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Site-specific conjugation of novel fluorophores and tags to accelerate immunotherapy discovery and development

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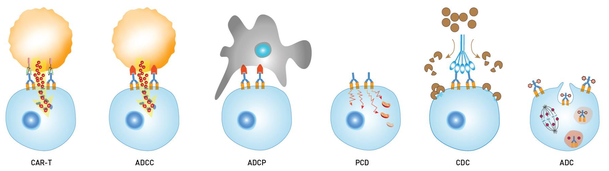
Abstract

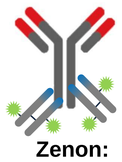
The basic cellular mechanisms of internalization and trafficking are important to many areas of cell biology, and especially to the proper function of therapeutic antibodies. Those antibodies intended for use as antibody drug conjugates should specifically bind to target cells and rapidly internalize 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 endocytosis of therapeutic antibodies in live cells using three approaches. These approaches utilize site specific antibody labeling, as well as covalent and non-covalent attachment of fluorophores to reference antibodies. We use quantitative, automated fluorescence microscopy to study anti-HER2 antibodies and ADCs in breast cancer models. Using these approaches we are able to demonstrate time and dose-dependency of uptake, correlative induction of cell death following conjugate incubation and dissect trafficking routes. The tools described herein will significantly benefit those working in bio therapeutic lead generation and development.

Antibody drug conjugates as therapeutic agents



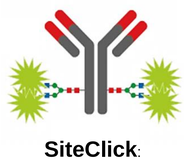
A. Increasingly biologics are being pursued as therapeutic agents. In the case of antibody-drug conjugates (ADC) antibody internalization is a critical test. Internalization may remove the ADC from the site of action at the plasma membrane. Furthermore many ADC require internalization and subsequent to trafficking to the lytic environment of the lysosome to activate the attached toxin

Novel conjugation techniques



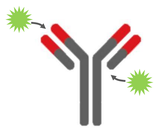
Zenon:

Fab fragments for fast, scalable screening



SiteClick:

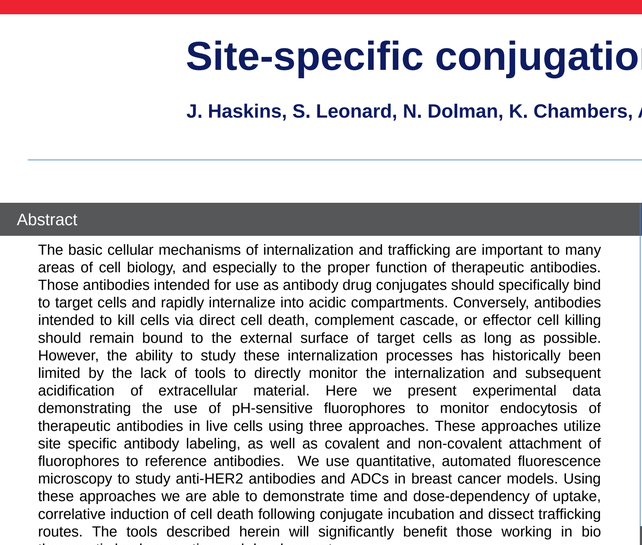
Efficient, site-specific, reproducible labeling



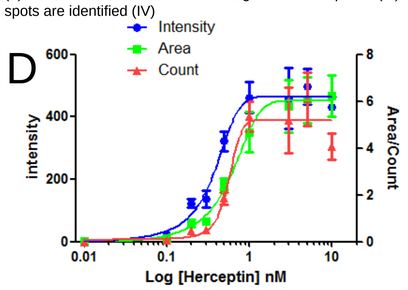
New pHrodo:

Stable, soluble, reactive, covalent labeling

High content imaging and analysis to investigate antibody-drug conjugates



Use of High content imaging and analysis to characterize antibody-conjugation approaches in cell-based assays. High content imaging and analysis allows the rapid automated acquisition and analysis of fluorescent images from millions of cells. A. Images are taken in the relevant channels. B. Image features are then analyzed. C. Cells are identified based on nuclear staining (I) Form the nuclear area a ring representing the cytoplasm is constructed (II) Which then overlaid onto the image form the reporter (III) and within the cytoplasmic ring spots are identified (IV)

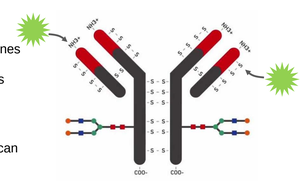


D. Automated analysis of these images allow for the characterization of numerous cellular properties. In the case of antibody-drug conjugate internalization spot features are analyzed, for example ring spot count, mean average intensity and mean average area. Data from the **CellInsight™ CX5 High Content Screening (HCS) Platform**

