Multiplexed Iterative FISH Experimental Protocol SOP002.v3.12 Shepherd Lab

Saber Method for PaintSHOP probes

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Document Summary: This document, SOP002 - Multiplexed Iterative FISH Experimental Protocol, describes the process for in-situ fluorescence labeling of RNA transcripts in cells and tissues using a layered probe design, which allows for identity barcoding (MERFISH or similar) and/or signal amplification (Branched DNA [bDNA] amplification), along with a cleavable disulfide (S-S) reporter molecule, attached to a readout oligo, to allow for iterative rounds of labeling and imaging of the same sample with minimal disruption to sample integrity between rounds. This document also describes cell and tissue handling for the labeling process, and the RNA labeling process which uses an mRNA binding using a specialized poly-t (locked nucleic acid, LNA) probe with an acrydite linker to bind mRNAs to a polyacrylamide matrix and clearing techniques used to reduce cellular autofluorescence and increase the signal to noise ratio of the final data.

Quick Overview:

Part 1 – Tissue or Cell-Based Experiment Preparation

Step 1 – Coverslip functionalization
Step 2 - Mount, SDS pretreat and permeabilize sample
Step 3 - Hybridize linker (optional)
Step 4 - Wash away residual linker
Step 5 - Gel embed sample (optional)
Step 6 - Digest & clear sample (optional)
Step 7 - Hybridize encoding probes
Step 8 - Wash away residual encoding probes
Step 9 – Hybridize amplifiers (optional)

Part 2a – Multiplexed Iterative FISH Imaging with Fluidics System

Step 1 - Prepare solutions for imaging
Step 2 - Assemble fluidics system
Step 3 – MULTIPLEXED ITERATIVE FISH imaging protocol

Part 2b – Alternate MULTIPLEXED ITERATIVE FISH Imaging without Fluidics System

Step 1a: MULTIPLEXED ITERATIVE FISH imaging protocol – using coverslip mounted sample.
Step 1b: MULTIPLEXED ITERATIVE FISH Imaging Protocol - multiple hybridizations using chamber slip.
Step 2: Dapi stain the sample
Step 3: Proceed to imaging of the sample

v3.12revision notes

i. *Minor revisions to language and formatting to make compatible with current protocol changes per current fluidics programming (flow speeds, solution priming). Also adjusted some formatting to improve flow and readability.*
ii. *Updated tissue rehydration time.*

References:

-Hershberg, E. A., Close, J. L., Camplisson, C. K., Attar, S., Chern, R., Liu, Y., ... & Beliveau, B. J. (2020). PaintSHOP enables the interactive design of transcriptome- and genome-scale oligonucleotide FISH experiments. *bioRxiv*.
-Moffitt, J. R., Hao, J., Bambah-Mukku, D., Lu, T., Dulac, C., & Zhuang, X. (2016). High-performance multiplexed fluorescence in situ hybridization in culture and tissue with matrix imprinting and clearing. *Proceedings of the National Academy of Sciences*, *113*(50), 14456-14461. https://doi.org/10.1073/pnas.1617699113
-Moffitt, J. R., & Zhuang, X. (2016). RNA imaging with multiplexed error-robust fluorescence in situ hybridization (MERFISH). In *Methods in enzymology* (Vol. 572, pp. 1-49). Academic Press. https://doi.org/10.1016/bs.mie.2016.03.020.
-Stellaris RNA FISH protocol for frozen tissues: https://biosearchassets.blob.core.windows.net/assets/bti_stellaris_protocol_frozen_tissue.pdf

Adapted from: Hershberg 2020, *Moffitt, 2016 and Biosearch Technologies Stellaris RNA FISH Protocol for Frozen Tissue*
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Full Protocol:

****All reagents for this protocol should be prepared sterile and RNase-free.*
***All incubation periods should be done in the dark.*
***Reagent/buffer preparation instructions follow protocol below.*
***Safety note: This protocol uses formamide which is a teratogen and can cause developmental malformation. Always work in a fume hood with formamide to avoid inhalation and avoid physical contact.*

Part 1 – Tissue or Cell-Based Experiment Preparation

Description: Part 1 of this protocol describes the steps to setup a multiplexed iterative FISH experiment for tissue or cell-based samples. These steps are focused on the biochemical requirement for tissue or cell preparation, probe hybridization and imaging. This protocol does not cover the requirements of the microscope for imaging. Additional detail can for the imaging setup can be found at https://doi.org/10.1016/bs.mie.2016.03.020.

