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Correlating internalization and potency to accelerate antibody discovery and development

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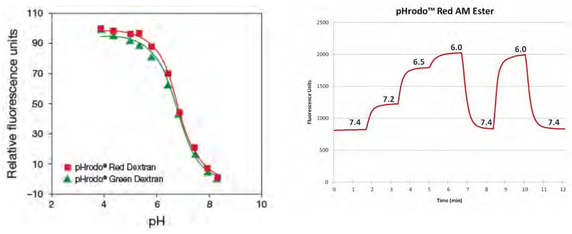
ABSTRACT

The basic cellular mechanisms of internalization and trafficking are important to many areas of cell biology, and especially to the proper function of therapeutic antibodies. Those antibodies intended for use as antibody drug conjugates should specifically bind to target cells and rapidly internalize into acidic compartments. Conversely, antibodies intended to kill cells via direct cell death, complement cascade, or effector cell killing should remain bound to the external surface of target cells as long as possible. 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 material.

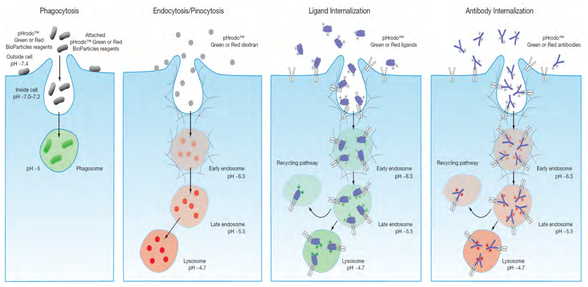
Here we present experimental data demonstrating the use of pH-sensitive fluorophores to monitor the three primary mechanisms of endocytosis in live cells. Additionally we demonstrate three approaches to directly visualize internalization of therapeutic antibodies and ADCs in live cells. Two of these techniques involve site-specific antibody labeling to screen for and further characterize internalizing clones without affecting antibody structure or function. These techniques are demonstrated using quantitative and widefield imaging to study anti-Her2 antibodies and ADCs in breast cancer models. The internalization of ADCs into Her2-positive breast cancer cells shows a strong correlation with target cell killing. We believe the insights provided by these approaches have the potential to accelerate therapeutic antibody lead generation and development.

Figure 1. pHrodo™ pH Sensor Dyes



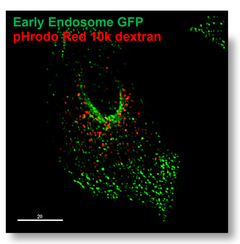
A. pH response profile of pHrodo Red and pHrodo Green dextrans. **B.** pHrodo fluorescence dynamically responds to pH changes.NIH-3T3 cells loaded with 5 µM **pHrodo™ Red AM Intracellular pH Indicator** were washed with a series of HEPES-based pH standard buffers containing 10 µM nigericin and 10 µM valinomycin to clamp intracellular pH to the indicated values.

Figure 2. Fluorogenic pH Sensing of Cellular Internalization Processes



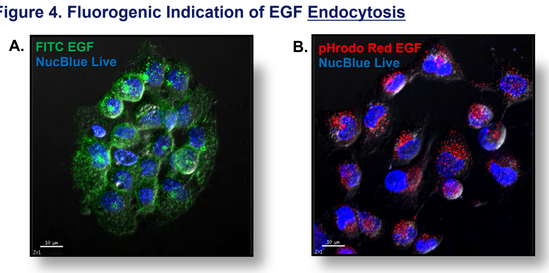
The fluorescence intensity of pHrodo™ Red and Green dyes dramatically increases as pH drops from neutral to acidic, making them ideal tools to study endocytosis, phagocytosis, and ligand and antibody internalization.

Figure 3. Monitoring pinocytosis with fluorescently-labeled dextran



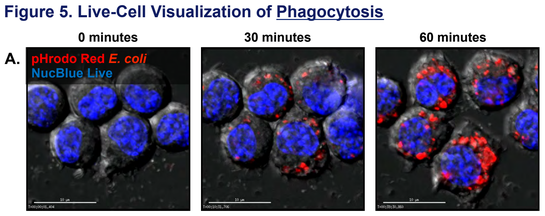
HeLa cells were treated overnight with CellLight® Early Endosome GFP, then loaded with 10 µg/ml **pHrodo Red 10k dextran** to visualize pinocytosis. pHrodo™ Red dextran appeared in GFP+ vesicles 5 minutes after loading, indicating both internalization of dextran and acidification of early endosomes.

