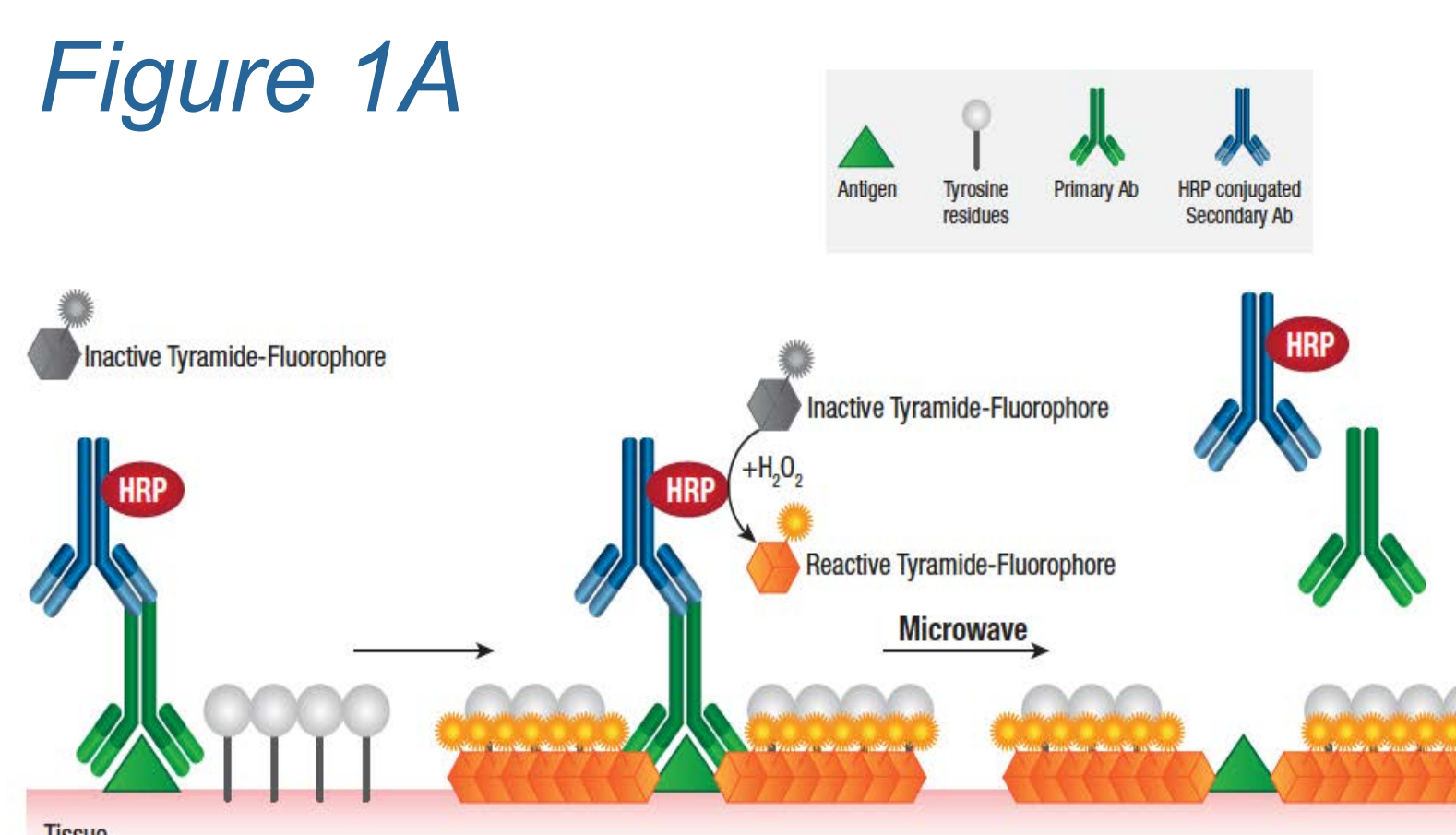
Panel	Targets
Targeted Therapies	
Xalkori	ALK (D5F3®) XP® Rabbit mAb #3633 ROS1 (D4D6®) Rabbit mAb #15027 Met (D1C2) XP® Rabbit mAb #8198 Krt (C11) Mouse mAb #4545
Prostate	PSA (D11E1) XP® Rabbit mAb #2475 PSMA (D7I8E) XP® Rabbit mAb #12815 AR (D6F11) XP® Rabbit mAb #5153 Krt (C11) Mouse mAb #4545
EGFR	EGFR (D38B1) XP® Rabbit mAb #4267 Her-2 (D8F12) XP® Rabbit mAb #4290 Her-3 (D22C5) XP® #12708 Krt (C11) Mouse mAb #4545

Panel	Targets
Immunotherapy	
Effector Cell/T_{reg} Ratio	CD8α (C8/144B) Mouse mAb #70306 FoxP3 (D2W8E™) Rabbit mAb #98377 Krt (C11) Mouse mAb #4545
Co-regulatory Ligand/T-cell Panel	PD-L1 (E1L3N®) XP® Rabbit mAb #13684 B7-H4 (D1M8I) XP® Rabbit mAb #14572 CD8 (C8/144B) Mouse mAb #70306 FoxP3 (D2W8E™) Rabbit mAb #98377 Krt (C11) Mouse mAb #4545
Her-2/Co-Regulatory Ligand Co-expression	PD-L1 (E1L3N®) XP® Rabbit mAb #13684 B7-H3 (D9M2L) XP® #14058 B7-H4 (D1M8I) XP® Rabbit mAb #14572 Her-2 (D8F12) XP® Rabbit mAb #4290 Krt (C11) Mouse mAb #4545
Anti-Mouse	PD-L1 (E1L3N®) XP® Rabbit mAb #13684 CD-8 (C8/144B) Mouse mAb #70306 Tim-3 (D5D5R™) XP® Rabbit mAb #45208 Arginase-1 (D4E3M™) XP® #93668 Krt (C11) Mouse mAb #4545
Exhausted T-cell	Tim-3 (D5D5R™) XP® Rabbit mAb #45208 PD-1 (D4W2J) XP® Rabbit mAb #86163 CD8 (C8/144B) Mouse mAb #70306 Lag-3 (D2G4O™) XP® Rabbit mAb #15372 CD68 (D4B9C) XP® #76437 Krt (C11) Mouse mAb #4545
PD-1:PD-L1 Ligand Axis	PD-L1 (E1L3N®) XP® Rabbit mAb #13684 PD-1 (D4W2J) XP® Rabbit mAb #86163 CD68 (D4B9C) XP® #76437 CD8 (C8/144B) Mouse mAb #70306 Tim-3 (D5D5R™) XP® Rabbit mAb #45208 Krt (C11) Mouse mAb #4545

