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Simple Method To Prepare Oligonucleotide-Conjugated Antibodies and Its Application in Multiplex Protein Detection in Single Cells

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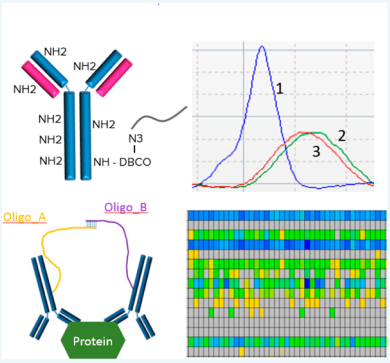
Supporting Information

ABSTRACT: The diversity of nucleic acid sequences enables genomics studies in a highly multiplexed format. Since multiplex protein detection is still a challenge, it would be useful to use genomics tools for this purpose. This can be accomplished by conjugating specific oligonucleotides to antibodies. Upon binding of the oligonucleotide-conjugated antibodies to their targets, the protein levels can be converted to oligonucleotide levels. In this report we describe a simple method for preparing oligonucleotide-conjugated antibodies and discuss this method's application in oligonucleotide extension reaction (OER) for multiplex protein detection. Conjugation is based on strain-promoted alkyne−azide cycloaddition (the Cu-free click reaction), in which the antibody is activated with a dibenzocyclooctyne (DBCO) moiety and subsequently linked covalently with an azide-modified oligonucleotide. In the functional test, the reaction conditions and purification processes were optimized to achieve maximum yield and best performance. The OER assay employs a pair of antibody binders (two antibodies, each conjugated with its own oligonucleotide) developed for each protein target. The two oligonucleotides contain unique six-base complementary regions at their 3′ prime ends to allow annealing and extension by DNA synthesis enzymes to form a DNA template. Following preamplification, the DNA template is detected by qPCR. Distinct oligonucleotide sequences are assigned to different antibody binders to enable multiplex protein detection. When tested using recombinant proteins, some antibody binders, such as those specific to CSTB, MET, EpCAM, and CASP3, had dynamic ranges of 5−6 logs. The antibody binders were also used in a multiplexed format in OER assays, and the binders successfully detected their protein targets in cell lysates, and in single cells in combination with the C1 system. This click reaction-based antibody conjugation procedure is cost-effective, needs minimal hands-on time, and is well-suited for the development of affordable multiplex protein assays, which provides the potential to accelerate proteomics research.



INTRODUCTION

While rapid advances in nucleic acid detection technologies allow genomics studies to be conducted in highly multiplexed formats, multiplex analysis is still a daunting task in the proteomics field. Therefore, there is great interest in using genomics tools for protein detection. One way to accomplish this feat is to use oligonucleotide-conjugated antibodies to bind to specific protein targets, and to monitor conjugated-oligonucleotide levels rather than measure protein levels directly. The earliest attempt to use oligonucleotide-attached antibodies to detect protein is immuno-PCR.[1] The assay has been improved by the combination of two antibody binders (two antibodies, each conjugated with its own oligonucleotide). The signal is produced only when the two antibodies bind to the same protein molecule and bring the two oligonucleotides in proximity, and form a DNA template through ligation or extension reaction.[2−4] One significant improvement resulting from using two antibody binders is that the excess antibodies in the solution will not produce signal because they are not in proximity to their respective partners. This feature, which is reminiscent of Forster resonance energy transfer (FRET), converts the assay to a homogeneous format. As a result, a washing step is no longer needed, which offers many advantages for assay development.

Various methods have been developed to conjugate oligonucleotides to antibodies. Heterobifunctional cross-linkers, such as succinimidyl 4-hydrazinonicotinate acetone hydrazone (SANH)[5] and succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC),[4] are often used to introduce a bridge between the oligonucleotide and the antibody. Commercial kits are also available for the production of oligonucleotide-conjugated antibodies. Examples include the Solulink Antibody-Oligonucleotide All-in-One Conjugation Kit, and the Innova Thunder-Link kit. However, these methods are either too labor intensive or too costly, and are not suitable for large-scale production of oligonucleotide-conjugated antibodies needed for multiplex protein detection.

The copper-catalyzed alkyne−azide cycloaddition (CuAAC), or click reaction, has been used to modify biomolecules in various applications.[6] Although robust, CuAAC is not suitable

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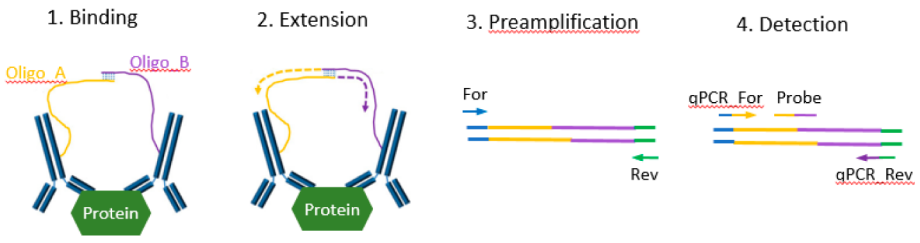
A



B

Name	Sequence
Oligo_A	actgacgacatggttctacaGACTCTCATTCCCCTCCTCTAGGTC
Oligo_B	cggtagcagagacttggtctTAGAGGCTTTACTGGACCGGACCTA
Extension product	ACTGACGACATGGTTCTACAGACTCTCATTCCCCTCCTCTAGGTCCGGTCCAGTAAA GCCTCTAAGACCAAGTCTCTGCTACCG
Preamp_For	actgacgacatggttctaca
Preamp_Rev	ggtagcagagacttggtct
qPCR_For	ggttctacaGACTCTCATTC
qPCR_Rev	acttggtctTAGAGGCTTTA
qPCR_probe	CCCTCCTCtaggtcCGGTCCAG

Figure 1. Protein detection based on oligonucleotide extension reaction (OER). (A) The principle of OER.OER contains four steps. The first two steps (1—Binding, 2—Extension) convert protein levels to DNA template levels. After preamplification, the DNA template level, which corresponds to the protein level in the sample, is measured by qPCR. (B) Set of sequences used in OER, which includes oligonucleotide pair, extension product, preamplification primer pair, qPCR primer pair, and qPCR probe.

