Multiplexed Iterative FISH Experimental Protocol SOP002.v3.12 V.3

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ABSTRACT

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). This protocol also provides the option for signal amplification using Branched DNA [bDNA] amplification. Iterative rounds of labeling and imaging are possible through the use of a readout probe with a cleavable disulfide (S-S) reporter molecule, a method that allows for minimal disruption to sample integrity between rounds. This document also describes cell and tissue preparation for RNA FISH as well as a system of mRNA anchoring using a specialized poly-t (locked nucleic acid, LNA) probe with an acrydite linker to bind mRNAs to a polyacrylamide (gel) matrix. Clearing and digestion techniques are used to reduce cellular autofluorescence and increase the signal to noise ratio of the final data. This protocol is *strongly* derived from Moffitt 2016 (https://doi.org/10.1016/bs.mie.2016.03.020) with some modifications with credit for a majority of this protocol due to Moffitt et al 2016.

ATTACHMENTS

SOP002.v3.12 Multiplexed Iterative FISH_Paintshop_Saber.pdf

GUIDELINES

v3.12 revision 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 re-hydration time.

MATERIALS

MANUSCRIPT CITATION:
- 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

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Solution Preparation:

Wash Buffer A (40% Formamide Wash Buffer)

- 2x SSC (20X), RNase-free **Thermo Fisher Catalog #AM9763**
- 1% (vol/vol) Tween 20 **Sigma Aldrich Catalog #P9416-100ML**
- 40% (vol/vol) Formamide **Thermo Fisher Scientific Catalog #AM9342**
- Nuclease-free water

Wash Buffer A Master Mix, 45 ml:

- 36.75 ml nuclease-free water
- 7.5 ml SSC (20X), RNase-free **Thermo Fisher Catalog #AM9763**
- 750 µl Tween 20 **Sigma Aldrich Catalog #P9416-100ML**
- Add 40% Formamide **Thermo Fisher Scientific Catalog #AM9342** to prepare on demand

Wash Buffer B

- 2x SSC (20X), RNase-free **Thermo Fisher Catalog #AM9763** prepared in nuclease-free water

Wash Buffer C (10% Formamide Wash Buffer)

- Nuclease-free water
- 2x SSC (20X), RNase-free **Thermo Fisher Catalog #AM9763**
- 10% (vol/vol) Formamide **Thermo Fisher Scientific Catalog #AM9342**

Wash Buffer C Master Mix, 49.5 ml:

- 44 ml nuclease-free water
- 5.5 ml SSC (20X), RNase-free **Thermo Fisher Catalog #AM9763**
- Add 10% Formamide **Thermo Fisher Scientific Catalog #AM9342** formamide to prepare on demand

Saber Encoding Hybridization Buffer

- Nuclease-free water
- 2x SSC (20X), RNase-free **Thermo Fisher Catalog #AM9763**
- 40% (vol/vol) Formamide **Thermo Fisher Scientific Catalog #AM9342** Formamide

Keywords: 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

- 0.1% (wt/vol) Yeast tRNA **Thermo Fisher Catalog #15401011**
- 1% (vol/vol) RNase Inhibitor, Murine - 15,000 units **New England Biolabs Catalog #M0314L**
- 1% (vol/vol) Tween 20 **Sigma Aldrich Catalog #P9416-100ML**
- 10% (wt/vol) Dextransulfate **Sigma Aldrich Catalog #D8906-100g**
- Add 5-200 µM encoding probes depending on the size of the pool
- Prepare on demand

Saber Encoding Hybridization Buffer Master Mix, 4.8 ml (for 8.0 ml prep with formamide added)

- Nuclease-free water
- 800 µl SSC (20X), RNase-free **Thermo Fisher Catalog #AM9763**
- 320 µl Yeast tRNA **Thermo Fisher Catalog #15401011** reconstituted to 25mg/ml
- 80 µl RNase Inhibitor, Murine - 15,000 units **New England Biolabs Catalog #M0314L**
- 80 µl Tween 20 **Sigma Aldrich Catalog #P9416-100ML**
- 0.8 g Dextransulfate **Sigma Aldrich Catalog #D8906-100g**
- Aliquot mix and store at -20°C
- To prepare on demand, add 40% (vol/vol) Formamide **Thermo Fisher Scientific Catalog #AM9342** to master mix at time of use
- Add 5-200 µM encoding probes depending on the size of the pool

Encoding Buffer Rinse (SSC-tw)

