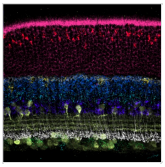

Aug 21, 2020

5: User-friendly protocol: SABER RNA FISH in cells

In 1 collection

Jocelyn Y. Kishi[1,2,3], Sylvain W. Lapan[4,3], Brian J Beliveau[1,2,5,3,6], Emma R. West[4,3], Allen Zhu[1,2], Hiroshi M. Sasaki[1,2], Sinem Saka[1,2], Yu Wang[1,2], Constance L Cepko[4,7,6], Peng Yin[1,2,6]

[1]Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA, USA;
[2]Department of Systems Biology, Harvard Medical School, Boston, MA, USA; [3]These authors contributed equally;
[4]Department of Genetics, Blavatnik Institute, Harvard Medical School, Boston, MA, USA;
[5]Present address: Department of Genome Sciences, University of Washington, Seattle, WA, USA;
[6]Correspondence: py@hms.harvard.edu (P.Y.), cepko@genetics.med.harvard.edu (C.L.C.), beliveau@uw.edu (B. J. B.);
[7]Howard Hughes Medical Institute, Chevy Chase, MD, USA

1 *Works for me* This protocol is published without a DOI.

Human Cell Atlas Method Development Community

Jocelynkishi

ABSTRACT

This protocol describes the SABER RNA FISH in cells.

This protocol is part of the SABER-FISH collection.

EXTERNAL LINK

http://saber.fish/

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Kishi, J.Y., Lapan, S.W., Beliveau, B.J. et al. SABER amplifies FISH: enhanced multiplexed imaging of RNA and DNA in cells and tissues. Nat Methods 16, 533–544 (2019). https://doi.org/10.1038/s41592-019-0404-0

ATTACHMENTS

SABER amplifies FISH_enhanced multiplexed imaging of RNA and DNA in cells and tissues.pdf

PROTOCOL CITATION

Jocelyn Y. Kishi, Sylvain W. Lapan, Brian J Beliveau, Emma R. West, Allen Zhu, Hiroshi M. Sasaki, Sinem Saka, Yu Wang, Constance L Cepko, Peng Yin 2020. 5: User-friendly protocol: SABER RNA FISH in cells. **protocols.io** https://protocols.io/view/5-user-friendly-protocol-saber-rna-fish-in-cells-bh9kj94w

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

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EXTERNAL LINK

http://saber.fish/

COLLECTIONS

SABER-FISH – Signal amplification for multiplexed fluorescence in situ hybridization assays

KEYWORDS

cells, SABER, RNA, Hybridization

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PROTOCOL INTEGER ID

38924

PARENT PROTOCOLS

Part of collection

SABER-FISH – Signal amplification for multiplexed fluorescence in situ hybridization assays

MATERIALS

NAME	CATALOG #	VENDOR
Dextran sulfate, sodium salt	D8906	Sigma Aldrich
Tween-20	P9416	Sigma Aldrich
µ-Slide 8 Well Glass Bottom	80827	Ibidi
UltraPure™ DNase/RNase-Free Distilled Water	10977023	Thermo Fisher
UltraPure™ SSC, 20X	15557044	Thermo Fisher
SlowFade™ Gold Antifade Mountant with DAPI	S36939	Thermo Fisher
PBS	10010023	Gibco - Thermo Fischer
0.5% (vol/vol) Triton X-100		
fluorescent oligos		
Formamide Deionized	S4117	Merck Millipore

MATERIALS TEXT

Solution preparation:

ddH2O 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).

UltraPure™ DNase/RNase-Free Distilled Water
by Thermo Fisher
Catalog #: 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:

- [M]1 X PBS
- [M]0.1 % (v/v) Tween-20

Example mix for 1×PBSTw: 1 mL 10×PBS , 10 µl 100% Tween-20 , 9 mL H2O .

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:

- [M]1 X PBS
- [M]0.5 % (v/v) Triton X-100

Example mix for 1×PBS + Triton: 1 mL 10×PBS , 50 µl 100% Tween-20 , 9 mL H2O .

