Published in final edited form as:

Anal Biochem. 2010 June 15; 401(2): 173–181. doi:10.1016/j.ab.2010.02.015.

A Differential Cell Capture Assay for Evaluating Antibody Interactions with Cell Surface Targets

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Abstract

Many biological and biomedical laboratory assays require the use of antibodies and antibody fragments that strongly bind to their cell-surface targets. Conventional binding assays such as the enzyme-linked immunosorbent assay (ELISA) and flow cytometry have many challenges, including capital equipment requirements, labor intensiveness, and large reagent and sample consumption. Although these techniques are successful in mainstream biology, there is an unmet need for a tool to quickly ascertain the relative binding capabilities of antibodies/antibody fragments to cell-surface targets on the bench top at low cost. We describe a novel cell capture assay that enables several candidate antibodies to be evaluated quickly as to their relative binding efficacies to their cell-surface targets. We used chimeric rituximab and murine anti-CD20 monoclonal antibodies as cell capture agents on a functionalized microscope slide surface to assess their relative binding affinities based on how well they capture CD20-expressing mammalian cells. We found that these antibodies' concentration-dependent cell capture profiles correlate with their relative binding affinities. A key observation of this assay involved understanding how differences in capture surfaces affect the assay results. This approach can find utility when an antibody or antibody fragment against a known cell line needs to be selected for targeting studies.

Keywords

antibody; biotin; CD20; cell capture; ELISA; fluorescence microscopy; flow cytometry; mammalian cell; streptavidin

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INTRODUCTION

The engineering and production of intact antibodies, fragments, and conjugates has become an important area of therapeutics and diagnostics development [1-4]. The broad use of antibodies as reagents for a variety of *in vitro* and *in vivo* applications, alongside the widespread use of genetically modified cell lines for studying an extensive range of biological phenomena, has led to the development of antibody libraries against a diversity of cell surface markers [5-9]. This in turn has led to the experimental requirement of quickly selecting a strongly binding antibody against a known cell type or a cell-surface target, whether it is for cell characterization [10, 11], tissue staining [12], or *in vivo* imaging [13, 14].

The cell-based enzyme-linked immunosorbent assay (ELISA) has historically been the prominent technique used to determine whether a particular antibody or select group of antibodies is a strong binder against specific cell lines [15-18]. More recently, due to its considerable multiplexing characteristics, flow cytometry has become the preferred technique for selecting antibodies on the basis of binding affinity [19-22]. In instances where a cell-surface antigen can be recombinantly expressed while retaining antibody recognition, surface plasmon resonance (SPR), a powerful, high-throughput and quantitative technique [23, 24], is also used.

Although these techniques are understood and lead to well-defined analysis, they are labor- and reagent-intensive and can require significant investments in equipment. By taking advantage of some of the attributes and incorporating specific benefits of ELISA and flow cytometry, we have developed an inexpensive, reagent efficient cell capture assay that enables statistical comparisons of the binding selectivity and specificity of different antibodies against a cell surface target (Fig. 1). Microfluidic and other low-volume cell capture techniques have proven useful as ways of isolating circulating tumor cells or leukocyte populations from the blood for diagnostic purposes [25-27]. Additionally, cell capture using microarray technology has been used to spatially separate specific types of immune cells and to induce or measure distinct cellular responses to antibody or peptide-MHC complexes [28, 29]. In all cases, cells are typically captured via characteristic cell-surface protein markers that are recognized by antibodies immobilized on a substrate. These approaches are often employed to preserve cell viability, as the cells are sometimes studied after capture for signal transduction analysis [29], drug perturbation studies, or “small systems biology” pathway analysis purposes [30].

We believe that sensitive cell capture assays have many applications beyond the capture of novel cells or cell sorting and can be employed to study the interactions between cells and the capture agent. In the present work, we use immobilized antibodies for cell capture to compare three candidate binders against a well-characterized cell line. Cell-capture efficiency – the captured cell density in a given area – across an antibody concentration range is used as an approximate measure of binding selectivity and specificity. Other capture-based single cell techniques have been reported to efficiently capture cells with as few as 2,000 antigen molecules per cell [27]. It is therefore reasonable to consider capture

assays as a means of sensitively comparing the relative capture efficacies of different antibodies under specific and characterized immobilization conditions.