Step 1: Coverslip Functionalization *Refer to current version of SOP003 for protocol on Coverslip Functionalization. PDL-coated coverslips are preferable as tissue can be post-fixed to the coating using 4% PFA.*

Step 2: Mount, SDS Pretreat and Permeabilize Sample

a. *Using 4% PFA-fixed tissue.* ***Note:*** *For some tissue, it is simpler to mount directly to the coverslip after slicing. In this case, mount and post-fix first then follow the remaining steps in order.*
    i. Slice tissue and place slices in 1xPBS for 5 min. Remove PBS and repeat this for a second wash.
    ii. Pretreat tissue by washing in 4% SDS Clearing Solution (SDS-CS), once for 5 minutes.
    iii. To permeabilize the tissue, immerse the slip mounted tissue in 70% (vol/vol) ethanol overnight at 4°C (recommended) in a Pyrex 60mm petri dish (Fisher 08-747A) or similar. (For faster results, sample can be incubated in EtOH for 1 hour at RT).
    iv. Move tissue slices to functionalized (PDL-coated) coverslip, aspirate off the 70% ethanol and incubate with PBS buffer for 30 min to rehydrate the sample.
    v. To bring the sample in sufficient contact with the coverslip surface, aspirate the PBS buffer and place in 37-45°C oven for ~5-10 mins to dry any excess PBS buffer. Monitor the progress closely. You will want to ensure the sample lying flat on the coverslip surface without drying the sample.
    vi. Post-fix tissue to the coverslip by incubating in 4% PFA at RT for 10 mins.
    vii. Remove PFA from the sample and rinse with 1x PBS for 5 mins at RT, two times.

b. *Using 4% PFA-fixed cells grown on coverslip (optionally, use 8-chamber well or similar)*
    i. To permeabilize the cells, immerse the slip mounted sample in 70% (vol/vol) ethanol overnight at 4°C (recommended) in a Pyrex 60mm petri dish (Fisher 08-747A). (For faster results, sample can be incubated in EtOH for 1 hour at RT).
    ii. Alternatively, pipette 100µL permeabilization buffer (PBS-t) to each well and incubate at RT for 10 min with gentle rocking.
    iii. Rinse with permeabilization buffer rinse (PBS-tw).
    iv. Aspirate rinse from the sample and let dry.

c. Using a hydrophobic pen, draw a barrier around your sample and let dry before hybridizations. You may want to add a very small volume of PBS during this process to tissue samples to prevent sample desiccation.

Step 3: Hybridize Linker (optional – use when gel embedding)

1. Wash & equilibrate sample by immersing slip-mounted sample in 37°C pre-heated 200µL Wash Buffer A for 30 min.
2. Assemble humidified chamber (empty pipette box with lid or otherwise that can house the sample-mounted coverslip with a single, saturated, and folded paper used to line the inner edge of the chamber to prevent evaporation of probe solution).
3. Remove slip from Wash Buffer A and carefully wipe away excess buffer surrounding sample.
4. Dispense 125µL of Encoding Hybridization Buffer containing 1µM** linker to your sample, replace the petri dish lid, parafilm the dish and place the dish with the sample in the humidified chamber.
   *****Adjust concentration of the linker according to sample size.***
5. Incubate at 37°C in a humidified chamber for 18-24 hours (up to 36 hours).

Step 4: Wash Away Residual Linker

1. Remove the hybridization buffer and carefully remove excess buffer surrounding sample.
2. Wash sample in pre-heated 37°C Wash Buffer A for 30 min, two times.
3. Wash two times in 37°C pre-heated Encoding Wash Buffer (SSC-tw) for 5 mins, two times.
4. Wash two times in RT 1x PBS.

Step 5: Gel Embed Sample (optional – gel embed when clearing and digesting)

1. Incubate sample for 2min** with de-gassed PA monomer solution.
   **adjust time based on sample size. For 100µm tissue slices, increase this to 3 hours.
2. Incubate sample for 2 min with PA Gel Solution and remove.
3. Cast a thin PA film by adding ~50-100µL gel solution to the sample and by inverting a smaller (25mm) gel-slick coated coverslip onto the gel solutions, being careful to avoid air bubbles. Adjust the volume and make sure your gel film is thin. Aspirate any extra gel solution away.
4. Allow casting for 1.5 hour at RT.
5. After casting, carefully remove the smaller coverslip from your sample. If the coverslip is stuck, you can loosen the coverslip by immersing SDS-CS at 37°C.

