17.05.2011 by Christian Steigerwald and André Heinen, IMM Mainz, Ag Waisman

# Intracellular staining protocol (ink. preparation of organs)

## Preparation

- For organ extraction label falcon tubes, fill with 3ml DPBS-2%FCS, prepare your workspace
- Score your mice, note each abnormality
- Sacrifice mice with Isoflurane
- Extract spleen and lymph nodes (inguinal, axillar, brachial, mesenteric), etc.

## Mash organs

- Mash extracted organs
- Flush dish and tissue strainer with each 5ml DPBS-2%FCS
- Centrifuge at 250g for 7:00 minutes

## Red blood cell lysis

- Resuspend with 1ml ACK per normal sized spleen
- 3 min on ice
- Fill up to 11ml with PBS-2%FCS
- Centrifuge at 250g for 7:00 minutes
- Remove supernatant, fill up to 250µl LN and 3ml spleen (firstly resuspend in 1 ml PBS then fill up to 3ml)

## Count cells

- Dilute cells with trypan blue (1:4 diluted with PBS)
  1:10 LN , 1:40 spleen
- Neubauer chamber:
  counted cells x 10000 x dilution factor = cells per ml

## Stimulate the cells for intracellular staining

- Use 5 mio spleen cells and xxx mio LN cells
- Stimulate with Brefieldin A, Ionomycin and PMA in T-Cell-Media
- Dilution factors:

| | | | |
|---|---|---|---|
| PMA | → | 1:1000 | final 50ng/ml |
| BrefA | → | 1:1000 - 1:200 | final 1-5µg/ml |
| Ionomycin | → | 1:1000 | final 500ng/ml |

- Resuspend spleen cells in 2,5ml and LN cells in xxxml T-cell medium
- Incubate 4 hours at 37° Celsius

## Transfer to 96-Well-V plate or eppies

- Centrifuge at 250g for 7:00 minutes
- Resuspend in 150µl FACS-Buffer I (be sure to rinse the inner side of the falcon tip accurately)
- Centrifuge at 250g for 5:00 minutes

## Surface staining

- Do Fc' block if needed (10 min)
- Stain with specific antibodies in FACS-Buffer I
- Incubate 10-15 minutes on ice and dark
- Washing 1: Fill up to 200µl with FACS-Buffer I
- Centrifuge at 250g for 5:00 minutes
- Washing 2: Resuspend in 200µl FACS-Buffer I
- Centrifuge at 250g for 5:00 minutes
- Remove supernatant

## Fixation

- Resuspend Pellet in 200µl 2%PFA (diluted with DPBS)
- Inkubate 20 minutes on ice and dark (in this case GFP/EYFP/RFP will resist) or fix over night covered in the fridge
- Centrifuge at 300g for 5:00 minutes
- Remove supernatant

## Intracellular staining

- Wash with 200µl Saponine-Buffer (optional: leave cells 5 min in Buffer)
- Centrifuge at 300g for 5:00 minutes
- Remove supernatant
- Fc' block if needed (10 min)
- Stain with specific antibodies in Saponine-Buffer
- Incubate for 30 minutes on ice and dark
- Washing 1: fill up to 200µl with Saponine-buffer
- Centrifuge at 300g for 5:00 minutes
- Remove supernatant
- Washing 2: resuspend in 200µl Saponine-Buffer
- Centrifuge at 300g for 5:00 minutes
- Resuspend pellet in xxxµl FACS-Buffer II
- Record at a FACS