- Nuclease-free water
- 2x SSC (20X), RNase-free **Thermo Fisher Catalog #AM9763**
- 0.1% (vol/vol) Tween 20 **Sigma Aldrich Catalog #P9416-100ML**
- Store at Room Temperature

PA Solution

- Nuclease-free water
- 4% (vol/vol) 40% Acrylamide/Bis Solution 19:1 **Contributed by users Catalog #1610144**

- 60 mM Tris (1 M), pH 8.0, RNase-free **Thermo Fisher Catalog #AM9856**
- 0.3 M NaCl (5 M) RNase-free **Thermo Fisher Scientific Catalog #AM9759**
- One of the following:

1. For four-color experiments: 1:500 dilution 0.1µm-diameter light yellow beads Fluorescent Light Yellow Particles **SpheroTech Catalog #FP-0245-2**
2. For two-color experiments:1:200,000 dilution of 0.1µm-diameter carboxylate-modified orange fluorescent beads FluoSpheres™ Carboxylate-Modified Microspheres **Thermo Fisher Scientific Catalog #F-8800**

- De-gas solution before use
- Prepare on demand

PA Gel

- PA Solution including polymerizing agents:

1. 0.03% (wt/vol) Ammonium Persulfate **Sigma Catalog #A3678**
2. 0.15% (vol/vol) TEMED
3. 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) RNase Inhibitor, Murine - 15,000 units **New England Biolabs Catalog #M0314L**
- Store in aliquots at -20°C

Amplifier Hybridization Buffer

- Nuclease-free water
- 2x SSC (20X), RNase-free **Thermo Fisher Catalog #AM9763**
- 10% (vol/vol) Formamide **Thermo Fisher Scientific Catalog #AM9342**
- 0.1% (wt/vol) Yeast tRNA **Thermo Fisher Catalog #15401011**
- 1% (vol/vol) RNase Inhibitor, Murine - 15,000 units **New England Biolabs Catalog #M0314L**
- 10% (wt/vol) Dextransulfate **Sigma Aldrich Catalog #D8906-100g**
- Prepare on demand

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

- Nuclease-free water
- 800 µl SSC (20X), RNase-free **Thermo Fisher Catalog #AM9763**
- 320 µl Yeast tRNA **Thermo Fisher Catalog #15401011** reconstituted to 25mg/ml
- 80 µl RNase Inhibitor, Murine - 15,000 units **New England Biolabs Catalog #M0314L**
- 0.8 g Dextransulfate **Sigma Aldrich Catalog #D8906-100g**
- Aliquot mix and store at -20°C
- To prepare on demand, add 10% (vol/vol) Formamide **Thermo Fisher Scientific Catalog #AM9342** to master mix at time of use
- Add 5 nM amplifiers

Readout Hybridization Buffer

- 2x SSC (20X), RNase-free **Thermo Fisher Catalog #AM9763**
- 10% (vol/vol) Ethylencarbonat 98% **Sigma Aldrich Catalog #E26258**
- 0.1% (vol/vol) RNase Inhibitor, Murine - 15,000 units **New England Biolabs Catalog #M0314L**
- Nuclease-free water
- 3 nM readout probes
- Prepare on demand

Wash Buffer D (Readout Wash Buffer)

- 2x SSC (20X), RNase-free **Thermo Fisher Catalog #AM9763**
- 10% (vol/vol) Ethylencarbonat 98% **Sigma Aldrich Catalog #E26258**
- Store at Room Temperature

Imaging Buffer

- 2x SSC (20X), RNase-free **Thermo Fisher Catalog #AM9763**
- 50 mM Tris (1 M), pH 8.0, RNase-free **Thermo Fisher Catalog #AM9856**
- 10% (wt/vol) D-( )-Glucose **Sigma Aldrich Catalog #DX0145-1**
- 2 mM Trolox **Sigma Aldrich Catalog # 238813**

- 0.5 mg/ml Glucose oxidase **Sigma Aldrich Catalog #G2133**
- 40 µg/ml Catalase **Sigma Catalog #C30**
- 0.1% (vol/vol)
  RNase Inhibitor, Murine - 15,000 units **New England Biolabs Catalog #M0314L**
- Nuclease-free water
- Prepare on demand
- Store under layer of Mineral Oil when using fluidics system
  Mineral oil **Sigma Aldrich Catalog #330779**

