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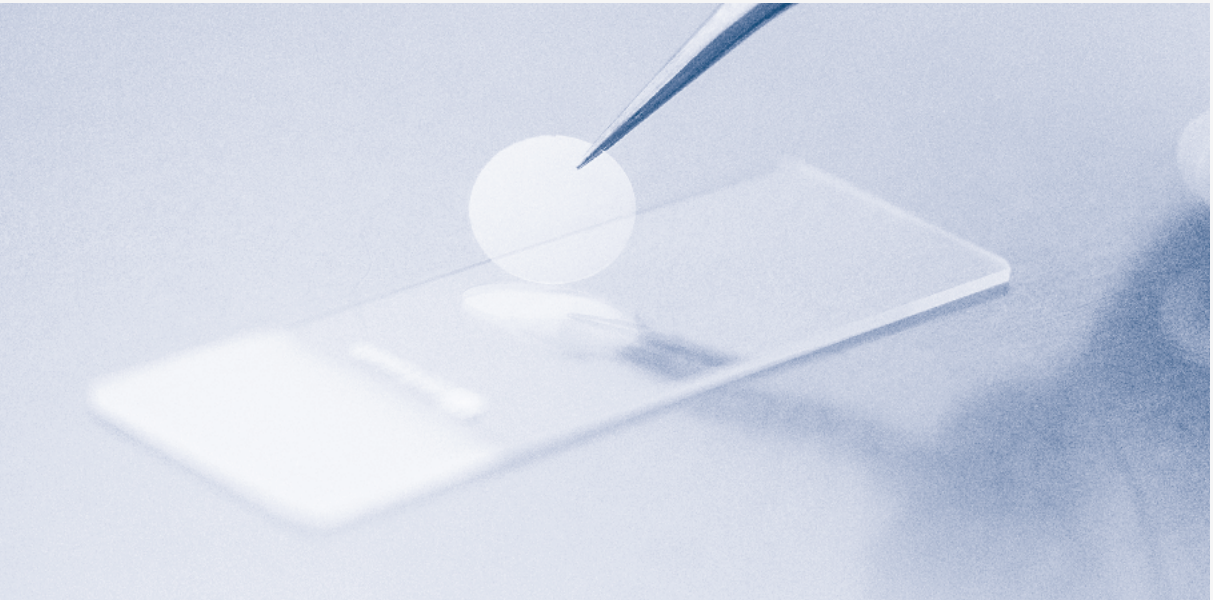
How To Optimize Your

IMMUNOFLUORESCENCE STAINING

Technical Tips and Troubleshooting



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CONTENTS

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FUNDAMENTAL PRINCIPLES

- Widely used technique in biological research/clinical diagnostics.
- The location, expression and pattern of the target antigen can be identified.
- IF utilizes fluorescent-labelled antibodies to detect specific target antigens.

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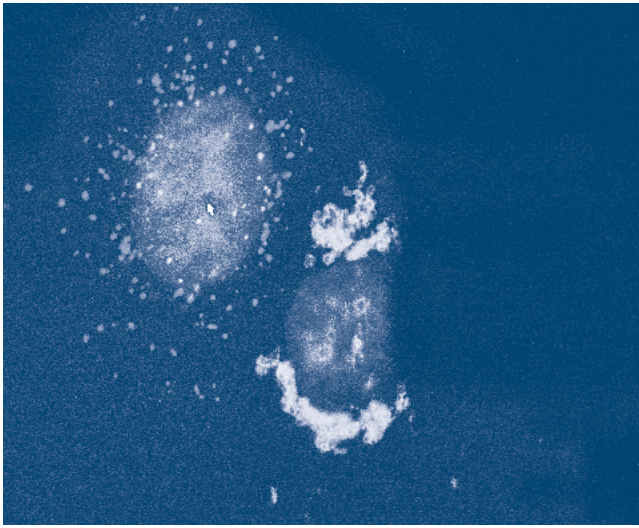
FUNDAMENTAL PRINCIPLES

Drawbacks

Besides the many advantages of IF staining, there are also disadvantages:

- Fixation results in cell killing.
- Dynamic and fast processes cannot be monitored.
- Snapshot image.
- Fixation/permeabilization results in artefacts.
- Several controls are needed .
- IF samples are not suitable for long time storage.



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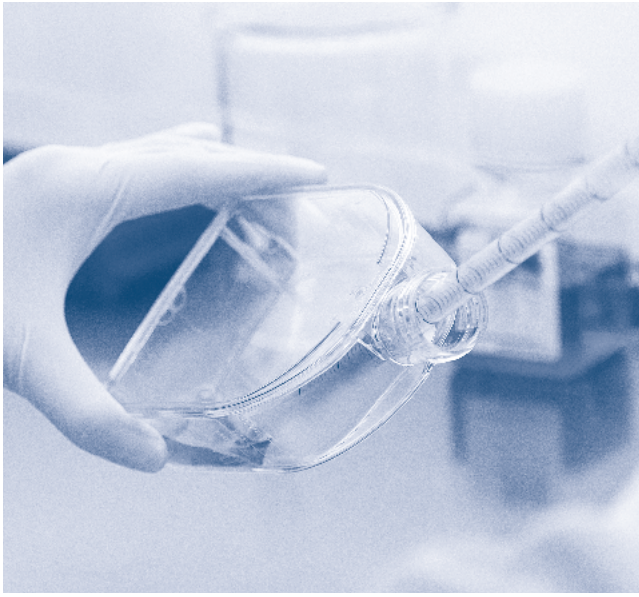
SAMPLE PREPARATION

Cell Culture

- Up-front literature research about protein localization and cell confluency.
- Too high or too low cell density might influence the normal cell structure.
- Seed cells in 12- or 24 well plate on coverslips.
- Let them grow until the desired cell density is reached.
- If performing stimulation experiments, recalculate cell number and density.

