Endosomal pH Measurement in Bone Marrow Derived Dendritic Cells

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[Abstract] Endosomes embraces different set of compartments such as early endosomes, intermediate endosomes and late endosomes or lysosomes. They become acidic as they mature. This acidification is generated by the vacuolar membrane proton pump V-ATPase that is recruited in late endosomes. This protocol described the measurement of endosomal pH using dextran molecules labelled with pH sensitive and insensitive dyes.

Materials and Reagents

1. CO_2 independent medium (Invitrogen, catalog number: 18045054)
2. Iscove's Modified Dulbecco's Medium (IMDM) (Sigma-Aldrich, catalog number: I3390)
3. 10% Fetal Bovine Serum (FBS) (Hyclone/PAA, catalog number: sv143-03)
4. Penicillin-streptomycin (100 Units/ml, 100 µg/ml) (Sigma-Aldrich, catalog number: P11-010)
5. Glutamine (Sigma-Aldrich, catalog number: G75013)
6. 2-mercaptoethanol (Sigma-Aldrich, catalog number: M6250)
7. Granulocyte-macrophage colony stimulating factor (GM-CSF) (Peprotech, catalog number: 315-03)
8. 40,000 MW Dextran fluorescein (10 mg/ml) (Molecular Probes)
9. 40,000 MW Dextran Alexa 647 (10 mg/ml) (Molecular Probes)
10. 5 mM EDTA (Invitrogen, catalog number: 15575-038)
11. 1% Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A2153)
12. Triton X-100 (Sigma-Aldrich, X-100) kept at room temperature
13. 1x PBS (see Recipes)
14. Conditioned complete medium (see Recipes)

Equipment

1. Water bath (37 °C)

2. Incubator (37 °C)
3. The FACSCalibur flow cytometer (Becton Dickinson)
4. Hemocytometer
5. Centrifuge

Procedure

1. Detach bone marrow-derived dendritic cells (BMDCs) with 1x PBS-5 mM EDTA (10 min at 37 °C).
2. Wash the cells with 1x PBS twice by centrifugation at 367 *x g* for 10 min.
3. Count cells. A total of 3 x 10^6 cells are required for pH measurement at different time points (kinetics of 10 min, 20 min, 30 min and 60 min for example). Additionally, 6 x 10^6 cells are needed to acquire the pH standard curve.
4. For measurement of pH at different time points, resuspend the cells in 100 µl total volume of prewarmed conditioned complete medium containing 1 mg/ml of fluorescein- and 0.5 mg/ml Alexa-647-labeled 40,000 MW dextrans. Pulse cells in a water bath set at 37 °C for 10 min.
5. Stop the reaction by adding a large volume (1 ml) of cold 1x PBS-1% BSA and wash cells extensively (6 times) with the same buffer to get rid of the non-internalised dextrans.
6. After washing, resuspend the cells in prewarmed conditioned complete medium (1 ml) and incubate at 37 °C for different time points (chase). The pulse (10 min) corresponds to early endosomes (EE), the chase of 40 min corresponds to intermediate endosomes (IE) and the 110 min of chase corresponds to the lysosomes.
7. Stop the reaction each time by immediately adding cold PBS and placing the tubes on ice.
8. Rapidly, analyse the cells by FACS, via a FL1/FL4 gate selective for cells that have endocytosed both fluorescent probes and determine the ratio of the mean fluorescence intensity (MFI) emission between the two probes.
9. For the pH standard curve, resuspend the cells in 200 µl total volume of prewarmed conditioned complete medium containing 1 mg/ml of fluorescein- and 0.5 mg/ml Alexa-647-labeled 40,000 MW dextrans and pulse the cells in a water bath set at 37 °C for 20 min. Repeat step 5 and split cells into nine 1.5 ml Eppendorf tubes for a pH measurement ranging from 4 to 8.
10. Prepare several buffers that differ in pH by 0.5 units using prewarmed CO_2 independent medium. Adjust the pH with citric acid or NaOH.
11. Resuspend each cell pellet in a different pH solution supplemented with 0.001% of Triton X-100 to slightly permeabilise the cells and to give access to the external prefixed pH solution into the cell.

12. Analyse immediately by FACS and determine the ratio of the mean fluorescence intensity (MFI) emission between the two fluorescent probes at each pH. Make the standard curve by plotting the different MFI ratio values that correspond to each pH and apply this formula to the MFI ratio values obtained before (steps 1-8, Figure 1).

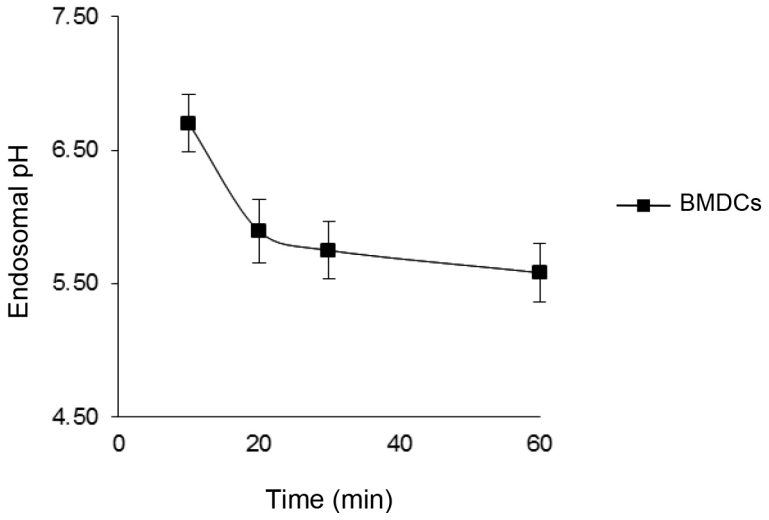

Figure 1. Kinetic of endo-lysosomal pH in BMDCs pulsed with a mixed of fluorescein- and Alexa-647-labeled 40,000 MW dextrans for 10 min and then chased for different times

Recipes

1. 1x PBS
   137 mM NaCl
   2.7 mM KCl
   8 mM Na_2HPO_4
   1.46 mM KH_2PO_4
   Keep 1x PBS cold
2. Conditioned complete medium
   IMDM supplemented with
   10% FBS
   100 Units/ml, 100 µg/ml penicillin-streptomycin
   2 mM glutamine
   50 µM 2-mercaptoethanol
   10 ng/ml GM-CSF

Acknowledgments

This protocol is adapted from Savina *et al.* (2010); Maschalidi *et al.* (2012) and Sepulveda *et al*. (2009).

References

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