Immuno-SABER enables highly multiplexed and amplified protein imaging in tissues

Supplemental Protocols

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Contents

6. General recommendations

1. Oligos and concatemer synthesis by PER

User-friendly protocols for sequence design, ordering and preparing oligo nucleotides, performing PER and storing or purifying the concatemers are available in our recent publication (Kishi, Lapan, West et al., Nature Methods, 2019 - **Reference 30**).

Conjugation oligos

The conjugation involves crosslinking of thiol-modified DNA oligonucleotides (bridges) to lysine residues on antibodies in a non-sequence-specific way.

Bridge oligos are designed as follows: /5ThioMC6-D/ tt Bridge sequence (42mer)

The oligos can be ordered from IDT either in desalted format (economical) or with purification (higher quality) and resuspended in nuclease-free water at 1 mM, and stored at -20°C.

2. Antibody-DNA conjugation

Not all commercial antibodies are provided in a formulation readily available for conjugation (for example antibodies may be provided in unpurified whole serum form or formulated with stabilizers or protectors that interfere with conjugation). Hence customized carrier-free formulation of antibodies (especially without BSA and other peptides/proteins) may be required.

We currently utilize non-specific conjugation to Lys residues and provide a simple protocol to prepare custom conjugation of antibodies (see also Agasti et al., Chem Sci, 2017). Although multiple DNA oligos can be attached to each antibody molecule for further signal amplification, our reaction conditions are optimized to achieve 1-3 oligos per antibody, in order to prioritize conserving the antigen recognition capability upon conjugation. Alternatively, other unspecific conjugation approaches or site-specific conjugation chemistries can be utilized.

The protocol below is divided into 4 days for convenience, but it can be shortened down to 1 day when a faster preparation is desired.

Materials

1. Amicon Ultra 0.5 Centrifugal Filter Unit, 50kDa (Merck Millipore, #UFC505096)
2. Amicon Ultra 2 Centrifugal Filter Unit, 50kDa (Merck Millipore, #UFC205024)
3. illustra NAP-5 Columns (GE Healthcare, # 17085302)
4. Zeba™ Spin Desalting Columns, 7K MWCO, 0.5 ml (Thermo Fisher, #89882)
5. Pierce™ DTT (Dithiothreitol), No-Weigh™ Format (Thermo Fisher, #20291)
6. DMF (N,N-Dimethylformamide) (SIGMA-ALDRICH, #227056)
7. $SM(PEG)_2$ (PEGylated SMCC crosslinker) (Thermo Fisher, #22102)
8. 3 ml Sub-Q Syringe
9. Nuclease-free water (Invitrogen 10977-015)
10. 1× PBS (diluted from the 10× stock, Thermo Fisher AM9625)
11. 0.5 M EDTA, pH 8.0 (Ambion AM9260G)
12. 1× PBS-EDTA (1 mM EDTA in 1× PBS, prepare with 500 mM pH 8.0 EDTA stock)
13. 100 µg Antibody (with carrier-free formulation) (we recommend saving a few µg of the unconjugated antibody for the gel and staining controls)
14. DNA-thiol oligo (1 mM, resuspended in nuclease-free water)
15. Tris-acetate SDS running buffer (20×) (Novex, #LA041)
16. NuPAGE 3-8%Tris-Acetate gel (1.0 mm × 10 wells) (Novex, # EA0375BOX)
17. NuPAGE LDS Sample Buffer (4×) (Thermo Fisher, NP0007)

18. PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa. (Thermo Fisher, 26619)
19. Gel scanner (such as Typhoon FLA 9000)
20. NanoDrop Spectrophotometer (Thermo Fisher)

DAY 1

DNA-thiol reduction

1. Thaw DNA-thiol oligo at room temperature (RT).
2. Rinse Amicon Ultra centrifugal filter (50k, 500 μl) with 400 μl 1× PBS, centrifuge at 5000 g for 6 min at RT.

   Note: In general, about 50 μl solution remains in the filter.
3. Dissolve DTT with 100 μl 1× PBS-EDTA to prepare 500 mM DTT solution.
4. DNA-thiol reduction reaction system: 55 μl 1× PBS-EDTA + 25 μl DNA (1 mM) + 20 μl DTT (100 μl in total), wrap the tube with aluminum foil and let the reaction to proceed for 2 h at RT.