Step 6: Digest and Clear Sample

Note: *For lung tissue start at step 1. Skip ahead to step 3 for brain tissue.*

1. Incubate sample in 3mL PBS with 10% collagenase/elastase at 20,000U/mL for 3 hours at 37°C.
2. Wash sample with a quick rinse of RT 1x PBS followed by two 5min washes of 1x PBS at RT.
3. Wash the sample on the coverslip twice with 1mL SDS Clearing Solution (SDS-CS) for 5 min each wash at 37°C.
4. Incubate with 3mL of SDS Clearing Solution with 1% Proteinase K in a humidified chamber for 1-12h at 37°C, depending on the sample. For best results, monitor the sample clearing and halt clearing as soon as the sample clearing is complete. For 100µm brain tissue slices, this can be as little as 1 hour.
5. Wash the sample by immersing it in Wash Buffer B 3 to 4 times for 5 min each.

Step 7: Hybridize Encoding Probes

1. Wash & equilibrate sample by immersing slip-mounted sample in 41°C pre-heated 250-500µL Wash Buffer A for 30 min.
2. Assemble a humidified chamber (An empty pipette box with lid or otherwise that can house the sample-mounted coverslip with a single, saturated, and folded paper used to line the inner edge of the chamber to prevent evaporation of probe solution).
3. Remove the slip from Wash Buffer A and carefully wipe away the excess buffer surrounding the sample.

4. Dispense 125µL of Encoding Hybridization Buffer containing 5-200µM encoding probes (depending on the number of unique encoding probes in the probe set and sample size) to your sample, replace the petri dish lid, parafilm the dish and place the dish with the sample in the humidified chamber.
5. Incubate at 41°C in a humidified chamber for 18-24 hours, up to 36 hours.

Step 8: Wash Away Residual Encoding Probes

1. Remove the hybridization buffer and carefully remove the excess buffer surrounding the sample.
2. Wash the sample in pre-heated 41°C Wash Buffer A for 30 min, two times.
3. Wash two times in a 41°C pre-heated Encoding Wash Buffer (SSC-tw) for 5 mins, two times.
4. Wash two times in RT 1x PBS.

Step 9: Hybridize amplifiers (optional)

*Amplifier concentration may need to be increased based on the thickness of your sample

******Adjust incubation time based on sample size. For a 100µm tissue section, an overnight incubation is preferable for the primary amplifiers while the secondary amplifiers can be incubated for 5-6 hours, on the following day.

To label the gel embedded and cleared samples with primary and secondary amplifiers.

1. Incubate sample in Wash Buffer C at 37°C for 30min.
2. Aspirate to remove Wash Buffer C.
3. Hybridize primary amplifier. Incubate the sample in a 125µL droplet of 5nM* primary amplifier in amplifier hybridization buffer for 15 min** in humidity-controlled 37°C incubator.
4. Wash 3x with Wash C for 5-10 min each at RT, depending on sample size.
5. Hybridize secondary amplifier. Invert sample onto a 125µL droplet of 5nM* secondary amplifier in amplifier hybridization buffer for 15 min** in humidity-controlled 37°C incubator.
6. Wash twice in Wash C for 5-10 min at RT followed by a 15-30min wash in Wash C at 37°C.
7. Perform MULTIPLEXED ITERATIVE FISH Imaging (Part 2) immediately or store sample for up to 24hr in storage buffer at 4°C

aPart 2a – MULTIPLEXED ITERATIVE FISH Imaging with Fluidics System ***The following steps are used for the Full MULTIPLEXED ITERATIVE FISH protocol. For experiments that don't use the fluidics system, move to Part 2B below.*

Description: Imaging for MULTIPLEXED ITERATIVE FISH involves multiple rounds of fluid exchange to hybridize, image, cleave and rinse samples. Automated fluid exchange and imaging approach is recommended. For setups lacking an automated fluidics exchange system, proceed to Part 2b.

