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Real-Time 96-Well Antibody Internalization Assays Using Incucyte® Fabfluor-pH Antibody Labeling Dye

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Introduction

Monoclonal antibodies are now widely used as anti-cancer, anti-inflammatory, and anti-viral therapeutic agents. An important property of antibodies is the extent, location, and rate of internalization into cells. For example, antibody-drug conjugates (ADCs) deliver cytotoxic payloads to cells and the specificity and degree of cellular uptake is critical to the efficacy and safety of the molecule. Monoclonal antibodies are also used in immunotherapy to label tumor cells for immune cell clearance (e.g., ADCC, ADCP[1,2,3]) and to drive internalization of receptors associated with tumor survival (e.g., EGFR[4,5]).

In each case, the rate of removal from the cell surface will strongly influence the therapeutic profile of the antibody. More generally, internalization of antibodies by either specific (target mediated) or non-specific (e.g., pinocytosis in endothelial cells) mechanisms is a key determinant of the half-life in the body, and small modifications to monoclonal antibody structure can have profound effects on duration of activity. For these reasons, understanding the internalization of antibodies into cells, and being able to compare the uptake of different antibodies, is a key requirement in antibody selection and optimization for biologics drug discovery.

Current methods to directly quantify antibody internalization are laborious, time-consuming, and not amenable to testing multiple antibodies. These assays generally require labeling each antibody with a fluorescent tag—in most cases the labeled antibody must be separated from the free label via a column or wash step. The analysis requires that the signal from the internalized antibody can be robustly isolated from that outside the cells. Wash steps, blocking dyes and/or a reduction in temperature to slow cellular activity are common. Almost all (e.g., flow cytometry, high content imaging) provide only end point assays and multiple experiments are required to follow internalization over time.

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In this application note, we describe an integrated assay solution, based on Incucyte® live-cell imaging and analysis and a novel pH-sensitive dye coupled antibody fragment (Incucyte® Fabfluor-pH Antibody Labeling Dyes), that is designed to address these limitations. This solution provides a simple, turnkey method for comparing the internalization of large numbers of antibodies and is amenable for use throughout the industrial antibody drug discovery process and basic scientific research.

Assay Principle

Incucyte® Fabfluor-pH Antibody Labeling Dyes are Fc-region targeting Fab fragments conjugated to a pH-sensitive fluorescent probe. These reagents enable a generic one-step, no-wash, labeling protocol for all isotype matched, Fc-containing test antibodies. At pH 7.0, the Fab-Ab complex has little or no fluorescence. When labeled antibodies are added to cells, a fluorogenic signal is observed as the Fab-Ab complex is internalized and processed via acidic (pH 4.5–5.5) lysosomes and endosomes (Figure 1). The full time course of internalization is visualized and automatically quantified using Incucyte® real-time live-cell analysis. The labeling method and assay workflow is commensurate with comparing internalization rates of large numbers of antibodies (10–100's) in miniaturized (96/384-well) plate formats.

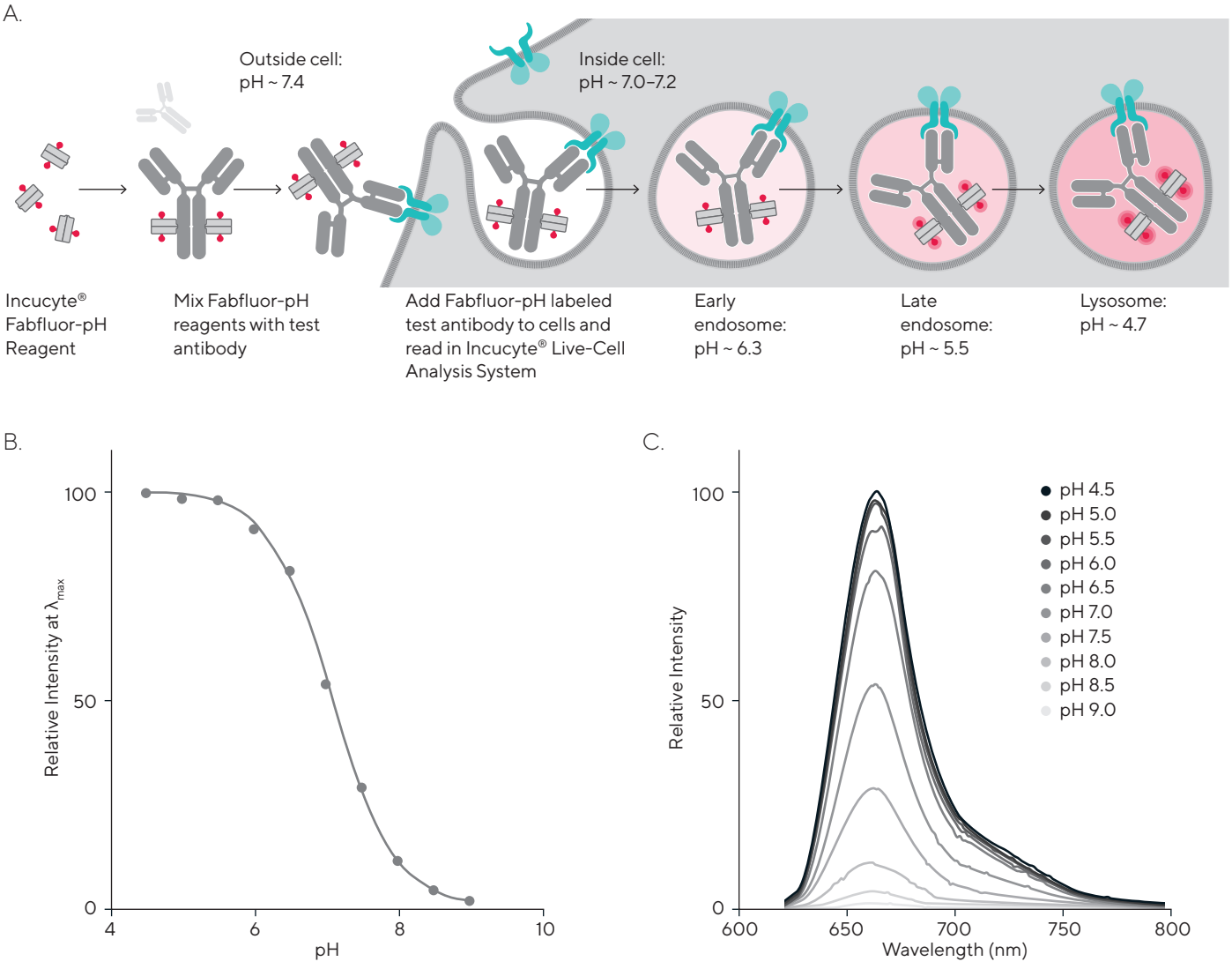
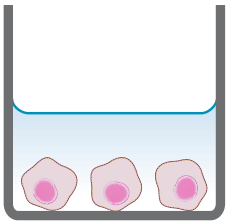


