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Digital Receptor Occupancy Assay in Quantifying On- and Off-Target Binding Affinities of Therapeutic Antibodies

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Author Contributions

C.-K. C. conceived the project; C.-K. C., P.-J. H. and P.-H. T. performed the DRO analysis; C.-T. C. supported the tissue sample collection; C.-K. C., H.-H. L., Y.-N. W., and J.-F. L. performed cell culture and sample preparation; Y.-L. L., Y.-I. C., T. E. Y., and H.-C. Y. helped the optical system and performed data analysis; Y.-I. C. conducted the experiments of flow-cytometry and fluorescence imaging. C.-K. C., Y.-L. L., H.-C. Y., J. L. H., and P.-J. H. wrote the manuscript; J. K., H.-C. Y., and M.-C. H. supervised the project.

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Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssensors.9b01736>.

Detailed information about materials, methods, DRO optical system, microchannel fabrication, and quantification of DRO assay (PDF)

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Abstract

While monoclonal antibodies are the fastest-growing class of therapeutic agents, we lack a method that can directly quantify the on- and off-target binding affinities of newly developed therapeutic antibodies in crude cell lysates. As a result, some therapeutic antibody candidates could have a moderate on-target binding affinity but a high off-target binding affinity, which not only gives a reduced efficacy but triggers unwanted side effects. Here, we report a single-molecule counting method that precisely quantifies antibody-bound receptors, free receptors, and unbound antibodies in crude cell lysates, termed digital receptor occupancy assay (DRO). Compared to the traditional flow cytometry-based binding assay, DRO assay enables direct and digital quantification of the three molecular species in solution without the additional antibodies for competitive binding. When characterizing the therapeutic antibody, cetuximab, using DRO assay, we found the on-target binding ratio to be 65% and the binding constant (K_d) to be 2.4 nM, while the off-target binding causes the binding constant to decrease by 0.3 nM. Other than cultured cells, the DRO assay can be performed on tumor mouse xenograft models. Thus, DRO is a simple and highly quantitative method for cell-based antibody binding analysis which can be broadly applied to screen and validate new therapeutic antibodies.

Graphical Abstract

Keywords

antibody binding affinity; receptor occupancy; on-target binding; on-target quantification; single-molecule detection; microfluidics

Achieving a high on-target binding affinity but a low off-target affinity is always at the center of all therapeutic monoclonal antibody development.¹ With a vast amount of new monoclonal antibodies produced every year,² it is critically important to rapidly screen these antibodies and identify the ones with the highest on-target binding affinity and specificity.³ Currently, several methods are routinely used for characterizing the antibody binding metrics in the cell-free condition, including enzyme-linked immunosorbent assay (ELISA),^{4,5} surface plasmon resonance (SPR),^{6,7} and biolayer interferometry.⁸ While these cell-free measurements are simple and reliable, the results may not reflect the true binding affinities in the cellular environment.⁹ To bypass this limitation and evaluate the antibody's pharmacodynamics, flow cytometry-based receptor occupancy assay is often used to quantify the antibody-bound receptors and the unbound receptors in cells, revealing the binding status during the treatment.^{10–12} However, flow cytometry analysis has two major problems. First, the number of unbound receptors is usually estimated from the amount of a competitive antibody that binds to the receptors previously unoccupied by the therapeutic antibody. Not only is the application of competitive antibody a cumbersome labeling process, but it is also difficult to have a “perfect” competitive antibody that binds to the same epitope on the target and reaches identical binding strength (as compared with the

