Application Note

Cell Analysis

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Real-Time Antibody Internalization Assays with Agilent xCELLigence RTCA eSight

For live-cell analysis and kinetic quantification

Authors

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Abstract

In recent years, antibody-drug conjugates (ADCs) have emerged as a significant advancement in tumor treatment. These therapies rely heavily on target-directed internalization. This has increased the need for improved antibody internalization detection methods that are precise, convenient, and cost-effective to select high efficacy monoclonal antibodies, evaluate mechanisms of action, and enhance safety. Antibody internalization detection and quantification methods aid biological therapeutic advancement by validating biomarkers, understanding receptor dynamics, and optimizing intracellular delivery systems. This application note presents a robust method to perform real-time, quantitative antibody internalization assays using the Agilent xCELLigence RTCA eSight system combined with CypHer5E-NHS-ester-labeling dyes. CypHer5E is a pH-sensitive, red-excited cyanine dye that fluoresces in acidic pH environments, enabling precise tracking of antibodies moving into different intracellular compartments. We demonstrated that CypHer5E-labeled trastuzumab is selectively internalized in human epidermal growth factor receptor 2 (HER2) positive cell lines through receptor-mediated uptake. We confirmed that the primary CypHer5E-labeling and secondary CypHer5E-Fab-conjugated-labeling methods effectively enable visible internalization of trastuzumab using the xCELLigence RTCA eSight system. Furthermore, secondary Fab-CypHer5E was used to analyze the internalization of multiple therapeutic antibodies targeting HER2. Overall, the method presented offers a robust, integrated tool to study antibody internalization, providing valuable insights to optimize therapeutic antibody efficacy, safety, and pharmacokinetics in biopharmaceutical discovery stages.

Introduction

Antibody internalization refers to the process in which antibodies bind to specific cell surface receptors, inducing receptor-mediated endocytosis and bringing the antibody-receptor complex into the intracellular space. This phenomenon represents the primary mechanism by which ADCs enter cells and is a prerequisite for ADC drugs to exert therapeutic effects on tumors.[1] Considered the "missiles" of ADC drugs, antibodies possess targeting capabilities that enable them to recognize and bind to tumor cell surface antigens specifically. Highly cytotoxic compounds are covalently linked to targeting antibodies to facilitate their uptake into tumor cell lysosomes for degradation. An active form of the compound is then released, leading to the destruction of the tumor cells.[1,2] The magnitude and rate of internalization may strongly vary between antibodies, even when they recognize the same target molecule epitope on a cancer cell.[3]

Therefore, accurate targeting and efficient antibody internalization are crucial indicators during early-stage screening and engineering in ADC drug development. These characteristics also significantly contribute to the therapeutic effect and safety of ADC drugs. Additionally, beyond their significance in ADC development, internalization studies are vital in understanding receptor dynamics, validating biomarkers, and optimizing intracellular delivery systems for targeted therapies. Here, we present a solution to asses antibody internalization using real-time, live-cell imaging with the xCELLigence RTCA eSight instrument. Unlike endpoint detection, which only provides data at a single point and requires cell destruction, the xCELLigence RTCA eSight enables continuous real-time measurement of antibody internalization for more than 72 hours. Another distinct advantage shown is automated, live-cell kinetic data generation inside a tissue culture incubator, and simple quantitative analysis at various concentrations and time points. In this application note, we used a conventional labeling reagent known as CypHer5E to directly label trastuzumab or immunoglobulin G (IgG) to detect antibody internalization in different cell lines. We also prepared secondary Fc-targeted CypHer5E-labeled Fab binding protein to bind and form complexes with trastuzumab or trastuzumab-based ADC drugs to screen and compare large antibody libraries within a simple internalization assay. These methods offer an effective, reproducible blueprint for biopharmaceutical discovery and optimization, especially in screening and engineering fast, efficient internalized antibodies in ADC discovery, greatly contributing to developing potent ADCs.

Assay principle

CypHer5E NHS Ester (Cytiva, part number PA15401) is a pH-sensitive cyanine dye derivative that is minimally fluorescent at basic pH and shows maximal fluorescence at an acidic pH. It can label amines of lysine residues on both proteins and antibodies. When an antibody conjugated with CypHer5E dye binds to antigens on the cell membrane surface, it exhibits minimal fluorescence. Upon receptor-mediated internalization, antibody-CypHer5E conjugates traffic through the endosome (pH ~ 6.3) and the lysosomal (pH ~ 4.7) system, where pH becomes more acidic, causing the antibody-CypHer5E complex to fluoresce (Figure 1A). The fluorescence signal intensity that reflects internalization efficiency is measured and quantified by the xCELLigence RTCA eSight in real time. CypHer5E can directly label the antibody being tested, or it can first label Fab fragments (here the AffiniPure Fab fragment goat anti-human IgG (H+L), Jackson ImmunoResearch, part number 109-007-003, was used), which then binds to the Fc fragment of the antibody being tested to provide indirect labeling.