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

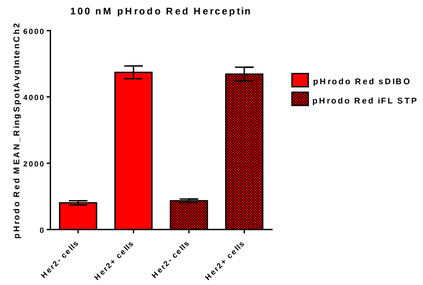


Thiol Labeling

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

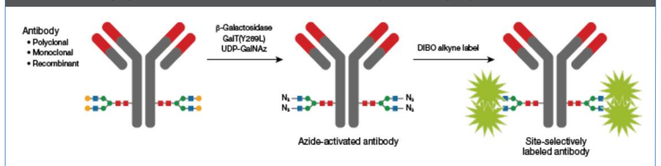
Amine- and thiol-reactive dyes are commonly used to label antibodies. However, the lack of specificity of these bioconjugation reactions can threaten immunoreactivity and lead to poorly defined constructs.

Herceptin labeled with pHrodo Red via amine-reactive or SiteClick chemistry both give bright, specific signals



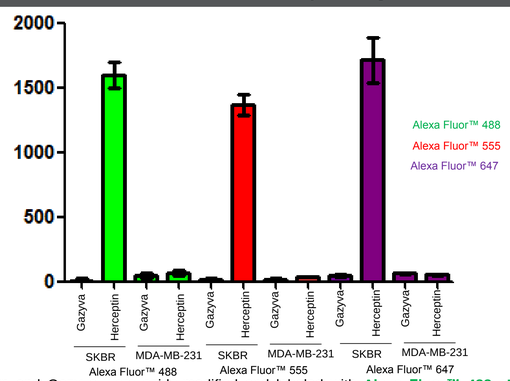
Trastuzumab was amine-labeled with **pHrodo™ iFL Red STP** or with **pHrodo™ Red-sDIBO** using the SiteClick labeling system. SKBR-3 (Her2$^+$) or MDA-MB-231 (Her2$^-$) cells were treated with 100 nM Herceptin conjugates, 16 hours at 37ºC. Cells from triplicate samples were analyzed on the **CellInsight™ CX5 High Content Screening (HCS) Platform**. **pHrodo™ Red labeled Herceptin specifically internalizes into Her2$^+$ cells, regardless of conjugation chemistry.**

Defined Conjugation with the SiteClick™ Antibody Labeling System



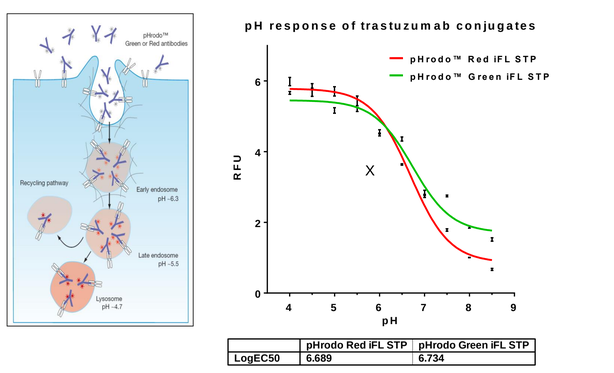
IgG antibodies contain two Fc N-glycans that provide an ideal target for conjugation. The SiteClick™ labeling system involves three steps: 1, enzymatic removal of terminal galactose residues on heavy chain glycans; 2, enzymatic addition of galactose-azide (GalNAz); 3, the covalent click conjugation of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

Updated Detection Molecules for SiteClick Antibody Labeling



Herceptin and Gazyva were azide-modified and labeled with **Alexa Fluor™ 488 sDIBO, Alexa Fluor™ 555sDIBO or Alexa Fluor™ 647 sDIBO** SK-BR-3 (Her2$^+$) or MDA-MB-231 (Her2$^-$) cells were treated with 10nM Herceptin/Gazyva, 24 hours at 37ºC. Cells from triplicate samples were analyzed on the **CellInsight™ CX5 High Content Screening (HCS) Platform**. Conjugated Herceptin specifically binds to Her2$^+$ cells. All negative controls provide minimal signal.