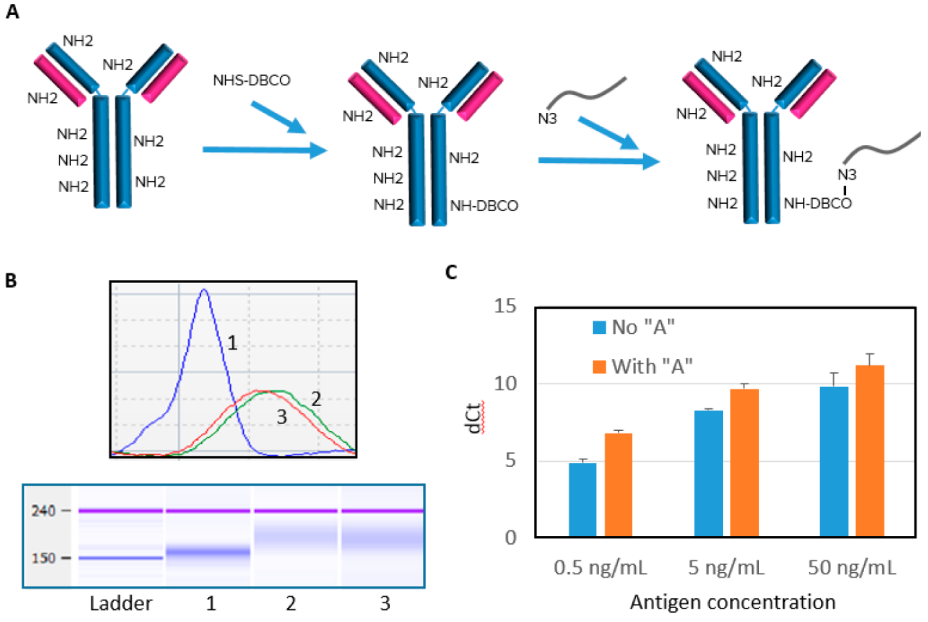


Figure 2. Conjugation of azide-modified oligonucleotide to antibody. (A) Schematic representation of the conjugation process using click chemistry. The antibody was first functionalized using DBCO-PEG5-NHS. Azide-modified oligonucleotide was then added for conjugation. (B) Analysis of anti-CSTB antibody conjugated with azide-modified oligonucleotides: upper panel, electropherogram graph; lower panel, gel image; 1, unconjugated antibody; 2, anti-CSTB antibody conjugated with oligo A (CSTB-A); 3, anti-CSTB antibody conjugated with oligo B (CSTB-B). The band-size shift to a higher molecular weight (2 and 3) indicates that the oligonucleotides were successfully conjugated on the antibody. (C) Test of antibody binders CSTB-A and CSTB-B using OER. The dCt values were calculated by subtracting background Ct by antigen Ct. Results are expressed as the means ± SD. Note that higher concentrations of antigen produced higher dCt values. The addition of an extra adenosine (A) also increased the dCt value.

for applications involving functional biomolecules because copper ions may be detrimental to these molecules. For example, copper ions can cause protein denaturation.[7] To circumvent this issue, a Cu-free click reaction based on strain-promoted alkyne−azide cycloaddition (SPAAC) has been developed.[7,8] In SPAAC, cyclooctynes such as difluorinated cyclooctyne (DIFO) and dibenzocyclooctyne (DBCO) are used to react with azide-functionalized molecules. The reaction is performed under physiological conditions and has no adverse effects on macro-biomolecules such as antibodies.

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In this report, we describe the development of oligonucleotide-conjugated antibody binders for multiplex protein detection employing SPAAC. The oligonucleotide was attached to the antibody through the sequential addition of a DBCO moiety and an azide-modified oligonucleotide. The reaction condition and purification process were optimized to achieve maximum yield and best performance in the functional test using oligonucleotide extension reaction (OER). In the OER assay a pair of antibody binders (two antibodies, each conjugated with its own oligonucleotide) is developed for each protein target. The two oligonucleotides contain a six-base complementary region at their 3′ prime ends to allow annealing and extension by DNA synthesis enzymes to form a DNA template. The template is then detected by qPCR. Distinct oligonucleotide sequences are assigned to different antibody binders to enable multiplex protein detection. The assays were tested using recombinant proteins and cell lysates, and in single cells using the C1 system. The SPAAC-based conjugation method is simple and cost-effective and is well-suited for the preparation of oligonucleotide-conjugated antibodies for multiplex protein assays, which has the potential to accelerate proteomics research.

