Supplementary Protocols

SABER amplifies FISH: enhanced multiplexed imaging of RNA and DNA in cells and tissues

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1 User-friendly protocol: Probe set design

For full documentation on the application of Oligominer, check the initial publication by Brian Beliveau and the Yin Lab[1] and Github: https://github.com/brianbeliveau/OligoMiner.

Genome-wide probe sets have already been generated for a variety of stringency levels. The probe set files with medium probes end in 'b', for instance the 'mm10_chr16b.bed' file. B stands for 'balanced.' Probe sets by chromosome for a variety of organisms are available on The Wu lab Oligopaints website: https://oligopaints.hms.harvard.edu/.

We also recommend installing:

1. Biopython[2] (http://biopython.org/DIST/docs/install/Installation.html)
2. BEDTools[3] (https://bedtools.readthedocs.io/en/latest/content/installation.html)

Step by step instructions for mouse probe design (standard mRNA detection):

1. Retrieving BED file for gene or gene region of interset from UCSC genome browser:[4]
    - Select appropriate genome (e.g. mm10 for mouse)
    - Bring gene (or region) of interest into field of view
    - tools → table browser
    - Fill in as follows:
        - Group: genes and gene predictions
        - Track: e.g. NCBI RefSeq
        - Table: e.g. RefSeq
        - Region: Position
        - Output format: BED
    - Get output → exons (for exon FISH)
    - Check file
2. If there are multiple isoforms (e.g. different names that start with NM), then manually delete all but the desired one
3. **Check the strand orientation**. If (-), then no need for extra steps. If (+), then you must get the reverse complement after probe design.
4. Example UNIX commands for identifying overlapping probes:
    - Ensure Biopython and BEDTools are installed and loaded (see above), or transfer BED file and probeset files to a cluster/machine that has these set up
    - A symbolic link to the chromosome probe set file can be placed in the working directory using:
      `ln -s ../oligopaints/mm10_chrNb.bed`
    - For the gene of interest (GOI) bed file, run intersectbed (bedtools):
      `intersectBed -a mm10_chrNb.bed -b GOI.bed -f 1 > GOI_probes.bed`
    - If the gene is the (+) strand, take the reverse complement:
      `python ../bin/probeRC.py -f GOI_probes.bed -o GOI_probesRC`
5. Primer sequences are appended to the 3' end of the primers with a linker of `TTT`, e.g.:
   `(probe sequence)TTT(9-mer primer sequence)`

The optimal number of required probes for each mRNA target varies based on considerations such as which fluor will be used for detection and whether branching will be employed as well as transcript length and sequence (e.g. homology to the genome). In tissues, we successfully detected RNAs across a variety of fluors using anywhere from 24 to 50 probes. We recommend starting with this range of probe set sizes.

2 User-friendly protocol: Oligo ordering and preparation

Probe oligos are ordered from IDT in a 96 well format with standard desalting. Cost is significantly reduced by ordering plates at 10nM synthesis scale. Additional ordering specs are: Resuspended in IDTE pH7.5, v bottom plate, normalized in nanomoles. Individual wells from the plate can be pooled by multichannel pipetting equal volumes from all wells into a trough. IDTE pH7.5 is used for dilutions of probe primers down to 10uM.

PER[5] hairpins typically function well if synthesized with standard desalting and a polyT of 7 T's on the 3' end of the hairpin (unless the primer sequence ends in a A bases, in which case the 3' tail should be designed to not hybridize to the concatemer sequence). They can be resuspended in IDTE (pH 7.5) and stored as 100 µM stocks. Dilutions of hairpin down to 5µM for extension are also done in IDTE. Certain hairpins, however, require the addition of an Inverted dT (InvdT) at the 3' end and HPLC purification (see below).

Fluorescent oligos are ordered with a 5' fluorescent adduct and require HPLC purification. Yield is variable and cost is significantly reduced by ordering in bulk. These are resuspended in ddH_2O or IDTE to 100uM for storage, or as 10µM dilutions (diluted in ddH_2O or IDTE). A 3' InvdT on fluorescent oligo is not essential. A full list of primers, hairpins, and branches are available in Supplementary Table 1.

To generate a catalytic (telomerase-like) hairpin corresponding to the primers sequence (RC = reverse complement):

`A(primer sequence)GGGCCTTTTGGCCC(RC of primer sequence)T(RC of primer sequence)/3InvdT`

For example, given the primer 27 sequence of `CATCATCAT`, the catalytic hairpin h.27.27 sequence would be:

A**CATCATCAT**GGGCCTTTTGGCCC**ATGATGATG**T**ATGATGATG**/3InvdT/

The `/3InvdT/` can be replaced with `TTTTTTT` for most hairpins to save cost. We have found empirically however, that some hairpins (including those for primers 25, 32, and 41 seem to require the InvdT modification).

To change the primer appended to a probe set, a hairpin for primer switching ('re-mapping') can be used (Fig. S1a-c). Remapping hairpin is introduced in the same reaction as the catalytic hairpin, and the concentration is flexible (0.05µM-0.25µM). Reactions involving primer switching may require additional time for extension to equivalent lengths.

