Ca^{2+} Measurements in Primary Mouse Lymphocytes

Procedure:

Design experiment

- Decide which tissues and cell types to analyze in how many mice
  ==> Calculate sample numbers and prepare sufficient reagents and solutions
- Include the following compensation controls to set compensations and optimize PMV voltage settings:
  - unstained cells
  - PE AB only
  - APC AB only
  - {Third color} AB only
  - Indo-1 (Ca^{2+} dye mix) only

  Collect baseline data in MMF first, then add Ca^{2+}-add back and Ionomycin to collect data for maximal Ca^{2+} influx.Adjust PMV voltage to optimally separate blue and violet Indo-signal. Then compensate the other channels under maximal Ca^{2+} influx.

Prepare lymphocyte suspensions

- conduct this part at RT
- harvest desired organs (spleen, LN, BM, thymus) in 5 ml M199-2 (10mM Hepes, 2% FCS, 1X Pen/Strep)
- gently disperse cells in M199-2 through a cell strainer with the plunger of a 1 or 3 ml syringe into a 50 ml tube
- wash strainer with 1ml M199-2 and add the remaining cells to the tube
- spin 5 min at 1500 RPM, discard supernatant

  a) If working with spleen or BM, or if significant contamination with red blood cells occurs:
  - resuspend cells gently in 2 ml 1x BD Pharmlyse (cat # 555899); incubate at RT for 2-3 min.
  - add 10 ml M199-2 and mix gently
  - immediately spin 5 min at 1500 RPM, discard supernatant
  - resuspend cells in 5 ml M199-2

  b) If no RBC contamination occurs, then proceed to cell counting
- count cells
- spin 5 min at 1500 RPM, discard supernatant
- resuspend cells required for assay in MFC at 10^7 cells/ml; for remaining cells, keep in M199-2

==> At this point, cells can be stored at RT for a few hours (but not > 6 hrs)

Prepare Ca^{2+} dye solutions

- for 2µg/µl (=2 mM)**Indo-1 stock**, reconstitute 50 µg Indo-1in 25 µl DMSO at RT, vortex, spin briefly.
- you will need 5 µl stock solution per sample
- keep at RT in the dark (drawer, closet)

- make fresh **2x CDLS** from this stock for each sample batch (2.5 µl Indo-1 stock in 1 ml MC, use within 1 hr). You will need 250 µl of this solution per sample.

Loading the cells with Ca^{2+} dye and staining for surface markers

Ca^{2+} flux profiles may vary significantly between cells that are run immediately after loading and cells from the same batch that have been loaded >2 hrs before. Therefore, never load more samples than you can run within 2hrs (typically 4-6 samples plus loading controls)

- For the following, use 2.5 x 10^6 cells (250 µl of the above cell stock) per sample. Samples that will be stained for the same surface markers can be pooled.
- make the 2x CDLS fresh for each sample batch (One freshly made dye stock solution in DMSO can be used for all samples on the same day)
- transfer 2.5 x 10^6 cells into individual microtubes
- add 1 vol of CDLS
- incubate for 30-45 min at 37 ºC in the dark (bacterial incubator). Invert gently to mix every ~10 min.

- During this incubation time, prepare surface marker staining and stimulation mixes (see below)

- spin loaded cells for 5 min at 1500 RPM at RT, discard supernatant
- add 900 µl MFC to loaded cells to wash
- repeat wash (MFC) and resuspend in 200 µl MFC

- stain the cells on ice for surface markers, use cold buffers and treat very gently to preserve membrane integrity

- add 1/10 vol (25 µl) FACS-AB premix

- Stimulation variant A: Add 1/10 vol (25 µl) Stimulation-AB Mix1A here
(In the alternativestimulation variant B, this is instead done during FACS analysis)

- incubate 15 min on ice in the dark

- during this time, go to Flow facility, turn on 37 ºC waterbath and UV laser on FACS machine

- wash cells 1-2x in MFC
- wash cells 1x in MMF
- resuspend in 500 µl MMF and transfer into FACS tube, store on ice in the dark

