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Advanced Endocytosis Assays: Sensitive Detection and Parameter Optimization for HER2/CD20 Antibody Internalization Studies

Dong Zhicheng, Wang Chao, Xiao Zhu, Yuan Yang, Wang Yu, Liu Yuwei
ACROBiosystems, Beijing, China

ABSTRACT

The therapeutic efficacy of antibody-drug conjugates (ADCs) relies on efficient cellular internalization, necessitating robust screening methods. We have developed and validated a novel detection reagent for monitoring antibody internalization based on pH-sensitive dye. Confocal microscopy analysis confirmed the intracellular localization of fluorescent signals, demonstrating specific endosomal activation rather than surface membrane binding. Through systematic optimization using flow cytometry, we established key working parameters including reagent concentration (1-4μg/mL) and incubation time (30min-1h). While increasing reagent concentration enhanced the positive signal, it also elevated background signals, necessitating careful concentration optimization. Time-course experiments revealed distinct internalization kinetics between adherent and suspension cell lines, with fluorescence intensity showing time-dependent increases that varied by cell type. These findings collectively demonstrate the reagent's utility for antibody or ADC internalization assessment, while highlighting the importance of target-specific optimization for concentration and incubation conditions to achieve optimal signal-to-noise ratios.

Keywords:

ADC, Internalization detection reagent, pH-sensitive fluorescent dye, Flow cytometry

INTRODUCTION

Antibody-drug conjugates (ADCs) are an emerging class of targeted cancer therapeutics composed of a monoclonal antibody covalently linked to a cytotoxic payload. The antibody is designed to specifically recognize and bind to antigens expressed on the surface of cancer cells, enabling selective delivery of the cytotoxic agent to tumor tissues while minimizing systemic toxicity.[1]

A key step in the mechanism of action (MOA) of ADCs is receptor-mediated endocytosis. Upon binding to its target antigen on the cell surface, the ADC-antigen complex is internalized through endocytic pathways, forming early endosomes. The early endosomes are subsequently trafficked to late endosomes and lysosomes, where the linker is cleaved—either enzymatically or chemically—to release the active cytotoxic drug. The released payload then exerts its anti-tumor activity, typically by disrupting DNA replication or microtubule function, leading to cell apoptosis or death.[1,2]

Given this mechanism, efficient internalization of the ADC is a prerequisite for therapeutic efficacy. The internalization process is influenced by several factors, including antigen density on the cell surface, antibody affinity for the antigen, and epitope accessibility. Therefore, screening antibodies with high-efficiency internalizing capabilities is critical during early-stage development.[3]

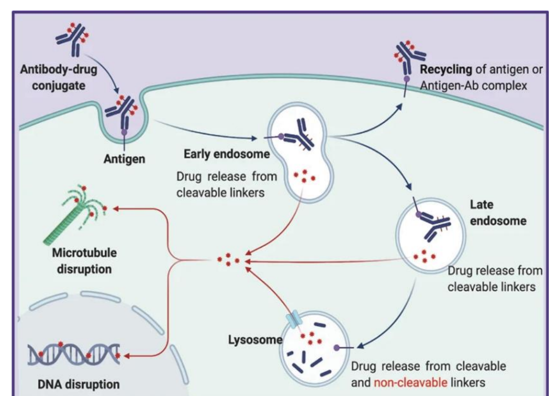

Figure 1. *Classic mechanism of action of ADC drugs.*[2]

Endocytosis assessment is necessary across multiple stages of ADC development:

- **Discovery stage:** To identify antibodies that not only bind specifically to tumor-associated antigens but also internalize efficiently.
- **Lead optimization:** To evaluate how variations in antibody structure or conjugation chemistry affect internalization and intracellular trafficking.
- **Preclinical development:** To confirm that the selected ADC candidate exhibits consistent internalization behavior across relevant cell models.
- **Mechanism-of-action studies:** To validate the intracellular delivery of the payload and its correlation with cytotoxic effects.

