Technical Note

A Simple and Sensitive High-Content Assay for the Characterization of Antiproliferative Therapeutic Antibodies

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Abstract

Monoclonal antibodies (mAbs) have become a central class of therapeutic agents in particular as antiproliferative compounds. Their often complex modes of action require sensitive assays during early, functional characterization. Current cell-based proliferation assays often detect metabolites that are indicative of metabolic activity but do not directly account for cell proliferation. Measuring DNA replication by incorporation of base analogues such as 5-bromo-2′-deoxyuridine (BrdU) fills this analytical gap but was previously restricted to bulk effect characterization in enzyme-linked immunosorbent assay formats. Here, we describe a cell-based assay format for the characterization of antiproliferative mAbs regarding potency and mode of action in a single experiment. The assay makes use of single cell–based high-content-analysis (HCA) for the reliable quantification of replicating cells and DNA content via 5-ethynyl-2′-deoxyuridine (EdU) and 4′,6-diamidino-2-phenylindole (DAPI), respectively, as sensitive measures of antiproliferative mAb activity. We used trastuzumab, an antiproliferative therapeutic antibody interfering with HER2 cell surface receptor-mediated growth signal transduction, and HER2-overexpressing cell lines BT474 and SKBR3 to demonstrate up to 10-fold signal-to-background (S/B) ratios for treated versus untreated cells and a shift in cell cycle profiles indicating antibody-induced cell cycle arrest. The assay is simple, cost-effective, and sensitive, providing a cell-based format for preclinical characterization of therapeutic mAbs.

Introduction

Biological drugs such as therapeutic antibodies are in the process of replacing chemical compounds as the major class of future medicines. Therapeutic antibodies are often characterized by complex modes of action, such as inhibition of cell proliferation, induction of apoptosis, and targeted immune recruitment. Moreover, antibody drug conjugates (ADCs) that combine chemotherapeutic cytotoxicity with antibody-mediated tumor specificity even increase the diversity of potential modes of action.[1] Thus, the functional characterization during early drug development requires sensitive cell-based high-throughput assays that address this complexity and measure multiple cellular parameters.[2] One of the major modes of action of therapeutic antibodies is based on inhibition of target cell growth by, for example, blocking growth signaling pathways in cancer cells.[3] For assessing the antiproliferative potency of such candidates, several methods have been described.[4] A simple approach to quantify the number of cells that survive treatment consists of automated cell counting.[5] However, a significant proportion of remaining cells is likely to have entered apoptosis or cell cycle arrest, leading to an overestimation of the proliferating cell population. A more precise approximation of proliferation can be achieved by detecting metabolic activity in viable cells and thus excluding apoptotic cells. Compounds such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) are converted to a colored product by NAD(P)H-dependent cellular oxidoreductases, providing a quantifiable measure for metabolic activity.[6] An alternative approach to assess viability is the detection of intracellular adenosine triphosphate (ATP), which is maintained only at high levels in metabolically active cells and declines rapidly upon cell death or apoptosis. The release of intracellular ATP and its

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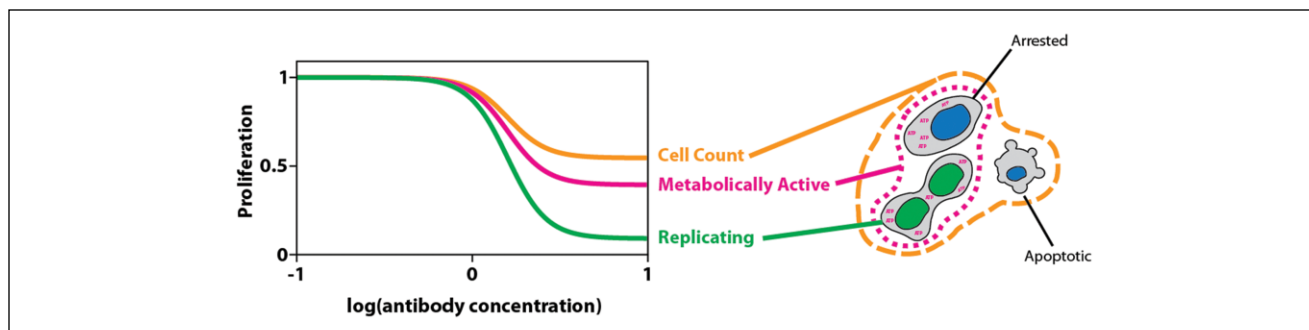Email: helma@biologie.uni-münchen.de

