Effectively monitor antibody internalization and trafficking

Improved labeling tools for antibody–drug conjugate screening and characterization.

In recent years, biotherapeutics (or biologics) have emerged as an important class of treatments that complement traditional, small-molecule drugs. Biotherapeutics comprise any therapy that utilizes a biological entity to produce it. Predominant among biotherapeutics are antibodies that can be used to modulate a specific cellular process relevant in a disease state. These antibodies confer specificity based on an extracellular epitope found only on the subset of diseased cells. Once bound to the cell surface, the antibodies can act directly to initiate apoptosis or trigger cell-mediated or complement-dependent cytotoxicity. Alternatively, the targeted antibody can be coupled to a small cytotoxic molecule to create an antibody–drug conjugate (ADC) that specifically binds an epitope on the plasma membrane and is then brought into the cell via endocytosis. Following internalization, the ADC can be trafficked to the lysosome where the drug is liberated, resulting in a highly targeted chemotherapeutic agent (Figure 1).

Given that an ADC requires endocytosis and subsequent acidification to be effective, pH-sensitive labels are very useful for following the internalization of an antibody conjugate. Fluorescein, the conventional pH-sensitive label, exhibits bright fluorescence that is quenched as the pH drops, making it a negative indicator of the acidic conditions of the endocytic pathway. In contrast, the Invitrogen™ pHrodo™ dyes display very low fluorescence at neutral pH and exhibit increasing fluorescence as the pH becomes more acidic, providing a positive indication of endocytosis

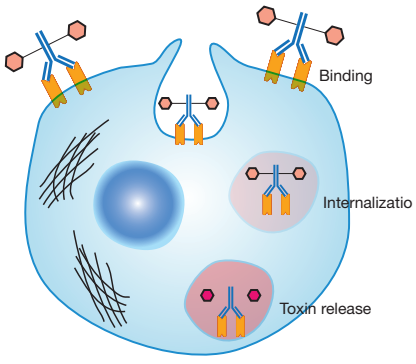

Figure 1. Internalization of an antibody–drug conjugate. An antibody–drug conjugate (ADC) comprises a monoclonal antibody directed against a tumor cell antigen coupled to a small cytotoxic molecule. An ADC is designed to specifically bind to target cells, where it is rapidly internalized. Typically the drug is liberated following trafficking to the lysosome, resulting in a highly targeted chemotherapeutic agent.

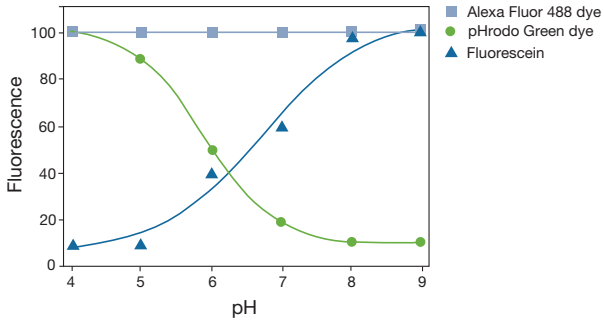

Figure 2. Comparative pH response of pH-insensitive Alexa Fluor 488 dye and pH-sensitive pHrodo Green dye and fluorescein. The Invitrogen™ Alexa Fluor™ 488 dye exhibits relatively constant fluorescence across a range of pH levels, whereas the fluorescence of fluorescein and Invitrogen™ pHrodo™ Green dye is significantly affected by pH. As the pH becomes more acidic, the fluorescence of fluorescein is quenched while the fluorescence of the pHrodo dye increases.

(Figure 2). When monitoring the internalization of a pHrodo dye–labeled antibody, the increase in pHrodo fluorescence is an effective tool, both for screening antibody candidates when creating new ADCs and for characterizing existing ADCs. An additional advantage of labeling with pHrodo dyes is that they eliminate the need for wash steps and quencher dyes to block any non-internalized dye fluorescence because they are not significantly fluorescent at the neutral pH found outside of cells.