Figure 1A.
A schematic of the Tyramide Signaling Amplification (TSA) protocol. FFPE tissue is deparaffinized and rehydrated. After antigen retrieval, tissue is stained with a 1° antibody followed by an HRP conjugated 2° antibody. The HRP enzyme catalyzes the reaction of tyramide (PerkinElmer) into a reactive form, which binds tyrosine residues on and near the target. The tyramide is conjugated to a fluorophore which can then be detected and imaged.
Products used:
SignalStain® Boost IHC Detection Reagent (HRP, Rabbit) #8114
SignalStain® Boost IHC Detection Reagent (HRP, Mouse) #8125
SignalStain® Antibody Diluent #8112
SignalStain® Mounting Medium #14177

Figure 1B.
A schematic illustrating the TSA protocol as applied to the serial staining process necessary for multiplexing. To multiplex, the tissue is then heated such that the weaker hydrogen bonds formed by the primary and secondary antibodies are broken, but the covalent bonds between the deposited tyramide and tyrosine residues remain. Multiplex staining is achieved in a serial fashion, with another staining cycle started once the first primary/secondary antibody pair has been removed.

Table 1A.
A list of the mIHC panels that have been constructed to date, grouped according to their research area. CST's highly specific and validated antibodies serve as the foundation for clarifying distinct signals.
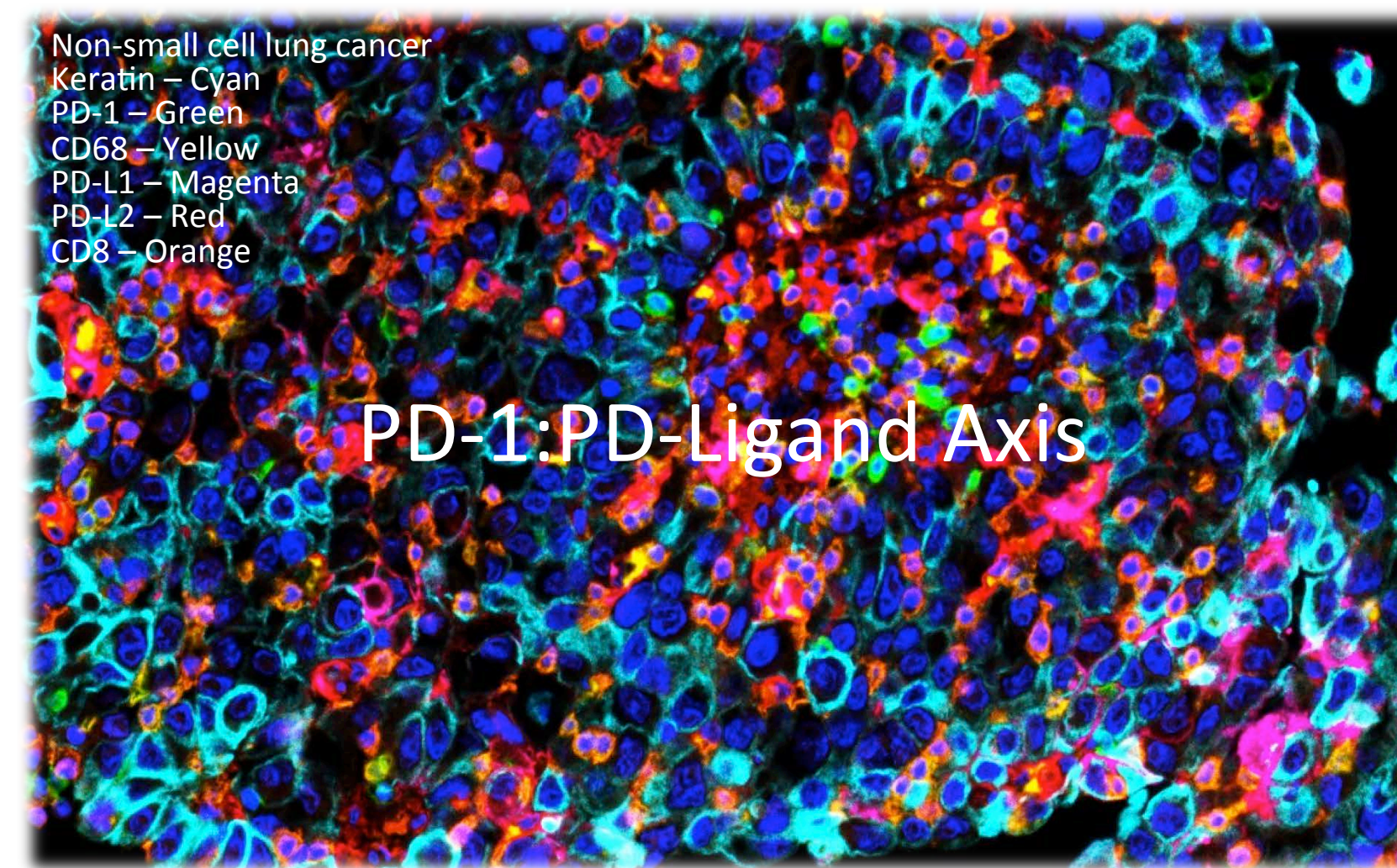
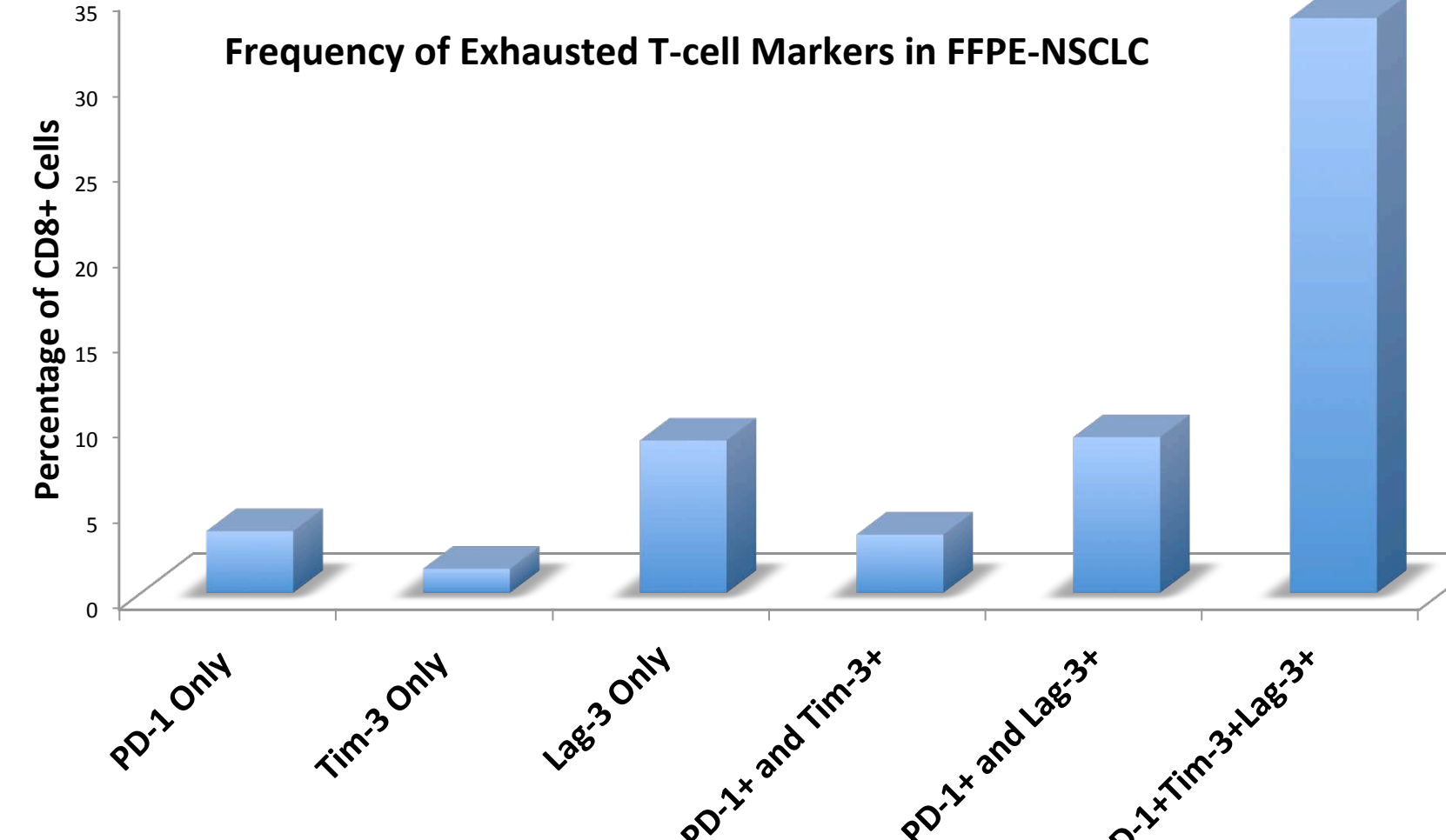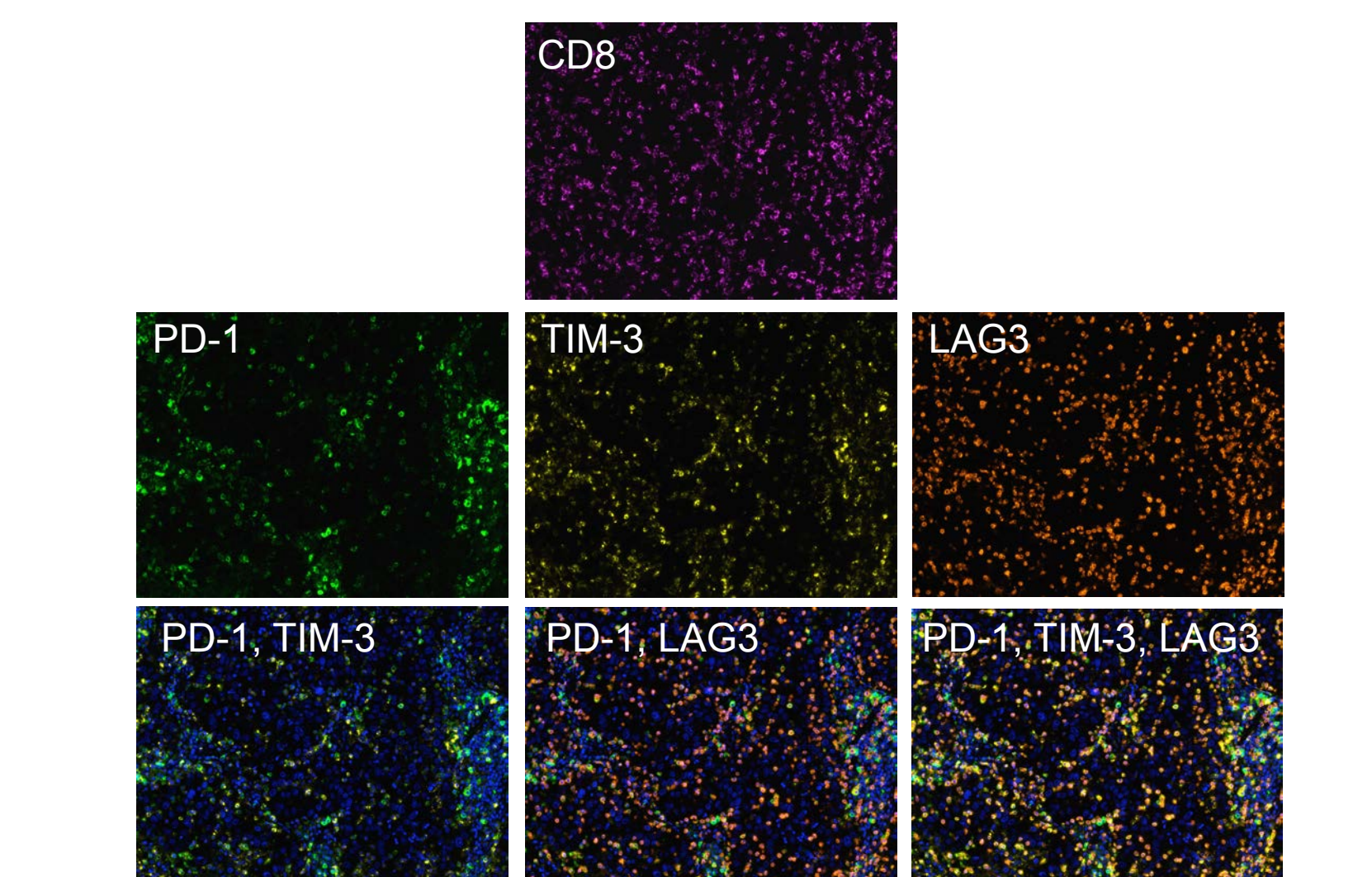**Spatial localization and frequency of exhausted T cells expressing immune checkpoint receptors**