To generate a hairpin for primer switching:

`A(new primer)GGGCCTTTTGGCCC(RC of new primer)T(RT of old primer sequence)/3Invdt/`

A detailed schematic for designing such a hairpin can be seen in Fig. S1c. Here, as above, `/3InvdT/` can generally be replaced with `TTTTTTT` for most hairpins to save cost.

Fluorescent oligos are designed as follows:

`/5dye/TT(RC of primer sequence)T(RC of primer sequence)T`

This represents a 20mer binding sequence with a 5' conjugated dye linked to the binding region by a TT' linker

PER primer re-mapping

Probe sets with one primer (e.g. primer sequence **a**) that have to be used in the same experiment with another set that has the same primer (**a**) can be re-mapped to alternate PER concatemer sequences using a two-hairpin reaction (Fig. S1a-b). An example for designing re-mapping hairpins given a starting primer sequence and desired concatemer sequence is depicted in Fig. S1c. (The original 9 nt primer sequence is not predicted to hybridize to the complementary 20mer imager sequence at 37°C in 1×PBS, so one of the probe sets can usually be concatemerized with the original primer sequence. In this case it is good to have a control condition with just that probe set missing to verify no cross-talk between channels.)

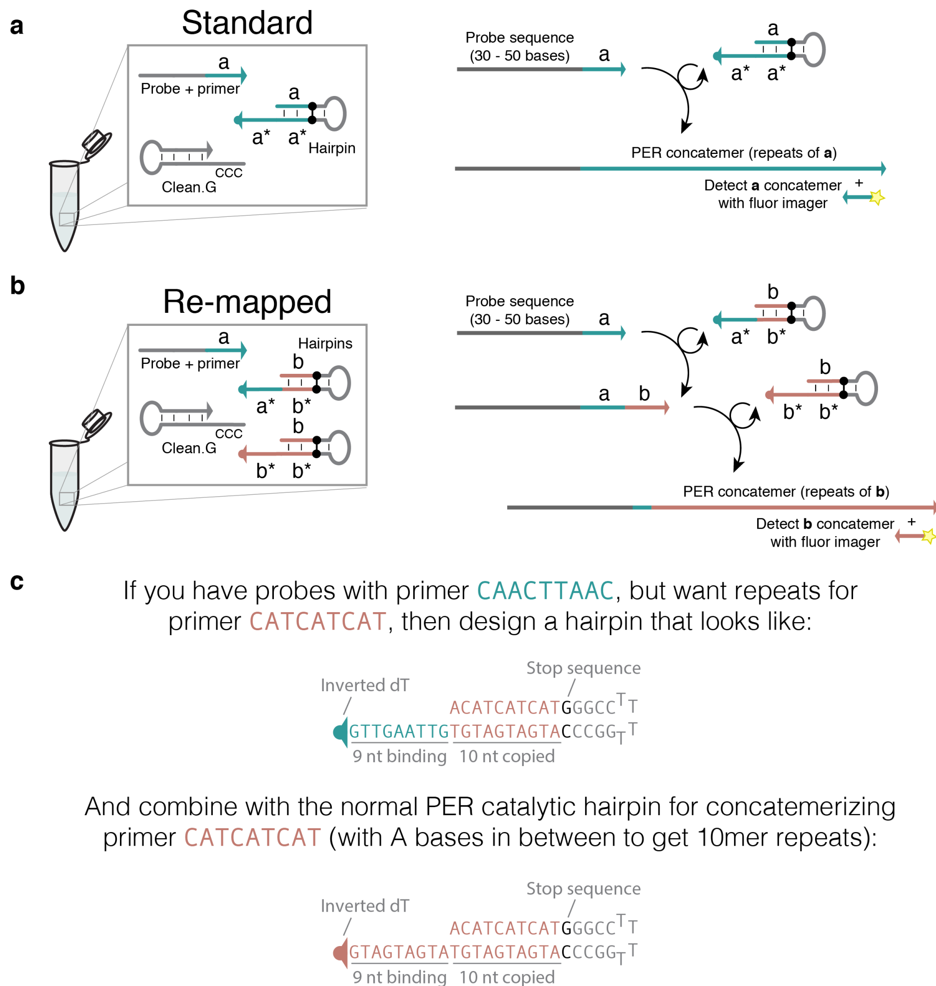

Figure S1: **Designing PER primer re-mapping reactions. a**, Standard PER setup, which uses one PER hairpin to concatemerize the **a** sequence. **b**, Re-mapping PER setup, which uses an additional re-mapping hairpin to swap one sequence for another (in this case, **b** for **a**). **c**, Design example for primer re-mapping primer `(A)CAACTTAAC` to repeats of sequence `(A)CATCATCAT`.

3 30mer branch melting temperatures

We recommend using a temperature at least 1 degree lower than the lowest melting temperature of all branch sequences you plan to use (see plot of melting temperatures Fig. S2). You can find these melting temperature curves reported for each sequence, as well as those computed for 20mer imagers, 42mer barcode sequences, and an example set of FISH probes, reported in Supplementary Table 2.