Figure 4. Fluorogenic Indication of EGF Endocytosis



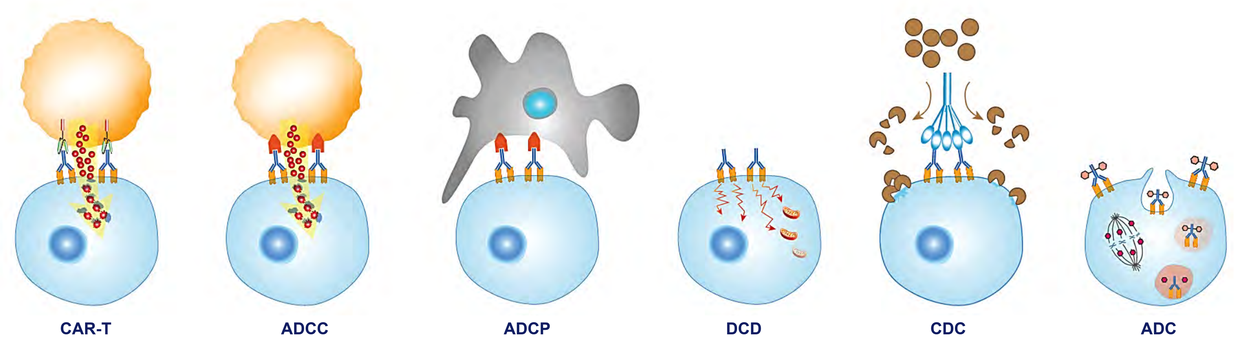
A431 cells were labeled with 5 µg/mL of FITC or **pHrodo Red** Epidermal Growth Factor conjugates and NucBlue™ Live Cell Stain at 37ºC for 30 minutes. **A.** FITC fluorescence is seen on the cell surface and throughout the endocytic pathway. **B.** pHrodo Red fluorescence is limited to punctate spots, indicating endocytic vesicles with low pH.

Figure 5. 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. 100 µg of **pHrodo Red E. coli BioParticles®** conjugates were added to MMM cells and imaged after 0, 30, and 60 minutes. The pHrodo BioParticles conjugates dramatically increase in fluorescence upon internalization and acidification.

Figure 6. Mechanisms of tumor cell killing by antibodies.

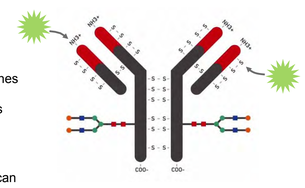


Antibodies participate in tumor cell killing by a variety of mechanisms, including: chimeric antigen receptor T cells (CAR-T), antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), direct cell death (DCD), complement-dependent cytotoxicity (CDC), or by the action of antibody-drug conjugates (ADC). Determination of antibody internalization kinetics is a critical step when developing therapeutic antibodies intended to participate in one of these mechanisms.

Figure 7. Conventional Antibody Labeling Strategies

Amine Labeling

- Stable covalent bond to lysines and terminal amines
- Degree of labeling varies between antibody and fluorophore
- Labeling of binding site can affect immunoreactivity

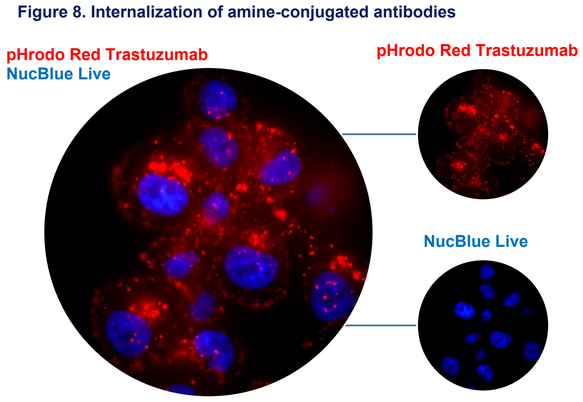


Thiol Labeling

- Stable covalent bond to cysteine residues
- Degree of labeling varies for each antibody and fluorophore
- Disulfide reduction can affect antibody structural integrity

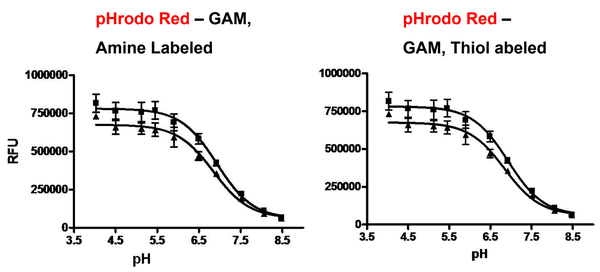
Amine- and thiol-reactive dyes are commonly used to label antibodies. However, the lack of specificity of these bioconjugation reactions can threaten immunoreactivity and lead to poorly defined constructs.