Figure 1. Addressing proliferation at different layers. Antiproliferative antibodies interfere with a cell's ability to replicate. Directly, detecting replicating cells (green) allows for the largest separation between maximal and minimal number of affected cells. Indirectly, restrained DNA replication also reduces the amount of metabolically active cells and the total number of cells remaining after treatment. However, the detection of metabolically active cells (magenta) includes arrested cells, resulting in an overestimation of proliferating cells. This effect is even more drastic when further generalizing the detection to all remaining cells (orange), which also includes apoptotic cells.

detection via ATP-dependent luciferase activity is widely used in proliferation assays.[7]

However, cells that have undergone cell cycle arrest are still metabolically active and consequently not distinguishable from proliferating cells by above-described assays. A major characteristic of proliferating cells is the replication of DNA during S phase. Thus, the incorporation of nucleotide analogues such as 5-bromo-2′-deoxyuridine (BrdU) into chromosomal DNA during replication allows for the distinction between proliferating and arrested cells. BrdU can be detected by antibodies and thus may be implemented with highly sensitive enzyme-linked immunosorbent assay (ELISA)–based multiwell assays.[8] It has been shown that a wider separation between signals from treated and untreated samples (signal-to-background [S/B] ratio) can be achieved with BrdU incorporation compared with assays detecting metabolic activity.[9] 5-Ethynyl-2′-deoxyuridine (EdU), an alternative nucleotide analogue, enables a simpler, milder, and more efficient detection via copper-catalyzed azide alkyne cycloaddition (CuAAC) of fluorescent dyes, such as 6-FAM–azide. The use of EdU coupled to fluorescent dyes simplifies the assay procedure and in addition improves compatibility with other nuclear stains such as 4′,6-diamidino-2-phenylindole (DAPI), thus representing the method of choice for sensitive microscopy-based detection of proliferation.

Accurate distinction between proliferating and nonproliferating cells improves the sensitivity of an antiproliferative potency assay (**Fig. 1**). Changing the mode of signal detection, on one hand, can further improve sensitivity but also provide additional information about the antiproliferative effect. Plate reader–based readouts are commonly used in screening experiments to validate lead candidates and produce statistically relevant data. Commonly used colorimetric multiwell proliferation assays are restricted to single-course parameters such as mean metabolic activity per well. To better understand the mode of action underlying an antiproliferative effect, cellular or subcellular information on signal localization and intensity may prove useful, which is usually not accessible with plate reader systems. Fluorescence microscopy is the method of choice to gain information about single cells with a variety of microscopic high-content screening (HCS) platforms developed in recent years that allow for automated image acquisition and analysis in a high-throughput manner.[10]

In the present study, we describe a simple and sensitive microscopic high-content assay for the quantification and characterization of the antiproliferative potency of therapeutic antibodies. The quantification of replicating cells, via EdU incorporation, as a measure for proliferation allows for most sensitive distinction between proliferating and nonproliferating cells. In addition to quantifying the antiproliferative potency of a monoclonal antibody (mAb), the mode of action can be investigated in the course of the same experiment. For example, potential induction of cell cycle arrest can be studied by cell cycle profiling based on nuclear DNA content quantification.

Materials and Methods

Cell Lines and Cell Culture

Antibodies were produced in FreeStyle HEK 293-F cells (Thermo Fisher Scientific, Waltham, MA, USA) cultured in FreeStyle 293 Expression Medium and maintained at cell densities from 3×10^5 to 3×10^6 cells/mL in a shaker flask at 37 °C, 5% CO_2, shaking at 120 rpm.