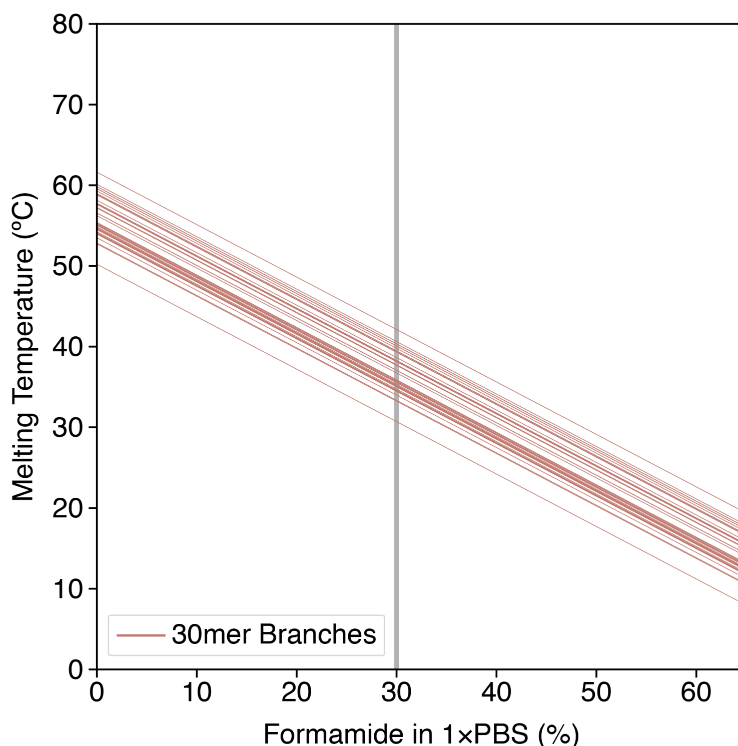

Figure S2: **Melting temperatures of branches under different formamide conditions.** Melting temperatures of 30mer branch binding sequences are shown for the 50 designed PER primers in 1×PBS with different concentrations of formamide. Modeled with Biopython.[2]

4 User-friendly protocol: Cost-efficient Primer Exchange Reaction (PER) concatemerization

Component	Volume (µL)
10×PBS	10
100mM MgSO4* (NEB)	10
dNTP mix* (A,C,T only 6mM each, NEB)	5
Clean.G (1µM)	10
Bst LF polymerase* (McLab)	0.5
Hairpin* (5µM)	10
Probe oligo pool (10µM)	10
H_2O	44.5

Table S1: **Example 100 µL PER mix.** *Indicates component concentration can easily be varied to control reaction kinetics and therefore concatemer length, see comments below.

First make mix without hairpin or probe oligo on ice, adding the polymerase last. Add mix to strip tube with hairpin, mix. Incubate for 15 minutes at 37°C (see below), then pause cycler, add probe oligos, mix, and return to cycler for extension.

Extension program:

1. Heat cycler to 37°C
2. Pause
3. Insert tube with reaction mix plus hairpin, 37°C incubation for 15 minutes
4. Remove tube, add probe (unextended probe oligo mix)
5. Incubate 37°C for desired extension time (see below)
6. Heat to 80°C 20 minutes to inactivate the Bst polymerase
7. Cool to 4°C

Clean.G (`CCCCGAAAGTGGCCTCGGGCCTTTTGGCCCGAGGCCACTTTCG`) is ordered with standard desalting and diluted in H_2O.

Note 1: dGTP nucleotides are excluded from the reaction as a string of C's in the hairpin is used as a stop sequence. The Clean.G oligo incorporates contaminating dGTPs (see Fig. S6 from ref[5]). The dNTP mix is generated by ordering dNTP's in separate tubes and mixing A, C, T to a final concentration of 6mM each. Bst large fragment polymerase NEB (M0275L) can be used instead.

Note 2: Hairpin final concentration may be adjusted depending on the desired probe length and properties of the specific hairpin. Extension rates can be quite variable depending on the specific hairpin. As a starting point try .5µM final hairpin concentration for a 60 minute extension reaction. Hairpin concentration, extension time, and other reaction conditions can be adjusted to modify concatemer length (see Fig 1b and Fig. 1a).

Note 3: We recommend using ddH_2O or ideally molecular grade water such as UltaPure DNase/RNase-free distilled water (Invitrogen #10977023), especially if you will be using the concatemers for RNA FISH.

To check the lengths of extensions, load 10 µL of reaction with loading dye on a dense agarose gel (~1.25%). You can also load samples into 1% E-Gel EX agarose gels (Thermo Fisher G402001).

Note 5: We recommend using primary probes extended to ~500-650 nt and branch probes extended to ~250-450 nt.

Note 6: Because there aren't G bases in the concatemer sequences, and they are highly single-stranded, intercalating dyes are not as effective as they are at staining other types of sequences (we recommend using the Sybr Gold dye). This means the bands may look faint on the gel but still be efficiently elongated, so don't be surprised if you need to turn up the contrast quite a bit.