RESULTS

Design of Oligonucleotides. A pair of oligonucleotides (oligo A and oligo B, 45 nucleotides each) is designed for each protein target. The two oligonucleotides contain a six-base complementary region at the 3′ end to allow annealing and extension by DNA synthesis enzymes. The oligonucleotides also contain primer sequences for preamplification and qPCR detection. A cleavage qPCR probe that covers the six base complementary region is also included (Figure 1A). A set of oligonucleotide pair, extension product sequence, preamplification primer pair, qPCR primer pair, and qPCR probe is shown in Figure 1B. For SPAAC conjugation, an azide group is added to the 5′ end of the oligonucleotide sequence.

To allow multiplexing, different oligonucleotide pairs were designed for different protein targets. These oligonucleotide sequences were designed using the proprietary Fluidigm assay design pipeline. They are distinguished by the unique six-base complementary region, and have different qPCR probe sequences. Each qPCR primer for one protein target contains at least one nucleotide difference compared to any other qPCR primers, but all qPCR primers have similar T_ms. The preamplification PCR primer sequences are the same for all assays. A total of 146 pairs of oligonucleotides were obtained, among which 55 pairs were selected for antibody conjugation.

Conjugation of Azide-Modified Oligonucleotide to the Antibody. The conjugation process is illustrated in Figure 2A. The DBCO moiety and the azide-modified oligonucleotide were attached to the antibody sequentially. For DBCO functionalization, we used DBCO-PEG5-NHS to react with the NH_2 groups on the antibody. Inclusion of a PEG5 linker increases the water solubility of the DBCO moiety and introduces a spacer between the bulky antibody and the oligonucleotide. The anti-CSTB polyclonal antibody was conjugated with oligo A or oligo B to produce a pair of antibody binders (CSTB-A and CSTB-B). The successful conjugation of oligo A and oligo B to the anti-CSTB antibody was demonstrated by the band shift to a higher molecular weight compared to the unconjugated antibody (Figure 2B).

During the OER experiment, the antibody binders CSTB-A and CSTB-B were incubated with samples containing the CSTB recombinant protein. No-protein controls containing just buffer (i.e., no CSTB protein) were performed to measure background. Because a polyclonal antibody was used to produce the pair of antibody binders, CSTB-A and CSTB-B can bind to different epitopes of the same molecule, resulting in bringing oligonucleotides A and B into proximity. When the two oligonucleotides are in proximity, the six-base overlap on oligonucleotide A and oligonucleotide B anneals and forms a template for extension reaction. The extension product can then be detected by qPCR. The qPCR signal intensity will reflect the CSTB protein level in the sample. As shown in Figure 2C, an antigen-dose-dependent response was obtained with the oligonucleotide-conjugated anti-CSTB antibody.

We reasoned that adding some extra space between the bulky structure formed by DBCO/azide reaction and the oligonucleotide may improve the performance of the OER assay. To test this hypothesis, an extra adenosine (A) was added to the 5′ end of the oligonucleotide. The results showed that the oligonucleotide with an extra A produced a higher signal-to-noise (i.e., delta Ct) readout.

Effect of Degree of Labeling. To obtain different degrees of labeling (DOL), varied amounts of input DBCO-PEG5-NHS (2, 4, 6, and 8 mol equiv) were used for DBCO functionalization, resulting in 1.5, 2.3, 3.1, and 4.2 DBCO molecules per antibody after reaction. The DBCO-functionalized antibodies were then reacted with 2, 4, 6, and 8 mol equiv of oligonucleotides (oligo A or oligo B), respectively. The resulting conjugates were named CSTB2A, CST2B, CSTB4A, CSTB4B, CSTB6A, CSTB6B, CSTB8A, and CSTB8B, respectively. Figure 3A demonstrates that the sizes of the labeling products shifted to higher molecular weights with the increase of DOL, presumably due to the different numbers of oligonucleotides attached.

OER assay revealed that the background signal increased (lower Ct) with the increase of DOL (Figure 3B). However, the signal from the recombinant protein also increased (data not shown). As a result, the differences between dCts were not significant, with CSTB8 having slightly lower dCt (Figure 3C). We chose to use a molar equivalent of 4 as the oligonucleotide input for the subsequent labeling reactions. The resulting number of oligonucleotides attached to the antibody was determined by fast protein liquid chromatography (FPLC) analysis of antibodies conjugated with a Cy5-modified oligonucleotide. The number of oligonucleotides per antibody was 1.2 when a molar equivalent of 4 oligonucleotides was used in the conjugation reaction (Figure S1).

Effect of Unlabeled Antibody. To investigate the effect of unlabeled antibody, various amounts of unlabeled antibody were spiked in the oligonucleotide-conjugated anti-CSTB antibody binders and evaluated using OER. CSTB recombinant protein at a concentration of 10 ng/mL was used as the antigen. As shown in Figure 4, the background Ct (CSTB0) was not affected by the unlabeled antibody. For signals produced in the presence of CSTB antigen (CSTB10), when the concentration of unlabeled antibody was low (at 12.5% of oligonucleotide-conjugated antibody or lower), it did not affect the Ct significantly. The Ct began to increase when the unlabeled antibody reached a level of 25% of oligonucleotide-conjugated antibody or higher.

Dynamic Ranges of OER Assays Determined Using Recombinant Proteins. The antibody binders were evaluated in OER assays using a series of diluted recombinant proteins with concentrations ranging from 10^{-2} pg/mL to 10^{7} pg/mL.