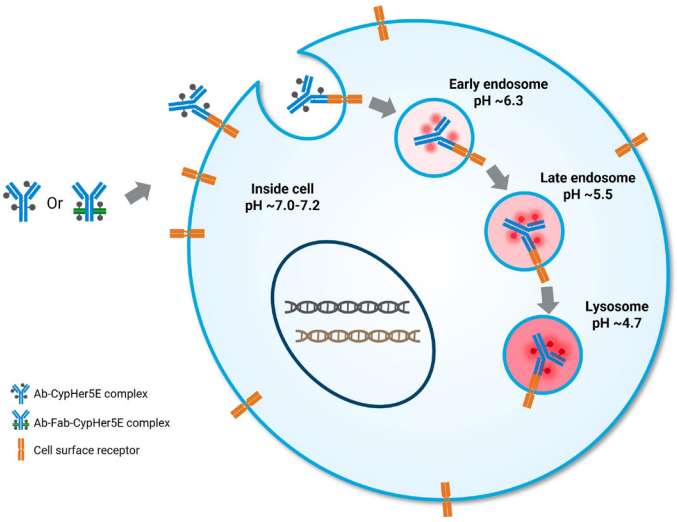


Figure 1. Antibody (Ab) internalization assay principle using CypHer5E NHS ester and the Agilent xCELLLigence RTCA eSight imaging system. CypHer5E is a red-excited fluorescent, pH-sensitive cyanine dye. It is nonfluorescent at basic pH and is fluorescent at acidic pH. The fluorescence signal of the internalized antibody can be observed when the Ab-CypHer5E or Ab-Fab-CypHer5E complex is transported and processed through acidic endosomes and lysosomes.

Experimental

Cell maintenance and assays were conducted in a 37 °C incubator with 5% CO_2. Cell lines and their growth media are shown in Table 1. Fetal bovine serum (FBS) (Gibco, part number 10099-141) and penicillin/streptomycin (pen/strep) (HyClone, part number SV30010), were used as the media supplements. Sodium bicarbonate 7.5% solution was purchased from Gibco (part number 25080094).

Table 1. Cell lines and their growth media

Cell Lines	Base Media	Media Supplements
MCF7	DMEM (Gibco, p/n 12430-054)	10% FBS + 1% pen/strep
HCC1954	RPMI 1640 (Gibco, p/n 11875-093)	10% FBS + 1% pen/strep
SKOV3	Moccy's 5A (Gibco, p/n 16600-082)	10% FBS + 1% pen/strep
SKBR3	RPMI 1640 (Gibco, p/n 11875-093)	10% FBS + 1% pen/strep
BT474	Hybri-Care (ATCC, p/n 46-X)	10% FBS + 1% pen/strep

Antibodies being tested (Table 2) were purchased and dissolved according to the manufacturer's protocol. CypHer5E N-hydroxysuccinimide (NHS) ester (Cytiva, part number PA15401) was conjugated to antibodies according to the manufacturer's protocol.

Table 2. Tested antibodies

Antibody	Supplier	Part Number
Trastuzumab	MedChemExpress	HY-9907
Human IgG1 Kappa	MedChemExpress	HY-9901
Trastuzumab deruxtecan (T-DXd/DS-8201)	Selleck	E0200
Trastuzumab Emtansine (T-DM1)	MedChemExpress	HY-9921

Measurement of cell surface HER2 expression using flow cytometry

To detect cell surface HER2 expression, cells were harvested from T25 cell culture flasks. Cells were stained with either the anti-HER2 BV421 antibody (Biolegend, part number 324420) or IgG isotype BV421 antibody (Biolegend, part number 400157) for 30 minutes on ice in the dark. Cells were then washed twice using PBS containing 2% FBS and analyzed using flow cytometry (Agilent, NovoCyte flow cytometer).

**Note: As highlighted in this application note, we utilized an enhanced Agilent xCELLigence RTCA eSight filter set to further improve performance of the red fluorescent channel, including extended wavelength. Exposure times may need to be adjusted by the user, as needed, to further increase and optimize red fluorescent intensity.*

Direct labeling antibodies by CypHer5E

Antibodies were diluted to 1 mg/mL in 7.5% sodium bicarbonate, pH 8.3. A 20-fold molar excess of CypHer5E dye was added to the antibody solutions and incubated for one hour at room temperature in the dark. The unconjugated dye and low molecular weight contaminants were removed by dialyzing against PBS overnight at 4 °C or fractionating over a PD minitrap G-25 desalting column (Cytiva, part number 28922529). The fraction containing the antibody-CypHer5E conjugation was collected and adsorption was measured at 280 and 500 nm, A280 and A500, respectively. The antibody concentration and the average number of CypHer5E molecules conjugated to the antibodies, known as the dye-to-protein ratio (D/P), were calculated based on A280 and A500 (Figure 2A). For the CypHer5E conjugates purified by dialyzing overnight used in this study, the D/Ps were: trastuzumab-CypHer5E D/P = 3.9, Isotype IgG1-CypHer5E D/P = 3.4, and goat anti-human IgG (H+L) Fab-CypHer5E D/P = 10.5. Labeled antibodies can be stored at 4°C in the dark for 24 hours. For long-term storage, it is recommended to be stored at –15 to –30 °C. See CypHer5E NHS ester product specification sheet for additional information.[4]

Indirect labeling antibodies by CypHer5E

For step one, the CypHer5E-conjugated Fab fragment was prepared and quantified following the CypHer5E direct labeling protocol. In step two, antibodies (Table 2) were mixed with Fab-CypHer5E at a 1:3 molar ratio in complete growth media and incubated for 30 minutes at 37 °C (Figure 2B). Any required dilutions were performed after conjugation to maintain the molar ratio in the labeling step. Fab-CypHer5E enables for an easy, nonpurified labeling method for all various antibodies.

