Immunofluorescent Staining of Intracellular Cytokines (ICS)

Immunofluorescent staining with antibodies against intracellular cytokines and cell surface markers provides a high resolution method to identify the nature and frequency of cells which express a particular cytokines. Optimal intracellular cytokine staining can be achieved using a combination of fixation with paraformaldehyde (PFA) and subsequent permeabilization of cell membranes with the detergent saponin. PFA-fixation allows preservation of cell morphology and intracellular antigenicity, while also enabling the cells to withstand permeabilization by detergent. Membrane permeabilization by saponin allows the cytokine-specific monoclonal antibody to penetrate the cell membrane.

The protocol uses brefeldin a to block intracellular transport processes and results in the accumulation of cytokines in the rough endoplasmic reticulum or Golgi complex, which leads to an enhanced ability to detect cytokine-producing cells.

NOTE: *Since transport inhibitors such as Brefeldin A have a dose- and time-dependent cytotoxic effect, exposure must be limited.*

Buffers

Cell culture media:	IMDM containing 10% (v/v) FCS
FACS Buffer:	1x PBS containing 2 mM EDTA, 2 mM NaN3 and 2% (v/v) FCS
Permeabilization Buffer:	1x PBS containing 2 mM EDTA, 2 mM NaN3 and 2% (v/v) FCS and 0.1% (w/v) saponin
Brefeldin A:	10 mg/ml in DMSO
Paraformaldehyde:	4% (w/v) in 1x PBS

Staining Protocol

Prepare single cells suspension e.g. of splenocytes in 5 ml of cell culture media.

- ✓ Adjust cell concentration to $1x10^7$/ml.
- ✓ Incubate 100 µl of the cell suspension in a 96-well round bottom plate, corresponding to ~$1x10^6$ cells.

- ✓ Restimulate cells by adding 100 µl of media containing polyclonal activators (e.g. anti-CD3 and anti-CD28; LPS) or a corresponding antigen. Restimulation should be performed in the presence of a protein transport inhibitor e.g. Brefelding A (final concentration 10 µg/ml).
- ✓ Incubate cell suspension for 5 h at 37°C.
- ✓ Wash the cells twice with FACS buffer.
- ✓ Label cells in 50 µl of FACS Buffer (100 µl for staining in tubes) with a fluorochrome-conjugated monoclonal antibody specific for a cell surface antigen, such as, CD3, CD4, CD8 for 20-30 min, 4° in the dark.
- ✓ Wash cells 2x with FACS Buffer, pellet by centrifugation (1500 rpm), and remove supernatant.
- ✓ Thoroughly resuspend cells in 75 µl (250 µl for staining in tubes) of 4% PFA solution and incubate for 5 min at 4°C.

  ***NOTE*:** Cell aggregation can be avoided by vortexing prior to the addition of the PFA solution.

- ✓ Wash the cells twice with permeabilization buffer (1 ml/wash for staining in tubes), pellet, and remove supernatant.
  ***NOTE*:** Perm buffer is required in washing steps to maintain cells in a permeabilized state.

- ✓ Thoroughly resuspend fixed/permeabilized cells in 50 µl of Perm buffer (100 µl for staining in tubes) containing fluorochrome-conjugated anti-cytokine antibody (diluted according to manufacturer's instruction). Incubate at 4°C for at least 30 min or overnight in the dark.

- ✓ Wash cells 2 times with Perm buffer (1 ml/wash for staining in tubes) and once with FACS buffer
- ✓ Resuspend in 200 µl FACS Buffer prior to flow cytometric analysis