Figure 1: Principle of antibody Internalization assay using Incucyte® Fabfluor-pH labeling dye (A). Fluorogenic signal as internalized antibody is processed into the acidic endosome and lysosome. Data to show the pH sensitivity of the labeling probe (B and C). Note the relatively low fluorescence of Fabfluor-pH at pH 7.0.

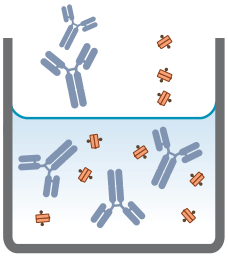
Quick Guide

1. Seed cells



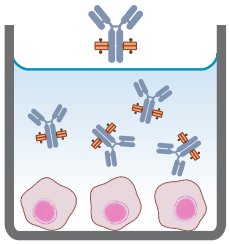
Seed cells (50 µL/well, 5,000–30,000 cells/well), into 96-well plate and leave to adhere (2–24 h, depending on cell type).

2. Label test antibody



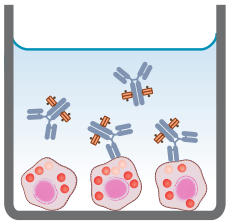
Mix antibody and Incucyte® Fabfluor-pH Dye at a molar ratio of 1:3 in media, 2x final assay concentration. Incubate for 15 minutes at 37° C to allow conjugation.

3. Add to cells



Add Fabfluor-antibody mix (50 µL/well) to cell plate.

4. Live-cell fluorescent imaging



Capture images every 15–30 minutes (10x or 20x) in Incucyte® Live-Cell Analysis System with appropriate fluorescent module configured for 24-48 hours. Analyze using integrated software.

General Methods and Materials

Cell Culture and Reagents

BT-474 cells (DMEM+10% FCS), HT-1080 cells (F12K+10% FCS, 1% glutamax, 1u/mL pen/strep), Jurkat and Raji cells (both RPMI 1640+10% FCS) were grown in 75 cm^2 tissue culture treated flasks. All cell lines were provided by ATCC and culture reagents were obtained from Life Technologies. Commercially available test antibodies were CD71 clone 1a, 2, CD20 (Sigma), clone 3-5 (Abcam), CD3, CD45 (Biolegend), human IgG isotype control (Absolute Antibody), mouse IgG1 isotype control (R&D Systems).

Antibody Labeling

Test antibody at known concentration was mixed with isotype matched, Incucyte® Fabfluor-pH Dye at a molar ratio of 1 to 3 in complete growth media. Reactions were performed at twice the final required assay concentration in either a round bottom 96-well plate (Costar Cat. No. 3799) or an amber microtube, depending on the volumes required, and incubated for 15 min at 37° C. Any required dilutions (e.g., for construction of concentration response-curves) were performed post conjugation to maintain the labeling ratio. Fabfluor-pH labeled antibody (50 µL) was then added directly to pre-plated cells (in 50 µL).

Incucyte® Antibody Internalization Experiments

Cells were plated (BT-474 10K per well, 16 h, HT-1080 8K per well 4 h) on flat-bottom 96-well plates (Costar Cat. No. 3595) prior to assay. For assays using non-adherent cell types, plates were coated with poly-L-ornithine (PLO, Sigma Cat. No. P4957) for 1 h and allowed to dry for 30 min prior to cell seeding. Cells were seeded at 30 K/well, 1 h prior to assay. Following addition of Ab/Fab complex assay plates were immediately placed in an Incucyte® Live-Cell Analysis System and scanned for HD phase and red fluorescence images at 10X or 20X magnification every 15–30 minutes for up to 48 h. Images were automatically analyzed using the integrated Incucyte® software for the following metrics: (1) Phase Confluence (measure of cell area), (2) Red Fluorescence Object area (index Fabfluor-pH labeled internalized antibody). Top Hat subtraction was used to minimize any background fluorescence signal.