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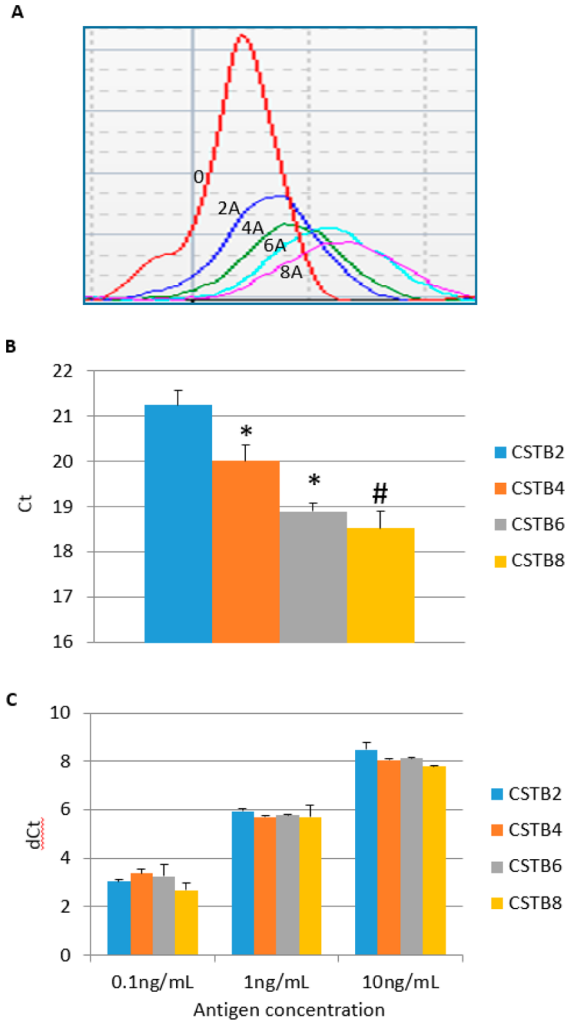

Figure 3. Effect of degree of labeling. (A) Electropherogram graph of anti-CSTB antibody conjugated with different numbers of oligonucleotides. Only oligo A data were shown. Curves shifting to the right represent higher molecular weights. (B) Background Ct of CSTB binders with different degrees of labeling. A lower Ct value represents a higher background signal. * $p < 0.05$; # $p > 0.05$. (C) Performances of CSTB binders with different degrees of labeling as determined by dCt values. The results in (B) and (C) are expressed as the means ± SD. Ct: cycle threshold.

As shown in Figure 5, the CSTB-, MET-, and CASP3-specific antibody binders detected their respective antigens in the concentration range between 10^0 pg/mL and 10^5 pg/mL, with a dynamic range of 5 logs; the EpCAM-specific antibody binder detected the EpCAM recombinant protein between 10^{-1} pg/mL and 10^5 pg/mL, with a dynamic range of 6 logs. When the antigen concentrations were higher than 10^5 pg/mL, a hook effect was noticed for all antibody binders, where the increase of antigen concentration caused a decrease in dCt. This hook effect is likely due to the competition of antibody binders by excessive antigen molecules, resulting in fewer antigen molecules binding with both antibody binders A and B—a condition needed for successful OER. It is noteworthy that the performance of antibody binders depends on the quality of the original antibodies used in the conjugation reaction. Although the antibody binders shown in Figure 5 have a similar performance, variation in performance was noted for other antibody binders (data not shown).

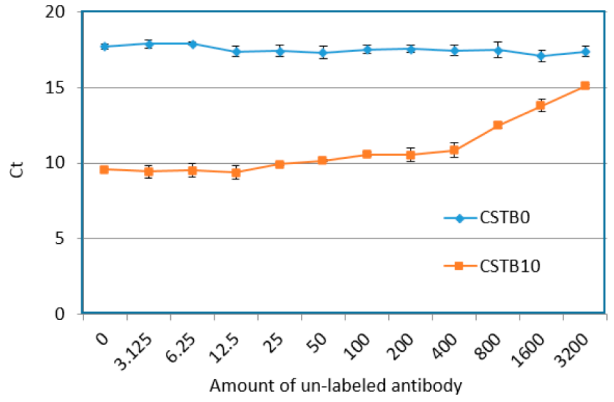

Figure 4. Effect of unlabeled antibody on the performance of OER. Different amounts of unlabeled anti-CSTB antibody (shown as percentages of the oligonucleotide-conjugated antibody) were spiked in the conjugated antibody. The performances of antibody binders containing unlabeled antibody were determined using OER assay. Either 0 ng/mL (background) or 10 ng/mL of CSTB was used as the antigen. Ct values are shown as the means ± SD. Ct: cycle threshold.

Multiplex OER Assays in Cell Lysates. The performances of antibody binders were also evaluated in multiplexed format. A mixture of 24 different pairs of antibody binders was used in OER assay to detect their respective protein targets in MCF7, K562, A549, and A431 cell lysates. The cell lysates were diluted to a series of concentrations ranging from 5 ng/mL to 5×10^5 ng/mL (total protein). As shown in Figure 6, CSTB was detected in all four cell lysates (decreased Ct compared to control C0), while CASP3 was only detected in K562, A549, and A431; K_i-67 detected in K562 and MCF7; GATA3 detected in MCF7. For those antibody binders that produced positive signals, the signal intensities were dependent on the concentrations of the cell lysates. The lack of signal in cell lysates without the respective antigens demonstrates the high specificity of the OER assay.

