

Separation of Intracellular Vesicles for Immunoassays

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[Abstract] The endosome/lysosome systems play important roles in immune cell functions as signaling platforms. Immune cells utilize these endosome/lysosome for signal transduction or intercellular communication to elicit the proper immune responses, regulating the localization or the association of the signaling complexes. Here we introduce the procedures to separate the intracellular vesicles such as endosomes or lysosomes, which could be useful to identify the subcellular localization of the signaling complexes.

Materials and Reagents

1. Cells (e.g. RAW264.7, Raji, mouse naïve B cells)
2. Trypan blue staining (0.4% solution) (Life Technologies, catalog number: 15250-061)
3. CD43-microbeads (Miltenyi Biotec, catalog number: 130-049-801)
4. Blocking One (Nacalai tesque, catalog number: 03953-95)
5. Anti-mouse LAMP1 (eBioscience, catalog number: 1D4B)
6. Anti-GM130 (BD biosciences, catalog number: 35/GM130)
7. Anti-Rab5 (Santa Cruz Biotechnologies, catalog number: D-11)
8. Anti-Calnexin (Enzo Life Sciences, catalog number: ADI-SPA-860)
9. Anti-COX IV (Cell Signaling Technology, catalog number: 4844)
10. Anti-rat Ig(H+L)-HRP (Jackson ImmunoResearch Laboratories, catalog number: 712-036-153)
11. Anti-mouse Ig(H+L)-HRP (Jackson ImmunoResearch Laboratories, catalog number: 115-035-062)
12. Anti-rabbit Ig(H+L)-HRP (Jackson ImmunoResearch Laboratories, catalog number: 111-035-144)
13. ECL substrate (Thermo Fisher Scientific, catalog number: 34095)
14. Protease Inhibitor Cocktail (Thermo Fisher Scientific, catalog number: 87785)
15. Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, catalog number: 78420)
16. 60% OptiPrep (Axis-Shield plc, catalog number: 1114542)
17. Solution A (see Recipes)
18. Solution B (see Recipes)
19. Solution C (see Recipes)
20. Solution D (see Recipes)

21. Solution E (see Recipes)
22. 30%, 23%, 17%, 11%, and 5% Optiprep (2 ml each) (see Recipes)
23. TBS-T (see Recipes)

Equipment

1. 10 cm dish
2. Centrifuge tube (Beckman Coulter, catalog number: 347357)
3. Hemocytometer
4. Centrifuge (with cooling function will be recommended)
5. 1 ml syringe with 29-gauge needle

Procedure

Protocol A should be chosen when regular cell-line (e.g. RAW264.7, Raji, or HEK293T) or cells having large cytosol in volume are analyzed whereas Protocol B should be chosen for small cells or cells having small cytosol, such as lymphocytes.

A. Procedures for cell extraction from 10 cm dish (2 dishes for each sample)

1. Layer 360 μ l of the each gradient solution (30%, 23%, 17%, 11%, and 5% OptiPrep) in the Centrifuge tube(s) with P200 micropipette, as shown in Figure 1.

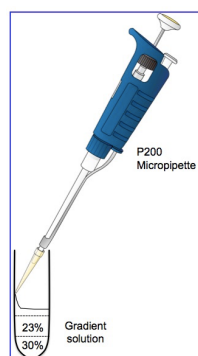


Figure 1. Illustration showing the way to layer the gradient solution using P200 micropipette. Pour 360 μ l of each gradient solution carefully into a centrifuge tube using P200 micropipette (180 μ l x 2 times), not to make bubbles nor disturb the other layers. Layer in a centrifuge tube from the heaviest (30%) to lightest (5%) solution.

2. Leave tubes at 4 °C for more than one hour and keep it still until use.
3. Confirm that cells (e.g. RAW264.7, or Raji) are healthy and 75-80% confluent under a microscopy.
4. (Optional) Treat with stimulants for the desired period (e.g. LPS at 100 ng/ml for 30 min).
5. Wash twice with 10 ml of ice-cold PBS, transfer to suitable tubes, and determine cell

density and viability with a hemacytometer and trypan blue staining.

