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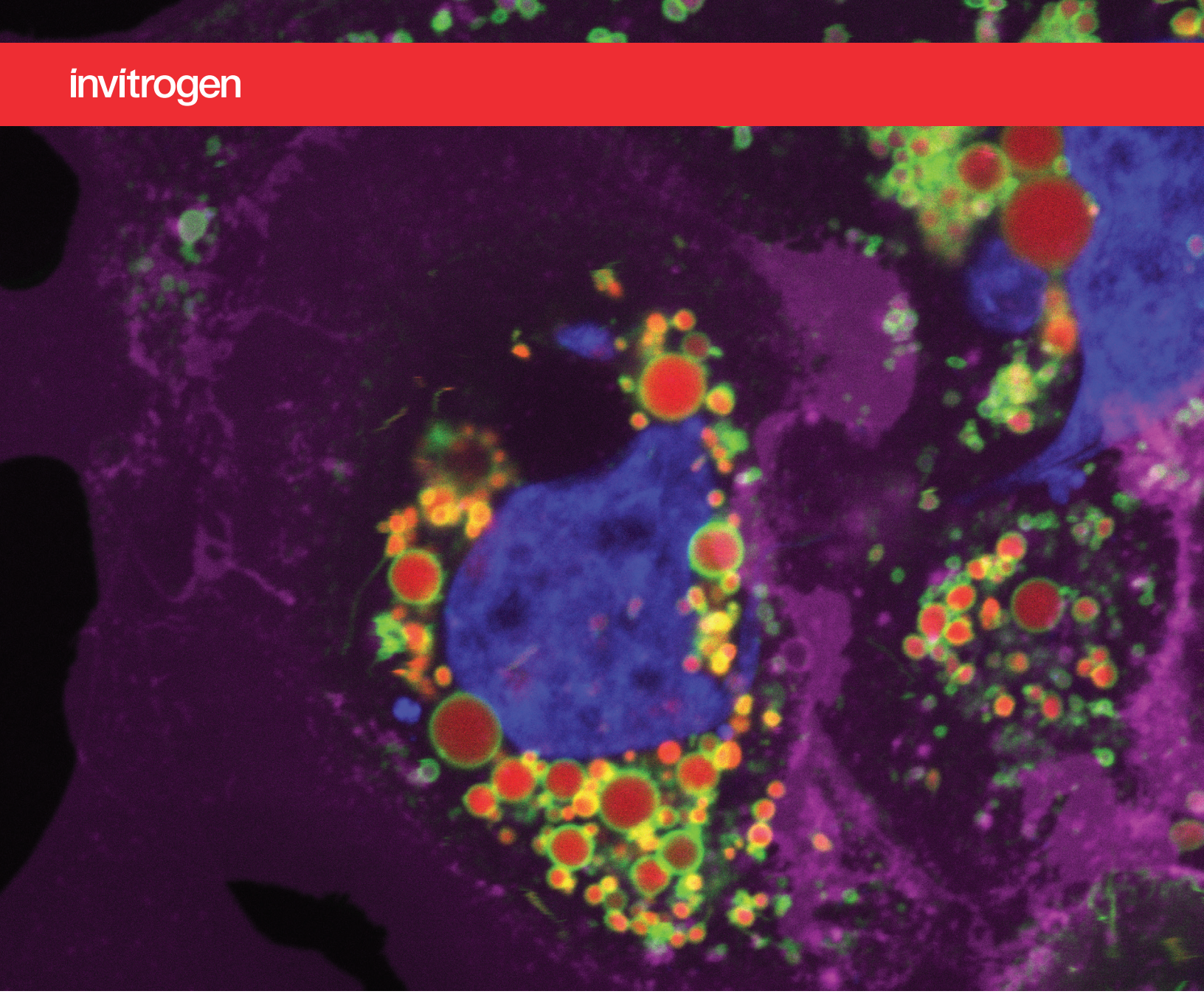

Antibody–drug conjugate screening and characterization

Simple and effective tools for monitoring antibody internalization

ThermoFisher SCIENTIFIC

Antibody–drug conjugates

Visual confirmation of internalization

Over the past decade, biologics have been increasingly pursued as therapeutic agents. In the case of antibody–drug conjugates (ADCs), internalization of the antibody is a powerful mechanism of action. Internalization moves the ADC from its binding site at the plasma membrane of the target cell to the lytic environment of the lysosome, resulting in activation of the attached toxin (Figure 1).

The efficiency of this internalization process is directly linked to the therapeutic index of the ADC. Under the Invitrogen™ brand, we have combined powerful pHrodo™ iFL dyes with Zenon™ and SiteClick™ antibody-labeling technologies to provide easy-to-use antibody labeling tools to study internalization.

