Amamoto Lab, 2023

Protocol: SABER FISH on Tissue Sections

SABER FISH on tissue sections (retina)

Note: This protocol uses normal Superfrost Plus slides, which is not suitable for multiplexing SABER. If multiplexing is desired, use 8-well Ibidi chamber slides (Ibidi cat. # 80827). Refer to original Kishi et al. official protocol.

DAY 1 (~60 minutes)

1. Pre-warm the incubator to 43°F.
2. Pre-warm 200 uL of 40% wHyb per sample to 43°F for at least 15 minutes.
3. Prepare the Probe Mix and incubate at 43°F for at least 30 minutes.
4. Take out cryosectioned slides from the -80°C freezer and place in a slide container, face-up.
    a. Note: SABER FISH requires proper fixation. When fixing your tissue of interest, use freshly prepared 4% PFA, made from a fresh 16% PFA ampule.
5. Using a plastic transfer pipette, put ~3 mL of PBS on the slide and incubate for ~1 minute to remove the OCT.
6. Drain the PBS and dab onto a paper towel.
7. Repeat 1x with PBS.
8. Dry the slide and the section by keeping the slide at a 45° angle.
    a. Note: Drying at this step makes sure that the OCT is completely gone and that the section adheres to the slide. After this step, the section cannot dry until the very end.
9. Clean the slide around the tissue with a KimWipe.
10. Adhere a SecureSeal Hybridization Chamber (Grace BioLabs, catalog # 621502)
    a. Note: There are many options for the chamber size. Choose according to the size of your tissue. The volumes indicated in this protocol are for SA-50.
11. Using a P200 pipette, add 100 uL of PBS into the chamber to rehydrate the tissue. Incubate for 1 minute.
12. Using a P200 pipette, aspirate the PBS.
13. Add 100 uL of pre-warmed 40% wHyb into the chamber. Do not seal the holes. Incubate the slide at 43°F for 30 minutes.
14. Aspirate the 40% wHyb.
15. Quickly, add 100+ uL of Probe Mix into the chamber. Seal the 2 holes with the stickers. Press down on the stickers softly to make sure they're adhered. Incubate at 43°F overnight (16 hours).
    a. Note: It is crucial that the Probe Mix and the sample are at 43 °C when it is added to the sample. If the Probe Mix is added at a lower temperature, significant off-target binding of probes can occur causing unwanted background. This is also important for subsequent addition of wHyb solution for washes.
    b. Note: The goal here is to make sure that the Probe Mix fully covers your tissue of interest. Overnight, there will be some evaporation. Try to fill the chamber with the Probe Mix with no air bubbles. If there are air bubbles, tilt in such a way that it is not near your tissue.

DAY 2 (~90 minutes)

16. Pre-warm 40% wHyb (300 uL per sample) to 43°F.

17. Pre-warm 2x SSC (300 uL per sample) to 43°F.
18. Pre-warm Fluorescent Oligo Mix to 37°F.
    a. Note: Given that this is a different temperature, you can use a heat block or water bath.
19. Quickly, remove the slide from the incubator and take off the stickers.
20. Aspirate the Probe Mix completely.
21. Perform a quick wash by adding 100 uL of 40% wHyb into the chamber and repeat pipette several times to remove the residual Probe Mix. Aspirate completely.
22. Add 100 uL of pre-warmed 40% wHyb and incubate for 30 minutes at 43°F.
23. Aspirate and add 100 uL of new pre-warmed 40% wHyb. Incubate for 30 minutes at 43°F.
24. Aspirate and add 100 uL of pre-warmed 2XSSC. Incubate for 5 minutes at 43°F.
25. Aspirate and add 100 uL of new pre-warmed 2XSSC. Incubate for 5 minutes at 43°F.
26. Aspirate and add 100 uL of pre-warmed (37°F) Fluorescent Oligo Mix. Incubate for 10 minutes at 37°F.
27. Take off the hybridization chamber using forceps.
28. Wash 3x with PBS, 5 minutes each, RT.
29. If you want DAPI, apply DAPI (1:50000) for 5 minutes and wash 1x with PBS.
30. Dry the slide.
31. Place 5 drops of Fluoromount-G (without DAPI) across the slide, continuous.
32. Place one end of a 24x50mm No. 1.5 coverslip into the Fluoromount-G and press down from one end to the other, making sure that no air bubble gets in.
33. For best results, image on a confocal microscope within several hours. You should be able to see puncta with a 20x objective.