Labeling antibodies with pH-sensitive pHrodo dyes

Traditional antibody labeling protocols can require significant optimization and often result in heterogeneous dye attachment. Moreover, when using reactive forms of fluorophores (e.g., thiol- or amine-reactive fluorescent dyes), the antigen binding site can be blocked during the labeling process, rendering the antibody ineffective. To overcome these complications, we have developed a suite of antibody labeling tools that incorporate the classic pH-insensitive, bright and photostable Invitrogen™ Alexa Fluor™ dyes, as well as the new pH-sensitive pHrodo iFL dyes. Labeling antibodies with Alexa Fluor dyes can provide a useful control in internalization experiments because the Alexa Fluor label will remain brightly fluorescent throughout the endocytosis process, from binding to internalization, regardless of the pH (Figure 2). The next-generation pHrodo iFL Green and pHrodo iFL Red dyes →

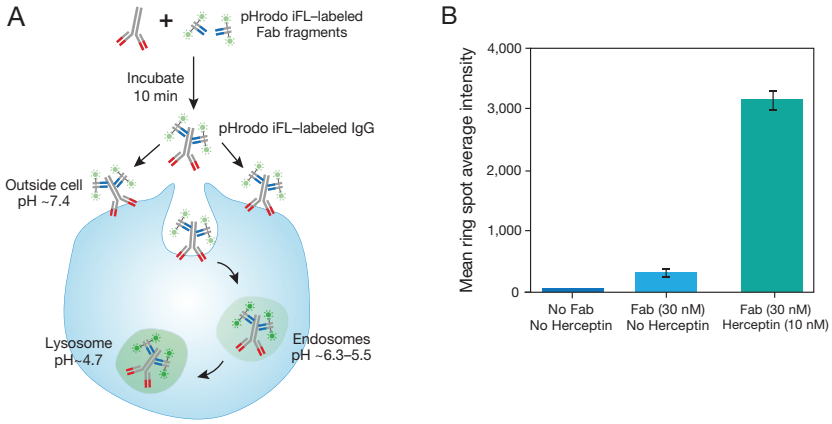

Figure 3. Zenon antibody labeling for following antibody internalization. (A) Invitrogen™ Zenon™ Antibody Labeling Kits can be used to noncovalently couple a fluorescent dye to an antibody in ~10 min. Unconjugated antibodies are incubated with fluorescently labeled Fab fragments directed against the Fc portion of a human or mouse IgG antibody, leaving the antigen binding site unmodified. **(B)** The Zenon antibody labeling method was used to label Herceptin™ (trastuzumab) with Invitrogen™ pHrodo™ iFL Red–conjugated Fab fragments (Fab). After incubation with HER2-positive SKBR3 cells (expressing human epidermal growth factor receptor 2, to which Herceptin binds), the pHrodo iFL Red–labeled Herceptin conjugate began to fluoresce, indicating internalization and acidification of the pHrodo label. No significant fluorescence was seen in vehicle-treated control cells or in cells incubated with the pHrodo iFL Red–conjugated Fab fragments alone, confirming the signal specificity.

are more soluble than the original pHrodo Green and pHrodo Red dyes, making them useful for labeling antibodies that may otherwise precipitate out of solution during conjugation.

Here we will focus on two methods for labeling antibodies with the pH-sensitive pHrodo iFL dyes: the extremely fast and noncovalent Invitrogen™ Zenon™ antibody labeling technology, and the recently updated Invitrogen™ SiteClick™ click chemistry–based antibody labeling kits. Though different in their mechanisms, both of these methods label antibodies at a specific site on the Fc portion of the heavy chain, far from the antigen binding site, thus preserving the binding properties of the antibody. We also offer amine-reactive STP esters of the pHrodo iFL dyes for traditional protein conjugation at available lysine residues.

