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Original Research

High-Throughput Screening for Internalizing Antibodies by Homogeneous Fluorescence Imaging of a pH-Activated Probe

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Abstract

Antibody-drug conjugates (ADCs) represent a rapidly growing class of biotherapeutics that deliver drugs specifically to target cells by binding of the antibody component to surface receptors. The majority of ADCs require receptor internalization depending on intrinsic features of the specific ADC-antigen interaction. The development of potent ADCs would greatly benefit from the identification of efficiently internalizing antibodies at early stages of discovery. We developed a highly sensitive and rapid antibody internalization assay using an indirect Cypher5E label. The pH-activated CypHer5E label becomes fluorescent upon internalization into the acidic environment of endocytic organelles, whereas background fluorescence of noninternalized CypHer5E is minimal. The pH-dependency of the CypHer5E signal enables robust discrimination of antibody internalization from surface binding. The favorable signal-over-background ratio allows a homogeneous assay design with high-throughput fluorescence imaging in 384- and 1536-well formats. The biophysical readout of the primary internalization event substantially shortens incubation times compared to killing assays using toxin internalization. The assay was validated with tumor-relevant targets, including receptor tyrosine kinases (EGFR and HER2) and a class II cytokine receptor (TF) expressed by A431, AU565, and SKOV-3 cells and transient expression systems (CHO-S). Our method enables functional screening of large antibody libraries to identify therapeutic antibody candidates with internalization characteristics favorable for the development of ADCs.

Keywords

antibody-drug conjugates, antibody internalization, pH-dependent dye, homogenous assay, high-throughput screening

Introduction

Antibodies are taking an increasingly important role in the arsenal of therapeutic drugs and are finding their way into the clinic for treatment of many different diseases, including cancer, inflammatory diseases, transplantation, and infectious disease. More than 30 monoclonal antibody-based therapies have been approved worldwide and over 300 are in clinical trials.[1] Growing insights into the mechanisms of action of therapeutic antibodies demand a greater scrutiny of their specific functional activities very early in development to select the best possible drug candidates. This trend, combined with the desire to screen very large numbers of candidates, requires the development of novel sensitive high-throughput assays suitable for assaying antibody function.

Therapeutic antibodies may engage different mechanisms of action to induce a therapeutic effect. Most antibodies developed for cancer therapy are selected on their ability to induce antigen-specific cytotoxicity or specific cellular immune responses involving naturally occurring effector mechanisms.[2] Another emerging therapeutic approach is antibody-drug conjugate (ADC) therapy in which the therapeutic antibody is covalently linked to a cytotoxic compound.[3] In most cases, the conjugated drug needs to be transported over the cancer cell membrane to exert its cytotoxic function. The internalization of the ADC-cell surface receptor complexes thereby is a crucial step for efficient drug conjugate delivery. The magnitude and rate of internalization may strongly vary between antibodies, even when they recognize the same epitope on the target

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molecule on a cancer cell.[4,5] To enable the identification of antibody candidates suitable for development as internalizing ADCs, it is therefore desirable to assess antibody internalization during early discovery. Different technologies are currently employed to determine the internalization of surface antigen-directed antibodies. Widely used techniques to study antibody internalization include flow cytometry usually in combination with surface quenching[6,7] and radiolabeled antibody studies.[8,9] Other methods make use of assessing internalization by image analysis using either macro-confocal imaging or high-content readers.[10–12] Finally, direct and indirect cytotoxic assays using antibody drug conjugates are used.[13,14] Although these assays have proven to be reliable in internalization and cytotoxicity testing, their application for screening of large antibody libraries is limited due to complexities in assay handling, limitations in throughput, and relatively high costs.

Here we describe the development of an image-based homogeneous high-throughput assay for antibody internalization that is applicable to the screening of large antibody libraries on various imaging platforms. In this assay, the internalization of antibodies is detected by a fluorescence signal that is conditional on entry of the label into endosomal, acidic compartments. For the screening of human antibody libraries, our assay employs a secondary Fab-CypHer5E conjugate specific to human IgG, which is endocytosed by piggybacking on internalizing antibodies. CypHer5E is a pH-sensitive cyanine dye derivate that is minimally fluorescent at basic pH and shows maximal fluorescence at an acidic pH.[15] Accordingly, labeling of antibodies with CypHer5E should allow quantitation of the relocation of receptor-binding antibodies from cell surface to acidic compartments by assessing CypHer5E fluorescence in a homogeneous (no-wash) assay format. CypHer5E has been previously shown to be a powerful pH-sensitive label for studying trafficking of G protein–coupled receptors and phagocytosis with high assay sensitivity and throughput without a requirement for surface quenching.[12,16] The CypHer5E label has also been successfully used for screening of scFvs in periplasmic extracts and antibodies in hybridoma supernatants for internalization.[17] Indeed, with the homogeneous assay design reported here, antibody internalization can be detected with the CypHer5E label at as little as 20 ng/mL antibody sample concentration. The low antibody concentration required enables the screening of hybridoma as well as transiently expressed recombinant antibody libraries. Due to its minimalist two-step liquid handling and the feasibility to miniaturize the assay to a 1536-well format, the assay is well suited for integration into early antibody discovery.

Materials and Methods

Antibodies

The generation of human IgG1κ mAbs specific to human EGFR (zalutumumab), HER2 (HER2-005, -025, -084, -091, -098, -129, -153 -169), and tissue factor (TF-011, -098, -111) by immunizing HuMAb mice (Medarex)[18] has been described previously.[19–21] All antibodies were expressed in HEK cells as a human IgG1 (allotype IgG1m(f)) antibody with a κ light chain, purified using protein A affinity chromatography (MabSelect SuRe; GE-Healthcare, Eindhoven, the Netherlands) and formulated in phosphate-buffered saline (PBS). Cetuximab (Erbitux), trastuzumab (Herceptin), and panitumumab (Vectibix) were purchased from Merck (Whitehouse Station, NJ), Roche (Basel, Switzerland), and Amgen (Thousand Oaks, CA), respectively. Human ChromPure IgG (009-000-003) and Fab fragments of goat anti–human IgG (H+L) (109-007-003) were purchased from Jackson ImmunoResearch Europe Ltd. (Newmarket, Suffolk, UK).

CypHer5E Conjugation of Antibodies

Purified human IgG1 mAbs and Fab fragments of goat anti–human IgG (H+L) from primary amine-free stock solutions were diluted to approximately 1 mg/mL in PBS containing 50 mM $NaHCO_3$ (pH 8.3), and final antibody concentration was determined by absorption measurement at 280 nm. CypHer5E N-hydroxysuccinimide (NHS) ester (PA15405) was obtained from GE Healthcare (Eindhoven, the Netherlands) and reconstituted according to the manufacturer's protocol. A 20-fold molar excess of CypHer5E was added to the antibody solutions and incubated for 1 h at room temperature (RT) in the dark followed by dialysis against PBS overnight. Any unbound dye and low molecular weight (MW) contaminates were removed by fractionation over a PD-10 desalting column. Fractions containing antibody and CypHer5E dye were detected by measuring absorption at 280 nm (A280) and 500 nm (A500), respectively, and were pooled. The average amount of CypHer5E (C5E) molecules conjugated to antibodies (dye to protein ratio, D/P) was estimated in pooled fractions by final A280 and A500 measurements, and samples were stored at 4 °C in the dark. For the C5E conjugates used in this study, the following D/Ps were obtained: cetuximab-C5E D/P = 2.3, HER2-153-C5E D/P = 6.6, huChromPure-C5E D/P = 6.1, and goat anti–human IgG (H+L) Fab-C5E D/P = 3.8 and 1.7, respectively (two batches used in this study).

