

Modular, defined assembly of antibody-based constructs using click chemistry

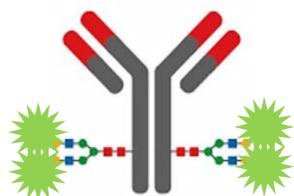
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ABSTRACT

Immunoglobulin antibodies contribute to a broad range of research assays and also provide the foundation for a diverse variety of biopharmaceutical agents. Here we describe the use of bio-orthogonal click chemistry reactions to synthesize several common antibody-based structures and demonstrate their use in cell-based applications. First, site-specific antibody-fluorophore conjugates were synthesized using SiteClick™ conjugation chemistry to attach Alexa Fluor™ and iFL pHrodo™ dyes to common therapeutic antibodies. Analytical characterization of these antibody conjugates confirmed efficient, reproducible payload attachment without affecting antibody functionality. Flow cytometry and high content analysis experiments demonstrate performance of these antibodies in receptor occupancy, antibody internalization, and effector function assays. Next, a double conjugation strategy was employed to synthesize antibody drug conjugates which become brightly fluorescent upon endocytosis. ADC uptake and targeted cell killing were demonstrated using cell line and spheroid models. All applications are demonstrated using flow cytometry to analyze suspension cells. Alternatively for adherent cells and spheroid models high content analysis or live cell microscopy were employed.

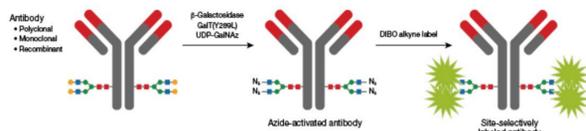
SiteClick Antibody Labeling System

- Standards:**
 Alexa Fluor 488 sDIBO alkyne
 Alexa Fluor 555 sDIBO alkyne
 Alexa Fluor 647 sDIBO alkyne
- Tool kit:**
 Biotin sDIBO alkyne
 Amine sDIBO alkyne
 amine-reactive sDIBO alkyne
- pH sensitive:**
 iFL pHrodo Red sDIBO alkyne



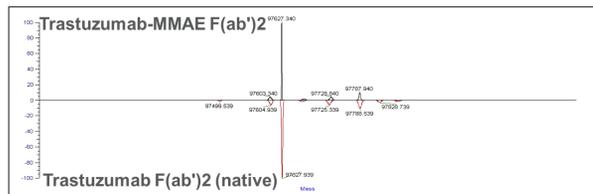
SiteClick antibody-labeling technology enables simple and site-specific attachment of compounds, including fluorescent dyes or toxins, to the carbohydrate domains present only on the heavy chains of IgG antibodies regardless of isotype and host species

Three-step Labeling Process



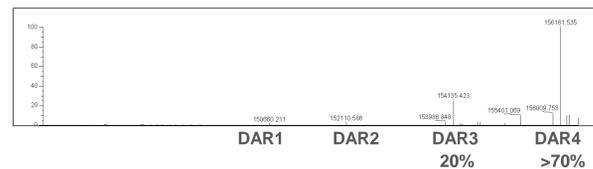
- SiteClick antibody-labeling process begins with removal of terminal galactose residues from heavy-chain N-linked glycans using β -galactosidase, exposing modifiable GlcNAc residues.
- Free terminal GlcNAc residues are activated with azide tags by enzymatic attachment of GalNAz using the GalT(Y289L) enzyme.
- Azide residues are reacted via click chemistry with a dibenzocyclooctyne (sDIBO)-functionalized probe of choice (e.g., Alexa Fluor 488 sDIBO alkyne).

SiteClick conjugation does not modify F(ab')₂ domains



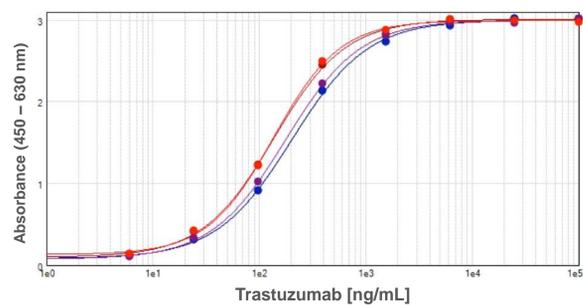
Mass spectra of F(ab')₂ fragments of DIBO-modified trastuzumab species compared to unmodified trastuzumab F(ab')₂ fragments

SiteClick Labeling: Consistent Conjugate-Antibody Ratio



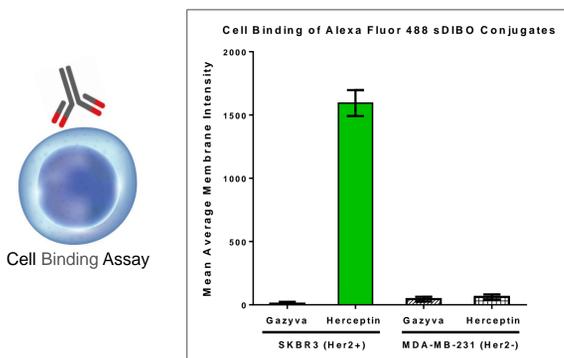
Mass Spectra of Trastuzumab – MMAE SiteClick conjugate. ADCs were made from SiteClick labeling of Trastuzumab with DIBO-MMAE toxin. MMAE conjugated mAb analysis was conducted by LC-MS on an Orbitrap Fusion system using a Thermo Scientific™ MAbPac™ RP (2.1x 100mm, 4.0 μ m, heated at 60°C) column.