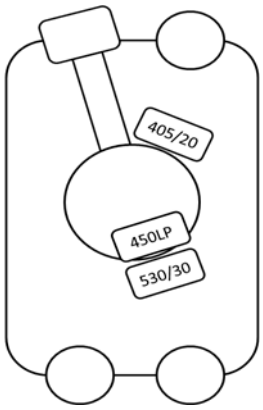
==> the cells are now ready for FACS analysis in a Ca^{2+} re-addition protocol

FACS analysis

(~15 min per sample, stimulation mixes must be pre-warmed to 37 ºC)

- Take the following to the FACS facility (unless already available there):
  - Heat block (37 ºC)
  - Stimulation mixes
    Variant A:Mock, SA, Tg, Ca^{2+} add-back, Ionomycin
    Variant B: Mock, Stimulation-AB Mix 1B, xABMix, Tg, Ca^{2+} add-back, Ionomycin
  - Cells on ice in the dark (Aluminum foil on top of ice bucket)
  - Pipets (P200, P1000), yellow & blue tips or better filter tips

- 10% bleach
- deionized H_2O



- Check/adjust filter settings (Here shown for LSRII):
  FSC: Linear
  SSC: Linear
  PE: Logarithmic
  APC: Logarithmic
  Indo-1 violet: Linear
  Indo-1 blue: Linear
- Set compensations (not needed for combination PE/APC/Indo)
- Read Violet/Blue ratio over time (and plot for monitoring progress)

- place one cell sample at 37 ºC for 5 min (during which you bleach, prime and flush with water)
- run bleach for 2 min, fast speed
- run water for 2 min, fast speed
- prime
- set speed to low
- make sure FACS software is set to new sample with correct annotation
- collect **30 sec** *(baseline)*
  - **Stimulation variant B:***This is not done if stimulation variant A is used!*
  - add 100 µl Stimulation-AB Mix 1B (5 µg/ml final in MMF), vortex or swirl briefly
  - collect **1 min***(AB baseline)*.
- add 50 µl Stimulation Mix 2 (Mock or SA or xAB or Tg), vortex or swirl briefly
  (If machine runs out of sample before time course over, change SA addition to adding 400 µl of 1:10 diluted Stimulation Mix 2, which gives ~ the same final conc in a lerge enough volume for a 15 min run)
- collect **3 min***(intracellular store release)*
- add 50 µl Ca^{2+} add-back, vortex or swirl briefly
- collect **5 min***(plasma membrane influx)*
  - **If SOC/CRAC modulating compounds are being tested**
    - collect only **3 min***(plasma membrane influx)*
    - add 50 µl mock or compound at desired concentration in MFC, vortex or swirl briefly
    - collect **2 min***(modulated plasma membrane influx)*
- add 50 µl Ionomycin
- collect **1-2 min**until peak flux reached *(loading & viability control)*
- place next cell sample at 37 ºC for 5 min and start again by bleaching, flushing and

- If last sample done: Bleach, flush, prime and then place sample capillary into water, set machine on standby.

- Export FCS files onto server, memory stick or CD/DVD and analyze offline in Flowjo.

- If more samples will be run on same day, keep machine on standby.
  Otherwise, turn of machine.

Media and solutions: *(FCS can be replaced with FBS)*

CM: RPMI + 10 mM HEPES + 10% FCS + 1% Glu (+ Pen/Strep/Glu = PSG)

M199: M199 + EBSS + 20 mM HEPES + 2% FCS + 1% Glu + 2.2 g/L Na-Bicarbonate (+ PSG)

MFC: HBSS with Ca^{2+} and Mg^{2+} (~ 1 mM each) + 10 mM HEPES + 1% FCS (phenol-red free)

MC: MFC without FCS

MMF: HBSS without Ca^{2+} and Mg^{2+} + 10 mM HEPES + 1% FCS + 1 mM $MgCl_2$ + 1 mM EGTA (phenol-red free)

Indo-1 stock, 2 µg/µl (=2 mM):Reconstitute 50 µg Indo-1 in 25 µl DMSO at RT, vortex, spin briefly, store at RT in the dark.