We have explored this concept by capturing CD20-expressing mammalian cells with two biotinylated anti-CD20 monoclonal antibodies (mAbs): chimeric (mouse/human) rituximab (Rituxan®, Genentech) and a commercially available murine anti-CD20 mAb. Both antibodies demonstrate nanomolar dissociation constants with their cell-surface antigen, as measured using flow cytometry (see Figure 5). CD20-expressing Daudi B lymphoblast cells were used in these cell capture experiments, and Jurkat T lymphocytes were used as a negative control. We demonstrated that concentration-dependent capture of Daudi cells by both antibodies generated cell capture density curves with differing cell capture maxima, corresponding to their relative binding affinities. In this work, we describe this novel single-cell resolution assay that is executed on the bench top with few reagents, processing steps, and negligible capital equipment beyond the ability to count cells. The small number of processing steps in particular and the fact that cells are not immobilized prior to read-out significantly increases the reproducibility of this assay for non-adherent cells and offers a performance advantage compared to cell-based ELISA.

MATERIALS AND METHODS

Antibodies, cell lines and cell culture

The antibodies used for cell capture assays were rituximab (Rituxan®, Genentech Inc., South San Francisco, CA), murine anti-CD20 mAb, and biotinylated murine anti-CD20 mAb (both from Abcam Inc., Cambridge, MA, Catalog #'s ab10326 and ab27579, respectively). Daudi B lymphoblasts and Jurkat T lymphocytes (both from ATCC, Manassas, VA, ATCC #'s CCL-213 and TIB-152, respectively) were cultured in 1× RPMI 1640 medium (Mediatech, Inc., Manassas, VA) supplemented with 15% fetal bovine serum (FBS) (Lonza, Basel, Switzerland), 1% Penicillin/Streptomycin (Mediatech, Inc.), and 10 mM HEPES (Omega Scientific, Inc., Tarzana, CA). Alkaline-phosphatase (AP) conjugated avidin was purchased from Pierce (Rockford, IL). R-Phycoerythrin (PE) conjugated streptavidin was purchased from Sigma-Aldrich (St. Louis, MO); fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG AffiniPure F(ab')₂ fragment (Fc_γ-specific) and FITC-conjugated goat anti-human IgG AffiniPure IgG (Fc_γ-specific) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Biotin conjugation

Both rituximab and murine anti-CD20 mAb were biotinylated using an EZ-Link Micro Sulfo-NHS-LC-Biotinylation Kit (Pierce) according to the manufacturer's recommendations. Reactions were performed in phosphate buffered saline (PBS) for 2 hours on ice with a 50-molar excess of biotinylating reagent. Excess biotin was then removed using Zeba Desalt Spin Columns (Pierce). Final protein concentrations were calculated based on A_{280 nm} measurements with a molar extinction coefficient of 210,000 M⁻¹cm⁻¹.

Gel electrophoresis and Western blot

Murine anti-CD20 mAb, commercially biotinylated murine anti-CD20 mAb, rituximab, and biotinylated rituximab were analyzed by SDS-PAGE (under non-reducing conditions) and Western blot. The gel was stained with InstantBlue (Expedeon, Cambridgeshire, UK). For the Western blot, the nitrocellulose membrane was stained with avidin-AP and color was developed using the AP substrates NBT and BCIP (Promega, Madison, WI) in AP buffer (100 mM Tris-HCl at pH 9.6, 100 mM NaCl, and 5 mM MgCl₂).