Zenon antibody labeling with pHrodo iFL dyes

The Zenon Antibody Labeling Kits provide a means of very rapidly labeling IgG antibodies (in ~10 minutes) for use in cell binding or internalization experiments. The fast, scalable Zenon labeling method employs isotype-specific Fab fragments conjugated with either pH-sensitive pHrodo iFL dyes or classic Alexa Fluor dyes. These Fab fragments are directed against the Fc portion of a human or mouse IgG antibody, leaving the antigen binding site of the target antibody intact and free from obstruction while also providing a consistent degree of labeling. Zenon technology can label as little as 1 µg of antibody, and unlike traditional labeling methods using amine- or thiol-reactive labels, the Zenon antibody labeling is compatible with bovine serum albumin (BSA) and other stabilizing proteins.

Although the interaction between the Zenon Fab fragment and the primary antibody is noncovalent, the binding is sufficiently stable to allow detection of internalization and trafficking of the conjugates within cells. This method was used to quickly and specifically label the biotherapeutic antibody Herceptin™ (trastuzumab), which is internalized in HER2-positive cells (expressing human epidermal growth factor receptor 2, to which Herceptin binds). Because of its efficient internalization, Herceptin has been used to create the HER2-targeted ADC Kadcyla™ (ado-trastuzumab emtansine), which consists of the trastuzumab antibody linked to a cytotoxic maytansinoid.

Using the Invitrogen™ Zenon™ pHrodo™ iFL Red Human IgG Labeling Kit, we labeled Herceptin with pHrodo iFL Red–conjugated Fab fragments (Figure 3A) in order to follow its internalization in HER2-positive SKBR3 cells. pHrodo iFL Red–labeled Herceptin is nonfluorescent at neutral pH outside of cells, enabling a no-wash, no-quench assay of its internalization. No nonspecific uptake was seen in cells incubated with the pHrodo iFL dye–conjugated Fab fragments alone, confirming the specificity of the signal seen in cells treated with the pHrodo iFL dye–labeled Herceptin (Figure 3B).

Expanded options for SiteClick antibody labeling

SiteClick antibody labeling technology enables simple and site-selective attachment of compounds, including fluorescent dyes or toxins, to the carbohydrate domains present on the heavy chains of essentially all IgG antibodies, regardless of isotype and host species. We recently introduced the Invitrogen™ SiteClick™ Antibody Azido Modification Kit, which can be used to create a label-ready, site-specific azido-modified antibody, without lengthy and often inefficient genetic modification. The site-specific method employed in the SiteClick modification kit uses the enzymes β-galactosidase and β-1,4-galactosyltransferase to modify the carbohydrate domain and then attach an azide-modified sugar on the