Antibody internalization assay

Cell lines were seeded into flat-bottom, 96-well plates. BT474 and SKBR3 were added at 10,000 to 15,000 cells per well while A549, HCC1954, and MCF7 were plated at 8,000 to 10,000 cells per well. The culture media was replaced with complete media containing either directly- or indirectly-labeled antibodies overnight after cell seeding. The plates were immediately placed in the xCELLigence RTCA eSight system and scanned for brightfield and red fluorescence images at 10x magnification every hour for up to 48 hours (Figure 2C).

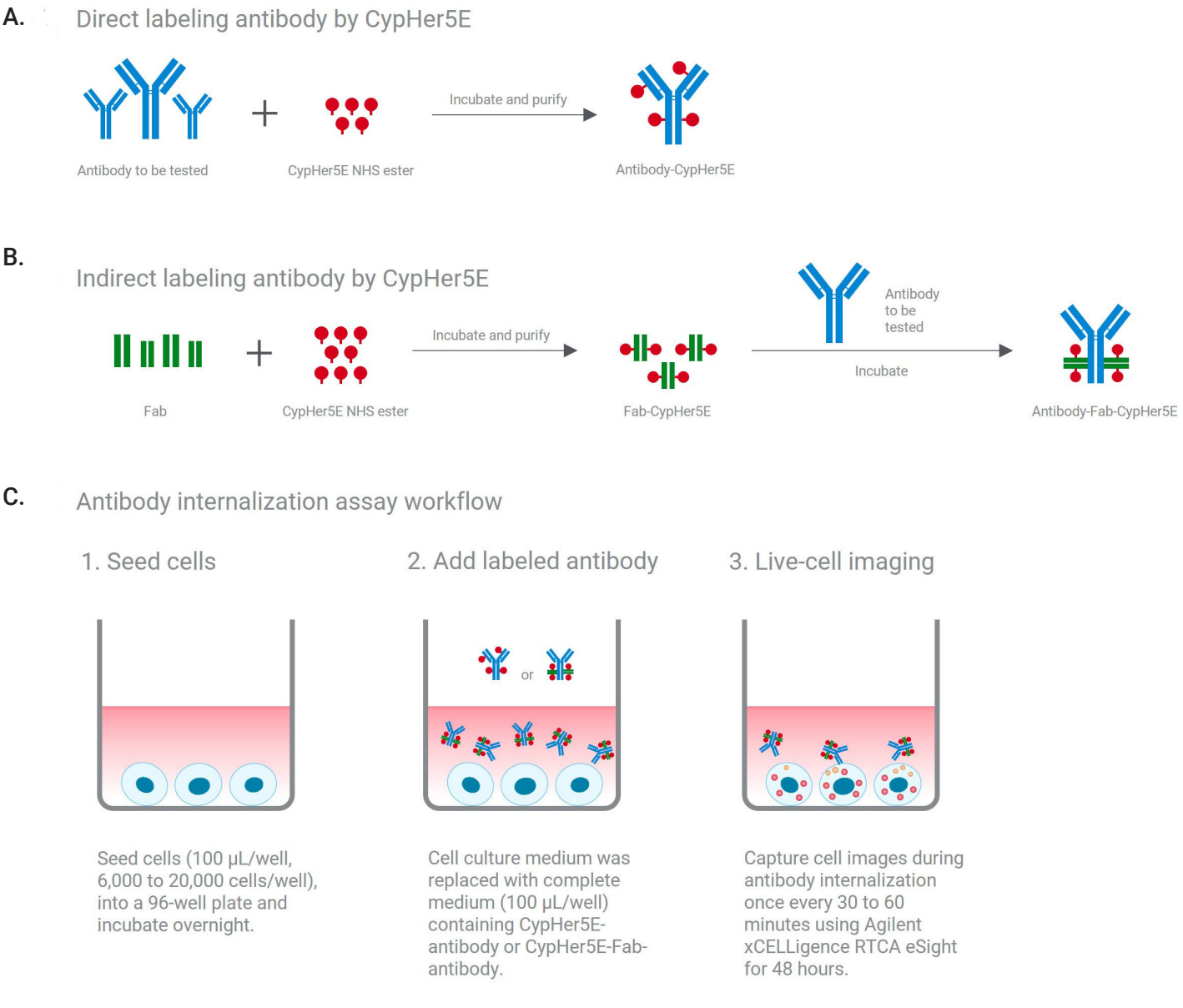


Figure 2. Brief workflows for antibody labeling methods and antibody internalization assay. The antibody to be tested was labeled with CypHer5E (A) directly or (B) indirectly. (C) Antibody internalization assay workflow.