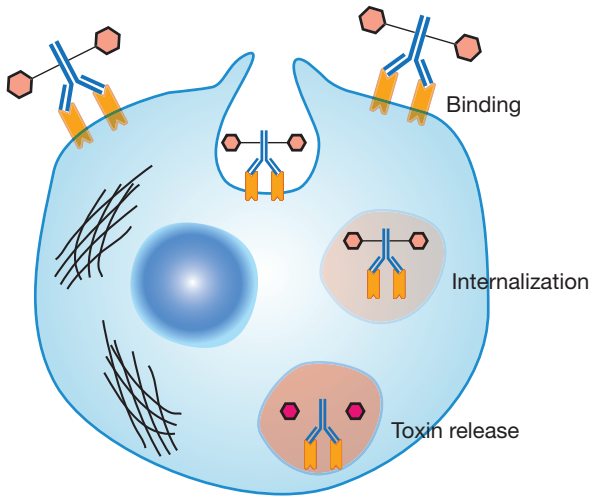

Figure 1. Internalization of an antibody–drug conjugate. An antibody–drug conjugate (ADC) comprises a monoclonal antibody directed against a tumor cell antigen coupled to a small cytotoxic molecule. An ADC is designed to specifically bind to target cells, where it is rapidly internalized. Typically the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

pHrodo iFL technology

pHrodo iFL reagents are ideal for monitoring internalization of biomolecules

Fluorophores whose fluorescence is unaffected by the environment (e.g., Invitrogen™ Alexa Fluor™ dyes), require the use of quenchers in internalization. In contrast, pHrodo iFL dyes are nonfluorescent at neutral pH and become brightly fluorescent in the acidic environment as they are internalized, thus eliminating the need for a quencher.

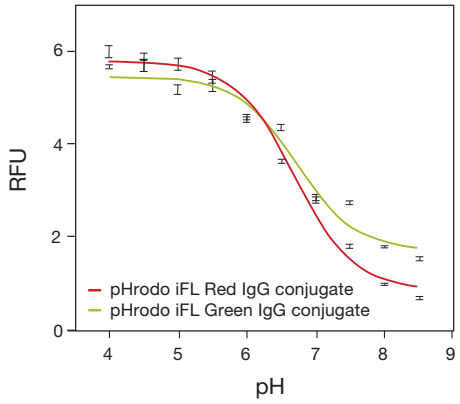

Figure 2. pH response profiles of trastuzumab conjugates prepared with amine-reactive Invitrogen™ pHrodo™ iFL Red and pHrodo™ iFL Green reagents. These conjugates will be minimally fluorescent at a neutral pH outside of cells, but they will become brightly fluorescent upon internalization into cells.

Biomolecules labeled with pHrodo iFL dyes are essentially nonfluorescent outside of cells. This feature enables a no-wash, no-quench assay for detection of endocytosis and trafficking of ADCs in live cells. Fluorescent signals are only detectable for antibodies that have been specifically internalized (Figure 3). In addition, the location of the internalized antibody in the endocytic pathway can be studied. Figure 4 shows the signal from the pHrodo iFL dye being colocalized with GFP in lysosomes.

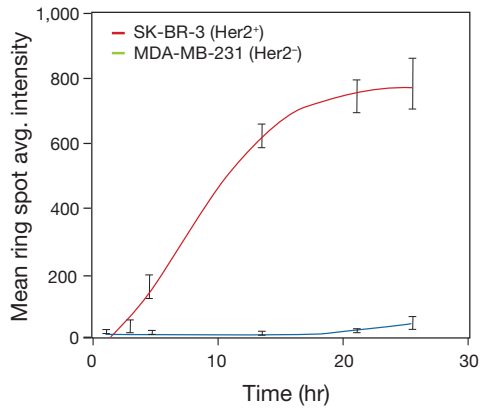

Figure 3. The fluorescent signal from trastuzumab–pHrodo iFL Red conjugates is only detected in Her2⁺ cells, indicating specific internalization. SK-BR-3 (Her2⁺) or MDA-MB-231 (Her2⁻) cells were treated with 5 nM trastuzumab–pHrodo iFL Red conjugate at 37°C for the indicated time. Cells were analyzed on the Thermo Scientific™ CellInsight™ CX5 High Content Screening (HCS) Platform. Conjugated trastuzumab is internalized into Her2⁺ cells over time; Her2⁻ cells give minimal signal.

pHrodo iFL antibody-labeling methods

pHrodo iFL Red and Green amine-reactive dyes and labeling kits

pHrodo iFL products are available for traditional antibody-labeling of available lysines on antibodies. These amine-reactive pHrodo iFL STP esters are available in green and red versions, as either stand-alone packages or microscale protein labeling kits that contain everything you need to label and purify your protein or antibody.