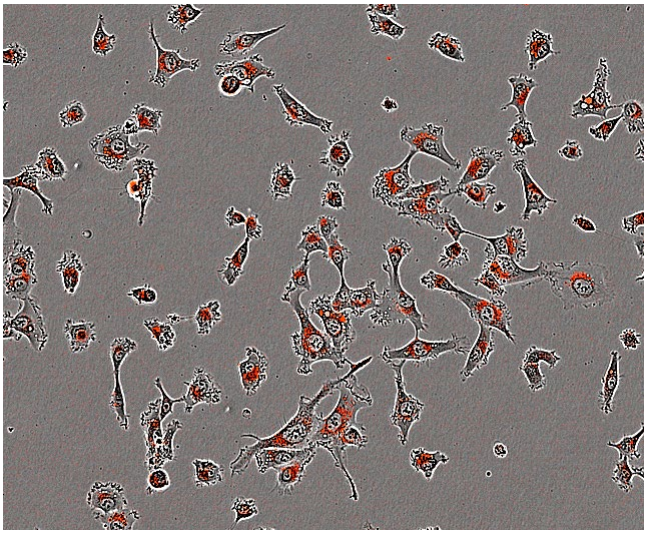
Validation Data

Proof of Concept

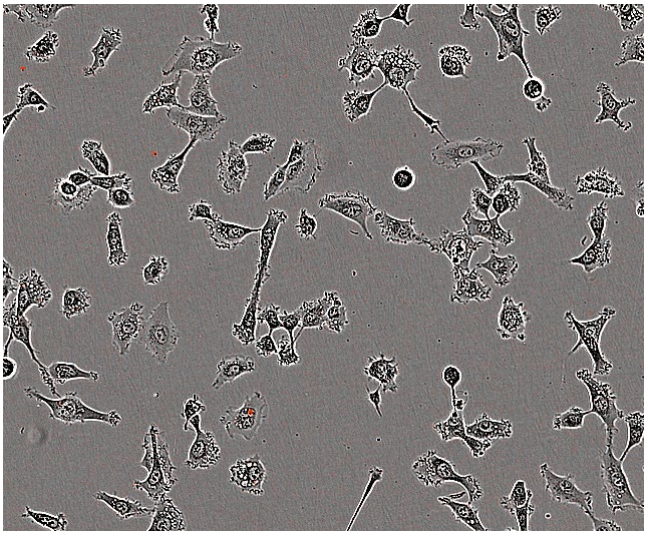
As a proof of concept, internalization of an antibody to CD71, the transferrin receptor, was measured in HT-1080 fibrosarcoma cells. The anti-CD71 antibody (clone MEM189) and a mouse IgG1 isotype control were labeled with isotype matched Incucyte® Fabfluor-pH Red Mouse IgG1 Antibody Labeling Dye as described. Fabfluor-pH labeled antibodies (4 µg/mL) were added to cells and monitored (phase contrast, red fluorescence) for 12 h in an Incucyte® Live-Cell Analysis System.

A rapid time-dependent increase in red fluorescence was observed with anti-CD71, but not isotype or media control, from the first time point (15 min) of the assay (Figure 2). At 12 h, the signal:background of the assay was > 15 fold. The red signal was observed in the cytosolic compartment of the cells but not in the nucleus, consistent with the expected localization of the internalized antibody to lysosomes and endosomes. Fluorescence area (µm^2/well) was used to quantify the specific signal. Similar proof of concept assays were conducted using the Incucyte® Fabfluor-pH Orange Antibody Labeling Dye as well.

A.
CD-71-Fabfluor-pH



B.
IgG-Fabfluor-pH



C.

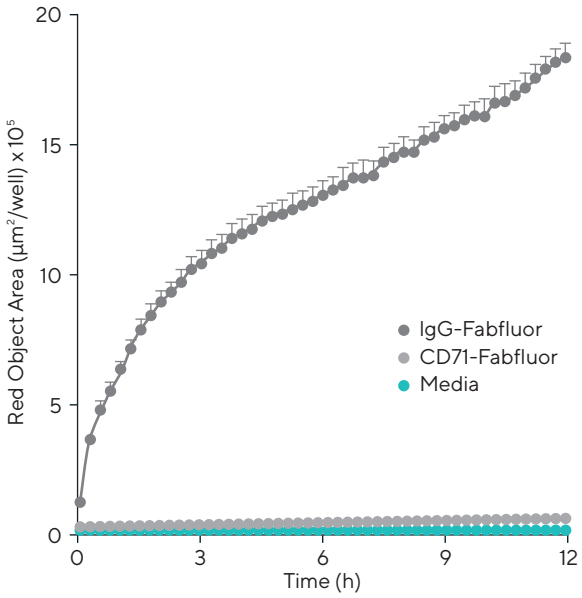


Figure 2: Internalization of Incucyte® Fabfluor-pH labeled α-CD71 in HT-1080 cells. HT-1080 cells were treated with either Incucyte® Fabfluor-pH labeled α-CD71 or IgG1 isotype control (4 µg/mL), HD phase and red fluorescence images (10X) were captured every 15 min over 12 h. Images of cells treated with Fabfluor-pH-α-CD71 display red, cytosolic fluorescence (A). Cells treated with labeled isotype control display no cellular fluorescence (B). Time course data shows a rapid increase in red object area over time in cells treated with labeled α-CD71 but not with isotype control (C). Images shown taken at 6 h post treatment, data shown as mean of 3 wells ± SEM.

Normalization for Cell Number

In theory, the antibody internalization signal should increase as a function of cell number. To verify this, and to understand the contribution of cell proliferation to the signal over time, experiments were conducted on cells plated at different densities (1–20 K per well). Anti-CD71 internalization (red fluorescence area) was detectable at 1 K cells per well and markedly increased at higher plating densities (Figure 3 A and B). Over time (0–12 h) the internalization signal continued to rise. When the red fluorescence area was normalized to the total cell area (Phase Confluence) to account for proliferation, the time course signals were highly similar. Importantly, after 4 h there was no further increase in the normalized signal, indicating that antibody internalization had reached equilibrium. This normalization method helps minimize the impact of well-to-well cell seeding variation and isolate the true internalization rate signal from cell proliferation.

