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)
- Cytofix/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% CO_2 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 @ 10^7 cells/mL in **cold** growth media + test antibody (5μg/mL, samples 3&5) and incubate 30' on ice.

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