Technical Innovations in Immunology

Expanded tools for spatial biology applications

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Abstract

Fluorescence labelling is a highly versatile approach to immunohistochemistry (IHC), offering the ability to detect numerous targets simultaneously with excellent sensitivity and specificity. Currently, the fluorescent labelling method utilized for IHC is the use of secondary antibodies. This multi-step process is restricted by targets that share the same species isotype, limiting the number of targets possible in one sample. Another method used for multiplex staining is cyclic labeling, the process of staining and stripping a tissue sample with different antibodies to detect multiple targets. This process is time-consuming and increases the risk of tissue damage or loss of antigenicity. Our new reagents will enable researchers to stain in fewer steps with preserved antigenicity, ensuring accurate and reliable detection of target antigens in tissue samples. One of the key benefits of these products is the ability to achieve higher-plex labeling, allowing researchers to multiplex with 8-10 biological targets on a single sample in one staining mix. This reduces the need for cyclic labeling, streamlines the workflow, and reduces overall time required for analysis. We provide a process that enables successful detection of multiple protein markers across diverse tissue organs while simultaneously detecting transcriptomic targets, providing comprehensive insights into complex biological processes and disease mechanisms. Intended for research use only.

Introduction and methods

- Spatial biology in IHC allows researchers to understand the spatial organization, function, and disease-related changes within tissues.
- Multiplex IHC techniques enable the simultaneous analysis of multiple protein expression patterns in a single experiment.
- Innovative IHC primary antibody conjugates offer a convenient solution for multiplex staining, saving time and resources.
- Invitrogen™ Aluora™ Spatial Amplification Kits enhance the multiplexing capabilities by providing highly sensitive detection of low-abundant targets, enabling the detection of multiple targets with exceptional sensitivity and specificity.

A) Primary Antibody Conjugates

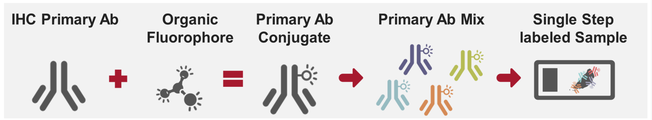


Figure 1A. Primary antibodies are conjugated to fluorophores then added into a primary antibody multiplex mix. These are added to tissue and incubated for 1hr.

B) Aluora Spatial Amplification Labeling

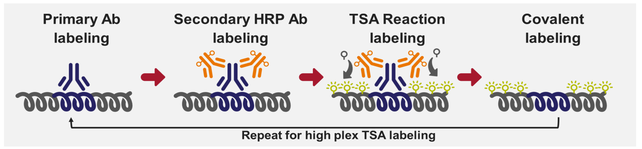
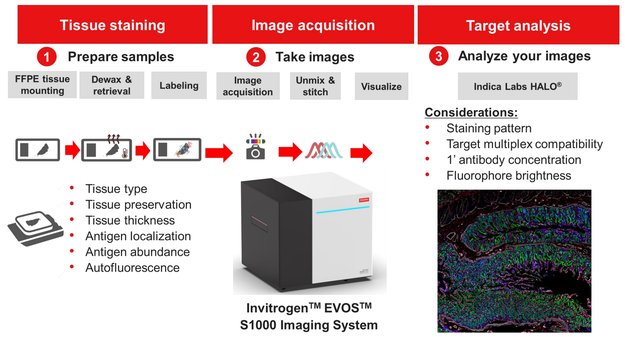


Figure 1B. Multiplex Aluora spatial amlilfication labeling workflow uses horseradish peroxidase (HRP) to enzymatically deposit fluorophores on and surrounding protein epitope targeted primary antibody. Automated Stainer = ~12 hours; Manual staining = ~2-3 days.

Workflow



Fluorophores for spatial biology

Our primary antibody conjugates and Aluora Spatial Amplification Kits allow for sensitive and specific antibody multiplexing using an array of fluorophores that can be spectrally unmixed using a variety of imaging platforms. Below are the spectra of the dyes we are providing with our new conjugates.

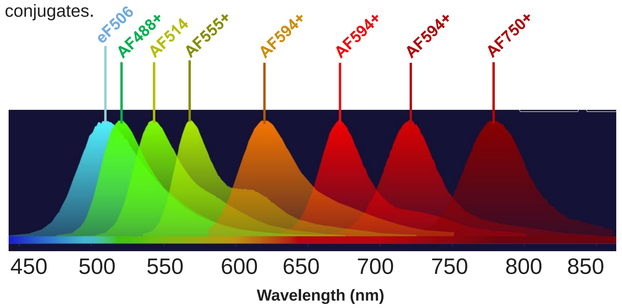


Figure 2. Emission spectra of primary antibody conjugates for spectral imaging, offered in 7 varieties of Invitrogen™ Alexa Fluor™ and Alexa Fluor™ Plus dyes, as well as Invitrogen™ eBioscience™ eFluor™ 506 dyes. Conjugates of different colors can be multiplexed together and easily unmixed on the EVOS S1000 and other spectral imaging systems.

