Troubleshooting Forum

Molecular Biology Techniques Q&A

Immunology and Immunochemistry: Fluorescence Activated Cell Sorting (FACS)

This month's question from the Molecular Biology Forums (online at molecularbiologyforums.biotechniques.com) comes from the "Immunology and Immunochemistry Methods" section. Entries have been edited for concision and clarity. Mentions of specific products and manufacturers have been retained from the original posts, but do not represent endorsements by, or the opinions of, *BioTechniques*.

Can I prevent cell death when preparing cells for FACS? (Thread 20362)

Q I am trying to perform an indirect flow cytometry protocol for a cell surface antigen using HeLa cells. First, I detached the cells with 2 mM ethylenediaminetetraacetic acid (EDTA)/PBS while tapping the flask for 15 min on ice. After 2 washes in PBS, I resuspended them in the FACS wash buffer, which is PBS + 0.1% sodium azide (NaN_3), and stained them with my antibodies. All centrifugation steps were done at 1300 rpm for 5 min at 4°C, and I kept the cells on ice throughout the procedure except during the 2 15-min blocking steps, which were done at room temperature. Then I fixed the cells in 2% paraformaldehyde (PFA)/PBS solution. Upon analysis, almost all of the cells were dead. The whole flow cytometry procedure takes about 5 h for me since I am new to the method. Could that be the cause of the poor cell viability?

A I am puzzled. Do you expect the cells to be alive after fixing them with PFA, or did you test the viability before fixation?

Q As far as I know, cells that die during the procedure can still be roughly distinguished from healthy ones even after fixation based on their different forward and side scatter properties. They also take up an excessive amount of the fluorescent dye.

A The NaN_3 in your FACS buffer is toxic. I recommend using PBS plus 2% FCS. Here is my basic protocol for working with viable cells:

1. Spin down $\sim 10^5$ cells.
2. Add 500 μL of your primary antibody diluted in PBS-FCS and incubate on ice for 30 min.
3. Add 2 mL PBS-FCS, spin it down, and remove the supernatant.
4. Add 500 μL of your secondary antibody, also diluted in PBS-FCS, and incubate on ice for 30 min.
5. Add 2 mL PBS-FCS and spin.
6. Add 500 μL PBS-FCS, plus 10 μL 1 mg/mL propidium iodide (PI), if desired, and analyze.

Q You were right. Four to five hours in the sodium azide was too toxic for the cells to survive; they were viable when I left the sodium azide out of the washing buffers. But I am nervous about omitting it altogether since it helps to prevent capping of the receptors, which is particularly useful for me. My aim is to detect any potential quantitative differences in the expression of my receptor in samples treated under different experimental conditions.

I also tried something else that ended up being successful. I fixed the cells in 1% PFA at the beginning of the procedure, which did not influence the antigenicity of my antibody. Then I stained the cells and the whole procedure was much easier since I did not need to use ice or sodium azide. My cells were nicely preserved throughout the process, and I got good signals for analysis.

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A If capping is important, you could make Fab fragments of your primary antibody for a monovalent reagent. Bind your primary antibody and then fix the cells. Then you will not need to worry about retaining the antigenicity of your various targets.

Q It is useful to know that I have that option for future experiments. In my case, I saw that my primary antibody was designed mainly for immunofluorescence, so that is why I thought it would work with fixed cells.

Could you please suggest a method for making Fab fragments from my primary antibodies? How difficult or expensive would this protocol be for a small lab to accomplish? Why do companies manufacture Fab fragments only for secondary antibodies, but not primary antibodies?

A There are standard protocols available for making Fab fragments. Try looking at "Current Protocols in Immunology" or "Antibodies: A Laboratory Manual" by Harlow and Lane. Basically, you do a papain digest of your intact IgG to release Fab and Fc. Then purification with protein A removes Fc and undigested IgG, leaving only Fab. The digestion conditions need troubleshooting to customize the procedure for your particular antibody. Try doing a pilot run with a time course to check on different digestion times and starting amounts of antibody then monitor the fragments from these reactions by non-reducing SDS-PAGE. Once you have the digest working to your satisfaction, you can scale things up to prepare the fragments for your FACS experiments.

Secondary antibodies are offered as F(ab')₂ fragments for cell binding experiments so that researchers can take advantage of the better binding of bivalent reagents, while avoiding the nonspecific background of Fc binding to Fc receptors on the cell surface. Since not everyone needs Fab fragments for primary antibodies, there isn't really a market for them.

A You should be sure to inactivate complement at 56°C for 30 min in any serum you might be using in your blocking solutions.

Since you are doing indirect immunofluorescence, it would be more useful to have Fab fragments for your secondary antibody, not your primary. Secondary antibodies as Fab fragments are monovalent, not divalent like your typical antibody. They are usually available commercially. Crosslinking receptors with whole antibodies can sometimes prompt physiological effects such as cell death since some primary antibodies have ligand-like properties of their own. For example, some anti-CD3 antibodies actually stimulate CD3, so make sure your antibody cannot act as an agonist for the receptor that it is detecting.

To detect living and dead cells, forward-scatter versus side-scatter comparisons work well. There are also live-dead stains for flow cytometry. PI, like trypan blue dye, needs energy to exclude it from the cell. Once cells are fixed or remain on ice too long with the dye, these dyes will no longer give informative signals. 7-aminoactinomycin D (7-AAD), a fluorescent chemical compound, can be taken up by dead cells and post-fixed.

Selected and edited by Kristie Nybo, Ph.D.

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