Antibody concentration and antibody-crosslinker conjugation

5. Rinse an Amicon 0.5 Ultra centrifugal filter (add 500 μl 1× PBS and centrifuge at 5000 g for 6 min at RT making sure one side of the membrane is always facing towards the center of the centrifuge. Discard the flow-through).
6. Add 100-120 μg antibodies to the Amicon filter. Complete the volume to ~450 μl by adding 1x PBS to the filter. Centrifuge at 5000 g for 6 min at RT.
7. If the original antibody contains sodium azide or is in a different buffer, 2-3 more washes with 400 μl 1× PBS should be performed with centrifugation at 5000 g for 6 min at RT.
8. Weigh $SM(PEG)_2$ (crosslinker), and add DMF to prepare the 10 mg/ml $SM(PEG)_2$ solution.

   Note: $SM(PEG)_2$ is sensitive to moisture, hence the air exposure of both the crosslinker and the DMF stock should be minimized.
9. (Optional) Aliquot 20 μl $SM(PEG)_2$ to 2-5 new tubes and store them at -80. These $SM(PEG)_2$ stock should be used within 10 days. Alternatively, the no-weigh format pre-aliquoted crosslinker (Thermo Fisher, #A35397) can be used.
10. Reverse the Amicon 0.5 Ultra centrifugal filter and put it into a new Eppendorf tube. Centrifuge at 1000 g for 3 min at RT to collect antibody.
11. Measure the concentration of antibody with a NanoDrop (Thermo Fisher). Measure its volume with a 100 μl pipette. If the concentration is over 2 mg/ml, dilute the antibody to 2 mg/ml with 1× PBS. Calculate the mass of antibody.

    Note: In general, the volume is about 50 μl and the concentration is around 2 mg/ml.

12. Dilute 17 μl 10 mg/ml $SM(PEG)_2$ with 183 μl DMF, to prepare 200 μl diluted crosslinker solution in total at 2 nmol/ μl (0.85 mg/ml).
13. Add 1.25 μl or 1.7 μl diluted crosslinker (from the 0.85 mg/ml) to each 100 μg antibody. Vortex briefly, then add another 1.25 μl or 1.7 μl diluted crosslinker and vortex.

    Note: 1.25 μl (2.5 μl in total, 7.5 molar excess) crosslinker gives conjugated products with a distribution that has the majority of antibodies with 1 to 2 DNA oligos per antibody. 1.7 μl (3.4 μl in total, 10.3 molar excess) crosslinker shifts the distribution to 2 to 3 DNA oligos per antibody and reduces the amount of antibodies that are not conjugated with DNA oligos. The trade-off is that higher amount of DNA oligos per antibody may increase the risk of disrupting antibody's affinity and cause nonspecific binding. When fresh batches of crosslinker or no-weigh format single aliquots are used, the crosslinking efficiency might vary, hence if the level of conjugation is higher than desired, the molar excess of the crosslinker can be decreased further accordingly, to obtain 1-3 oligos per antibody.
14. Seal the antibody tube with parafilm, wrap with aluminum foil and react at 4°C for 1.5-2 h on a rotator.

Removing DTT from DNA oligonucleotides

15. Wash the NAP-5 column with 10 ml nuclease-free water. Add the water when the column stops dripping.
16. Prepare 10 labeled 1.5 ml tubes in a rack.
17. Carefully add the reduced DNA oligos to the column.
18. Slowly add 100 μl nuclease-free water for four times and collect the flow-through in the 1st tube. The water should be added carefully and slowly to avoid disturbing DNA oligos in the column.
19. For elution add 500 μl nuclease-free water directly.
20. Immediately collect the eluate following this scheme: 3 drops in the 2nd and 3rd tubes, and 2 drops in the remaining tubes.
21. Measure the concentration of DNA oligos starting with the 3rd tube using the NanoDrop in the ssDNA mode until the tube that the concentration decreases substantially.
22. Identify the tubes with the highest ssDNA concentration and mix them together (typically 2 tubes with the highest concentration are pooled together).
23. Measure the concentration of the mixed solution.

Removing excess crosslinker

24. Prepare Zeba Spin Desalting Columns in 2 ml Eppendorf tubes. Centrifuge at 1500 g for 1 min at RT. After centrifugation, mark the side of the column where the resin is slanted upward.

25. Wash the column three times with 350 µl 1x PBS. Centrifuge at 1500 g for 1 min at RT (place the column in the centrifuge with the marked side facing outward).
26. Put the column to a new 1.5 ml Eppendorf tube.
27. Add the antibody into the column.
28. Rinse the tip and the tube with 100 µl 1× PBS and add to column.
29. Centrifuge at 1500 g for 2 min at RT.

Combining Antibody-Crosslinker with reduced DNA oligos

30. Add 7.5 nmol DNA to each 100 µg antibody (Molar ratio of antibody/ssDNA ~ 1:11).

    Note: In general, the volume of DNA added to antibody is 50-100 µl.