therapeutic antibody).¹¹ In addition, the assessment of unbound receptors can be obstructed by the interfering factors that neutralize competitive antibody.¹¹ Second, flow cytometry analysis employs additional antibodies to label the receptors and the therapeutic antibodies separately. The off-target binding affinities of these additional labeling antibodies can bias the estimate.^{3,13,14} There is, therefore, an urgent need to create a new quantification method that not only enables “digital” on- and off-target binding quantification in cells but bypasses the use of any additional antibodies for measurement. Microfluidics-based single-molecule detection provides a direct method to observe biomolecule interactions and count molecular species one by one. For instance, Yeh et al. developed a two-color fluorescence coincidence detection method in a microcapillary and digitally counted wild-type and mutant DNA.¹⁵ Wadhwa et al. invented a microfluidic immunoassay for distinguishing bacterium-caused Johne’s disease.¹⁶ Recently, we also developed a microchannel-based flow-proteometric technique that enables digital quantification of target proteins and related complexes from fixed biological samples.¹⁷ In our flow-proteometric analysis, each photon burst represents a fluorescently labeled molecule or molecular complex which flows through the detection volume in the microchannel (Figure 1A). As the microchannel width (2 μm) is only 2.5-fold larger than radial dimension of the detection volume (0.8 μm), and the channel height (500 nm) is smaller than the axial dimension of the detection volume (3 μm) (Figure 1B), our mass detection efficiency can be as high as 31%. Here, we extended this flow-proteometric assay to a “cell-based” assay that enables direct single-molecule quantification of therapeutic antibodies binding to the target receptors in cells, which we term digital receptor occupancy assay (DRO). It is known that epidermal growth factor receptor (EGFR) deregulation causes cancer progression, and anti-EGFR antibody (cetuximab, EGFRab hereinafter)¹⁸ has been used to treat colorectal cancer,¹⁹ non-small cell lung cancer,²⁰ and head and neck cancer.²¹ Using EGFRab as an example, here we digitally count the numbers of EGFRab, EGFR, and EGFRab-EGFR complexes in DRO assay after treating cancer cells with EGFRab (Figure 1A). Without the need to use competitive and labeling antibodies, DRO assay provides bias-free on- and off-target binding affinities of EGFRab in cancer cells. Not only is it a highly quantitative cell-based assay, but DRO can also complete antibody binding measurement in a few hours using a minute amount of sample ($\sim 10^4$ cells).

In a typical DRO assay, cells expressing the target receptors fused with the green fluorescent protein (i.e., EGFR^{GFP}) were treated with Alexa Fluor 647 (A647)-conjugated therapeutic antibody (i.e., EGFRab^{A647}). After treatment, the unbound antibodies were removed by phosphate-buffered saline (PBS) rinse and the cells were sequentially lysed and diluted for microfluidic measurements. In this protocol, only the antibodies ever associated with cells can be detected in the DRO assay. In the EGFRab binding experiment, we first generated a stable cell line that expresses EGFR^{GFP} in the EGFR-null Chinese hamster ovary cells (CHO-EGFR^{GFP}) and treated the cells with A647-conjugated EGFRab (EGFRab^{A647}) under the designated condition, followed by a collection of the crude cell lysate for DRO analysis. The coincident detection method is able to identify EGFR^{GFP}, EGFRab^{A647}, and the bound complex. A green photon burst represented EGFR^{GFP} without EGFRab^{A647} binding (Figure 1C, top), whereas a red photon burst represented EGFRab^{A647} without EGFR^{GFP} binding (Figure 1C, middle). Coincident green and red photon bursts thus indicated an EGFRab^{A647}-EGFR^{GFP} binding complex (Figure 1C, bottom). As the sample

flowed through the detection volume, individual photon bursts were seen in the two fluorescence time traces (Figure 1D). EGFR occupancy ratio and dissociation constant (K_d) of EGFRab–EGFR complex could then be computed from the photon burst counting results. The binding fractions (BF) based on EGFR^{GFP} or EGFRab^{A1547} were derived through the DRO assay to reveal in an unbiased manner the interaction between EGFR and anti-EGFR antibody (Figure 1E). The EGFR^{GFP} BF indicates the portion of EGFR^{GFP} recognized by EGFRab^{A647}, and the EGFRab^{A647} BF represents the percentile of EGFRab^{A647} binding to EGFR^{GFP}. Please see Supporting Information for the detailed description.

To evaluate the treatment dynamics, we measured EGFRab^{A647}–EGFR^{GFP} binding affinity in CHO cells treated with 20 nM EGFRab^{A647} for 5 to 60 min. From the 5 min treatment sample, 1,861 EGFRab^{A647} events per 5,000 EGFR^{GFP} events were detected (on average, Figure 2A). Out of these 1,861 EGFRab^{A647} events, 1,038 were EGFRab^{A647}–EGFR^{GFP} complex events. Extending the treatment time increased the number of EGFRab^{A647} events, but the number peaked at 30 min. The fraction of EGFRab^{A647} bound to EGFR^{GFP} was nearly 60% at 5 min, but reduced to 40% at 60 min (Figure 2B). Interestingly, the fraction of EGFR^{GFP} involved in EGFRab^{A647} binding remained the same during the 60 min treatment. These results reflected the intriguing dynamic process of EGFRab^{A647} in targeting EGFR^{GFP}.