Mass spectrometry

Mass spectrometry samples were prepared by: 1) desalting five antibodies (rituximab, biotinylated rituximab, murine anti-CD20 mAb, murine anti-CD20 mAb purchased as a biotin conjugate, and murine anti-CD20 mAb that was biotinylated “in lab”); 2) buffer exchanging with MilliQ water; and 3) concentrating down to ~30-50 µL using a Microcon ultrafiltration device (MilliPore, Billerica, MA). Samples were directly spotted (0.3 µL sample plus 0.5 µL matrix solution) and analyzed by matrix-assisted laser desorption ionization (MALDI) coupled to delayed extraction time-of-flight (TOF) mass spectrometry in the reflector mode (Voyager DE STR, Applied Biosystems). The matrix was α-cyano-4-hydroxycinnamic acid (10 mg/ml of solution in 30/70/0.1 water/acetonitrile/trifluoroacetic acid). Average biotinylation levels were computed from the relative mass differences between biotinylated and non-biotinylated antibodies. Calculated biotinylation levels were confirmed using an EZ Biotin Quantitation Kit (Pierce).

Flow cytometry

Flow cytometry was employed to confirm the CD20 antigen positive and negative cell lines, i.e. Daudi and Jurkat cells, respectively, and to measure relative binding affinities for biotinylated rituximab and biotinylated murine anti-CD20 mAb. Cells were incubated with the antibody of interest in PBS/1% FBS at 4°C for 1 h. The cells were washed and subsequently incubated with a secondary antibody (or Streptavidin-PE) in PBS/1% FBS (1 : 200 dilution) for 1 h. Antibody binding was quantified using a modified FACScan analytic flow cytometer (Becton Dickinson, UK), and data were analyzed using CellQuest software (BD).

Cell capture assay

At least two hours prior to performing a cell capture experiment, a streptavidin substrate slide (ArrayIt, TeleChem International, Inc., Sunnyvale, CA) was stamped with hydrophobic circles of 2.5 mm radius using an ImmEdge Hydrophobic Barrier pen (Vector Laboratories, Burlingame, CA). The substrate slide was then stored at 4°C until needed for the experiment to allow the circles to dry (at least 2 h).

In each circle, 10 µL of biotinylated antibody was added. The substrate slide was placed in a Petri dish with water surrounding it to maintain humidity. This preparation was incubated at 37°C for 30 to 45 min. After antibody coating, the slide was washed 4-5 times with PBS, and 10 µL commercial blocking buffer (ArrayIt, TeleChem International, Inc.) was added to each spot. The slide was placed back in the Petri dish and incubated at room temperature for 1 h. Following 4-5 washes with PBS, the area surrounding the spots was dried to a thin

residue, and the slide was then either immersed in 10-11 mL of a cell suspension with 10^6 cells/mL or the cell suspension was spotted directly into the circles (10 μ L/circle). The slide was incubated with cells in the Petri dish at 37°C for 45 min before gently washing the slide 4-5 times with PBS. The area surrounding the spots was dried, and 10 μ L per spot of 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) was applied prior to placing the slide in the Petri dish and incubating at room temperature for 15 min. After this step, the slide was washed 1-2 times with PBS, and 10 μ L of a 1 μ g/mL DAPI staining solution (Invitrogen) was added to each spot, followed by incubation at room temperature for 2 min. After 2 washes with PBS, a coverslip was placed on the slide, and the spots were imaged using a Nikon TE2000 fluorescence microscope (Nikon Inc., Melville, NY). MetaMorph software (Molecular Devices, Downingtown, PA) was used to count the nuclei in each DAPI image, and bright field images were used to confirm the cell counts.

Empirical evaluation of potential biotinylation sites

An intact human IgG1 (Protein Data Bank accession code 1HZH) and murine IgG2a (Protein Data Bank accession code 1IGT), the subclasses of rituximab and murine anti-CD20 mAb, respectively, were rendered in PyMOL Molecular Viewer (www.pymol.org) with the lysine residues in the constant domains selected for visualization. The coordinate geometries of these antibodies were transferred to MATLAB R2009 (MathWorks), where centroid and eigenvector data were calculated to determine the PyMOL viewing coordinates to superimpose the two sets of residues. The domains were visually compared to determine the likelihood of off-axis orientations on the streptavidin substrate based on the locations of potentially biotinylated lysines (see Figure 8).

