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Conjugation of Antibodies for Simultaneous Detection of Surface Proteins and Transcriptome Analysis on a Single-cell Level.

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Abstract

Transcriptome analysis at a single-cell level with single-cell RNA sequencing (scRNA-seq) is a powerful method for detailed characterization of heterogeneous cell populations. Recent developments have enabled parallel analysis of both transcript and protein levels by using antibodies conjugated to barcoded oligonucleotides. These antibodies allow protein levels to be converted into nucleotide format, permitting the sequencing-based detection of both modalities at single-cell level. Here we present a simple and reliable method for conjugation of oligonucleotides with antibodies and a protocol for their use in single-cell transcriptome sequencing.

Key words

scRNA-seq, CITE-seq, transcriptome analysis, transcriptomics, proteomics

1. Introduction

Transcriptome analysis of cell populations at the level of individual cells by single-cell RNA-sequencing (scRNA-seq) allows data-driven characterization of cell type identities and regulatory states. This recent methodological advancement is revolutionizing many fields of biology, and the impact on immunology has been particularly high due to the heterogeneity typically associated with immune cell populations [1–3]. Recent studies have revealed diverse novel subsets and cell states within T cells [4,5], dendritic cells [6], and macrophages [7]. At the same time, biological and functional interpretation of newly identified cell populations presents a significant challenge. Typical starting point for such efforts is characterization of the expression of classical cell surface CD molecules that are used to define a cell type or state and are backed by decades of research. However, in some cases these gold standard surface

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markers are not robustly detected by sequencing [8]. Moreover, mRNA abundances only partially predict the levels of the corresponding proteins [9,10]. Finally, certain markers such as CD45 are regulated at the level of splicing, and the analysis of such markers are inherently beyond the capabilities of 3'-scRNA-seq.

These limitations have been addressed by recent methodological advances in the field of single-cell sequencing by adding modalities to the analysis including detection of proteins [8,11]. In these applications, antibodies (or other feature recognizing molecules) are conjugated with oligonucleotides containing barcode sequences and A-nucleotide stretch (or other sequences compatible with the scRNA-seq chemistry). Following single cell capture and lysis, the sequences are converted into cell-barcoded double stranded cDNA by the reverse transcriptase in parallel with the cellular mRNAs. The subsequent processing steps result in DNA sequencing libraries containing information on cellular transcriptomes and levels of antigens recognized by the antibody-oligo-conjugates. Bioinformatic analysis of the sequenced libraries resolves this information into single cell transcript and protein profiles for biological analyses.

While commercial oligo-conjugated antibodies are already available for many clones, they are expensive, may not be available for the clones of interests, or oligo-labeling may not be optimal for the chosen scRNA-seq application. Here, we describe a simple and reliable method for conjugation of oligonucleotides with antibodies and for their use along with single cell transcriptome sequencing. In this protocol, DBCO-PEG₄-NHS molecules are first covalently attached to the antibody via amine groups. The activated antibodies are then conjugated to 5'-azide-modified oligos. Finally, the antibody-oligo conjugates are cleaned up of the remaining free oligo.