Materials and Reagents

Note: Deviate away from these exact catalog items at your own risk. There have been several instances when the same (seemingly) item from another company led to a failed SABER FISH attempt.

Reagents for Probe Synthesis

1. 10x PBS (Thermo Fisher Scientific, catalog number: AM9624). Store at RT.
2. 100 mM $MgSO_4$ (NEB, catalog number: B1003S). Store at -20 °C.
3. dNTP (A, C, T 6 mM each) (NEB, catalog number: N0446S). Store at -20 °C. **Do not mix dGTP.**
4. Clean.G (1 µM), store at -20 °C.
   Clean.G is ordered through IDT with the following sequence (standard desalting, diluted with UltraPure water):

   CCCCGAAAGTGGCCTCGGGCCTTTTGGCCCGAGGCCACTTTCG

5. Bst DNA Polymerase (McLab, catalog number: BPR-200). Store at -20 °C.
6. IDTE pH 7.5 (IDT, catalog number: 11-05-01-05). Store at RT.
7. Sterile reagent reservoir (*e.g.*., MilliporeSigma, catalog number: CLS4870). Store at RT
8. MinElute PCR Purification Kit (Qiagen, catalog number: 28004). Store columns at 4 °C,

everything else at RT. Other purification kits have been tested, but this kit has provided the most reliable results.

Reagents for Probe Hybridization

1. SecureSeal Hybridization Chamber (Grace BioLabs, catalog number: 621502).
2. 16% Paraformaldehyde Ampule (Thermo Fisher Scientific, catalog number: 28908). Store at RT.
   Prepare 4% PFA by diluting in PBS before each experiment. Mix whole ampule (10 ml) with 30 ml of PBS. Dilute fresh 4% PFA before experiment and discard after use.
3. 20x SSC (Thermo Fisher Scientific, catalog number: 15557044). Store at RT.
4. Deionized formamide (MilliporeSigma, catalog number: S4117). Aliquot and store at -20 °C. Do not thaw and refreeze.
5. Dextran sulfate sodium salt (MilliporeSigma, catalog number: D8906). Store powder at 4 °C.
   To make a 50% solution, dissolve 5 g of powdered dextran sulfate in 8 ml of UltraPure water overnight, then bring total volume to 10 ml. Store 50% dextran sulfate at -20 °C.
6. Tween-20 (MilliporeSigma, catalog number: P9416).

Recipes:

1. **40% wHyb**

20x SSC	5 ml
10% Tween-20	5 ml
UltraPure Water	20 ml
Deionized formamide	20 ml
Total Volume	50 ml

   Store at -20 °C. This solution should not freeze at -20 °C

2. **Hyb1**

20x SSC	1 ml
10% Tween-20	1 ml
Deionized formamide	4 ml
50% Dextran sulfate	2 ml
Total Volume	8 ml

   Store aliquots of 120 µl at -20 °C. This solution should not freeze at -20 °C

3. **Probe Mix**

Hyb1	96 µl
Purified Probe set	1 µg
UltraPure Water	up to 120 µl

   Pre-warm to 43 °C before use
   Notes:
   a. *Increasing the amount of purified probe set to 2 µg has shown to increase fluorescence intensity. It is unclear if, with 2 µg, a point of saturation has been achieved. Therefore, more probe set may improve fluorescence intensity.*
   b. *The total volume of purified probe sets cannot exceed 24 µl.*

4. **Fluorescent Oligo Mix**

PBS	100 µl
10 µM Fluorescent oligo	2 µl (each)

Pre-warm to 37 °C before use

Notes:

a. *Fluorescent oligos are ordered from IDT. The sequences and the conjugated fluorophores (on the 5' end) can be found in the Oligonucleotide_List file* on the Amamoto et al., Probe-Seq Bio-Protocols*. Listed are 2 different fluorophores per hairpin, but other fluorophores can be used as well. They are resuspended in UltraPure water as 100 µM stocks, and 10 µM working stocks (diluted in water) can be aliquoted and stored at -20* °C.

