

Flow Cytometry FAQs

Detailed troubleshooting techniques for flow cytometry.

1. No signal/weak fluorescence intensity

Signal not correctly compensated

Check positive single color control is set up correctly on flow cytometer and gated/compensated correctly to capture all the events.

Insufficient antibody present for detection

Increase amount/concentration of antibody.

Intracellular target not accessible

Check if target protein is intracellular. For internal staining, ensure adequate permeabilization. To prevent internalisation of cell surface proteins, everything must be done on ice or at 4°C, with ice cold reagents, to stop all reactions. Adding sodium azide will prevent the modulation and internalization of surface antigens which can produce a loss of fluorescence intensity. For staining of cell lines, trypsin can often induce internalisation of cell surface proteins, particularly cell surface molecules and more gentle detachment methods may be required.

Intracellular staining – fluorochrome conjugate too large

Fluorochromes for intracellular staining experiments should have low molecular weight. Large molecular weight fluorochromes can reduce antibody motility and possibly its entry into the cell.

Lasers not aligned

Ensure lasers on flow cytometer are aligned correctly by running flow check beads and adjusting alignment if necessary. If the lasers do not align correctly or if drift occurs, you may need to consider having the machine serviced.

Target protein not present/expressed at low level

Ensure tissue/cell type expresses target protein and that it is present in a high enough amount to detect.

Soluble/secreted target protein

Is the target protein soluble and secreted from the cell? It needs to be membrane bound or cytoplasmic to be detected easily by flow cytometry. A golgi-block step, such as with Brefeldin A, may improve the signal achieved for intracellular staining.

Offset too high/gain too low

Use the positive control to set up the flow cytometer correctly again, using the offset to ensure the fluorescent signal from cells is not being cut off, and increase the gain to increase the signal (within reason – care should be taken).

Fluorochrome fluorescence has faded

Antibody may have been kept for too long or left out in the light. Fresh antibody will be required.

The primary antibody and the secondary antibody are not compatible

Use secondary antibody that was raised against the species in which the primary was raised (e.g primary is raised in rabbit, use anti-rabbit

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secondary).

2. High fluorescence intensity

Antibody concentration too high

This will give high non-specific binding or very high intensity of fluorescence. Reduce the amount of antibody added to each sample.

Excess antibody trapped

This can be a particular problem in intracellular staining where large fluorochrome molecules on the antibody can be trapped. Ensure adequate washing steps and include tween or triton in wash buffers.

Inadequate blocking

Add 1% to 3% blocking agent with antibody as well as a blocking step.

3. High background/high percentage of positive cells

Gain set too high/offset 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.

Excess antibody

Decrease the antibody concentration. You can also add detergent to the wash buffers to ensure washing away of excess antibody.

4. Two or more cell populations observed when there should be just one

More than one cell population present expressing target protein

Check expected expression levels from the cell types contained in the sample and ensure adequate cell separation if necessary.

Cell doublets present

Doublets of cells will show as a second cell population at approximately twice the fluorescence intensity on the plot. Mix cells gently before staining and again before running on the cytometer using a pipette. Cells can also be sieved or filtered to remove clumps (30 µl Nylon Mesh).

5. High side scatter background (from small particles)

Cells lysed

Ensure cells in the sample have not lysed and broken up. Samples should be fresh and prepared correctly. Do not centrifuge cells at a high rotor speed or vortex too violently.

Bacterial contamination

Ensure sample is not contaminated. Bacteria will auto fluoresce at low level. This will also give a high event rate.

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6. Low event rate

Low number of cells/ml

Run 1×10^6 cells/ml. Ensure cells are mixed well (but gently).

Cells clumped, blocking tubing

Ensure a homologous single cell suspension by pipetting gently several times before staining. Ensure you mix again before running. In extreme cases, cells can be sieved or filtered to remove clumps (30 μ l Nylon Mesh).

7. High event rate

High number of cells

Dilute to between 1×10^5 and 1×10^6 cells/ml

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