RESULTS

Biochemical characterization of antibodies

Initially, we compared two biotinylated anti-CD20 antibodies: in-lab biotinylated rituximab and a commercially available biotinylated murine anti-CD20 mAb. For the lab biotinylation, a kit that modifies the ϵ -amino group of exposed lysine residues with biotin molecules was used. The purity of the antibodies was assessed by SDS-PAGE (Fig. 2a). Following Coomassie staining, all antibodies (biotinylated and non-biotinylated) exhibited similar purities, with the major bands around ~150,000 Da, as expected for an intact antibody under non-reducing conditions. The biotinylation of rituximab and the anti-CD20 mAb was analyzed by Western blot where biotin was detected (Fig. 2b). The biotinylated antibodies showed distinct bands following staining with avidin-AP (lanes 1 and 3), and there was no staining of the unbiotinylated antibodies (lanes 2 and 4).

To determine the extent of biotinylation, MALDI-TOF mass spectrometry was performed on the four antibodies described above in addition to our own in-lab biotinylated murine anti-CD20 mAb (Fig. 3). The in-lab biotinylated murine anti-CD20 mAb was used as a biotinylation control to assess the reproducibility of the in-lab biotinylation procedure and to compare the cell capture efficiencies of both rituximab and murine anti-CD20 mAb when subjected to the same biotinylation procedure. The calculated biotinylation levels are given in Table 1. The two in-lab biotinylated antibodies exhibit comparable levels of biotinylation

(1.1 – 2.4 biotins on average per antibody), whereas the commercial biotinylated murine anti-CD20 mAb contains 7.4 biotin molecules on average per antibody. This is consistent with the technical specifications from the manufacturer, which claims between 4 and 7 biotin molecules per antibody.

Cell line characterization

Prior to performing cell capture experiments, flow cytometry was conducted to confirm binding of the biotinylated antibodies as well as CD20 expression on the Daudi B cells and non-expression on the Jurkat T cells. The flow scatter plots (Fig. 4) demonstrate antibody binding to the Daudi cells only, confirming that they are markedly positive for CD20 expression, whereas the Jurkat cells are negative for CD20 expression. This experiment confirms the suitability of Daudi and Jurkat cells as positive and negative controls in a differential binding assay for the CD20 target.

We also sought to quantitate the relative binding affinities of the in-lab biotinylated rituximab and the commercially available murine anti-CD20 biotin conjugate mAb by varying the antibody concentrations from very low to the saturation point at which all antigens are theoretically bound by antibodies, while keeping the cell number constant at each antibody concentration [31-34]. The titration curves (Fig. 5) show that rituximab, despite the presence of biotins on various lysine residues on the antibody surface [35], binds Daudi cells much better than the murine anti-CD20 mAb. The relative binding constants are estimated based on the theoretical antibody concentrations at which half of the cellular epitopes are bound by antibody [31-34]. The relative locations of the curves were confirmed in an experiment using Streptavidin-PE for secondary detection of both antibodies (data not shown). It should be noted that there are no exposed lysine residues in the antigen recognition site of rituximab, and affinity is therefore probably not compromised [31-33]. Indeed, biotinylated rituximab exhibited a relative binding constant of less than 10 nM, which is in the range of previously reported binding constants for the non-biotinylated antibody [34].

Cell capture

Cell capture experiments were performed with in-lab biotinylated rituximab and murine anti-CD20 mAb, as well as the commercially biotinylated murine anti-CD20 mAb. In order to measure the sensitivity of the assay and to confirm concentration-dependent capture, various antibody concentrations were applied to different spots on the streptavidin substrate slide. Additionally, to avoid any biases or edge effects due to spot locations or circle size variations on the substrate slide, each condition was repeated 4 times with random spotting. Cell capture was more homogeneous within the circles when the slide was immersed in the cell suspension as opposed to spotted with the cell suspension, but otherwise the results were comparable when the two techniques were used for cell capture.

