Real-Time Imaging for Quantitative Assessment of Antibody-Drug Conjugate Internalization

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Abstract

Antibody-drug conjugates (ADCs) represent a major advancement in cancer therapy, relying on efficient target-mediated internalization for optimal efficacy.[1] This underscores the need for precise, convenient, and cost-effective methods to monitor antibody internalization for candidate selection, mechanism-of-action studies, and safety evaluation.

We present a robust, real-time quantitative assay using the Agilent xCELLigence RTCA eSight system combined with CypHer5E-NHS-Ester labeling. CypHer5E is a pH-sensitive cyanine dye that fluoresces in acidic environments, enabling dynamic tracking of antibody trafficking into intracellular compartments.[2] We demonstrated selective internalization of CypHer5E-labeled trastuzumab in HER2-positive cell lines via receptor-mediated uptake. Both primary CypHer5E labeling and secondary Fab-CypHer5E conjugation enabled clear visualization of internalization events. Furthermore, secondary Fab-CypHer5E labeling facilitated comparative analysis of multiple HER2-targeting therapeutic antibodies.

This integrated platform provides a powerful tool for mechanistic studies and drug-screening assays, offering critical insights to optimize antibody efficacy, safety, and pharmacokinetics in biopharmaceutical development.

Assay Principle

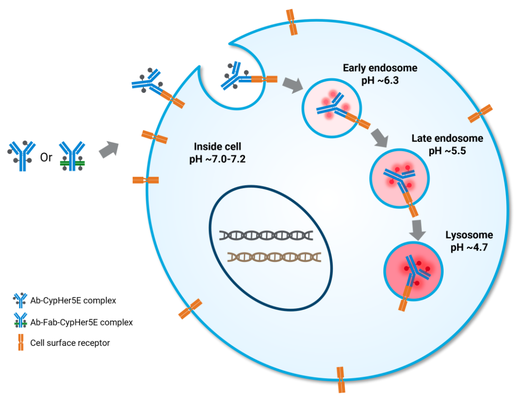


Figure 1. Principle of antibody internalization assay using Cytiva CypHer5E Mono NHS Ester and xCELLigence RTCA eSight system. CypHer5E is a red-excited fluorescent, pH-sensitive cyanine dye. It is nonfluorescent at basic pH and is fluorescent at acidic pH. The fluorogenic signal of the internalized antibody can be observed when the CypHer5E-Ab or CypHer5E-Fab-Ab complex is transported and processed through acidic endosomes and lysosomes.

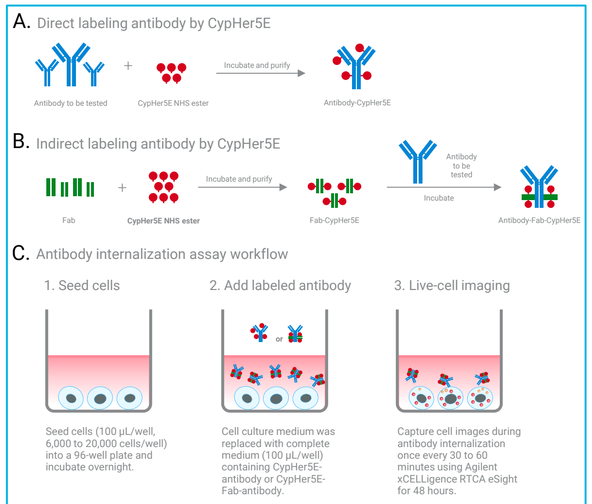
References

[1]Hamamichi, S.; Fukuhara, T.; Umeda, I. O.; Fujii, H.; Hattori, N. Novel method for screening functional antibody with comprehensive analysis of its immunoliposome. *Sci. Rep.* **2021**, *11*(1) 4625. DOI:10.1038/s41598 -021-84043-w2.

[2]Cytiva, Molecular and Immunodiagnostics, Western blotting and other genomics reagents, CypHer5E NHS Ester Product Specification Sheet. https://www.cytivalifesciences.com/en/us/shop/molecular-and-immunodiagnostics/genomic-consumables/ cypher5e-nhs-ester-p-00341

Experimental

Brief workflow for antibody labelling methods and antibody internalization assay



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 by fractionating over a PD minitrap G-25 desalting column (Cytiva, part number 28922529).

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 were mixed with Fab-CypHer5E at a 1:3 molar ratio in complete growth media and incubated for 30 minutes at 37 °C. Any required dilutions were performed after conjugation to maintain the molar ratio in the labeling step.

