Illuminating Endocytosis with Targeted pH-sensitive Fluorescent Compounds

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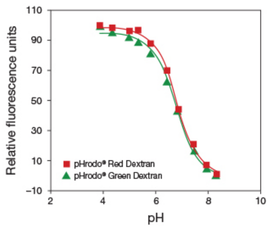
ABSTRACT

The basic cellular internalization mechanisms of endocytosis are important to many areas of cell biology, and especially to the proper function of therapeutic antibodies. However, the ability to study these internalization processes has historically been limited by the lack of tools to directly monitor the internalization and subsequent acidification of extracellular matter.

Here we present experimental data demonstrating the use of novel pH-sensitive fluorescent compounds to track the three primary mechanisms of endocytosis in live cells. pH-sensitive conjugates of EGF and transferrin were used to monitor and quantify receptor-mediated endocytosis. Nonspecific pinocytosis was studied using dextran covalently labeled with pH-sensitive fluorescent dyes. Phagocytosis was visualized in live-cell time-lapse microscopy using lyophilized bacteria labeled with pH sensing dyes.

Preliminary results are presented for quantification of cytosolic pH under normal and pharmaceutically modified conditions using novel probes for intracellular pH. Additionally, we present two new approaches to directly visualize endocytosis and trafficking of therapeutic antibodies and ADCs in live cells without affecting antibody structure or function.

Figure 1. pHrodo™ pH Sensor Dyes

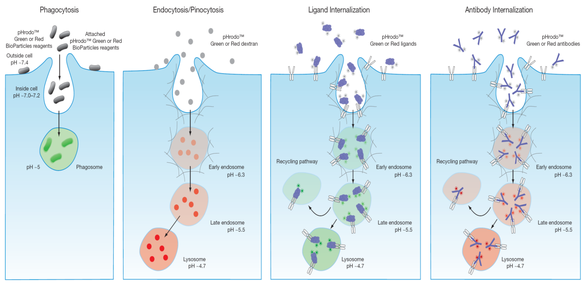


pH response profile of pHrodo™ Red and pHrodo™ Green dextrans. Citrate, MOPS, and borate buffers were used to span the pH range from 4 to 8.5.

pHrodo™ Green
Ex/Em max 505/525 nm

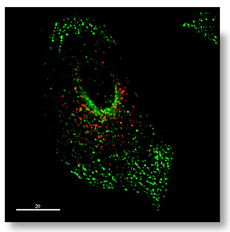
pHrodo™ Red
Ex/Em max 550/585 nm

Figure 2. Fluorogenic pH Sensing of Cellular Internalization Processes



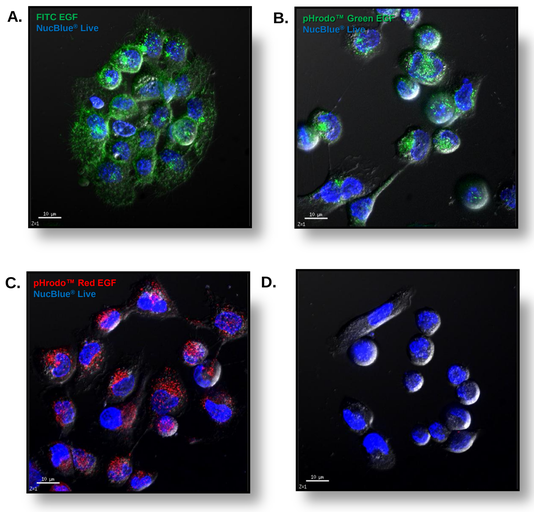
The fluorescence intensity of pHrodo™ Red and Green dyes dramatically increases as pH drops from neutral to acidic, making them ideal tools to study endocytosis, phagocytosis, and ligand and antibody internalization.

Figure 3. Monitoring endocytosis with fluorescently-labeled dextran



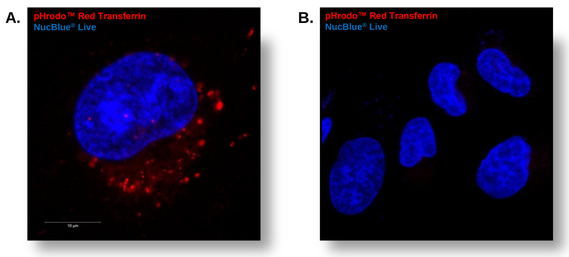
HeLa cells were treated overnight with CellLight® Early Endosome GFP and then loaded with 10 µg/ml pHrodo™ Red 10,000 MW dextran to visualize pinocytosis. pHrodo™ Red dextran appeared in GFP+ vesicles approximately 5 minutes after loading, indicating both internalization of dextran and acidification of early endosomes.