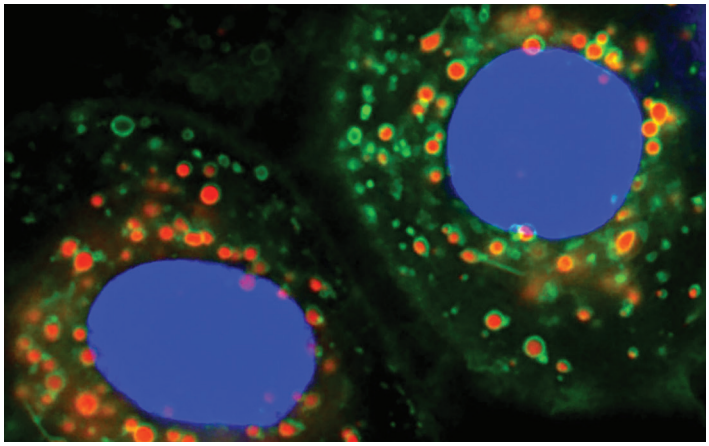

Figure 4. Trastuzumab conjugates prepared with amine-reactive pHrodo™ iFL Red STP ester become fluorescent only when internalized into acidic organelles. These Her2⁺ SK-BR-3 cells were labeled with Invitrogen™ CellLight™ Lysosomes-GFP and NucBlue™ Live ReadyProbes™ Reagent, treated for 18 hours with pHrodo™ iFL Red–Herceptin conjugate, and imaged on an Invitrogen™ EVOS™ FL Auto Imaging System.

Fast, simple, and reproducible antibody-labeling methods

SiteClick antibody-labeling technology

Making reproducible, site-specific modifications of IgG antibodies easy

Invitrogen™ 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 essentially all IgG antibodies regardless of isotype and host species (Figure 5). SiteClick labeling is based upon click chemistry. The Invitrogen™ SiteClick™ Antibody Azido Modification Kit uses enzymes to specifically attach azido moieties to the antibody carbohydrate domains. Once the azide is attached, a variety of sDIBO alkyne labels are available to conjugate with a simple incubation step. For example, pHrodo iFL Red sDIBO alkyne was attached to azido-modified antibodies to evaluate internalization in suspension and adherent cancer cell models (Figures 6, 7).

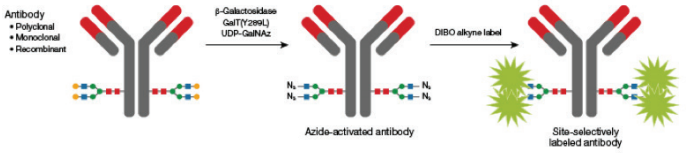

Figure 5. SiteClick labeling is a 3-step process using enzymatic modification followed by label attachment via click chemistry. First, in the SiteClick antibody-labeling process involves removal of terminal galactose residues from heavy-chain N-linked glycans using β-galactosidase, exposing modifiable GlcNAc residues. Second, the free terminal GlcNAc residues are activated with azide tags by enzymatic attachment of GalNAz using the GalT(Y289L) enzyme. Third, the azide residues are reacted via click chemistry with a dibenzocyclooctyne (sDIBO)-functionalized probe of choice (e.g., Alexa Fluor 488 sDIBO alkyne).

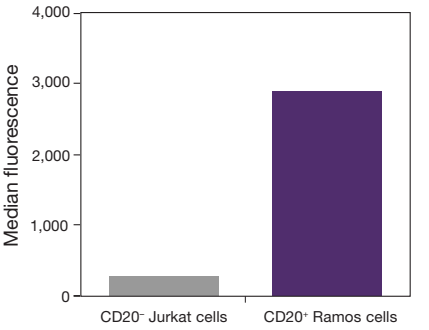

Figure 6. Internalization assay using SiteClick labeled antibodies and suspension cells, analyzed using the Attune NxT Flow Cytometer. CD20⁺ Ramos cells or CD20⁻ Jurkat cells were treated with rituximab–pHrodo iFL Red conjugate for 16 hr and analyzed using an Attune NxT cytometer. CD20⁺ cells have significantly higher median fluorescence, indicating specific antibody internalization.

