Calcium Mobilisation Assay in Response to Chemokine Stimulation

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[Abstract] This assay is used to measure calcium mobilization in lymphocytes (either in primary cells or cell lines) in response to chemokine stimulation using ratiometric analysis. It has also been used for measuring TCR mediated calcium flux. In addition, the same labeling procedure with the addition of brilliant black (a quenching agent) (Sigma-Aldrich, catalog number: 211842) in the loading buffer (at 100 μM) allows for quantification using the FLIPR system on poly-D-lysine plates. Probenicid is an anion-exchange protein inhibitor and prevents the extrusion of the dyes by organic ion transporters.

Materials and Reagents

1. Cells of choice
2. RPMI 1640 media (Life Technologies, Invitrogen™, catalog number: 11875-119)
3. Fetal bovine serum (FBS) (Life Technologies, Invitrogen™, catalog number: 10437-028)
4. PTX (List Biological Labs, catalog number: 183)
5. HBSS (Life Technologies, Invitrogen™, catalog number: 14185-052)
6. BSA (Sigma Aldrich, catalog number: A7030)
7. HEPES (Life Technologies, Invitrogen™, catalog number: 15630-080)
8. Probenecid (Sigma Aldrich, catalog number: P8761)
9. Fluo-4 (Life Technologies, Invitrogen™, catalog number: F14201)
10. Fura-red (Life Technologies, Invitrogen™, catalog number: F3021)
11. Pluronic F-127 (Life Technologies, Invitrogen™, catalog number: P-3000MP)
12. DMSO (Sigma Aldrich, catalog number: D2650)
13. Ionomycin (Sigma Aldrich, catalog number: I0634)
14. NaOH (Thermo Fisher Scientific, catalog number: BP359-212)
15. Assay buffer (see Recipes)
16. Loading buffer (see Recipes)

Equipment

1. FCM (FACS LSR II machine)
2. FACS tube

Procedure

1. Cells are collected, washed and resuspended at ~1-2 x 10^6 cells/ml in RPMI 1640 media (no FBS).
2. If treated with PTX, then samples are split in 2 with one receiving 100 ng/ml PTX and the other PBS. Tubes are rotated at 37 °C for 3-4 h.
3. If cells are not pretreated with PTX, then cells are rotated for 1 h at 37 °C.
4. Cells are then washed twice with assay buffer+ 2.5 mM probenecid.
5. Resuspend cells in loading buffer (assay buffer + probenecid + 2.28 µg/ml Fluo-4 + 0.91 µg/ml Fura-red) at ~4 x 10^6 cells/ml. N.B. In both cases, 50 µg of Fluo-4 and Fura-red are dissolved in 22 µl pluronic F-127 (20% solution in DMSO) and 22 µl DMSO.
6. Light protected tubes are rotated for 30 min at 37 °C.
7. Cells are then washed once and resuspended with assay buffer + probenecid at ~ 5 x 10^6 cells/ml.
8. For SDF-mediated calcium flux analysis, 450 µl of cells are aliquotted into a FACS tube and basal ratio of FITC/PerCP-Cy5.5 levels are recorded. At 1 min, cells are stimulated with 50 µl 10 µg/ml (~125 nM final) SDF-1 and responses recorded for a further 3 min. Finally, cells are stimulated with 50 µl 20 µM ionomycin.
   Note: SDF-1 can be replaced by other chemokines as you desire. However, optimal dose needs to be determined.

Notes

1. Assay buffer can be stored at 4 °C. Allow to reach room temperature before use.
2. Probenecid is prepared fresh each time in 1 N NaOH (stock = 500 mM).
3. Unused Fluo-4 and Fura-Red, once reconstituted in DMSO/Pluronic acid, can be refrozen once for use on a future date.

Recipes

1. Assay buffer (pH 7.4)
   HBSS

0.1% BSA
HEPES (pH 7.4) 25 mM
Probenecid 2.5 mM

2. Loading buffer
   Assay buffer
   Probenecid
   2.28 µg/ml Fluo-4
   0.91 µg/ml Fura-red

Acknowledgments

This protocol was adapted from previously published reports (Cronshaw *et al.*, 2006 and Cronshaw *et al.*, 2010).

References

1. Cronshaw, D. G., Kouroumalis, A., Parry, R., Webb, A., Brown, Z. and Ward, S. G. (2006). Evidence that phospholipase-C-dependent, calcium-independent mechanisms are required for directional migration of T-lymphocytes in response to the CCR4 ligands CCL17 and CCL22. *J Leukoc Biol* 79(6): 1369-1380.
2. Cronshaw, D. G., Nie, Y., Waite, J. and Zou, Y. R. (2010). An essential role of the cytoplasmic tail of CXCR4 in G-protein signaling and organogenesis. *PLoS One* 5(11): e15397.