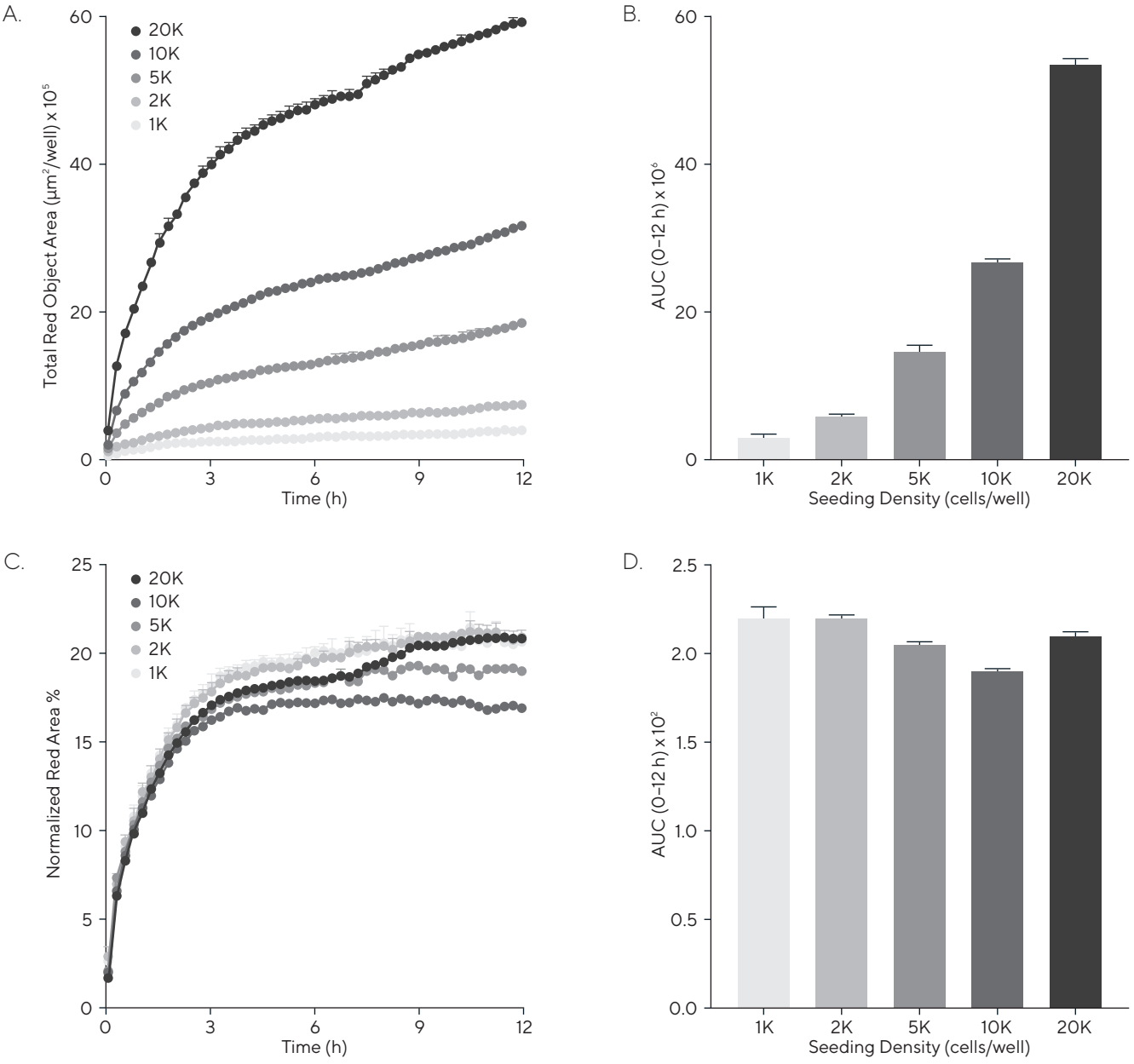
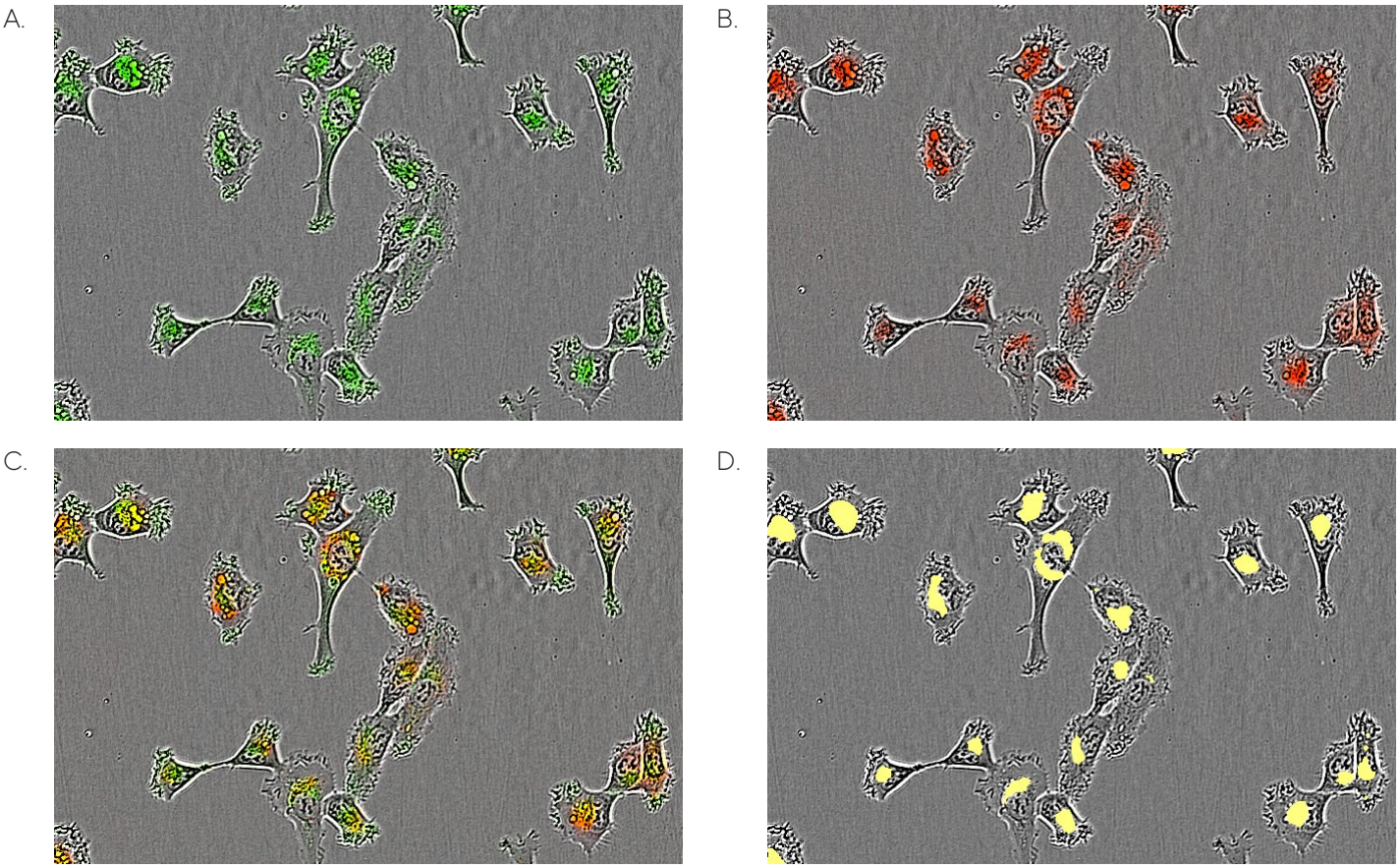


