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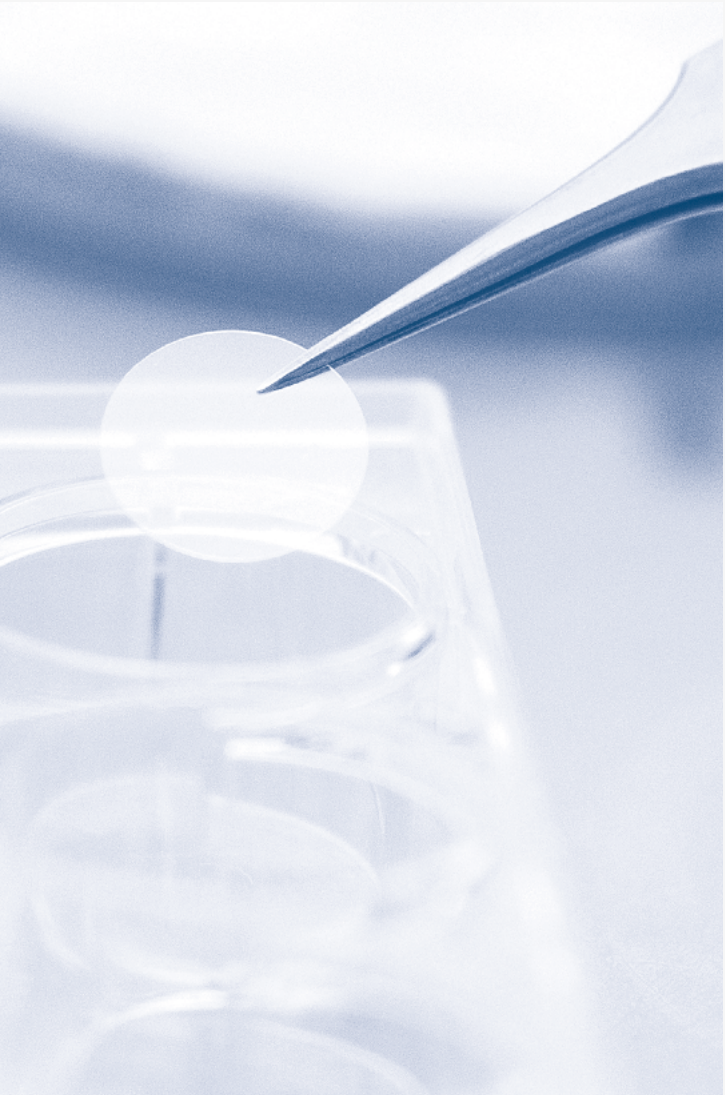
The Complete Guide To Optimizing

IMMUNOFLUORESCENCE STAINING



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FOREWORD

Immunofluorescence (IF) staining is a widely used technique in biological research and clinical diagnostics. IF utilizes fluorescent-labeled antibodies in order to detect specific target antigens. Followed by imaging, it is a very direct technique as you can actually see something. Although it is a well-established tool, multiple factors have to be considered and various optimization steps have to be taken to ensure successful staining.

This guide provides not only an introduction to immunofluorescence staining, but also includes protocols and detailed troubleshooting. We discuss and present useful tips for preparing optimal samples that produce the best signal-to-noise ratios for immunofluorescence staining signals.

What's Inside

THE BENCHMARK IN ANTIBODIES

Since the day it was founded, Proteintech has been making all of its products to the highest standards possible whilst taking complete responsibility for the quality of each product.

- Proteintech makes every single antibody in its 12,000+ catalog.
- Each Proteintech product is unique and cannot be bought under a different label.
- Antibodies are detected with siRNA-treated samples to demonstrate specificity.
- It works in every single species and application or get a full money-back refund.

Proteintech has over 12,000 antibodies in its extensive catalog, all fully validated and available for next day delivery.