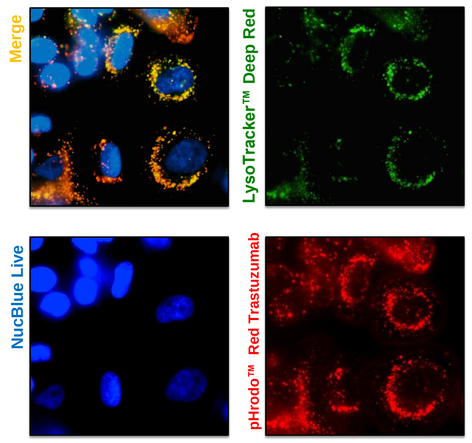
Novel pH sensitive-conjugates to study antibody internalization and trafficking



	pHrodo Red iFL STP	pHrodo Green iFL STP
LogEC50	6.689	6.734

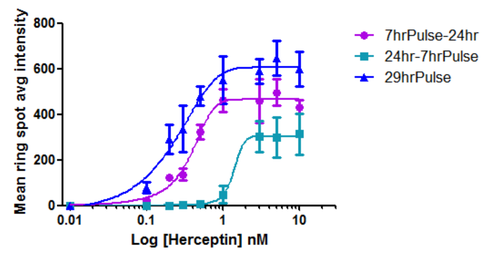
A. pH response profile of Herceptin conjugates of amine-reactive pHrodo™ Red iFL STP and pHrodo™ Green iFL STP. **B.** These conjugates will be minimally fluorescent at neutral pH outside of cells but become brightly fluorescence upon internalization.

Defined conjugation with pHrodo™ Red sDIBO for antibody internalization



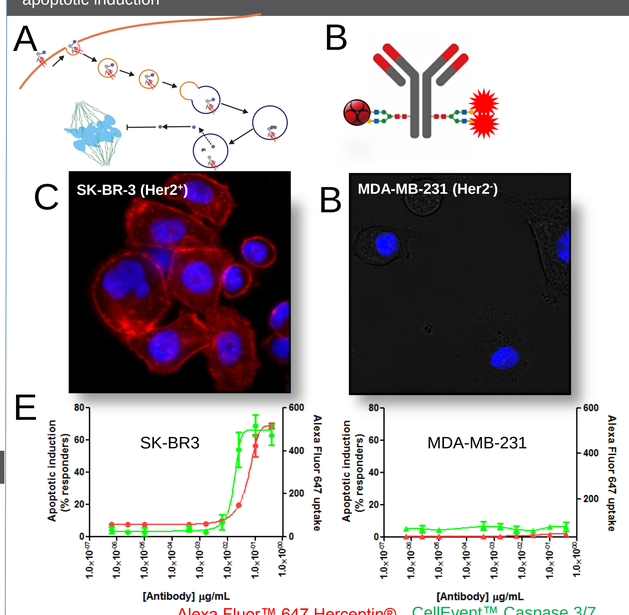
Trastuzumab was azide-activated with the SiteClick labeling system, then click conjugated with **pHrodo™ Red-sDIBO**. Cells were treated with 10 nM pHrodo Red-labeled Trastuzumab for 16 hours at 37ºC, 5% CO2. Media was replaced with Live Cell Imaging Solution with 1% BSA, **NucBlue Live Cell Stain**, 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. Cells imaged on **EVOS™ FL Auto cell imaging system**.

Time and dose-dependent uptake of pHrodo™ iFL Red STP Herceptin



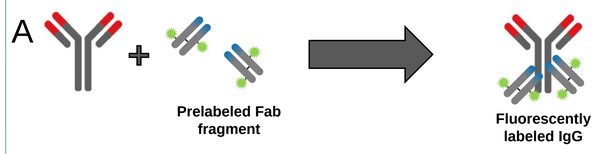
SK-BR-3 (Her2$^+$) or MDA-MB-231 (Her2$^-$) cells were treated a dose-response of **pHrodo™ iFL Red STP Herceptin** at 37ºC. Experiments were conducted in duplicate wells across two plates. Conjugates were incubated with cells for either 29 hours continuously or for a 7 hr pule followed by a 24 hour incubation in the absence of the conjugate. Alternatively cells were incubated in the presence of the conjugate for 7 hours at the very end of the experiment. Cells from triplicate samples were analyzed on the **CellInsight™ CX5 High Content Screening (HCS) Platform**. Conjugated Herceptin internalizes into Her2$^+$ cells over time. Her2$^-$ negative cells provide minimal signal.