Data analysis

Images were first processed with optimized parameters. Cells were well identified using brightfield images (Figure 3B). The red fluorescence signals, representing the internalized antibodies, were recognized as shown in Figure 3D. Next, the brightfield total area, measuring cell area, the red total area, and the red total integrated intensity, were automatically quantified and provided by the software, reflecting the degree of antibody internalization. Additionally, the ratio of red total area to brightfield total area referred to as normalized red area (normalized red area = red total area / brightfield total area), represents the antibody internalization rate. This parameter helps eliminate well-to-well cell seeding or cell proliferation variation, providing a robust antibody internalization evaluation.

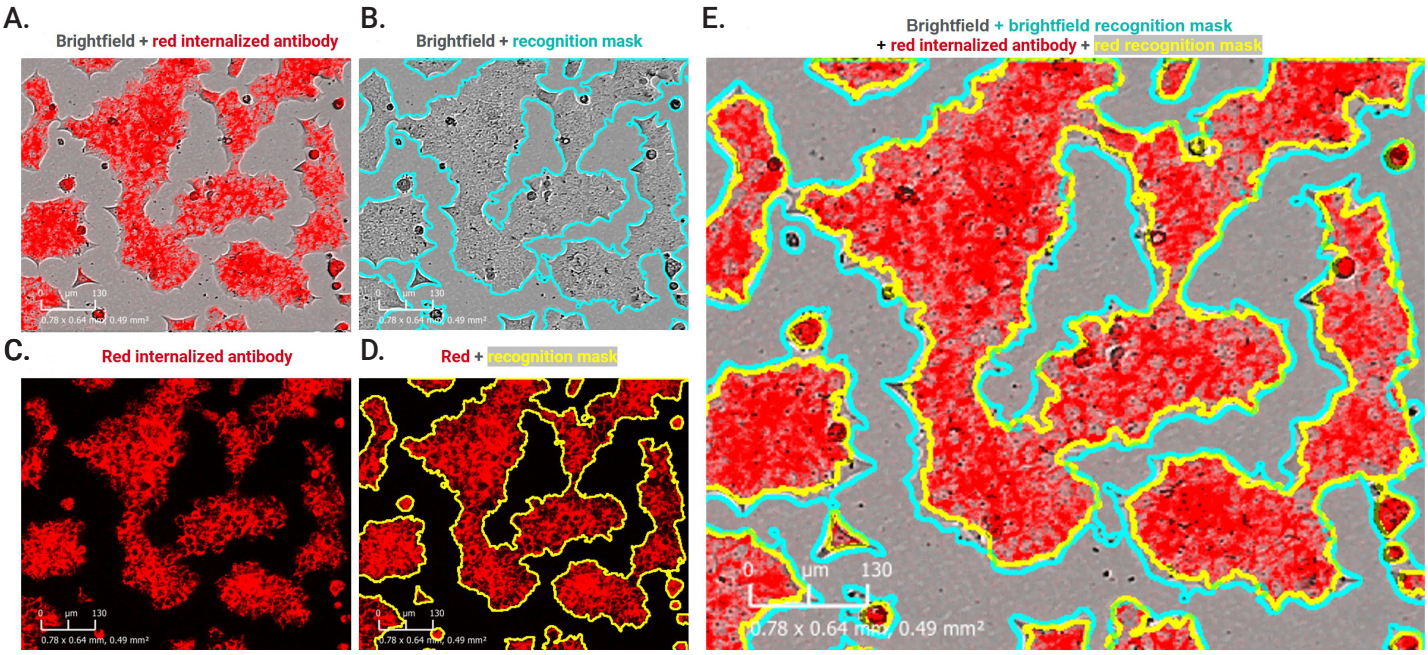


Figure 3. Representative images show the analysis principle for trastuzumab-CypHer5E internalization by BT474 cells. Images were captured 48 hours post Ab-CypHer5E addition, using 10x magnification, through brightfield and red fluorescence channels on the Agilent xCELLigence RTCA eSight.

Results and discussion

Quantification of trastuzumab internalization in real-time using the xCELLigence RTCA eSight system

Trastuzumab is a monoclonal antibody that targets HER2, inhibiting the growth of HER2-positive cancer cells by blocking the attachment of an epidermal growth factor. It is used in treating HER2-positive metastatic breast cancer and gastric cancer.[5,6] Here, we detect the internalization of trastuzumab in tumor cell lines with varying levels of HER2 expression. We first quantified HER2 expression on the membrane of a panel of cancer cell lines using flow cytometry (Figure 4A). All cell lines, except for MCF7, exhibited high levels of HER2 expression with more than 99% of cells being HER2-positive.