6. Transfer $1.5 \sim 2.0 \times 10^7$ cells to new tubes and centrifuge at 1,100 rpm for 5 min at 4 °C.
7. Aspirate the supernatant, tap tubes to loosen cells, and add 450 μ l of ice-cold Solution E per tube.
8. Homogenize cells by 80-100 passages through a 29-gauge needle until cells show roughly 70-80% trypan blue positive. Cells should be kept cold.
9. Centrifuge the homogenate at $1,000 \times g$ for 5 min at 4 °C and transfer the whole supernatant (the post-nuclear fraction) to new 1.5 ml tubes and centrifuge at $1,000 \times g$ for 5 min at 4 °C again. Repeat this step until pellet of cell debris can not be seen after centrifugation (usually 3 times).

Note: The whole supernatant should be taken for the second centrifugation, and the same for the third centrifugation. Initial volume of supernatant after centrifugation should be roughly 400 μ l, and after 2 times centrifugation it will be reduced to ca. 350 μ l.

10. Carefully layer 300 μ l of the supernatant on the top of the gradient solution made in step A1 (Figure 3).
11. Set the centrifuge tubes in the chilled ultracentrifuge rotor TLS-55.
12. Centrifuge for 4 h at 39,000 rpm ($130,000 \times g$) at 4 °C by Optima MAX-TL.
Note: Set acceleration (Accel) and deceleration (Decel) at 8 and 0 (free deceleration), respectively.
13. Harvest 185 μ l per one fraction to make 11 fractions using P200 micropipette, and transfer to 1.5 ml microtubes.
14. Store at -80 °C until use.

B. Procedures for mouse naïve B cells

1. Layer 360 μ l of the each gradient solution (30%, 23%, 17%, 11%, and 5% OptiPrep) in the Centrifuge tube(s) with P200 micropipette.
2. Leave tubes at 4 °C for more than one hour and keep it still until use.
Separate the naïve splenic B cells by using CD43-microbeads and MACS according to the manufacturer's instruction (please see the following link:
<http://www.miltenyibiotec.co.jp/~media/Images/Products/Import/0001200/IM0001263.ashx>).
3. (Optional) Treat $1.5 \sim 2.0 \times 10^8$ of B cells with stimulants for the desired period (e.g. CpG-1668 at 100 nM for 1 h).
4. Wash twice with 10 ml of ice-cold PBS in suitable tubes, and determine cell density and viability with a hemacytometer and trypan blue staining.
5. Transfer the cells to new tubes and centrifuge at 1,100 rpm for 5 min at 4 °C.
6. Aspirate the supernatant, tap tubes to loosen cells, and add 450 μ l of ice-cold Solution E per tube.
7. Homogenize cells by passing roughly 200 times through a 29-gauge needle until cells

show 70-80% trypan blue positive. Cells should be kept cold on ice.

8. Centrifuge the homogenate at 1,000 x g for 5 min at 4 °C and transfer the supernatant (the post-nuclear fraction) to new 1.5 ml tubes. Repeat this step three times.
9. Carefully layer 300 µl of the supernatant on the top of the gradient.
10. Set the tubes in the chilled ultracentrifuge rotor TLS-55.
11. Centrifuge for 4 h at 39,000 rpm (130,000 x g) at 4 °C by Optima MAX-TL.
Note: Set acceleration (Accel) and deceleration (Decel) at 8 and 0 (free deceleration), respectively.
12. Harvest 290 µl per one fraction to make 7 fractions using P200 micropipette, and transfer to 1.5 ml microtubes.
13. Store at -80 °C until use.

C. Evaluation of the fractionation

1. Take 30 µl of the fractions and mix with the equal volume of 2x SDS-PAGE sample buffer.
2. After brief denaturing at 95 °C, apply 15 µl of the samples to run SDS-PAGE and transfer to PVDF membrane.
3. Block the blot with Blocking One, followed by incubation with the primary antibodies against organelle markers. Following antibodies used in this protocol; anti-LAMP1 (dilution 1:1,000) for the detection of the late endosome/lysosome, anti-GM130 for cis-Golgi, anti-Rab5 for endosome, anti-Calnexin for ER, and anti-COX IV for mitochondria, respectively.
4. Wash the blots 3 times with TBS-T to remove excess antibodies (5 min x 3 times).
5. Incubate with the proper secondary antibodies conjugated with HRP for 1 h [Anti-rat Ig (H+L) for anti-LAMP1, anti-mouse Ig (H+L) for anti-GM130 or anti-Rab5, anti-rabbit Ig (H+L) for anti-Calnexin or COX IV, respectively. Concentration of secondary antibodies is roughly at 50 ng/ml].
6. Wash the blots with TBS-T to remove excess antibodies (15 min x 3 times).
7. Incubate with ECL substrate, and detect the luminescence signals (Figure 2).