The main purposes of measuring endocytosis include quantitative analysis of ADC internalization rate; analysis for intracellular localization of ADC; and high-throughput screening of candidate ADC molecules, etc. Over the years, several techniques have been used to study antibody internalization including radiolabels, fluorescent microscopy, flow cytometry and cellular toxicity assays.[4]

Confocal Imaging

Confocal imaging using fluorescence-labeled antibodies is one of the most commonly employed methods for studying antibody internalization, offering the capability to localize antibodies within specific subcellular compartments such as endosomes and lysosomes.[4] This technique enables high-resolution visualization of ADC localization and internalization in cells.

Flow cytometry

Flow cytometry is widely employed for the quantitative analysis of ADC internalization in cells, using techniques such as pH probe assays and cell surface fluorescence quenching. In these approaches, fluorescently labeled antibodies are incubated with cells and internalized, enabling the distinction between internalized and surface-bound probes based on fluorescence changes resulting from acidic pH or quenching effects.[4]

Toxin-based cytotoxicity assay

Toxin-based cytotoxicity assays provide a functional method to evaluate antibody internalization by linking cytotoxic payloads to target antibodies. These assays offer three key advantages for screening applications: (1) high sensitivity due to amplified toxin-mediated signals, (2) compatibility with high-throughput formats (96/384-well plates), and (3) Close to the cytotoxic MOA. [3]

While powerful, the method requires careful optimization to minimize nonspecific toxicity and remains limited to endpoint measurements.[3]

Multiple analytical approaches are available for investigating ADC internalization, each offering distinct advantages: live-cell imaging facilitates real-time visualization of fluorescently labeled ADCs, radioactive labeling enables accurate quantification of antibody uptake, electron microscopy provides nanometer-scale localization details, while toxin-based assays establish a direct correlation between internalization and cytotoxic activity, delivering functionally relevant data.

In this application note, we present a newly developed **endocytosis detection reagent** designed for sensitive and reliable measurement of antibody internalization in both **HER2+ adherent cells** and **CD20-positive suspension cells**. We explore key experimental parameters, including dye concentration and incubation time, and evaluate their impact on fluorescence signal and internalization efficiency.

METHODS AND MATERIALS

Experimental Reagents & Materials

Lyophilized Reagent were reconstituted using sterile deionized water into the mother solution (200 ug/mL) and diluted into 4× working solution(4 μg/ml) before use.

Table 1. *Materials used for FACS*

Materials	Catalog No.	Vendor
Antibody Internalization Detection Reagent	IGG-PZF2001	ACROBiosystems
Trastuzumab Biosimilar	AM598	ACROBiosystems
Rituximab biosimilar–Research Grade (MALS verified)	CD0-M36	ACROBiosystems
Human IgG1 Kappa Isotype Control(mAb, carrier free, MALS verified)	DNP-M2	ACROBiosystems
Fetal Bovine Serum (FBS)	SA211.02	CellMax
Fetal Bovine Serum (FBS)	A5669701	ThermoFisher Gibco
DMEM media	L120KJ	BasalMedia
PRMI-1640 Medium	30-2001	ATCC

Table 2. *Materials used for Fluorescence Imaging*

Materials	Catalog No.	Vendor
Antibody Internalization Detection Reagent	IGG-PZF2001	ACROBiosystems
Trastuzumab Biosimilar	AM598	ACROBiosystems
Human IgG1 Kappa Isotype Control(mAb, carrier free, MALS verified)	DNP-M2	ACROBiosystems
CellLight™ Lysosomes-GFP	C10596	ThermoFisher-Invitrogen
NucBlue™ Live ReadyProbes™	R37605	ThermoFisher-Invitrogen
Fetal Bovine Serum (FBS)	SA211.02	CellMax
DMEM media	L120KJ	BasalMedia

Table 3. *Reagents used in FACS & Fluorescence Imaging*

Conditions	Description
SK-BR-3	Used to evaluate Anti-Human Her2 Antibody Internalization.
Raji	Used to evaluate Anti-Human CD20 Antibody Internalization.
PBS	Used as washing buffer.
FACS Buffer	2% bovine serum albumin (BSA) in phosphate buffered solution (PBS), pH 7.2-7.4

Table 4. *Instruments for Cell Culture & FACS & Fluorescence Imaging*

Type	Instrument
Incubator	CO_2 Incubator
Cell Counter	Monwei
Flow Cytometer	BD Biosciences FACSLyric
Data Visualization Software	FCS Express 7
Fluorescence Imaging System	Invitrogen™ EVOS™ M7000

FACS Analysis of Antibody Internalization Protocol

1. Cell Culturing
SK-BR-3 cells were cultured in DMEM medium with 10% FBS (CellMax) in the CO_2 incubator at 37°C, 5% CO_2.
Raji cells were cultured in PRMI-1640 medium with 10% FBS (Gibco) in the CO_2 incubator at 37°C, 5% CO_2.