HER2 overexpression cell lines BT474 (ATCC HTB20) and SKBR3 (ATCC HTB30) and a control cell line with neglectable HER2 expression levels (1000-fold less than SKBR3), MDA-MB-468 (ATCC HTB-132), were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 + Gibco Glutamax-I (Thermo Fisher Scientific, Waltham,

MA, USA) supplemented with 10% fetal calf serum (FCS) at 37 °C, 5% CO_2.

Protein Expression and Purification

Trastuzumab was expressed in FreeStyle HEK 293-F cells as described previously from the pVITRO1-trastuzumab-IgG1/κ vector (Addgene plasmid 61883; Addgene, Cambridge, MA, USA).[11]

Antibody purification from cleared and sterile filtered cell culture supernatants was performed with an Äkta purifier system equipped with a 1-mL HiTrap Protein A HP column (GE Healthcare, Piscataway, NJ). The system was operated with a constant flow rate of 1 mL/min. After sample application, the column was washed with 10 column volumes (CVs) of wash buffer (20 mM phosphate buffer, 150 mM NaCl, pH 7.3). Bound antibody was eluted with a one-step pH decrease to 3.0 (10 mM Na-citrate buffer, pH 3.0). Eluted fractions of size 0.2 mL or 0.5 mL were collected followed by immediate neutralization of the pH with one-third volume 1 M Tris HCl, pH 8.0. Peak fractions were pooled and concentrated using an Amicon Ultra 4-mL Centrifugal Filter NMWL 10 kDa (Merck Millipore, Billerica, MA, USA) and stored at 4 °C or snap frozen in liquid nitrogen and transferred to −80 °C for long-term storage.

Antibody Treatment, EdU Incorporation, and Nuclear Staining

In total, 1×10^4 cells were seeded in each well of a 96-well optical cell culture plate supplemented with 100 μL culture media. To ensure proper attachment, cells were incubated for 4 h prior to addition of antibody. The 1:3 serial dilutions of trastuzumab in culture media were performed at threefold the desired final concentration, ranging from 50 nM to 0 nM. Then, 50 μL of each dilution was added in triplicates to individual wells. Cells were incubated with antibody for 4 days followed by the addition of EdU to a final concentration of 10 μM. To guarantee labeling of all proliferating cells, EdU treatment was done for 20 h followed by fixation of cells in phosphate-buffered saline (PBS) + 4% paraformaldehyde (PFA), permeabilization in PBS + 0.5% Triton X-100, and blocking of the well surface with PBT (PBS, 2% BSA, and 0.02% Tween 20). EdU was labeled via CuAAC by the addition of 30 μL of staining reagent (4 mM $CuSO_4$, 20 μM 6-FAM–azide, 50 μM Na-ascorbate in 100 mM Tris/HCl, pH 7.0) per well and incubated for 30 min at room temperature. Remaining unconjugated dye was removed by washing three times with 100 μL PBST (PBS + 0.02% Tween 20). Then, 100 ng/mL DAPI in PBST was added for 10 min at room temperature to counterstain nuclear DNA, followed by three washing steps with PBST and one additional wash with ddH$_2$O.

Image Acquisition and Data Analysis

Images were acquired with an Operetta High-Content Imaging system (PerkinElmer, Waltham, MA, USA) equipped with a 40× high NA objective. The 380/40-nm excitation and 410- to 480-nm emission filters were used to image DAPI, and the 475/30-nm excitation and 500- to 550-nm emission filters were used to image 6-FAM–EdU.

DAPI images were used to segment and count the total number of nuclei for each well, representing the total cell count. Each antibody concentration was tested in technical triplicates. Total cell counts of triplicates were averaged and normalized to the cell count of an untreated control (c(trastuzumab) = 0). Averaged and normalized cell counts were plotted against $\log_{10}$-transformed antibody concentrations. Fitting a nonlinear four-parametric model equation

$$y = \min + \frac{\max - \min}{1 + \left(\dfrac{IC_{50}}{x}\right) \text{Hill slope}}$$ to the normalized cell

counts y and antibody concentration x yielded inhibition curves with the descriptive parameters IC_{50} (concentration of half-maximal inhibition) and Hill slope.