Following this, 2 µg/mL of trastuzumab-CypHer5E or IgG-CypHer5E was added to various cell lines 24 hours after cell seeding. Brightfield and red fluorescence images were captured for 48 hours at one-hour intervals.
The results showed that trastuzumab-CypHer5E was internalized by the HER2 high-expression cell lines (HCC1954, SKBR3, SKOV3, and BT474) (Figure 4B), showing intense red fluorescence. Furthermore, trastuzumab-CypHer5E was not internalized by HER2-negative or low expression cell line MCF7, as expected. For the HER2 high-expression cell lines, trastuzumab was internalized, but not the control (IgG).

Taken together, this demonstrates the specificity of the internalization signal as measured by the assay. Red total area (µm²/image), red total integrated intensity (RRi × µm²/image), and normalized red area (%) were used to quantify the efficiency of internalization (Figure 4C, 4D, and 4E). As shown, internalization happened almost immediately, as soon as the HER2-positive cell lines were exposed to trastuzumab-CypHer5E. The red total area (Figure 4C), red total integrated intensity (Figure 4D) and normalized red areas (Figure 4E) increased rapidly within about six hours of adding trastuzumab-CypHer5E and stabilized after 12 hours, indicating an internalization equilibrium after rapid uptake. At 72 hours (48 hours posttreatment), the normalized red area of BT474, SKBR3, HCC1954, and SKOV3 (Figure 4E) reached 74.15, 41.65, 37.66, and 19.69%, respectively, showing that BT474 cells exhibited the most effective trastuzumab-CypHer5E internalization.

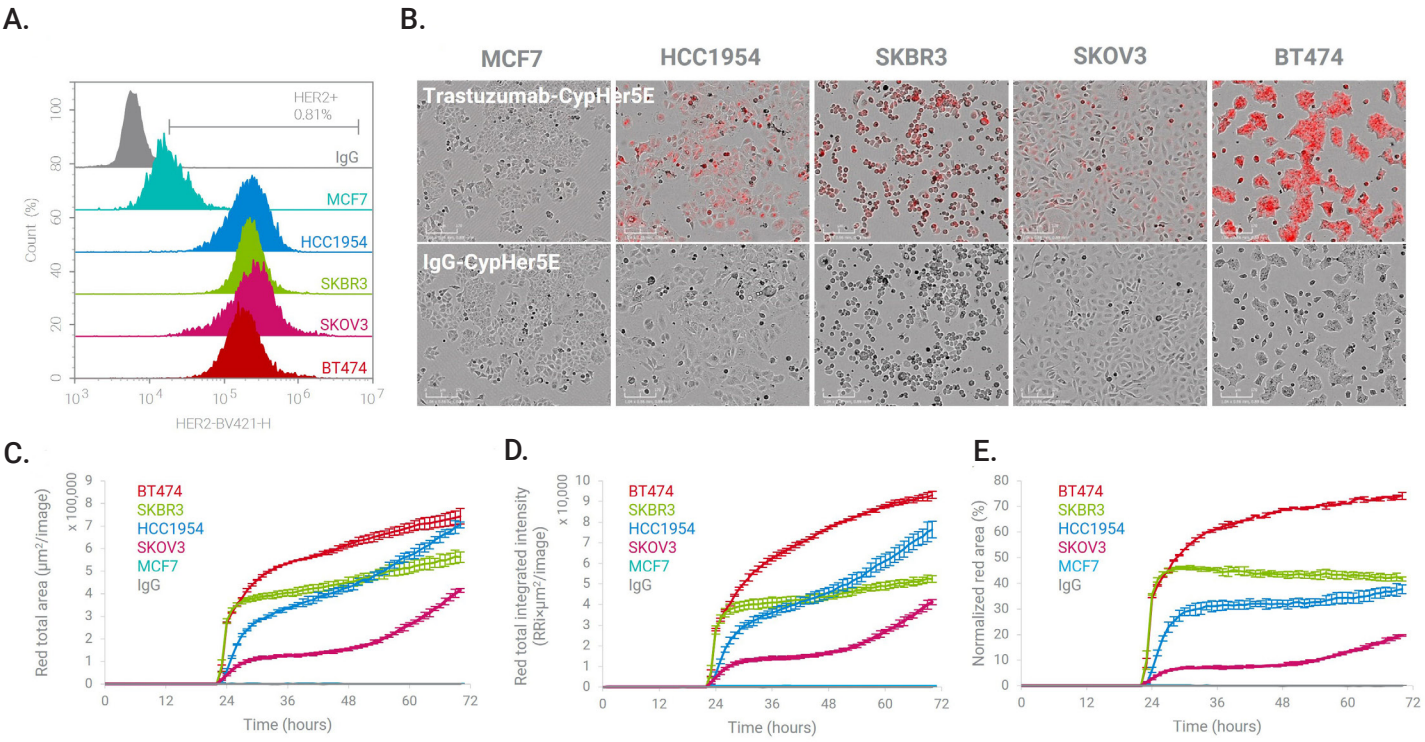


Figure 4. Internalization of trastuzumab-CypHer5E by different cell lines. (A) HER2 expression levels of MCF7, HCC1954, SKBR3, SKOV3, and BT474 cells were measured and compared to the steady state (determined by IgG isotype BV421 antibody staining) using a flow cytometer. (B) MCF7, HCC1954, SKBR3, SKOV3, and BT474 cells were treated with either trastuzumab-CypHer5E or IgG1-CypHer5E at a concentration of 2µg/mL. Brightfield and red fluorescence images (10x) were captured 12 hours posttreatment. The time-dependent red total area (C), red total integrated intensity (D), and normalized red area (E) show the kinetic antibody internalization process.