31. Wrap in aluminum, and react at 4°C overnight, on a rotator.

    Note: The reaction times can be substantially shortened for a faster protocol (especially when incubation is performed at RT or 37°C).

DAY 2

Removing unconjugated DNA oligos

32. Add 1 ml 1× PBS to an Amicon Ultra 2 ml 50 kDa centrifugal filter unit, centrifuge at 2000 g for 10 min at 4°C.
33. Pipette the antibody (~200 µl) into the filter.
34. Add 1 ml 1× PBS into the filter.
35. Centrifuge at 2000 g for 20 min at 4°C.

    Note: In general, ~50 µl solution remains in the filter at this point.

36. Discard the liquid in the collection tube. Add 1.9 ml 1× PBS and centrifuge at 2000 g for 20 min, at 4°C.
37. Repeat step 36 for 5 times.
38. Add 50 µl 1× PBS into the filter carefully.
39. Discard the tube at the bottom. Reverse the filter unit, and centrifuge at 1000 g for 3 min at 4°C.
40. Recover the antibody (~100 µl) and take it into a new tube.
41. (Optional) To collect any leftover antibody, add 100 µl 1× PBS into the filter, and centrifuge at 1000 g for 3 min at 4°C, add the collected solution onto on the antibody from the previous step (~200 µl antibody solution in total).
42. Temporarily store the antibody-DNA conjugate at 4°C.

DAY 3 (quality control)

Running the denaturing non-reducing PAGE gel

1. Prepare the NuPAGE 3-8% TA gel for loading, with 1× Tris-acetate SDS running buffer.
2. Pre-run the gel at 100 V for 10 min.
3. Prepare 12 µl loading mix with 2-3 µg antibody and 4× LDS Sample Buffer.

   Note: We recommend also running the unconjugated antibody on a separate lane for reference. It is expected that ~50% of the antibody may be lost throughout the process, so an adjustment of the concentrations may be necessary.
4. Denature the proteins at 98°C for 3 min to denature the proteins using a benchtop heating block.
5. Load 10 µl sample per well and load 3 µl protein ladder.
6. Run the gel at 30 V until the sample enters the gel. Then increase the voltage to 100 V and run for 3 h (until the heavy orange band of the ladder reaches close to the bottom). After the run, transfer the gel to a big container.
7. Wash with tap water and microwave ~1.5-2 min.
8. Take it out when the water is close to boiling. Put the container on the shaker and shake the gel for 5 min.
9. Discard the water and repeat the steps 7-8 twice.
10. Discard the water and add enough SimpleBlue Safe stain to merge the gel and microwave 30 s. Place it on a shaker and stain the gel for 10 min.
11. Discard the staining solution and wash the gel with water. It is ideal to wash the gel overnight but it can also be scanned after 2-3 h.

DAY 4

Image the gel using a gel scanner.

Successful conjugation is visualized in the form of a band shift for the antibody band. Multiple higher bands may be visible, showing the antibodies conjugated with 1, 2, or 3 oligos. Also, a faint band for the unconjugated antibody might be visible. This fraction can be optionally further removed ion exchange chromatography or by the DNA-affinity pull-down purification protocol as below.

3. Purification of DNA-conjugated antibodies using toehold displacement-mediated DNA affinity pull-down (optional)

Materials:

1. Pierce High Capacity Streptavidin Agarose (Thermo Fisher, 20357)
2. Amicon Ultra 2 Centrifugal Filter Unit, 50 kDa (Merck Millipore, #UFC205024)
3. Biotin-modified capture DNA oligos (prepared by standard desalting)
4. Toehold displacement DNA oligos (prepared by standard desalting) (1 mM, resuspended in nuclease-free water)
5. Bovine Serum Albumin (BSA) (nuclease and protease-free) (AmericanBio, CAS 9048-46-8)

Blocking buffer: 2% BSA + 0.1 % Triton X-100 (v/v) in 1× PBS

Design of capture and toehold DNA oligos

The purification is based on the pull-down of the antibody DNA-conjugate onto streptavidin beads coated with the biotin-capture oligos to remove the unconjugated antibodies, followed by elution of the conjugates from the beads by toehold-mediated strand displacement (Guo et al., 2017, Quantitative Biology; Zhang et al., 2009, *Journal of the American Chemical Society*). The DNA oligos are designed using NUPACK Design tool (www.nupack.org) (**References 36, 51, 52**). The pull-down DNA oligo ('capture oligo') is made of 31 nucleotides which constitute a 16-nt domain ('B1') that binds to the DNA conjugated onto the antibodies and a 15-nt domain ('B2') that functions as the toehold binding sequence. The toehold displacement oligo is designed to be complementary to the capture oligo.