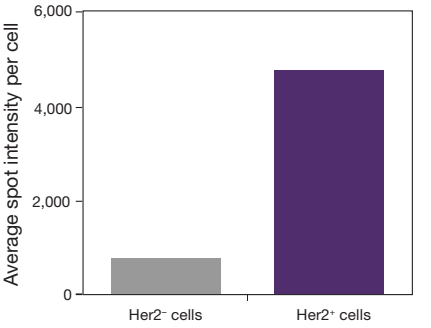

Figure 7. Internalization assay using SiteClick labeled antibodies and adherent cells, analyzed using the CellInsight CX5 HCS Platform. Her2⁺ SK-BR-3 cells or Her2⁻ MDA-MB-231 cells were treated with trastuzumab–pHrodo iFL Red conjugate for 18 hr and analyzed using a CellInsight CX5 HCS Platform. Her2⁺ cells have significantly brighter average spot intensity.

Fast, scalable ADC screening

Zenon pHrodo iFL kits

Zenon pHrodo iFL reagent—a fast, easy antibody-labeling method that is optimal for screening internalization

Zenon labeling technology provides a reliable method for producing fluorescently labeled antibodies with pHrodo iFL Green or Red labels in 10 minutes. Zenon fragments are specifically designed to target and bind to the Fc portion of a human or mouse IgG antibody (Figure 8). The Zenon labeling method offers unique flexibility for screening various antibodies or even antibody concentrations (Figure 9). Using Zenon technology you can label as little as 1 µg of antibody, and unlike traditional labeling methods using amine- or thiol-reactive labels, the Zenon labeling procedure is compatible with BSA and other stabilizing proteins.

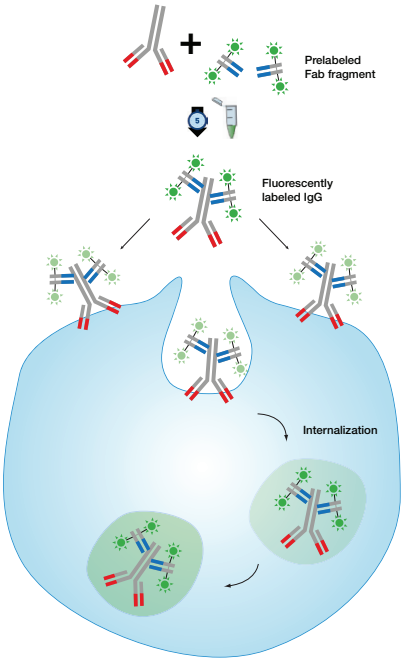

Figure 8. The Zenon pHrodo iFL labeling reagents can be complexed with human or mouse IgG to provide rapid labeling for fast, scalable screening of antibody internalization for many antibody samples.

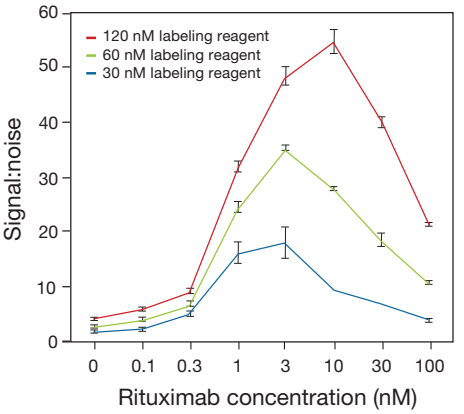

Figure 9. Rituximab labeled with Zenon pHrodo iFL Red reagent becomes brightly fluorescent when internalized by CD20⁺ cells, providing a clear indication of specific antibody internalization in a wide range of antibody concentrations. After being labeled with three different concentrations of the Zenon pHrodo iFL Red reagent, labeled rituximab was added to CD20⁺ cells at varying concentrations for 16 hr at 37°C, and the signal-to-noise ratio (S/N) was measured using an Attune NxT Flow Cytometer.

On the cover:

Trastuzumab labeled with amine-reactive pHrodo™ iFL Red reagent becomes fluorescent only when internalized into acidic organelles. Live Her2⁺ SK-BR-3 cells are also labeled with CellLight™ Lysosomes-GFP, NucBlue™ Live ReadyProbes™ Reagent, and CellMask™ Deep Red Plasma Membrane Stain.

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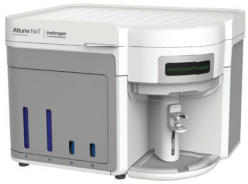
Compiling data sets from multiple instrument platforms allows you to better understand, predict and, ultimately, influence the factors that underlie antibody internalization and drug development research.



Attune NxT Flow Cytometer

Varioskan™ LUX Plate Reader

CellInsight CX5 HCS Platform

EVOS FL Auto 2 Imaging System

Ordering information

Product	Size	Cat. No.
pHrodo iFL Red STP ester, amine reactive	1 mg	P36010
	3 x 100 µg	P36011
pHrodo iFL Green STP ester, amine reactive	1 mg	P36012
	3 x 100 µg	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
SiteClick Biotin Antibody Labeling Kit	1 kit	S20033
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
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

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