Step 1: Prepare Solutions for Imaging. *Prepare the following solutions with volumes corresponding to your experiment size.*

i. *Readout Hybridization Buffer*
ii. *Readout Wash Buffer*
iii. *Imaging Buffer (store under mineral oil)*
iv. *TCEP Cleavage Buffer*
v. *2x SSC Wash Buffer (Wash B)*
vi. *DAPI Staining Solution*

Step 2: Assemble Fluidics System

1. Make sure that all tubing is properly connected. MULTIPLEXED ITERATIVE FISH probes and sample preparation time are costly so leaks need to be avoided.
2. Ensure the system is fully assembled, plugged in and turned on.
3. Double-check correctness of the details for the pump protocol for the MULTIPLEXED ITERATIVE FISH Fluidics for the current project.
4. Load the sample to the flow cell and connect.
5. Carefully load all solutions to the proper reservoirs.

Step 3: MULTIPLEXED ITERATIVE FISH Imaging Protocol. Once the fluidics system is setup, solutions are prepped and loaded and the sample is in place in the chamber, an automated program should run the following cycle:

1. Readout hybridization buffer (with readout probes)
    a. Run 2mL over 3 min to prime buffer to the sample.
    b. Run additional 2.5mL over sample for 4 min.
    c. Pause flow for 15-120 mins, depending on sample size (10 µm = 15 min, 30 µm = 60 min, 100 µm = 120 min).
2. Readout Wash Buffer (Wash D)
    a. Run 2mL over 3.5 min to flush.
    b. Run additional 2mL over 10 min to wash.
3. Imaging Buffer –
    a. Run 2mL over 10 min to run remaining wash D over sample and prime imaging buffer to the sample.
4. 2x SSC Wash Buffer (Wash B)
    a. Run 1mL in 3 min to move 1mL imaging buffer over the sample.
5. Imaging. Pause fluidics and proceed with imaging.
6. TCEP Cleavage Buffer
    a. 2mL in 3.5 min to prime cleavage buffer to the sample.
    b. 1mL in 5 min.
    c. Pause flow for 10 min.
7. 2x SSC Wash Buffer (Wash B)

a. 2mL in 10 min to run cleavage buffer over sample and prime SSC buffer.
b. 2mL over 4 min to rinse off cleavage buffer.

8. Repeat steps 1-7 for each readout round.

When all readout rounds are complete proceed with steps 9-12

9. DAPI Stain – Wash 2mL DAPI in 2xSSC (Wash B) for 60 min.
    a. Use 50µg/mL for thick (100µm) samples.
    b. Use 1-10µg/mL for 10µm samples.
10. 2xSSC (Wash B) - 2mL for 4min.
11. Imaging Buffer - 2mL in 6 min then halt flow.
12. Proceed with Imaging.

Part 2b – Alternate MULTIPLEXED ITERATIVE FISH Imaging without Fluidics ***The following steps are used for manual, iterative FISH without a fluidics system. For trial that uses the fluidics system, move to Part 2a (above).*

Description: For some MULTIPLEXED ITERATIVE FISH experiments, it may be simpler to proceed without the fluidics system for imaging. Once you have hybridized probes and amplifiers if desired, readout probes can be hybridized and imaged in a single round or in multiple rounds if necessary. If you are hybridizing more than one round of readouts, proceed to Steps 1b-3.

Step 1a: MULTIPLEXED ITERATIVE FISH Imaging Protocol - Single Hybridization using coverslip mounted sample.

1. Readout Probe Hybridization.
    a. Pipette 200µL of 3nM readout probes in Readout Hybridization Buffer to sample and incubate at RT for 10min.
    b. Aspirate Readout Hybridization Buffer from the sample.
2. Wash away unbound probe by adding 200µL RT Readout Wash Buffer D to sample for 5min, two times. Additional washes may improve the result.
3. Dapi Stain. Add 200µL Wash Buffer B with DAPI nuclear stain (at 1µg/mL) to sample and incubate for 30 min at 37°C.
4. Remove the Dapi stain and wash with Wash Buffer B for 5 min, two times.
5. Add 100µL-200µL Imaging buffer to sample and mount to glass plate with clear nail polish.
6. Proceed with imaging.