While purification of probes is not usually required for cell applications, we have found it helpful for SABER-FISH in tissue. Probes are typically purified by running 90 µL of reaction (for 500 nt probe) over one MinElute PCR purification column (Qiagen #28004). Add PB buffer to 7×the volume of reaction being purified. Elute in 25 µL of ddH_2O. Concentration after purification are determined by nanodrop using ssDNA setting. Store probes at -20°C.

5 User-friendly protocol: SABER RNA FISH in cells

Preparing the slide:
Seed 8-well Ibidi chamber slides (Cat #80827) with cells and grow in tissue culture incubator (typically 37°C with 5% CO_2) to desired confluency.

Notes: You may need to adjust the deposition protocol (e.g. what concentration of cells, how long to let them grow on the slide) in order to achieve the desired confluency of your cell type for imaging. Some cell types will not adhere well through the fixation protocol, so the chamber should be quickly imaged in Brightfield after fixation to ensure the proper density of cells.

If this is your first time doing this protocol, we recommend using some of the wells for controls and a couple length conditions. For example, you might seed 4 wells and use one as a no probe control well (which receives all other treatments) where you should not see signal, and then 3 different concatemer length conditions. Or if you have 3 targets, you might do 3 single-color wells each receiving one of the concatemer species, and then one multi-color well. We have found that some microscope stages are not amenable to imaging the leftmost and rightmost wells in the chamber, so this is a good thing to check before you plan on using all of the wells.

Depending on the type of fluid, the minimum volume for each well is around 120-150 µL. We recommend using 250 µL for washes. If you are using a different type of chamber with smaller volume, we recommend performing several more washes at each step to ensure complete removal of previous elements. The easiest method of aspiration is with an unfiltered pipette tip attached to a vacuum line. We recommend cleaning the line with ethanol and changing the tip a several times throughout the experiment. Good lab technique for avoiding RNase contamination and use of RNase-free water is important throughout the protocol.

Fixation:
At room temperature, rinse cells in 1×PBS and then immediately fix in 4% (wt/vol) paraformaldehyde for 10 minutes. Rinse again in 1×PBS and store at 4°C. We stored chambers for DNA FISH up to a couple of weeks before use, but for RNA integrity we recommend waiting no longer than a few days.

Hybridization (RT = room temperature):

- Wash in 1×PBS for 1' at RT
- Permeabilize in 1×PBS + 0.5% Triton X-100 for 10' at RT
- Wash in 1×PBSTw for 1' at RT
- Wash in 2×SSCT for 1' at RT
- Add hybridization solution and denature at 60°C for 3'
- Return temperature to 42°C (see below) for at least 4 hours (typically overnight is easiest)
- Add 200 µL pre-warmed (see below) 2×SSCT to wells and aspirate
- Wash 4×5' in pre-warmed 2×SSCT at 60°C
- Wash 2×2' in 2×SSCT at RT

If you are going directly to the fluorescent protocol, rinse in 1×PBS for 1 minute and transfer to fresh 1×PBS (at RT).

Pause point: sample can be stored at 4°C overnight to several days.

Note: if the probes are retrieved using the Oligominer[1] pipeline as described or from the Wu lab database (see section above), then 42°C hyb temperature should be sufficient for RNA FISH. If you are using custom designed and especially shorter probes, you will need to check their melting temperatures in 2×SSC + 50% formamide. We suggest setting your oven temperature to a couple degrees lower than those melting temperatures. We have found that thermocyclers especially with heated lids versus ovens set to the same temperature can actually be up to a few degrees higher at the sample plane. This can cause a big difference in yield because we are operating close to the melting temperatures, so if you feel that your signal is too low you can try reducing the temperature of the hyb a bit. Alternatively, if there seems to be a lot of off target binding of probes, you can try increasing a bit.

For pre-warming 2×SSCT, we suggest you first make enough for all washes and then aliquot the amount needed for the hot washes into 2 mL tubes. Put these tubes on a heated tube rack set to 65°C and allow them to heat for at least 30-40 minutes. Remove only the amount of tubes you need at each wash step, so that the rest can stay hot.

Branching:

- Wash in 2×SSCT for 2' at RT

- Add branch hybridization solution and hold at 37°C (see notes below) for at least 30'
- Add 200 μL pre-warmed 2×SSCT to wells and aspirate
- Wash 4×5' in pre-warmed 2×SSCT at 60°C
- Wash 2×2' in 2×SSCT at RT
- Rinse in 1×PBS for 1'
- Transfer to fresh 1×PBS (at RT).

Note: See note below about adjusting branch hybridization temperature/formamide conditions according to branch sequence melting temperatures.

Fluorescent detection:

- Rinse once in 1×PBS at RT
- Add fluor hybridization solution and hold at 37°C for 1 hour (see note in Solution section below)
- Wash for 5' with pre-warmed (see below) 1×PBS at 37°C
- Wash 2×2' in pre-warmed 1×PBS
- Rinse once in 1×PBS at RT
- Load SlowFade + DAPI (Thermo Fisher S36939) mountant to completely cover cells
- Image

Pause point: Samples can be stored at 4°C before imaging for a few days, although we recommend doing the fluorescent hyb closer to your imaging session time (day of) where possible.

