TROUBLESHOOTING IMMUNOFLUORESCENCE

Troubleshooting *This troubleshooting document gives a guideline to the problem, possible cause and suggested solution for problems during the immunofluorescence application:*

Problem: Weak or no staining

The primary antibodies are inactive	Use a new batch of antibodies.
The primary/secondary antibodies may have lost its activity due to improper storage	Store antibodies according to manufacturer's instructions. Aliquot antibodies into smaller volumes and store in freezer (-20 ºC to -70 ºC), avoid repeated freeze and thaw cycles.
Improper dilution has taken place, or extensive freezing/thawing	Run positive controls to make sure that the primary/secondary antibodies are working properly.
The protein of interest is not present in the sample	Run an appropriate positive control.
The protein of interest is not abundantly present in the sample	Maximize the signal with an amplification step.
The antibody concentration is too low	Increase the concentration of primary/secondary antibodies. Or determine the optimal dilution, by running a serial dilution test, to find the best signal to noise ratio.
The antibody incubation time is inadequate	Increase antibody incubation time, incubate longer (e.g. overnight) at 4℃.
The fixation is inadequate	Try different fixatives or increase the duration of post-fixation.
The epitope that the antibody recognizes may be modified by fixation procedures, which use formalin and paraformaldehyde fixatives	Reduce the duration of post-fixation. If the sample has already been overfixed, perform an appropriate or recommended antigen retrieval procedure.
The antibody may not be suitable for IF procedures which reveal the protein in its native (3D) form	Test the antibody in a native (non-denatured) form on a Western Blotting to ensure it is not damaged.
The primary antibody and the secondary antibody are not compatible	Use secondary antibody that will interact with primary antibody, antibodies that are raised against the species in which the primary was raised. For example, if primary antibodies are raised from rabbits, use anti-rabbit secondary antibodies.
The secondary antibody is inactive	Use a new batch of antibodies.
The enzyme substrate system is defect or incompatible	Use a new batch of reagents.
The substrate incubation time is inadequate	Increase the substrate incubation time.
The protein is located in the nucleus (nuclear protein) and the antibody cannot penetrate the nucleus	Add a permeabilizing agent to the antibody dilution buffer and blocking buffer.
Incorrect mounting medium	Choose correct mounting medium.
The reagents are applied in wrong order or steps omitted	Check the protocol used.
The fluorescent antibody was not stored in the dark.	Always prevent the fluorescent-antibody from exposure to light.

Problem: Non-specific staining

The concentration of primary/ secondary antibodies is too high	Reduce the concentration of primary/secondary antibodies. Or determine the optimal dilution, by running a serial dilution test, to find the best signal to noise ratio.
The incubation time is too long	Reduce the incubation time.
The incubation temperature is too high	Reduce the incubation temperature.
The substrate incubation time	Reduce the substrate incubation time.

is too long	
The primary antibody is raised against the same species as the sample stained (e.g. mouse primary antibody tested on mouse sample). When the secondary antibody is applied it binds to all the sample as it is raised against that species	Use secondary antibody that will interact with primary antibody, antibodies that are raised against the species in which the primary was raised. For example, if primary antibodies are raised from rabbits, use anti-rabbit secondary antibodies. Treat sample with e.g. Mouse-On-Mouse blocking reagent prior to the primary antibody incubation.
The samples have dried out	Avoid drying out of the samples.

Problem: High Background

The sample is not washed enough, the fixative is still present.	Wash the sample at least 3 times in PBS between all steps.
The sample contains endogenous enzyme such as peroxidase or alkaline phosphatise	Block endogenous enzyme activities using 3% H_2O_2 in methanol or levamisole solution before the incubation of the primary antibodies.
The sample contains endogenous biotin activity	Block endogenous biotin activity using avidin/biotin blocking reagent prior to incubation of primary antibodies.
Blocking of non specific binding might be absent or insufficient	Increase the blocking incubation period and consider changing blocking agent. 10% normal serum 1hr for sections or 1-5% BSA for 30 min for cells in culture.
The primary antibodies bind non-specifically to the sample Antibody concentration was too high	Non-specific binding may be reduced by using higher dilution of the primary antibodies. Titrate the antibody to the optimal concentration, incubate for longer but in more dilute antibody (a slow but targeted binding is best).
The incubation temperature is too high	Incubate sections or cells at 4℃.
The secondary antibodies bind non-specifically to the sample	Run a secondary control without primary antibody. Treat sample with normal serum from the same species as secondary antibodies, or use pre-adsorbed secondary antibody.
The chromogen reacts with the PBS present in the cells	Use Tris buffer to wash samples.
Inadequate fixation causes diffusion of the antigen	Increase the duration of post-fixation.
Pemeabilization has damaged the membrane and removed the membrane protein.	Use buffers without permeabilizing agent.
The primary antibody is raised against the same species as the sample stained (e.g Mouse primary antibody tested on mouse sample). When the secondary antibody is applied it binds to all the sample as it is raised against that species.	Use secondary antibody that will interact with primary antibody, antibodies that are raised against the species in which the primary was raised. For example, if primary antibodies are raised from rabbits, use anti-rabbit secondary antibodies. Treat sample with e.g. Mouse-On-Mouse blocking reagent prior to the primary antibody incubation
The sections have dried out	Avoid drying out of the sections.

Helpful links / references

www.protocol-online.org
www.ihcworld.com