Mammalian Cells

Human SKOV-3 and AU565 (epithelial ovary and breast cancer cell lines, respectively) were from the American Type Culture Collection (ATCC, Manassas, VA). Human A431 (epithelial squamous carcinoma) cells were from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany). A431 cells were cultured in RPMI 1640 (BE12-115F; Lonza, Basel, Switzerland) containing 10% heat-inactivated calf serum. SKOV-3 and AU565 cells were cultured in RPMI 1640, containing 10% heat-inactivated calf serum, 2% sodium bicarbonate (BE17- 613E; Lonza), 1%

sodium pyruvate (BE13-115E; Lonza), and 0.5% glucose (G8769; Sigma-Aldrich, St. Louis, MO). Cell lines were tested for mycoplasma contamination, and dedicated cell banks were prepared from early passage cultures.

For the transient expression of human EGFR in CHO-S cells (CHOs-EGFR) using the Freestyle MAX CHO Expression System (Life Technologies, Grand Island, NY), the codon-optimized (GeneArt; Life Technologies Europe BV, Bleiswijk, the Netherlands) coding sequence of human EGFR was cloned into pcDNA3.3 (Life Technologies).

EGFR, HER2, and TF cell surface expression was quantified using the QiFi flow cytometry bead-based indirect immunofluorescence kit (Dako, Glostrup, Denmark). Secondary conjugate $F(ab')_2$ fragments of goat anti–mouse immunoglobulins conjugated with FITC (F0479; Dako) were used according to the supplier's guidelines. As primary antibodies, mouse anti–human EGFR (555996; BD Biosciences, San Jose, CA), mouse anti–human HER2 (MAB1129; R&D Systems, Minneapolis, MN), and mouse anti–human TF (CLB, MW1838; Sanquin, Amsterdam, the Netherlands) were used for the quantification of human EGFR, HER2, and TF, respectively.

CypHer5E Internalization Assay

Cells from freshly thawed cryo cell banks were seeded at 5000 cells per well in 384-well microtiter plates or 2000 cells per well in 1536-well microtiter plates in PBS containing 240 ng/mL Fab fragments of goat anti–human IgG conjugated with CypHer5E (Fab-CypHer5E). Serially diluted antibodies (5000 to 4.9 ng/mL) were added as 1/5 of the final assay volume, resulting in a final Fab-CypHer5E concentration of 192 ng/mL. Plates were incubated protected from light at RT unless stated otherwise. Plates were imaged by either the Celigo Cell Cytometer (Nexcelom Bioscience, Lawrence, MA) at ex/em 628(/40) nm/688(/31) nm or the 8200 Cellular Detection System (8200 CDS; Applied Biosystems, Foster City, CA) at ex/em 633 nm/650 to 685 nm. The macro-confocal 8200 Cellular Detection System with FMAT technology (Fluorometric Microvolume Assay Technology) images and quantifies fluorescence in a well section with a depth of focus of ~100 μm from the plate bottom. Any out-of-focus background fluorescence in the remaining volume of the well remains undetected, resulting in increased signals over background in homogeneous assay designs.[22] The number of segmented fluorescently labeled objects (counts) per well and their mean fluorescence (FL) intensities were recorded. Total FL intensity per well was obtained by multiplying counts with their mean FL intensity.

A count cutoff of 50 counts was generally applied to raw data sets before data analysis, resulting in mean FL intensity and total FL intensity values of zero for samples with fewer than 50 counts. Resulting data were plotted as total FL intensity against final antibody concentration in the assay.

LysoTracker Red Labeling

The labeling of acidic organelles in living A431 cells was performed with LysoTracker Red DND-99 (L-7528; Life Technologies). Cells were seeded in 96-well microtiter plates the day before labeling to allow cell attachment. Culture medium was removed and cells were incubated for 1 h at 37 °C with 75 nM LysoTracker Red in culture medium followed by a medium refresh. Cells were imaged and analyzed using the Celigo Cell Cytometer (Nexcelom Bioscience) at ex/em 531(/40) nm/629(/53) nm in addition to brightfield imaging.

Data Analysis

Data analysis was performed with GraphPad Prism software V6.04 (GraphPad Software, La Jolla, CA). The 8200 Cellular Detection System images were analyzed with 8200 software V3.0 (Applied Biosystems). Celigo images were analyzed using Celigo software version 2.1.1.4 (Nexcelom Bioscience).

The lower limit of detection (LLOD) for total fluorescence intensity was estimated as a 95% one-sided confidence limit and calculated by the formula LLOD = mean blank + 1.645 * SD blank + 1.645 * SD low concentration sample. The low concentration sample was hereby identified as the lowest concentration of positive control antibody tested yielding a total fluorescence intensity signal higher than the negative control sample with a coefficient of variation (CV) (total fluorescence intensity) equal to or smaller than 15%. The upper limit of detection (ULOD) was estimated as the positive control antibody concentration yielding the highest top signal.

The signal-to-background ratio (S/B) was calculated as S/B = mean positive control signal/mean negative control signal. In the absence of mean negative control signal (mean negative control signal = 0), the mean negative control signal was set to 1 for S/B calculation. The signal-to-noise ratio (S/N) was calculated as S/N = (mean positive control signal – mean negative control signal)/quadratic mean of standard deviations. Z factor was calculated according to Zhang et al.[23]

Results

Homogeneous CypHer5E Antibody Internalization Assay

To prove that CypHer5E can be used to assess antibody internalization, we conjugated the EGFR-specific antibody cetuximab and the HER2-specific antibody HER2-153 as well as a nonspecific IgG negative control (huChromPure) with CypHer5E by primary amine coupling. A431 cells expressing high copy numbers of EGFR and low copy numbers of HER2, as well as SKOV-3 cells expressing low copy numbers of EGFR and high copy numbers of HER2 (**Fig.**

