APPLICATION NOTE

Anti-FluorTag Antibodies Enable Immunohistochemistry with Flow Cytometry Antibodies

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INTRODUCTION

The KPL anti-FluorTag antibodies are a set of goat-derived polyclonal antibodies that can be used to detect Fluorescein (FITC)-, Phycoerythrin (PE)-, Allophycocyanin (APC)- and Peridinin Chlorophyll (PerCP)-labeled antibodies. They are available unconjugated, as Horseradish Peroxidase (HRP) conjugates and as High Potency Alkaline Phosphatase (ReserveAP™) conjugates. FITC-, PE-, APC- and PerCP-labeled antibodies are used in flow cytometry. These fluorescent tags are chosen due to their brightness and compatibility with lasers commonly found on flow cytometers. While flow cytometry is very effective at determining what cells express a given protein at a given level, it provides no information about where those cells are located in relation to one another. For this, microscopy-based immunohistochemistry (IHC) techniques are frequently required. Ideally, this should not require the purchase and optimization of an entirely new set of primary antibodies.

THE CHALLENGES TRANSITIONING DIRECTLY FROM FLOW CYTOMETRY TO IHC

With the appropriate filter sets and cameras, it is possible to detect FITC-, PE-, APC- and PerCP-labeled antibodies using a fluorescence microscope. However, the standard DAPI/FITC/Texas Red filter sets are generally not ideal for the visualization of PE, APC and PerCP. Also, these tags are very susceptible to photobleaching, making it difficult to obtain good images. This can be mitigated by purchasing new primary antibodies with fluorescent tags that are suitable for fluorescence microscopy. However, new fluorescently-labeled primary antibodies require a new round of optimization. Background fluorescence can be an issue – especially, when flow cytometry data indicates that something is expressed at a low level. Additionally, the detection of some tandem dyes requires a camera that detects near infrared light.

In contrast, IHC is not susceptible to photobleaching and background fluorescence and requires no special filter sets. Enzyme-labeled species- and isotype-specific secondary antibodies can sometimes be used in order to detect primary antibodies. However, most monoclonal antibodies used in flow cytometry for the study of human and rat cells are of the mouse IgG1κisotype, making it very difficult to use and detect two different primary antibodies simultaneously.

Figure 1 compares the sensitivity of fluorescence microscopy to immunohistochemistry. Sections of a mouse spleen were first stained for dendritic cells using different concentrations of a FITC-labeled anti-CD11c antibody and then imaged with a fluorescence microscope. Afterwards, HRP-labeled anti-FITC and TrueBlue™ substrate were used to detect the FITC-labeled antibodies. At 93 ng/ml, background signals from autofluorescence began to exceed the specific signal and at 20 ng/ml, very little FITC fluorescence was visible above background. However, the dendritic cells could still be detected with the anti-FITC antibody in conjunction with TrueBlue peroxidase stain.

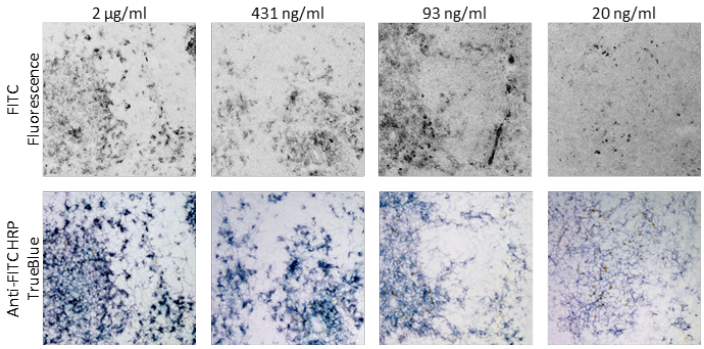

Figure 1. – Fluorescence Microscopy vs. Immunohistochemistry
Frozen sections of a mouse spleen were fixed in ice-cold acetone and dried. Endogenous peroxidase was inactivated with 0.1% sodium azide in water for 5 minutes followed by 0.1% sodium azide with 0.3% hydrogen peroxide in water for 10 minutes. Slides were blocked for 15 minutes with 1% Normal Mouse Serum and 1% Normal Goat Serum in TBST buffer (blocking solution) and then stained for 30 minutes with 2 µg/ml, 431 ng/ml, 93 ng/ml or 20 ng/ml of FITC-labeled anti-CD11c (clone HL3, BD Biosciences) in blocking solution. Slides were washed in TBST buffer and imaged with a fluorescence microscope under TBST. 4 second exposures were used for the highest two concentrations and 8 second exposures were used for the lower two and the images were converted to black and white and inverted (top row).

Afterwards, the sections were incubated for 30 minutes with 0.5 µg/ml of HRP-labeled anti-FITC in blocking solution where TBST buffer was replaced by TBS buffer, washed, incubated with TrueBlue substrate for 5 to 20 minutes, washed, dried and mounted. Brightfield images were taken of the regions that had been imaged for FITC fluorescence (bottom row).