Binding to Fc γ RIIIA – V158 by SiteClick-modified IgG1 antibody



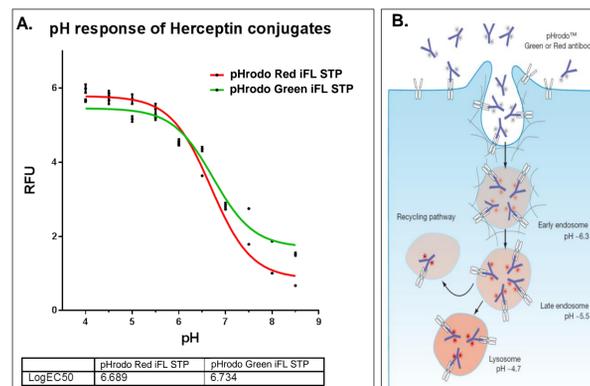
- Native Trastuzumab
 - Galactose
 - + GalNAz
 - + sDIBO-Biotin (Unpurified)
 - + sDIBO-Biotin (Purified)
- Fractions of trastuzumab at indicated steps of SiteClick antibody labeling were tested in Fc γ R binding ELISAs. Shown is a representative curve depicting the SiteClick variants binding to Fc γ RIIIA – V158 ELISA. SiteClick antibody labeling has minimal impact on IgG affinity for Fc γ receptors.

SiteClick Labeled Antibodies Specifically Bind to Cells



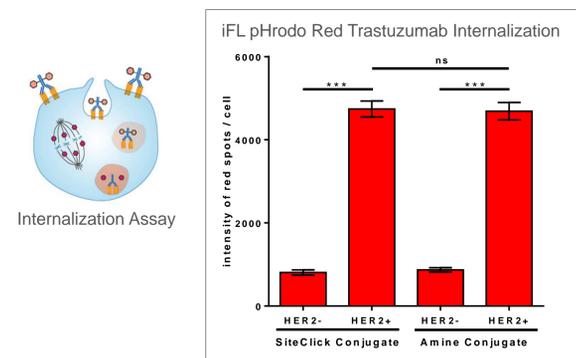
Trastuzumab (Herceptin®, anti-Her2) and obinutuzumab (Gazyva®, anti-CD20) were conjugated with Alexa Fluor 488 sDIBO using SiteClick antibody labeling. SKBR-3 (Her2+) or MDA-MB-231 (Her2-) cells were treated 1 hour with 10 nM antibody and analyzed on the CellInsight™ CX5 HCS Platform.

New pHrodo™ iFL Sensor Dyes Report pH Change



A. Cell-free pH response profile of trastuzumab labeled with amine-reactive pHrodo Red iFL STP or pHrodo Green iFL STP. B. These conjugates will be minimally fluorescent at neutral pH outside of cells but become brightly fluorescent following specific endosomal internalization.

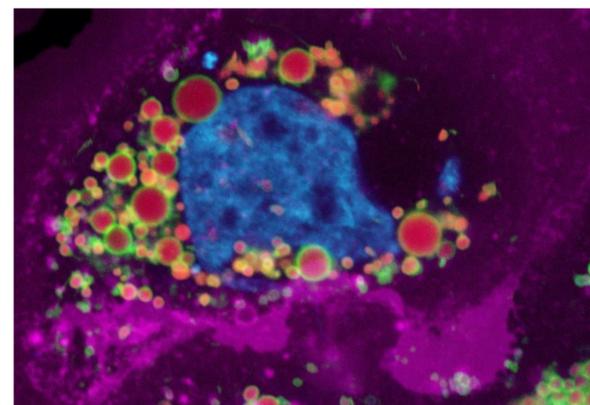
Bright, Specific Signal Indicates Antibody Internalization



Trastuzumab was labeled with iFL pHrodo Red using amine or SiteClick conjugation chemistry. HER2⁺ or HER2⁻ cells treated 16 hours with 100 nM antibody. Analyzed on the CellInsight CX5 HCS Platform.

