Cell Analysis

# Advancements in multiplexed spatial phenotyping

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**Poster #3798/21**


## Abstract

Spatial omics is an expanding research area focused on integrating spatial knowledge of tissue with transcriptomics (RNA) and proteomics (protein). Understanding the complexity of the biological structure(s) is an important biological process in cancer immunotherapy research which requires accurate target classification. Translational profiling of 4+ targets on a single sample at one time can be difficult in many ways, because of the complexity involved with panel design, staining protocols, and data analysis. To design a reliable multi-target biomarker panel, the impact of protein abundance and localization, fluorophore compatibility, varying tissue types, and data characterization need to be considered, to name a few. To address the needs of high target multiplex-ability, labeling methods have advanced in cyclic detection enabling iterative labeling and detection using automated fluidics, however these approaches suffer from low simultaneous target throughput. Thermo Fisher Scientific can now provide a streamlined process for tissue labeling; a process that enables sufficient labeling with high plex panels completed within a couple of hours. We show successful detection of a wide range of various protein markers across numerous tissue organs used in various biological research applications with our uniform technique of labeling.

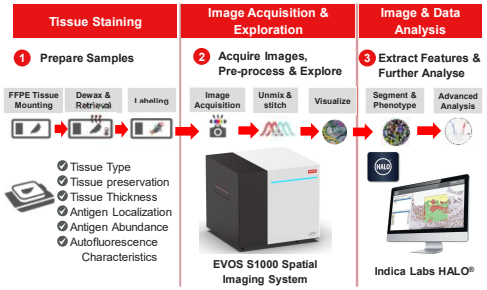

## Introduction

### Spatial Biology Provides Deep Insights

Ability to analyze single cells in spatial context provides a variety of information
- Cellular identity in time and space
- Cell types, cell states and cell functions
- Cell-cell interactions
- Cellular neighborhoods
- Tissue microenvironments and architecture

### Workflow

Immunology targets labeled with antibody-based detection, to characterize how cells interact across complex tissues imaged on a high-resolution spatial slide scanner.




### Cellular Phenotyping Characterization

Visualize and quantify cellular traits of innate and adaptive immune cell populations.

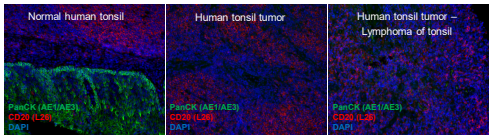



**Figure 1.** Different types of FFPE Human Tonsil labeled using our IHC validated primary antibody conjugates. (A) Normal Human Tonsil consist of distinct tissue structure with a clear separation between the epithelial layer and B cells. (B) Human tonsil tumor exhibits a loss of structure organization and the epithelial layer. (C) Lymphoma of human tonsil is characterized by a complete absence of normal tissue structure and a disarray of B cells.

## Multiplex Labeling for Spatial Biology

### Aluora Spatial Amplification Kits

Traditional signal amplification labeling technique for enhanced sensitivity. Combining the brightness of Invitrogen Aluora fluorescent dyes with polyHRP-mediated tyramide-like labeling permits high-fidelity multiplexing of a variety of validated antibody clones.

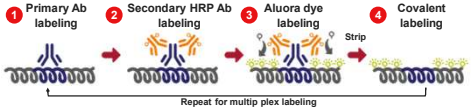



**Figure 2.** Multiplex Aluora spatial amliification labeling workflow uses horseradish peroxidase (HRP) to enzymatically deposit fluorophores on and surrounding protein epitope targeted primary antibody. Automated Stainer = ~12 hours; Manual staining = ~2-3 days.

## Spatial Biology

- Analyzing spatial relationships within organs and between cells on tissue to investigate organization of biomolecules, cellular structures, cellular communication, and how their arrangement influences biological function and behavior

### Factors to keep in mind when multiplexing

- Relative target abundance
- Antibody specificity
- Target localization
- Fluorescence output per dye
- Fluorophore photostability
- Spectral separation of dyes

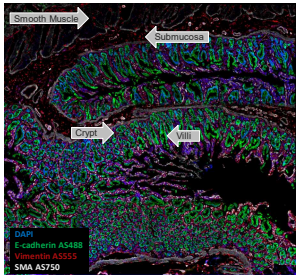



**Figure 3.** FFPE Human Small Intestine labeled with our new Spatial dyes and Invitrogen Aluora Spatial Amplification Kit reagents

### 9-plex Aluora spatial amplification staining with UNMIXING

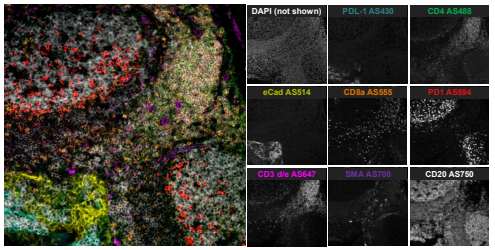



**Figure 4.** FFPE Human Tonsil labeled with our new Spatial dyes using our Invitrogen Aluora Spatial Amplification Kits. The 9-plex tissue label allows us to investigate the immune responses within the human tonsil and differentiate between T cells, B cells, and tissue structure.