DAPI and bright field images of Daudi cells captured by biotinylated rituximab and commercially biotinylated murine anti-CD20 mAb at different concentrations are shown in Fig. 6. The cell capture follows a clear pattern of concentration-dependence with high concentrations corresponding to conditions at which the amount of antibody spotted greatly

exceeds the number of streptavidin molecules in the spot area. Thus, it is expected that cell capture levels should reach a plateau. In addition, at very low antibody concentrations, differences in cell capture yield are too small to make a noticeable difference in the cytometry images used to quantify capture efficacy. Images of the entire spot areas were used to count the total number of cells in each spot. To correct for variations in the circle sizes, the total number of cells in each circle was divided by the actual average circle area. Graphs showing the cell capture densities (following background subtraction) for each antibody and each condition are given in Fig. 7a. The upper plateau and bottom “leveling off” are clearly seen. These graphs also illustrate the cell capture densities for the CD20-negative Jurkat cells. The Jurkat cells were captured at levels equal to or even lower than the negative PBS control condition in which only PBS was applied during the substrate-coating step.

To account for the differences in biotinylation levels between the in-lab biotinylated rituximab and the commercially biotinylated murine anti-CD20 mAb, murine anti-CD20 mAb was also biotinylated in-lab and used in cell capture experiments. MALDI-TOF data confirmed that these two antibodies, when subjected to the same biotinylation protocols, had similar biotinylation levels (Table 1). When this antibody cell capture data set was compared with that of the biotinylated rituximab, a greater cell capture maximum was achieved with biotinylated rituximab (Fig. 7b). This indicates that rituximab is a better capture agent for Daudi cells compared to the murine anti-CD20 mAb, which is consistent with the trend seen in the relative antigen dissociation constants (see Fig. 5). The fact that the commercially biotinylated murine anti-CD20 mAb appears to be a better capture agent than the in-lab biotinylated rituximab suggests that different biotinylation levels and locations of the biotin molecules on the antibodies affect capture efficacy.

DISCUSSION

In this study, we compared two antibodies, one chimeric (mouse/human) and one mouse, against the cell-surface target CD20 using cell capture as a measure of relative binding capability. These biotinylated antibodies were immobilized on a dense streptavidin substrate slide and used as capture agents for CD20-positive Daudi cells and CD20-negative Jurkat cells. Both Daudi and Jurkat cells are non-adherent immune cells, which we thought would be suitable for proof-of-concept experiments, as the issues of cell spreading and non-specific interactions with the streptavidin substrate slide [28] were avoided.

Initially, we characterized the antibodies by SDS-PAGE, Western blot, and MALDI-TOF. We validated our cell lines as target-positive and -negative using flow cytometry. We also used flow cytometry to construct binding curves in order to measure the relative binding affinities of the two antibodies. The relative binding constant of the biotinylated rituximab appeared to be between 5 and 10 nM, which is in accordance with the reported literature values [36,37]. The commercially biotinylated murine anti-CD20 mAb has a noticeably lower relative binding affinity (higher dissociation constant based on the binding curve). We expected this result, as rituximab is a clinically available drug that was optimized to bind to its target cancer cells. This type of rigorous affinity maturation is likely unnecessary for laboratory-grade commercial non-human antibodies, such as the murine anti-CD20 mAb,

which do not require as high of a binding affinity to elicit signal in flow cytometry and Western blotting. Despite this difference, the relative dissociation constants were both still in the nanomolar range.

The cell capture assay demonstrated a higher maximum cell capture density for the commercially biotinylated murine anti-CD20 mAb compared to that of in-lab biotinylated rituximab. At first glance, this seemingly contradicts the flow cytometry cell binding data. However, Student *t*-tests indicate that for the data points along the maximum plateau on the graphs, there is no statistically significant difference between the two antibodies (data not shown).

In order to fully compare the relative binding affinities of the two antibodies by the cell capture assay, we believe that the extent of biotinylation and, more importantly, the location of the biotins should be comparable on the different capture agents. Once we established differential cell capture based on the capturing antibodies, we sought to further explore how biotin levels and locations affect cell capture efficacies. The location of biotins on the antibody surface has been known to control antibody orientation on streptavidin-coated substrates [35]. The binding constant of the rituximab is probably not affected too drastically by the biotinylation reaction because there are no lysines in the key epitope recognition site [36,37]. However, this does not discount the possibility of nearby lysine residues that can sterically hinder binding. The more important factor in this case is the location of exposed lysine residues (where possible biotinylation can occur) on the areas surrounding the Fab.