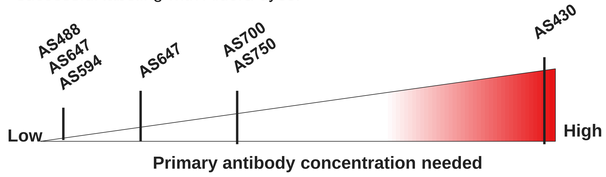
Considerations in IHC experimental design

Primary antibody conjugates

Optimization step	Impact
Heat-Induced Epitope Retrieval (HIER)	Modifying incubation duration, adjusting buffer pH, and selecting specific heating equipment can influence antigen accessibility.
Primary antibody conjugate concentration	Too much or too little antibody can affect labeling. Some fluorophores, such as eF506, may require higher antibody concentration.
Initial testing	Testing at least 3 different concentrations and starting with HIER of pH=9 for 10min can yield higher success rates.
Adjusting antibody concentration and HIER if necessary	Fine-tuning antibody concentration and HIER parameters may be required for optimal results.

Aluora spatial amplification staining

- **All optimization should be done at the primary antibody concentration step** – this is the easiest to adjust and the rest of the workflow is standardized from here.
- Fluorophore has a big impact on primary antibody concentration needed for successful labeling with Aluora dyes.



Aluora spatial amplificaiton vs primary antibody conjugate labeling

Consideration	Aluora spatial amplification	Primary antibody conjugate
Labeling time for 8-plex	12+ hours	Single step labeling ~1 hour
Antigen expression level	Low abundance	Medium/High abundance
Workflow order	First	Last
Amplification over autofluorescence	Yes	Wavelength dependent
Stripping compatibility	No (covalent)	Yes (can be stripped)
Antibody concentration required for labeling	~0.01-0.5 µg/mL	~1-20 µg/mL

Aluora spatial amplification

Signal amplificaiton is a valuable tool in IHC multiplexing, amplifying even the lowest abundance targets for detection with exceptional sensitivity and spatial resolution. Signal amplification can be used with a variety of fluorophores and tissues to multiplex in IHC.

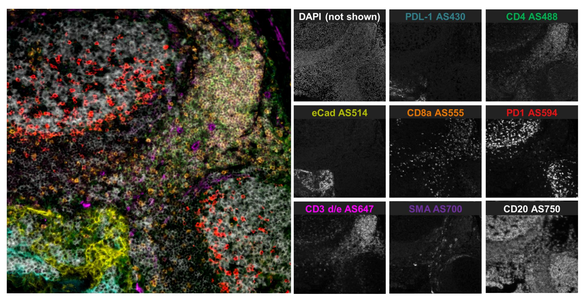


Figure 3. FFPE human tonsil labeled with our new spatial dyes using Aluora Spatial Amplification Kits. The 9-plex tissue label allows investigation of the immune responses within the human tonsil and differentiate between T cells, B cells, and tissue structure.

Primary antibody conjugates

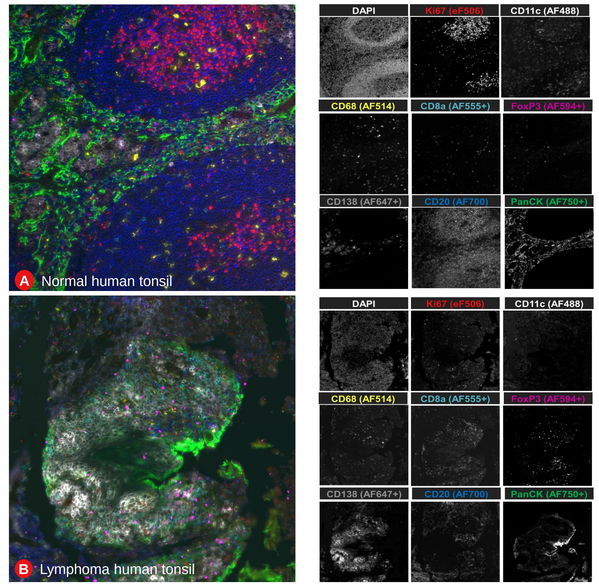


Figure 4. FFPE normal human tonsil (A) and tonsil lymphoma (B) samples have been labeled with primary antibody conjugates. With this advanced 9-plex technology, the enhanced expression of CD138 (tumor marker in myelomas, lymphomas), FoxP3 (immunosuppressive T-regulatory cells), and other biologically relevant markers in the lymphoma tissue can be visualized, allowing mapping of the tumor microenvironment.

Labeling with Aluora dyes + primary antibodies

IHC products that offer a unique advantage by allowing researchers to combine the use of primary antibody conjugates and Aluora dyes to identify their specific targets of interest. This powerful capability empowers researchers to successfully multiplex, regardless of the epitope abundance.

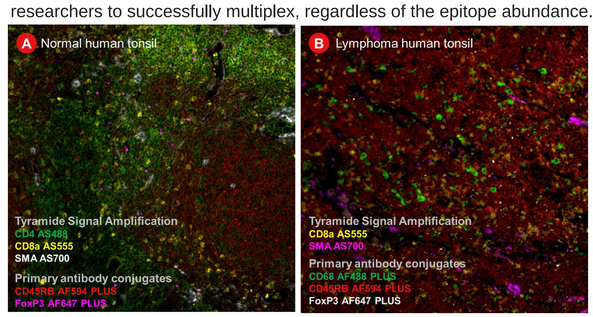


Figure 5. Both Aluora spatial amplification and primary antibody conjugate labeling methods were used to multiplex on a single tissue sample. The labeling process began with multiple rounds of Aluora spatial amplification labeling to achieve covalent fluorophore labeling on the sample, ensuring no cross-reactivity with upcoming primary antibody labeling; su+bsequently, the primary antibody labeling mix was added in a single step. The figure above illustrates the contrasting cellular phenotypes between two tissue types.

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