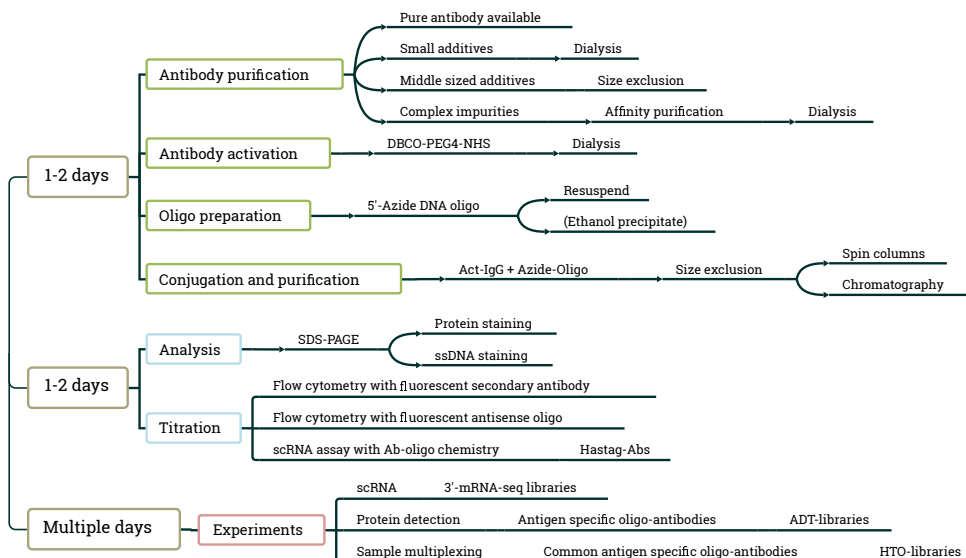


Figure 1: Overview of the steps of the antibody-oligo conjugation with optimal case timing. The timing is tentative and heavily depends on the steps needed to purify and/or concentrate the target antibody from additives and on steps needed to purify the final conjugate off from remaining free oligo.

2. Materials

1. Phosphate-buffered saline (PBS).
2. Target antibody in a suitable format.
3. Dibenzocyclooctyne-PEG₄-N-hydroxysuccinimidyl ester (DBCO-PEG₄-NHS).
4. HPLC purified 5'-Azide-DNA-oligo (available from oligo manufacturers).
5. Dialysis membrane cups: Thermo's Slide-A-Lyzer MINI Dialysis Units 3500 MWCO or equivalent.
6. Optional: Dry polyethylene glycol (PEG), MW 10 000 or higher.
7. Size exclusion spin columns: Amicon Ultra 0.5 ml 35 000 and 100 000 MWCO or equivalent.
8. Bovine serum albumin (BSA).
9. Microcentrifuge tubes, preferably low protein retention (e.g. Eppendorf Protein LoBind tubes).
10. Microcentrifuge tubes, preferably low DNA retention (e.g. Eppendorf DNA LoBind tubes).
11. Optional: desalting columns for DNA oligo purification.
12. Bio-Rad TGX stain-free precast gradient gel 4-15 % or equivalent.
13. Silver staining kit (Bio-Rad Silver Stain Plus) or equivalent.
14. Magnetic stirrer for mixing the dialysis solution.
15. Floating microtube rack to hold the dialysis cups.
16. Microcentrifuge.
17. NanoDrop or spectrophotometer for antibody solution analysis.

18. Agilent Bioanalyzer, TapeStation, Biorad LabChip, or equivalent equipment for nucleic acid analysis.
19. SDS-PAGE equipment.
20. Hemocytometer or an automated cell counter
21. Droplet or microwell based single cell processing platform (e.g. 10x Genomics Chromium Controller).
22. PCR cyclers.
23. Access to a next generation sequencing services.

3. Methods

3.1. General guidelines

1. Keep antibodies cooled and use an ice bath when possible.
2. Store antibodies according to manufacturer's instructions.
3. Avoid excessive agitation of antibodies. Mix by gentle pipetting or tapping.
4. A fraction of the antibody is lost in every step due to binding to tubes and tips, and in the steps required for monitoring / quality control of the reactions. A good amount of an IgG antibody to start with is 0.1-0.2 mg. Starting with less than 0.05 mg of the antibody for labeling is not recommended.
5. Use autoclaved or sterile filtered solutions in every step to avoid microbial contamination.
6. When processing cells, work as gently and quickly as feasible to prevent stress-induced transcriptomic changes. Avoid excessive centrifugation speeds ($>400 \times g$). Pipette slowly and use wide orifice tips whenever possible to prevent damage to the cells from shearing forces. However, regular-bore tips are usually needed to resuspend cell pellets after centrifugation. Keep the cells on ice and start cell capture as soon as the sample is ready, preferably within 30 minutes.

3.2. Azide-modified oligo in conjugation

1. 5'-azide-modified oligos are readily available from manufacturers. We have successfully used HPLC purified 5'-azide-oligos without additional desalting or buffer exchange steps.
2. Rehydrate the lyophilized oligo with PBS to 200 μ M concentration.
3. The azide-oligos are stable in PBS for several days in fridge 4 °C. For longer term storage keep frozen at -20 °C.