A sample code is attached below to enable users design new sequences by changing the DNA sequence in domains B1 and B2. Other capture and toehold sequences used for the manuscript are listed in **Supplementary Table 4**.

Sample code for designing the capture and toehold displacement DNA oligos for bridge strand bc42_0

```
#
# design material, temperature, and trials
#
material = dna
sodium = 0.167
```

```
temperature[K] = 296.15 # optional units: C (default) or K
trials = 1
#
# target structures
#
structure s1 = U42
structure s2s3 = D31 +
structure s3 = U31
#
# sequence domains
#
domain B1 = AATTCTATGACACCGCCACGCCCTATATCCTCGCAATAACCC
domain B2 = ATCCTCGCAATAACCC
domain toehold = N15
#
# strands (optional, used for threading sequence information
# and for displaying results)
#
strand strands1 = B1
strand strands2 = toehold B2*
strand strands3 = B2 toehold*
#
# thread strands onto target structures
#
s1.seq = strands1
s2s3.seq = strands2 strands3
s3.seq = strands3
#
# target test tubes
# (htube should contain only target structures hairpin1 and
# hairpin2, itube should contain only target structures
# initiator1 and initiator2)
tube htube = s1 s2s3 s3
# target concentrations for target structures in test tubes
```

```
# default concentration: 1.0e-6 M
#
htube.s1.conc[uM] = 1.0 # optional units: M (default), mM, uM, nM, pM
htube.s2s3.conc[uM] = 10
htube.s3.conc[uM] = 10
#
# design against all off-target ordered complexes of up to this
# number of strands (design against homodimers and heterodimers)
#
htube.maxsize = 2
#
# prevent sequence patterns
#
prevent = AAAA, CCCC, GGGG, UUUU, KKKKKK, MMMMMM, RRRRRR, SSSSSS,
      WWWWWW, YYYYYY
```

Purification of conjugated antibodies

1. Spin down high capacity streptavidin agarose resin (100 μl beads for each antibody, equivalent to 200 μl from the 50% slurry). Centrifuge at 500 g for 1 min at RT.
2. Remove supernatant carefully, without perturbing the beads and wash with 1000 μl 1× PBS 3 times, vortex and spin down each time.
3. Add 300 μl 1× PBS to the beads.
4. Add 10 μl 1 mM biotin-labeled DNA oligos to the beads and vortex. Rotate the tube in a rotator for 30 min and spin down. Carefully discard the supernatant.
5. Wash the beads with 500 μl 1× PBS twice.
6. Add 200 μl **blocking buffer** into beads, and rotate for 1 h at RT.
7. Spin down and discard supernatant.
8. Add 200 μl blocking buffer to the beads.
9. Add antibodies to beads, and vortex gently. Rotate at 4°C in the cold room for 1 h or overnight.
10. Transfer the supernatant that contains the unconjugated antibodies into a new tube. Wash with **blocking buffer** (precooled at 4°C) and spin down at 500 g for 1 min at 4°C. This step is ideally performed in the cold room but is not required.
11. Repeat the previous step one more time.
12. Add 200 μl blocking buffer to the beads.

13. Add 20 μl 1 mM toehold displacement DNA oligos and rotate at RT for 3 h.
14. Centrifuge at 500 g for 1 min at RT and collect the supernatant (the eluate).
15. Wash with 300 μl blocking buffer 3 times and pool the supernatants with the eluate in step 14.

Cleaning up and concentrating the purified DNA-conjugated antibodies

1. Add 1 ml 1× PBS to an Amicon Ultra 2 ml 50 kDa centrifugal filter unit, centrifuge at 2000 g for 10 min at 4°C.
2. Add the eluted antibodies (pooled from steps 14 and 15 above) into the filter unit.
3. Centrifuge at 2000 g for 20 min at 4°C.
4. Discard the flow-through in the collection tube. Add 1.9 ml 1× PBS and centrifuge at 2000 g for 20 min at 4°C.
5. Repeat step 4 for 5 times.
6. Add 50 μl 1× PBS into the filter carefully.
7. Discard the bottom tube and reverse the filter unit, centrifuge at 1000 g for 3 min at 4°C.
8. Add the antibody (about 100 μl) into a new tube.
9. Use 100 μl 1× PBS to rinse the filter once more, centrifuge at 1000 g, 3 min, 4°C.
10. Mix the two antibody solutions (about 200 μl in total).
11. Store the antibody-DNA conjugates at 4°C for short term (a few weeks).

For longer term storage, a few different alternatives exist and the ideal buffer and storage conditions may be different from antibody to antibody.