Figure 4. Fluorogenic Indication of Epidermal Growth Factor Internalization



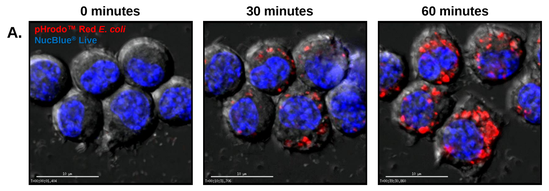
EGF internalization is typically monitored using fluorescent dye conjugates. A431 epidermoid carcinoma cells were pretreated with 20 µg/mL native EGF or vehicle for 60 minutes. Cells were labeled with NucBlue™ Live Cell Stain and treated with 5 µg/mL of FITC, pHrodo™ Green, or pHrodo™ Red EGF conjugates at 37ºC for 30 minutes. Cells were washed twice and imaged with standard DAPI, FITC, and TRITC filters. **A.** FITC fluorescence is seen on the cell surface and throughout the endocytic pathway. **B-C.** pHrodo™ Red and Green fluorescence is limited to punctate spots, indicating endocytic vesicles with low pH. **D.** Preloading cells with unlabeled EGF resulted in a greatly diminished internalization of fluorescently-labeled EGF, an important control.

Figure 5. Visualization of Transferrin Receptor Internalization



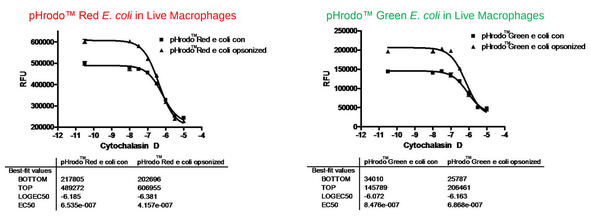
Fluorescent transferrin conjugates are frequently used to visualize recycling endosomal pathways. A431 epidermoid carcinoma cells were pretreated with 100 µg/mL transferrin or vehicle for 60 minutes. Cells were treated with 50 µg/ml pHrodo™ Red transferrin and incubated at 37ºC for 30 minutes. Cells were washed twice with LCIS and imaged with standard DAPI and TRITC filters. **A.** Punctate red spots are visible where labeled transferrin was internalized and entered acidic endosomes. **B.** Preloading with unlabeled transferrin to saturate transferrin receptors greatly diminished internalization of the labeled transferrin.

Figure 6. Live-Cell Visualization of Phagocytosis



The dynamic pH-response of pHrodo™ dyes enable visualization of acidification processes such as the phagocytosis of bacteria by macrophages. Mouse monocyte/macrophage cells (MMM cells, ATCC) were labeled with NucBlue™ Live Cell Stain for 15 minutes in Live Cell Imaging Solution (LCIS, cat #A14291DJ). 100 µg of pHrodo™ Red E. coli BioParticles® conjugates were added and imaged with standard DAPI and TRITC filters after 0, 30, and 60 minutes. Nonfluorescent at neutral pH outside the cell, the pHrodo™ BioParticles® conjugates dramatically increase in fluorescence as they are internalized and acidified in lysosomes.

Figure 7. HTS Analysis of Phagocytosis Inhibition

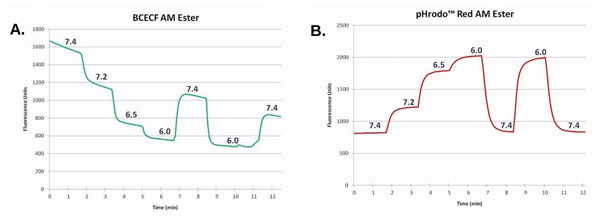


Best-fit values	pHrodo™ Red e coli con	pHrodo™ Red e coli opsonized
BOTTOM	217805	202696
TOP	489272	606955
LOGEC50	-6.185	-6.381
EC50	6.535e-007	4.157e-007