Figure 8. Internalization of amine-conjugated antibodies



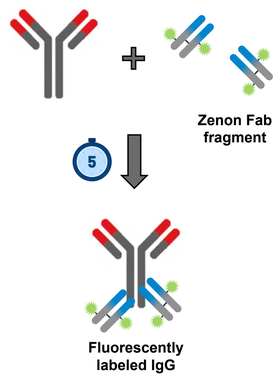
Trastuzumab was labeled with amine-reactive pHrodo Red (DOL 2.8). Her2+ SK-BR-3 cells were plated in DMEM + 10% FBS in a 35 mm glass-bottom dish. Cells were treated with 10 nM pHrodo Red-labeled Trastuzumab for 16 hours at 37ºC, 5% CO2. Media was removed and replaced with Live Cell Imaging Solution supplemented with 1% BSA and NucBlue Live Cell Stain for 30 minutes at 37ºC. Images were captured on an EVOS FI Auto imaging system with 60x Olympus Plan Apochromat oil immersion objective and DAPI and TRITC light cubes. Red spots indicate internalization of trastuzumab into acidic vesicles.

Figure 9. pH-sensitive pHrodo-Antibody Conjugates



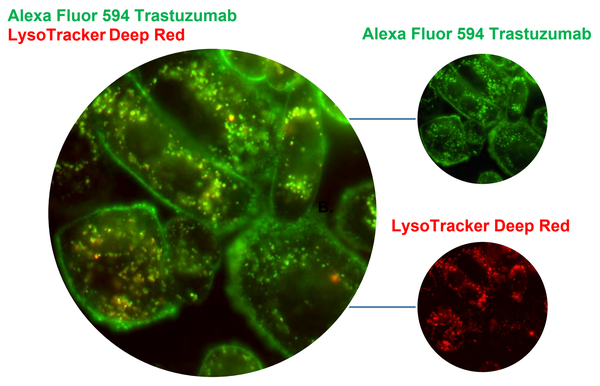
Antibodies were labeled with two reactive forms of pHrodo™ Red. **A.** First, the amine-reactive pHrodo™ Red, succinimidyl ester was used to label lysine residues on Goat anti Mouse IgG at molar ratios of 10 and 20. **B.** Second, the thiol-reactive pHrodo™ Red maleimide was used to specifically label 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.

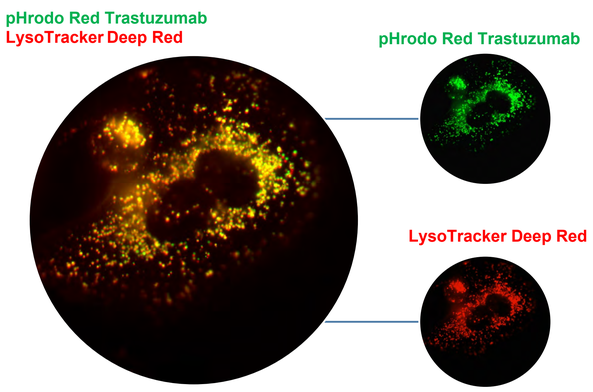
Figure 10. Fast, scalable screening of antibody internalization with pHrodo Fab fragments



Any IgG antibody can be non-covalently coupled with fluorescent dyes using the Zenon labeling system. Unconjugated primary antibodies are incubated with fluorescently-labeled Fc-specific Fab fragments. 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).

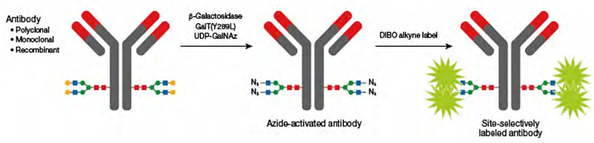
Figure 11. Trastuzumab labeled with pHrodo Red-Zenon is Internalized and Trafficked to Lysosomes