Multiplex Protein Detection in Single Cells. The antibody binders were tested for single-cell protein detection in the C1 system. Twenty antibody binders together with four controls (a total of 24-plex) were used for multiplex protein detection. The single-cell isolation, live/dead cell staining, cell lysis and antibody binding, oligo extension, and preamplification were all conducted in the C1 system. For comparison, two sets of in-tube controls, one set composed of three negative controls, and one set composed of three positive bulk-cell controls were included. As shown in Figure 7, CSTB, EpCAM, CASP3, VEGFA, BSG, CCNA2, CCNB1, TRAIL-R1, TRAIL-R2, Lin-28, and MUC1 produced signals in both in-tube positive control and C1 single cell preparations. Distinct patterns of protein expression in single cells were noticed. CSTB and CASP3 were expressed at similar levels in different cells. However, the protein levels of EpCAM, BSG, CCNA2, CCNB1, TRAIL-R1, and MUC1 varied dramatically in individual cells. Live cells and dead cells also showed diverse protein expression patterns for different targets. For example, EpCAM, BSG, TRAIL-R1, and TRAIL-R2 had a similar expression pattern in dead cells as that in live cells, whereas CSTB, CASP3, CCNA2, and CCNB1 had very low or no protein expression in dead cells, which is similar to negative controls.

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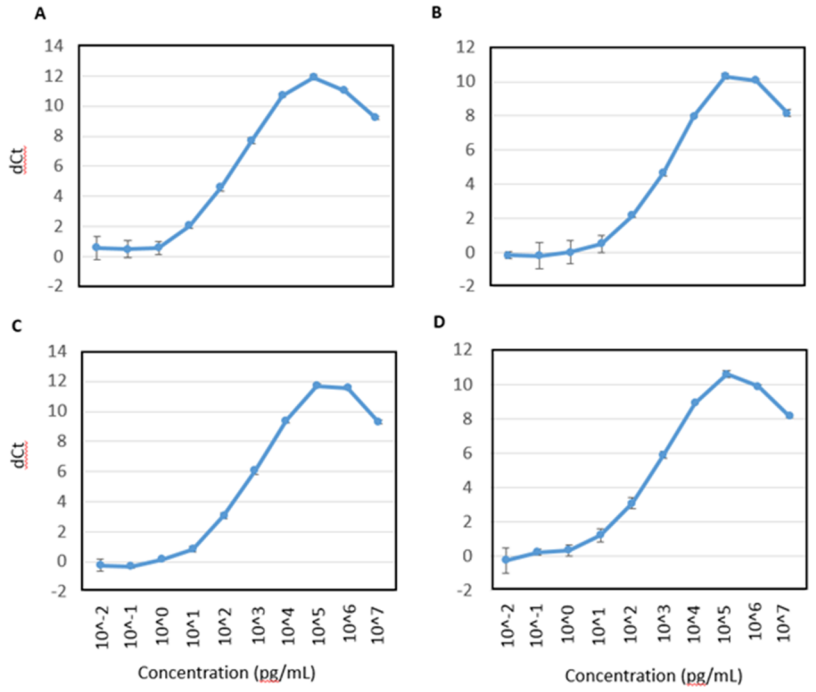

Figure 5. Dynamic ranges of proteins measured using their respective oligonucleotide-conjugated antibody binders. Different concentrations of recombinant proteins CSTB (A), MET (B), EpCAM (C), and CASP3 (D) were used in the OER assay and reacted with their respective antibody binders. The assay buffer that does not contain any antigen was included as background. The dCt values were calculated by subtracting background Ct by recombinant protein Ct. Results are expressed as the means ± SD. Ct: cycle threshold.

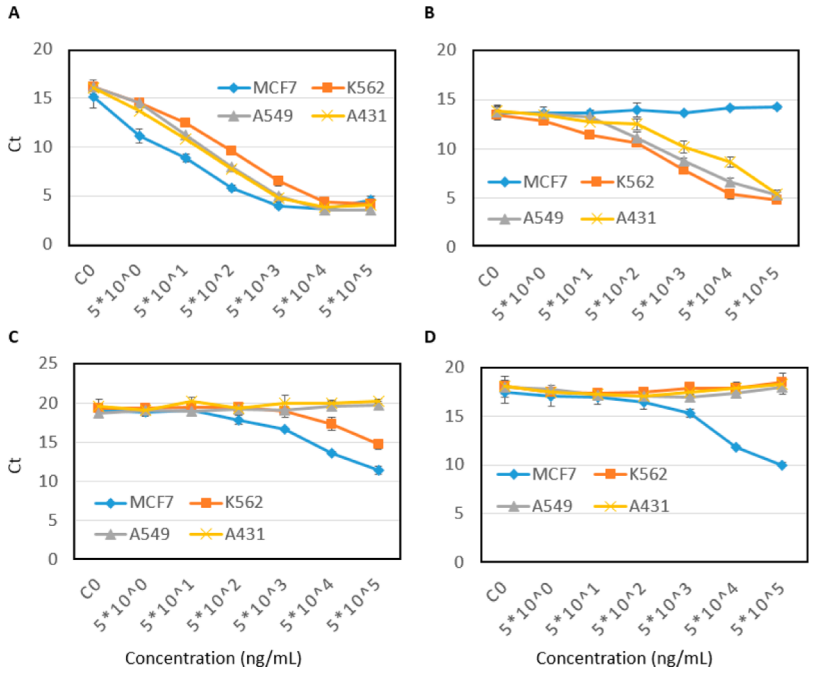

Figure 6. Detection of proteins in cell lysates. A panel of 24-plex antibody binders was used to detect their respective target proteins in MCF7, K562, A549, and A431 cell lysates. The cell lysates were diluted to different concentrations and reacted with a mixture of antibody binders. Only selected examples of protein targets CSTB (A), CASP3 (B), K_i-67(C), and GATA3 (D) are shown. Ct values were shown as the means ± SD. Ct: cycle threshold.