Best-fit values	pHrodo™ Green e coli con	pHrodo™ Green e coli opsonized
BOTTOM	34010	25787
TOP	145789	206461
LOGEC50	-6.072	-6.163
EC50	8.476e-007	6.868e-007

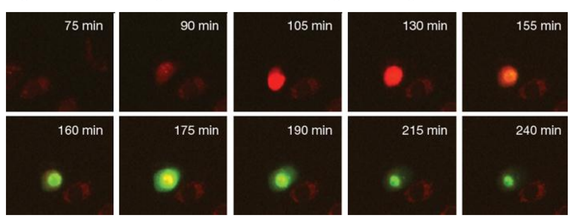
A 384-well dish of MMM cells was incubated with cytochalasin D (10 µM to 3 pM) and incubated for 15 minutes at 37ºC in triplicate rows. pHrodo™ Green *E. coli* BioParticles® conjugates were resuspended in LCIS at 2X working concentration (2 mg/mL) and added to cells. Cells were incubated at 37ºC for 90 minutes to allow phagocytosis to run to completion. The plates were scanned on a microplate reader with 490Ex/525Em, 515 cutoff.

Figure 8. Real-time Intracellular pH measurement



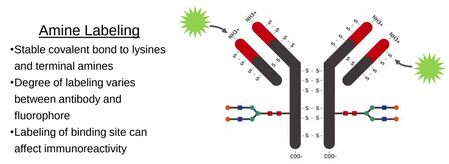
NIH-3T3 cells were loaded with 5 µM BCECF AM Ester or 5 µM pHrodo™ Red AM Intracellular pH Indicator. Cells were washed with a series of HEPES-based pH standard buffers containing 10 µM nigericin and 10 µM valinomycin to clamp intracellular pH to the indicated values. BCECF AM fluorescence decreases stepwise as pH is reduced, and decreases continuously due to photobleaching. pHrodo™ Red AM fluorescence increases as pH is reduced. Further, pHrodo™ Red AM shows no apparent photobleaching after twelve minutes of imaging.

Figure 9. Visualizing intracellular pH changes during apoptosis



Cytosol acidification occurs early in apoptosis, followed by caspase activation and the eventual breakdown of plasma membrane integrity. Here an intracellular pH indicator (pHrodo™ Red AM) is used with a fluorogenic caspase substrate (CellEvent® Caspase-3/7 Green) to monitor a population of HeLa cells undergoing apoptosis. A single cell is shown over 4 hours after treatment with camptothecin. This cell first exhibits increasing pHrodo™ Red fluorescence, indicating cytosol acidification, followed by the appearance of green fluorescence from the caspase-3/7 probe.

Figure 9. Conventional Antibody Labeling Strategies



Amine- and thiol-reactive dyes are commonly used to label antibodies. However, the lack of specificity of these bioconjugation reactions can threaten immunoreactivity and lead to poorly defined constructs.

Figure 10. pH-sensitive pHrodo™-Antibody Conjugates

Antibodies were labeled with two reactive forms of pHrodo™ Red. **A.** First, the amine-reactive pHrodo™ Red, succinimidyl ester was used to label lysine residues on Goat anti Mouse IgG at molar ratios of 10 and 20. **B.** Second, the thiol-reactive pHrodo™ Red maleimide was used to specifically label cysteine residues of Goat anti Mouse IgG, with the goal of minimizing nonspecific and FC labeling. All conjugates were then tested for pH response and found to have the expected pKa of 6.8.