- For long-term storage at 4°C addition of EDTA, BSA and sodium azide is recommended.
- For long-term storage at -20°C glycerol stocks can be prepared.
- For long-term storage at -80°C aliquots could be prepared (single thaw) and flash-frozen with liquid nitrogen.

4. Immuno-SABER user-friendly protocols (for cultured cells and FFPE sections)

Materials

1. PBS
2. 4% Paraformadehyde (PFA)
3. 100 mM NH_4Cl (Sigma 09718) in PBS
4. $BS(PEG)_5$ (Thermo Fisher #21581)
5. Sheared salmon sperm DNA (Thermo Fisher #AM9680)
6. Dextran sulfate (Millipore #S4030)
7. EDTA (0.5 M), pH 8.0, RNase-free (Thermo Fisher, AM9260G)
8. Formamide (deionized, Ambion #AM9342)
9. Bovine Serum Albumin (BSA) (nuclease and protease-free) (americanBio, CAS 9048-46-8)
10. Mounting medium such as SlowFade with DAPI (Invitrogen #S36938) (optional)
11. DAPI (Invitrogen #D1306)
12. Triton X-100 (Sigma T8787)
13. 20× SSC (Ambion AM9763)
14. Tween-20 (Sigma P9416)
15. Mounting medium (optional) such as SlowFade with DAPI (Invitrogen #S36938)
16. Primary Concatemers
17. Secondary concatemers (optional)
18. Imagers (100 μM stock)
19. DNA-conjugated antibodies

4a. Buffers

4× Hyb mix:

- 8× SSC
- 40% Dextran Sulfate (wt/vol)
- 0.4% Tween-20

4x Hyb mix (40 ml): 16 g of Dextran Sulfate, 16 ml of 20x SSC, 160 µl Tween-20, H2O to 40 ml. First measure 16 g of Dextran Sulfate and transfer into a 50 ml Falcon tube. Then add the 20× SSC and Tween-20. Add H_2O to a volume of roughly 35-38 ml and rotate 1-2 days to mix all components. Finally, add H_2O to adjust the final volume to 40 ml and mix again. Can be stored at RT for several months.

Due to its high viscosity, it is recommended to use a positive displacement pipette to transfer the 4× Hyb mix, but if one is not available, a normal pipette with a blunted pipette tip can be used.

Concatemer hybridization buffer: to be prepared fresh before use

- 2× SSC
- 10% Dextran sulfate
- 0.1% Tween-20
  - These chemicals are included in the 1× Hyb mix, which can be prepared as above.
- 30% formamide (deionized, Ambion #AM9342).
- 0.2 mg/ml sheared salmon sperm DNA (Thermo Fisher #AM9680, diluted from 10 mg/ml stocks)
- 50-150 nM PER concatemers (each) (1:20 to 1:7.5 dilution from 100 µl PER mix)

Note: Starting primer concentration in the *in vitro* PER mix is considered a proxy for the concatemer concentration after the reaction.

Example hybridization mix (125 µl): 31.25 µl 4× FISH master mix, 37.5 µl 100% Formamide, 2.5 µl sheared salmon sperm DNA, 12.5 µl 1 µM concatemer 1, 12.5 µl 1 µM concatemer 2, 28.75 µl nuclease-free H_2O.

Displacement buffer: 1× PBS + 50-60 % Formamide

We recommend flowing displacement buffer through the chamber or over the tissue multiple times to ensure complete signal removal. Formamide concentration can be increased to 60% as long as you are not using 30 mer branches.

Note: Hybridization temperature might need to be further adjusted depending on the branches being used. We recommend using a temperature at least 1°C lower than the lowest melting temperature of your branch sequences (see Ref. 30). Note that formamide concentration can also be adjusted instead of oven/thermocycler temperature.

Post-fixation solution: 5 mM $BS(PEG)_5$ (Thermo Fisher #21581) in 1× PBS, pH 7.5

Prepare the 250 mM stock $BS(PEG)_5$ (Thermo Fisher #21581) in DMSO as described in manufacturer's protocol. Make 10 µl aliquots and store at -20°C. Before use dilute 1:50 in 1× PBS, pH 7.5.

BSA blocking buffer: 2% BSA + 0.1 % Triton X-100 (v/v) in 1× PBS

Antibody blocking buffer: 2% BSA + 0.1 % Triton X-100 (v/v) in PBS supplemented with 0.2 mg/ml sheared salmon sperm, 4 mM EDTA (diluted from 0.5 M stock), up to 0.05% Dextran sulfate (diluted from 10 or 50% stock, see General Recommendations Section below).