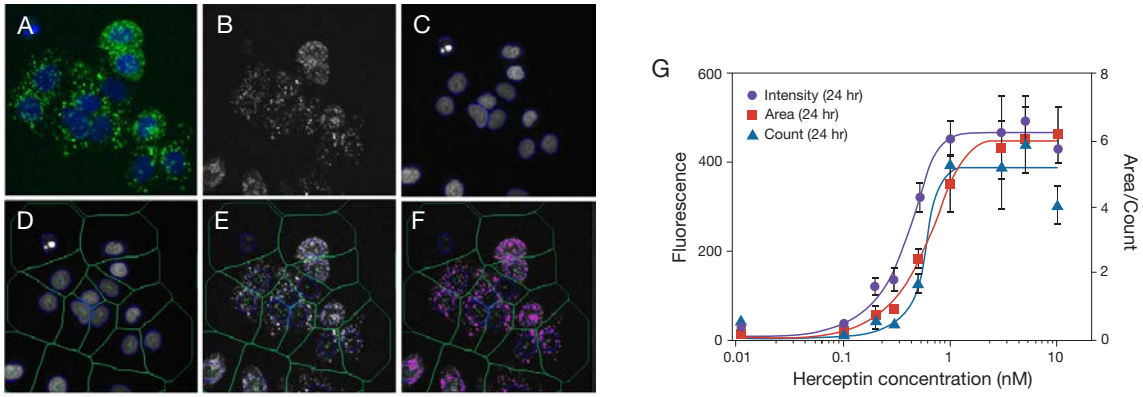

Figure 4. High-content analysis of HER2-positive cells incubated with pHrodo iFL Red–labeled Herceptin. Herceptin™ (trastuzumab) was labeled with the pH-sensitive pHrodo iFL Red dye using the Invitrogen™ SiteClick™ Antibody Azido Modification Kit in conjunction with Invitrogen™ Click-iT™ pHrodo™ Red iFL sDIBO Alkyne. HER2-positive SKBR3 cells incubated with a dose range of pHrodo iFL Red–labeled Herceptin were analyzed after 24 hr on a Thermo Scientific™ CellInsight™ CX5 High-Content Screening (HCS) Platform. **(A)** Overlay of Herceptin conjugate (green) and Hoechst™ 33342 (blue) fluorescence. **(B)** Herceptin conjugate staining alone. **(C)** Hoechst 33342 staining alone. **(D)** Cytoplasmic (ring) and nuclear (circ) segmentation with Hoechst 33342 staining. **(E)** Ring and circ overlay with Herceptin conjugate staining. **(F)** Ring, circ, and ring spot with Herceptin conjugate staining. **(G)** Over 400,000 cells were analyzed for a number of spot features using Thermo Scientific™ HCS Studio™ Cell Analysis Software—mean ring spot average intensity, mean ring spot area, mean ring spot count—in order to quantitate the fluorescence of the internalized pHrodo iFL Red–labeled Herceptin at different doses across the cell population.

heavy chains of an IgG antibody. Because the modification takes place only on the Fc portion of the heavy chains, the location and degree of labeling is very consistent and the antigen binding domains remain unaltered.

Once azido-modified, the antibody can be covalently labeled with an Invitrogen™ Click-iT™ sDIBO alkyne using copper-free click chemistry. Click-iT sDIBO alkynes are available for the pH-sensitive pHrodo iFL Red dye and the pH-insensitive Alexa Fluor dyes. Figure 4 shows HER2-positive SKBR3 cells incubated with a dose range of pHrodo iFL Red–labeled Herceptin (prepared using SiteClick labeling methods) and analyzed using the Thermo Scientific™ CellInsight™ CX5 High-Content Screening Platform, which allows quantitation of a number of parameters at a single-cell level on a large population of cells. In this experiment, over 400,000 cells were analyzed, producing a robust and reproducible data set that is particularly valuable when evaluating the performance of novel biotherapeutic antibodies across a cell population.

Find the best solution for your antibody labeling experiments

We offer a number of ready-to-use protein labeling kits for the noncovalent and covalent attachment of a broad range of intensely fluorescent dyes to your antibody or other protein, at scales from as little as 1 µg up to 3 mg IgG, including the recently introduced pHrodo iFL Microscale Protein Labeling Kits for labeling 20–100 µg of purified antibody with the amine-reactive pHrodo iFL STP ester. To learn more, visit **thermofisher.com/antbodylabelingbp76**. To see more applications of high-content imaging analysis, visit **thermofisher.com/hcabp76**. ■

Product	Quantity	Cat. No.
Zenon antibody labeling kits		
Zenon™ pHrodo™ iFL Green Human IgG Labeling Kit	1 kit	Z25611
Zenon™ pHrodo™ iFL Green Mouse IgG Labeling Kit	1 kit	Z25609
Zenon™ pHrodo™ iFL Red Human IgG Labeling Kit	1 kit	Z25612
Zenon™ pHrodo™ iFL Red Mouse IgG Labeling Kit	1 kit	Z25610
SiteClick antibody labeling kit and Click-iT sDIBO alkynes		
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™ pHrodo™ iFL Red sDIBO Alkyne for Antibody Labeling	1 kit	C20034
Amine-reactive pHrodo iFL dyes		
pHrodo™ iFL Green Microscale Protein Labeling Kit	3 labelings	P36015
pHrodo™ iFL Green STP Ester (amine-reactive)	3 x 100 µg 1 mg	P36013 P36012
pHrodo™ iFL Red Microscale Protein Labeling Kit	3 labelings	P36014
pHrodo™ iFL Red STP Ester (amine-reactive)	3 x 100 µg 1 mg	P36011 P36010