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Multiplexed Iterative FISH Experimental Protocol SOP002.v3.5 V.2

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protocol .

Human Cell Atlas Method Development Community

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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.

Multiplexed Iterative FISH Experimental Protocol SOP002.v3.5.pdf

Rory Kruithoff, Lei Zhou, Douglas Shepherd 2021. Multiplexed Iterative FISH Experimental Protocol SOP002.v3.5. **protocols.io** https://protocols.io/view/multiplexed-iterative-fish-experimental-protocol-s-byxzpxp6 Rory Kruithoff

protocol

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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

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v3.5 revision notes

1. *Minor edits and typo corrections.*
2. *Updated solution preparations with manufacturer and catalog numbers as well as adding Amplifier and Saber Encoding Hybridization Buffer master mixes.*
3. *Updated linker hybridization and wash temperatures from 41°C to 37°C.*
4. *Added adjustment note for amplifier hybridizations.*

Solution Preparation:

Wash Buffer A (40% Formamide Wash Buffer)

SSC (20X), RNase-free **Thermo**

- 2x **Fisher Catalog #AM9763**

Tween 20 **Sigma**

- 1% (vol/vol) **Aldrich Catalog #P9416-100ML**

Formamide **Thermo Fisher**

- 40% (vol/vol) **Scientific Catalog #AM9342**
- Nuclease-free water

Wash Buffer A Master Mix, 45 ml:

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

Wash Buffer B

SSC (20X), RNase-free **Thermo**

- 2x **Fisher Catalog #AM9763** prepared in nuclease-free water

Wash Buffer C (10% Formamide Wash Buffer)

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

Wash Buffer C Master Mix, 49.5 ml:

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

Saber Encoding Hybridization Buffer

- Nuclease-free water
  SSC (20X), RNase-free **Thermo**
- 2x **Fisher Catalog #AM9763**
  Formamide **Thermo Fisher**
- 40% (vol/vol) **Scientific Catalog #AM9342** Formamide
  Yeast tRNA **Thermo**
- 0.1% (wt/vol) **Fisher Catalog #15401011**
  RNase Inhibitor, Murine - 15,000 units **New England**
- 1% (vol/vol) **Biolabs Catalog #M0314L**

Tween 20 **Sigma**

- 1% (vol/vol) **Aldrich Catalog #P9416-100ML**

Dextransulfate **Sigma**

- 10% (wt/vol) **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

SSC (20X), RNase-free **Thermo**

- 800 µl **Fisher Catalog #AM9763**

Yeast tRNA **Thermo**

- 320 µl **Fisher Catalog #15401011** reconstituted to 25mg/ml

RNase Inhibitor, Murine - 15,000 units **New England**

- 80 µl **Biolabs Catalog #M0314L**

Tween 20 **Sigma**

- 80 µl **Aldrich Catalog #P9416-100ML**

Dextransulfate **Sigma**

- 0.8 g **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

SSC (20X), RNase-free **Thermo**

- 2x **Fisher Catalog #AM9763**

Tween 20 **Sigma**

- 0.1% (vol/vol) **Aldrich Catalog #P9416-100ML**
- Store at Room Temperature

PA Solution

- Nuclease-free water

40% Acrylamide/Bis Solution 19:1 **Contributed by**

- 4% (vol/vol) **users Catalog #1610144**

Tris (1 M), pH 8.0, RNase-free **Thermo**

- 60 mM **Fisher Catalog #AM9856**

NaCl (5 M) RNase-free **Thermo Fisher**

- 0.3 M **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:
  Ammonium

1. 0.03% (wt/vol) 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
  RNase Inhibitor, Murine - 15,000 units **New England**
- 0.1% (vol/vol) **Biolabs Catalog #M0314L**
- Store in aliquots at -20°C