Relative nucleic DNA quantities were obtained by calculating total DAPI intensities of segmented nuclei. Absolute DAPI intensities per nucleus were subgrouped by binning and plotted as a probability histogram to analyze probability distributions. A 10-parameter model function $p_x = P(x) = G_1(x) + G_2(x) + S(x)$, comprising the sum of two Gaussian terms $G_1(x) = a_{G1} * exp(-\dfrac{1}{2}\dfrac{(x - \mu_{G1})^2}{\sigma_{G1}^2})$ and

$G_2(x) = a_{G2} * exp(-\dfrac{1}{2}\dfrac{(x - \mu_{G2})^2}{\sigma_{G2}^2})$ representing G1 and G2/M cell cycle phases, and a constant term with Gaussian fadeout,

$$S(x) = \begin{cases} h * exp(-\dfrac{1}{2}\dfrac{(x - x_{lower})^2}{\sigma_S^2}) & x < x_{lower} \\ h * exp(-\dfrac{1}{2}\dfrac{(x - x_{upper})^2}{\sigma_S^2}) & x > x_{upper}, \\ h & else \end{cases}$$

modeling S phase, were fitted to the DAPI intensity probability densities p_x and histogram bin centers x to model the DNA content distribution throughout the cell cycle. The function was fitted by globally minimizing the squared error via simulated annealing using the GenSA package in R. By integrating over the respective term of the derived fit equation representing the G1, S, or G2/M phase, the relative proportion of each phase of the whole cell population was calculated—for example,

$$P(\text{G1}) = \frac{\int_{-\infty}^{\infty} G_1(x)}{\int_{-\infty}^{\infty} P(x)}.$$

Based on 6-FAM–EdU signal, nuclei were classified as proliferating or nonproliferating. Data averaging, normalization, and curve fitting were done in a similar manner as described above for total cell counts.

All image processing was performed with the Harmony software (PerkinElmer); data analysis and curve fitting were done in MATLAB and R (2016, https://www.R-project.org). The R script used for the estimation of cell cycle distributions from DAPI intensity distributions is available at https://github.com/hoerldavid/CellCycleFit.

Results and Discussion

In the field of biologics, therapeutic antibodies have emerged as an especially promising drug format over the past years.[2] A role model for this class of drugs is trastuzumab, which binds the extracellular domain of the HER2 cell surface receptor. In a subset of breast cancers, the growth factor receptor HER2 is overexpressed and mediates increased proliferation.[12] Trastuzumab counteracts this accelerated growth by reducing HER2-mediated signaling and therefore acting as an antiproliferative drug on HER2-overexpressing cells.[13] To assess the antiproliferative potency of a therapeutic antibody, cells are subjected to a range of antibody concentrations. Higher antibody concentrations are expected to lead to lower numbers of viable cells and an even more pronounced decrease in proliferating cells (**Fig. 1**).

In the described assay, HER2-overexpressing cells (BT474 and SKBR3) and control cells (MDA-MB-468) were supplemented with EdU after 4 days of trastuzumab treatment. The proliferating fraction of the cell population incorporates EdU molecules into newly synthesized DNA during S phase. Surviving cells are stained with DAPI, whereas the incorporated EdU is labeled by CuAAC-mediated coupling of the fluorescent dye 6-FAM–azide. Imaging of stained cells on an Operetta system facilitates the detection and segmentation of nuclei, DNA content analysis using the DAPI signal, and definition of the proliferation status according to the EdU signal. Testing multiple antibodies over a range of concentrations is conveniently done in a multiwell tissue culture plate, which is compatible with the Operetta HCS imaging system. With this setup, an inhibition curve with 10 data points as technical triplicates can easily be generated for two individual antibodies in a 96-well format. Quantification of counted nuclei and detected proliferating cells can readily be done with the built-in software package of the Operetta system (Harmony), whereas statistical analysis and curve fitting are conveniently handled with respective MATLAB toolboxes.

Besides the quantification of total cell counts and proliferating cells, the relative intensities of the DAPI and/or EdU signal per nucleus provide additional information with regard to cell cycle phase distributions.