2. Prepare 4× antibody working solution
Prepare sufficient volume of 4× working solution(8μg/ml) of antibody in cell culture medium and add 25μl of 4× working antibody solution to each well of a 96-well plate.

3. Prepare 4× Internalization Detection Reagent working solution
Prepare 4× working solution(4 μg/ml) of Antibody Internalization Detection Reagent and add 25μl of 4× working solution to each well of the 96-well plate from Step 2. Incubate at room temperature for 10 minutes to allow the labeling complexes to form.

4. Harvesting & Cell Counting
Harvest the cells and count cell number and viability. Cell viability must exceed 95% at this stage.

5. Label cells
Prepare suspension cells at 2× 10^6 cells/mL in cell culture medium. Add 50μL of cells to each well of the 96-well plate containing the Antibody-Internalization Detection Reagent conjugate(from Step 3). Incubate at 37°C for 2h.

6. Washing
Wash the cells 3 times using the FACS buffer.

7. Cell Resuspension
Resuspend the cell pellet in 200uL of PBS per sample.

8. Transferring to FACS
Transfer the cell suspension into the flow tube and detect the cells using flow cytometry.

9. Analysis
Analyze the result data using FCS Express 7 software.

Fluorescence Imaging of Antibody Internalization Protocol

1. Cell Culturing
SK-BR-3 cells were cultured in DMEM medium with 10% FBS(CellMax) in the CO_2 incubator at 37°C, 5% CO_2.

2. Harvesting & Cell Counting
Harvest the cells and count cell number and viability. Cell viability must exceed 95% at this stage.

3. Cell Plating
Harvest the cells and plate cells at a density of $7×10^4$ cells/well in 24-well culture plates in 1 mL of assay medium (DMEM containing 10% FBS). Incubate cells at 37°C with 5% CO_2 overnight.
Remove medium and add 500μL of assay medium to wells.

4. Prepare 4× antibody working solution
Prepare sufficient volume of 4× working solution(8μg/ml) of antibody in cell culture medium and add 270μl of 4× working antibody solution to 1.5ml centrifuge tube.

5. Prepare 4× Internalization Detection Reagent working solution
Prepare 4× working solution(4μg/ml) of Antibody Internalization Detection Reagent and add 270μl of 4× working solution to 1.5ml centrifuge tube from Step 4. Incubate for at room temperature 10 minutes to allow the labeling complexes to form.

6. Label cells
Add 500μL of the labeling complex (from Step 5) to each well of the 24-well plate. Incubate at 4°C for 60min. Remove the supernatants and wash three times with PBS. Add 1 ml of assay medium to each well of the 24-well plate.

7. Lysosomes Marking
Add CellLight™ Lysosomes-GFP to each well and incubate cells at 37°C with 5% CO2 for 16 hours.

8. Cell Nucleus Marking
Add NucBlue™ Live ReadyProbes™ to each well and incubate cells at room temperature for 20 minutes.

9. Fluorescence Imaging
Remove the supernatants and 1 ml of PBS to each well of the 24-well plate. Analyze cells using EVOS M7000 Imaging System.

RESULTS AND DISCUSSION

Specificity Validation of the Internalization Detection Reagent

To validate the specificity of the internalization detection reagent, we performed detection in HER2+ cell lines and use the HER-2 specific antibody Trastuzumab Biosimilar as well as a nonspecific IgG1 isotype control. Fluorescence microscopy revealed bright fluorescent signals exclusively in the endosomal compartments of test group cells (Fig. 2C), while control groups (either antibody-free or with isotype control antibody) showed minimal background signal (Fig. 2A-B). This antibody-dependent fluorescence pattern clearly demonstrates that the internalization signal is specifically mediated by target antibody binding. Z-stack scanning further confirmed this conclusion, demonstrating high colocalization between the internalization reagent signal (Red) and lysosomal markers (Green), with no extracellular signal detected (Fig. 2D).