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

2×SSCT buffer:

- [M]2 X SSC
- [M]0.1 % (v/v) Tween-20

Example mix for 2×SSCT: 1 mL 20×SSC , 10 µl 100% Tween-20 , 9 mL H2O .

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

Displacement buffer:

- [M]1 X PBS
- [M]50 % to [M]60 % Formamide
- [M]0.1 % (v/v) Tween-20

Example master mix: 1 mL 10×PBS , 5 mL to 6 mL Formamide , 10 µl 100% Tween-20 , 3 mL H2O .

Prepare fresh from [M]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 [M]**60 % formamide** as long as you are not using 30mer branches.

4×FISH master mix:

- [M]**8 X SSC**
- [M]**40 % (wt/vol) Dextran Sulfate**
- [M]**0.4 % Tween-20**

Example mix for 4×FISH master mix: **16 g Dextran Sulfate** , **16 mL 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 **16 g Dextran Sulfate** and put into a 50 mL Falcon tube. Then add the [M]**20 X SSC** and Tween-20. Add H_2O to a volume of roughly **35 mL** - **38 g** 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 **Room temperature** for up to several months.

Primary hybridization solution:

- [M]**2 X SSC**
- [M]**10 % Dextran sulfate**
- [M]**0.1 % Tween-20**
- [M]**50 % Formamide**
- ~ [M]**100 Nanomolar (nM)** 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 ddH2O** .

Notes: The PER step generally produces concatemers at a concentration of [M]**1 Micromolar (µM)** , and we recommend using the highest concentration you can fit into the solution to start. We often dilute 15× (to a final concentration of ~ [M]**67 Nanomolar (nM)**). 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 ([M]**1.34 Nanomolar (nM)**). 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 hyb**). It is helpful to use a positive displacement pipettor to transfer the [M]**4 X 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 **00:00:10** 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:

- [M]**2 X SSC**

- [M]10 % Dextran sulfate
- [M]0.1 % Tween-20
- [M]30 % Formamide *
- [M]100 Nanomolar (nM) 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 ddH2O .

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 00:30:00 in [M]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, protocol 3). Note that formamide concentration can be adjusted instead of oven/thermocycler temperature.

Fluorescent hybridization solution:

- [M]1 X PBS
- [M]1 Micromolar (µM) 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 ddH2O .

Notes: We recommend starting with the 1 hour hybridization with [M]1 Micromolar (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 00:15:00 (at Room temperature e.g. for Fig. 6 (see Nature Methods article)). Side-by-side testing with two probes in tissue indicate [M]0.2 Micromolar (µM) fluorescent oligo is also sufficient (see Fig. S3, protocol 6).

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

BEFORE STARTING

Prepare Solutions as described in section 'Materials'.

Preparing the slide

1 Seed 8-well Ibidi chamber slides (Cat #80827) with cells and grow in tissue culture incubator (typically 37 °C with [M]5 % CO2) 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 probe 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 µl** - **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

2

At **Room temperature** , rinse cells in [M]**1 X PBS** and then immediately fix in [M]**4 % (wt/vol) paraformaldehyde** for **00:10:00** .

3

Rinse again in [M]**1 X 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

4

Wash in [M]**1 X PBS** for **00:01:00** at **Room temperature** .

5

Permeabilize in [M]**1 X PBS** + [M]**0.5 % Triton X-100** for **00:10:00** at **Room temperature** .

6

Wash in [M]**1 X PBSTw** for **00:01:00** at **Room temperature** .

7

Wash in [M]**2 X SSCT** for **00:01:00** at **Room temperature** .

8

Add hybridization solution and denature at **60 °C** for **00:03:00** .

9

Return temperature to **42 °C** for at least **04:00:00** (typically **Overnight** is easiest).

Note: if the probes are retrieved using the Oligominer[33] pipeline as described or from the Wu lab database, 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 [M]**2 X SSC** + [M]**50 % formamide** . We suggest setting your oven temperature to a couple degrees lower than those melting temperatures (See protocol 3). 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.

10

Add **200 µl** *pre-warmed* [M]**2 X SSCT** to wells and aspirate.

Note: For pre-warming [M]**2 X 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 **00:30:00** - **00:40:00** . Remove only the amount of tubes you need at each wash step, so that the rest can stay hot.