In order to compare antibodies with similar biotinylation levels, we performed cell capture experiments with in-lab biotinylated rituximab and murine anti-CD20 mAb. The cell capture results demonstrate that, with comparable biotinylation levels, rituximab is a better capture agent than the murine anti-CD20 mAb (see Figure 7b). This corresponds to the measured relative binding constants and clearly illustrates how similar biotinylation (and distribution of lysine residues) is necessary in order for two antibodies to be objectively compared as cell capture agents (as a proxy for binding) [35,38,39].

We also acknowledge that the distribution of exposed lysine residues on rituximab and murine anti-CD20 mAb likely differs. Rituximab is a chimeric mAb with a human IgG1 Fc constant region, whereas the murine anti-CD20 mAb is a fully murine antibody with a murine IgG2a Fc constant region. Thus, one might suspect differences in the primary and secondary structures of the two antibodies. To model these differences, we tagged the locations of the exposed lysine residues in the 3D structures of the two antibody constant domains (Fig. 8), and despite the different secondary structures, there is fairly good consistency between the antibodies in terms of possible orientation variations through different biotinylation locations. We hypothesize that there is a greater likelihood of off-axis orientations for rituximab based on the spatial distribution of exposed lysines in the human constant domain. This could account for the relatively lower capture efficacy of the biotinylated rituximab when compared to commercially biotinylated murine anti-CD20 mAb, where the additional biotins on the mouse antibody may also contribute to a higher likelihood of an axial orientation.

Here, we have demonstrated the intricacies of a cell capture assay as a means of comparing different antibodies as capture agents. There is no known direct relationship between cell capture efficiency and binding affinity, but this low-cost, bench top assay can at least give preliminary, semi-quantitative information about comparative binding. Cells can be counted manually using a bright field microscope (somewhat arduous) although the process can be streamlined through the use of automated software tools capable of segmenting DAPI-stained nuclei. Further experiments involve creating a library of mAbs with a series of different biotin levels and determining the point at which biotin levels affect cell capture. Additionally, we would like to try to use more antibodies and cell-surface targets. We believe that this assay is a simple, low-cost alternative to flow cytometry and ELISA when relative binding information (or binder selection) is required for an application.

ACKNOWLEDGEMENTS

This study was funded by a UCLA Crump Institute for Molecular Imaging faculty seed grant. We would like to thank Felix B. Salazar, Dr. Tove Olafsen, Dr. Shannon J. Sirk, and Dr. Jing Sun for their help with troubleshooting the ELISA, Western blot, and other biochemical assays used in this study. We are especially grateful to Dr. Julian Whitelegge and Dr. Christopher Ryan from The Pasarow Mass Spectrometry Laboratory, The NPI-Semel Institute, David Geffen School of Medicine, UCLA, for their assistance with mass spectrometry. We are thankful for the flow cytometry resources from the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research Flow Cytometry Core Facility that is supported by National Institutes of Health awards CA-16042 and AI-28697, and by the JCCC, the UCLA AIDS Institute, and the David Geffen School of Medicine at UCLA.

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Fig. 1. Schematic diagram of a cell capture assay used to measure the relative binding affinity of antibodies. A grid is first stamped onto a dense streptavidin substrate slide, after which varying concentrations of biotinylated antibodies are added to different grid elements. Spots are blocked with a blocking buffer containing biotin in order to saturate the streptavidin binding sites. The substrate slide is subsequently incubated with a suspension of cells expressing a particular antigen, after which the cells are fixed and counted using a

microscope. Cell capture by different immobilized antibodies is quantitated and used as a measure of relative binding affinities of the capture agents.

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Fig. 2.
 (A) SDS-PAGE and (B) Western blot results. The four antibodies analyzed were biotinylated rituximab (1), rituximab (2), commercially biotinylated murine anti-CD20 mAb (3), and murine anti-CD20 mAb (4). Antibodies were detected in the Western blot with avidin-AP.