2x Ca^{2+} dye loading solution (2x CDLS): 2.5 µl Indo-1 stock in 1 ml MC, use within 1 hr, store at RT in the dark. The final loading conc will be 5 µg/ml. *If loading is not good, try 10 µg/ml.*

10x FACS AB mixes:(need 25 µl per sample)

a) T cell mix: αCD4-APC, clone GK1.5, [1:25] + αCD8-PE, clone 53-6.7, [1:25] in MFC

b) B cell mix:αCD4-APC, clone GK1.5, [1:25] + αCD8-APC, clone 53-6.7, [1:25] + αB220-PE (or CD90-PE) [try 1:25] in MFC

c) B/T cell 5-color stain: αCD4-APC, clone GK1.5, [1:25] + αCD8-PE, clone 53-6.7, [1:25] + αB220 or αCD90-*{determine suitable color, must be compatible with PE, APC, Indo-blue and Indo-violet}* [try 1:25] in MFC. This can then replace (a) and (b).

10x Stimulation-AB Mix 1A:(need 25 µl per sample)

a) CD3 stimulation, mild: αCD3-Bio, clone 2C11, [1:36] in MFC

b) CD3 stimulation, strong: αCD3-Bio, clone 2C11, [1:4] in MFC

c) CD3/CD4 co-stimulation: αCD3-Bio, clone 2C11, [1:4] + αCD4-Bio, clone RM4-4, [1:10] in MFC

d) BCR stimulation: αIgM-Bio, [1:xxx] or αIgD-Bio, [1:xxx] in MFC *{Optimize, start at 1:4}*

$F(ab')_2\alpha$-mouse IgM at a final concentration of 30 µg/ml in MFC.

10x Stimulation-AB Mix 1B:(need 100µl per sample)

a) CD3 stimulation, mild: αCD3-Bio, clone 2C11, [1:xxx] in MMF

{Optimize - try 1:36 to start}

b) CD3 stimulation, strong: αCD3-Bio, clone 2C11, [1: xxx] in MMF

{Optimize - try 1:4 to start }

c) CD3/CD4 co-stimulation: αCD3-Bio, clone 2C11, [1: xxx] + αCD4-Bio, clone RM4-4, [1: xxx] in MMF

{Optimize - try 1:4 respectively 1:10 to start}

d) If unbiotinylated, purified ABs are used: Make a stock in MMF that yields 5 µg/ml final *{Optimize}*

d) BCR stimulation: αIgM-Bio, [1:xxx] or αIgD-Bio, [1:xxx] in MMF*{Optimize, start at 1:4}*

10x Stimulation Mix 2:

Mock: MMF

10x SA to crosslink Bio-ABs: 50 µg/ml streptavidin in MMF, need 50 µl/sample, final conc in tube ~5 µg/ml). SA stock is 10 mg/ml.

xAB mix to crosslink purified non-biotinylated ABs: Make an anti-isotype AB stock in MMF that yields 5 µg/ml final

10x Tg:20 µM Thapsigargin in MMF, need 50 µl/sample (final conc in tube ~2 µM)

10x Ca^{2+}add-back:20 mM Ca^{2+} in MFC (will reconstitute ~1 mM Ca^{2+}due to presence of EGTA in MMF. 20 mM Ca^{2+} can be made by diluting 40 µl 1 M Cacl2 into 2 ml MFC. If it doesn't work well, try 50 mM Ca^{2+} in MFC.)

Ca^{2+}Influx modulating compound: Add xy x desired final concentration in MFC. *Determine factor xy by measuring remaining cell volume in a mock Flow experiment! Tentatively, try 10x cpd in MFC (for example 5 mM or 1 mM IP_4/AM or IP_4/PM)*

Ionomycin:50 µg/ml Ionomycin in MFC, need 50 µl/sample(final conc in tube ~5 µg/ml)

Reagents

Bio-CD3	BD#553060 (2C11), 0.5 mg/ml
Bio-CD4	BD#557443 (RM4-4), 0.5 mg/ml
CD4-APC	Other epitope for FACS: eBiosc #17-0041-83 (GK1.5) 0.2 mg/ml
CD8a-PE	eBiosc #12-0081-83 (53-6.7) 0.2 mg/ml
B220-xxx	xxx
F(ab')$_2\alpha$-mouse IgM	Jackson Immuno Research xxx (?)