As the highest EGFRab^{A647}–EGFR^{GFP} binding ratio was achieved within the first 5 min of treatment, we set this 5 min time point as the basis for generating the EGFRab^{A647}–EGFR^{GFP} binding curve. The Scatchard plots were used to determine the dissociation constants (K_d) and the maximum number of targetable EGFRs (B_{max}). Please see Supporting Information for a detailed description of the Scatchard equation. When plotting the binding saturation curve based on the EGFRab^{A647} event numbers (which represent the EGFRab^{A647} ever associated with cells), we found the K_d to be 2.126 nM (Figure 2C), similar to the value reported previously.²¹ To compare the DRO assay with other conventional receptor occupancy assay, we conducted flow cytometry-based receptor occupancy assay, and the measured K_d was 0.68 nM (Figure S1). Kang's group has reported that the binding affinity of Cetuximab to EGFR on the cell surface was 5 nM and 0.38 nM based on SPR and flow cytometry-based occupancy assay, respectively.²² Besides, Tao's group reported that the binding affinity of an anti-EGFR monoclonal antibody assessed by with SPR was between 0.1 and 0.7 nM.²³ The discrepancy might be attributed to different instruments, cell samples, and antibodies. In addition, the discrepancies can be due to the differences in antigen presentation and cell surface receptor density, which were reported by Chiu's group.²⁴ However, we thought that our DRO assay is comparable to other occupancy assays because the difference of dissociation constants was within 1 order of magnitude.

We found that the K_d slightly increased by 0.298 nM to become 2.424 nM (Figure 2D) when plotting the curve based on the EGFRab^{A647}–EGFR^{GFP} event numbers (which represent the actual receptor occupancy). This increase in K_d implied off-target binding of EGFRab to other molecules in cells. The capability to dissect on- and off-target bindings is advantageous over conventional receptor occupancy assays, such as flow cytometry, SPR. We next calculated the fractions of EGFRab^{A647} and EGFR^{GFP} involved in EGFRab^{A647}–EGFR^{GFP} binding. Based on the total EGFR^{GFP} numbers, the K_d of EGFRab^{A647}–

EGFR^{GFP} complexes was 2.436 nM (Figure 2E), which was identical to the K_d calculated from the number of binding events (Figure 2D). Interestingly, the fractions of the complex in the EGFRab^{A647} events were all around 65%, regardless of the EGFRab^{A647} treatment concentration (Figure 2F). This result suggested that 65% of EGFRab^{A647} remained bound to EGFR^{GFP} even after PBS wash (mimicking posttreatment in patients), and the bound antibodies would consistently provide the blockade effect (Figure 2G). As a result, we hypothesize that the EGFRab^{A647} binding fraction could serve as an indicator to evaluate the binding efficacy of therapeutic antibodies, which could be applied for antibody selection and treatment dosage optimization. In summary, our results demonstrated that the DRO assay is able to accurately access the antibody's ability in binding targets and maintaining the occupancy after treatment, which leads to the blockade of EGFR signaling.

As EGF-stimulated EGFR endocytosis can prevent EGFR from binding the therapeutic antibody, we also evaluated the influence of EGF on EGFRab^{A647}-EGFR^{GFP} binding. CHO-EGFR^{GFP} cells were pretreated with or without 50 ng/mL EGF for 30 min, then followed by 15 nM EGFRab^{A647} treatment for 5 min. As expected, after EGF treatment, the fraction of EGFR^{GFP} involved in EGFRab^{A647} binding was significantly decreased (Figure 2H). To confirm that EGF-induced EGFR endocytosis, as reported previously,²⁵ was indeed responsible for the reduced surface EGFR for EGFRab^{A647} targeting, we quantified the EGF-stimulated EGFR internalization using fluorescence imaging and also suppressed endocytosis by lowering the treatment temperature to 4 °C. The surface EGFR significantly decreased after EGF stimulation (Figure S2), and the DRO result did show that EGF had no influence on the on-target binding at 4 °C (Figure 2H). We also found the fraction of EGFRab^{A647} bound with EGFR^{GFP} was slightly increased upon the EGF treatment (Figure 2I); however, no obvious difference was seen in the fraction of EGFRab^{A647} binding when endocytosis was suppressed (Figure 2I). These results indicated that environmental alterations could affect the number of receptors available for targeting.