Cell Survival and Cell Cycle Progression

Treatment of HER2-overexpressing cell lines with trastuzumab leads to a reduction in cell growth, but BT474 cells have been reported to be more susceptible than SKBR3 cells.[14] After 4 days of treatment, fluorescence microscopy of DAPI-stained nuclei indicates a clear reduction in cells with increasing concentrations of trastuzumab for BT474 (**Fig. 2A**) as well as SKBR3 cells. Next, we performed high-content image analysis by nuclei segmentation and subsequent quantification of surviving cells as a function of antibody concentration. By fitting a four-parametric nonlinear model to the obtained data points, we calculated inhibition curves. These fits revealed a decrease in total cell number with increasing antibody concentration and S/B ratios lower than 3 for BT474 (**Fig. 2B**) and SKBR3 (**Fig. 2C**). The maximal induction of cell death is 64% with a concentration of half maximal inhibition (IC_{50}) of 1.8 nM for BT474 cells and 65% with an IC_{50} value of 1.9 nM for SKBR3 cells. The low S/B values can be explained by the specific mode of action mediated by trastuzumab, decelerating cell proliferation rather than actively promoting cell death.[14] Therefore, cells that have already passed G1 phase will further progress in cell cycle. With BT474 and SKBR3 cells exhibiting long doubling times (2–3 days), S/B ratios greater than 4 (two doublings) are not to be expected in the time course of the assay, which holds also true for other assays merely detecting survival or viability.[9] Moreover, a very low Hill slope could be observed for SKBR3 cells compared with BT474, which is linked to the lower susceptibility of SKBR3 to trastuzumab.[9,14] Consistently, an unsusceptible cell line (MDA-MB-468) showed no difference in the number of viable cells between treated and untreated conditions (**Fig. 2B,C**). These results indicate that exclusively measuring cell survival is limiting the S/B ratio of proliferation assays, since arrested cells, which are still metabolically active, cannot be distinguished from proliferating cells.