Results and Discussion

Analysis principle for the antibody internalization assay

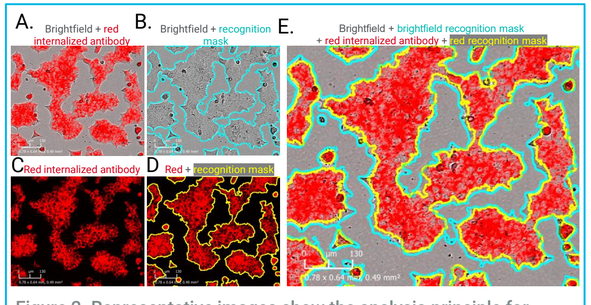


Figure 2. Representative images show the analysis principle for trastuzumab-CypHer5E internalization by BT-474 cells. Images were captured 48 hours post antibody-CypHer5E addition, using 10x magnification, through brightfield and red fluorescence channels on the Agilent xCELLigence RTCA eSight. Crucially, the key quantitative parameter, the normalized red area, is calculated by dividing the red total area **(D)** by the brightfield total area **(B)**. This value reflects the antibody internalization rate **(E)**.

Results and Discussion

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

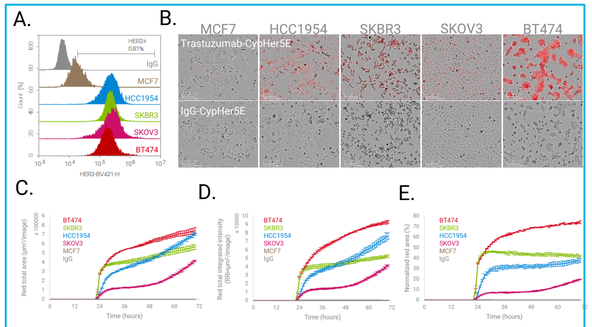


Figure 3. Internalization of trastuzumab-CypHer5E by different cell lines. (A) HER2 expression levels of MCF-7, HCC1954, SKBR3, SK-OV-3, and BT-474 cells were measured and compared to the steady state (determined by IgG isotype BV421 antibody staining) using a flow cytometer. **(B)** MCF-7, HCC1954, SKBR3, SK-OV-3, and BT-474 cells were treated with either trastuzumab-CypHer5E or IgG1-CypHer5E at a concentration of 2 µg/mL. Brightfield and red fluorescence images (10x) were captured 12 hours post-treatment. 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 the antibody internalization assay

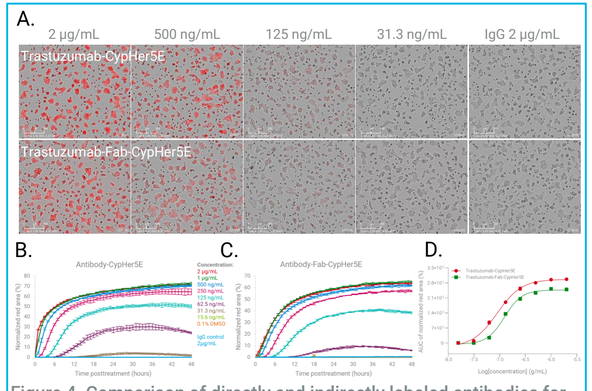
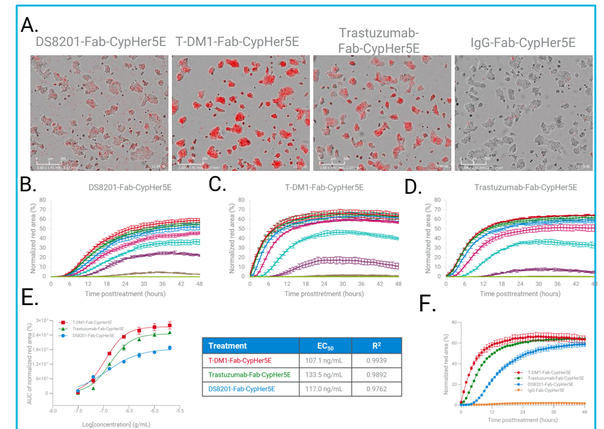


Figure 4. Comparison of directly and indirectly labeled antibodies for the antibody internalization assay. (A) BT-474 cells were treated with various concentrations of trastuzumab-CypHer5E (the upper panel) or trastuzumab-Fab-CypHer5E (the lower panel). IgG-CypHer5E and IgG-Fab-CypHer5E served as the 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 EC_{50} values for direct labeling with trastuzumab-CypHer5E and indirect labeling with trastuzumab-Fab-CypHer5E were 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.

Results and Discussion

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



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 5. Visualization and measurement of the internalization of trastuzumab and trastuzumab-conjugated drugs driven by HER2 on BT474 cell surfaces. BT-474 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 post-treatment. **(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 EC_{50} 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 (2 µg/mL) in BT-474 cells.

Conclusions

- The Agilent xCELLigence RTCA eSight system enables reliable, quantitative measurement of antibody internalization kinetics using a pH-sensitive dye.
- Distinct internalization profiles can be resolved across cell lines with different HER2 expression levels and cellular characteristics.
- The xCELLigence RTCA eSight assay differentiates internalization kinetics among HER2-targeting antibody-drug conjugates (ADCs).
- Fab-CypHer5E indirect labeling provides a convenient approach for labeling multiple antibodies simultaneously, yielding results comparable to direct CypHer5E labeling.
- Precise quantification of antibody internalization using RTCA eSight supports the advancement of antibody efficacy, safety, and pharmacokinetic evaluations.