ANTI-FLUORTAG ANTIBODIES ENABLE REUSE OF FLOW PRIMARIES

By detecting the tags commonly found on antibodies in flow cytometry, the anti-FluorTag antibodies enable the reuse of

many primary antibodies from flow cytometry in immunohistochemistry – provided that the epitopes are preserved in sections. This is frequently the case with frozen sections that have been fixed in acetone. The HRP and AP conjugates of the anti-FluorTag antibodies enable dual color staining and are readily paired with various chromogenic stains to produce permanent IHC results (Figure 2; see the other Application Note Appendix for a protocol for dual color staining).

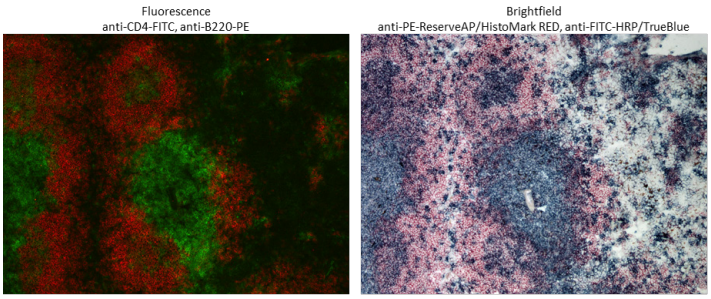

Figure 2. Dual Color Stain with HRP and AP Conjugates
A frozen section of a mouse spleen was stained with two primary antibodies of the rat IgG2a isotype: FITC-labeled anti-CD4 to detect CD4+ T cells (clone RM4-5; BD Biosciences) and PE-labeled anti-CD45R/B220 to detect B cells (clone RA3 6B2; BD Biosciences) and imaged with a fluorescence microscope. Afterwards, a mix of HRP-labeled anti-FITC and ReserveAP-labeled anti-PE was applied in order to bind the fluorescent tags. The AP substrate HistoMark RED was used first followed by the HRP substrate TrueBlue. After mounting, the section was imaged under brightfield.

THE BENEFIT OF POLYCLONAL ANTIBODIES

All anti-FluorTag antibodies are polyclonal, which provides three major benefits. First, they are likely to detect fluorescent proteins from many suppliers. PE, APC and PerCP are proteins that are each found in what may be hundreds to thousands of different species within a phylum. As can be expected, these proteins are not 100% conserved between species (see Figure 3). For flow cytometry, manufacturers of such proteins may choose to isolate them from proprietary species that grow well and express particularly bright and stable variants and at a high concentration. Because the binding of a monoclonal antibody is dependent on the conservation of a particular epitope, the ability of a monoclonal antibody to detect a particular current or future variant of PE, APC or PerCP is not a given. This is mitigated by using polyclonal antibodies that recognize many different epitopes.

Second, polyclonal antibodies are likely to detect tandem dyes and otherwise modified proteins. For example, APC is commonly treated with N-Ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC) in order to stabilize its subunits, which is also likely to change surface carboxyl groups. The activation of proteins for their attachment to antibodies (e.g., via SMCC or glutaraldehyde) can also lead to the loss of surface epitopes. This is again mitigated by using polyclonal antibodies that recognize many different epitopes.

Third, multiple different polyclonal antibodies can potentially bind to a given molecule at the same time while monoclonal antibodies are limited to the amount of repeating epitopes and subunits. Thus, signals with polyclonal antibodies can be higher than with monoclonal antibodies.

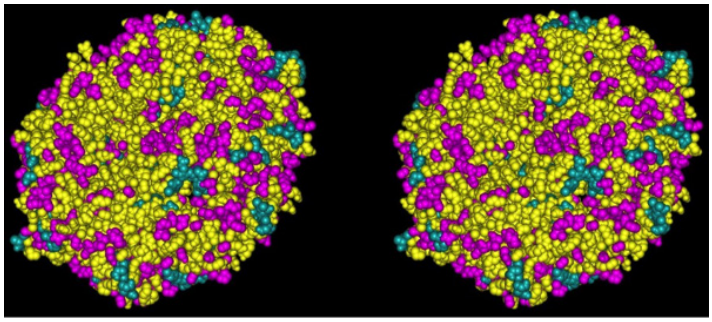

Figure 3: Conserved Phycoerythrin Structure
A stereo image of R-PE based on the 1B8D crystal structure[1] was generated. Chromophores are shown in blue-green. Amino acids that are conserved in R-PE and B-PE from 10 different species are shown in yellow. Amino acids that are not conserved are shown in magenta. The image was generated with Cn3D[2].

SPECIFICITY

A potential problem with polyclonal antibodies is a lack of specificity. Anti-FluorTag antibodies are affinity purified in order to ensure that they bind to a specific fluorescent tag and exhibit minimal crossreactivity. Figure 4 shows the amount of signal with each antibody that is obtained in a representative ELISA experiment.