11

Wash 4× **00:05:00** in *pre-warmed* [M]**2 X SSCT** at **60 °C** .

11.1 Wash **00:05:00** in pre-warmed [M]**2 X SSCT** at **60 °C** . (1/4)

11.2 Wash **00:05:00** in pre-warmed [M]**2 X SSCT** at **60 °C** . (2/4)

11.3 Wash **00:05:00** in pre-warmed [M]**2 X SSCT** at **60 °C** . (3/4)

11.4 Wash 00:05:00 in pre-warmed [M]2 X SSCT at 60 °C . (4/4)

12

Wash 2× 00:02:00 in [M]2 X SSCT at Room temperature .

12.1 Wash 00:02:00 in [M]2 X SSCT at Room temperature . (1/2)

12.2 Wash 00:02:00 in [M]2 X SSCT at Room temperature . (2/2)

13

If you are going directly to the fluorescent protocol, rinse in [M]1 X PBS for 00:01:00 and transfer to fresh [M]1 X PBS (at Room temperature).

14

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

Branching

15

Wash in [M]2 X SSCT for 00:02:00 at Room temperature .

16

Add branch hybridization solution and hold at 37 °C for at least 00:30:00 .

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 [M]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, protocol 3). Note that formamide concentration can be adjusted instead of oven/thermocycler temperature.

17

Add 200 µl *pre-warmed* [M]2 X SSCT to wells and aspirate.

18

Wash 4× 00:05:00 in *pre-warmed* [M]2 X SSCT at 60 °C .

18.1 Wash 00:05:00 in *pre-warmed* [M]2 X SSCT at 60 °C . (1/4)

18.2 Wash 00:05:00 in *pre-warmed* [M]2 X SSCT at 60 °C . (2/4)

18.3 Wash 00:05:00 in *pre-warmed* [M]2 X SSCT at 60 °C . (3/4)

18.4 Wash 00:05:00 in *pre-warmed* [M]2 X SSCT at 60 °C . (4/4)

19

Wash 2× 00:02:00 in [M]2 X SSCT at Room temperature .

19.1 Wash 00:02:00 in [M]2 X SSCT at Room temperature . (1/2)

19.2 Wash 00:02:00 in [M]2 X SSCT at Room temperature . (2/2)

20

Rinse in [M]1 X PBS for 00:01:00 .

21

Transfer to fresh [M]1 X PBS (at Room temperature).

Fluorescent detection

22

Rinse once in [M]1 X PBS at Room temperature .

23

Add fluor hybridization solution and hold at 37 °C for 01:00:00 .

Notes: We recommend starting with the 1 hour hybridization with [M]1 Micromolar (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 **00:15:00** (at **Room temperature** e.g. for Fig. 6 in the Nature Methods article). Side-by-side testing with two probes in tissue indicate **0.2 Micromolar (µM) fluorescent oligo** is also sufficient (see Fig. S3, protocol 6).

24

Wash for **00:05:00** with *pre-warmed* **1 X PBS** at **37 °C** .

Note: For pre-warming **1 X 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** .

25

Wash 2× **00:02:00** in pre-warmed **1 X PBS** .

25.1 Wash **00:02:00** in pre-warmed **1 X PBS** . (1/2)

25.2 Wash **00:02:00** in pre-warmed **1 X PBS** . (2/2)

26

Rinse once in **1 X PBS** at **Room temperature** .

27

Load SlowFade + DAPI (Thermo Fisher S36939) mountant to completely cover cells.

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

28

Image.

29

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.

Serial detection (complete before repeating fluorescent detection)

30 

Wash 2× **00:01:00** in **PBS** at **Room temperature** to remove mountant.

30.1 Wash **00:01:00** in **PBS** at **Room temperature** to remove mountant. (1/2)

30.2 Wash **00:01:00** in **PBS** at **Room temperature** to remove mountant. (2/2)

31 

Add displacement buffer and incubate for **00:15:00** at **Room temperature** .

Notes: the signal should be stripped almost immediately with the displacement buffer, but we recommend letting it sit for at least **00:05:00** - **00:15:00** 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).