Figure 2A

Figure 2B
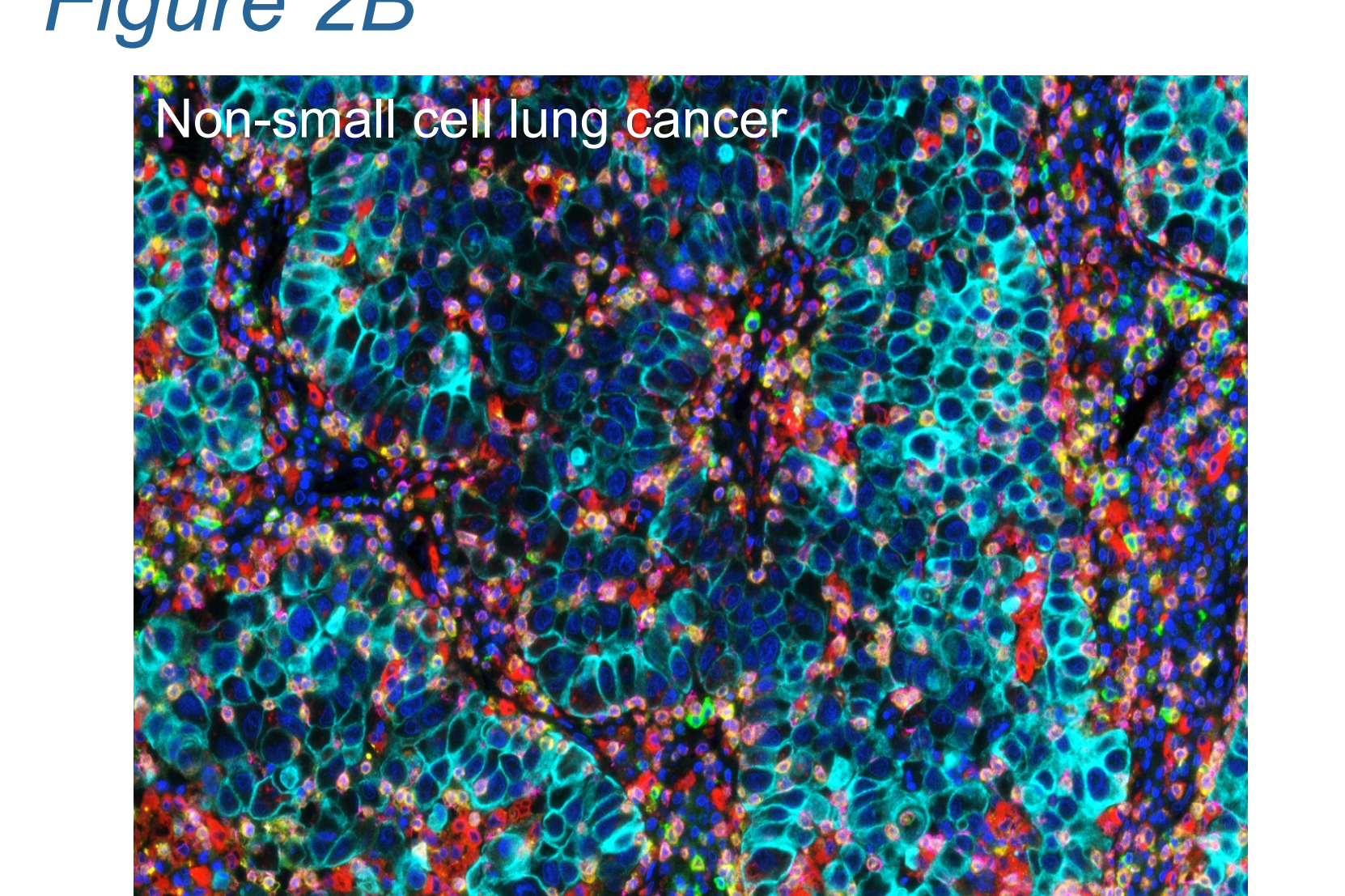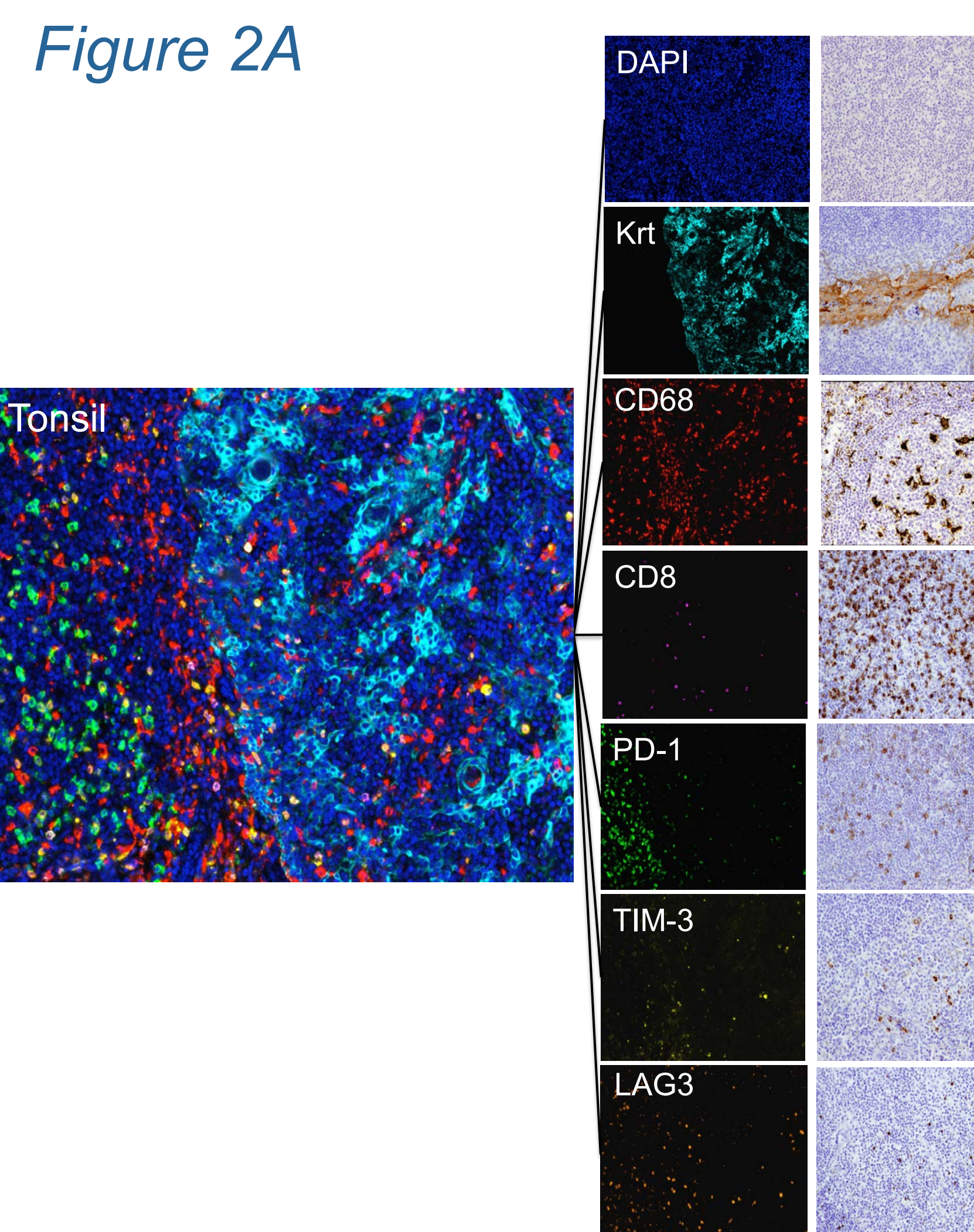