Triton wash buffer: 0.1 % Triton X-100 (v/v) in 1× PBS

4b. Preparing the cultured cells

Additional Materials

1. Cultured cells on coverslips (we typically use ibidi glass-bottom µ-slides (ibidi #80826).

Preparing the cell chamber

1. Plate cells on 8-well ibidi glass-bottom µ-slides.
2. Grow until 50-60% confluency (1-3 days depending on the cell type and seeding cell count). Proceed to fixation when the desired confluency is achieved.
3. Wash cells with PBS 2 times at RT.
4. Fix with 4% PFA in PBS pH 7.4 for 30-45 min at RT.
5. Wash with PBS for 5 min at RT.
6. Quench with 100 mM NH_4Cl in PBS for 20 min.
7. Wash with PBS for 2×2 min.
8. Block and permeabilize with **BSA blocking buffer** for 3 x 10 min.

4c. Preparing the FFPE tissue sections

Additional materials

1. ImmEdge Hydrophobic Barrier PAP Pen, Vector Laboratories #H4000
2. (optional) Removable chamber (ibidi, #80381)
3. FFPE tissue block, microtome, antigen retrieval system

Tissue preparation

1. Cut 5 µm sections with a rotary microtome, collect the sections in a water bath at 30˚C, and transfer onto positively charged glass slides.
2. Bake section at 60˚C for 2 h.
3. For antigen unmasking, place slides placed on a PT-Link instrument (Agilent), which allows the entire pre-treatment process of deparaffinization, rehydration and epitope retrieval (with citrate buffer) to be combined into a single step. After antigen retrieval, sections can be optionally stored in PBS at 4˚C up to 1-3 weeks.
4. For staining, wash sections in PBS for 15 min.
5. Briefly dry the periphery to outline the sample with a hydrophobic pen or enclose in a removable chamber.
6. Block samples for 3×20 min with **BSA blocking buffer**.

4d. Immuno-SABER staining for cells and FFPE sections

Antibody staining

1. Dilute DNA-conjugated primary antibodies in **antibody blocking buffer**, and apply onto the sample. Apply the antibodies on the samples overnight at 4˚C in a humidified chamber. Depending on the sample and antibody this step can be shortened to 1 h when performed at room temperature or at 37°C.
2. Wash with **BSA blocking buffer** for 3 ×10 min.
3. Wash with PBS 2×5 min to remove the excess BSA.
4. Post-fix with **post-fixation solution** for 30 min.
5. Wash with PBS for 2 min.
6. Quench in 100 mM NH_4Cl in PBS for 5 min.
7. Wash with PBS for 2 min.
8. Wash with **Triton wash buffer** for 15 min.

Concatemer hybridization

9. Dilute the previously prepared 600-650 nt long primary PER concatemers in **concatemer hybridization buffer** following the example above. Incubate with the samples at 37°C for 1 h to overnight in a humidified chamber.
10. After concatemer hybridization, wash samples for 5 min at RT with 45-50% formamide in PBS.

    Note: This step helps to reduce the background binding. It can be optionally skipped, if branching will be performed.
11. Wash 3×10 min with PBS + 0.1% Triton X-100 at 37°C.

(Optional) Branching

12. Dilute the previously prepared 400-450 base long secondary PER concatemers in **concatemer hybridization buffer** following the example above. Incubate with the samples at 37°C for 1 h to overnight in a humidified chamber.
13. After concatemer hybridization, wash samples for 5 min at RT with 45% formamide in PBS.
14. Wash 3×10 min with **Triton wash buffer** at 37°C.
15. (Optional: Iterative amplification) Perform a third round of concatemer hybridization for higher signal by repeating steps 12-14 for the tertiary concatemer. We recommend shorter (<250 bases) lengths for the tertiary concatemer.

    Note: For iterative amplification the wash temperatures after concatemer hybridizations can be raised to 42°C and the 45-50% formamide in PBS wash step can be performed once as a final wash at the end of all iterations (rather than after each amplification round).

Imager hybridization

16. Hybridize imagers at 0.2-1.5 µM final concentration in **Triton wash buffer** for 1 h at RT in a humidified chamber (hybridization duration with the imagers can be significantly decreased for faster preparation).
17. Wash for 5 min wash with **Triton wash buffer** at 37°C.
18. Wash 2 × 5 min wash with PBS at RT.
19. Stain with 4 µg/ml DAPI in PBS for 10 min.
20. Wash twice for 1 min with PBS.
21. Embed as appropriate for the experiment.

    For experiments with a single round of imager hybridization (no multiplexing or only spectral multiplexing) samples can be embedded in SlowFade with DAPI.