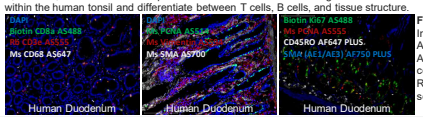
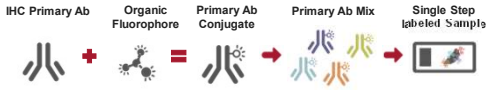


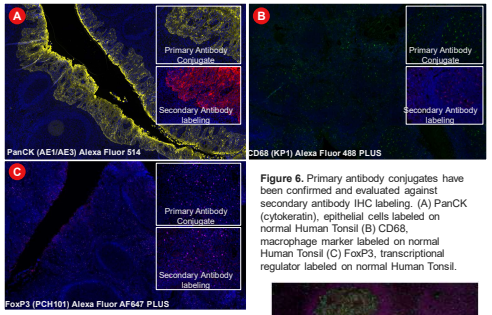

**Figure 5.** Invitrogen™ Aluora Spatial Amplificaiton Kits contain Mouse, Rabbit, and Biotin secondaries

## Antibodies and Fluorophores for Spatial Biology

### Primary Antibody Conjugate Mix

Convenient labeling method offered in a variety of fluorophores, including Alexa Fluor and Alexa Fluor PLUS dyes conjugated to highly validated IHC antibody clones for single-step labeling.

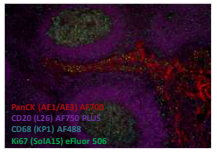



Primary antibody conjugates have been validated across various tissue types, including normal and certain cancerous human tissues: spleen, appendix, duodenum, tonsil, thymus, cerebellum, liver, colon etc…

**Right Figure 7.** Multiple primary antibody conjugates can be combined in a single mixture for multiplexing on a single sample. Human Tonsil

**Figure 6.** Primary antibody conjugates have been confirmed and evaluated against secondary antibody IHC labeling. (A) PanCK (cytokeratin), epithelial cells labeled on normal Human Tonsil (B) CD68, macrophage marker labeled on normal Human Tonsil (C) FoxP3, transcriptional regulator labeled on normal Human Tonsil.




### Multiplex staining with Primary antibody conjugates

Primary antibody conjugates yield comparable results to those achieved with Aluora Spatial Amplification labeling.

Primary antibody conjugates have the same staining pattern with same positive and negative regions on designated tissue when compared to established IHC unconjugated antibody of the same clone.

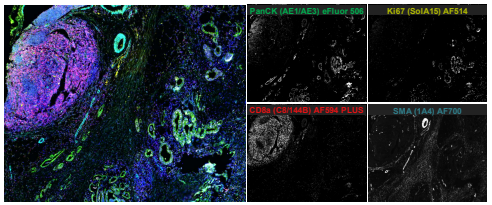



**Figure 8.** FFPE Human colon adenocarcinoma labeled using our Primary IHC validated antibody conjugates. The 5-plex (DAPI included) tissue label allows us to investigate the immune responses within the human colon and differentiate between T cells, tissue structure, and proliferative cells.

## Primary antibody conjugates can be used in combination with signal amplification labeling methods

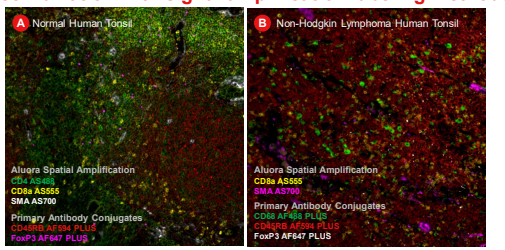


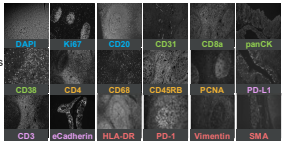

**Figure 9.** Both Aluora spatial amplification and primary antibody conjugates mix labeling methods were used to multiplex on a single tissue sample. The labeling process began with multiple rounds of Aluora dye labeling to achieve covalent fluorophore labeling on the sample, ensuring no cross-reactivity with upcoming primary antibody labeling; subsequently, the primary antibody labeling mix was added in a single step. The figure above illustrates the contrasting cellular phenotypes between two tissue types (A) Normal Human Tonsil (B) Non-Hodgkin lymphoma Human Tonsil.

| Consideration | Aluora Spatial Amplification | Primary Antibody Conjugate |
|---|---|---|
| 1. Labeling time for 8-plex | 12+ hours | Single step labeling ~1 hour |
| 2. Antigen expression level | Low/Medium Abundance | Medium/High Abundance |
| 3. Workflow Order | First | Last |
| 4. Amplification over autofluorescence | Yes | Wavelength dependent |
| 5. Stripping Compatibility | No (Covalent) | Yes (Can be stripped) |

**Table 1.** Strategies for optimal reagent selection

## Steps in determining fluorophore selection

1. Identify main target of interest
2. Determine antigen expression level
3. Save brightest fluorophores for dimmest markers
   - Use on the most important targets
   - Use on worst resolved targets
   - Low/unknown expression
   - Poor access to antigen
4. Minimize spillover using known expression patterns
   - Space out co-expressed markers
   - Mutually exclusive markers in adjacent channels
5. Plan for autofluorescence
6. Avoid using dim or low expressing targets in channels with wide spectrums
   - The ability to resolve populations is a function of autofluorescence, background, and co-expressing markers.




## EVOS S1000 Spatial Imaging System

Designed to capture high-resolution images, utilizing advanced fluorescence microscopy technology for spectral imaging. Capturing images across a wide variety of channels – enabling spectral unmixing for high multiplex sample analysis.

- Multiplex spectral fluorescence, transmitted brightfield, phase-contrast, and color brightfield
- Fast and Robust Imaging (<1 hour/cm²)
- 9-plex spectral unmixing (8 targets plus DAPI)
- Image up to 4 tissue slides in one sitting
- Robust and fast laser based autofocus
- Precise visualization of cellular structures
- Highly intuitive graphical user interface

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