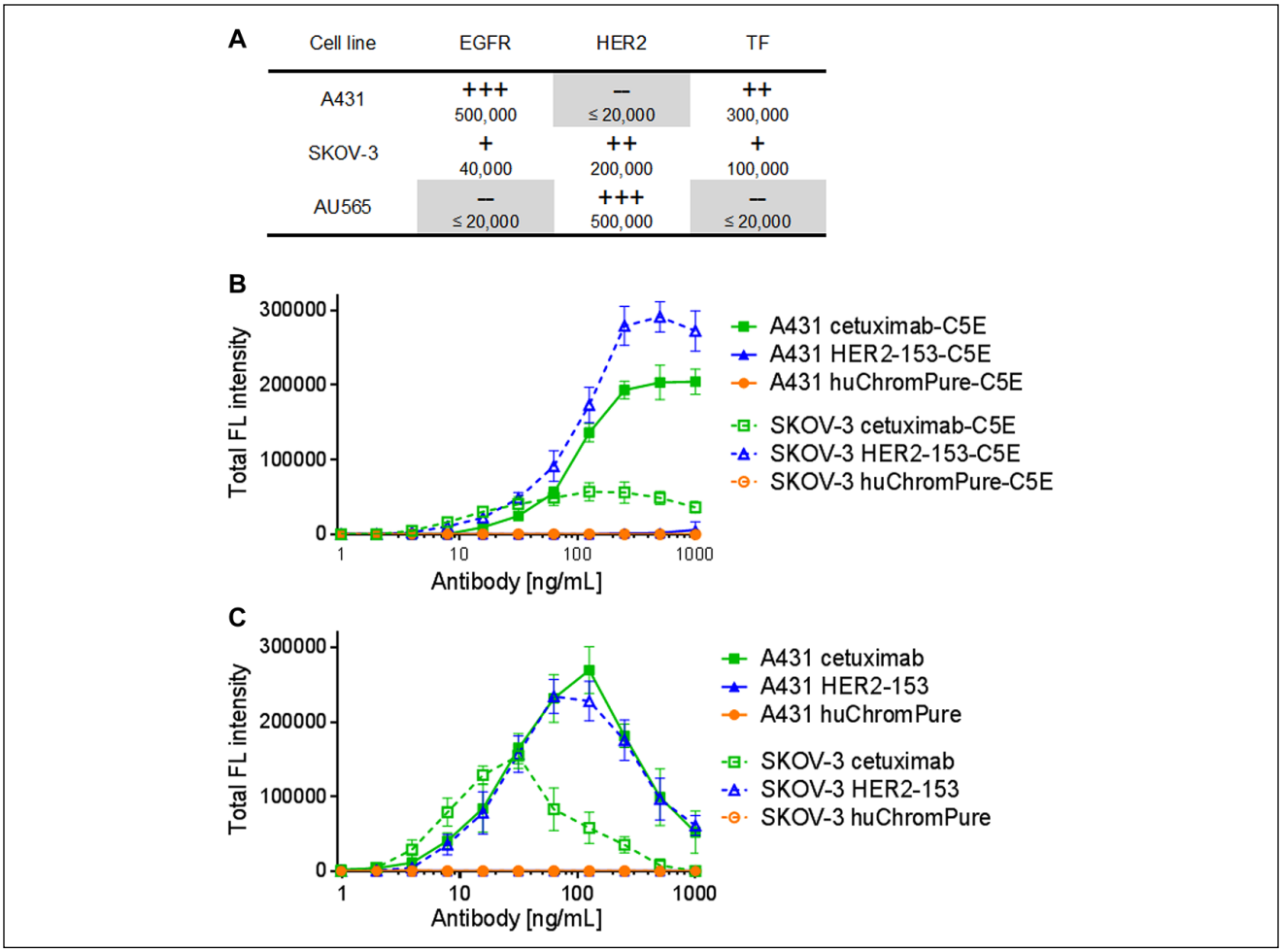

Figure 1. Dose response in direct and indirect CypHer5E antibody internalization assays correlates with target expression. (**A**) Table summarizing approximate surface copy numbers of EGFR, HER2, and tissue factor (TF) antigens expressed in molecules per cell detected on A431, SKOV-3, and AU565 cells used in this study. Antigen cell surface expression was quantified by flow cytometric QiFi analysis as described in the Materials and Methods section. (**B**) Direct assay format: cetuximab, HER2-153, and the negative control antibody huChromPure were conjugated with CypHer5E, resulting in the following dye to protein ratios (D/Ps): cetuximab-C5E D/P = 2.3, HER2-153-C5E D/P = 6.6, and huChromPure-C5E D/P = 6.1. A dose titration of the resulting CypHer5E (-C5E) conjugates was incubated with A431 and SKOV-3 cells. (**C**) Indirect assay format: unconjugated cetuximab, HER2-153, and the negative control antibody huChromPure were incubated with A431 and SKOV-3 cells in the presence of anti–human IgG Fab-CypHer5E conjugate (D/P = 3.8) at a final concentration of 192 ng/mL. The total cell-associated fluorescence (FL) intensities (± SD, n = 8) were measured on the 8200 Cellular Detection System after 6 h of incubation at room temperature. Representative data of two (**A**) and three (**B**) independent experiments are shown.

1A), were incubated with the CypHer5E conjugates for 6 h at RT and imaged without any washing procedure (**Fig. 1B**).

We did not detect any fluorescence signal with the huChromPure-C5E negative control for both cell lines. Incubation of A431 cells with the EGFR-specific cetuximab-C5E conjugate resulted in a strong dose-dependent fluorescence signal, whereas the HER2-specific HER2-153-C5E conjugate only showed a marginal fluorescence signal at the highest concentration tested. Using SKOV-3 cells, in contrast, we observed the highest fluorescence signal with the HER2-specific HER2-153-C5E conjugate and a significantly lower signal with the EGFR-specific cetuximab-C5E conjugate. The correlation between the observed fluorescence signals and antigen expression levels (**Fig. 1A**) clearly indicates appropriate antigen specificity of the assay, although absolute fluorescence signals obtained with CypHer5E-conjugated antibodies cannot be directly compared due to variable dye to protein ratios (D/Ps) of cetuximab-C5E (D/P = 2.3) and HER2-153-C5E (D/P = 6.6).

To enable the screening of large antibody panels, we reformatted the assay into an indirect homogeneous setup. In this format, the internalization of nonconjugated