Evaluation of direct and indirect antibody labeling methods for antibody internalization assay

To verify the effectiveness of both the direct and indirect labeling methods to study trastuzumab internalization kinetics, serially diluted trastuzumab-CypHer5E or trastuzumab-Fab-CypHer5E (2,000 to 31.25 ng/mL) was added to BT474 cells in a 96-well plate, and antibody internalization was monitored for 48 hours.

With both labeling methods, trastuzumab internalization by BT474 cells was observed and red fluorescence markedly increased over time (0 to 12 hours) when trastuzumab concentrations were higher than 31.3 ng/mL (Figure 5A, 5B, and 5C). The EC50 values for trastuzumab-CypHer5E and trastuzumab-Fab-CypHer5E were very close (89.37 and 124.00 ng/mL, respectively), demonstrating that both direct and indirect labeling methods can be used to visualize and accurately evaluate trastuzumab internalization kinetics.

Of note, in the indirect labeling assay, we first labeled the anti-human Fab with CypHer5E, which reacts with whole molecule human IgG and light chains of other human immunoglobulins.

Internalization of trastuzumab and trastuzumab-conjugated drugs by BT474 cells using indirect labeling

Secondary Fab-CypHer5E offers a cost-efficient way to label multiple antibodies simultaneously, eliminating the potential disturbance from variations in the D/P ratio. To illustrate the quantitative convenience of the indirect labeling method and its flexibility for analyzing multiple therapeutic antibodies and drugs, we compared the internalization ability of BT474 cells against trastuzumab and trastuzumab drug conjugates T-DM1 and DS8201 (in parallel) to determine the EC50 values for internalization (Figure 6). T-DM1 and DS8201 are ADCs generated by trastuzumab, conjugated with microtubule inhibitors or cytotoxic topoisomerase inhibitors, respectively. After labeling with the secondary Fab-CypHer5E reagent, each drug was serially diluted (1:2, 2,000 to 31.25 ng/mL) and added to cells at 24 hours post-cell-seeding. Labeled IgG was added as an isotype control.