5. **Stripping Buffer**

UltraPure Water	5 ml
10X PBS	1 ml
Deionized formamide	4 ml
Total Volume	10 ml

Oligo and hairpin design and ordering

Note: The oligos without the primer sequence contain the sequences for hybridization to the RNA, but not the hairpin concatemers that are hybridized by fluorescent oligos. Specific hairpin primer sequences are appended to the oligo sequences to attach hairpin concatemers during the probe set synthesis step (see below). Therefore, at this step, determine which hairpin will be used for which gene. If using two genes on one tissue, two different hairpin primer sequences should be added because fluorescent oligos will hybridize to the hairpin region. For example, in order to sort two populations using Vsx2 and Grik1, for Vsx2, add the 25.25 hairpin primer sequence ("CCAATAATA") to each oligo sequence and extend Vsx2 oligos with the 25 hairpin during the probe set synthesis step, while for Grik1, add the 27.27 hairpin primer sequence ("CATCATCAT") to each oligo sequence and extend Grik1 oligos with the 27 hairpin during the probe set synthesis step. Then, hybridize 25.488 nm fluorescent oligo and 27.565 nm fluorescent oligo to detect Vsx2 and Grik1, respectively.

1. For oligo design for gene-specific probe sets, we use PaintSHOP (https://oligo.shinyapps.io/paintshop/)
2. Click on RNA Probe Design in the header.
3. Choose probe set: look for your species of interest (mm10 for mice, hg38 for human, etc). For SABER FISH, newBalance is appropriate.
4. Look up the RefSeq ID of your gene of interest. For example, mouse Arr3 is NM_133205. Easy to look up through NCBI.
5. Enter the RefSeq ID in PaintSHOP.
6. Click Submit.
7. It will output a list of oligos for your probe set.

   Note: *Note: In general, you want long transcripts that are highly expressed. A sufficient number of oligos is crucial for successful SABER FISH. We have used as few as 12 oligos for high-expressing transcripts and saw a nice separation by FACS. However, in general, more is better. For low expressing transcripts, or if fewer than < 20 oligos, Probe-Seq may not work well.*

8. Click on Download in the header.
9. Used Appending: No, Design Scheme: RNA Probe Design, Choose a file to download: Order File.
10. Click Download.
11. This will generate a .txt file with the list of oligos for the probe set.
12. For ordering the oligos, we use IDT. Oligos from other companies have not been tested.
13. Go to www.idtdna.com and under “Products & Services” click on “Custom DNA oligos.”
14. Under “DNA oligos,” click on “Order Now” and “All ordering options.”
15. Under “Ordering,” click on “Plates” and order 25 nmole DNA Plate oligo in a 96 well format.
    Note: For most experiments, 25 nmole permits thousands of Probe-Seq experiments, which is likely excessive. If possible, order less DNA (i.e., 10 nmole) in order to decrease cost. If you don’t see 10 nmole as an option (you probably won’t if this is your first time), email your IDT rep for your university to request the option.

16. Click on “Upload Plates” and download the sample ordering template.
17. Open the template with Excel.
18. Copy and paste the contents of the gene-specific probe set file (.txt file from PaintSHOP) into a region of the Excel template that is not being used.
19. Copy the oligo sequences into the column under “Sequences.” Delete the other pasted information.
20. For each oligo sequence, add “TTT” and a primer sequence. From our experience, the hairpin primer sequences that have worked well are:
    - 25.25 CCAATAATA
    - 27.27 CATCATCAT
    - 28.28 CAACTTAAC
    - 36.36 AACTAATCT

    Note: Other hairpins have been used by our group and others. However, these are the hairpins that have worked most consistently. For other hairpins, please see the Oligonucleotide_List file.

21. For each line, the sequence should read, “(oligo sequence)TTT(primer sequence)”.
    Note: You can find examples of sequences in the Oligonucleotide_List file.
22. Name each line with the name of the gene, hairpin used, and a number (*i.e.*, Grik1_25_1, Grik1_25_2...).
23. Many gene-specific probe sets can fit on one plate, but it’s advisable that a new gene starts in a new row every time (*i.e.*, If Grik1 oligos use 30 wells, and thus 3 rows: 2 full rows + 6 wells, then the Grm6 oligos should start on the 4th row).
24. Upload the Excel file on IDT.