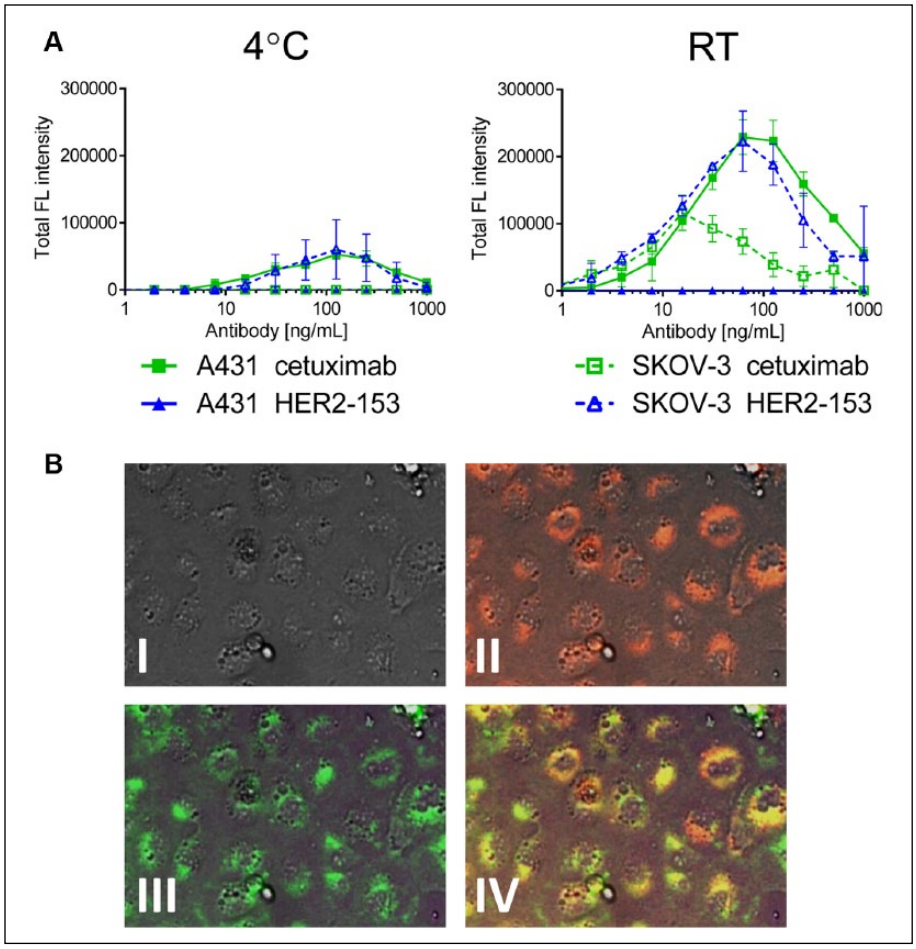

Figure 2. Antibody-mediated endocytosis of a secondary anti–human IgG Fab-CypHer5E conjugate. (**A**) Indirect internalization assay performed at room temperature and 4 °C. A431 and SKOV-3 cells were incubated with cetuximab and HER2-153 in the presence of anti–human IgG Fab-CypHer5E conjugate. The total cell-associated fluorescence (FL) intensities (± SD, n = 4) were measured on the 8200 Cellular Detection System after 6 h of incubation. Representative data of n = 3 experiments is shown. (**B**) Colocalization of LysoTracker Red staining and internalized CypHer5E label. A431 cells were counterstained with the acidotropic LysoTracker Red probe prior to an indirect internalization assay at room temperature with cetuximab (anti–EGFR) in the presence of anti–human IgG Fab-CypHer5E conjugate. Subsequent imaging of live cells was performed with the Celigo Cytometer. **I**, brightfield image; **II**, LysoTracker Red (red); **III**, CypHer5E (green); **IV**, merge of II and III.

antibodies is detected by the binding of a Fab fragment of a goat anti–human IgG antibody conjugated with CypHer5E with a D/P of 3.8 (**Fig. 1C**). A Fab format for the conjugate was chosen to prevent receptor cross-linking by the secondary antibody that might affect the internalization rate.[7] In this indirect format, we observed specific, dose-dependent binding to A431 and SKOV-3 cells of the EGFR- and HER2-specific antibodies as described above. However, at high concentrations, we observed decreased signals (prozone effect) due to depletion of the excess of the Fab-CypHer5E conjugate at a final concentration of 192 ng/mL in the assay.

It has been previously shown that clathrin-dependent receptor endocytosis is decreased at reduced temperature due to microtuble depolymerization.[24] To test whether the observed CypHer5E fluorescence is indeed correlated with the internalization of receptor-bound antibodies, we also performed internalization assays at 4 °C. For all target-antibody combinations tested, we observed a significant reduction of the fluorescence signal after incubation at 4 °C compared to the incubation at RT (**Fig. 2A**). Finally, we performed indirect antibody internalization experiments with A431 cells in which acidic organelles, in particular lysosomes, were labeled with LysoTracker Red prior to antibody treatment. Incubation with the EGFR-specific antibody cetuximab in the presence of goat anti–human IgG-C5E Fab fragments resulted in a strong increase of CypHer5E fluorescence in intracellular regions or compartments that counterstained with the LysoTracker Red dye (**Fig. 2B**). The observed pattern of the CypHer5E signal supports the specificity of the assay for antibody internalization.

Antigen and Cell Type–Specific Antibody Internalization

To address the effects of target expression on the CypHer5E assay, we identified three epithelial tumor cell lines (A431, SKOV-3, and AU565; isolated from different tissues) that express three antigens (EGFR, HER2, and TF) at different levels on their cell surface (**Fig. 1A**). With each cell line, indirect internalization assays were performed with three or four EGFR-, HER2- or TF-specific antibodies, respectively

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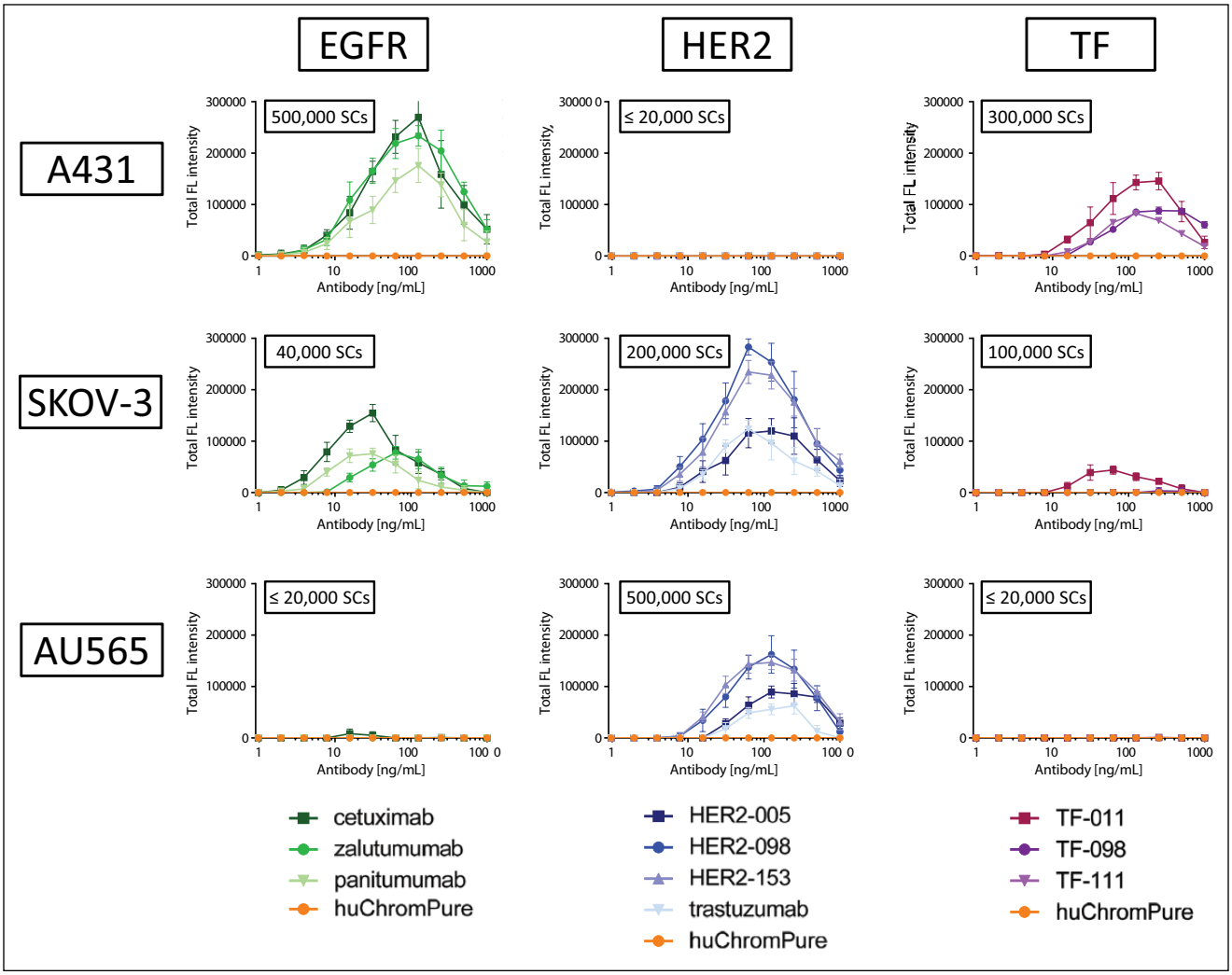