Frequency of Exhausted T-cell Markers in FFPE-NSCLC

Figure 2A.
Normal human tonsil was stained with an exhausted T cell phenotype mIHC panel demonstrating seven fluorescent signals can be clearly unmixed via spectral imaging. The fluorescent multiplex staining in each case mirrors the corresponding chromogenic staining performed on serial sections from the same tissue block.

Figure 2C.
A NSCLC section was stained with an exhausted T cell phenotype mIHC panel including PD-1, TIM-3 and LAG3. Co-expression of these markers was imaged and quantified.
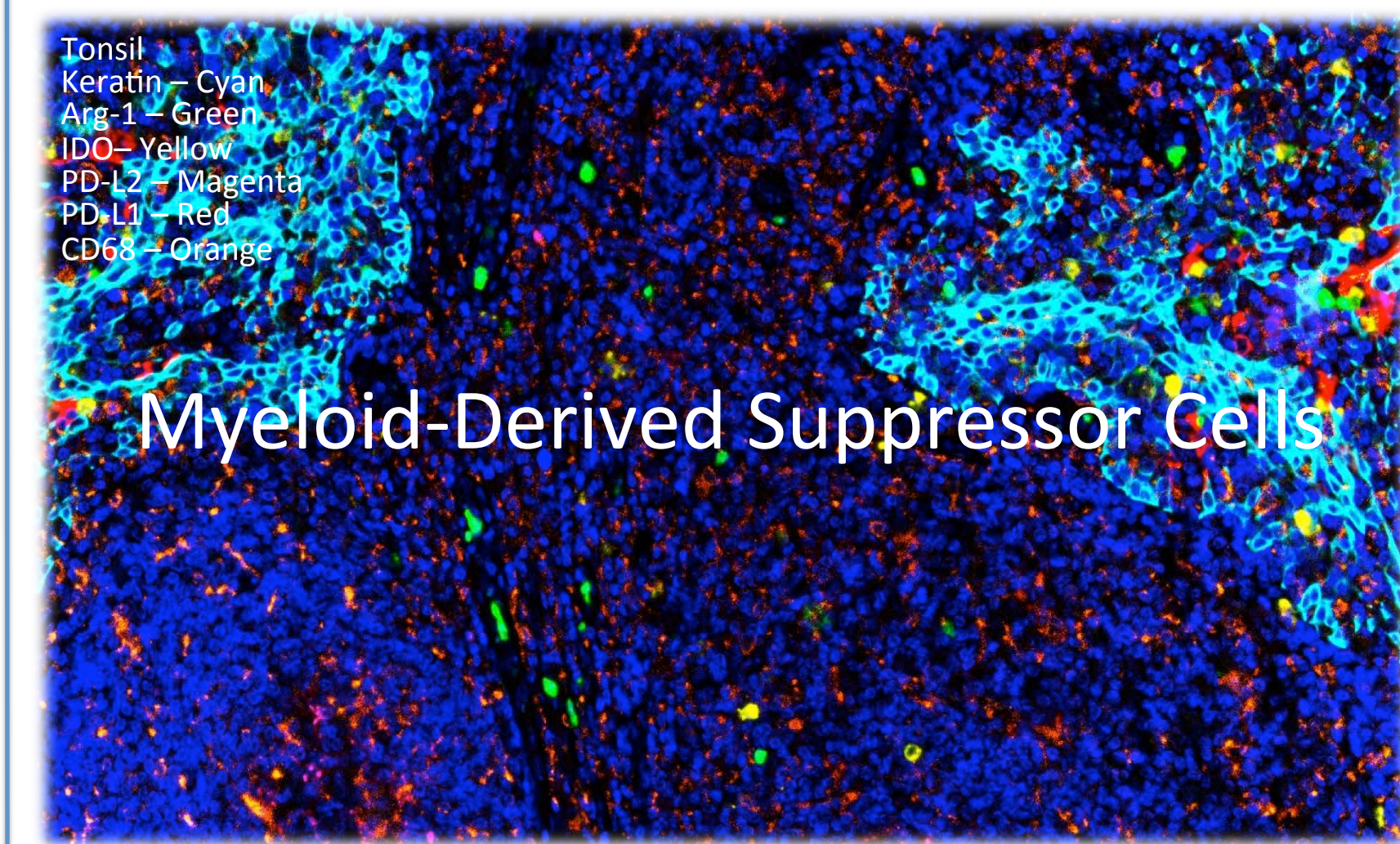
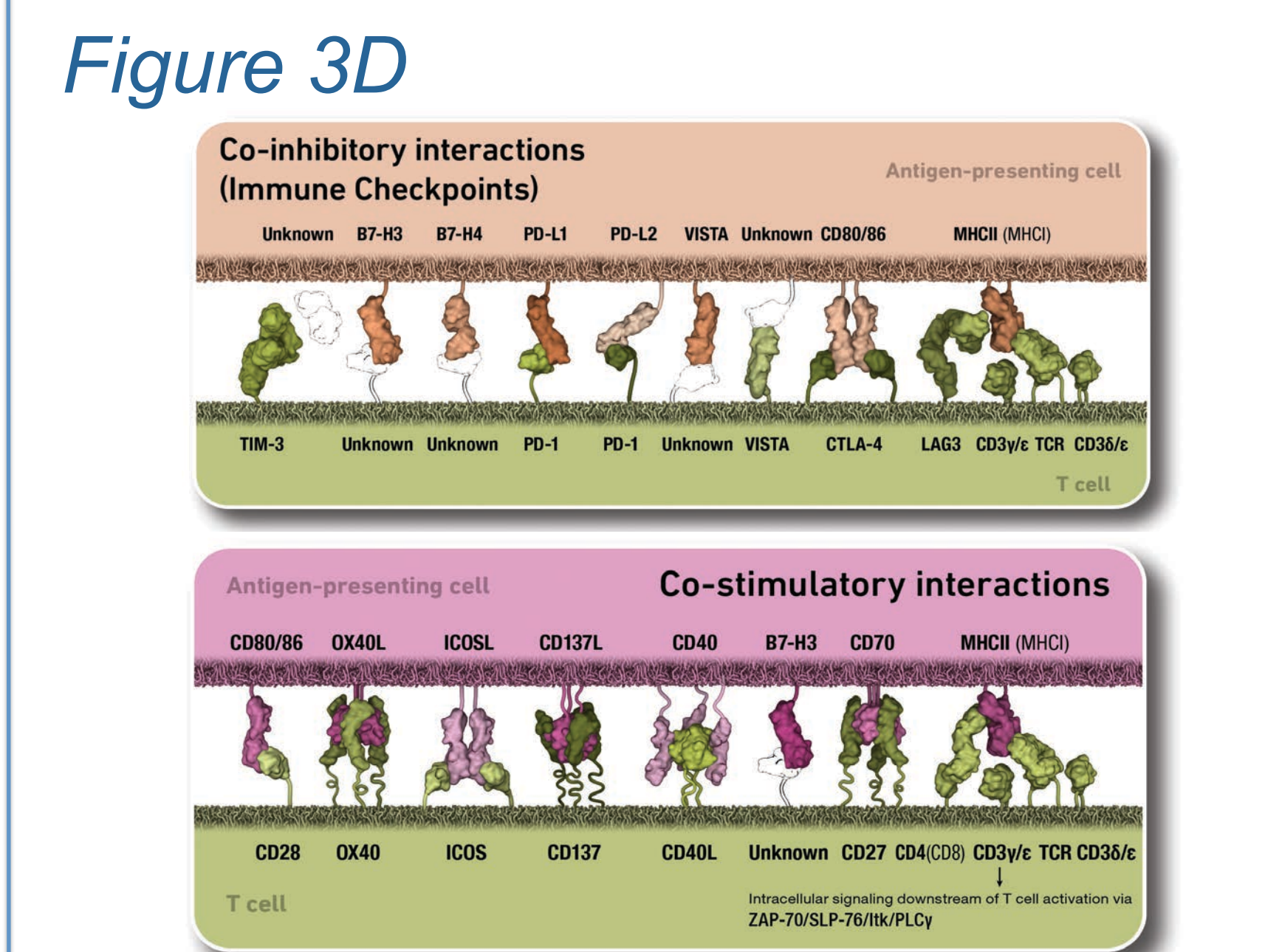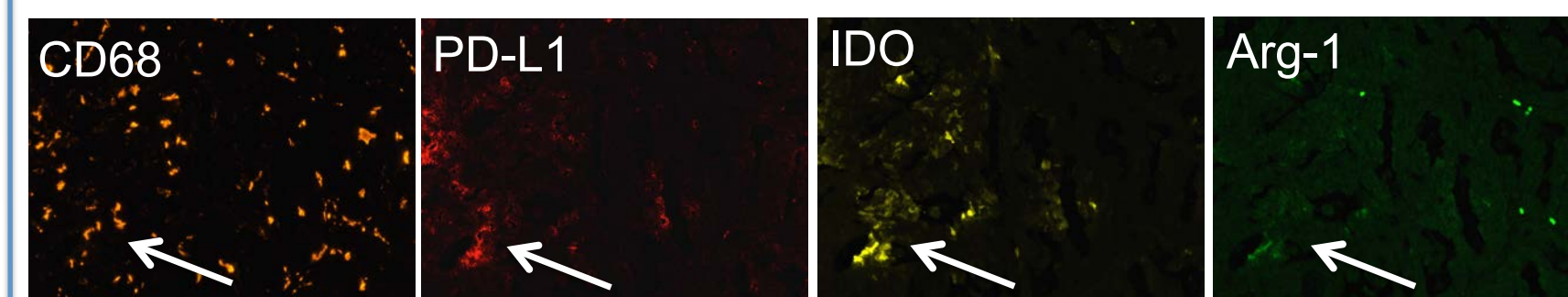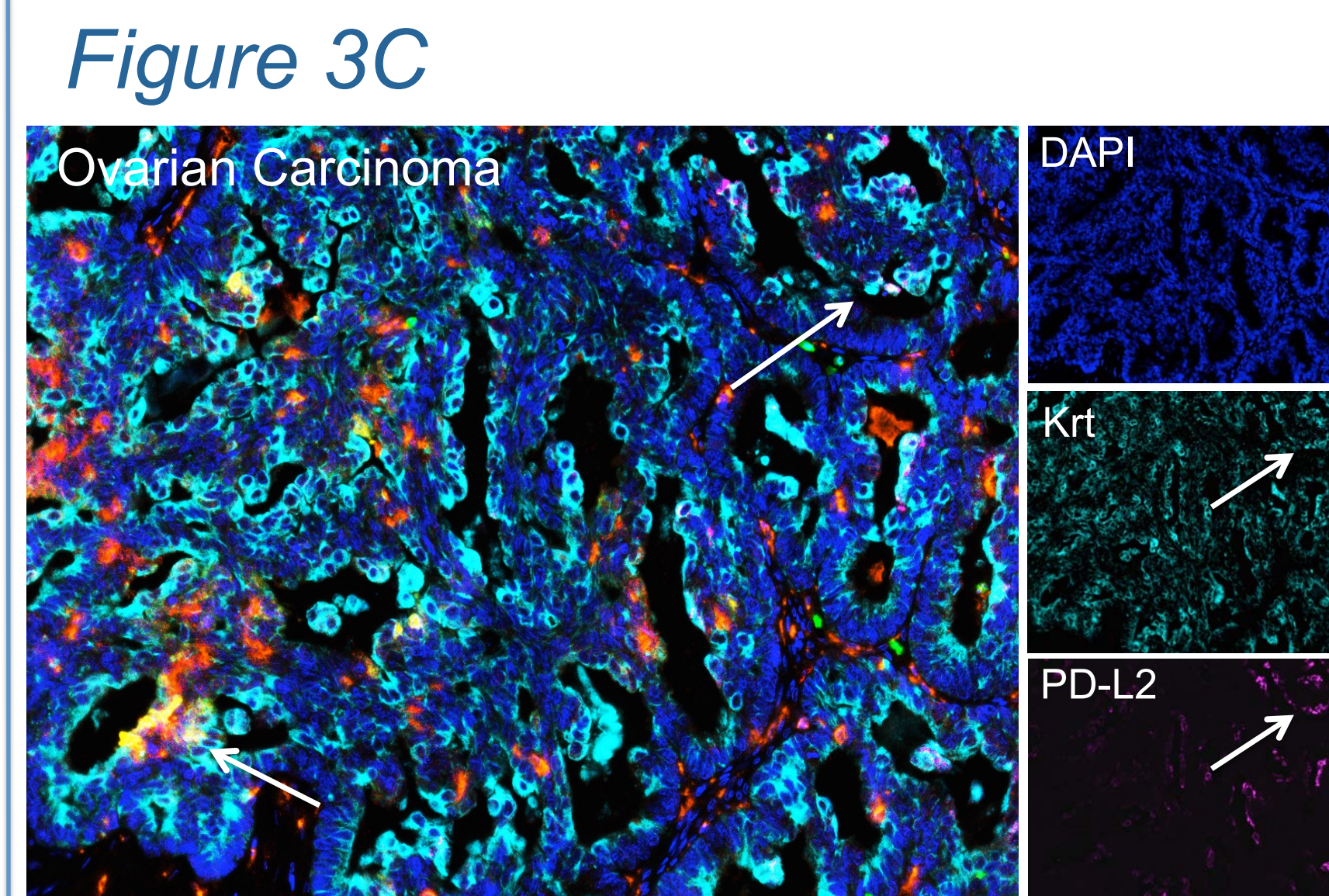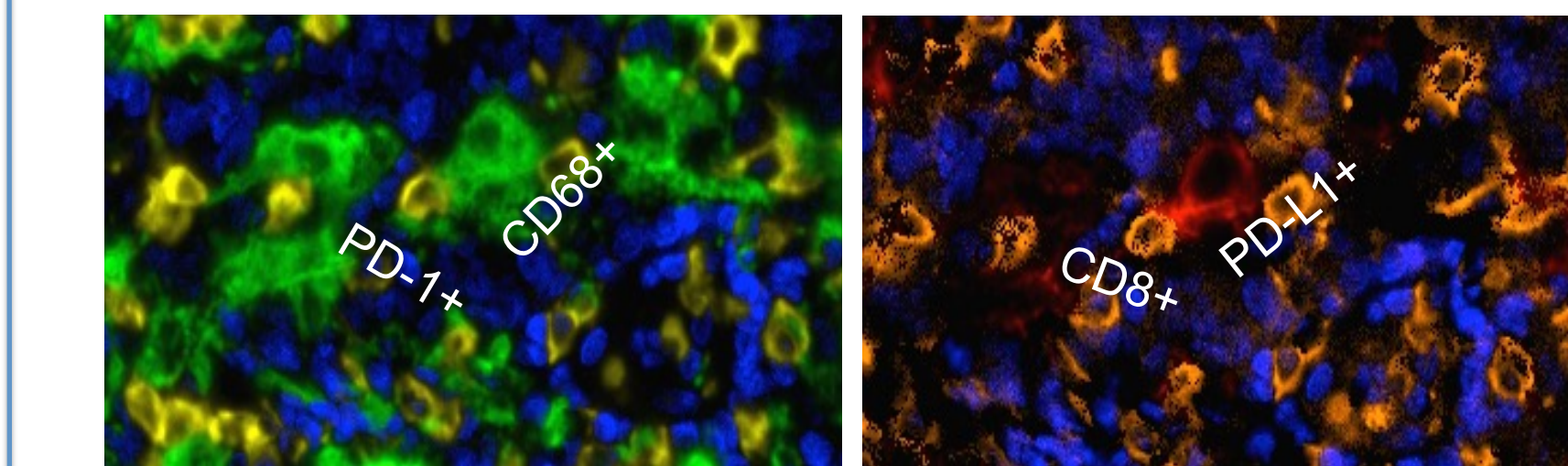**Proximity and co-localization of immunosuppressive cells within the tumor microenvironment**

