TROUBLESHOOTING FLOW CYTOMETRY (FC)

Troubleshooting *This troubleshooting document gives the problem, possible cause and suggested solution for problems during the flow cytometry application:*

Problem: No signal or weak fluorescence intensity

Incorrect storage	Ensure that all antibodies have been stored correctly according to the manufacturer's instructions.
The antibodies are too old	Make sure that the antibodies have not exceeded their date of expiration.
The incubation time and temperature are not optimal	Optimize the incubation time and temperature of the primary and secondary antibody.
The signal is not correctly compensated	Check whether the positive single colour control is set up correctly on the flow cytometer and is compensated correctly to capture all the events.
There is insufficient antibody present for detection	Antibodies are over diluted, make sure to use the correct concentration by titrating the antibodies before use.
The intracellular target is not accessible	Check if the target protein is really intracellular. Ensure adequate permeabilization for internal staining. To stop all reactions to prevent internalisation of cell surface proteins, all the steps should be done at 4℃, with ice cold reagents.
The fluorochrome conjugate is too large for intracellular staining	The fluorochromes used for intracellular staining experiments should have low molecular weight. Larger flourochromes can reduce the antibody motility and possibly its entry into the cell.
The lasers are not aligned	Make sure that the lasers on the flow cytometer are aligned correctly by running flow check beads and adjust the alignment if necessary. You may need to consider having the machine serviced when the lasers do not align correctly or if drift occurs.
The target protein is not present or the expression is too low	Check the literature for antigen expression and incorporate a positive control of the known antigen expression alongside test material. If antigen expression is weak, select an antibody that is conjugated to a brighter fluorochrome.
The target protein might be soluble/secreted	To be detected easily by flow cytometry, the target protein needs to be membrane bound or cytoplasmic. For intracellular staining a golgi-block step, such as with Brefaldin A, may improve the signal achieved
The antigen is not recognized by the antibody	Check if the antibody will react with the species being used. Not all antibodies will cross-react across species.
The offset is too high or the gain is too low	A positive control should be used to set up the flow cytometer correctly. Use the offset to make sure that the fluorescent signal from cells is not being cut off. Increase the gain to increase the signal but do this within reason.
The fluorescence of the fluorochrome has faded	The antibody may have been kept in the light too long, a fresh antibody is required.
The primary and the secondary antibody are not compatible	Use a secondary antibody that was raised against the species in which the primary was raised (e.g primary is raised in rabbit, use anti-rabbit for secondary antibody).
Secondary antibody is not active/not conjugated to a fluorochrome	Make sure that the secondary antibody is active. Ensure that the appropriate fluorochrome-conjugated secondary antibody is used.

Problem: The fluorescence intensity is too high

The antibody concentration is too high	A high concentration of antibody will give high non-specific binding or very high intensity of fluorescence. The amount of antibody added to each sample should be reduced.
The excess antibody is trapped	This can be a particular problem in the intracellular staining where large fluorochrome molecules on the antibody can be trapped. Ensure that adequate washing steps are taken and include Tween or Triton in the wash buffers.

Problem: There is a high background

The gain set is too high, the offset is too low	Use the positive control to set up the flow cytometer correctly again, using the offset to reduce background from small particles and reduce the gain to decrease the signal but do this within reason.
Excess antibody	The antibody concentration should be decreased. You can also add a detergent to the wash buffers to ensure washing away of excess antibody.

Problem: Non-specific staining

The sample gives autofluorescence	The level of autofluorescence can be checked by including a tube of cells without any antibody into your panel.
Non-specific staining may be due to the secondary antibody	Select a secondary antibody that will not cross-react with the target sample.
The washing is incorrect	Make sure that correct washing steps are included.

Problem: Two or more cell populations are observed when there should be only one population

There is more than one cell population present which is expressing the target protein	Check the expected expression levels from the cell types that are present in the sample and ensure adequate cell separation if necessary.
Cell doublets are present in the sample	Cell doublets will show as a second cell population at approximately twice the fluorescence intensity on the plot. Mix the cells gently before the staining process and again before running them on the cytometer using a pipette. Sieve or filter cells to remove clumps (30µl Nylon Mesh).

Problem: High side scatter background (from small particles)

The cells are lysed	A high side scatter background can be caused by cells in the sample that have been lysed and broken up. Samples should be fresh and prepared correctly, so do not centrifuge at a high rotor speed or vortex too violently.
Bacterial contamination	Make sure that the sample is not contaminated. Bacteria will auto fluoresce at a low level, and will give a high event rate.
Unusual scatter profile	The cells must be used as fresh as possible. The scatter profile may be showing dead cells and debris. Activation methods may affect scatter characteristics of cells.

Problem: Low event rate

Number of cells/ml too low	Run the sample with $1x10^6$ cells/ml. Make sure that the cells are mixed well, but gently.
The cells are clumped and blocking the tube	Pipette the sample gently several times before staining for a homologous single cell suspension. Make sure you mix the sample again before the running process. In extreme cases, sieve or filter the cells to remove clumps (30µl Nylon Mesh).

Problem: High event rate

Number of cells/ml too high	Dilute the sample to $1x10^5$ or $1x10^6$ cells/ml.

Helpful links / references

www.protocol-online.org