Figure 3. Antigen and cell type–specific antibody internalization. Indirect internalization assays were performed with antibodies against EGFR, HER2, and TF for A431, SKOV-3, and AU565 cells representing cells with distinct target expression. The target expression is indicated in the left top corner of each particular chart in molecules per cell (SC: surface copy numbers). Cell-associated total fluorescence (FL) intensities (± SD, n = 8) were measured on the 8200 Cellular Detection System after 6 h of incubation at room temperature. The data shown are representative of at least two independent experiments, respectively.

(**Fig. 3**). The EGFR- and TF-specific antibodies used bind to overlapping but nonidentical epitopes on EGFR and TF, respectively.[21,25,26] The four HER2-specific antibodies used represent three distinct cross-block groups with HER2-098 and HER2-153 belonging to the same cross-block group.[20]

In agreement with high cell surface target expression, we observed the highest CypHer5E fluorescence signals on A431 cells for EGFR- and TF-specific antibodies and for SKOV-3 and AU565 cells for Her2-specific antibodies. Lower fluorescence signals were observed for EGFR- and TF-specific antibodies on SKOV-3 cells with lower EGFR and TF expression compared to A431 cells. Virtually no CypHer5E signal was observed on AU565 cells with EGFR- and TF-specific antibodies and on A431 cells for Her2-specific antibodies, in agreement with very low levels of antigen expression (<20,000 surface copy numbers per cell). For the studied antibodies and targets, the assay sensitivity thus requires target cell surface expression higher than 20,000 copy numbers per cell to detect antibody internalization. Although our results underline the antigen specificity of the CypHer5E internalization assay, we were not able to correlate antigen surface expression levels with the obtained CypHer5E signal intensity. Indeed, despite about 10-fold lower EGFR surface expression on SKOV-3 cells compared to A431 cells, we obtained only about 2-fold lower CypHer5E fluorescence signals for the EGFR-specific antibodies cetuximab, zalutumumab, and panitumumab. Similarly, although HER2 surface expression is 2.5-fold higher on AU565 cells than on SKOV-3 cells, we observed lower CypHer5E fluorescence signals for HER2-specific antibodies with AU565

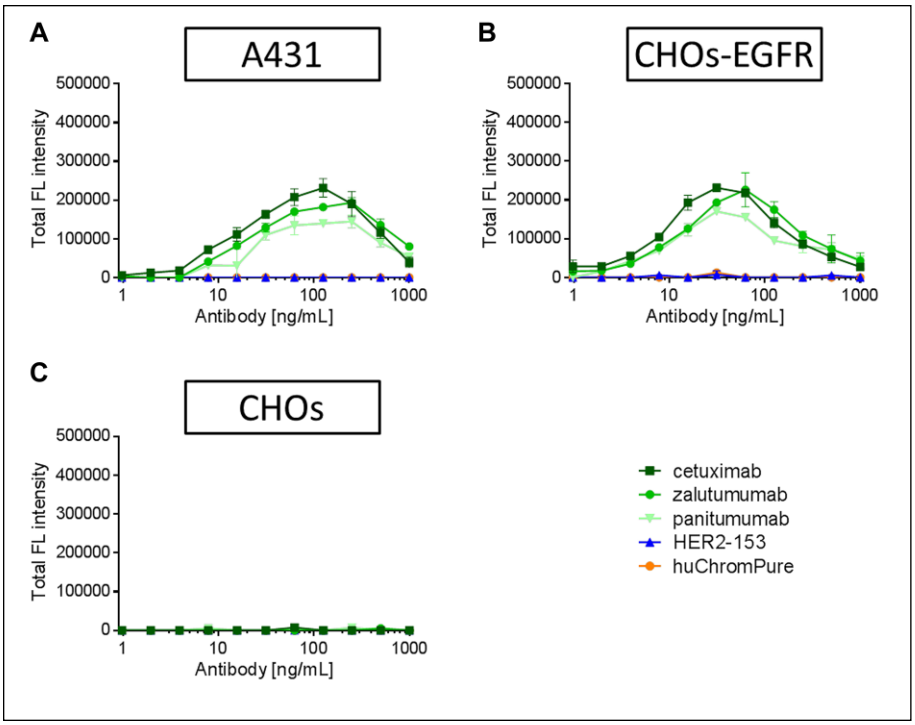

Figure 4. Antibody internalization by recombinant surface receptors. A431 cells (**A**) were subjected to dose-response internalization assays along with CHOs-EGFR cells transiently expressing human EGFR (**B**) and wild-type CHOs cells (**C**). A431 cells show significant surface expression of EGFR and minimal expression of HER2. A431, CHOs-EGFR, and CHOs cells were incubated with EGFR-specific antibodies (cetuximab, zalutumumab, panitumumab), a HER2-specific antibody (HER2-153), and a negative control antibody (huChromPure) in the presence of anti–human IgG Fab-CypHer5E conjugate. Total fluorescence (FL) intensities measured by the 8200 Cellular Detection System (± SD) were derived from duplicate wells.

cells than with SKOV-3 cells. These results suggest that the observed CypHer5E internalization signal cannot be directly correlated to variations in target surface expressions on distinct cells (**Fig. 3**). Moreover, analyzing the dose-dependent internalization of EGFR-specific antibodies in A431 cells and SKOV-3 cells in more detail, we noticed also differences in the lower limit of detection and apparent EC_{50}, suggesting that cell line–specific factors may affect internalization (**Suppl. Table S1**).

Antibody Cointernalization with Overexpressed Receptors

Engineered cell lines are frequently used in high-throughput screening (HTS) campaigns to introduce highly defined reporter systems that are easily obtained in large quantities. We therefore addressed whether defined host cells such as CHO-S that recombinantly express the surface receptor of interest might be used for initial screening in high-throughput campaigns for internalizing antibodies.