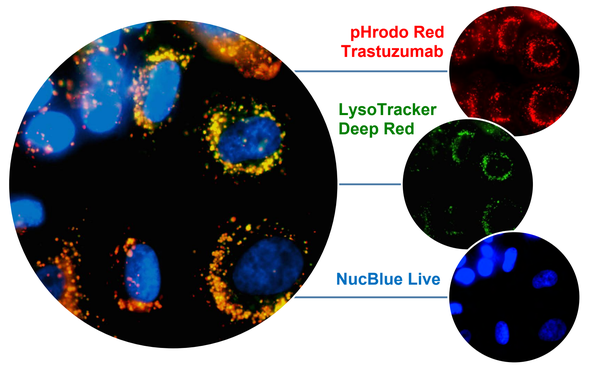
5 µg aliquots of trastuzumab were labeled with Alexa Fluor 594 or pHrodo Red human IgG Zenon conjugates. HER2+ SK-BR-3 cells were loaded with 1 µg/ml of fluorescently-labeled Trastuzumab and 50 nM LysoTracker Deep Red for 30 minutes at 37ºC. **A.** Alexa Fluor 594 Zenon - Trastuzumab complexes are visible both on the cell surface and where they have trafficked to lysosomes. **B. pHrodo Red Zenon - Trastuzumab complexes are brightly fluorescent when internalized to acidic lysosomes but not on the cell surface**.

Figure 12. Site-specific labeling of heavy-chain N-linked glycans



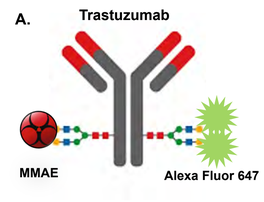
IgG antibodies contain two Fc N-glycans that provide an ideal target for conjugation. The SiteClick™ labeling system involves three steps: 1, enzymatic removal of terminal galactose residues on heavy chain glycans; 2, enzymatic addition of galactose-azide (GalNAz); 3, the covalent click conjugation of fluorophore-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 13. SiteClick antibody labeling with pHrodo dye for site-specific labeling and antibody internalization



Trastuzumab was azide-activated with the SiteClick labeling system, then click conjugated with pHrodo Red-DIBO. Cells were treated with 10 nM pHrodo Red-labeled Trastuzumab for 16 hours at 37ºC, 5% CO2. Media was replaced with Live Cell Imaging Solution with 1% BSA, NucBlue Live Cell Stain, and 50 nM LysoTracker Deep Red for 30 minutes at 37ºC. Red spots indicate internalization of trastuzumab into acidic intracellular vesicles that are positive for LysoTracker Deep Red.

Figure 14. Direct Monitoring of ADC Internalization in Live Cells Using Dual SiteClick Alexa Fluor 647/MMAE Trastuzumab Conjugates



A. Trastuzumab was azide-activated with the SiteClick labeling system, then conjugated with DIBO-Alexa Fluor 647 and DIBO-MMAE (Monomethyl auristatin E). HER2+ SK-BR-3 cells and HER2- MDA-MB-231 cells were treated with this fluorescently-labeled antibody-drug conjugate for one hour. B. Membrane-bound and internalized antibody was visible in SK-BR-3 cells. C. No signal was observed in MDA-MB-231 cells.

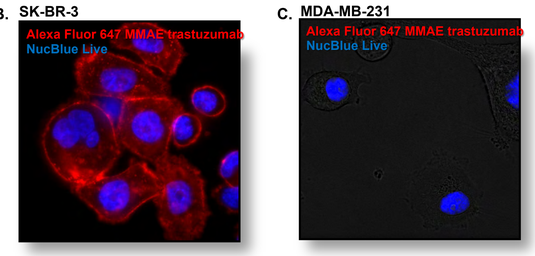
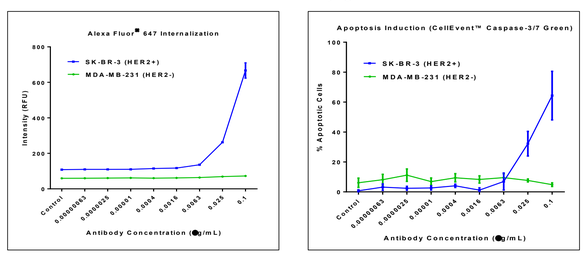


Figure 15. Specific Internalization and Cell Killing Response of Trastuzumab Alexa Fluor 647/MMAE Conjugates



HER2+ SK-BR-3 cells and HER2- MDA-MB-231 cells in 96 well plates were treated with Trastuzumab 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 antibody-drug conjugate, while MDA-MB-231 cells had minimal internalization. **B.** Apoptosis was observed in SK-BR-3 cells at higher ADC concentrations, while MDA-MB-231 cells had minimal apoptosis.

Conclusion

Studies of endocytosis, pinocytosis, and phagocytosis benefit from the ability to monitor pH changes in live cells with fluorogenic, pH-sensitive pHrodo dyes. Additionally, the internalization of antibodies can be analyzed using pHrodo dyes attached via three mechanisms.

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