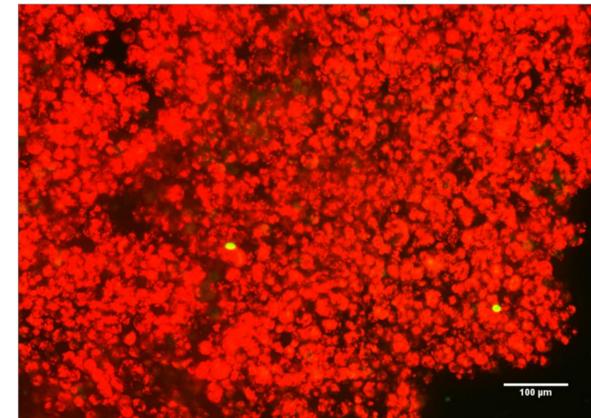
iFL pHrodo Red labeled trastuzumab specifically internalizes into HER2⁺ cells, regardless of conjugation chemistry.

Antibody internalization in HER2⁺ SKBR3 Cells



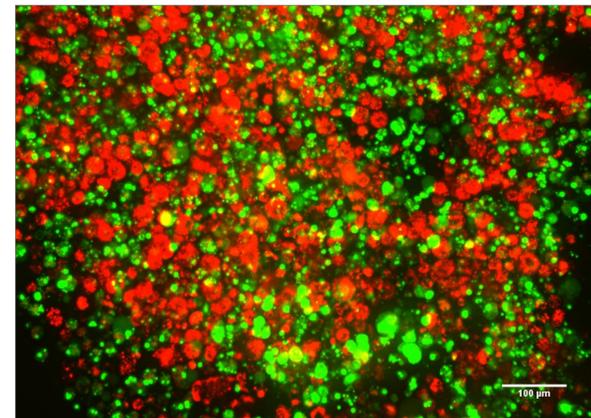
iFL pHrodo Red - Trastuzumab
 CellLight™ Lysozymes-GFP
 NucBlue™ Live ReadyProbes® Reagent
 CellMask™ Deep Red Plasma Membrane Stain
 Live confocal image of SKBR3 cells treated 16 hours with 30 nM trastuzumab – iFL pHrodo Red conjugate.

Visualize Antibody Penetration of Spheroids



Trastuzumab was labeled with iFL pHrodo Red using SiteClick conjugation. A spheroid of HER2⁺ SKBR3 cells was treated 72 hours with 30 nM antibody. Live cell imaging on the CellInsight CX7 HCS Platform. Red fluorescence indicates antibody penetration.

Direct Indication of ADC Cell Penetration and Killing



Trastuzumab was labeled with MMAE and iFL pHrodo Red via SiteClick conjugation. SKBR3 spheroid was treated 72 hours with 30 nM ADC. Live cell imaging with CellInsight CX7 HCS Platform. Red indicates antibody penetration, green indicates apoptosis with CellEvent Caspase 3/7 Green.

Conclusions

- SiteClick IgG antibody-labeling technology enables reproducible site-specific attachment of compounds without altering F(ab')₂ structure or compromising affinity for Fc γ receptors.
- When conjugated to antibodies, iFL pHrodo Red sDIBO alkyne provides a direct fluorescent indication of antibody penetration into cells.
- Dual-labeled antibodies, such as fluorescent antibody-drug conjugates, can be created using a mixture of two different sDIBO alkyne species.

Product	Size	Cat. No.
pHrodo™ iFL Red STP ester, amine reactive	1mg	P36010
	3 X 100 ug	P36011
pHrodo™ iFL Green STP ester, amine reactive	1mg	P36012
	3 x 100 ug	P36013
pHrodo™ iFL Red Microscale Protein Labeling Kit	1 Kit	P36014
pHrodo™ iFL Green Microscale Protein Labeling Kit	1 Kit	P36015
SiteClick™ Antibody Azido Modification Kit	1 Kit	S20026
Click-iT™ Alexa Fluor™ 488 sDIBO Alkyne for Antibody Labeling	1 Kit	C20027
Click-iT™ Alexa Fluor™ 555 sDIBO Alkyne for Antibody Labeling	1 Kit	C20028
Click-iT™ Alexa Fluor™ 647 sDIBO Alkyne for Antibody Labeling	1 Kit	C20029
Click-iT™ Biotin sDIBO Alkyne for Antibody Labeling	1 Kit	C20030
Click-iT™ Amine sDIBO Alkyne for Antibody Labeling	1 Kit	C20031
Click-iT™ SDP Ester sDIBO Alkyne for Antibody Labeling	1 Kit	C20032
Click-iT™ pHrodo™ iFL Red sDIBO Alkyne for Antibody Labeling	1 Kit	C20034
SiteClick™ Biotin Antibody Labeling Kit	1 Kit	S20033
Zenon™ pHrodo™ iFL green mouse IgG labeling kit	1 Kit	Z25609
Zenon™ pHrodo™ iFL red mouse IgG labeling kit	1 Kit	Z25610
Zenon™ pHrodo™ iFL green human IgG labeling kit	1 Kit	Z25611
Zenon™ pHrodo™ iFL red human IgG labeling kit	1 Kit	Z25612