

Assessment of Human Dendritic Cell Antigen Uptake by Flow Cytometry

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[Abstract] Antigen uptake by dendritic cells is the first key step towards induction of antigen-specific T-cell responses. This flow cytometry-based protocol describes the analysis of dendritic cell uptake of soluble antigens through two different mechanisms: non-specific macropinocytosis (using Lucifer Yellow CH), and receptor-mediated endocytosis (using DQTM Ovalbumin). The protocol is generated based on data presented in Olivar *et al.* (2013).

Materials and Reagents

1. Whole blood
2. RPMI 1640 Medium, GlutaMAXTM (Gibco[®], catalog number: 61870)
3. DPBS without Ca²⁺ and Mg²⁺ (Gibco[®], catalog number: 14190-169)
4. 100x liquid Penicillin-Streptomycin (Gibco[®], catalog number: 15140-122)
5. 200 mM L-Glutamine solution (Gibco[®], catalog number: 25030-024)
6. Fetal Bovine Serum (FBS) (Gibco[®], catalog number: 10270106)
7. Lipopolysaccharide from *Escherichia coli* 026:B6 (10 mg) (Sigma-Aldrich, catalog number: L2654)
8. Lucifer Yellow CH dilithium salt (25 mg) (Sigma-Aldrich, catalog number: L0259)
9. DQTM Ovalbumin (1 mg) (Molecular Probes[®], catalog number: D-12053)
10. Ficoll-Paque PLUS (General Electric Company, catalog number: 17-1440-03)
11. GMP Recombinant Human Interleukin-4 (50 µg, 13 x 10⁶ IU/mg) (Gentaur Molecular Products, catalog number: 04-GMPhuL4-50 µg)
12. Recombinant Human GM-CSF (300 µg, 3.88 x 10⁶ IU/vial) (Gentaur Molecular Products, catalog number: 04-RHUGM-CSF-300 µg)
13. IL-4
14. Bovine Serum Albumin Fraction V (BSA) (Roche Diagnostics, catalog number: 10735078001)
15. FITC-conjugated anti-CD14 (RMO52) (Beckman Coulter, catalog number: IM0645U)
16. FITC-conjugated anti-IgG2a (7T4-1F5) (Beckman Coulter, catalog number: IM0645U)
17. Perfect-Count MicrospheresTM (Cytognos S.L., catalog number: CYT-PCM-50)
18. NaN₃ (Sigma-Aldrich, catalog number: 71289)

19. FACS buffer (see Recipes)
20. Complete medium (see Recipes)
21. DQ-OVA (1 mg/ml) (see Recipes)
22. Lucifer Yellow (10 mg/ml) (see Recipes)
23. rHuIL-4 (500 IU/ml) (see Recipes)
24. rHuGM-CSF (800 IU/ml) (see Recipes)
25. LPS (1 mg/ml) (see Recipes)

Equipment

1. 15 ml Ficoll-Paque PLUS
2. 60-mm cell culture plates (Corning, catalog number: 15430166)
3. Cytometer tubes (BD Falcon tubes, round-bottom) (Becton, Dickinson and Company, catalog number: 352052)
4. Centrifuge Heraeus Multifuge 3 L-R (Heraeus Holding, catalog number: 75004370)
5. 37 °C, 5% CO₂ cell culture incubator
6. BD FACSCalibur flow cytometer (Becton Dickinson, catalog number: 342975)

Software

1. CellQuest Pro software (Becton, Dickinson and Company, catalog number: 643436)

Procedure

1. Dilute 25 ml of buffy coat (initial leukocyte concentrate from a whole blood donation) with the same volume of DPBS.
2. Prepare two 50 ml tubes with 15 ml Ficoll-Paque PLUS. Carefully layer 25 ml of the diluted blood sample on Ficoll-Paque PLUS. Important: When layering the sample do not mix Ficoll-Paque PLUS and the diluted blood sample.
3. Centrifuge at 400 x g for 25 min at 18-20 °C. Important: Brakes off.
4. Soak up the white interphase between the diluted plasma fraction and the transparent ficoll fraction with a pipette and transfer it into a fresh tube.
5. Wash twice with DPBS.
6. Resuspend the pellet in 5 ml DPBS.
7. In a cytometer tube mix 3 µl of FITC-conjugated anti-CD14 antibody plus 60 µl DPBS and 20 µl of cellular suspension.
8. Incubate 15-18 min at room temperature.

