Cell isolation protocol for T reg sorting

(with/out enrichment)

Protocol based on stroma isolation protocol: Fletcher, AL et al. (2011), *Frontiers in Immunology*

Required reagents:

- 1x PBS, pH 7.4
- 1x PBS SE, pH 7.4 (PBS, 2%(v/v) FCS, 1-**5**mM EDTA (as you wish))
- RPMI, 2%(v/v) FCS
- Collagenase **D** or P (both work equally, from Roche *11088882001*)
- DNase I, grade II (from Roche *10104159001*)
- 0.5M EDTA
- PBC lysis buffer (Biolgend *420301*) or any ACK Buffer

Procedure:

- Prepare fresh digestion Medium:

  RPMI, 2%(v/v) FCS
  + 0.2mg/ml Collagenase D
  + 0.1mg/ml DNase I

  15ml/mouse, keep at 37°C in a water bath

- Sacrifice mice, take out spleen/LNs, put into ice cold PBS (**without** EDTA!)
- Put spleen/LNs into a petri dish. Cut spleen/LNs into 4-6/2-4 pieces and transfer into a fresh 15ml falcon
  (To make things easier: if you are only interested in LNs, a 1.5ml reaction tube is sufficient. Incubate then at 1000rpm on a thermo-shaker, 37°C)
- Add 3ml of 37°C Digestion buffer & incubate for 10min, shake tube every 3min

- After 10min, medium should change from clear to turbid:
  Then put the **Supernatant** (containing the already dissociated cells) into a **fresh 50ml falcon**, add to the SN EDTA qs 5mM (e.g. for 2.5ml, 25µl of 0.5M EDTA), mix and spin 5min at 380g. Discard the digestion medium and resuspend in normal and ice cold RPMI 2%(v/v) FCS, keep on ice.
  At the same time, refill **the digestion tube** with 3ml of digestion buffer and put it back into the 37°C water bath, shake tube every 3min.

- After another 10min, try to pipette smoothly the debris with a p1000 (cut the tip)
  Take out the SN and pool it to the already isolated cells. Add EDTA qs 5mM and spin and resolve pellet in ice cold RPMI 2%(v/v) FCS. Keep on ice.
  At the same time, add 3ml of fresh digestion buffer to the digestion tube containing the organ debris.

  ↺ Repeat this **3 more times** (total 60-70min). All debris should be digested after 50-70min. Do not forget to pipette every 5min with a cut tip.

- Adjust the volume to 50ml RPMI 2%(v/v) FCS, filter isolated cells through a 70µm cell strainer, spin 10min at 380g (washing step/filtering step)

- Resuspend the pellet in 5ml of 1x RBC lysis buffer, incubate for 4-5min, add PBS SE qs 30ml, spin at 380g, 10min.

- Discard supernatant, pellet should be brownish/white. Resolve pellet in 10ml per mouse in PBS SE. Eventually filter again.

- Count cells in Trypan blue (pre-dilute 1:100)

- Adjust volume to 1ml per 20 x 10^6 cells with PBS SE

- Add Fc-block (anti-CD16/CD32, 2.4G2, BD Pharmingen) at 1µg/ml (1/500) and incubate for 5min on a roller shaker at RT

- Without wash, proceed to staining:
  CD4-A647 (RM4-5, Biolegend) at 0.33µg/ml (1/1500)
  CD25-PE at 0.4µg/ml (PC61, Biolegend; not for in vivo, depleting via ADCC)

  Incubate 10-15min on a roller shaker at 4°C

- Spin at 380g, 10min, discard SN, fill tube with PBS SE, spin at 380g, 10min, discard SN

- Take up cells in 40 x 10^6 cells per ml

- Proceed to sort

Scheme-Digestion: