

Fixing Stained Cells for Subsequent Flow Cytometric Analysis (BD Biosciences and Alternate Protocol)

Materials:

Staining medium (SM) [1X HBSS; 2% (v/v) calf serum; 10mM NaN₃, 10 mM HEPES [pH 7.2]

1X PBS

BD Cytotfix (contains 4% paraformaldehyde), Cat # 554655

OR

Paraformaldehyde solution, 16%, Canemco Inc, Cat #017B

Filtered calf serum (CS) [0.45µm TC sterile filtered to avoid bacterial growth]

0.1% Trypan Blue

Nitex mesh, 85-µm mesh size

4 ml conical tubes [Diamed STK 8550]

5 ml round bottom tubes [Falcon 2052]

Antibodies as required for experiment

Sample Note:

For each FACS sample use 0.5-2x10⁶ cells/tube. A considerable amount of cell loss will occur during the multiple washes and spins. The CS is used to underlay the cell suspension during washes. DO NOT add PI to the SM used in step 5 (BD protocol) or step 6 (alternate protocol), because the cells are now permeabilized and will all be PI positive.

BD Cytotfix Protocol:

- 1) Perform staining for extracellular markers according to our recommended Extracellular Staining Protocol (see FCF web page) or any other standard FACS staining protocol.
- 2) Wash cells by adding 500-1000µl SM, underlay with 250-300µl CS and collect by centrifugation at 400xg for 5 minutes at 4°C (1500 rpm in Beckmann GS6-KR). Aspirate supernatant.
- 3) Fix cells by re-suspending cell pellet in 200µl of BD Cytotfix Buffer™ per tube. Incubate cells for 20-30 minutes on ice. (Note: Cell aggregation can be avoided by briefly vortexing cells prior to addition of the fixation buffer).
- 4) Wash cells by adding 500-1000µl SM (omit the CS underlay at this point because of the fixative in the buffer) and collect by centrifugation as described above. Aspirate supernatant.
- 5) Re-suspend cells in 500µl SM, filter through Nitex mesh and store at 4°C, protected from light until ready to run samples on the cytometer.

Alternative Protocol:

- 1) Perform staining for extracellular markers according to our recommended Extracellular Staining Protocol (see FCF web page) or any other standard FACS staining protocol.
- 2) Wash cells in SM by adding 500-1000µl SM, underlay with 250-300µl CS and collect by centrifugation as described above. Aspirate supernatant.

- 3) In the meantime, prepare 2% paraformaldehyde solution in 1xPBS (from 16% paraformaldehyde stock). This working solution is stable for a week when stored at 4°C, in an airtight container, protected from light, provided that the solution remains at pH 7.
- 4) Fix cells by re-suspending the pellet in 500-1000µl of 2% paraformaldehyde. Alternatively, clumping may be best avoided first resuspending cells in 900µl 1xPBS and then adding 100µl 16% paraformaldehyde for a final concentration of 1.6%. Incubate at room temperature for 10 minutes or for 20-30 minutes on ice.
- 5) Wash cells in 2ml of 1xHBSS or 1xPBS SM (omit the CS underlay at this point because of the fixative in the buffer) and collect by centrifugation. Aspirate supernatant.
- 6) Re-suspend cells in 500µl SM, filter thru Nitex mesh and store at 4°C, protected from light until ready to run samples on the cytometer.

Note: works well with all fluorochromes, including PE and APC based tandem conjugates. It is recommended that fixed samples be run on the cytometer within 1 week, preferably the next day. Autofluorescence tends to increase and sample quality generally declines with long-term storage of fixed samples.