Cleavage Buffer

- 2x SSC (20X), RNase-free **Thermo Fisher Catalog #AM9763**
- 50 mM
  Tris(2-carboxyethyl)phosphine hydrochloride solution **Sigma Aldrich Catalo #646547-10X1ML**
- Prepare on demand

DAPI Staining Solution

- 50 µg/ml
  4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) **Thermo Fisher Scientific Catalog #D1306**
  in Wash Buffer B for thick (40 µm) tissue
- 1-10 µg/ml
  4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) **Thermo Fisher Scientific Catalog #D1306**
  in Wash Buffer B for thin (10 µm) tissue
- Prepare on demand

Permeabilization Buffer (PBS-t)

- Nuclease-free water
- 1x
  PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Invitrogen - Thermo Fisher Catalog #AM9625**
- 0.5% (v/v) Triton™ X-100 **Sigma Aldrich Catalog #T8787-100ML**
- Store at Room Temperature

Permeabilization Buffer Wash (PBS-tw)

- Nuclease-free water

- 1x
  PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Invitrogen - Thermo Fisher Catalog #AM9625**
- 0.1% (v/v) Tween 20 **Sigma Aldrich Catalog #P9416-100ML**
- Store at Room Temperature

SDS Clearing Solution (SDS-CS)

- Nuclease Free Water
- 4% SDS **Sigma Catalog #75746**
- 200 mM Boric Acid **Sigma Catalog #B0394**
- pH to 8.5
- Store at Room Temperature

SAFETY WARNINGS

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.

BEFORE START INSTRUCTIONS

All reagents for this protocol should be prepared sterile and RNase-free.
All incubation periods should be done in the dark.
Find reagent/buffer preparation instructions at index "Materials".

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 - Clear & digest 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.

Document Summary

1 **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.

Part 1 - Tissue or Cell-Based Experiment Preparation

2 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 requirements 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.

Part 1 - Step 1: Coverslip Functionalization

3 *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.*

Part 1 - Step 2: Mount, SDS Pretreat and Permeabilize Sam...

1h 5m

4 If using [M] 4 % PFA-fixed tissue , follow step 4. For [M] 4 % (v/v) PFA-fixed cells , skip below to step 5.

5 ***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.*

1h 15m

i. Slice tissue and place slices in 1xPBS for 00:05:00 . Remove PBS and repeat this for a second wash.

ii. Pretreat tissue by washing in 4% SDS Clearing Solution (SDS-CS), once for 00:05:00 .

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 in Room temperature PBS buffer for 00:30:00 to rehydrate the sample.

v. To bring the sample in sufficient contact with the coverslip surface, aspirate the PBS buffer and

place in 37 °C – 45 °C oven for 00:05:00 - 00:10:00 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 Room temperature for 00:10:00 .

vii. Remove PFA from the sample and rinse with 1x PBS for 00:05:00 at RT, two times.

6 ***Using 4% PFA-fixed cells*** *grown on coverslip (optionally, use 8-chamber well or similar)* 1h 15m

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 01:00:00 at Room temperature).

ii. Alternatively, pipette 100µL permeabilization buffer (PBS-t) to each well and incubate at 0 °C for 00:10:00 with gentle rocking.

iii. Rinse with Room temperature permeabilization buffer rinse (PBS-tw).

iv. Aspirate rinse from the sample and let dry.

7 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.

Part 1 - Step 3: Hybridize Linker (optional; use when ge... em... 1d 12h 30m

8 Wash & equilibrate sample by immersing slip-mounted sample in 37 °C pre-heated ~ 200 µL Wash Buffer A for 00:30:00 . 30m

9 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).

10 Remove slip from Wash Buffer A and carefully wipe away excess buffer surrounding sample.

11 Dispense 125 µL Encoding Hybridization Buffer containing [M] 1 micromolar (µM) 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.*

12 Incubate at 37 °C in a humidified chamber for 18:00:00 - 24:00:00 up to 36:00:00 . 1d 12h

Part 1 - Step 4: Wash Away Residual Linker 1h 10m

13 Remove the hybridization buffer and carefully remove excess buffer surrounding sample.

14 Immerse slip in pre-heated 37 °C Wash Buffer A for 30 min , two times.

15 Wash two times in 37 °C pre-heated Encoding Wash Buffer (SSC-tw) for 5 min each.

16 Wash two times in Room temperature 1x PBS.

Part 1 - Step 5: Gel Embed Sample (optional - gel embed wh... 1h 34m

17 1. Wash sample for 00:02:00 ** with de-gassed PA Solution. **adjust time based on sample size. For 100µm tissue slices, increase this to 3 hours. 2m

18 Wash sample for 00:02:00 with PA Gel Solution and then remove. 2m

19 1. Cast a thin PA film by adding 50 µL gel solution - 100 µL gel solution to the sample and invert a smaller (25 mm) 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.