Figure 3: Antibody internalization response is cell number dependent. An increasing density of HT-1080 cells were seeded (1–20 K/well) and treated with Incucyte® Fabfluor-pH labeled α-CD71 (4 µg/mL). HD phase and red fluorescence images (10X) were captured every 30 min over 12 h. The time-course of red object area data demonstrates an increasing internalization signal with increasing cell number (A and B). When the red object signal is normalized for phase area, it is clear the internalization response size is dependent on cell number (C and D). All data shown as a mean of 3 wells ± SEM, bar graphs show area under the curve (AUC) calculated from time course data.

Signal Co-Localization With Lysosomal Marker

To confirm the presence of the internalized antibody in the lysosome, we conducted dual labeling experiments with LysoSensor® Green (Thermo Scientific), an end-point lysosomal marker. HT-1080 cells were treated with Fabfluor-pH-labeled-CD71 for 3 h and monitored for antibody internalization. LysoSensor Green was added, and the plate then returned to the Incucyte® to measure red (CD71) and green (LysoSensor) fluorescence. A strong co-incident signal was observed in the two signal channels, 74% of the red signal was co-localized with green, supporting the premise that the Fabfluor labeled antibody was internalized to the lysosome.



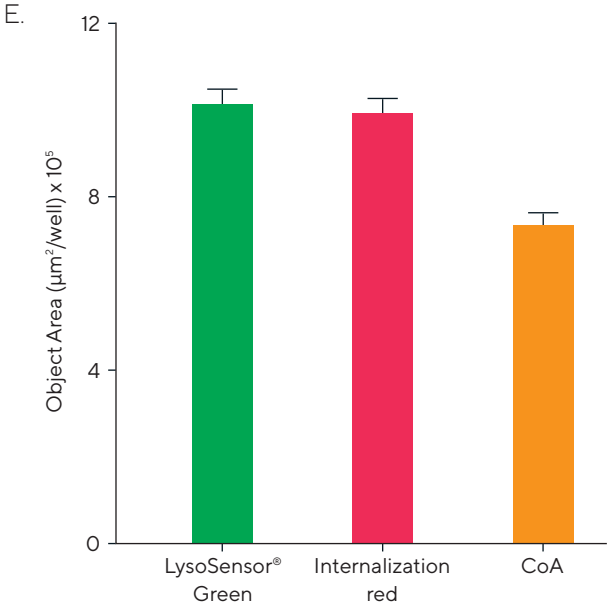


Figure 4: Co-localization of Incucyte® Fabfluor-pH labeled α-CD71 and LysoSensor® Green in HT-1080 cells. Internalization of Incucyte® Fabfluor-pH labeled α-CD71 (4 µg/mL) was established for 3 h in HT-1080 cells before addition of LysoSensor® Green DND-189 (Thermo, 0.25 µM). Images show individual LysoSensor® Green and Fabfluor-pH labeled α-CD71 red signal (A and B), co-localization of red and green signals (C), and the co-localized analysis mask shown in yellow (D). (E) Incucyte® analysis of the coincidence of the red and green fluorescence confirms co-localization of 74% of the red signal with the green signal. Images captured at 20X magnification, 30 min post LysoSensor® addition, data shown as mean of 4 wells ± SEM.

Applicability Across Different Cell Types and Antibodies

To demonstrate the broad application and specificity of the method, internalization was assessed for a range of test antibodies targeted against specific CD markers expressed in different cell lines (Figure 5). Anti-CD20 was internalized in the B cell line Raji, but not Jurkat, a T cell line. Conversely, anti-CD3 was internalized in Jurkat but not Raji. Antibodies to CD71 and CD45, general lymphocyte markers, were internalized in both cell types. Importantly, IgG was not internalized in either. These data are in alignment with the known CD surface marker expression of these cell lines, and provide strong confidence in the signal specificity and generic utility of the method.