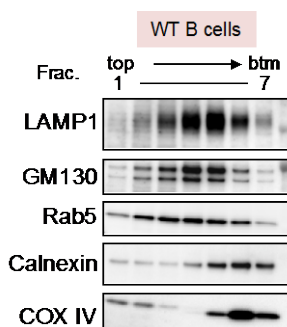


Figure 2. Intracellular vesicles of mouse naïve B cells were fractionated and their distribution was analyzed by western blotting with the specific antibodies against the following organelle markers; late endosome/lysosome with LAMP1, cis-Golgi

with GM130, pan-endosome with Rab5, ER with Calnexin, and mitochondria with COX IV, respectively.

Notes

1. For homogenization of cells, Dounce homogenizer or other devices can be used, as the degree of homogenization difficulty depends on the cell types.
2. For small cells such as mouse naïve B cells, number of passage with 29G syringe may differ depending on the cell density.

Recipes

1. Solution A
60% OptiPrep
2. Solution B
OptiPrep diluent

(Components)	100 ml	(Final conc.)
H ₂ O	~ 42.8 ml	
1 M Hepes (pH7.5)	15 ml	150 mM
1 M KCl	23.5 ml	235 mM
1 M MgCl ₂	1.2 ml	12 mM
1 M CaCl ₂	2.5 ml	25 mM
0.2 M EGTA	15 ml	30 mM

Adjust to pH 7.0 with 1 M KOH and make up to 100 ml

3. Solution C
40% OptiPrep working solution
2 vol. of Solution A + 1 vol. of Solution B
4. Solution D
Working solution diluent

(Components)	100 ml	(Final conc.)
H ₂ O	~ 81 ml	
1 M Hepes (pH7.5)	5 ml	50 mM
1 M KCl	7.8 ml	78 mM
1 M MgCl ₂	400 µl	4 mM
1 M CaCl ₂	840 µl	8.4 mM
0.2 M EGTA	5 ml	10 mM

Adjust to pH 7.0 with 1 M KOH and make up to 100 ml

5. Solution E
Homogenization medium

(Components)	100 ml	(Final conc.)
H ₂ O	~ 81 ml	
1 M Hepes (pH7.5)	5 ml	50 mM
1 M KCl	7.8 ml	78 mM
1 M MgCl ₂	400 µl	4 mM
1 M CaCl ₂	840 µl	8.4 mM
0.2 M EGTA	5 ml	10 mM
Sucrose (FW 342.30)	8.56 g	250 mM

Adjust to pH 7.0 with 1 M KOH and make up to 100 ml

Add Halt Protease Inhibitor Cocktail and Halt Phosphatase Inhibitor Cocktail before use

6. 30%, 23%, 17%, 11%, and 5% Optiprep (2 ml each)

(Components)	30%	23%	17%	11%	5%
Solution C (40% Optiprep)	1500 µl	1150 µl	850 µl	550 µl	250 µl
Solution D	500 µl	850 µl	1150 µl	1450 µl	1750 µl
Halt Protease Inhibitor*	20 µl	20 µl	20 µl	20 µl	20 µl
Halt Phosphatase Inhibitor†	20 µl	20 µl	20 µl	20 µl	20 µl

* Halt Protease Inhibitor Cocktail

† Halt Phosphatase Inhibitor Cocktail

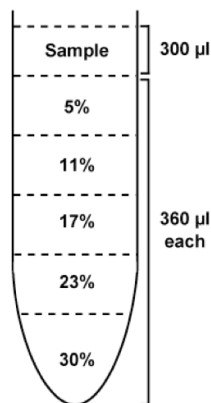


Figure 3. Diagram of completed Opti-prep gradient before ultracentrifugation

7. TBS-T

(Components)	1 L	(Final conc.)
1 M Tris-HCl (pH7.5)	20 ml	20 mM
NaCl	8 g	137 mM
Tween 20	1 ml	0.1%

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