4. Phosphorothioate nucleotides at 3'-end will increase nuclease resistance of the conjugated oligos.
5. The exact sequence design of the conjugated oligo depends on the scRNAseq platform and chemistry and it is out of the scope to go through them in this protocol see [11].
6. Uniformity, size, and integrity of the oligo can be checked with for example Agilent Bioanalyzer or TapeStation, Biorad LabChip, or polyacrylamide gel electrophoresis with appropriate staining (e.g. Sybr Green II, silver staining). Follow the manufacturer's or labs own protocols for using the above mentioned methods.
7. If suspecting problems to arise from additives in the oligo prep, ethanol precipitation or desalting column purification can be used for clearing the oligo prep.

3.3. Preparation of the antibody for activation

The first step in conjugation is to make sure that the target antibody is free of conjugation-interfering additives, is in a suitable buffer, and is in the right concentration for the activation (see **Note 1**). The removal of molecules close in size to the antibody, including gelatin and BSA, require affinity purification, which is beyond the scope of this chapter. Small molecules such as azides or tris can be removed with a simple size-exclusion purification, for which our preferred method is dialysis in a dialysis cup. This requires very little hands-on time, is very affordable and easy, allows simultaneous buffer exchange, and ensures that not much antibody is lost in dialysis. Desalting columns or size-exclusion spin filters can also be used.

1. If the antibody is free of additives (purchased as pure or conjugation ready), adjust concentration to 1 mg/mL with PBS and proceed directly to the activation step. Otherwise, start by recording the absorbance profile of the target antibody and a control pure antibody with the NanoDrop or other spectrophotometers. A280 absorbance provides an estimate of the antibody concentration, and the deviations in the shape of the absorbance curve compared to the pure antibody may reveal the presence of some additives or impurities in the antibody solution. Compare absorbance profiles of the pure and target antibodies. Azide and many other additives absorb light at wavelengths that will show in the absorbance curve.
2. Prepare the dialysis vessel with pre-cooled PBS and a magnetic stirrer in a cold room or in a fridge.
3. Transfer the antibody solution to a 3.5 kDa MWCO dialysis cup and attach it to a floating rack. Place the rack into the dialysis tank with the dialysis cup membrane in the PBS and start stirring.

4. Dialyze for a minimum of 1 h on 1 L of PBS (overnight dialysis works as well).
5. Change the PBS to fresh.
6. Dialyze for a minimum of 1 h on 1 L of PBS.
7. Depending on the volume of the antibody (>1 mL) and the concentration of the removed molecule (>100 mM), a third round of dialysis may be required, as the removal is dependent on the volume ratios of the antibody solution and the dialysis solution (see **Note 2**).
8. Measure the concentration and purity of the antibody with the NanoDrop or other available spectrophotometer. If the concentration is close to 1 mg/mL and no obvious impurities are present, proceed to the activation step.
9. If the concentration is lower than 0.75 mg/mL, concentrate the antibody with the 35,000 MWCO spin column (see **Note 3**).
10. Alternatively, concentrate the antibody by placing the dialysis cup on a 10 kDa or higher MW dry PEG-bed dialysis membrane, touching the PEG. The PEG will absorb water from the antibody reservoir and concentrate the antibody without antibody loss (a slow process, not recommended) (see **Note 4**).
11. Measure the concentration with a NanoDrop or other available spectrophotometers. If the concentration is close to 1 mg/mL (6.67 μ M), proceed to the activation step or store the antibody in fridge. If the antibody is too concentrated, dilute with sterile PBS.

3.4. Antibody activation with DBCO-PEG₄-NHS

To activate the antibody for azide-oligo conjugation, DBCO-PEG₄-NHS molecules are covalently attached to the antibody. The NHS group reacts with the primary amines in lysine residues and the amino-termini of the polypeptide chains. The NHS-amine reaction is very efficient in the right buffer, pH, and concentration of the components (see **Note 5** for NHS stability and storage). The strategy in this protocol is to regulate the overall oligo conjugation level by limiting the number of DBCO groups incorporated per antibody. The standard 30 times molecular ratio of DBCO-PEG₄-NHS to the antibody recommended in some protocols produces antibodies with a very high number of active DBCO groups. And, if not limited by the availability of the oligo in the conjugation step, will result in antibodies with very high oligo labeling. While this could increase sensitivity, and generally be desired for weak antibody-epitope pairs, we have opted to regulate the level of activation by using the

DBCO-PEG₄-NHS-to-antibody ratio in the range of 1 to 10–20.

