

ImageStream® Endosome Lysosome Protocol

Probing for Colocalization to Endosomes & Lysosomes

Materials:

- Ramos cells
- Test protein (Rituximab-ADC @ 1.8 mg/mL)
- Mouse anti-human EEA1 (BD Biosciences cat# 610457)
- Biotinylated mouse anti-human CD107a (Lamp-1) (BD Biosciences cat# 555799)
- Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) (Molecular Probes cat# A11013)
- PE-conjugated F(ab')₂ fragments goat anti-human IgG, Fcγ fragment specific (Jackson ImmunoResearch cat# 109-116-088)
- Cychrome-Streptavidin (BD Biosciences cat# 554062)
- Cytotfix/Cytoperm Kit (BD Biosciences cat# 554714)
- 1% paraformaldehyde in PBS

Samples (3 x 10 ⁶ cells per test):	timepoint	temperature
1. unstained control	0.5 h	4°C
2. AF488-EEA1	0.5 h	4°C
3. PE-labeled test antibody (human Rituximab)	0.5 h	4°C
4. Cychrome-Lamp1	0.5 h	4°C
5. AF488-EEA1 + PE-test antibody + Cychrome-Lamp1	0.5 h	4°C
6. AF488-EEA1 + PE-test antibody + Cychrome-Lamp1	2 hours	37°C
7. AF488-EEA1 + PE-test antibody + Cychrome-Lamp1	4 hours	37°C
8. AF488-EEA1 + PE-test antibody + Cychrome-Lamp1	8 hours	37°C

Cell preparation

We used Ramos cells cultured in RPMI supplemented with 10% fetal calf serum in an incubator containing 5% CO₂ at 37° C.

Staining protocol

Staining done in polypropylene (NOT POLYSTYRENE) microcentrifuge tubes. All washes done at 300 x g 10' 4° C in a swinging bucket rotor.

1. Culture cells to mid-exponential growth
2. For samples 1-5, Wash/Resuspend cells @ 10⁷ cells/mL in **cold** growth media + test antibody (5µg/mL, samples 3&5) and incubate 30' on ice.

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3. For samples 6-8, Wash/Resuspend cells @ 10^7 cells/mL in **warm** growth media + test antibody ($5\mu\text{g}/\text{mL}$) and incubate @ 37°C for times indicated above.
4. Wash, then fix and permeabilize cells with $100\mu\text{L}$ of Perm/Fix solution for 20' RT.
5. Wash cells with 1mL of Perm/Wash buffer.
6. Resuspend in $100\mu\text{L}$ Perm/Wash + $10\mu\text{g}/\text{mL}$ PE-goat anti-human IgG for 20' on ice.
7. Wash cells with 1mL of Perm/Wash buffer.
8. Resuspend in $100\mu\text{L}$ Perm/Wash + $10\mu\text{g}/\text{mL}$ of EEA1 for 20' on ice.
9. Wash cells with 1mL of Perm/Wash buffer.
10. Resuspend in $100\mu\text{L}$ Perm/Wash + $10\mu\text{g}/\text{mL}$ AF488-goat anti-mouse IgG for 20' on ice.
11. Wash cells twice with 1mL of Perm/Wash buffer.
12. Resuspend in $80\mu\text{L}$ Perm/Wash + $20\mu\text{L}$ biotinylated anti-Lamp-1 antibody for 20' on ice.
13. Wash cells with 1mL of Perm/Wash buffer.
14. Resuspend in $100\mu\text{L}$ Perm/Wash containing $10\mu\text{g}/\text{mL}$ of Cychrome-Streptavidin antibody for 20' on ice.
15. Wash cells with 1mL of Perm/Wash buffer.
16. Fix cells in $50\mu\text{L}$ of 1% paraformaldehyde.