We expressed human EGFR transiently in CHO-S cells (CHOs-EGFR; about 200,000 EGFR surface molecules per cell), and anti-EGFR and anti-HER2 antibody internalization was compared to A431 cells expressing high EGFR and very low HER2 levels (**Fig. 1A**). With A431 cells, we observed antibody internalization for all antibodies against EGFR but not for the HER2-specific HER2-153 or the negative control huChromPure (**Fig. 4A**). Similarly, with CHO-S cells expressing recombinant EGFR (CHOs-EGFR), we only found internalization of anti–EGFR antibodies but not of anti–HER2 antibodies or the negative control (**Fig. 4B**). The background control with nontransfected CHOs cells did not yield an internalization signal for any of the antibodies tested (**Fig. 4C**).

Effects of Incubation Time on Assay Results

With the aim to transfer the CypHer5E antibody internalization assay to an automated liquid handling environment, we assessed its robustness for incubation time. To this end, we compiled a test panel of eight HER2-specific antibodies comprising strongly internalizing antibodies (e.g., HER2-153 and HER-098), weakly internalizing antibodies (e.g., HER2-169), and antibodies with very similar internalization characteristics such as HER2-091 and HER2-129 (**Fig. 5**, de Goeij et al.[20]). The test antibodies show similar apparent affinity and belong to different cross-blocking groups (CBs), indicating their binding to different nonoverlapping epitopes (HER2-084, HER2-169: CB1; HER2-025, HER2-091, HER2-129: CB2; HER2-098, HER2-153: CB3; HER2-005: CB4).[20] Since the homogenous assay design is prone to hook effects (**Fig. 1C**), we performed time course experiments in dose-response analyses (**Fig. 5A**). For all antibodies tested, the top signal intensities increased with incubation time (**Fig. 5A,B**). For some antibodies (e.g., HER2-025 and HER2-098), we observed a very fast initial increase of top signals occurring

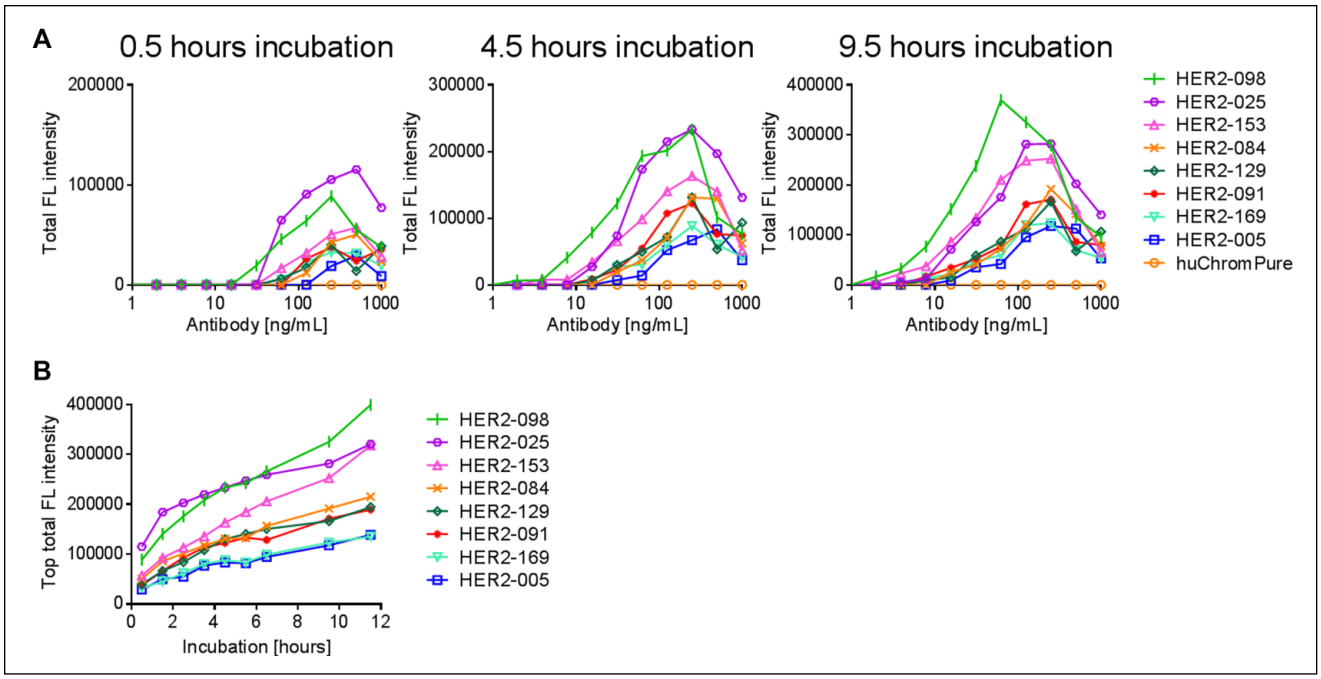

Figure 5. Time-resolved indirect CypHer5E internalization assay. A test panel of eight HER2-specific antibodies and a negative isotype control (huChromPure) were analyzed in an indirect CypHer5E internalization assay with AU565 cells using the 8200 Cellular Detection System macro-confocal scanner. The cellular total fluorescence (FL) intensities were assessed at the incubation times indicated. The average total intensity values of each eight wells are plotted. (**A**) CypHer5E internalization signals (Total FL) recorded at 0.5, 4.5, and 9.5 h of assay incubation, respectively. It should be noted that the presented graphs use different Y-axis scaling. (**B**) Increase of observed CypHer5E top fluorescence signals over time.

during the first 1.5 h of incubation, while internalization was more constant in time for other antibodies (**Fig. 5B**). Longer incubation times led to a lower limit of detection for all antibodies and could be reduced at least 5-fold for incubation times of 4.5 h or longer (**Fig. 5A**).

Although the CypHer5E top signal increased for all antibodies continuously with incubation time, the top signal-based ranking changed with incubation time for some antibodies such as HER2-098 and HER-025 (**Fig. 5B**). At the beginning of the time course, the CypHer5E fluorescence top signal for HER2-025 increased very rapidly and faster than the top signal for HER2-098. With longer incubation times, the rate of signal increase changed for both antibodies and eventually resulted in a higher CypHer5E top signal for HER2-098 over HER2-025 after 6.5 h of incubation (**Fig. 5B**).

We also noticed that for antibodies with very similar internalization characteristics such as HER2-129 and HER2-091, a ranking by the obtained top signal alone would not allow a clear differentiation (**Fig. 5B**). Nevertheless, when analyzing the obtained dose-response curves of HER2-129 and HER2-091 at every time point, we noticed that HER2-091 consistently reached the fluorescence top signal at a lower concentration than HER2-129 (**Fig. 5A**), indicating a lower EC_{50} value.

Taken together, our results indicate that an incubation time of at least 4.5 h would result in stable assay results when using a single-point measurement under primary screening conditions.