Step 1b: MULTIPLEXED ITERATIVE FISH Imaging Protocol - Multiple Hybridizations Using Chamber-slip

1. Readout Probe Hybridization.
    a. Pipette 200µL of 3nM readout probes in Readout Hybridization Buffer to sample and incubate at RT for 10min.
    b. Aspirate Readout Hybridization Buffer from the chambers.
2. Wash away unbound probe by adding 200µL RT Readout Wash Buffer D to sample for 5min, two times. Additional washes may improve the result.
3. Add 100µL-200µL Imaging buffer to sample. Proceed with Imaging.
4. TCEP Cleavage Buffer – 100µL for 15 min.
5. 2x SSC Wash Buffer (Wash B) – 250µL each well, three times.
6. Repeat steps 1-6 for each probe set
7. Move on to step 2 when complete.

Step 2: Dapi Stain the Sample

1. Add 200µL Wash Buffer B with DAPI nuclear stain (at 1µg/mL) to each chamber and incubate sample in this for 30 min at 37°C.
2. Wash sample in Wash Buffer B for 5 min two times.

Step 3: Proceed to Imaging of the Sample

Solution Preparation:

Wash Buffer A (40% Formamide Wash Buffer)
- 2x SSC Buffer (Fisher, AM9763)
- 1% (vol/vol) Tween 20 (Sigma, P9416-100ML)
- 40% (vol/vol) Formamide (Fisher, AM9342)
- Nuclease-free water

Wash Buffer A Master Mix, 45mL (for 75mL final volume with FA):
- 36.75mL nuclease-free water
- 7.5mL 20x SSC Buffer (Fisher, AM9763)
- 750µL Tween 20 (Sigma, P9416-100ML)
- Add 40% formamide (FA) to prepare on demand (Fisher, AM9342)

Wash Buffer B
- 2x SSC buffer (Fisher, AM9763) prepared in nuclease-free water.

Wash Buffer C (10% Formamide Wash Buffer)
- Nuclease-free water
- 2x SSC Buffer (Fisher, AM9763)
- 10% (vol/vol) Formamide (Fisher, AM9342)

Wash Buffer C Master Mix, 45mL (for 50mL final volume with FA)
- 40mL nuclease-free water
- 5mL 20x SSC Buffer (Fisher, AM9763)
- Add 10% formamide (FA) to prepare on demand (Fisher, AM9342)

Saber Encoding Hybridization Buffer
- Nuclease-free water
- 2x SSC Buffer (Fisher, AM9763)
- 40% (vol/vol) Formamide (Fisher, AM9342)
- 0.1% (wt/vol) Yeast tRNA (Life Technologies, 15401011)
- 1% (vol/vol) Murine RNase Inhibitor (New England Labs, M0314L)
- 1% (vol/vol) Tween 20 (Sigma, P9416-100ML)
- 10% (wt/vol) Dextran sulfate (Sigma, D8906-100g)
- Add encoding probes at 5-200µM final concentration, depending on the size of the pool.
- Prepare on demand.

Saber Encoding Hybridization Buffer Master Mix, 4.8mL (for 8.0mL prep with formamide added)
- Nuclease-free water
- 800µL 20x SSC Buffer (Fisher, AM9763)
- 320µL Yeast tRNA solution (Life Technologies, 15401011, reconstituted to 25mg/mL)
- 80µL Murine RNase Inhibitor (New England Labs, M0314L)
- 80µL Tween 20 (Sigma, P9416-100ML)
- 0.8g Dextran sulfate (Sigma, D8906-100g)
- Aliquot mix and store at -20°C.
- To prepare on demand, add 40% (vol/vol) Formamide (Fisher, AM9342) to master mix at time of use.
- Add encoding probes at 5-200µM final concentration, depending on the size of the pool.

Encoding Buffer Rinse (SSC-tw)
- Nuclease-free water
- 2x SSC (Fisher, AM9763)
- 0.1% (vol/vol) tween 20 (Sigma, P9416-100ML)
- Store at RT

PA Solution

- Nuclease-free water
- 4% (vol/vol) 19:1 acrylamide/bis-acrylamide (Bio-Rad Laboratories, 1610144)
- 60mM Tris-HCl pH8 (Fisher, AM9856)
- 0.3M NaCl (Fisher, AM9759)
- One of the following:
  - For four-color experiments: 1:500 dilution 0.1µm-diameter light yellow beads (Spherotech, FP-0245-2)
  - For two-color experiments: 1:200,000 dilution of 0.1µm-diameter carboxylate-modified orange fluorescent beads (Life Technologies F-8800)
- De-gas solution before use.
- Prepare on demand.