DISCUSSION

Protein detections are performed in various formats, with methods developed for intact cells and tissue sections, as well as for lysed cells and tissues. Currently the most widely used technologies for protein detection in lysed cells and tissues are still ELISA and Western blot, both of which were developed in the 1970s. Efforts have been made to improve these technologies. Examples include single-cell Western blot (scWestern)[9] and digital ELISA.[10] Although these studies improved the technology in one way or another, they do not resolve inherent limitations. For example, scWestern is still based on gel electrophoresis and has a limited dynamic range

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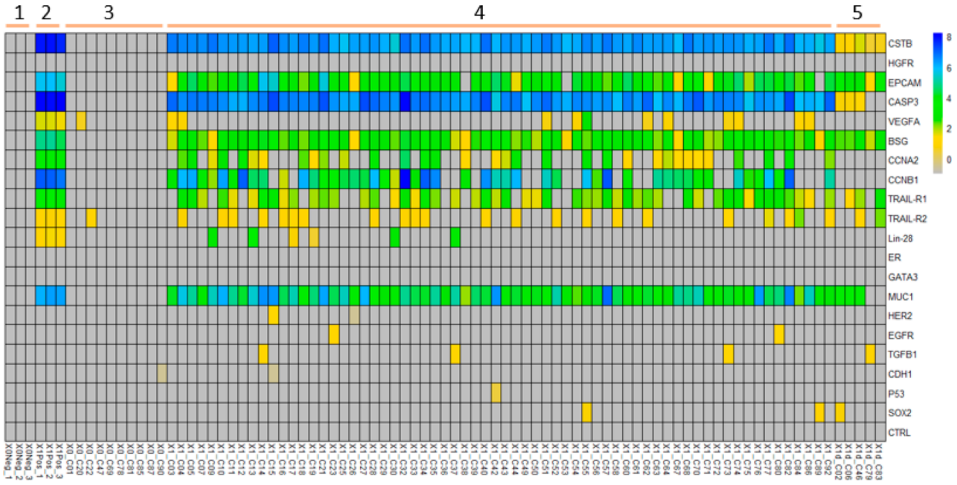

Figure 7. Multiplex protein detection in single K562 cells using the C1 system. Single cells were isolated in individual capture chambers of the C1 integrated fluidic circuit (IFC). A mixture of 24 pairs of antibody binders was used to detect their respective protein targets. The first three steps of OER (binding, extension, and preamplification) were conducted in the C1 IFC. The preamplification products were harvested and used for qPCR detection. The dCt values were calculated by subtracting the average Ct of all empty chambers by Ct values of individual chambers. Different levels of proteins are represented by different colors in the heat map: 1, In-tube negative controls; 2, In-tube positive controls; 3, C1 empty chambers; 4, C1 chambers with single live cells; 5, C1 chambers with single dead cells. Ct: cycle threshold.

(1.3−2.2 orders of magnitude) and sensitivity. Digital ELISA is sensitive, but its specificity is not superior to conventional ELISA. In addition, neither ELISA nor Western blot is compatible with multiplexed detection of proteins, which will be necessary in order to understand cellular processes under both physiological and diseased conditions[11]

Multiplex protein detection can be achieved by employing a DNA barcoding system. By attaching a distinct oligonucleotide sequence to a different antibody and converting the protein level to a surrogate DNA level, 90 protein targets were detected in the same reaction using a hybridization-based procedure.[12] Other nucleic acid detection technologies, such as qPCR and next-generation sequencing (NGS), could also be used for this purpose. Over two decades ago, Sano et al. invented a method called immuno-PCR with which as few as 580 antigen molecules can be reproducibly detected.[1] Different variants of immuno-PCR have been developed since then, and the limit of detection is reported to be 100- to 10^5-fold better than conventional ELISA.[13] To take advantage of DNA-based immunoassays and make multiplex protein detection affordable, it is essential to develop a simple, cost-effective procedure to produce oligonucleotide-conjugated antibody.

SPAAC has been used to join two molecules together in many applications, such as production of site-specific antibody−drug conjugates[14] and preparation of imaging agents for single-photon emission computed tomography (SPECT) and positron emission tomography (PET).[6] Due to the simple procedure and mild reaction condition, SPAAC could be a good option for attaching oligonucleotides to antibodies, where a DBCO moiety serves as a linker between the antibody molecule and the oligonucleotide. Different versions of DBCO modules, such as DBCO-NHS, sulfo-DBCO-NHS, and DBCO-PEG5-NHS, could be used in the reaction. We selected DBCO-PEG5-NHS, which contains a PEG5 linker. There are advantages to including a PEG linker in the DBCO moiety: (1) A PEG linker improves the water solubility of the hydrophobic DBCO; (2) it introduces a spacer between the antibody molecule and the oligonucleotide, which alleviates the steric effect of the bulky antibody on the enzymatic reactions;[15] (3) a PEG linker helps to restore the reactivity of a hydrophobic molecule by unmasking the interaction between the hydrophobic molecule and the antibody;[16] and (4) the OER assay becomes more flexible with a spacer between the antibody and the oligo, especially in situations where two distant epitopes are involved. This is advantageous to FRET where the distance between the two molecules is often limited to a few nanometers.[17]

The DBCO-functionalized antibody is then reacted with azide-modified oligo, resulting in the covalent attachment of the oligonucleotide to the antibody. Because the reaction between DBCO and azide is slow compared to CuAAC reaction, we prolonged the reaction time (16−18 h) to increase the final product yield. As the reaction was conducted in PBS at low temperature (4 °C), the longer reaction time is unlikely to exert any adverse effect on the antibody activity. In addition, the DBCO-functionalized antibody in the intermediate reaction is stable (data not shown), which makes the production process very flexible.