1. Aliquot 100 µg or more of the clean target antibody to a low-protein-binding microcentrifuge tube.
2. Add DBCO-PEG₄-NHS to reaction, mix, and incubate for 30 minutes at room temperature. The amount of reactive DBCO groups per antibody depends on molecular ratio of antibody to DBCO-PEG₄-NHS in the reaction.
 - a. **For low number of DBCO handles** use 5-10 molar excess. For 100 µg of IgG add 0.33-0.66 µl of 10 mM DBCO-PEG₄-NHS.
 - b. **For high number of DBCO handles** per antibody use 20 times molar excess of DBCO-PEG₄-NHS to antibody. For 100 µg of IgG add 1.32 µl of 10 mM DBCO-PEG₄-NHS.
3. To quench the NHS present in free DBCO-PEG₄-NHS still in the reaction add 1/10th of the reaction volume of 1 M tris pH 8.0.
4. Transfer the activated antibody to a 3.5 kDa MWCO dialysis cup, and place the cup into a floating rack in the dialysis tank with fresh, cold PBS, and start stirring.
5. Dialyse for 1 h at minimum in 1 L of PBS, or overnight for convenience.
6. Transfer the dialysis cup into 1 L of fresh PBS and dialyse for another 1 h.
7. Proceed to measurement of the activation.

3.5. Verifying DBCO incorporation with absorbance measurement

The DBCO absorbance curve at 235–400 nm differs from that of proteins allowing absorbance based measurement of the level of the DBCO-PEG₄-NHS incorporation. DBCO has an absorbance peak at the 309 nm wavelength (A₃₀₉) at which antibodies do not absorb. This can be used to measure the amount of DBCO in the protein-DBCO solution. To resolve the concentration of the antibody in the mix, the A₃₀₉ absorbance value with a correction factor (CF) of 1.089 is used to calculate DBCO absorbance at 280 nm (A₂₈₀), which is then subtracted from the combined A₂₈₀ to get the A₂₈₀ value for the antibody in the solution (see **Note 6** and Fig. 2 for the DBCO and antibody concentration calculation).

1. Measure the sample with a NanoDrop or other available spectrophotometers. If using a NanoDrop, record the A₂₈₀ and A₃₀₉ and/or save the antibody measurement results as a native NanoDrop data file with the .ndv ending. The .ndv file contains the absorbances of the whole measurement range and can be opened in a text editor for the recovery of the A₂₈₀ and A₃₀₉ values to be used

in calculations or to plot absorbance curves like in figure 2.

2. At the lowest suggested DBCO antibody activation ratio (1:5) the amount of attached DBCO is close to the reliable absorbance based detection limit and may not show up clearly in the A309.
3. The high DBCO derivatization attained with the 1:30 ratio may be above the linear DBCO correction factor range, and thus the calculated IgG concentration and the actual IgG/DBCO ratio values are not accurate in high DBCO incorporation level IgGs. The highest labeling shows up as a size shift in the 50 kDa antibody band in the SDS-PAGE gel (Fig. 3).
4. If the desired DBCO derivatization is detected, proceed to the oligo conjugation step. A sufficient DBCO-to-IgG ratio is 10–30 molecules of DBCO per IgG.
5. The DBCO in an activated antibody should not react with other biological molecules and is stable for short-term storage at 4 °C. However, we proceed to the oligo conjugation step immediately.

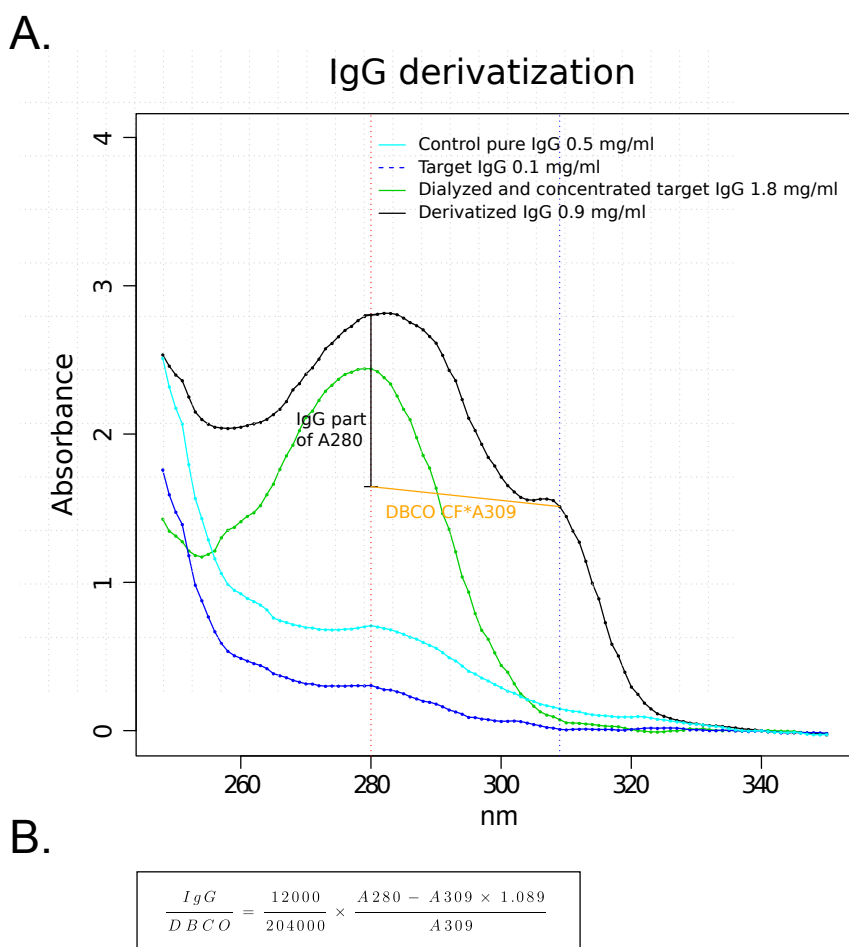


Figure 2: Example absorbance curves and DBCO ratio calculation. (a) Starting, purified, and derivatized IgG and column flow-through show different differing absorbance curves. DBCO’s unique absorbance peak at A309 is used to calculate the labeling efficiency. (b) Equation to calculate DBCO-to-IgG ratio after derivatization

3.6. Antibody conjugation

The presented strategy limits the oligo labeling at the derivatization step. Hence, it is not absolutely critical to limit the conjugation reaction by limiting the amount of oligo. However, a high concentration of free oligo is difficult to remove from the final conjugate, and therefore, the amount of oligo should be kept reasonable during the conjugation step. We recommend use of 2 to 5 times the molar excess of the oligo to DBCO as calculated from the antibody/DBCO absorbance measurement. The optimal amount of oligo depends on the number of incorporated DBCO groups in each antibody and the desired number of conjugated oligos per antibody. The detection of target antigens with low levels of expression may benefit from a higher number of oligos in the final antibody conjugate. A good starting point is to have 5–10 times more oligo than antibody in the conjugation reaction. The 1 $\mu\text{g}/\mu\text{L}$ IgG antibody concentration corresponds to 6.67 μM molar concentration as calculated with IgG MW of 150,000 g/mol. Hence, for each 100 μg of derivatized IgG, add 16.7–33.3 μL of 200 μM 5' azide-oligo.

1. Transfer the desired amount of the activated antibody to a protein low-binding microcentrifuge tube. Save a small sample of the activated antibody for SDS-PAGE gel analysis later.
2. Add 200 μM of the azide-oligo solution into the antibody solution, and mix by pipetting. Use 1.67–3.33 μL of the 200 μM azide-oligo solution per 10 μg of activated IgG.
3. Incubate for 3–4 h at room temperature, or for convenience overnight at 4 °C.
4. Proceed to the purification step.

3.7. Removal of free oligos from the antibody-oligo conjugate

To avoid a high background signal, the free oligo should be removed from the antibody-oligo conjugate. Also, to improve long term storage, the reaction buffer should be changed to an antibody/oligo storage buffer. In the presented 100 μg scale this may sometimes be problematic, due to losses in purification/exchange. Furthermore, as the chemical properties of the antibody have been modified by oligo addition, some techniques may not work as expected (see **Note 7**). Size-exclusion filtering with 100 kDa cutoff spin filters is our recommended option. To avoid losses due to the antibody binding to the filter, first block the filter with BSA. This should not interfere with the subsequent application and could also help in stabilization of antibodies in the storage. Antibody storage preservatives, such as trehalose and/or antimicrobial agents like sodium azide, can also be added.

1. Wash and block the 100 kDa MWCO spin filter with 1% BSA-PBS.

2. To wash, fill spin filter with 500 μ l PBS and spin at 12,000 \times g for 2 min.
3. To block, add 500 μ L of 1% BSA-PBS solution and spin for 5 min.
4. Remove excess blocking solution by spinning the filter shortly at 1000 \times g upside down in a microtube or by flicking the spin filter empty with hand.
5. Load the conjugated antibody into the spin filter and adjust the volume to 500 μ L with sterile PBS.
6. Spin at 12,000 \times g to concentrate the antibody-oligo conjugate. The time depends on the amount of the antibody, but 3–5 min is usually enough.
7. Discard the flow-through and add 500 μ L of fresh PBS into the filter/spin column and spin at 12,000 \times g until the surface reaches the collection mark. Discard the flow-through.
8. Repeat step 7 three times.
9. Collect the purified oligo-antibody to the microcentrifuge tube by spinning the filter/spin column at 1000 \times g for 1 min upside down in a clean low-protein-binding tube.
10. Proceed to the analysis of the oligo-conjugated antibody.

3.8. Analysis of the oligo-conjugated antibody by SDS-PAGE

The produced oligo-conjugated antibody should be analyzed for its level of labeling and if the labeling affects epitope recognition. The conjugation efficiency and the number of oligos per antibody can be estimated by separating the antibody and appropriate controls on a SDS-PAGE gel and staining the gel with protein and single-strand DNA-specific dyes. A convenient way is to have a fluorescent protein dye in the gel (for example stain-free gels with trihalo compound or SYPRO Orange), so that the proteins can be detected immediately after the gel is run and developed with a UV-light. Fluorescent ssDNA-labeling dyes like SYBR Green II or silver staining can be used to detect the oligos in the gel. We use silver staining, which is sensitive and will stain both polypeptides and single-stranded oligos, and which can be used directly after protein detection (Fig. 3).

1. Separate the purified antibody-oligo conjugates and appropriate controls in SDS-PAGE gel in standard running and loading buffers and protocol. Preferably use 4–15% gradient gel (e.g., Bio-Rad TGX stain-free precast gel), which separates the target molecules efficiently. Choose the appropriate samples to run from the following:

- a. 1 μg and/or 2 μg of unconjugated antibody for conjugate quantification:
 - Protein bands: antibody light 25 kDa and heavy 50 kDa chain bands.
 - Use these to estimate the concentration of the purified antibody conjugate.
 - b. Conjugated antibody 1-2 μg for estimation of conjugation efficiency, concentration, and clearance of the free oligo.
 - Protein bands: Should form higher molecular weight ladder above 50 kDa heavy chain. Still contain some non-labeled IgG light 25 kDa and heavy 50 kDa chain bands.
 - ssDNA bands: Non-purified antibody may still contain strong band of free oligo.
 - If already purified, sample should not have a strong oligo band. Note that ssDNA is only visible in appropriate staining. In our case the oligo runs as a ~ 20 kDa band.
 - If BSA was used in clearing step or was otherwise added to the storage buffer, it shows as 66.5 kDa band.
 - c. Optional: Activated non-conjugated antibody to see the level of derivatization.
 - Protein bands: Contains IgG light 25 kDa and heavy 50 kDa chain bands.
 - A detectable size shift in heavy chain compared to non-activated heavy chain indicates successful strong activation.
 - d. Optional: Use Free oligo for positive staining control and as a size marker for the oligo.
 - Oligo band is only visible after silver staining (or fluorescent ssDNA staining), in our case it runs as ~ 20 kDa band.
2. After running the gel, rinse it briefly with deionized water and document with appropriate fluorescent imaging or scanning apparatus. Alternatively, digital imaging allows for a computer-based quantification of the bands:
 - a. Develop the gel with fluorescent protein dye with UV-light and document it on UV-table with digital imager. Different Fluorescent dyes may require specific development. Follow the manufacturer's instructions.
 - b. Document the silver stained gel with incandescent light imager or a scanner.
 3. Quantify the bands from the gel with dedicated gel image software (commercial) or plugins in ImageJ (freely available from the NIH).
 4. The ladder-pattern in higher than 50 kDa molecular weight protein bands indicate strong labeling. Presence of less than 50% of non-conjugated bands (25 kDa and 50 kDa) in quantification already indicate very strong labeling.