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GENERAL PROTOCOLS

Fundamental Principles

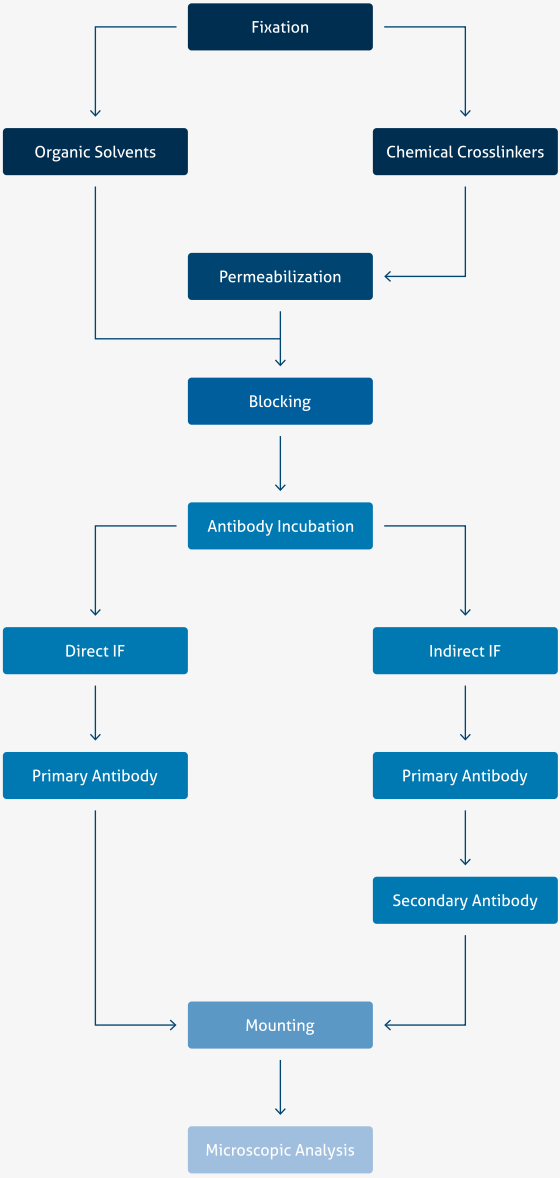
There is no one perfect protocol of how to fix, stain, and analyze cells. Producing beautiful, eye-catching, ready-to-publish IF stainings requires optimization of the best conditions for each single step of the protocol in accordance with sample type, target of interest, antibody used, antibody performance, and antigen-antibody combination.

These days, microscope software (e.g., deconvolution software, etc.) is advanced. It helps to enhance the quality of the image and can often be used to overcome the deficiencies of a poorly prepared sample. However, attention has to be paid to these techniques as they might lead to misinterpretation of results. Damaged cell structures or poor labeling of a suboptimal prepared sample can give false data. No microscopy time should be wasted on poorly prepared samples. Therefore, it is essential to appropriately prepare, fix, permeabilize, block, label, and mount your sample. Still, it is common in a lot of laboratories to use the same standardized protocol for all experiments once it has been established.

Therefore, it is important to understand the basics of an IF staining protocol and to know about the variation that could be implemented. Together with an up-front literature research about cell type, antigen, and antibody, you can save time and get closer to generating a beautiful staining for your research.

The Main Steps of IF

Overview of the main steps of IF.



IF Staining Protocols

Please note: This is a general protocol that provides a good start for testing a new sample, target, or antibody. The protocol has been developed by Proteintech's R&D Team. It may, however, require further adaptation for each specific experiment.

Fixation and Permeabilization

1. Aspirate medium, then briefly wash cells seeded on clean glass coverslips with 1X PBS.
2. Fix the cells for 5–7 min. Rinse coverslip with 1X PBS 3 times for 3 min each.
3. Treat coverslip with permeabilization buffer (e.g. TritonX-100, 0.1 to 1% in PBS) for 5 min at room temperature. Rinse coverslip 3 times with 1X PBS for 3 min each.

Blocking

4. Prepare blocking solution. Incubate the cells with blocking solution at room temperature for 1 h.

Primary Antibody Incubation

5. Aspirate blocking solution, apply primary antibody diluted in antibody dilution buffer. At the same time, set up a negative control without applying the primary antibody. Incubate for 1 h at room temperature or overnight at 4°C.
6. Wash coverslip with 1X PBS 3 times for 3 min each.
7. Apply corresponding fluorochrome-labeled secondary antibody diluted in antibody dilution buffer and incubate for 1 h at room temperature in a moist environment in the dark.
8. Wash coverslip with 1X PBS 3 times for 3 min each.