Notes: For pre-warming 1×PBS, we suggest you first make enough for all washes and then aliquot the amount needed for the warming washes into a separate Falcon tube and place into an oven or warm room at 37°C - 45°C.

You can also image samples directly in 1×PBS, in which case if you may need to do a short DAPI stain if you want to see that channel.

Serial detection (complete before repeating fluorescent detection):

- Wash 2×1' in PBS at RT to remove mountant
- Add displacement buffer and incubate for 15' (see below) at RT
- Wash 2×1' in PBS at RT

Notes: the signal should be stripped almost immediately with the displacement buffer, but we recommend letting it sit for at least 5-15 minutes to ensure complete removal. If you are using a smaller volume chamber or well, you may need to add fresh displacement buffer extra times in order to completely strip fluorescent signal. If you are doing the exchange in place on a microscope, you can image the same area before and after adding the displacement buffer to visualize the drop in signal.

If you are using DAPI, the displacement buffer will probably remove most of this signal. You will likely need to replace after each exchange step and before imaging, either by including it in the mountant or staining separately (see above).

Solution preparation

ddH_2O is used for all solutions, not DEPC. Where possible, such as for hybridization solutions, we recommend using UltaPure DNase/RNase-free distilled water (Invitrogen #10977023). In general, we recommend good lab technique like regularly aliquoting water, 10×PBS, 20×SSC, and other solutions to avoid constantly re-opening stock bottles. We recommend using plastic conical tubes and not lab glassware for solutions. Formamide should be stored at 4°C.

1×PBSTw buffer:

- 1×PBS

- 0.1% (vol/vol) Tween-20

Example mix for 1×PBSTw: 1 mL 10×PBS, 10 µL 100% Tween-20, 9 mL H_2O.

Note: It is helpful to use a positive displacement pipettor to transfer Tween-20 and other detergents, but if you don't have one available you can try to use a normal pipettor with a blunted pipette tip (cut off the bottom narrow part with scissors or a razor blade).

We recommend preparing this solution fresh the day you plan to use it.

1×PBS + Triton buffer:

- 1×PBS
- 0.5% (vol/vol) Triton X-100

Example mix for 1×PBS + Triton: 1 mL 10×PBS, 50 µL 100% Tween-20, 9 mL H_2O.

We recommend preparing this solution fresh the day you plan to use it.

2×SSCT buffer:

- 2×SSC
- 0.1% (vol/vol) Tween-20

Example mix for 2×SSCT: 1 mL 20×SSC, 10 µL 100% Tween-20, 9 mL H_2O.

We recommend preparing this solution fresh the day you plan to use it.

Displacement buffer:

- 1×PBS
- 50% (see below) Formamide
- 0.1% Tween-20

Example master mix: 1 mL 10×PBS, 6 mL Formamide, 10 µL 100% Tween-20 3 mL H_2O.

Prepare fresh from 100% Formamide held at 4°C. If you are using a flow chamber, we recommend flowing displacement buffer through the chamber many times to ensure complete signal removal and also increasing to 60% formamide as long as you are not using 30mer branches.

4×FISH master mix:

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

Example mix for 4×FISH master mix: 16g of Dextran Sulfate, 16 mL of 20×SSC, 160 µL Tween-20, H_2O to 40 mL.

Note: we recommend making 40 mL of this at a time, as it takes a while to incorporate all of the components but will last a long time. First measure 16g of Dextran Sulfate and put 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 overnight 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 up to several months.

Primary hybridization solution:

- 2×SSC
- 10% Dextran sulfate
- 0.1% Tween-20
- 50% Formamide
- ~100nM (see below) of each concatemer or concatemer pool

Example primary hyb mix: 31.25 µL 4×FISH master mix, 62.5 µL 100% Formamide 8.33 µL 1µM probe 1 concatemers, 8.33 µL 1µM probe 2 concatemers, 14.59 µL ddH$_2$O.

Notes: The PER step generally produces concatemers at a concentration of 1µM, and we recommend using the highest concentration you can fit into the solution to start. We often dilute 15× (to a final concentration of ~67nM). Keep in mind if you are using a probe pool (e.g. with 50 oligos), this means the actual concentration of each strand will be much lower (1.34nM). If you have a lot of PER concatemers you are trying to combine, or you would like to purify them, you can use a MinElute or similar column (see PER concatemerization protocol above).

We recommend creating a master mix for all of your wells (each of which should get at least 125 µL of hyb). It is helpful to use a positive displacement pipettor to transfer the 4×FISH master mix, but if you don't have one available you can use a normal pipettor with a blunted pipette tip (cut off the bottom narrow part with scissors or a razor blade). Mix the hyb solution very well by aggressively vortexing it for at least 10 seconds before spinning down. Once well mixed, a normal pipettor can be used to add the hyb solution to samples, but aspiration and pipetting must be done very slowly to ensure all of the material is transferred and reduce the chance of bubble formation.