High-content image analysis of DAPI-stained nuclei allows not only segmentation and quantification of nuclei but also the measurement of relative nuclear DNA contents. Since the amount of chromosomal DNA doubles through S phase from G1 to G2 phase, the absolute DAPI signal per nucleus can be used to analyze changes in cell cycle distributions. In this line, we generated frequency histograms of the absolute DAPI intensity per nucleus (**Fig. 3A** and **Suppl. Fig. S1**). Fitting a three-term model function to the data allowed us to determine the proportion of cells within each cell cycle phase (**Fig. 3B**). SKBR3 cells exhibited a clear change in cell cycle
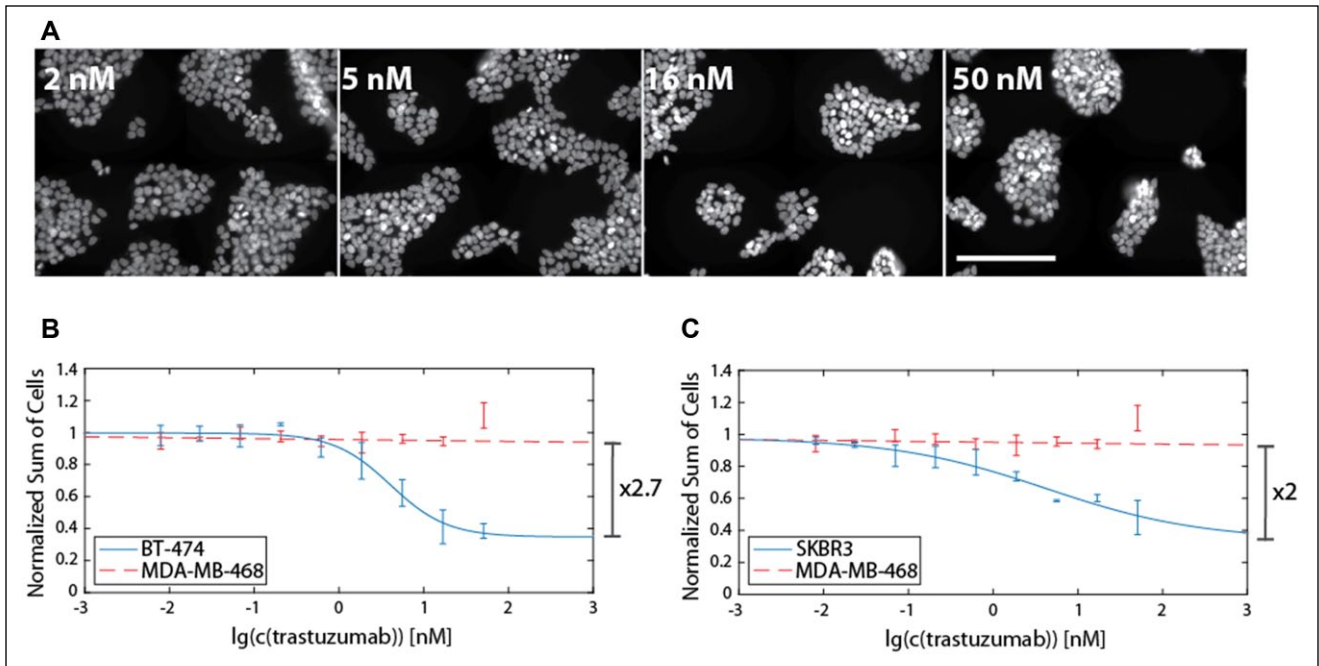
Figure 2. Quantification of antiproliferative potency by counting nuclei of surviving cells. 4′,6-Diamidino-2-phenylindole (DAPI)–stained nuclei were imaged with an Operetta high-content screening (HCS) system. Representative images of BT474 cells for four different antibody concentrations are shown in (**A**). Scale bar represents 100 μm. The observed decrease in surviving cells was quantified from technical triplicates for nine individual antibody concentrations (0.008–50 nM) and an untreated control. Averaged triplicates normalized to untreated control were plotted against $\log_{10}$-transformed trastuzumab concentrations for BT474 (**B**) and SKBR3 (**C**) and fitted to a four-parametric inhibition curve model equation (solid lines). Proliferation of a negative control cell line, MDA-MB-468, was unaffected by trastuzumab treatment (dashed line). The maximal difference in the number of surviving cells was 2.7-fold for BT474 as well as for SKBR3 cells.
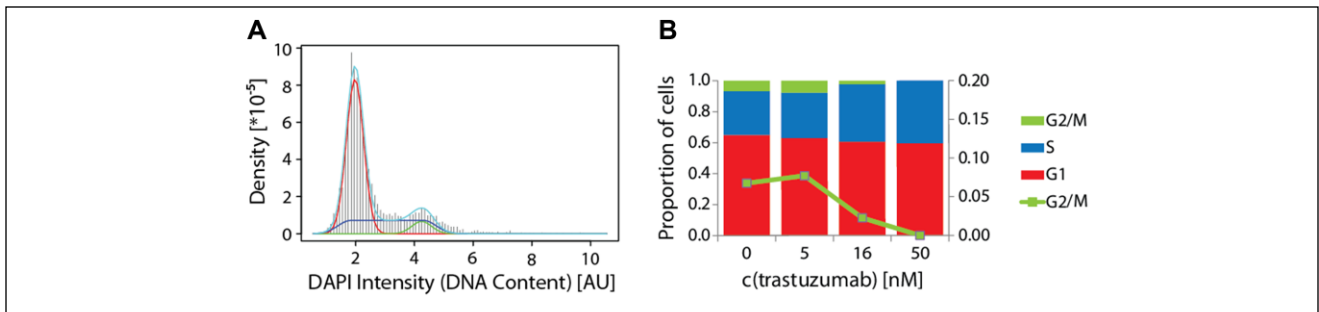