Fig. 3. MALDI-TOF results with rituximab, biotinylated rituximab, murine anti-CD20 mAb, and biotinylated murine anti-CD20 mAb (commercially and in-lab biotinylated). The full mass spectrum of rituximab is shown, along with highlights of the molecular ion peaks from the other spectra. The average extent of biotinylation for each antibody was calculated based on the molecular weights from the corresponding mass spectra. The average of the molecular ion peak, the doubly ionized mass peak, and the triply ionized mass peak was calculated to determine the mass of the antibody.

Fig. 4. Flow cytometry scatter plots of biotinylated rituximab and commercially biotinylated murine anti-CD20 mAb binding to CD20-positive Daudi and CD20-negative Jurkat cells. Fluorescence was detected with Streptavidin-PE. (A) Cells were incubated with no primary antibody and just Streptavidin-PE (negative control). (B) and (C) represent the binding of biotinylated rituximab and biotinylated murine anti-CD20 mAb, respectively. FL2-H, the vertical shift, measures the fluorescence intensity of PE.

Fig. 5. Antibody binding curve of biotinylated rituximab and commercially biotinylated murine anti-CD20 mAb to Daudi cells. The Y-axis represents the average of the mean fluorescence intensity (MFI) as measured by flow cytometry. Binding of biotinylated murine anti-CD20 mAb was detected with FITC-conjugated goat anti-mouse IgG AffiniPure F(ab')₂ fragment (Fc_γ-specific), and binding of biotinylated rituximab was detected with FITC-conjugated goat anti-human IgG AffiniPure IgG (Fc_γ-specific).

Fig. 6.

Microscope images of Daudi cells captured at different concentrations of (A) biotinylated rituximab and (B) murine anti-CD20 mAb (commercially biotinylated). The top (black background) image at each concentration point is a fluorescence microscope image based on a DAPI stain. The bottom is a bright field microscope image. (C) shows two bright field microscope images of Jurkat cell capture with biotinylated rituximab and murine anti-CD20 mAb (commercially biotinylated) at the highest capture concentration (54×10^{-3} mM) of antibody. All images are at $4\times$ magnification ($1 \text{ pixel} = 2.56 \mu\text{m}^2$).

Fig. 7. Cell capture assay results for biotinylated rituximab and murine anti-CD20 mAb (commercially biotinylated) (A) and a comparison of the cell capture profiles of the two biotinylated murine anti-CD20 mAbs (in-lab and commercially biotinylated) (B). Standard deviations in (A) represent the average of four data points from a single experiment. The experiment was repeated three times for validity. The data in (B) was normalized to the biotinylated rituximab curve to demonstrate the difference between the two biotinylated murine anti-CD20 mAbs. Only Daudi cell capture was used in (B). The “Cell Density” parameter represents a measure of the total number of cells captured divided by the average pixel area of the cell capture spots in a digital image of the capture surface.

Fig. 8.

An intact human IgG1 (Protein Data Bank accession code 1HZH) antibody showing solvent surface (A). A surface-rendering of this IgG with all domains visible and the Fc domain rendered for clarity (B). There are 88 lysines in 1HZH, 19 in the CH2/CH3 area that are exposed and possibly biotinylated (see insert) (C). An intact murine IgG2a (Protein Data Bank accession code 1IGT) zoomed in on the Fc domain (D). A demonstration of the 18 lysines in the CH2/CH3 area that are exposed and possibly biotinylated (E). An overlay showing that although there is some structural similarity and a fairly similar number of exposed lysines, there are localized areas (circled) where the ratio of potential biotins may tend to cause stronger off-axis orientations for the human IgG than the mouse IgG (F).

Table 1

Average number of biotin molecules per antibody based on MALDI-TOF molecular weight calculations.

Antibody	Ave. Molecular Weight (Da)	Ave. Number Biotins/Protein
Rituximab	147,414	0
Biotinylated Rituximab	148,011	2.4
Murine anti-CD20 mAb	148,077	0
Murine anti-CD20 mAb (commercial biotinylation)	149,886	7.4
Murine anti-CD20 mAb (lab biotinylation)	148,352	1.1