Branch hybridization solution:

- 2×SSC
- 10% Dextran sulfate
- 0.1% Tween-20
- 30% Formamide*
- ~100nM (see below) of each concatemer or concatemer pool

Example branch hyb mix: 31.25 µL 4×FISH master mix, 37.5 µL 100% Formamide 8.33 µL 1µM branch 1 concatemer, 8.33 µL 1µM branch 2 concatemer, 39.59 µL ddH$_2$O.

Note: Branching is performed similarly to primary probe incubation, but in a lower formamide solution. Branches are applied after primary probe washes are complete, and before fluorescent detection. For cells, branches are extended to length of ~250-450 nt and incubated for at least 30 minutes in 30% formamide Hyb solution. Hyb temperature must be adjusted depending on the branches being used. We recommend using a temperature at least 1 degree lower than the lowest melting temperature of your branch sequences (see Fig. S2). Note that formamide concentration can be adjusted instead of oven/thermocycler temperature.

Fluorescent hybridization solution:

- 1×PBS
- 1µM (see below) each fluor oligo

Example fluorescent hyb mix: 12.5 µL 10×PBS, 12.5 µL Fluor Oligo 1 (10µM), 12.5 µL Fluor Oligo 2 (10µM), 87.5 µL ddH$_2$O.

Notes: We recommend starting with the 1 hour hybridization with 1 µM fluor oligo if this is your first time running the protocol. Alternatively, we still see strong signal if the hybridization time is reduced to 15 minutes (at RT e.g. for Fig. 6). Side-by-side testing with two probes in tissue indicate 0.2µM fluorescent oligo is also sufficient (see Fig. S3).

6 Protocol optimization for SABER-FISH in tissues

We tested several variations to simplify the SABER-FISH protocol in tissues, and we found robust signal in most cases we tested. These experiments are summarized in Fig. S3.

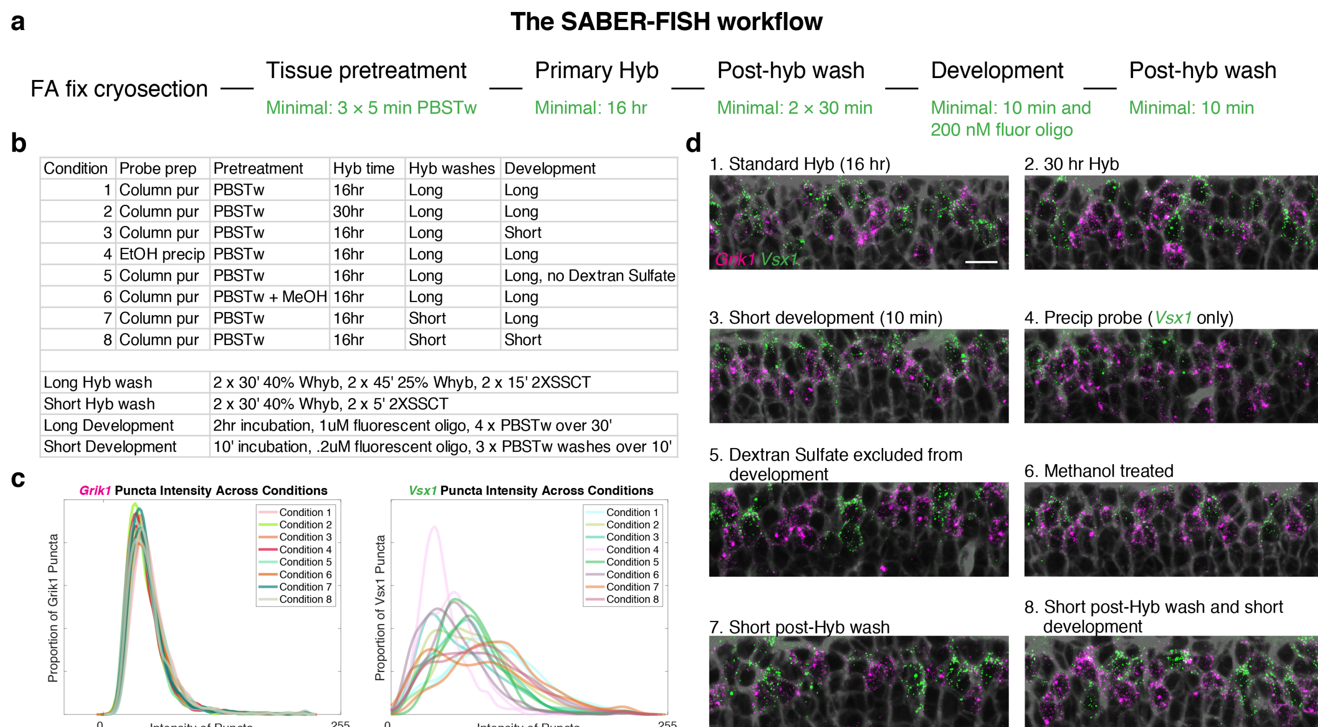

Figure S3: **Testing variations in the RNA FISH staining protocol.** **a**, Overview of main steps in the RNA FISH protocol. The most efficient condition tested in this experiment is shown below each step. **b**, Table of eight conditions tested in side-by-side comparison. See Methods for additional details. **c**, Quantification of signal intensity for conditions tested, with each line representing a replicate (N=2 retinal sections per condition). Each condition was tested using two-color FISH for *Vsx1* and *Grik1*. **d**, Representative images of conditions tested. Scale bar is 10 µm. All sections 40 µM, from P25 animals.