25. Under Plate Specification, use the following:
    - Scale: 10 nmole (or 25 nmole)
    - Purification: Standard Desalting
    - Plate Type: V-Bottom
    - Ship Option: Wet
    - Buffer: IDTE 7.5 pH
    - Normalization Type: Normalized Yield
    - Quantity: 5 nmol
    - Concentration: 100 µM
    - Volume: 50 µl

26. Order plate.
27. When the plate arrives, thaw the oligos, and use a P200 multichannel pipette to pipette the oligos into a sterile reservoir to pool the oligos. The pooled oligos can be stored at -20 °C.
28. Make a working stock of the pooled oligos (10 µM) by diluting the 100 µM stock with IDTE pH 7.5.
    Notes:
    a. *Do not use other TE buffers.*
    b. *The working stock can undergo freeze-thaw multiple times.*
29. Order hairpins as written in the Oligonucleotide_List or Supplementary Files of the SABER (Kishi *et al.*, 2019) or Probe-Seq (Amamoto *et al.*, 2019) paper. They can be ordered dry and resuspended in IDTE pH 7.5 as 100 µM stock solution. Working stock (5 µM) can be made using IDTE pH 7.5 and aliquoted for further use.
    Note: The 25 hairpin requires a InvdT at the 3' end and HPLC purification.

Probe set synthesis (2-3 hours)

30. Add the following reagents in a PCR tube on ice:

UltraPure Water.	44.5 µl
10x PBS	10 µl
100mM $MgSO_4$ (NEB)	10 µl
dNTP(A, C, T 6 mM each, NEB)	5 µl
Clean.G (1 µM)	10 µl
BST enzyme (McLab)	0.5 µl
Hairpin (5 µM)	10 µl
Total	90 µl

Notes:
a. *6 mM A, C, T dNTP is made by mixing dATP, dCTP, and dTTP to a final concentration of 6 mM.*
b. *Clean.G is ordered through IDT with the following sequence (standard desalting, diluted with UltraPure water):*
   CCCCGAAAGTGGCCTCGGGCCTTTTGGCCCGAGGCCACTTTCG

31. In a thermal cycler, incubate for 15 min at 37 °C.
32. Add 10 µL of the oligo pool (10 µM) and mix.
33. Incubate for 100 min at 37 °C, incubate for 20 min at 80 °C, incubate indefinitely at 4 °C. You can store this unpurified reaction at -20 °C.
34. Check the length of the extended probe set by taking 8 µl of the unpurified probe set and mix it with 1.6 µl of 6x Loading Dye (or any other Loading Dye should suffice). Run a 1.25% ethidium bromide agarose gel (2-3 µl of EtBr for 50 ml volume) at 150 V for 8 min. The band should be between 300-700 bp. See Figure 2 for an example gel.
    Notes:
    a. *Longer running time will lead to fainter band. Two bands are expected–the top band is your extended probe and the bottom band contains catalytic hairpins and the clean.G oligo. Even after purification, this bottom band remains and may even help stabilize the long ssDNA probes during storage.*
    b. *If the probe set is too short or too long, you can change the extension time from 100 min to 80 min (if too long) or 120 min (it too short). The hairpin amount can also be changed.*

For example, if you used 10 µl of hairpin and your probe runs at ~250 bp, you can try doubling the hairpin to 20 µl (and decrease the amount of water in the reaction) to theoretically double the length to 500 bp.

Figure 2. Example DNA gel of three gene-specific probe sets. The left lane is the DNA ladder with the 500 bp band indicated in red. For each lane, the bottom band is the unextended hairpin and other small DNA pieces. The top band is the extended probe set. The bands are always fuzzy because the probes are ssDNA.

35. Purify the remaining 92 µl of probe set using a MinElute PCR Purification Kit. We use 7x PB buffer (instead of 5x) to increase binding efficiency.
36. Elute in 25 µl of UltraPure water.
37. Determine the concentration by NanoDrop using the ssDNA setting. The concentration should be ~200-400 ng/µl.
38. Store purified probe set at -20 °C. Freeze-thaw is tolerated.