To demonstrate the use of DRO in receptor occupancy analysis *in vivo*, we generated a xenograft model of EGFR^{GFP}-expressing colorectal cancer as previously described²⁶ (Figure 3A). Mice with subcutaneous tumors arising from colorectal cancer cells stably expressing EGFR^{GFP} (GEO-EGFR^{GFP} cells with low-level endogenous EGFR expression) received intravenous injections of EGFRab^{A647} or isotype control IgG^{A647}, and small pieces of tumor tissue were collected at predefined time points (5 and 30 min after treatment). The tumor tissue samples were then degraded using tissue homogenizer with lysis buffer, centrifuged to remove large debris, and analyzed with DRO assay. Five minutes after injection, about 28% of the EGFR^{GFP} events were EGFRab^{A647}-EGFR^{GFP} binding events. Thirty minutes after injection, the fraction slightly increased to 33% (Figure 3B). The xenograft model showed EGFRab^{A647} binding dynamics similar to that of the cell culture model—a large portion of the available EGFR^{GFP} was targeted by EGFRab^{A647} in the first 5 min. Thus, we demonstrate that DRO assay is capable of directly quantifying antibody-receptor binding in both the crude cell lysate and tissue lysate.

As monoclonal antibody-based target therapy, including immune checkpoint therapy, has gained tremendous interest in cancer treatment in recent years,²⁷ here we developed a digital receptor occupancy (DRO) assay that can rapidly evaluate therapeutic antibody candidates

and provide highly quantitative, bias-free, and cell-based target binding characterization results based on a minute amount of sample. Not only identifying the on- and off-target binding affinity of therapeutic antibody,²⁸ our DRO assay also provides an accurate estimate of target molecules expressed in cancer cells, which is critical in effective antibody treatment.^{29,30} Whereas the surface-based methods, such as ELISA,^{4,5} SPR,^{6,7} and polarization-modulated oblique-incidence reflectivity difference (OI-RD),³¹ are still the dominant methods for K_d measurements, they require the antibodies to be captured by the surface-bound targets on a chemically functionalized microplate (with ELISA), metal-coated glass slide (with SPR), or glass slide (with OI-RD), which is not a physiologically relevant condition. The unknown surface effects may have altered the binding affinity and specificity of the antibody. In contrast, our DRO is completely a cell- and solution-based assay; in other words, our method preserves the molecule's conformational, rotational, and binding degrees of freedom, and eliminates any binding interference from the surface. Whereas the traditional flow cytometry-based receptor occupancy assay¹⁰ is also a cell-based, physiologically relevant method, it requires all target receptors in cells to be labeled with labeling antibodies. Similarly, free receptors have to be labeled with competitive antibodies, and therapeutic antibodies are labeled with secondary antibodies. In such an indirect binding measurement method, any interfering factors that prevent labeling and competitive antibodies from the binding target and therapeutic antibody can skew the binding measurement results. As we directly fuse the target receptor with a fluorescence protein and label the therapeutic antibody with an organic dye, our method bypasses any artifacts resulting from the use of competitive and labeling antibodies. As shown in Figure 2, DRO assay can easily identify and precisely quantify antibody-bound receptors, free receptors, and unbound therapeutic antibodies with a coincident detection method. From the single-molecule counts, the occupation ratio and on-target binding dissociation constant can be calculated. In addition, DRO also quantifies unbound therapeutic antibodies remaining in cells after wash. These unbound antibodies consist of both antibodies that bind other molecules (off-target binding) and antibodies dissociated from target receptors after treatment (Figure 2G). Simply using the number of unbound antibodies to represent the off-target binding can lead to an overestimated result. A better strategy to determine the off-target binding is to compare the dissociation constants K_d between antibody to the cell (Figure 2C) and antibody to the receptor (Figure 2D), where the difference (0.298 nM) is likely due to the off-target binding.

There is still space for improvement of the DRO assay. The Cetuximab is bivalent and can participate in avidity, and our current model assumes the binding of EGFR–EGFRab is monovalent. Therefore, the antibody binding affinity would be affected by antibody multivalency and avidity.³² As a regime of single-molecule detection, the DRO has the potential to recognize the number of Alexa 647 on single antibodies and the number of EGFRs captured by single antibodies using advanced single-molecule techniques.³³ However, more sophisticated instrumentation or analysis algorithms need to be implemented.