Mounting and Visualization

9. Mount coverslip. Optional counterstain with DAPI for nuclei detection.
10. Examine slides at a microscope.

Solutions Needed

1X PBS	1000 ml	Antibody dilution buffer	100 ml
10 mM Na_2HPO_4	3.58 g	1% BSA	1 g
1.8 mM NaH_2PO_4	0.28 g	Add 1X PBS to 100 ml	–
140 mM NaCl	8 g	–	–
Adjust pH to 7.4	–	–	–
Add ddH_2O to 1000 ml	–	–	–

SAMPLE PREPARATION

Cell Culture

To get the best staining pattern, it is worth conducting an up-front literature research about protein localization and cell confluency.

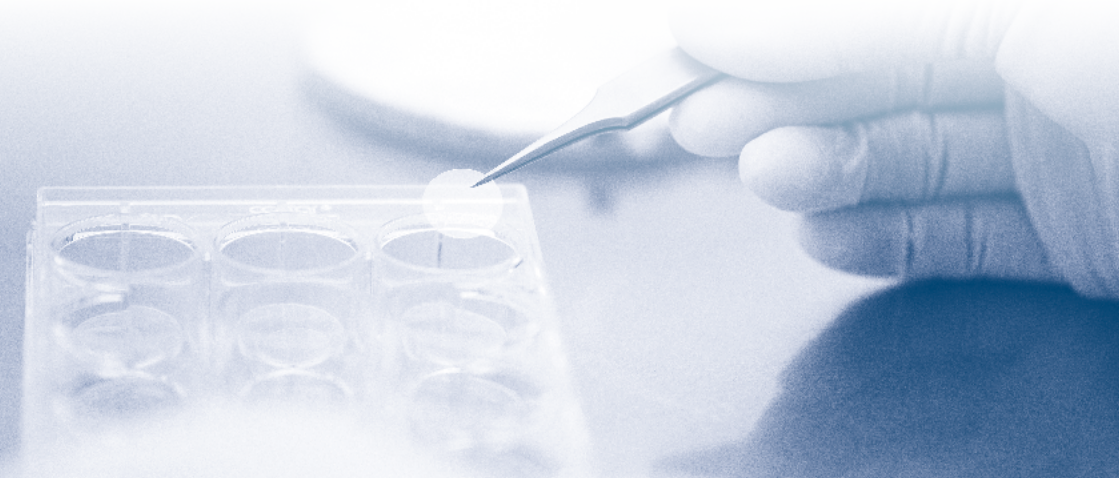
At time of staining, the cell density should be around 60–80%. Too high or low a cell density might influence the normal cell structure and architecture. If cell density is too low, it is more difficult to find a good area for imaging.

1. Seed cells in appropriate volume of media in 12- or 24-well plates on coverslips.
2. Let them grow until the desired cell density is reached.
3. If you are performing any stimulation experiments, recalculate cell number and time to reach cell density needed after the stimulation experiment.

Tip: Know your protein of interest. For instance, stainings for junction proteins need the appropriate cell confluency in order to represent the cell–cell contacts. Different cell densities might show different results here.

Coverslip Preparation

1. Sterilize coverslips using an autoclave.
2. Wash coverslips briefly with ethanol in a laminar flow hood.
3. Let coverslips dry completely before use.
4. Add coverslips to the well plate using tweezers.



Coating

Some cell types may not attach well to glass coverslips. In this case attachment can be improved by coating. The coating matrix always depends on the cell type. Most cell lines attach well to Poly-L-Lysine. Other matrix proteins are laminin (ESC, etc.), collagen (primary keratinocytes/hepatocytes, etc.), gelatin (microvascular endothelial cells, etc.), or fibronectin (NSC, etc.).

1. Add an appropriate volume of coating solution to the coverslip, covering the whole surface. Rock the well plate smoothly in order to ensure equal coating of the surface.
2. Incubate the coating solution according to the manufacturer's instructions (time, temperature).
3. Thoroughly wash the surface with 1x PBS or sterile cell culture grade water.

Fixation

1. When cells have reached the desired cell confluency, aspirate the cell culture media and wash 2x PBS.
2. Add fixative to each well, making sure the surface is well covered. Incubation time depends on the fixative used.
3. Remove the fixative and wash 2x PBS.
4. If not immediately proceeding to antibody staining, leave some PBS in the well and store the sealed plate at 4°C. Fixated cells can be stored for a few weeks in the cold. Make sure the coverslips don't dry out.