αIgM-Bio	xxx
αIgD-Bio	xxx

<u>Ca^{2+} dyes:</u>*(Make in MC buffer)*

Indo-1	Molecular Probes #I1223, 20 x 50 µg

OR

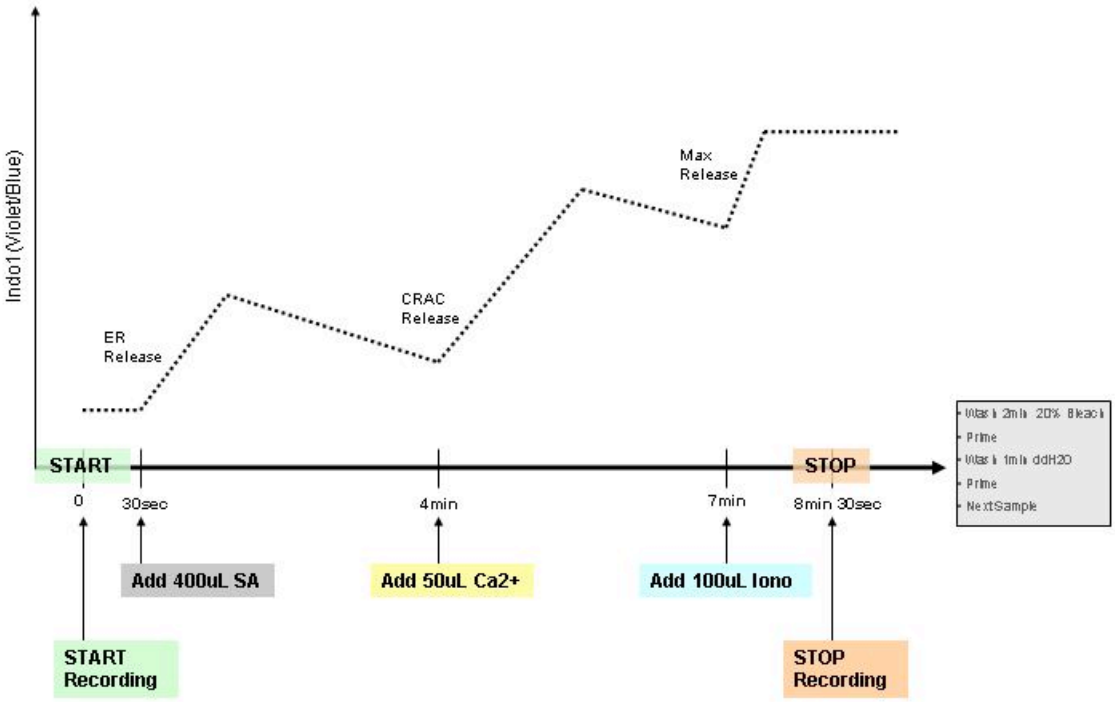
Fura Red, 2 mL/mL (1mM stock, Molecular Probes Cat: F-3021)
Fluo 4, 1 mL/mL (1mM stock, Molecular Probes Cat: F-14201)
Pluronic, 1 mL/mL (20% Solution, Molecular Probes Cat: F-127)

Use Fluo-4 at 3 mg/ml and Fura Red at 5 mg/ml, load in RPMI/3% FCS

<u>SOC Modulators:</u>

Tg: Thapsigargin, xxxx, Sigma (?)
Ionomycin: Ionomycin, xxxx, Sigma (?)

Exemplary reagent addition scheme for a Ca^{2+} add-back experiment
(Here, 400 ul Stimulation Mix 2 are added and the times are slightly different from above. They can be adjusted as needed.)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CRAC Release
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4min
7min
8min 30sec
Wash 2min 20% Bleach
Prime
Wash 1min ddH2O
Prime
NextSample
Add 400uL SA
Add 50uL Ca2+
Add 100uL Iono
START Recording
STOP Recording