Figure 3A

Figure 3B
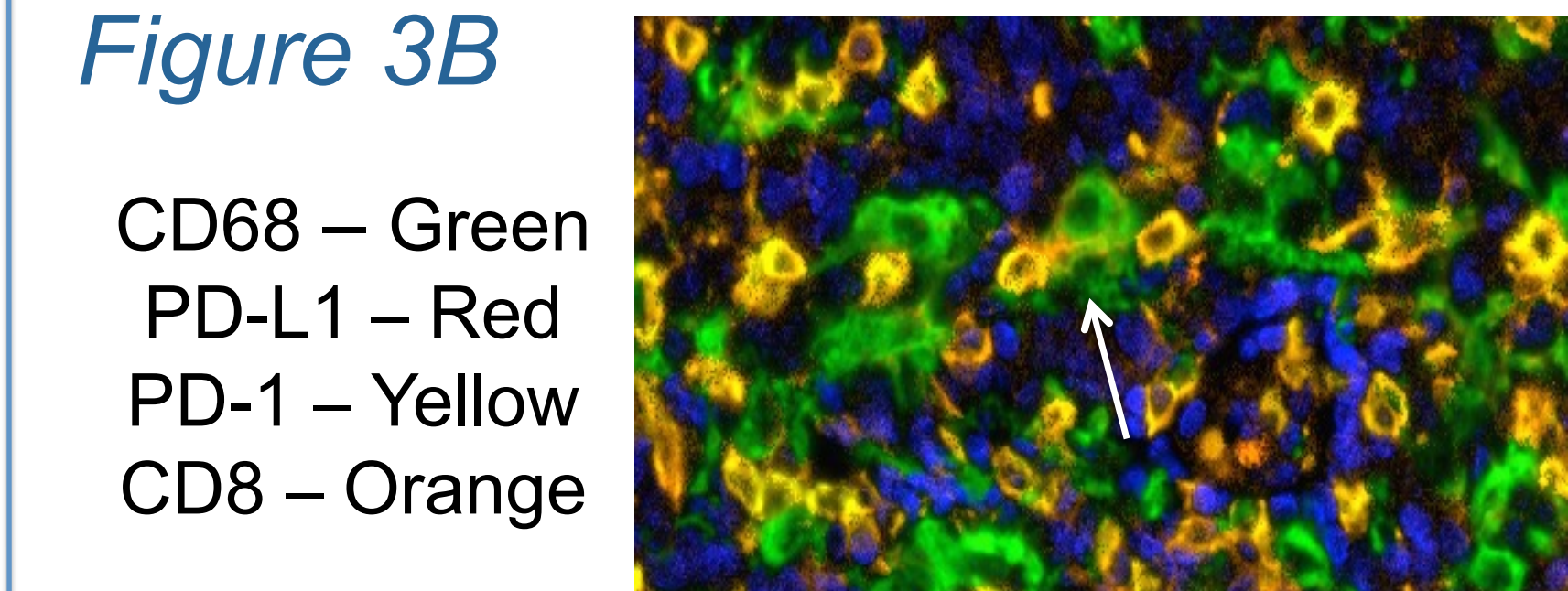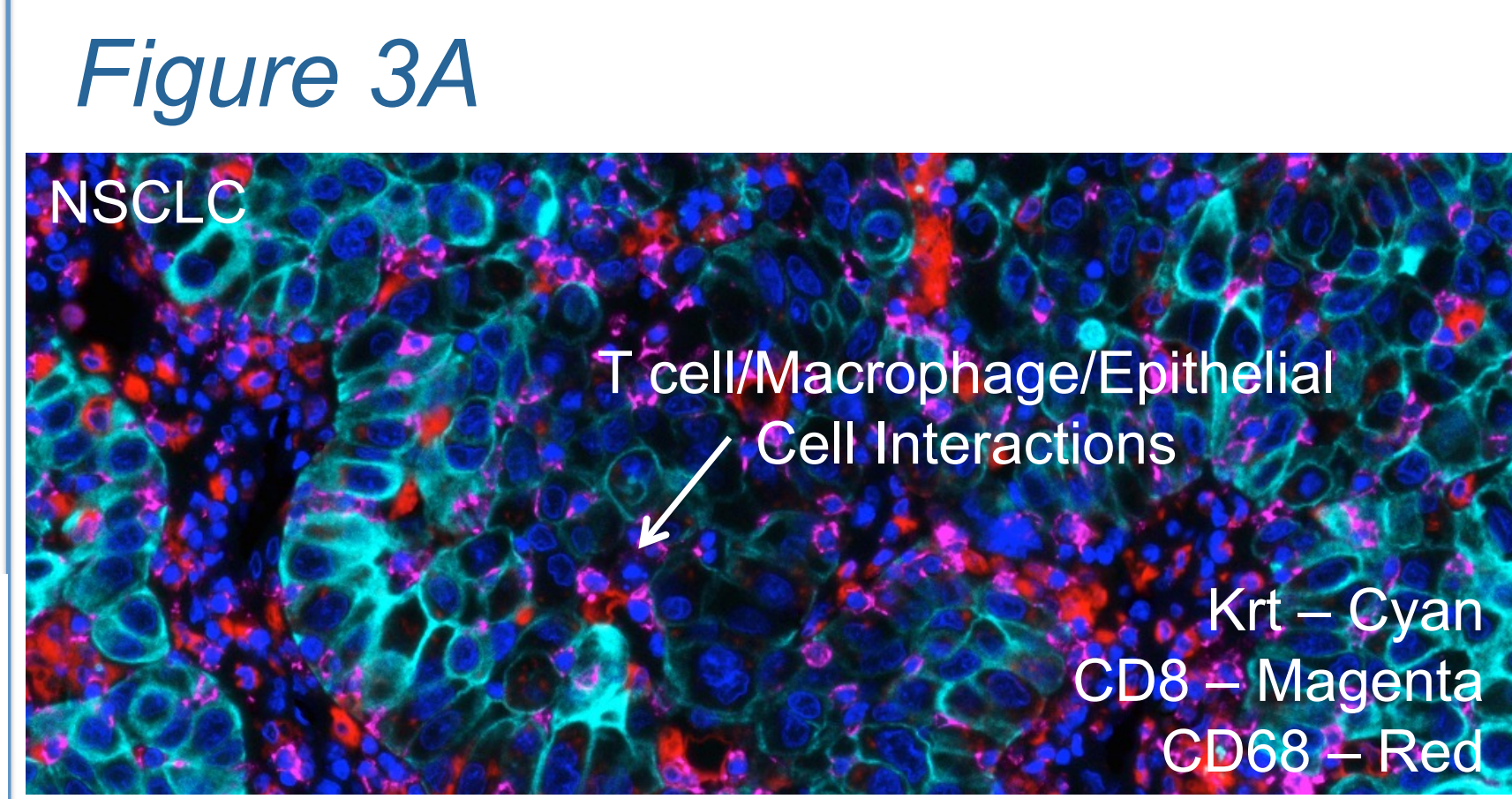

Figure 3C

Figure 3D

Figure 3A.
CD8+ T cells and CD68+ macrophages are shown in close proximity to tumor cells in a NSCLC section, suggesting possible interactions.

Figure 3B.
PD-L1+CD68+ macrophages interacts with a PD-1+CD8+ T cells in FFPE B cell Non-Hodgkin's lymphoma.

Figure 3C.
Ovarian carcinoma was stained with a MDSC-centric mIHC panel. Co-expression of PD-L1, IDO, and Arginase-1 was detected in CD68+ macrophages. PD-L2 co-localized with Krt+ tumor cells.

Figure 3D.
Future panels will continue to incorporate various co-inhibitory and co-stimulatory receptors that have been identified as being important in tumor immune evasion.

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