9. Add 120 μ l DPBS and count the number of CD14⁺ monocytes by flow cytometry using Perfect-Count Microspheres™ according to the manufacturer's instructions.
10. Plate monocytes at 1×10^6 cells/ml in 60-mm culture plates, in RPMI 1640 medium without serum, and allow to adhere for 2 h at 37 °C in 5% CO₂.
11. Remove the non-adherent cells by washing in DPBS. The final population of adherent cells contains 75-80% of monocytes, as demonstrated by flow cytometry of anti-CD14–stained isolates.
12. Generate monocyte-derived DCs by supplementing the monocyte cultures with 1 ml of complete RPMI 1640 medium plus GM-CSF (800 IU/ml) and IL-4 (500 IU/ml).
13. At day 3 add 1ml of complete RPMI 1640 medium plus GM-CSF (800 IU/ml) and IL-4 (500 IU/ml).
14. For DC maturation, at day 5 replace the old medium with fresh complete RPMI 1640 medium plus GM-CSF (800 IU/ml) and IL-4 (500 IU/ml) and stimulate the immature DCs for 48 h with 5 μ g/ml LPS.
15. Harvest the non-adherent cells floating in the culture medium in a 15 ml tube (at day 5 for immature DCs; at day 7 for mature DCs). Add 2 ml DPBS (37 °C), rinse and collect the adhered cells by pipetting. Wash twice more with DPBS and pool both floating and adherent cells. Centrifuge and resuspend the pellet in 500 μ l of complete medium.
16. Prepare two cytometer tubes with 60 μ l of complete medium plus 4 μ l DQ-OVA (stock: 1 mg/ml) at 37 °C or 0 °C.
17. Prepare two cytometer tubes with 60 μ l of complete medium plus 6 μ l Lucifer Yellow CH (stock: 10 mg/ml) at 37 °C or 0 °C.
18. Add 100 μ l of cell suspension ($\sim 2 \times 10^5$ cells/ml) to each cytometer tube.
19. Incubation time: 15 min for DQ-OVA; 120 min for Lucifer Yellow CH. The fluorescence of OVA labeled with BODIPY FL dye (DQ-OVA) is self-quenched until the OVA is taken up via the mannose receptor and degraded only by endolysosomal proteases. Lucifer Yellow CH (LY) is a hydrophilic tracer for fluid-phase macropinocytosis. LY is not degraded and is nontoxic at concentrations up to 6 mg/ml.
20. Stop the incubations by adding 1 ml cold FACS buffer.
21. Wash the cells two times with cold FACS buffer.
22. Analyze the incorporated fluorescence of both immature DCs (Figure 1) and mature DCs using flow cytometry. Compare the histograms and corresponding mean fluorescence intensities (MFI) between cells incubated at 37 °C (specific uptake) and cells incubated at 0 °C (non-specific uptake: passive diffusion...).

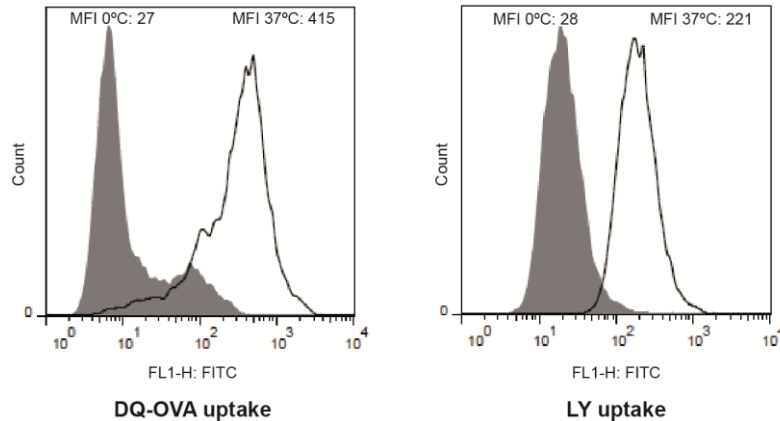


Figure 1. Analysis of the endocytic activity of immature DCs by flow cytometry. The endocytic activity of monocyte-derived immature DCs was assessed measuring the uptake of the fluorescent reporters DQ-OVA (receptor-mediated endocytosis) and Lucifer Yellow CH (fluid-phase endocytosis). Representative histograms are shown. Dye uptake controls are displayed in gray. The median fluorescence intensities (MFI) for the different fluorescent cell populations are indicated in each histogram.

Recipes

1. FACS buffer (500 ml)
 - Mix 5 g BSA and 0.5 g NaN₃ with 500 ml 1x DPBS
 - Filter sterilize (0.45 µm)
 - Stored at 4 °C
2. Complete medium
 - RPMI 1640 medium, GlutaMAX™
 - 100 µg/ml streptomycin
 - 100 IU/ml penicillin
 - 2 mM L-glutamine
 - 10% heat-inactivated FBS
 - 800 IU/ml GM-CSF
 - 500 IU/ml IL-4
 - Stored at 4 °C
3. DQ-OVA (1 mg/ml)
 - A 1 mg/ml solution can be prepared by dissolving the contents of one vial in 1 ml of DPBS.
 - Once reconstituted, the solution should be stored at -20 °C, protected from light.
4. Lucifer Yellow (10 mg/ml)

- A 10 mg/ml solution can be prepared by dissolving the contents of one vial in 2.5 ml of dH₂O. Once reconstituted, the solution should be stored at 4 °C, protected from light.
5. rHuIL-4 (500 IU/ml)
A 500 IU/ml solution can be prepared by dissolving the contents of one vial in 500 µl of dH₂O. Once reconstituted, the solution should be stored at -80 °C.
 6. rHuGM-CSF (800 IU/ml)
A 800 IU/ml solution can be prepared by dissolving the contents of one vial in 2 ml of dH₂O. Once reconstituted, the solution should be stored at -80 °C.
 7. LPS (1 mg/ml)
A 1 mg/ml solution can be prepared by dissolving the contents of one vial in 1 ml of DPBS. Once reconstituted, the solution should be stored at -20 °C.

Acknowledgments

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References

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