SIGNAL-TO-NOISE RATIOS

Autofluorescence

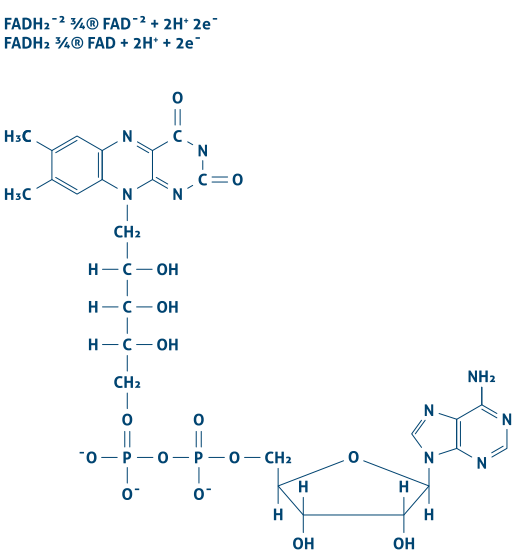
Autofluorescence complicates the analysis of IF stainings. In general, the spectra of autofluorescence are very broad, compared to those of fluorescent probes. Thus, it is challenging to distinguish between autofluorescence and the actual fluorescence of interest, leading to misunderstanding of the image analysis. To overcome the problem of autofluorescence, it is important to have an idea of its source before starting to optimize the protocol or playing around with the filter sets. Autofluorescence is often used as a collective term for "biological autofluorescence" or so-called "fixative-induced autofluorescence."

Biological Autofluorescence

Cells contain components that show fluorescent when excited by a suitable wavelength. This biological autofluorescence has its origin in endogenous fluorophores. Biological autofluorescence mainly comes from mitochondria, lysosomes, and aromatic amino acid components. The most important components causing intrinsic fluorescence are Flavin coenzymes (FAD, FMN) *(Figure 1)* or pyridine nucleotides (NADH) *(Figure 2)*.

Figure 1

Structural formula of flavin adenine dinucleotide (FAD), showing absorption at 450 nm and emission at 515 nm.



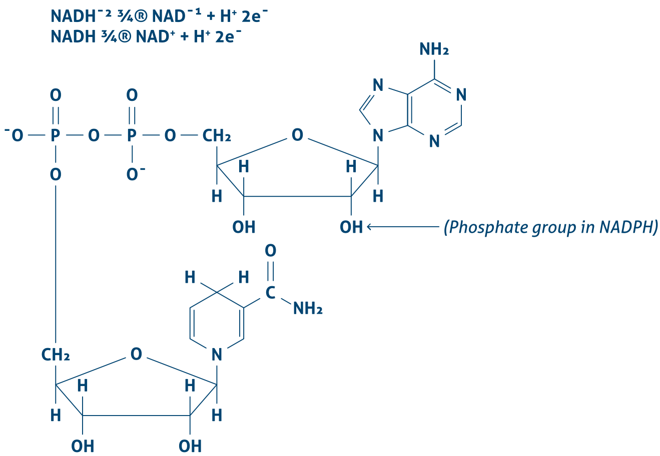


Figure 2

Structural formula of Nicotinamide adenine dinucleotide (NADH), showing absorption at 340 nm and emission at 460 nm.

Fixative-induced Autofluorescence

When fixing with aldehydes, these aldehydes can react with cellular amines or proteins, resulting in a fluorescent product. These issues can be diminished by reducing the aldehyde group to a hydroxyl group (e.g., by sodium borohydride).

Please note: These days, several commercial reagents are available to optimize and reduce background signal and autofluorescence. There are ready-to-use drop-in solutions on the market that suppress background signal and cell culture media suitable for live cell imaging. In case of a background suppressor that chemically reduces autofluorescence, over-dosage has to be avoided as it is always combined with a loss of the actual signal.

Most Commonly Used Fixative For IF Staining

Type of fixative	Name	Advantage	Disadvantage
Organic solvent	Methanol	Cellular architecture is conserved.	Damaging to several epitopes. Lipid components lost.
Organic solvent	Acetone	Gentle for epitopes.	Lipid components lost.
Chemical Cross-linker	Paraformaldehyde	Cellular morphology conserved.	Cross-linking of epitopes, autofluorescence.