Overall, the anti-FluorTag antibodies detect their tags with high sensitivity and specificity.

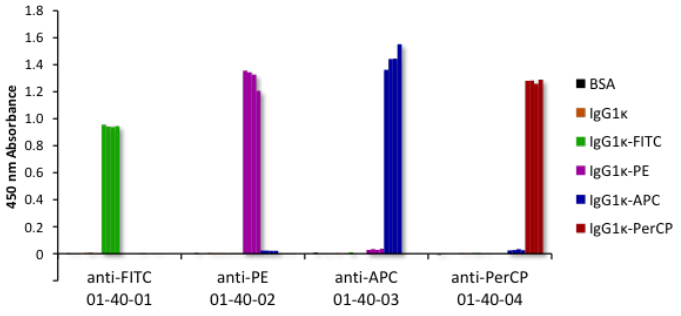

Figure 4 – Specificity of Anti-FluorTag Antibodies
Wells of an ELISA plate were first coated using either unlabeled or FITC-, PE-, APC- or PerCP-labeled mouse IgG1κ(1 µg/mL) or BSA (control). Wells were blocked with 1% BSA. A given unlabeled anti-FluorTag antibody was added at 0.1 µg/mL in 1% BSA and was tested in 4 wells each against all six coating conditions. Bound antibody was detected with biotinylated monoclonal mouse

anti-goat IgG, which was detected with streptavidin-HRP. Plates were developed with SureBlue™ TMB and stopped with TMB Stop solution. Absorbance was measured at 450 nm and the average signal from all 16 control wells was subtracted from each value.

CONCLUSIONS

IHC is a valuable tool for further understanding the location of targets studied in flow cytometry. The transition from flow cytometry to IHC can be difficult due to the fact that the fluorescent tags FITC, PE, APC, and PerCP commonly used in flow cytometry are prone to photobleaching and the standard fluorescent microscope filter sets are not ideal for these tags. Researchers must either attempt to manage these problems or purchase new primary antibodies with tags suitable for IHC – requiring time and money.

As an alternative, the line of KPL anti-FluorTag antibodies makes this transition easier by allowing for the reuse of many primary antibodies conjugates from flow cytometry in IHC. The anti-FluorTag antibodies are highly specific to FITC, PE, APC, and PerCP, yet recognize multiple epitopes. They are ideal for detecting fluorescent proteins from multiple suppliers and tandem dyes. The HRP and AP conjugates of the anti-FluorTag antibodies enable highly sensitive IHC dual labeling results.

PRODUCTS

Antibodies – Unlabeled

01-40-01	Goat anti-FITC, Affinity Purified Antibody, 1.0 mg
01-40-02	Goat anti-PE, Affinity Purified Antibody, 1.0 mg
01-40-03	Goat anti-APC, Affinity Purified Antibody, 1.0 mg
01-40-04	Goat anti-PerCP, Affinity Purified Antibody, 1.0 mg

Antibodies – HRP-Labeled

04-40-01	Anti-FITC, HRP-Labeled Antibody, 0.1 mg
04-40-02	Anti-PE, HRP-Labeled Antibody, 0.1 mg
04-40-03	Anti-APC, HRP-Labeled Antibody, 0.1 mg
04-40-04	Anti-PerCP, HRP-Labeled Antibody, 0.1 mg

Antibodies – ReserveAP™-Labeled

051-40-01	Anti-FITC, ReserveAP-Labeled Antibody, 0.1 mg
051-40-02	Anti-PE, ReserveAP -Labeled Antibody, 0.1 mg
051-40-03	Anti-APC, ReserveAP -Labeled Antibody, 0.1 mg
051-40-04	Anti-PerCP, ReserveAP -Labeled Antibody, 0.1 mg

Substrates

71-00-64	TrueBlue Substrate, 50 ml
54-75-00	HistoMark® BLACK, 1000 slides
55-69-00	HistoMark RED, 1000 slides
54-11-00	StableDAB Peroxidase Substrate, 500 slides
54-10-00	DAB Reagent Set, 500 slides

Related Reagents

71-00-27	10% Normal Goat Serum, 50 ml
71-18-01	10% Normal Mouse Serum, 10 ml
71-00-28	10% Normal Rabbit Serum, 50 ml
51-18-02	10X Tris-buffered Saline with 0.5% Tween 20, 1 liter

1. The 1B8D structure was published as: Ritter S, Hiller RG, Wrench PM, Welte W, Diederichs K. Crystal structure of a phycourobilin-containing phycoerythrin at 1.90-A resolution. J Struct Biol. 1999 Jun 15;126(2):86-97.

2. Wang Y, Geer LY, Chappey C, Kans JA, Bryant SH. Cn3D: sequence and structure views for Entrez. Trends Biochem Sci. 2000 Jun;25(6):300-2.

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