Exchange imaging

22. Image with an appropriate wide-field or confocal microscope. Depending on the experiment, one or more planes can be acquired to sample the targets in the tissue properly.
23. Remove imagers with 10 min incubation at RT in **displacement buffer** (several exchanges of the buffer is recommended for this step).
24. Wash 2 × 5 min wash with PBS at RT.
25. Perform a new round of imager hybridization as above.

4e. Immuno-SABER with secondary antibodies

For certain applications where signal amplification is desired with low levels of multiplexing, DNA-conjugated secondary antibodies can be utilized instead of primary conjugates. In this case, the conjugation protocol above can be applied on secondary antibodies. For the staining, the general protocol stays similar to above, but a 2-step indirect antibody staining is done as below after the initial blocking step.

Antibody staining

1. Perform the primary antibody incubation with unconjugated primaries as usual for the samples of choice (can be done with **BSA blocking buffer**, without the additional reagents in the **Antibody blocking buffer**).
2. Wash with **BSA blocking buffer** for 3×10 min.
3. Dilute DNA-conjugated primary antibodies in **antibody blocking buffer**, and apply onto the sample. Incubate the antibodies on the samples overnight at 4˚C in a humidified chamber. Depending on the sample and antibody this step can be shortened to 1 h when performed RT or at 37°C.
4. Wash with **BSA blocking buffer** 3 ×10 min.
5. Wash with PBS 2×5 min to remove the excess BSA.
6. Post-fix with **post-fixation solution** for 30 min.
7. Wash with PBS for 2 min.
8. Quench in 100 mM NH_4Cl in PBS for 5 min.
9. Wash with PBS for 2 min.
10. Wash with **Triton wash buffer** for 15 min.
11. Continue with the concatemer hybridization step as detailed above.

5. Immuno-SABER on formalin-fixed mouse retina cryosections

Additional Materials

1. ImmEdge Hydrophobic Barrier PAP Pen (Vector Laboratories #H4000)
2. Normal donkey serum (Jackson ImmunoResearch, 017-000-001)
3. Fixed cryopreserved tissue
4. Chambered coverglass (optional) (we typically use ibidi glass-bottom µ-slides (ibidi #80826) and treat with 0.3 mg/ml poly-D-Lysine for at least 30 min, followed by 3×1 min PBS washes.

Tris-buffered saline (TBS): 150 mM NaCl, 50 mM Tris-Cl, pH 7.5

TBST: TBS + 0.3% Triton X-100

Serum blocking buffer: 5% donkey serum + 0.1-0.3% Triton X-100 + 0.2 mg/ml sheared salmon sperm DNA in 1× PBS

Antibody incubation solution: 5% normal donkey serum + 0.1-0.3% Triton X-100 + 0.2 mg/ml sheared salmon sperm DNA + 5 mM EDTA in 1× PBS + 0.05% dextran sulfate (see the **General Recommendations** section below)

Antibody wash buffer: 1% normal donkey serum + 0.1-0.3% Triton X-100 + 5 mM EDTA

Primary hybridization buffer: 40% formamide + 10% Dextran sulfate + 0.1% Triton X-100 + 5 mM EDTA + 0.02% sodium azide in 1× PBS

Secondary hybridization buffer: 30% formamide + 10% Dextran sulfate + 0.1% Triton X-100 + 5 mM EDTA + 0.02% sodium azide in 1× PBS

Low formamide displacement buffer: 0.1× PBS + 30% formamide

Tissue preparation

1. Cut 40 µm sections using a cryotome.
2. Collect and adhere samples to glass slides, coverslips or chambered coverglass with the help of fine brushes.
3. Allow the chambers to completely dry at RT or on a heated stage.
4. Immobilize the tissue sections onto the glass (samples can be stored at -20°C).

5. Wash with TBST for 3×10 min.
6. Block samples with **serum blocking buffer** for >1 h at RT.
7. Dilute antibodies in **antibody incubation solution** (to final concentration individually optimized for each antibody) and incubate the samples with the antibodies in a humidified chamber at 4°C overnight.
8. Wash excess antibodies at RT 3 × 30 min with **antibody wash buffer**.
9. Wash 2×5 min with 1× PBS.
10. Post-fix the sample with post-fixation buffer for 30 min at RT.
11. Wash with PBS for 2 min.
12. Quench in 100 mM NH_4Cl in PBS or 1× TBS for 10 min at RT.
13. Wash with PBS for 2 min.

Primary concatemer hybridization

14. Dilute primary concatemers in the primary hybridization buffer 1:7.5 to 1:20 (depending on the target density)
15. Incubate the sample with SABER concatemers at RT overnight in a humidified chamber.
16. Wash the sample with 45% formamide + 0.1 % Triton X-100 + 5mM EDTA in 1× PBS for 30 min and twice with 30% formamide + 0.1 % Triton X-100 + 5mM EDTA in 1× PBS for 30 min each at RT.