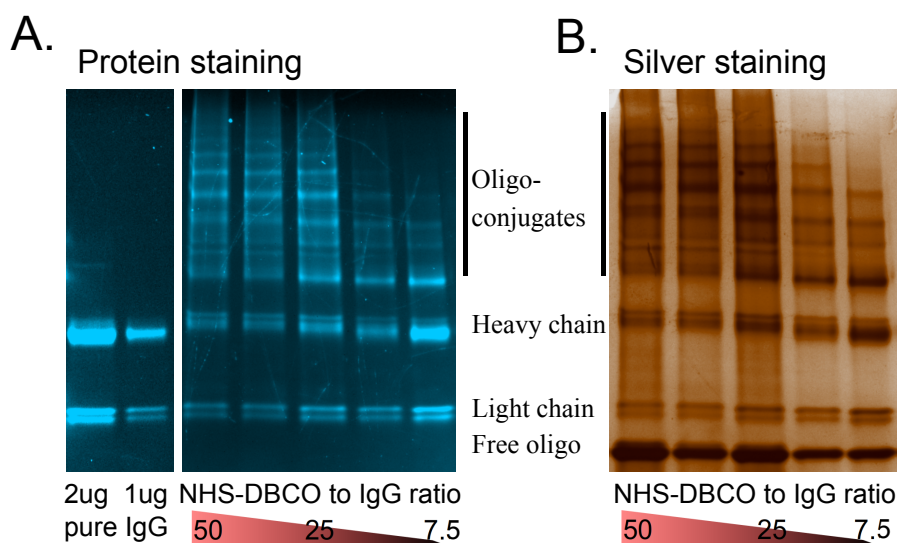


Figure 3: Oligo-IgG in SDS-PAGE. (a) Conjugated anti-CD38 IgG in Bio-Rad stain-free gel in pseudo-color. Left panel: pure-antibody controls; right panel: conjugated IgGs with constant oligo-to-IgG ratio and varying NHS-PEG₄-DBCO-to-IgG ratio as indicated with numbers below the gel image. (b) The same gel after silver staining. The free oligo travels below the light chain and is clearly visible in pre-purified samples in the gel.

3.9. Testing antibody binding on cells and initial titration

Before use in scRNA-seq experiments, the labeled antibody should be tested for antigen recognition specificity, and the right concentration for cell labeling should be determined. If the antibody's antigen recognition site contains lysine residues, it is possible that the antibody activation or oligo conjugation blocks the binding of the antibody to its epitope. This can be tested in flow cytometer assay by labeling the antigen-containing cells with the oligo-conjugated antibody, and then detecting cell bound oligo-IgGs with fluorescently labeled secondary antibody. This method can also be used to find a good antibody concentration for cell staining for scRNA-seq experiments. However, based on our experience, the flow cytometric signal is usually stronger than the standard oligo-antibody staining signal in single-cell sequencing applications. If using flow cytometry based titering, one should calibrate the staining concentration results with the used single-cell oligo-sequencing application. Methodologically the oligo-antibody cell surface staining is very similar compared to normal flow cytometry staining protocol. The critical difference is that for scRNA-seq applications, the buffers should not contain additives that would affect subsequent enzymatic steps, or that would compromise intracellular mRNA. These include divalent cations (that affect enzymatic reactions), agents that may compromise membrane integrity (mRNA leakage), and fixatives (mRNA availability). Use a staining buffer composed of Mg²⁺/Ca²⁺-

free PBS supplemented with 2% BSA and 0.02% Tween is recommended. Otherwise, for scRNA-seq one can use standard flow cytometry staining protocols such as the one described below:

1. Prepare the staining oligo-antibody master mix in staining buffer, with 50 μ L per sample. Add each antibody to the predetermined staining concentration and top up to a total volume of 50 μ L with the staining buffer. If titrating, make three- or fourfold dilution series of the tested antibody.
2. Prepare a single-cell suspension with your cell-specific protocol. We use peripheral blood mononuclear cells purified with a gradient centrifugation and then washed twice: first with PBS and then with the staining buffer.
3. Pellet the desired amount ($>200,000$ and $<2,000,000$) of cells to a microcentrifuge tube.
4. Resuspend the cells into staining buffer and incubate on ice for 30 min.
5. To wash, add 1 mL of staining buffer and spin cells down with $200 \times g$.
6. Repeat wash twice with 1 mL of staining buffer.
7. If doing a fluorescent detection, continue by staining with the appropriate fluorescent secondary antibody and flow cytometry protocol. Calculate the staining index to determine the optimal staining concentration: $(\text{MFI of positive population} - \text{MFI of negative population}) / (2 \times \text{SD of negative population})$ [12].
8. If doing single-cell mRNA sequencing with protein detection, resuspend the cells into scRNA-seq buffer ($\text{Mg}^{2+}/\text{Ca}^{2+}$ -free PBS supplemented with 0.04% BSA) and continue with the scRNA-seq protocol.

3.10. Preparation of cells for scRNA-seq

Single-cell transcriptomic analysis can be performed with any platform compatible with the oligonucleotide sequences. For example, the 10 \times Genomics Chromium cController with the 3'-single-cell solution can be used, and we recommend adhering to the manufacturer's instructions. A detailed protocol can be found in an earlier volume of this series [13].

1. Stain the cells using titrated concentrations of the antibodies as described above. Keep the cells on ice at all times.
2. Resuspend the cells to 1 \times PBS (calcium and magnesium free) containing 0.04% weight/volume BSA (400 μ g/mL), or another buffer compatible with the Chromium single-cell application (refer

to 10× Genomics demonstrated protocol CG000149). The optimal concentration range is from 700 to 1200 cells per L.

3. We recommend passing the cells through a filter, e.g., a Flowmi Tip Strainer to remove possible cell aggregates. However, note that this step can lead to the loss of some cells.

4. Count the cells using a hemocytometer or an automated cell counter. Measure the viability, e.g., using the trypan blue exclusion method. For improved accuracy, perform four counts from two independent draws for each sample and then calculate the average values. Make sure to include dead cells in the total cell count.

5. Proceed to the single-cell capture according to the manufacturer's instructions.

4. Notes

1. Target antibody: The simplest option is to buy the antibody in a conjugation-ready format. However, in many cases, the antibody clone of interest may not be available as a pure antibody, but rather is stored with stabilizing agents such as additional proteins, primary amine buffers, or azides, or a mixture of them. These additives either prevent the antibody activation step (NHS-primary amine reaction) or react with the DBCO and inhibit azide-oligo conjugation.

2. Dialysis: In each round of dialysis, the molecule to dilute out is diluted by a factor of diluent to the starting solution. If the dialysis buffer volume is 1 L and the starting material is 100 L, then the theoretical dilution is 1:10000 in this step. The sample volume usually goes up during the dialysis, causing the dilution ratio to change for the subsequent step. For example, if the volume rises to 250 L, the dilution against 1 L would be 1:4000. For these two steps, the theoretical dilution of the small molecule or the buffer exchange would hence be $1:10,000 * 4000 = 1:40,000,000$. Typically, antibodies are stored in a 3.08–7.69 mM sodium azide (0.02–0.05% (w/v)). This is ~1000 more azide than IgG in 1 mg/mL. To ensure the diluting of the sodium azide, we would suggest to dialyze 100 L of antibody solution at least two times against 1 L of PBS.

3. Spin filter: The use of spin filters is not recommended, as a significant amount of antibody is lost in each concentration step with the spin filter, due to let-through and IgG binding to the membrane.

4. NHS storage: NHS is highly reactive and hydrolyzes in aqueous solutions spontaneously in a

relatively short time. DMSO is very hygroscopic and condenses water molecules from the air. To prevent the spontaneous degradation of NHS, store DBCO-PEG-NHS dissolved in DMSO in -20 to -70 °C in a sealed container with silica gel beads, or equivalent, as a desiccative. Warm up the DBCO-PEG-NHS tube to the ambient temperature before opening to prevent water condensation on the tube.

5. DBCO measurement: The derivatization efficiency is calculated as the molar ratio of DBCO/IgG. The extinction coefficient for DBCO is $12,000 \text{ l}/(\text{M}\cdot\text{cm})$, and for IgG it is $204,000 \text{ l}/(\text{M}\cdot\text{cm})$ [14]; the DBCO A309 to A280 correction factor is 1.089. The simplified equation for the calculation of DBCO molecules per IgG is $17 * A309 / (A280 - 1.089 * A309)$.

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