The binding of therapeutic antibodies to target receptors is also greatly affected by environmental factors, which may reduce the therapeutic efficacy.³⁴ EGF stimulation not only activates the EGFR signaling cascade for cancer progression but also helps EGFR

escape from the therapeutic antibody targeting through endocytosis.²⁵ Therefore, strategies such as blocking EGF or suppressing EGFR endocytosis can be combined with the EGFRab treatment to increase the number of EGFR that can be effectively targeted. In summary, our platform bridges the gap between indirect and direct observation of receptor occupancy and *in vitro* and *in vivo* analysis of antibody binding dynamics, which will provide the needed support for therapeutic antibody development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Principle of digital receptor occupancy assay (DRO). (A) Procedure of DRO assay: first, cells were transfected with a target receptor fused with GFP (i.e., EGFR^{GFP}); second, the cells are treated with the A647-conjugated antibodies (i.e., A647-conjugated anti-EGFR IgG, EGFRab^{A647}); third, the cells are washed with PBS to remove the unbound antibodies and lysed subsequently; fourth, the lysates are loaded into the microchannel for DRO analysis. Photon bursts from individual target proteins (green) or the antibody (red) are detected; coincident photon bursts (red and green) indicate a receptor occupied by an antibody. (B) Photo of a microchannel and the SEM image of the cross section of the microchannel. (C) Representative photon bursts showing EGFR^{GFP} without EGFRab^{A647} binding (top), EGFRab^{A647} without EGFR^{GFP} binding (middle), and EGFRab^{A647}-EGFR^{GFP} complex (bottom). (D) Representative time traces of photon bursts of EGFR^{GFP} and EGFRab^{A647}. Each red photon burst represented a single EGFRab^{A647} event, while each green burst represented a single EGFR^{GFP} event. Thresholds were applied to exclude background noise and nonspecific autofluorescence signals. The dashed lines mark coincident burst events. (E) Binding fractions (BF) based on EGFR^{GFP} or EGFRab^{A647} events were derived through the DRO assay to reveal the interaction between EGFR and anti-EGFR antibody. EGFR^{GFP} BF indicates the portion of EGFR^{GFP} recognized by EGFRab^{A647}, and the EGFRab^{A647} BF represents the percentile of EGFRab^{A647} binding to EGFR^{GFP}.

Figure 2.

Digital quantification of EGFR-EGFRab binding in CHO-EGFR^{GFP} cells. (A) Numbers of EGFRab^{A647}-EGFR^{GFP} events and total EGFRab^{A647} events after 5–60 min of EGFRab^{A647} treatment. These EGFRab^{A647} events were associated with CHO-EGFR^{GFP} cells after PBS wash. (B) Binding fractions of EGFR^{GFP} and EGFRab^{A647} after treatments at different times. (C) Total EGFRab^{A647} events following treatment with different concentrations of EGFRab^{A647} are plotted, and the events include on- and off-target binding. (D) Receptor occupied EGFRab^{A647} events are plotted, and the bound events only include on-target binding. (E) Binding saturation curve was plotted on the basis of the EGFR^{GFP} binding fraction following treatment with different concentrations of EGFRab^{A647}. In (C–E), the Scatchard plots were used to determine the dissociation constants (K_d) and a maximum number of targetable EGFRs (B_{max}). (F) EGFRab^{A647} binding fraction involved in EGFRab^{A647}-EGFR^{GFP} events. Each data point was determined based on 5,000 EGFR^{GFP} events counted, $n = 3$. (G) Coincident detection method is able to dissect the amount of antibody effectively blocking the target. (H) CHO-EGFR^{GFP} cells were treated with or without 50 ng/mL EGF for 30 min at 37 or 4 °C, respectively. The cells were later treated with 15 nM EGFRab^{A647} and incubated for 5 min. After incubation, the cells were washed with PBS and lysed for DRO analysis. The results of EGFR^{GFP} binding fraction show that the EGF-induced endocytosis reduced the number of EGFR on the plasma membrane, so the binding of antibodies to EGFRs also decreased. In contrast, the low temperature suppressed EGFR endocytosis, and there is no significant difference between groups treated with or without EGF. (I) EGFRab^{A647} binding fractions with or without EGF stimulation at 37 or 4 °C. Each data point was determined based on 5,000 EGFR^{GFP} events counted with sample number $n = 3$. Statistical comparison is performed using paired t test, where the asterisk represents statistical significance: *** $p < 0.001$ and * $p < 0.05$. The error bars represent standard deviations.

Figure 3.

Quantification of the EGFRab^{A647} binding fraction in the tumor xenograft model. (A) Mice with subcutaneous GEO-EGFR^{GFP} tumors were treated with intravenous injection (IV) of 100 µg EGFRab^{A647} or isotype control IgG^{A647}. Small tumor samples were collected at injection and 5 and 30 min after injection and lysed for DRO analysis. (B) EGFR^{GFP} binding fraction in xenograft tumor tissue. Each data point was determined based on 5,000 overall EGFR^{GFP} events counted, $n = 3$. Statistical comparison is performed using paired t test, where the asterisk represents statistical significance: *** $p < 0.001$. The error bars represent standard deviations.