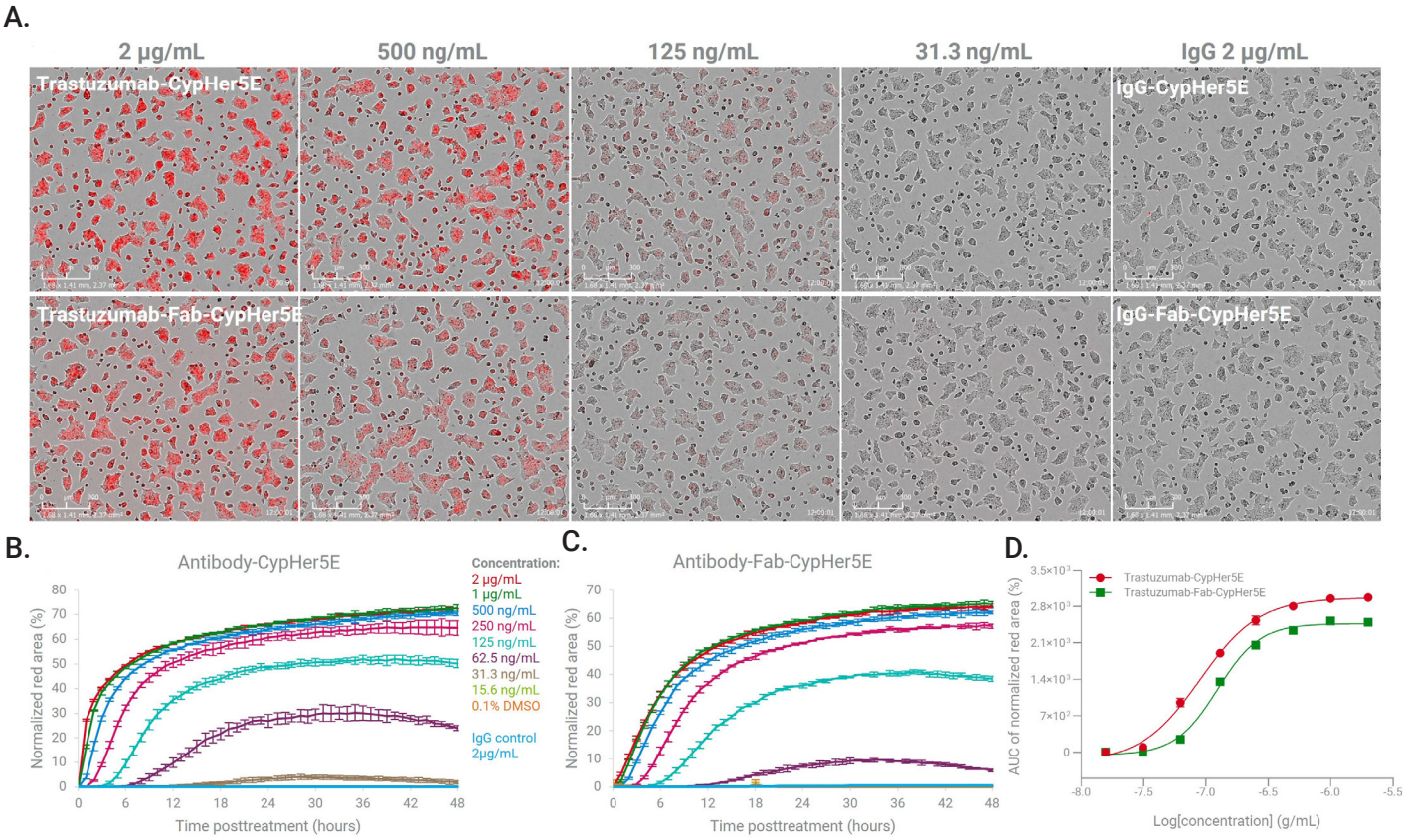
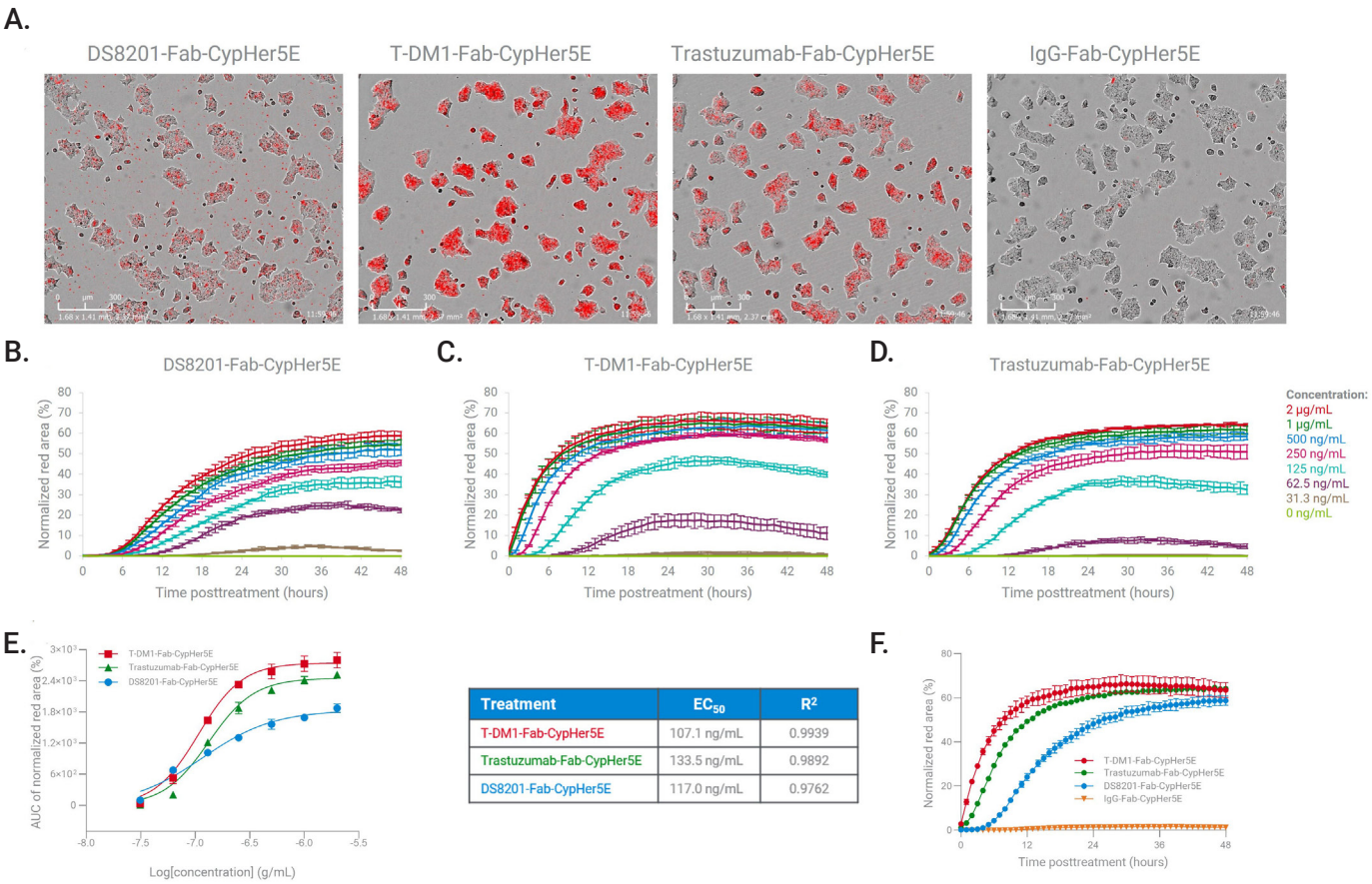