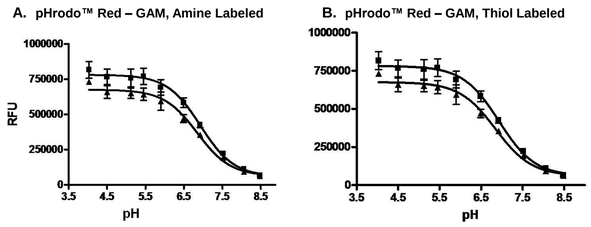
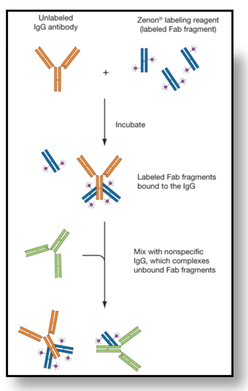
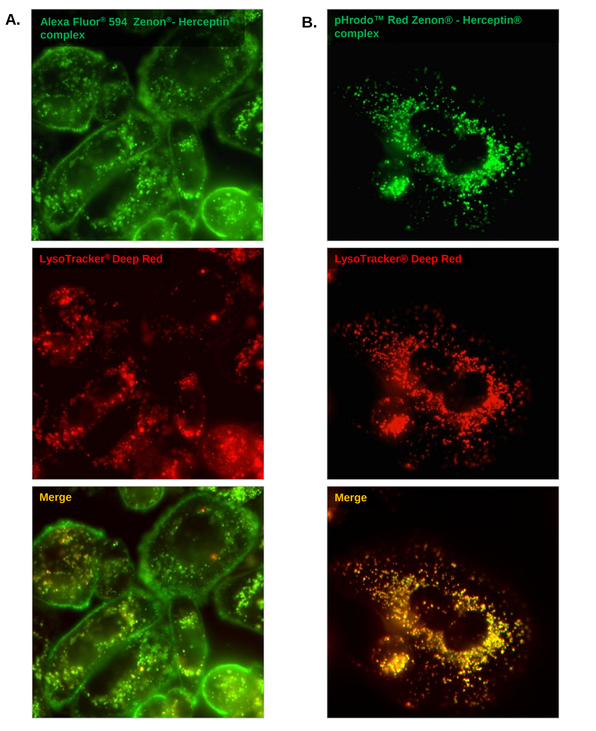


Figure 11. Fc-Specific Antibody Labeling with Zenon® Fab Fragments



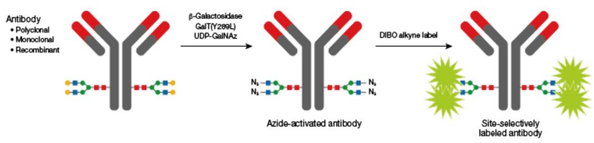
Any IgG1 antibody can be non-covalently coupled with fluorescent dyes using the Zenon® labeling system. **A.** Unconjugated primary antibodies are incubated with fluorescently-labeled Fc-specific Fab fragments, followed by an IgG blocking step to block any remaining unbound anti-Fc sites. This labeling reaction leaves the target antibody intact and free of antigen-binding site obstruction, while providing a consistent degree of labeling between 3 and 5. The Zenon® system is useful for labeling small amounts of antibodies (≤20 µg).

Figure 12. Herceptin® labeled with pHrodo™ Red-Zenon® is Internalized and Traffics to Lysosomes



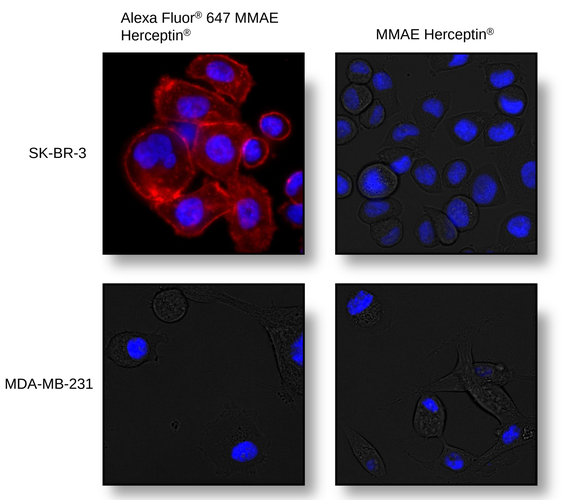
Alexa Fluor® 594 and pHrodo™ Red human IgG Zenon® conjugates were used to label 5 µg aliquots of Herceptin® (trastuzumab) at approximately three Zenon® Fab fragments per Herceptin® IgG. SK-BR-3 cells, which highly express the HER2/neu receptor, were loaded with 1 µg/ml Alexa Fluor® 594 Zenon® - Herceptin® and 50 nM LysoTracker® Deep Red for 30 minutes at 37ºC. **A.** Alexa Fluor® 594 Zenon® - Herceptin® complexes are visible both on the cell surface and where they have trafficked to lysosomes. **B. pHrodo™ Red Zenon® - Herceptin® complexes are brightly fluorescent when internalized to acidic lysosomes but not on the cell surface**.