VISUALIZATION

Depending on the target of interest, direct or indirect visualization is recommended.

Direct Visualization

Direct visualization requires the use of a labeled primary antibody:

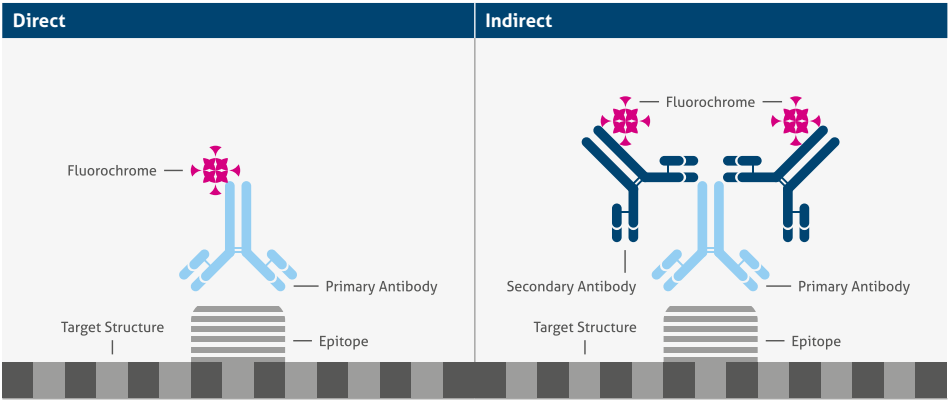
- **Short protocol, fast analysis, easy to handle.**
- **Expensive, a labeled primary antibody for each target is needed.**
- **A highly specific primary antibody is needed.**
- **No signal amplification, not suitable for low expressed targets.**

Indirect Visualization

Indirect visualization requires the use of a labeled secondary antibody:

- **Longer protocol.**
- **Less expensive, a labeled secondary antibody can be used for different primary antibodies.**
- **Higher background noise.**
- **Signal amplification, suitable for medium to high expressed targets.**

Direct Vs. Indirect Immunofluorescence



Most Commonly Used Fixative For IF Staining

Label	Characteristic	Example
Conventional fluorescent labels	Medium bright and photostable, replicate historic experiments	FITC, R-PE, TRITC, Cy3
Alexa® labels, DyLight	Cover the white range, common filter sets, bright, photostable, not pH sensitive	Covering different wavelengths
QDots	Single light source, multiplex, narrow emission	Covering different wavelengths

Mounting Media

Since a fluorescent signal fades away during time, mounting media is added at the end of the staining protocol to maintain the sample. It is also needed to optimize the refractive index for imaging. Mounting media are a solution of glycerol in a special buffer. This helps to maintain the fluorescence signal and slow down photobleaching. Mounting media are optimized for different conditions and dyes, and some questions to consider when selecting the right one are:

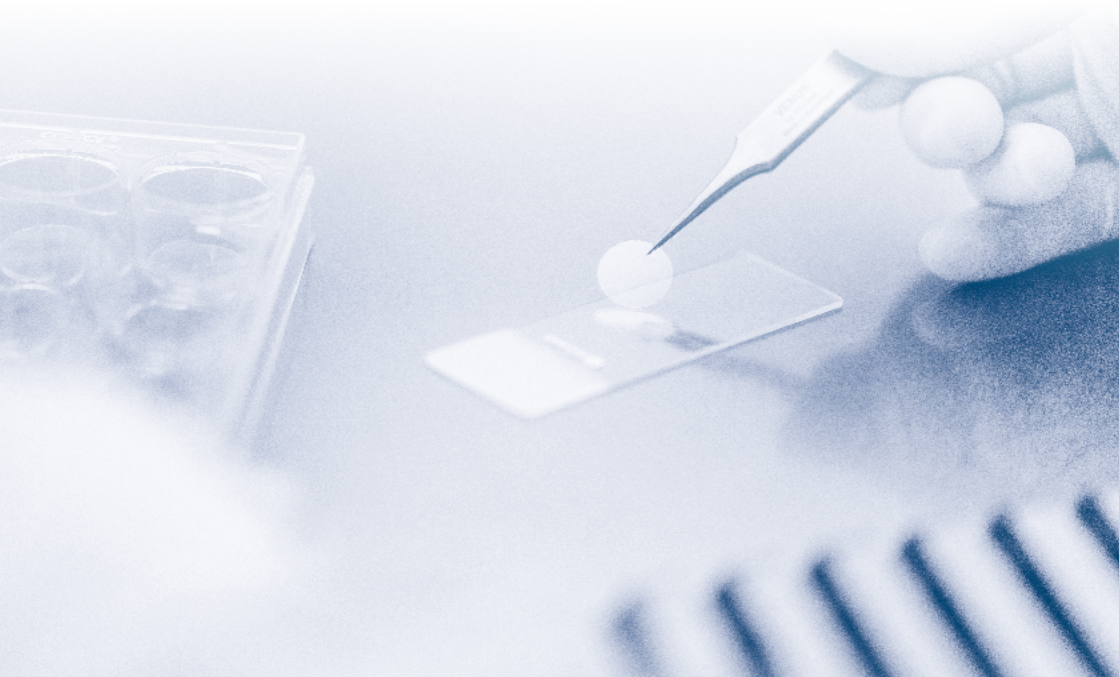
- **Fixed samples for long-term conservation?**
- **Fixed samples for immediate imaging?**
- **Which kind of dye/fluorophore?**
- **Multistaining?**