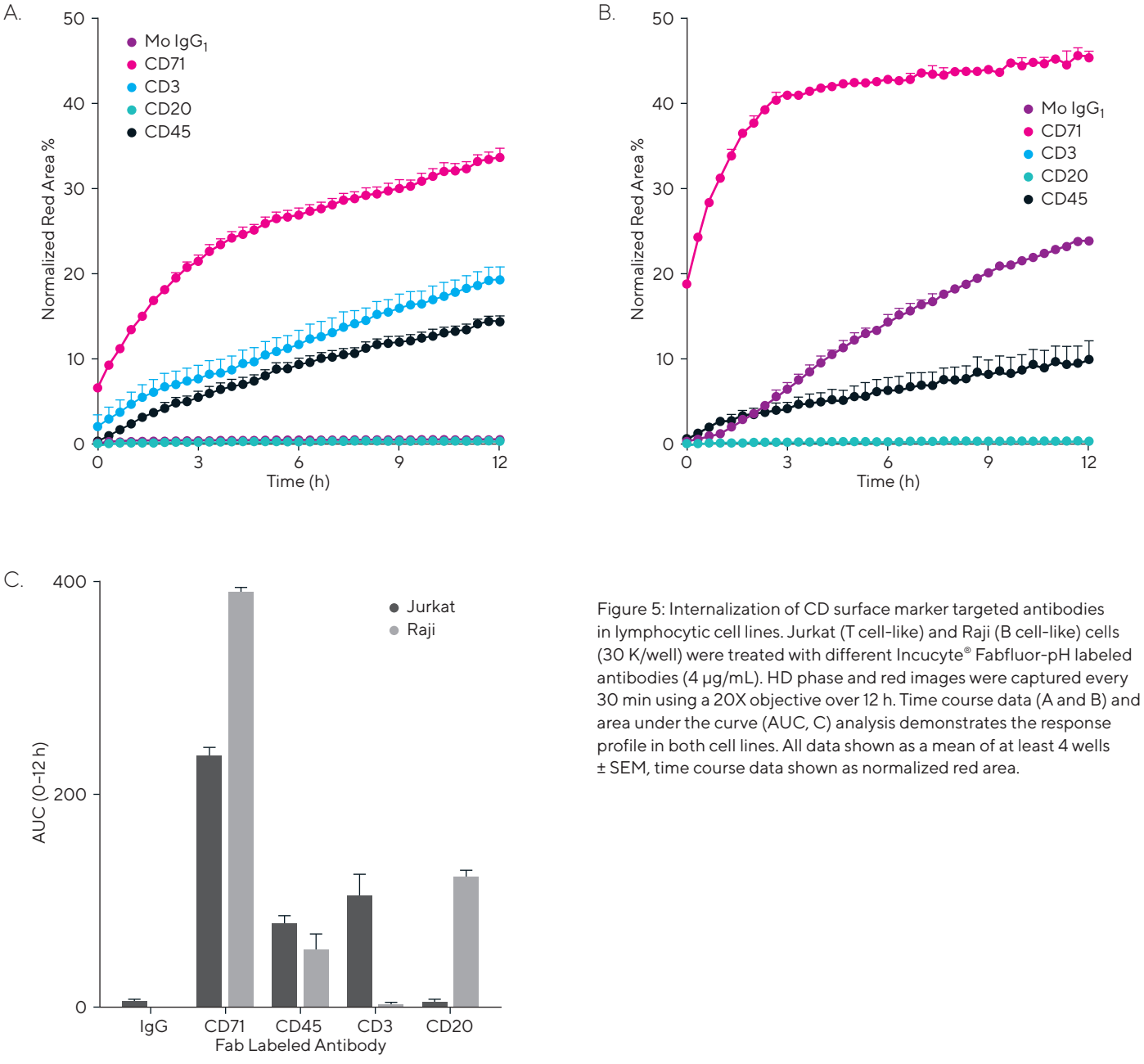


Figure 5: Internalization of CD surface marker targeted antibodies in lymphocytic cell lines. Jurkat (T cell-like) and Raji (B cell-like) cells (30 K/well) were treated with different Incucyte® Fabfluor-pH labeled antibodies (4 µg/mL). HD phase and red images were captured every 30 min using a 20X objective over 12 h. Time course data (A and B) and area under the curve (AUC, C) analysis demonstrates the response profile in both cell lines. All data shown as a mean of at least 4 wells ± SEM, time course data shown as normalized red area.

Quantitative Pharmacological Analysis

Pharmacological, Kinetic Quantification of Antibody Internalization

To illustrate the quantitative nature of the method and suitability for analyzing therapeutic antibodies, we sought to determine EC_{50} values for the internalization of Herceptin (Trastuzumab) and Rituxan (Rituximab), two clinically used monoclonal antibodies. After labeling of each antibody with the Fabfluor-pH reagent, the antibody was serially diluted (1:2) prior to addition to the cells to enable construction of a concentration-response curve. Handling the labeled antibody this way is good practice to eliminate any variation in Fab labeling efficiency which could occur across the concentration range. In BT-474 Her2-positive breast carcinoma cells, clear time and concentration dependent internalization of Herceptin was observed over 48 h. From an area under the time-course (AUC) analysis, the EC_{50} value for internalization was 323 ng/mL ≡ 2.1 nM, Figure 6). In Raji cells, the EC_{50} value for Rituxan was 426 ng/mL ≡ 2.6 nM, Figure 7). These EC_{50} values are similar to the known KD values for Herceptin and Rituxan for their target receptors (both approximately 5 nM).