7 User-friendly protocol: Retina Tissue Sections RNA FISH

Fixation:
Neural retinas are dissected in PBS and fixed in 4% FA (diluted from 16% FA ampule, Thermo Fisher #28908) for 25 minutes at room temperature. Retinas are embedded in a 1:1 (v/v) mixture of 30% sucrose (in 1×PBS) and OCT, frozen in ethanol bath. Frozen blocks are stored at -80°C prior to sectioning.

Preparing the slide:
8-well Ibidi chamber slides (Cat #80827) are PDL coated using 0.3 mg / mL PDL (sigma P7886) dissolved in 2×Borate buffer, diluted from 20×stock (Thermo Scientific, #28341) with ddH$_2$O. PDL is stored in aliquots at -20°C. Apply PDL to slide for at least 30 minutes. Then remove PDL, dry the slide, wash once in ddH$_2$O, dry again.

Sectioning:
The PDL coated, fully dried slide is placed inside the cryostat ahead of sectioning. Do not remove slide until all sections are in place or condensation will form that can impede adhesion. Place sections inside wells using brushes, and flatten sections as much as possible to prevent folding when the slide is transferred to room temperature. After removing the slide from the chamber, spin at 600g for 3 minutes (plate spinner centrifuge) 1-2 times to promote adhesion.

Hybridization:

- Wash sections 3×5' in PBSTw to remove OCT
- Replace PBSTw with Whyb
- Place in hybridization oven set to 43°C, minimum 10'

In oven, set to 43°C:

- Replace Whyb with Hyb1/Probe mixture pre-warmed to 43°C. Seal chamber with parafilm
- Incubate 16 hours minimum
- Replace Hyb1 with Whyb (quick wash to remove residual Hyb1)
- Wash 2×30' in Whyb
- Wash 2×5' in 2XSSCT

Return to room temperature (Pause point: sample can be stored in 2×SSCT or PBSTw at 4°C for several weeks)

Fluorescent detection:

- Replace 2×SSCTw with PBSTw (2 washes at room temperature)
- Set oven to 37°C
- Transfer slide to 37°C for hybridization and subsequent wash steps
- Once slide is warm, remove PBSTw and add Hyb2/fluor solution (prewarmed)
- Incubate at least 10 minutes at 37°C
- Replace Hyb2 with PBSTw (prewarmed, quick wash)
- Wash 2×5' in PBSTw

Return to room temperature (Pause point: Samples can be stored at 4°C without obvious signal loss for at least 1 week)

Imaging:
Wash once in PBS and add mounting media until sections are covered (See below for notes on imaging)

Serial detection (complete before repeating fluorescent detection):

- Remove glycerol mounting media with 3 or 4 PBSTw washes (~5 minutes)
- Wash 3×5' in Displacement buffer at room temperature
- Wash 3×2 minutes in PBSTw

Notes: This stepwise protocol corresponds to the most rapid version we have tested for 40μm sections. Experiments in the manuscript employ pretreatment steps and longer wash and development steps that we determined to be inessential in a test involving two 500 nt probes (see Fig. S3).

Fig. S3 demonstrates that the protocol is robust to certain variations. Tissues can be treated with methanol. Hyb1 and Hyb2 incubation time can be increased, and fluorescent oligo can be applied at a range of concentrations. To reduce viscosity for ease of solution exchange, dextran sulfate can be eliminated from Hyb2. Probes can be purified using ethanol precipitation; however, this resulted in a mild decrease in signal for unknown reasons.

After rehydrating sections, do not allow them to dry completely at any point in the protocol. Washes should be performed such that sections do not remain devoid of solution for more than a few seconds. For washes done at elevated temperature, solutions should be prewarmed and and added in or near the oven. Avoid letting samples cool during these wash steps. Volumes used are 120 μL for Hyb1 (minimal to cover tissue), and 150-200 μL for all other steps. When pipetting the hyb mixes with dextran sulfate, allow time for the viscous solution to fully accumulate in the pipette tip to avoid loss of volume.