HTS Assay Qualification and Miniaturization

To advance our homogeneous antibody internalization assay to primary screening applications, we analyzed the assay performance in a 384-well format. We tested three different cell lines (A431, SKOV-3, and AU565) with antibodies against EGFR, TF, and HER2. The upper part of the table in **Figure 6A** summarizes the pertinent assay parameters obtained with the 8200 CDS macro-confocal scanner for two antibodies per antigen. The lower part of the table in **Figure 6A** shows corresponding assay results when using an alternative imaging system such as a fluorescence microscope (Celigo Cell Cytometer). Regardless of the imaging technology used, we observed with only a single exception (zalutumumab on SKOV-3 cells) total fluorescence intensity signals at the ULOD with CV values below 20% and S/N ratios greater than 5 (**Fig. 6A**). This matches general HTS assay guidelines.[27] Sufficient quality of the assay for screening purposes was further documented by Z factors greater than or close to 0.5 (**Fig. 6A**). Only using SKOV-3

cells, we did not meet the criteria with all antibodies tested, possibly due to the low target surface expression (**Fig. 6A**). Notably, for all assay combinations tested, the LLODs ranged between 4 and 31 ng/mL with the 8200 CDS macro-confocal scanner. Slightly higher LLODs between 16 and 125 ng/mL were obtained with the Celigo Cell Cytometer, which thus appears to be a lower resolution platform for this assay (**Fig. 6A**).

The add-and-mix design of the homogeneous assay makes it perfectly suited to be transferred from the 384-well to a 1536-well format. To explore this opportunity, we performed automated test runs with A431 and AU565 cells with cetuximab and HER2-153, respectively. In both cases, we observed with the 1536-well format dose-response curves similar to the 384-well format, albeit with different absolute fluorescence values due to the changed plate format (**Fig. 6B**). The assay sensitivity as judged by observed lower limits of detection did not significantly change upon assay miniaturization (**Fig. 6B**). Importantly, we were able to obtain Z factors of 0.74 and 0.63 for A431/cetuximab and AU565/HER2-153, respectively, providing proof for the general applicability of the homogeneous CypHer5E internalization assay to screening campaigns in a 1536-well format.

Discussion

The success of internalizing ADCs in cancer therapy relies, among others, on specific tumor antigen recognition, efficient internalization of the antibody-drug conjugate, and the release of the functionally active drug within the tumor cell. The generation of antibody panels for ADC development would greatly benefit from a functional screening platform for antibody internalization that is applicable at early stages of antibody discovery. The assay should enable the screening of large numbers of samples with low antibody concentrations in complex matrices such as cell culture supernatants (**Suppl. Fig. S1**). To alleviate liquid handling requirements, the assay should be easy to perform and sufficiently robust to be compatible with high plate density formats that allow increased throughput at reduced costs.

We demonstrated that antibody internalization can be specifically assessed in a simple no-wash assay by the use of the pH-sensitive CypHer5E dye that converts to a fluorescently active state upon translocation to an acidic, endocytic compartment (**Figs. 1** and **2**). Using direct conjugation of the CypHer5E label is not feasible as throughput would be too low. Using a secondary CypHer5E Fab conjugate specifically binding the test antibody offers a homogeneous, indirect assay format that allows the application of the assay to large numbers of antibodies (e.g., de Goeij et al.[20]). The use of a Fab fragment as a secondary conjugate avoids receptor cross-linking that might affect the internalization rate.[7] Similar to the assay protocol presented here, the assay can be easily adapted to the detection of the internalization of mouse or rabbit IgGs by using appropriate Fab-CypHer5E conjugates. The use of the indirect assay format eliminates potential effects of differences in the dye to protein ratio (D/P) following CypHer5E conjugation. Indeed, we observed a significant increase of cetuximab CypHer5E internalization signal relative to HER2-153 when comparing the indirect with the direct assay format on SKOV-3 cells (**Fig. 1B,C**). This change in relative signal intensities might be explained by the different D/Ps of directly labeled HER2-153-C5E and cetuximab-C5E of 6.6 and 2.3, respectively, which is then eliminated in the indirect assay format. An alternative explanation for this apparent change in relative signal intensities could rely on the observed hook effect intrinsic to the indirect assay format (bell-shaped dose-response curves). This hook effect might cause the loss or, at least, the strong decrease of the observed CypHer5E fluorescence signal when antibody internalization is studied at high antibody concentrations. Moreover, data obtained from single-point screenings in particular with nonnormalized samples have to be evaluated very carefully and should rather be used for yes-no decisions than for antibody ranking.

The versatility of the CypHer5E antibody internalization assay was demonstrated by its applicability to various HTS platforms and different surface receptors in a variety of cell types. With different targets and various cell lines tested, we repeatedly demonstrated lower limits of detection in the range of 4 to 125 ng/mL (**Fig. 6A**), which makes the assay sensitive enough for the screening of antibodies in hybridoma and B-cell cell culture supernatants or transiently transfected antibody clones in early discovery (**Suppl. Fig. S1**). Assay qualities achieved as judged by S/N ratios and Z factors enabled transfer of the assay to an automated liquid handling platform (e.g., Tecan Freedom EVO200; Tecan Schweiz AG, Männedorf, Switzerland). The subsequent miniaturization to the 1536-well plate format further minimized sample consumption (2 μL of cell culture supernatant). When screening large antibody libraries with a large number of assay plates, an incubation time-dependent signal increase over plates (**Fig. 5B**) can be minimized using scheduled reading on an automated platform. Optimal assay results were obtained between 4.5 and 9.5 h of incubation, which makes the assay significantly faster than cytotoxicity-based internalization assays that require several days of incubation before being analyzed. The rapid sample turnover enables the use of the CypHer5E antibody internalization assay as a primary screening assay in flow-optimized B-cell selection and hybridoma generation processes that frequently demand a rapid hit selection to meet requirements of the cell culture work flow. Moreover, the rapid sample turnover and the low sample consumption of the

A

Reader	Cell line	Target	Antibody	LLOD [ng/mL]	ULOD [ng/mL]	Total FL intensity CV ULOD [%]	S/B	S/N	Z-factor
8200 CDS	A431	EGFR	cetuximab	8	125	11.6	269,832	8.6	0.65
			zalutumumab	8	125	8.6	233,577	11.7	0.74
		TF	TF-011	16	250	11.7	145,932	8.5	0.65
			TF-098	31	125	16.4	85,639	6.1	0.51
	SKOV-3	EGFR	cetuximab	4	31	10.9	154,732	9.2	0.67
			zalutumumab	16	31	22.6	53,945	4.4	0.32
		TF	TF-011	16	63	18.2	43,987	5.5	0.45
			TF-098	NA	NA	-	-	-	-
		HER2	HER2-005	16	125	6.5	137,632	15.5	0.81
			HER2-153	8	125	11.7	228,160	8.6	0.65
	AU565	HER2	HER2-005	31	125	12.8	89,350	7.8	0.62
			HER2-153	16	125	9.6	146,947	10.4	0.71
Celigo	A431	EGFR	cetuximab	31	500	16.5	46,566	6.0	0.50
		TF	TF-011	125	500	12.6	46,984	7.9	0.62
	SKOV-3	EGFR	cetuximab	16	63	16.7	34,412	6.0	0.50
	AU565	HER2	HER2-153	31	500	14.7	51,082	6.8	0.56