To improve the OER performance, we optimized the labeling process. A relatively low oligonucleotide-to-antibody ratio was used in the conjugation. This has two major benefits. First, it minimizes the impact of oligonucleotide modification on the antibody, and has less effect on the antibody affinity. Second, although the dCt did not change much at a lower oligonucleotide-to-antibody ratio (Figure 3), the lower signal level (high Ct) is desirable for OER assay, especially for highly multiplexed format, where different targets will compete with enzymes and reagents. A lower signal means that less enzyme and reagents are needed for that target. One caveat to using a low oligonucleotide-to-antibody ratio is that some antibodies will not be labeled. We tested the effect of unlabeled antibody, and found that the OER can tolerate at least 12.5% of unlabeled antibody.

Because OER utilizes antibody for detection, it is conceivable that the quality of antibodies affects assay performance. An assay LOD of about 0.06 pg/mL was obtained when a high affinity anti-CASP3 antibody was used. In contrast, an antibody with lower affinity could produce a LOD higher than 62.5 pg/

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mL. A study in mouse fibroblast cell line NIH 3T3 showed that the median copy number of proteins in a single cell is 50 000,[18] which is equivalent to 923 pg/mL in a C1 chamber (assume the protein has a molecular weight of 50 kDa; the volume of C1 cell capture chamber is 4.5 nL). Therefore, the OER assay is sensitive enough to detect most proteins in single cells using a C1 system.

One important advantage for OER is that the signal is produced only when both antibody binders bind to the antigen simultaneously. Similar to FRET, the assay can be performed in a homogeneous format, which makes novel applications possible. One example is the single-cell protein detection in the C1 system. Because it is hard to integrate a protein fixation step and multiple wash steps in the C1 protocol, a conventional ELISA would not work in the C1 system. In contrast, the OER protocol can be adapted to the C1 system easily. OER also has superior specificity compared to sandwich ELISA. Although the sandwich ELISA also utilizes two antibodies, the nonspecific binding of the detection antibody itself, or HRP-conjugated streptavidin, could produce background signal. Therefore, stringent wash steps are needed for sandwich ELISA. In contrast, binding of one antibody to the protein or supporting matrix will not produce any signal in OER. The high specificity of OER assay was demonstrated in the cell lysate experiment, where signals were only detected in cells that express the respective protein targets (e.g., GATA3 signal was only detected in MCF7, but not in K562, A549, and A431 cells).

Single-cell molecular analysis has become increasingly important in biomedical research, especially in the field of cancer research, where cell-to-cell heterogeneity is a characteristic feature.[19] Single-cell analysis can reveal the differences in cellular responses between individual cells that are masked by bulk-cell analysis.[20] As opposed to the proximity ligation assay (PLA)[4] which is widely used in fixed tissues and cells, and the proximity extension assay (PEA)[3,21] which focuses on detecting proteins in serum and plasma, OER is optimized for protein detection in lysed cells. In addition, the buffers and enzymes are compatible with the C1 system to allow streamlined single-cell protein detection. Single-cell OER assay demonstrated that the protein levels of EpCAM, BSG, CCNA2, CCNB1, TRAIL-R1, and MUC1 varied dramatically in individual cells. Because the expression of cyclins is tightly controlled with the progression of the cell cycle, it is not surprising that the cell cycle-related genes CCNA2 and CCNB1 are expressed at different levels in individual cells. CCNA2 is known to be absent or expressed at low levels in G1, and appears abundantly at the G1/S transition.[22] Cyclin B1 protein is also undetectable in early G1, but increases dramatically in G2 and M phases.[23] The detection of varied levels of CCNA2 and CCNB1 proteins in individual cells demonstrates that OER could be a valuable tool for single-cell molecular analysis.

In conclusion, we developed and optimized a simple and cost-effective method for preparing oligonucleotide-conjugated antibodies based on Cu-free click reaction. The oligonucleotide-conjugated antibodies were used in OER assays for multiplex protein detection. In combination with the C1 system, multiple protein targets can be detected in single cells. With the decrease of cost and increase of popularity of NGS, it would be of interest to combine NGS and OER for protein detection in the future.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. DBCO-PEG5-NHS was purchased from Click Chemistry Tools (Scottsdale, AZ). All antibodies and recombinant proteins were from R&D Systems (Minneapolis, MN) unless otherwise noted. MCF7, K562, A549, and A431 cell lysates were obtained from Novus (Littleton, CO). All oligonucleotides and qPCR probes were provided by Integrated DNA Technologies (Coralville, IA). All other reagents were from Fluidigm (South San Francisco, CA).

Production of Oligonucleotide-Conjugated Antibody. The antibody was first reacted with DBCO-PEG5-NHS following a standard procedure for antibody conjugation through the NH_2 group.[24] In brief, 100 μg of antibody was incubated with DBCO-PEG5-NHS in PBS for 2 h at room temperature. After reaction, the free DBCO-PEG5-NHS was removed using the Amicon Ultra-0.5 NMWL 50 kDa Centrifugal Filter (EMD Millipore, Billerica, MA). To determine the number of DBCO molecules on the antibody, the absorbance at 309 and 280 nm was measured. The molar concentrations of DBCO and antibody were determined using their respective molar extinction coefficient (12 000 M^{-1} cm^{-1} for DBCO at 309 nm and 204 000 M^{-1} cm^{-1} for antibody at 280 nm). The number of DBCO molecules per antibody was calculated by dividing the molar concentration of DBCO by the molar concentration of antibody.