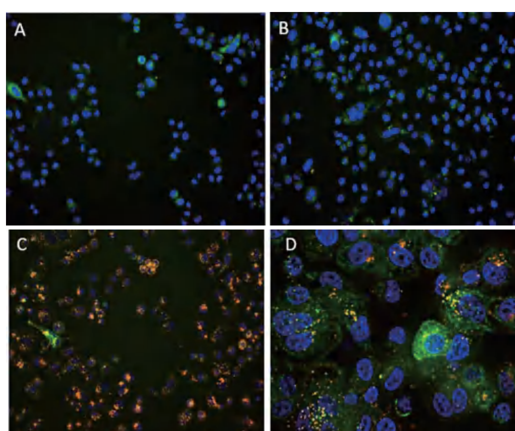

Figure 2. *SK-BR-3 cells were treated with CellLights Lysosome GFP (green) for 16 hours followed by treatment with Anti-Her2 Abs-Internalization Detection Reagent conjugate and IgG1 Isotype-Internalization Detection Reagent conjugate separately for 16 hours (red), then stained with NucBlue Live ReadyProbes (blue) for 20 minutes and imaged on the EVOS M7000. A. Antibody Internalization Detection Reagent (Cat.No.IGG-PZF2001). B. IgG1 Isotype-Internalization Detection Reagent conjugate. C. Anti-Her2 Abs-Internalization Detection Reagent conjugate. D. Anti-Her2 Abs-Internalization Detection Reagent conjugate(Z-stacking).*

Furthermore, flow cytometric analysis was performed in both HER2+ and CD20+ cell lines. The results demonstrated that this internalization detection reagent is not only applicable for antibody internalization assessment across multiple cell types, but also enables rapid and quantitative analysis through flow cytometry (Fig. 3A-B).

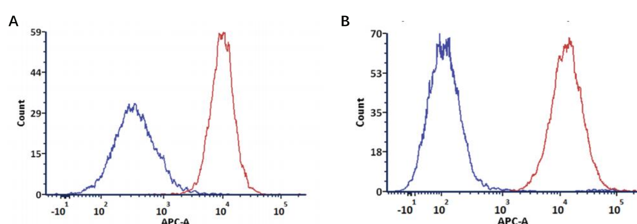

Figure 3. *FACS analysis of Antibody Internalization. A. HER2 specific antibody Trastuzumab (Red) in HER2+ cell line. B. CD20 specific antibody Rituximab (Red) in CD20+ cell line. Blue: IgG1 isotype control.*

Optimization of Internalization Detection Reagent Concentration

To establish the optimal working concentration, we systematically evaluated the internalization detection reagent in CD20+ and HER2+ cell lines using a fixed primary antibody concentration of 2μg/mL while detection reagent concentrations of 1μg/mL, 2μg/mL, and 4μg/mL. Dose-dependent analysis revealed that increasing the detection reagent concentration from 1μg/mL to 4μg/mL significantly enhanced positive signals though higher concentrations (4μ g/mL) concomitantly increased background signals in control groups. Through comprehensive signal-to-noise ratio (SNR) analysis, we determined that a 1:2 ratio (1μg/mL detection reagent to 2μg/mL antibody) provided the optimal balance between detection sensitivity and background suppression in both cell lines (Fig.4A-D). This optimized condition establishes a reliable and cost-effective protocol for subsequent internalization assays.