Tip: Poorly prepared samples show artifacts that could be misinterpreted. For instance, bubbles, insufficient washing, over-incubation with antibodies, or over-fixation can result in unspecific staining patterns and show false-positive results. Adding too much or too little mounting media can create artifacts and damage the sample.

IF STAINING CONTROLS

In order to understand and interpret the IF images obtained, different control stainings can be performed that help to reveal unspecific staining.

- **The unstained sample (just fixed, blocked, permeabilized) should be analyzed to understand the autofluorescence background signal.**
- **A sample (just fixed, blocked, permeabilized) that is incubated with the secondary antibody reveals whether the secondary antibody binding is specific.**
- **To ensure specific binding of the primary antibody, the sample can be blocked with a specific blocking peptide (used to raise the primary antibody). Binding of the primary antibody will be inhibited.**
- **In case of multistainings, the staining should also be performed separately to ensure no cross-reactions and appropriate labeling.**



AFTERWORD

As mentioned at the beginning of this guide, IF staining is a powerful tool with many benefits when used in/for analysis of a target of interest. Conversely, there are certain drawbacks about which you should be aware:

- **The fixation step results in the killing of cells and dynamic and fast processes cannot be monitored. An IF image is a snapshot of the cell at a certain point in time.**
- **Fixation or permeabilization also result in artifacts. In order to ensure that no artifacts are observed, it is necessary to perform a couple of additional time-consuming controls.**
- **The storage time for IF samples is short due to photobleaching and the limited stability of the fluorescent label.**

FAQs AND TIPS

No/Weak Staining

Potential cause	Recommended test
Conditions of antibody are not optimized.	Titrate the antibody concentration to optimize best working conditions. Incubate the primary antibody at room temperature or at 4°C overnight.
Protein of interest is low expressed in used cells.	Use signal amplification when visualizing.
Damaged epitope.	Over-fixation, reduce fixative step, or change to another fixative.
Antibody is not suitable for detection of protein in its native form in IF.	Perform a test on a native Western Blot (not-denaturated).

Non-specific Staining/ No Signal

Potential cause	Recommended test
Target of interest is a nuclear protein.	Use a permeabilization step.
Weak or no fluorescent signal.	Store fluorescent-labeled antibody in the dark. Use a direct-labeled primary antibody. Use signal enhancer.
Fading signal.	Store fluorescent-labeled antibody in the dark. Choose another mounting media.
Artifacts.	Can be due to: cell culture, cell density, insufficient washing, over-fixation, mounting issues.

Background Staining

Potential cause	Recommended test
Non-specific binding of primary/ secondary antibodies.	Run control. Prolong blocking step.
The sample is poorly washed.	Repeat or prolong washing step.
The antibody incubation temperature is too high.	Incubate at 4°C.
Inappropriate fixation causes artifacts or damages the antigen.	Reduce fixative step. Change fixative.
Permeabilization has damaged the cell or protein.	Decrease or skip the permeabilization step.
The slide has dried.	Always keep slide moist.

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