Figure 14. Site-specific labeling of heavy-chain N-linked glycans



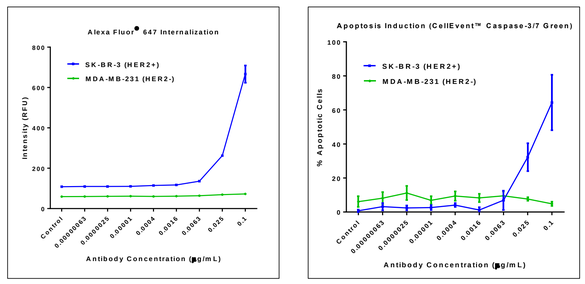
IgG antibodies contain two N-linked glycans on specific asparagine residues located in the antibody heavy chain Fc region. These are far from the antigen-binding domain and provide an ideal attachment point for antibody conjugation. The SiteClick™ labeling system involves three steps: 1, enzymatic removal of the terminal galactose residues on the heavy chain glycans; 2, the enzymatic addition of galactose-azide (GalNAz) residues into the heavy chain glycans; 3, the covalent click conjugation of fluorophore and chelator-modified dibenzocyclooctynes to the azide-modified sugars. This system allows efficient site-selective attachment of one or multiple fluorescent dyes, radiometal chelators, or small-molecule drugs to antibodies.

Figure 15. Direct Monitoring of ADC Internalization in Live Cells Using Dual SiteClick® Alexa Fluor® 647/MMAE Herceptin® Conjugates



Herceptin® was incubated for 6 hours at 30º C with β-(1-4) galactosidase, then overnight at 30ºC in non-phosphate buffer containing UDP-GalNAz and β-Gal-T1 (Y289L). Excess UDP-GalNAz was removed by washing antibodies in 2 mL Amicon 50 kD cut-off spin filters. The purified azide-activated antibodies were conjugated with 300 µM DIBO-MMAE, DIBO-Alexa Fluor® 647, or both (150 µM each) for 16 hours. HER2-positive SK-BR-3 cells and HER2-negative MDA-MB-231 cells were incubated with either Herceptin® MMAE or Herceptin® Alexa Fluor® 647/MMAE conjugates for one hour, counterstained with NucBlue® Live Cell Stain, and imaged with standard DAPI and Alexa Fluor® 647 filters. Membrane-bound and internalized Herceptin® Alexa Fluor® 647/MMAE conjugates were visible in SK-BR-3 cells. No signal was observed in MDA-MB-231 cells.

Figure 16. Specific Internalization and Cell Killing Response of Dual SiteClick® Alexa Fluor® 647/MMAE Herceptin® Conjugates



HER2+ SK-BR-3 cells and HER2- MDA-MB-231 cells were plated on 96 well plates at 2,000 cells per well. Cells were treated with varying concentrations of dual SiteClick® Herceptin® Alexa Fluor® 647/MMAE conjugates for 72 hours. Cells were then labeled with CellEvent® Caspase-3/7 Green detection reagent and analyzed on a Thermo Scientific™ ArrayScan™ VTI HCS reader **A.** SK-BR-3 cells internalized significant amounts of antibody-drug conjugate at higher concentrations, while MDA-MB-231 cells had minimal internalization. **B.** SK-BR-3 cells had dramatic cell death at higher ADC concentrations, while MDA-MB-231 cells had consistently low levels of apoptosis.

Conclusion

The study of physiological processes such as endocytosis, phagocytosis, and ligand internalization benefits from the ability to monitor pH changes in living cells. However, existing pH-sensitive dyes are prone to photobleaching and require tedious wash or quench steps. We have characterized two new pH-sensitive rhodamine-based dyes which are non-fluorescent at neutral pH, but become brightly fluorescent upon acidification. Because they are both fluorogenic and pH-sensitive, the pHrodo™ Green and Red dyes can be used as specific sensors of endocytic and phagocytic events. We have demonstrated the utility of these dyes for live-cell visualization of endocytosis and phagocytosis. Finally, the internalization and acidification of membrane receptor ligands and monoclonal antibodies were analyzed using pHrodo™ dyes.

References

1. Current Prot. Cytometry. 2002;9.19.1.
2. J Biol Chem. 2009;284:35926-38.
3. J Virol. 2010 Oct;84(20):10619-29.

Questions? Comments?
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