The DBCO-functionalized antibody was split into two tubes, each with equal amounts, and reacted with azide-modified oligonucleotide A or B, respectively. The azide-modified oligonucleotides were reconstituted in PBS before adding to DBCO-functionalized antibody. The click reaction was conducted at 4 °C for 16−18 h. The unreacted oligonucleotides were removed using the Amicon Ultra-0.5 NMWL 100 kDa Centrifugal Filter (EMD Millipore).

Analysis of Oligonucleotide-Conjugated Antibody. The oligonucleotide-conjugated antibody was analyzed on a 2100 BioAnalyzer (Agilent, Santa Clara, CA) using the protein 230 kit following the manufacturer's instructions. In brief, 4 μL of antibody was mixed with 2 μL of sample buffer without any denaturing reagents. After adding 84 μL of deionized water, 6 μL was loaded on a protein chip provided in the kit. The chip was then placed in the Agilent 2100 BioAnalyzer for electrophoresis and image acquisition. In the gel image, the band sizes indicate the molecular weights of the antibodies, with the upper band representing a higher molecular weight. In the electropherogram, the peak on the right indicates a higher molecular weight.

OER Assay in Tube. The oligonucleotide-conjugated antibody binders were reacted with their respective targets (recombinant proteins or cell lysates) at 37 °C on a thermal cycler for 30 min to allow binding of the antibody to the antigen, and annealing of oligos. Then, 1 μL of binding reaction mix was transferred to a tube containing 11.3 μL of Template Final Mix (see the Fluidigm C1 Protein Expression in Single Cells kit for components) for oligonucleotide extension to form a DNA template. The OER was conducted at 40 °C for 16.5 min before enzyme deactivation at 85 °C for 5 min. A preamplification PCR step was then performed to increase the copy number of the DNA template. The DNA template, whose abundance reflects the level of the corresponding protein target, was then detected by qPCR on the Biomark HD system (see Detection by qPCR on the Biomark HD system below). Please

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refer to the Fluidigm C1 Protein Expression in Single Cells protocol (In Tube Controls) for details.

Single-Cell Protein Sample Preparation and OER Assay in the C1 System. The human erythromyeloblastoid leukemia cell line K562 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Cells were maintained in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum (FBS).

The cells were washed twice with Cell Preparation Buffer provided in the Fluidigm C1 Protein Expression in Single Cells kit, and resuspended at a concentration of 70−80 cells/μL. If no empty chambers in the C1 IFC are wanted, a higher cell density (200 cells/μL) can be used. The cells were then mixed with C1 Cell Suspension Reagent at a 3:2 ratio, and loaded immediately on the C1 IFC along with 20 μL of freshly prepared LIVE/DEAD staining buffer (ethidium homodimer-1 and calcein AM, ThermoFisher Scientific, Waltham, MA) in their respective input inlets. Images of C1 chambers were collected with a Leica DMI 4000B microscope in the brightfield, GFP, and CY3 channels using Surveyor v 7.0.0.9 MT software (Objective Imaging, Cambridge, United Kingdom) for cell-occupying information (empty, live cell, or dead cell) assignment.[25]

The OER assay was conducted on the C1 IFC following the instruction described in the Fluidigm C1 Protein Expression in Single Cells kit protocol. In brief, the C1 Lysis & Ab Binding Final Mix, Template Final Mix, and Preamplification Final Mix were prepared using the reagents provided in the kit. Eight microliters of Lysis & Binding Final Mix, 27 μL of Template Final Mix, and 25 μL of Preamplification Final Mix were loaded on the C1 IFC in their respective input inlets. The IFC was then placed into the C1 system, which controls all OER steps automatically. After the program was completed, the reaction products were harvested into a 96-well plate for qPCR detection. For comparison, bulk cell reactions were conducted in tubes following the same procedure.

Detection by qPCR on the Biomark HD System. The qPCR was conducted on a 96.96 Dynamic Array IFC. Briefly, 3 μL of each sample was mixed with 3 μL of C1 Protein Detection Master Mix (the Fluidigm C1 Protein Expression in Single Cells kit) and loaded in the 96.96 Dynamic Array IFC inlets on the sample side (5 μL per well). The target-specific assays containing qPCR primers and probes were loaded on the assay side (5 μL per well). After loading and mixing on an HX Controller, the 96.96 Dynamic Array IFC was transferred to the Biomark HD system for PCR reaction and data acquisition.

Data Analysis. All experiments with recombinant proteins and cell lysates were conducted in triplicate. The results were expressed as the means ± SD. For single-cell protein detection in the C1 system, a recombinant protein of nonmammalian source was used as the OER assay control. Ct values of all protein targets were normalized against the OER assay control. The dCt values were calculated by subtracting average background Ct (Ct from empty C1 chambers) by the single-cell Ct for each protein target. A dCt value greater than 2 × SD of background Ct was considered significant and assigned a different color based on the dCt value. The heatmap was drawn using the pheatmap R package.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00613.

FPLC determination of the number of oligonucleotides conjugated to the antibody (PDF)

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Notes

The authors declare the following competing financial interest(s): The authors are employees at Fluidigm Corporation.

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