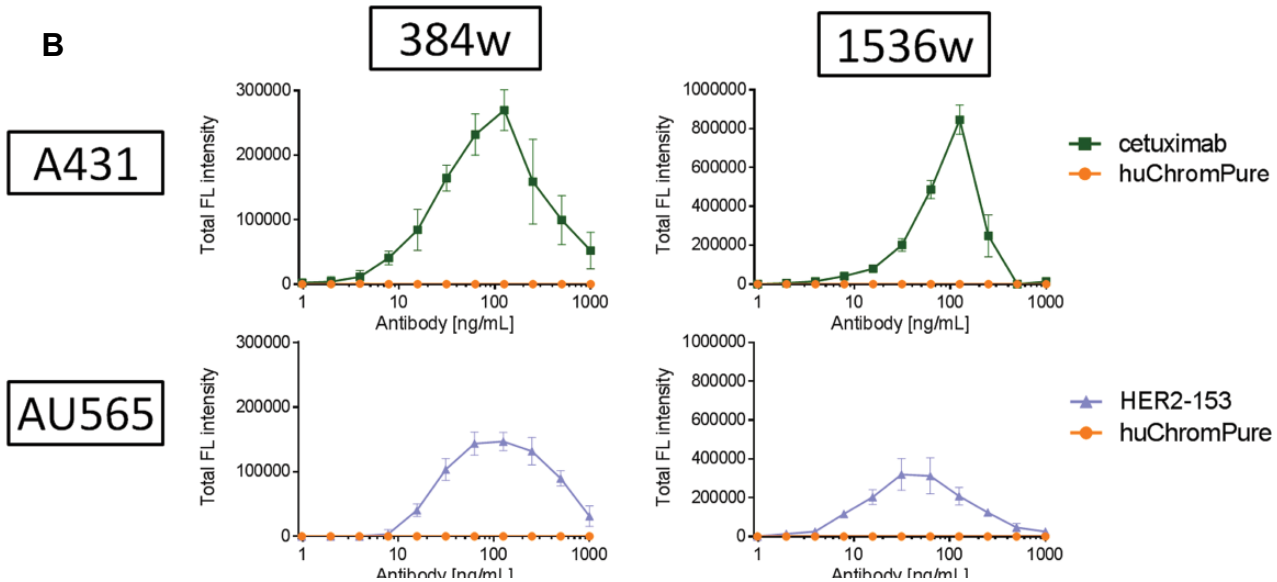

Figure 6. Indirect antibody internalization assay in a 384-well and 1536-well format. (**A**) Summary of assay parameters obtained in a 384-well format with different imaging platforms. Indirect CypHer5E internalization assays were performed with A431, SKOV-3, and AU565 cells with target-specific positive control antibodies indicated. HuChromPure served as negative control antibody. The reactions were analyzed using either the 8200 Cellular Detection System (8200 CDS) or Celigo Cell Cytometer (Celigo). Assay parameters were calculated as described in in the Materials and Methods section based on octuplet assay points. LLOD, lower limit of detection; ULOD, upper limit of detection; S/B, signal-to-background ratio; S/N, signal-to-noise ratio. S/B, S/N, and Z factor were calculated at the ULOD. (**B**) Positive and negative control antibodies cetuximab, HER2-153, and huChromPure were incubated in either a 384-well format or a 1536-well format with A431 and AU565 cells in dose response in the presence of anti–human IgG Fab-CypHer5E conjugate. The cell-associated total fluorescence (FL) intensities (± SD, n = 8) were measured after 6 h of incubation at room temperature on the 8200 CDS macro confocal scanner.

automated, miniaturized assay allow its ready application to different cell lines with various target surface expression levels already during primary screening campaigns.

The described CypHer5E internalization assay specifically assesses the ability of an antibody to cointernalize with its target, thereby delivering the CypHer5E label to

endocytic compartments. While ADCs frequently require their routing to lysosomes for drug release by specific proteases, both endosomes and lysosomes provide acidic environments for CypHer-5E fluorescence. Although we provide evidence that the observed CypHer5E signal colocalizes with a LysoTracker Red counterstain (**Fig. 2B**), we cannot conclude that observed signal intensities indeed reflect the amount of internalized target-specific antibodies routed to the lysosome. CypHer5E is minimally fluorescent at pH 7.4 and maximally fluorescent at pH 4.5 to 5.5.[12] The transport of a CypHer5E-conjugated molecule from early endosomes (pH 6.0–6.5) to lysosomes (pH 4.5–5.5) could yield a fluorescence enhance factor (change in fluorescent emission) of about 2- to 4-fold depending on the D/P ratio of the CypHer5E-labeled molecule.[12,28] This difference in fluorescence intensity would still allow CypHer5E-labeled molecules to be detected with 8200 CDS or Celigo, although when internalized to early endosomes and not further routed to lysosomes. Nevertheless, routing to the lysosome would result in higher fluorescence intensity per internalized molecule and would thus favor the detection of antibodies that are indeed transported to the lysosome over antibodies that remain in early or recycling endosomes. The discrimination of antibody internalization to endosomes or lysosomes should not be possible with the presented method since differences in signal intensities could rely on either the subcellular localization upon internalization or the number of internalized antibodies. The primary, target-specific antibody could undergo several rounds of recycling, delivering additional CypHer5E conjugates to endocytic vesicles with each of these rounds. This would result in an overestimation of the routing of an antibody to the lysosome. Consequently, the efficacy of an antibody in drug delivery to the lysosome would be overestimated. Indeed, studying the efficiency of TF-specific ADCs in delivering their cytotoxic drug, de Goeij and colleagues[29] found that the routing of the targeted receptor upon internalization to the lysosome has a major impact on ADC efficacy. In this study, it was demonstrated that TF-specific ADCs (anti–TF-duostatin3) might outperform ADCs targeting HER2 or EGFR due to a higher protein turnover rate and more efficient lysosomal targeting of TF than HER2 and EGFR.[29] Such TF-specific candidates would then also be identified in the CypHer5E internalization assay but possibly with lower signal intensities compared to antibodies specific to fast recycling HER2 or EGFR. Indeed, on A431 and SKOV-3 cells, we observed with our indirect CypHer5E internalization assay lower total fluorescence signals for TF-specific antibodies than, for example, EGFR-specific antibodies (**Fig. 3**).

Thus, similar to other high-throughput antibody internalization assays reported, including cytotoxicity assays (Klussman et al.[14] and references therein), the presented CypHer5E internalization assay will identify internalizing antibody candidates, which then will require a more elaborate characterization in lower throughput secondary assays for ADC development.

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Declaration of Conflicting Interests

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: T. Riedl, E. van Boxtel, M. Bosch, P. W. H. I. Parren, and A. F. Gerritsen are Genmab employees and own Genmab warrants and/or stock.

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