20 Allow casting for 01:30:00 at Room temperature . 1h 30m

21 After casting, carefully remove the smaller coverslip from your sample. If the coverslip is stuck, you can loosen the coverslip by immersing in SDS-CS at 37 °C .

Part 1 - Step 6: Digest & Clear Sample (optional) (buffer an... 15h 55m

22 ***Note:*** *For lung tissue start at step 22. Skip ahead to step 24 for brain tissue.*

23 Incubate sample in 3 mL PBS with [M] 10 % collagenase/elastase at [M] 20.000 U/mL for 03:00:00 at 37 °C . 3h

24 Wash the sample with a quick rinse of RT 1x PBS followed by two 5 min washes of 1x PBS at Room temperature .

25 Wash the sample on the coverslip twice with 1 mL SDS Clearing Solution (SDS-CS) for 00:05:00 each wash at 37 °C 5m

26 Incubate with 3 mL SDS Clearing Solution with [M] 1 % Proteinase K in a humidified chamber for 01:00:00 - 12:00:00 at 37 °C, depending on the sample. 13h

27 Wash the sample by immersing it in Wash Buffer B four times for 00:05:00 at Room temperature 5m

Part 1 - Step 7: Hybridize Encoding Probes 1d 12h 30m

28 Wash and equilibrate sample by immersing slip-mounted sample in 41 °C pre-heated 200-500 µL Wash Buffer A for 00:30:00. 30m

29 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).

30 Remove the slip from Wash Buffer A and carefully wipe away the excess buffer surrounding the sample.

31 Dispense 125 µL Encoding Hybridization Buffer containing [M] 5 micromolar (µM) - [M] 200 micromolar (µM) 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.

32 Incubate at 41 °C in a humidified chamber for 18:00:00 - 24:00:00 up to 36:00:00. 1d 12h

Part 1 - Step 8: Wash Away Residual Encoding Probes

1h 10m

33 Remove the hybridization buffer and carefully remove the excess buffer surrounding the sample.

34 Wash the sample in pre-heated 41 °C Wash Buffer A for 00:30:00, two times. 30m

35 Wash two times in 41 °C pre-heated Encoding Wash Buffer (SSC-tw) for 00:05:00. 5m

36 Wash two times in Room temperature 1x PBS.

Part 1 - Step 9: Hybridize amplifiers (optional)

1h 25m

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

38 Incubate sample in Wash Buffer C at 37 °C for 00:30:00. 30m

39 Aspirate to remove Wash Buffer C.

40 Hybridize primary amplifier. Incubate the sample in a 125 µL droplet of [M] 5 nanomolar (nM) primary amplifier* in amplifier hybridization buffer for 00:15:00** in humidity-controlled 37°C incubator. 15m

*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.

41 Wash 3 times with Wash C for 5-10 min each at Room temperature.

42 Hybridize secondary amplifier. Incubate the sample in a 125 µL droplet [M] 5 nanomolar (nM) secondary amplifier* in amplifier hybridization buffer for 00:15:00** in humidity-controlled 37°C incubator. 15m

*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.

43 Wash twice in Room temperature Wash C for 5 min each followed by a 00:15:00 - 00:30:00 wash in 37 °C Wash C. 45m

44 Perform MULTIPLEXED ITERATIVE FISH Imaging (Part 2) immediately or store sample for up to 24 hours in storage buffer at 4 °C.

Part 2a - MULTIPLEXED ITERATIVE FISH Imaging with Fluidi...

45

Note

***The following steps (Part 2a) are used for the Full MULTIPLEXED ITERATIVE FISH protocol. For experiments that don't use the fluidics system, move to Part 2B below.*

46 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.

Part 2a - Step 1: Prepare Solutions for Imaging

47 Prepare the following solutions with the corresponding volumes:

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

Part 2a - Step 2: Assemble Fluidics System

48 Make sure that all tubing is properly connected. MULTIPLEXED ITERATIVE FISH probes and preparation time are costly so leaks need to be avoided at all costs.

49 Ensure the system is fully assembled, plugged in and turned on.

50 Double-check correctness of the details for the pump protocol for the MULTIPLEXED ITERATIVE FISH
Fluidics for the current project.