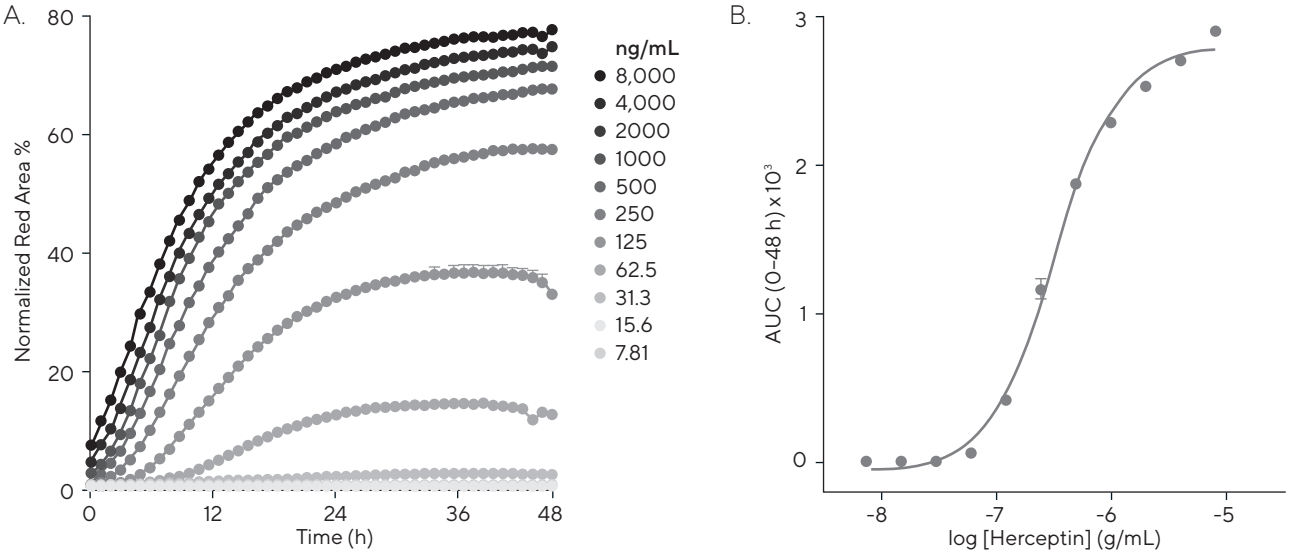


Figure 6: Quantitative pharmacological analysis of Incucyte® Fabfluor-pH labeled Herceptin. BT-474 Her2-positive cells were treated with increasing concentrations of Fabfluor-pH labeled Herceptin. The time course graph displays an increase normalized red area over time with increasing Herceptin concentrations (A). Area under the curve analysis of this response displays a clear concentration dependent response with an EC_{50} of 323 ng/mL (B). All data shown as a mean of 3 wells ± SEM, time course data shown as normalized red area.

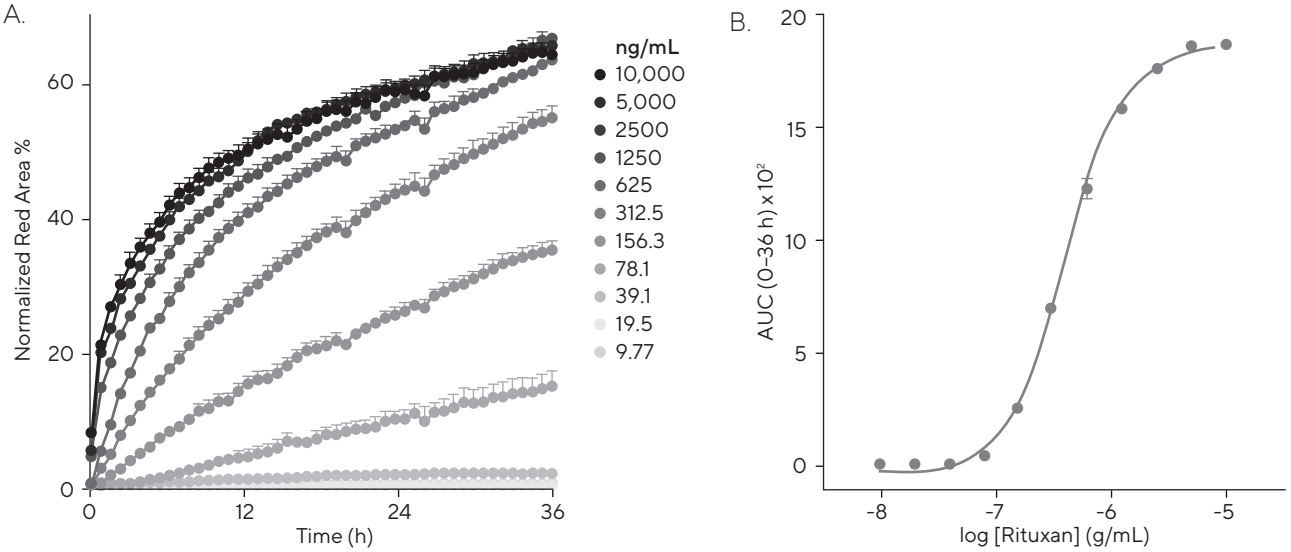


Figure 7: Quantitative pharmacological analysis of Incucyte® Fabfluor-pH labeled Rituxan. Raji cells were treated with increasing concentrations of Fabfluor-pH labeled Rituxan. The time course graph displays an increase normalized red area over time with increasing Rituxan concentrations (A). Area under the curve analysis of this response displays a clear concentration dependent response with an EC_{50} of 426 ng/mL (B). All data shown as a mean of 3 wells SEM, time course data shown as normalized red area.

Comparison of Multiple Test Antibodies for High-Throughput Screening

The features of the Incucyte® Antibody Internalization Assay are such that it should be facile to parallel label many antibodies and compare their internalization. To validate this, we took 6 different commercially available anti-CD71 antibodies and compared their internalization properties head-to-head. The antibodies were plated in 96-well plates and labeled in full media with the Incucyte® Fabfluor-pH Dye. Serial dilutions were performed in full media (8 point, 1:2). Labeled IgG and Fabfluor-pH alone were added to control wells. Labeled antibodies were then added to pre-plated HT-1080 cells and monitored for internalization for 12 h.