(Optional) Branching

17. Dilute secondary concatemers 1:7.5 or 1:20 in the secondary hybridization buffer.
18. Incubate the sample with SABER concatemers at RT overnight in a humidified chamber.
19. Wash the sample with 40% formamide + 0.1 % Triton X-100 + 5mM EDTA in 1× PBS for 30 min and twice with 30% formamide + 0.1 % Triton X-100 + 5mM EDTA in 1× PBS for 30 min each at RT.

Imager hybridization

20. Hybridize imagers at 0.2-1 μM final concentration in PBS + 0.1% Triton X-100 for 30 min at RT in a humidified chamber.

    Note: The hybridization duration might need further optimization depending on the sample thickness.
21. Wash with 0.5× PBS + 0.1% Triton X-100 at RT for 3×10 min.
22. Stain with 4 μg/ml DAPI in PBS for 10 min.
23. Proceed to imaging.

Exchange imaging

24. For multiplexed detection that requires buffer exchange, dehybridize the imager strands by washing in **low formamide displacement buffer** for 3×10 min.
25. Wash 3×5 min with 1× PBS to remove the residual formamide before rehybridization of imager strands as described above.

6. General recommendations

More information regarding future updates and a section addressing Frequently Asked Questions will be available at http://immuno-saber.net and http://saber-imaging.net.

Antibodies

- It is recommended that conjugated-antibodies are validated by comparing the staining pattern to indirect IF with fluorophore-conjugated secondary antibodies. After DNA-conjugation it may be beneficial to use higher antibody concentration than the unconjugated case.

- Purification of conjugated antibodies (via removal of unconjugated antibodies, and removal/dilution of unconjugated oligos) is recommended for better signal, especially if a significant unconjugated antibody fraction remains after conjugation (since these would compete with the conjugated antibodies).

- Post-fixation is critical to ensure that the antibodies are not washed off during further labeling and imaging. Alternative fixatives (such as mild PFA fixation) can be employed, but the concentration and incubation duration can be further optimized for the best signal.

Buffers and hybridizations

- Formamide concentration can be reduced or eliminated by balancing the salt concentration and temperature of the incubation buffer (refer to the simulations in **Reference 30**). Similarly, incubation temperatures can be adjusted, by balancing the kinetics with buffer salt concentration and formamide concentration.

- We use elongated hybridization times for convenience, but for faster protocols, antibody incubation times and hybridization duration for the concatemers and imagers can be significantly decreased. We had success with concatemer hybridizations shortened to 1-3 h, and imager hybridizations down to 30 min.

- In most of our demonstrations, PER products were diluted into concatemer hybridization solutions for binding to the bridge sequences. Alternatively, concatemers can be purified and concentrated using a MinElute (Qiagen #28004) kit with distilled water elution to reduce volume and salt concentration from the reaction condition, or any other appropriate ssDNA purification method.

- For different tissue types, and combination of targets for multiplexing, experimental conditions may need to be optimized to achieve the best signal level. For primary concatemers we recommend using sequences ≤650 nt (target length: 600 nt), for secondary concatemers ≤450 nt (target length: 400 nt), and for tertiary concatemers ≤250 nt (target length: 200 nt).

Unspecific background

- Dextran sulfate during antibody incubation: Small amounts of dextran sulfate help to reduce the unspecific binding of DNA-conjugated antibodies (especially nuclear background due to DNA-DNA interactions). If you encounter this problem, the concentration can be increased up to 0.05%. However, some antibodies might be sensitive to it. We usually use 0.05% dextran sulfate in the antibody incubation buffer, however this may be decreased down to 0.01% or 0, if a negative effect on the antigen recognition is observed. We recommend starting with 0-0.02% for the initial testing of the antibodies, and then increasing up to 0.05% if nuclear background is observed.

- Unspecific nuclear background may become more prominent when the target antigen is not present in the sample, or is very rarely present (not enough competition). In those cases, antibody concentrations should also be decreased to get better results.

DAPI staining

- It is observed that DAPI staining is partially lost during imager removal. If high staining level is required, it is recommended to re-stain with DAPI after rehybridization of imagers.

- Some autostainer protocols apply the DAPI stain early in the protocol (for example during blocking). At the moment it is unclear if this step interfere with the antibody staining or concatemer hybridizations at some level. We recommend testing that before application.

Imaging

- It is recommended that samples are imaged soon after preparation. If storage is necessary, we recommend keeping the samples up to 1-2 days at 4°C, with high-salt buffers (such as PBS + 500 mM NaCl), and optionally with 0.02% sodium azide to avoid bacterial growth.