Immunofluorescence Troubleshooting Tips

Immunofluorescence (IF) technique combines the immunological method (specific binding of antigen and antibody) with fluorescence labeling method. The fluorescence produced by fluorescein could be detected with the fluorescence microscope, thus IF can be used in the localization analysis of specific antigens in cells.

Here Elabscience lists the common Immunofluorescence troubleshooting.

1. Weak fluorescence signals or no fluorescence expression

Possible causes	Suggestions
The target protein not present or low expressed in the sample	Use cells or tissues with high content of target protein
The antigen epitope was destroyed by immobilization step before staining	Choose another immobilization method
Poor cell permeability	Increase the concentration or reaction time of permeability agent
Antigen was lost due to the permeation	Decrease the concentration or reaction time of permeability agent
The primary/ secondary antibody concentration may be too low	Increase the primary/ secondary antibody concentration
Inappropriate secondary antibody	Ensure that the species of the secondary antibody matches the species of the primary antibody

2. High background of fluorescence

Possible causes	Suggestions
The primary antibody has a poor quality	Use primary antibody with good specificity and high titer
The primary/ secondary antibody concentration may be too high	Decrease the primary/ secondary antibody concentration
Insufficient blocking	Increase the blocking time
BSA of blocking buffer contains IgG	Use highly purified BSA (IgG free)
Insufficient washing	Increase the time and frequency of washing
The cell section has dried out	Keep the cell section at high humidity and do not let them dry out
Antigen was lost due to the permeation	Decrease the concentration or reaction time of permeability agent
The parameters of fluorescence microscope were not set correctly	Adjust the parameters of the fluorescence microscope to reduce background

3. Fast fluorescence quenching

Possible causes	Suggestions
The fluorescein has poor stability	Use fluorescein secondary antibody with good photo stability
The sealing agents which can prevent fluorescence from quenching has	Use sealing agents to prevent fluorescence from quenching

4. Cell auto-fluorescence

Possible causes	Suggestions
No fluorescence quenching was performed after using glutaraldehyde as fixative	Detect the auto-fluorescence before staining, and operate the fluorescence quenching if there is
The sample itself (such as paraffin) has auto-fluorescence	Set negative control, and decrease the parameters of the fluorescence microscope to reduce
The cell components (e.g. riboflavin, cytochrome, etc.) produce auto-fluorescence	Try to avoid using samples with high concentration of riboflavin and cytochrome and other cell components with auto-fluorescence
The ratio of dead cells/living cells is too high	Avoid cell death

Notes for fluorescent double staining

For Indirect method:

1) It is recommended to use primary antibodies from two different species, and secondary antibodies with different fluorescence labeling.
2) It is recommended to incubate one primary antibody, then incubate the fluorescence secondary antibody which matches the primary antibody, then followed with incubation of another primary antibody and its matched fluorescence secondary antibody.
3) Set positive control and negative control.
4) Use blocking serum that was collected from the species in which the secondary antibody was raised. The other procedures are the same as conventional IF experiments.

For Direct method:

The species of primary antibodies are not specially required, and the fluorescent labels of two primary antibodies should be different.