Of the 6 antibodies, 3 (Ab 1a, Ab2 and Ab 1b) produced large internalization signals and were detected at low concentrations (< 0.05 µg mL-1). Reassuringly, Ab 1a and Ab 1b were the same antibody clone from different suppliers and gave similar internalization responses. Abs 3, 4, and 5 were internalized more weakly and only at higher concentrations (Figure 8). From the control responses, a mean Z' value of 0.82 was determined (2 plates 0.75, 0.87) indicating a microplate assay with high robustness. These data confirm the suitability of the method for comparing the internalization of multiple antibodies at a single target, and illustrate that the internalization profile is a property of the antibody per se. Indeed, the assay precision and workflows are such that hundreds of different antibodies could be compared at once and further throughput could be achieved through miniaturization to 384-well format.

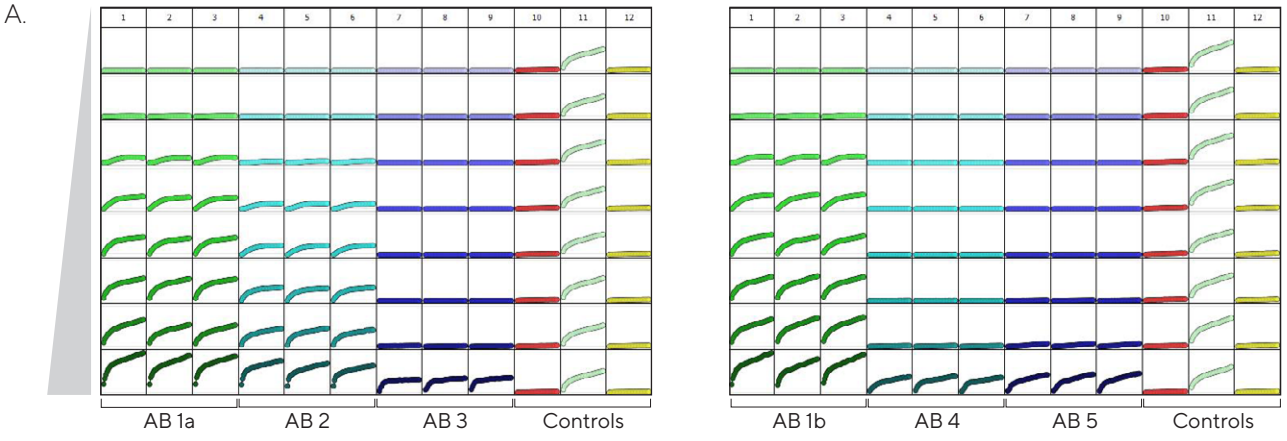


Figure 8: Screening test Abs for internalization. Six different CD71 antibodies including one clone from 2 different suppliers (clone 1a & 1b) were tested head-to-head in HT-1080 cells. The antibodies were labeled with Incucyte® Fabfluor-pH Dye prior to addition to cells and the internalization signal captured every 30 min over 12 h using a 10X magnification. Plate views taken from Incucyte® show clear positive and negative control responses in column 11 and 12 with concentration dependent responses for each antibody across two plates (A). Head-to-head analysis of antibody data shows a range of responses across these clones (B) control responses at 12 h display a clear positive response. All data shown as mean of 3 wells ± SEM, controls shown as mean of 8 wells.

Conclusions

The key features of the approach described in this application note are:

1. A single step labeling protocol for easily tagging antibodies of interest with an Fc-targeted Fab coupled pH-sensitive dye (Incucyte® Fabfluor-pH). The labeling method is conducted in full media and is suitable for purified antibodies and antibody supernatants.
2. An automated, image-based and real time analysis method (Incucyte® live-cell imaging and analysis) for monitoring internalization in multiple 96-well microplates at once. The format is amenable to both adherent and non-adherent cells.
3. An assay system that follows the full time course of the biology and reports internalization with high specificity, sensitivity, and morphological information. The use of a pH-sensitive dye provides for low background signal and obviates the need to separate out fluorescence arising from antibody on the cell surface or in bulk solution.

Taken together, these attributes provide a simple, integrated and quantitative solution for directly studying internalization of antibodies into cells that can easily be scaled to compare many antibodies (10–100's) in parallel. This method enables antibody internalization measurements to be implemented at earlier stages in the biologics discovery process, and will prove valuable in efficacy, safety, and pharmacokinetic optimization of novel therapeutic antibodies. In addition, the method is suited to understanding basic mechanisms of endocytosis, pinocytosis, and receptor turnover where antibodies can be employed.

References

1. Ochoa, M.C., Minute, L., Rodriguez, I., Garasa, S., Perez-Ruiz, E., Inogés, S., Melero, I., & Berraondo, P. (2017). Antibody-dependent cell cytotoxicity: immunotherapy strategies enhancing effector NK cells. *Immunology and Cell Biology, 95*(4), 347-355.
2. Weiskopf, K., & Wiessman, I. (2017). Macrophages are critical effectors of antibody therapies for cancer. *mABs, 7*(2), 303-310.
3. Beck, A., Goetsch, L., Dumontet, C., & Corvaïa, N. (2017). Strategies and challenges for the next generation of antibody-drug conjugates. *Nature Reviews Drug Discovery, 16*(5), 315-337.
4. Tomas, A., Futter, C.E., & Eden, E.R. (2014). EGF receptor trafficking: consequences for signaling and cancer. *Trends in Cell Biology, 24*(1), 26-34.
5. Harding, J., & Burtness, B. (2005). Cetuximab: An epidermal growth factor receptor chimeric human-murine monoclonal antibody. *Drugs Today, 41*(2), 107-127.

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