PA Gel

- PA Solution including polymerizing agents:
  - 0.03% (wt/vol) ammonium persulfate (Sigma A3678)
  - 0.15% (vol/vol) TEMED
  - ***Prepare on demand. Polymerizing agents will act rapidly. Make gel in small quantities (1mL) and right before use.*
- Prepare on demand.

Storage Buffer (SSC-SB)

- Wash Buffer B
- 0.1% (vol/vol) murine RNase Inhibitor (New England Labs, M0314L)
- Store in aliquots at -20°C.

Amplifier Hybridization Buffer

- Nuclease-free water
- 2x SSC buffer (Fisher, AM9763)
- 10% (vol/vol) Formamide (Fisher, AM9342)
- 0.1% (wt/vol) Yeast tRNA (Life Technologies, 15401011)
- 1% (vol/vol) Murine RNase Inhibitor (New England Labs, M0314L)
- 10% (wt/vol) Dextran sulfate (Sigma, D8906-100g)
- Prepare on demand
- Add amplifiers at 5nM final concentration.

Amplifier Hybridization Buffer Master Mix, 7.2mL (for 8.0mL prep with formamide added)

- Nuclease-free water
- 800µL 20x SSC buffer (Fisher, AM9763)
- 320µL Yeast tRNA solution (Life Technologies, 15401011, reconstituted to 25mg/mL)
- 80µL Murine RNase Inhibitor (New England Labs, M0314L)
- 0.8g Dextran sulfate (Sigma, D8906-100g)
- Aliquot mix and store at -20°C.
- To prepare on demand, add 10% (vol/vol) Formamide (Fisher, AM9342) to master mix at time of use.
- Add amplifiers at 5nM final concentration.

Readout Hybridization Buffer

- 2x SSC buffer (Fisher, AM9763)
- 10% (vol/vol) ethylene carbonate (Sigma, E26258)
- 0.1% (vol/vol) Murine RNase Inhibitor (New England Labs, M0314L)
- Nuclease-free water
- Add readout probes at 3nM final concentration
- Prepare on demand

Wash Buffer D (Readout Wash Buffer)

- 2x SSC Buffer (Fisher, AM9763)

- 10% (vol/vol) ethylene carbonate (Sigma, E26258)
- Store at RT.

Imaging Buffer
- 2x SSC Buffer (Fisher, AM9763)
- 50mM Tris-HCl, pH 8 (Fisher, AM9856)
- 10% (wt/vol) Glucose (Sigma DX0145-1)
- 2mM Trolox (Sigma, 238813)
- 0.5mg/mL Glucose Oxidase (Sigma, G2133)
- 40µg/mL Catalase (Sigma, C30)
- 0.1% (vol/vol) Murine RNase Inhibitor (New England Labs, M0314L)
- Nuclease-free water
- Prepare on demand
- **Store under layer of Mineral Oil when using fluidics system (Sigma, 330779)

Cleavage Buffer
- 2x SSC Buffer (Fisher, AM9763)
- 50mM Tris(2-carboxyethyl) phosphine [TCEP] (Sigma, 646547)
- Prepare on demand.

DAPI Staining Solution
- 50µg/mL DAPI stain (Fisher, D1306) in Wash Buffer B for thick (40µm) tissue.
- 1-10µg/mL DAPI stain (Fisher, D1306) in Wash Buffer B for thin (10µm) tissue.
- Prepare on demand.

Permeabilization Buffer (PBS-t)
- Nuclease-free water
- 1x PBS Buffer (Invitrogen, AM9625)
- 0.5% (v/v) Triton X-100 (Sigma, T8787-100mL)
- Store at RT

Permeabilization Buffer Wash (PBS-tw)
- Nuclease-free water
- 1x PBS Buffer (Invitrogen, AM9625),
- 0.1% (v/v) Tween 20 (Sigma, P9416-100ML)
- Store at RT

SDS Clearing Solution (SDS-CS)
- 1x PBS
- 4% SDS (Sigma, 75746)
- Nuclease Free Water
- Store at RT

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In-situ hybridization, FISH, fluorescence, RNA, iterative FISH, formamide, amplified probes, bDNA, branched DNA, fluidics, acrydite, linker probe, anchor probe, encoding probes, tissue, cells, thick tissue, clearing, digestion, polyacrylamide gel