Note

- **Junction proteins need appropriate confluency to represent cell–cell contacts.**



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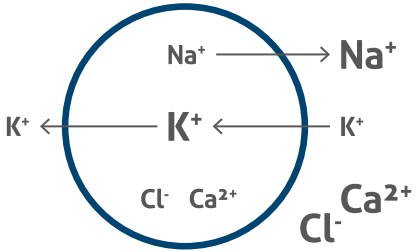
SAMPLE PREPARATION

Working Environment

- Antibodies are optimized by the immune system.
- PBS: Cell surface stainings.
- Potassium: Intracellular environment.



Coating

- Some cell types may not attach well to glass cover slides.
- The coating matrix always depends on the cell type.
- Most cell lines attach well to Poly-L-Lysine.

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FIXATION

Most Common Used Fixatives

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

Note

- **If not immediately proceeding to the staining, leave some PBS in the well and store sealed at 4°C.**

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AUTOFLUORESCENCE

Autofluorescence

- Complicates the analysis of IF.
- Spectra of autofluorescence is very broad.

Biological Autofluorescence

- Cells contain components that show fluorescence.
- Endogenous autofluorescence mainly comes from: mitochondria, lyosomes, aromatic amino acid components.

Fixative-induced Autofluorescence

- Aldehyde fixatives can react with cellular amines/proteins to form fluorescent products.
- Prevention: Aldehyde groups can be reduced.

Note

- **Several commercial reagents are available to optimize and reduce background signal and autofluorescence.**

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VISUALIZATION

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

Direct Visualization

- Labelled primary antibody.
- Short protocol, fast analysis, easy to handle.
- No signal amplification.
- Not suitable for low expressed targets.

Indirect Visualization

- Labelled secondary antibody.
- Longer protocol.
- More flexibility.
- Suitable for medium to high expressed targets.

Factor	Direct	Indirect
Time	Short	Longer
Complex	No	More steps
Flexible	Limits	Flexible
Sensitive	Weaker signal	Strong signal
bkg	Reduced	bkg
Cross-reaction	Reduced	Cross-reaction

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VISUALIZATION

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 wavelength.
QDots	Single light source, multiplex, narrow emission.	Covering different wavelength.

Note

– **Different labels are available for direct or indirect visualization.**

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MOUNTING MEDIA

Mounting media are:

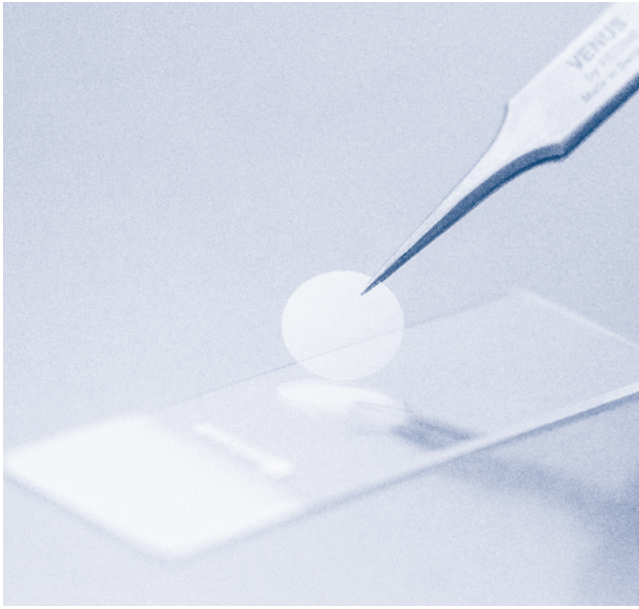
- PBS/ Glycerol mixture.

Why is a mounting media required:

- Refractive index
- Prevent photobleaching, conserve sample.

How to choose a mounting media:

- Type of imaging: fixed-longterm/ immediately, fluorophore, multi-fluorophores.
- Important factors: Toxicity, shrinking, durability, refractive index.



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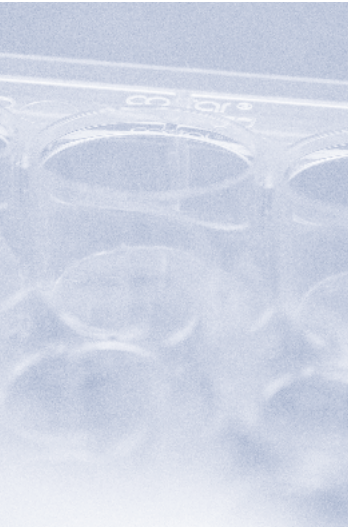
CONTROLS



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.

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FAQS AND TIPS

No/Weak Staining

Potential source	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.
Protein of interest is not expressed in used tissue.	Run a positive control.
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).

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FAQS AND TIPS

Unspecific 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.
Artefacts	Can be due to: cell culture, cell density, insufficient washing, over-fixation, mounting issues.

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FAQS AND TIPS

Background Staining

Potential cause	Recommended test
Unspecific binding of primary/ secondary antibodies.	Run control. Prolong blocking step.
The sample is poorly washed.	Repeat or prolong washing step.
Repeat or prolong washing step.	Incubate at 4°C.
Inappropriate fixation causes artefacts 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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