Figure 5. Comparison of directly and indirectly labeled antibodies for antibody internalization assay. (A) BT474 cells were treated with various concentrations of trastuzumab-CypHer5E (the upper panel) or trastuzumab-Fab-CypHer5E (the lower panel). IgG-CypHer5E and IgG-Fab-CypHer5E served as the control. Brightfield and red fluorescent images were captured 12 hours after treatment using 10x magnification. The normalized red area curves of cells were treated with increasing concentrations of (B) trastuzumab-CypHer5E, or (C) trastuzumab-Fab-CypHer5E. (D) Dose-response curves generated from panels B and C. The EC50 values for direct labeling with trastuzumab-CypHer5E, and indirect labeling with trastuzumab-Fab-CypHer5E were 89.37 and 124.00 ng/mL, respectively.

In HER2-positive BT474 cells, concentration-dependent internalization of Fab-CypHer5E conjugated DS8201 (Figure 6B), T-DM1 (Figure 6C), and trastuzumab (Figure 6D) was observed over 48 hours. From an area-under-curve (AUC) analysis, the EC50 value for internalization of Fab-CypHer5E conjugated DS8201, T-DM1, and trastuzumab was 117.0, 107.1, and 135.5 ng/mL, respectively (Figure 6E).

The kinetics of internalization of DS8201-Fab-CypHer5E, T-DM1-Fab-CypHer5E, trastuzumab-Fab-CypHer5, or IgG-Fab-CypHer5E (2µg/mL) by BT474 cells were compared by plotting the normalized red area versus the time of these conditions together (Figure 6F). T-DM1 was quickly internalized within six hours after treatment and reached a plateau. Trastuzumab showed a slightly slower internalized rate, while DS8201 was internalized with the slowest rate, achieving an internalization equilibrium in approximately 36 hours. IgG is not internalized due to the absence of antibody-receptor interaction (Figure 6F). These data confirm the applicability and certain advantages of the indirect labeling method for comparing the internalization of multiple antibodies and drugs targeting the same cell surface receptor.



Treatment	EC_{50}	R^2
T-DM1-Fab-CypHer5E	107.1 ng/mL	0.9939
Trastuzumab-Fab-CypHer5E	133.5 ng/mL	0.9892
DS8201-Fab-CypHer5E	117.0 ng/mL	0.9762

Figure 6. Visualization and measurement of the internalization of trastuzumab and trastuzumab-conjugated drugs driven by HER2 on BT474 cell surfaces. BT474 cells were treated with DS8201(cytotoxic topoisomerase inhibitor)-Fab-CypHer5E, T-DM1(microtubule inhibitor)-Fab-CypHer5E, or trastuzumab-Fab-CypHer5E at various concentrations, or 2 µg/mL of IgG-Fab-CypHer5E . Brightfield and red fluorescent images were captured every hour for 48 hours using 10x magnification. (A) Representative images measured at 12 hours posttreatment. (B) The normalized red area of cells treated with DS8201-Fab-CypHer5E, (C) T-DM1-Fab-CypHer5E, and (D) trastuzumab-Fab-CypHer5E increased over time with growing treatment concentration. The area under the curve analysis of response curves in panels B, C, and D displayed concentration-dependent internalization (0 to 48 hours post treatment) as EC50 values of 117.0, 107.1, and 133.5 ng/mL, respectively. (F) Time-varying internalization of DS8201-Fab-CypHer5E, T-DM1-Fab-CypHer5E, trastuzumab-Fab-CypHer5, or IgG-Fab-CypHer5E (2µg/mL) in BT474 cells.

Conclusion

This application note demonstrates that the Agilent xCELLigence RTCA eSight can kinetically monitor and quantify the rate of antibody internalization with a pH-sensitive dye. This dye fluoresces upon translocation to acidic endocytic compartments, and fluorescence can be measured by real-time imaging analysis. Both direct and indirect antibody labeling methods have certain advantages. The direct antibody labeling method has fewer experimental steps, simpler operations, and higher dye use. Detecting antibody internalization using CypHer5-coupled Fab adds additional steps. However, the secondary Fab-CypHer5E can be easily used to detect large antibody libraries, while offering a convenient way to label multiple antibodies at once and eliminate potential variation in the dye-to-protein ratio. Following this, the internalization ability of cells for different antibodies can be quantified and compared using metrics like red total area and red total integrated intensity.

In summary, this integrated, cost-effective method and assay on the RTCA eSight facilitates precise antibody internalization detection and quantification, while offering unique benefits across therapeutic antibody development, receptor biology, and biomarker validation. Ultimately, this aids in the advancement of antibody efficacy, safety, and pharmacokinetic-based studies.

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Products used in this application

Agilent products

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