*Branching is performed similarly to primary probe incubation, in Hyb1 solution. Branches are applied after primary probe washes are complete, and before fluorescent detection. Branches are extended to length of ~350-500 nt and incubated for at least 5 hours (longer may be required, depending on tissue penetration) in 40% formamide Hyb solution. Hyb temperature must be adjusted depending on the branches being used. We recommend using a temperature at least 1 degree lower than the Tm of the branch with the lowest melting temperature (see Fig. S2). Note that formamide concentration can be adjusted instead of oven temperature. Another option is to extend the branch annealing portion (the 30mer) by adding an additional full or partial repeat (primer sequence + T) to match the Tm of the branch with highest Tm. If doing serial multiplexing with branching, 30mers with lower melting temperatures may be partially displaced by displacement of the fluorescent oligos. For this reason we recommend using a branch with high Tm (e.g. 27*27*27*) and extending other branches as needed to match this melting temperature.*

Solution preparation

ddH_2O is used for all solutions, not DEPC. We recommend using sterile plastic tubes and not reusable glassware for solutions. Formamide and formamide-containing solutions are aliquoted and stored at -20°C.

Whyb (Wash hyb):

- 2×SSC (From 20×stock, pH 7, Invitrogen)
- 1% Tween-20 (Sigma P9416)
- 40% Formamide (Millipore, Deionized)

Example mix for 40% formamide Whyb: 1 mL 20×SSC, 1 mL 10% Tween, 4 mL Formamide (for 40% mix), 4 mL H_2O.

Hyb 1 (for primary probe hyb):

- 2×SSC
- 1% Tween-20
- 40% Formamide
- 10% Dextran sulfate (Sigma D8906)

Probes are generally used at a concentration of 1μg /120 μL volume.

Example Hyb1 master mix: 1 mL 20xSSC, 1 mL 10% tween, 4 mL Formamide, 2 mL Dextran sulfate (50% solution).

Example Hyb1/Probe mix: 96 μL Hyb1 master mix, 5 μL 200ng/ μL probe 1, 5 μL 200ng/ μL probe 2, 14 μL ddH_2O.

Notes: Stock of Hyb1 should be made accounting for the fact that probes, which are eluted in ddH_2O, have a water volume. The example Hyb1 mix above allows up to 24 μL of probe per 96 μL of Hyb mix for a total of 120 μL volume to add to the well. For higher multiplexing probes can also be concentrated to reduce water volume by using a SpeedVac or heat block. After addition of probe to Hyb1 mix, the solution should be mixed well by pipetting until uniform consistency is observed. Likewise, mix well by rocking after adding dextran sulfate to the Hyb1 mix. Dextran sulfate is usually added from 50% w/v stock made by dissolving powder in ddH_2O. Dextran sulfate takes time to dissolve, and volume will shrink as powder dissolves. Use tick marks on the side of the tube to add water to final volume after most of the powder has dissolved.

Hyb 2 (for fluorescent detection):

- 1×PBS

- 0.2% Tween-20
- 10% Dextran sulfate

Fluorescent oligos added to concentration of .2µM-1µM

Example Hyb2 master mix: 1 mL 10×PBS, 200 µL 10% Tween-20, 2 mL dextran sulfate, 4.8 mL H_2O.

Example Fluor/Hyb2 mix: 96 µL Hyb2 master mix, 2.4 µL Fluor Oligo 1 (10µM), 2.4 µL Fluor Oligo 2 (10µM), 19.2 µL ddH_2O.

Notes: Master mix aliquots are stored at 4°C. Side-by-side testing with two probes indicates 0.2µM fluorescent oligo is sufficient. Dextran sulfate can be excluded from the Hyb2 mixture (tested for fluorescent oligo at 1µM).

Displacement buffer:

- 1×PBS
- 50% formamide

Stored at -20°C.

Example master mix: 1 mL 10×PBS, 5 mL Formamide, 4 mL H_2O.

Glycerol mounting media:

- 80% glycerol
- 1×PBS
- 20mM Tris pH 8
- 2.5 mg / mL of propyl gallate

Example mix: 8 mL 100% glycerol, 1 mL 10×PBS, 200 µL 1M Tris , 25mg propyl gallate.

Stored at 4°C.

Note: Resuspend fully and spin in centrifuge before use (3 minutes on max in table top centrifuge) to remove undissolved propyl gallate specks that fluoresce.

PBSTw

Mg/Ca-free, RNAse/DNAse free PBS (Gibco #10010-023) with 0.1% Tween-20

8 Supplemental References

1. Beliveau, B. J. *et al.* OligoMiner provides a rapid, flexible environment for the design of genome-scale oligonucleotide in situ hybridization probes. *Proceedings of the National Academy of Sciences* (2018).
2. Cock, P. J. A. *et al.* Biopython: freely available Python tools for computational molecular biology and bioinformatics. *Bioinformatics* **25**, 1422–1423 (2009).
3. Quinlan, A. R. & Hall, I. M. BEDTools: A flexible suite of utilities for comparing genomic features. *Bioinformatics* **26**, 841–842 (2010).
4. Kent, W. J. *et al.* The Human Genome Browser at UCSC. *Genome Research* **12**, 996–1006 (2002).
5. Kishi, J. Y., Schaus, T. E., Gopalkrishnan, N., Xuan, F. & Yin, P. Programmable autonomous synthesis of single-stranded DNA. *Nature Chemistry* (2017).