Figure 3. Shift in cell cycle distribution of trastuzumab-treated SKBR3 cells. Nuclear 4′,6-diamidino-2-phenylindole (DAPI) intensities were analyzed to categorize cells into cell cycle phases according to their relative DNA content. Probability density histograms of DAPI intensities were used to fit a model equation to the observed distribution. An exemplary histogram for c(trastuzumab) = 16 nM is given in (**A**) with the fitted curve in cyan and respective cell cycle phase terms in red (G1), blue (S), and green (G2/M). Integration over the individual terms yields the proportion of cells in each cell cycle phase treated with different trastuzumab concentrations (**B**). High concentrations of trastuzumab lead to a reduction in the G2/M phase proportion, indicating cell cycle arrest.

profiles upon trastuzumab treatment. The quantification of these data shows a decrease in the G2 phase population with increasing antibody concentration, which suggests an arrest in either G1 or S phase. This is consistent with the proposed G1 arrest induced by trastuzumab.[15]

Cell cycle profiles are an additional readout of the described assay and provide supplementary information about the mode of action of an antiproliferative antibody. Investigation of potency and mode of action in a single experiment was facilitated by increasing resolution to the single-cell level combined with high-throughput sample and data handling implemented in HCS systems. Cell cycle analysis of the less susceptible SKBR3 cell line showed that we are able to analyze an antibody's mode of action even if the overall
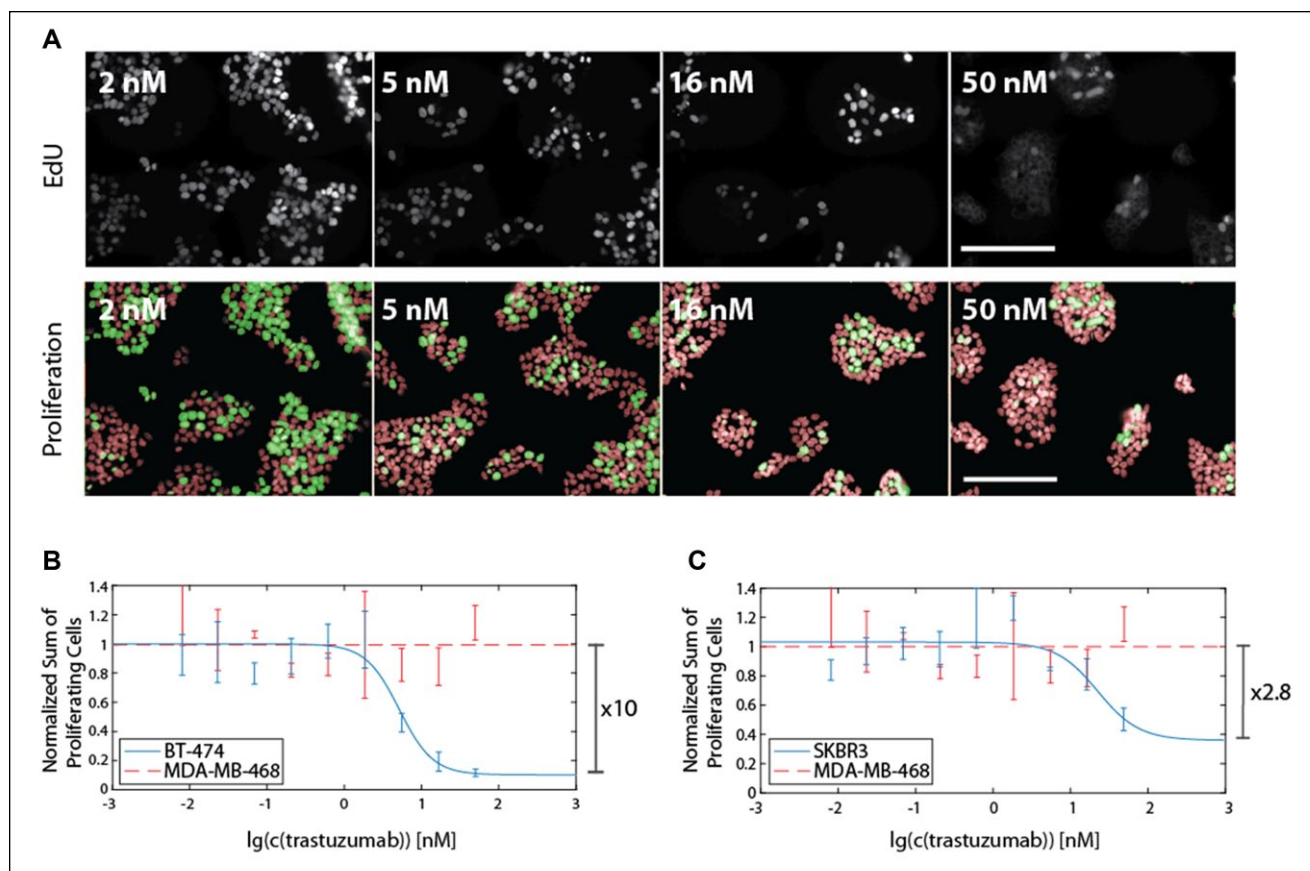
Figure 4. Improving assay sensitivity by detecting proliferating cells via 5-ethynyl-2′-deoxyuridine (EdU) incorporation. EdU, incorporated into chromosomal DNA during replication, was labeled by copper-catalyzed azide alkyne cycloaddition (CuAAC) with 6-FAM and imaged with an Operetta high-content screening (HCS) system. Representative images of BT474 cells are shown in (**A**). Scale bar represents 100 μm. Segmented nuclei from **Figure 2A** were classified as proliferating (green) or nonproliferating (red) based on EdU signal presence. It is clearly visible that only a small fraction of all surviving cells is still proliferating at high antibody concentrations. Results of quantification of proliferating cells and data fitting similar to data in **Figure 2** are shown for BT474 cells (**B**) and SKBR3 cells (**C**). The signal to background (S/B) ratio could be greatly improved for BT474 cells from 2.7 to 10 compared with surviving cell quantification (**Fig. 2**). SKBR3 cells exhibit an S/B ratio of 2.8, which is comparable to the value derived from 4′,6-diamidino-2-phenylindole (DAPI)–based quantification of surviving cells (2.7).