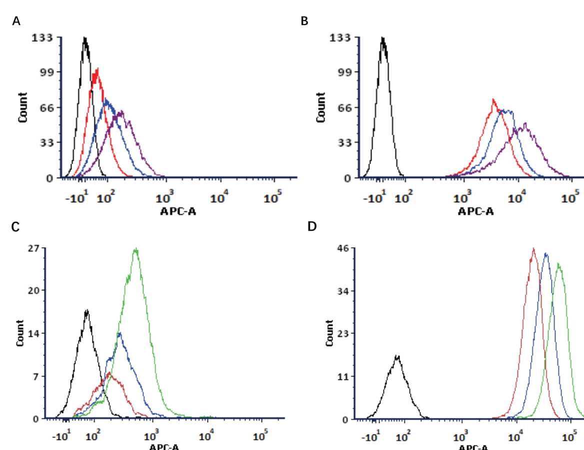

Figure 4. *ACS analysis of Antibody Internalization with different detection reagent concentration. A: Detection Reagent control in CD20+ cell line. B: Detection Reagent and CD20 specific antibody Rituximab in CD20+ cell line. Blank: Only cell. Red:1μg/mL Detection Reagent, Blue:2μg/mL Detection Reagent, Purple:4μ g/mL Detection Reagent. C: Detection Reagent control in HER2+ cell line. B: Detection Reagent and HER2 specific antibody Trastuzumab in HER2+ cell line. Blank: Only cell. Red:1μg/mL Detection Reagent, Blue:2μg/mL Detection Reagent, Green:4μ g/mL Detection Reagent.*

Temporal Dynamics of Antibody Internalization

We investigated the kinetic profiles of antibody internalization by incubating the antibody-reagent complex with CD20+ and HER2+ cell lines for 30 minutes to 4 hours followed by flow cytometric analysis. The results demonstrated cell type-dependent internalization kinetics, with CD20+ cells showing a linear increase in fluorescence intensity throughout the time course, while HER2+ cells exhibited a biphasic response characterized by minimal signal change during the initial 2 hours followed by a significant increase at 4 hours (Fig.5A-D). These differential kinetic patterns confirm that our internalization detection reagent can reliably monitor the dynamic internalization process through quantitative fluorescence changes while

revealing target-specific variations in internalization rates. The findings underscore the importance of establishing cell line-specific detection windows, as optimal timepoints may vary significantly depending on both the target antigen and cellular context. This temporal characterization provides critical guidance for experimental design in future applications, emphasizing that internalization kinetics should be empirically determined for each new antibody-cell system combination to ensure accurate assessment of internalization efficiency.

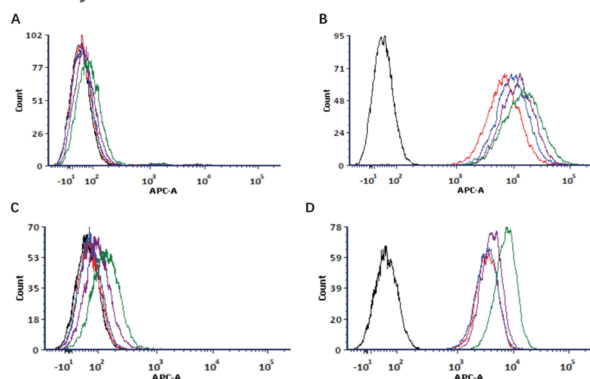

Figure 5. *FACS analysis of Antibody Internalization with different incubation time. A: Detection Reagent control in CD20+ cell line. B: Detection Reagent and CD20 specific antibody Rituximab in CD20+ cell line. C: Detection Reagent control in HER2+ cell line. B: Detection Reagent and HER2 specific antibody Trastuzumab in HER2+ cell line. Blank: Only cell. Red:30min, Blue:1h, Purple:2h, Green:4h.*

CONCLUSION

The field of antibody-drug conjugates (ADCs) has seen remarkable advancements in recent years, with multiple ADC therapies gaining regulatory approval. The therapeutic efficacy of ADCs in oncology stems from their ability to selectively internalize into target cells and release potent payloads within the tumor microenvironment. A critical factor in ADC development is the establishment of robust, high-throughput methods to assess antibody internalization efficiency. We have developed a pH-sensitive fluorescent dye-based internalization assay, which demonstrates strong signal amplification in acidic endosomal compartments while maintaining minimal background in both HER2+ and CD20+ cell models.

References

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About ACROBiosystems

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+1 800-810-0816 (US / Canada)
+86 400-682-2521 (Asia & Pacific)
techsupport@acrobiosystems.com
1 Innovation Way, Newark, DE 19711