51 Load the sample to the flow cell and connect.

52 Carefully load all solutions to the proper reservoirs.

Part 2a - Step 3: MULTIPLEXED ITERATIVE FISH Imaging Pro.

1h 5m

53 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:

54 Readout hybridization buffer (with readout probes) 2h 22m

a. Run 2 mL over 00:03:00 to prime buffer to the sample.
b. Run additional 2.5 mL over the sample for 00:04:00.
c. Pause flow for 00:15:00 - 02:00:00 depending on sample size (10 µm = 15 min, 30 µm = 60 min, 100 µm = 120 min).

55 Readout Wash Buffer (Wash D) 13m 30s

a. Run 2 mL over 00:03:30 to flush.
b. Run 2 mL over 00:10:00 to wash.

56 Imaging Buffer 10m

a. Run 2 mL over 00:10:00 to run remaining wash D over sample and prime imaging buffer to the sample.

57 1. 2x SSC Wash Buffer (Wash B) 3m

a. Run 1 mL over 00:03:00 to move 1mL imaging buffer over the sample.

58 Imaging. Pause fluidics and proceed with imaging.

59 TCEP Cleavage Buffer - 18m 30s

a. Run 2 mL in 00:03:30 min to prime cleavage buffer to the sample.
b. Run 1 mL over sample for 00:05:00

c.Pause flow for 00:10:00

60 2x SSC Wash Buffer (Wash B) - 14m

a. Run 2 mL in 00:10:00 to run cleavage buffer over sample and prime SSC buffer.

b. Run 2 mL in 00:04:00 to rinse off cleavage buffer.

61 Repeat steps 54-60 for each readout round.

62 When all readout rounds are complete proceed with step 63.

63 DAPI Stain 1h

a. Run 2 mL DAPI in 2xSSC (Wash B) for 01:00:00 .

i. Use [M] 50 µg/mL DAPI for thick (40 µm) samples.

ii. Use [M] 1 µg/mL - [M] 10 µg/mL DAPI for 10 µm samples.

64 2xSSC (Wash B) - 2 mL for 00:03:30 to flush. 3m 30s

65 Imaging Buffer - 2 mL in 00:06:00 then halt flow. 6m

66 Proceed with Imaging.

Part 2b - Alternate MULTIPLEXED ITERATIVE FISH Imaging ...

67

Note

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).

68 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.

Part 2b - Step 1a: MULTIPLEXED ITERATIVE FISH Imaging P...

1h

69 Readout Probe Hybridization. 10m

a. Pipette 200 µL 3nM readout probes in Readout Hybridization Buffer to sample and incubate at Room temperature for 00:10:00.
b. Aspirate Readout Hybridization Buffer from the sample.

70 Wash away unbound probe by adding 200 µL RT Readout Wash Buffer D to sample for 5 min, two times. Additional washes may improve the result.

71 Dapi Stain. Add 200 µL Wash Buffer B with DAPI nuclear stain (at 1µg/mL) to sample and incubate for 00:30:00 at 37 °C. 30m

72 Remove the Dapi stain and wash with Wash Buffer B for 5 min, two times.

73 Add 100 µL - 200 µL Imaging buffer to sample and mount to glass plate with clear nail polish.

74 Proceed with imaging.

Part 2b - Step 1b: MULTIPLEXED ITERATIVE FISH Imaging - ... 35m

75 Readout Probe Hybridization. 10m

a. Pipette 200 µL 3nM readout probes in Readout Hybridization Buffer to sample and incubate at Room temperature for 00:10:00.

b. Aspirate Readout Hybridization Buffer from the chambers.

76 Wash away unbound probe by adding 200 µL RT Readout Wash Buffer D to sample for 5 min, two times. Additional washes may improve the result.

77 Add 100 µL - 200 µL Imaging buffer to sample.

78 Proceed with imaging of the round.

79 TCEP Cleavage Buffer – 100 µL for 00:15:00. 15m

80 2x SSC Wash Buffer (Wash B) – 250 µL each well, three times.

81 Repeat steps 81-85 for each probe set round.

82 Move on to step 88 when all rounds are complete.

Part 2b - Step 2: Dapi Stain the Sample

40m

83 Add 200 µL Wash Buffer B with DAPI nuclear stain (at 1µg/mL) to sample and incubate for 30m 00:30:00 at 37 °C .

84 Wash sample in Wash Buffer B for 5 min two times.

Part 2b - Step 3:

85 Proceed to Imaging of the Sample.