antiproliferative effect is weak. Nevertheless, it is also desirable to detect this weak proliferation inhibition with greater resolution. To address this need, we chose EdU incorporation for sensitive detection of proliferating cells.

Increased Assay Sensitivity via Quantification of EdU Incorporating Cells

Since DNA replication is a major characteristic of proliferation, we decided to use EdU incorporation as a marker for proliferating cells. Labeling EdU with a fluorescent dye allowed the distinction between proliferating and nonproliferating cells by fluorescence microscopy. Automated quantification of EdU-positive cells increased the S/B ratio to 10 for treated versus untreated BT474 cells (**Fig. 4B**). A concentration of half maximal inhibition (IC_{50}) of 4.9 nM was obtained from the fitted inhibition curve, whereas the

maximal induction of proliferation inhibition was 90%. For SKBR3 cells, we observed a maximal induction of proliferation inhibition of 64% and IC_{50} of 3.9 nM. To ensure that the detected inhibition of proliferation was due to trastuzumab-mediated effects, we subjected a control cell line, MDA-MB-468, to the same treatment. As expected, we could not observe any difference in the proliferating fraction upon addition of trastuzumab (**Fig. 4B,C**). We could show that EdU incorporation-based detection of proliferating cells by microscopy greatly increases the S/B ratio compared with detecting surviving cells and improves the inhibition curve parameters such as Hill slope in the case of SKBR3 (**Fig. 4C**). A 10-fold change in proliferation has recently also been demonstrated with a DELFIA-BrdU–based assay.[9] However, the assay described in the present article uses the more sensitive and mild EdU staining method, provides the possibility for multiplexed readout of

various parameters, and increases the assay resolution by the detection of single cells instead of averaging over a bulk population.

In summary, we could show that EdU-based labeling of proliferating cells with subsequent automated imaging and analysis combined with DAPI-based cell cycle profiling is a simple and sensitive way for parallel investigation of anti-proliferative potency and mode of action of therapeutic antibodies.

Acknowledgments

Declaration of Conflicting Interests

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