Simultaneous, Multi-parametric imaging of antibody-drug conjugate uptake and apoptotic induction

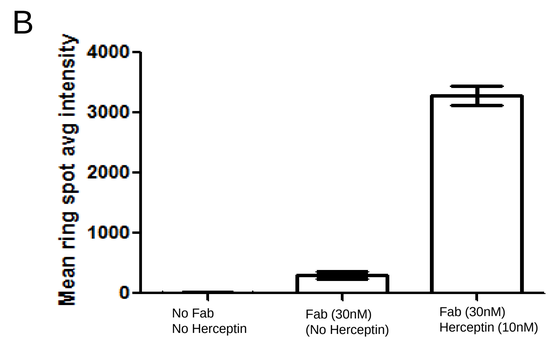


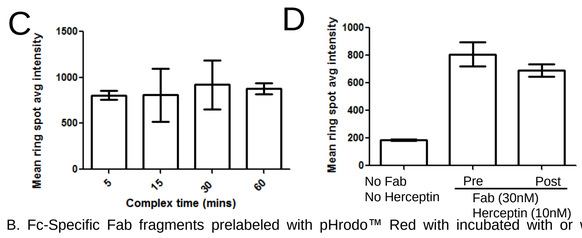
A. Uptake and trafficking of ADC to trigger apoptosis. ADC binding to extracellular epitopes results in endocytosis and subsequent delivery to the lysosome where the drug conjugate is released. The drug conjugate, in this case MMAE (Monomethyl auristatin E) acts to depolymerize microtubules. This arrests the cells in mitosis triggering apoptosis. B. Trastuzumab was azide-activated with the SiteClick labeling system, then conjugated with sDIBO-Alexa Fluor 647 and sDIBO-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. C. Membrane-bound and internalized antibody was visible in SK-BR-3 cells. D. No signal was observed in MDA-MB-231 cells. HER2$^+$ SK-BR-3 cells and HER2$^-$ MDA-MB-231 cells 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. E. Apoptosis was observed in SK-BR-3 cells at higher ADC concentrations, while MDA-MB-231 cells had minimal apoptosis.

Quick Antibody Labeling for Internalization Screening



A. Fc-Specific Fab fragments prelabeled with pHrodo™ Red or pHrodo™ Green can be complexed with human or mouse IgG to provide rapid labeling for fast, scalable screening of antibody internalization for many antibody samples.

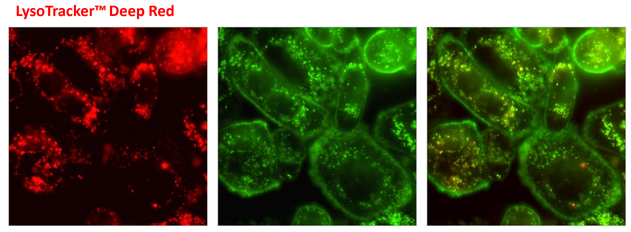




B. Fc-Specific Fab fragments prelabeled with pHrodo™ Red with incubated with or without Herceptin (10nM) for 15 minutes in complete media. The complexes were then added to wells containing SK-BR-3 cells and incubated overnight under regular culture condition. As a control SK-BR-3 cells labeled only with Hoechst were also imaged. C. Fc-specific Fab fragments labeled with pHrodo™ Green were complexed with Herceptin for the indicated times in complete media. Following this the complexes were added to wells containing SK-BR-3 cells and incubated overnight under regular culture conditions. D. Fc-specific Fab fragments were complexed with Herceptin either prior to cell labeling (pre) or following a 15 minute incubation of cells to Herceptin (Post). SK-BR-3 cells were then incubated overnight under regular culture conditions. As a control SK-BR-3 cells labeled only with Hoechst were also imaged. Cells from triplicate samples were analyzed on the **CellInsight™ CX5 High Content Screening (HCS) Platform**

Trastuzumab labeled with pHrodo™ Red Fabs are fluorescent in acidic organelles

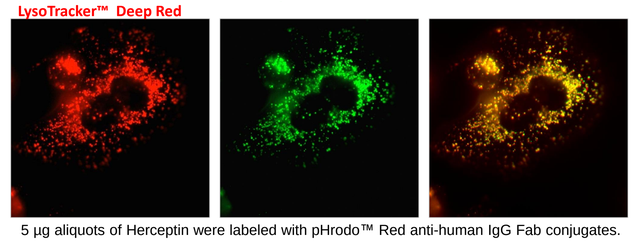
Alexa Fluor™ 594 Fab – Trastuzumab complex
LysoTracker™ Deep Red



5 µg aliquots of Herceptin were labeled with Alexa Fluor™ 594 anti-human IgG Fab 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. Alexa Fluor™ 594 Fab - Trastuzumab complexes are visible both on the cell surface and where they have trafficked to lysosomes.

Trastuzumab labeled with pHrodo Red Fabs are fluorescent in acidic organelles

pHrodo™ Red Fab – Trastuzumab complex
LysoTracker™ Deep Red



5 µg aliquots of Herceptin were labeled with pHrodo™ Red anti-human IgG Fab 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. B. pHrodo™ Red Fab - Trastuzumab complexes are brightly fluorescent when internalized to acidic lysosomes but not on the cell surface.

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