Amplifier Hybridization Buffer

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

SSC (20X), RNase-free **Thermo**

- 800 µl **Fisher Catalog #AM9763**

  Yeast tRNA **Thermo**

- 320 µl **Fisher Catalog #15401011** reconstituted to 25mg/ml

  RNase Inhibitor, Murine - 15,000 units **New England**

- 80 µl **Biolabs Catalog #M0314L**

  Dextransulfate **Sigma**

- 0.8 g **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

SSC (20X), RNase-free **Thermo**

- 2x **Fisher Catalog #AM9763**

  Ethylencarbonat 98% **Sigma**

- 10% (vol/vol) **Aldrich Catalog #E26258**

  RNase Inhibitor, Murine - 15,000 units **New England**

- 0.1% (vol/vol) **Biolabs Catalog #M0314L**
- Nuclease-free water
- 3 nM readout probes
- Prepare on demand

Wash Buffer D (Readout Wash Buffer)

SSC (20X), RNase-free **Thermo**

- 2x **Fisher Catalog #AM9763**

  Ethylencarbonat 98% **Sigma**

- 10% (vol/vol) **Aldrich Catalog #E26258**
- Store at Room Temperature

Imaging Buffer

SSC (20X), RNase-free **Thermo**

- 2x **Fisher Catalog #AM9763**

  Tris (1 M), pH 8.0, RNase-free **Thermo**

- 50 mM **Fisher Catalog #AM9856**

  D-( )-Glucose **Sigma**

- 10% (wt/vol) **Aldrich Catalog #DX0145-1**

Trolox **Sigma**

- 2 mM **Aldrich Catalog # 238813**

Glucose oxidase **Sigma**

- 0.5 mg/ml **Aldrich Catalog #G2133**
- 40 µg/ml Catalase **Sigma Catalog #C30**

RNase Inhibitor, Murine - 15,000 units **New England**

- 0.1% (vol/vol) **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

SSC (20X), RNase-free **Thermo**

- 2x **Fisher Catalog #AM9763**
- 50 mM

Tris(2-carboxyethyl)phosphine hydrochloride solution **Sigma**

Aldrich Catalog #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

Triton™ X-100 **Sigma**

- 0.5% (v/v) **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**
  Tween 20 **Sigma**
- 0.1% (v/v) **Aldrich Catalog #P9416-100ML**
- Store at Room Temperature

SDS Clearing Solution (SDS-CS)

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

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.

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.

Part 1 - Tissue or Cell-Based Experiment Preparation

1 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 for 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.

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> *If doing multiple hybridizations without using fluidics system (part 2b), defer to setting up cell samples in a chamber slip.It would be difficult to run multiple hybridizations on tissue samples without using fluidics either automated or manually.*

Part 1 - Step 1: Coverslip Functionalization

3 *Refer to current version of SOP003 for protocol on Coverslip Functionalization.*

Part 1 - Step 2: Mount, SDS Pretreat and Permeabilize Sample 1h 5m

4 If using [M]**4 % PFA-fixed tissue** , follow step 5. For [M]**4 % (v/v) PFA-fixed cells** , skip below to step 11.

5 If frozen, thaw tissue to **Room temperature** .

6 Wash tissue slices twice in nuclease-free 1xPBS for **00:02:00** - **00:05:00** each. 5m

7 Mount tissue slices (10-40µM sections typically) to functionalized (silanized) coverslip and dry in 37°C oven for 30mins.

8 Pretreat tissue by washing in [M]**4 % SDS Clearing Solution** (SDS-CS), for **00:05:00** minutes, four times. 5m

9 To permeabilize the tissue, immerse the slip mounted tissue in [M]**70 % (v/v) ethanol** **Overnight** at **4 °C** (recommended) in a Pyrex 60 mm petri dish (Fisher 08-747A).

> For faster results, sample can be incubated in ethanol for 1 hour at room temperatur.

10 Remove sample from ethanol, carefully wipe away excess ethanol surrounding tissue and let dry.

11 Using [M]**4 % PFA-fixed cells** grown on coverslip (optionally, use 8-well chamber slip).

12 To permeabilize cells, immerse the slip mounted sample in [M]**70 % (v/v) ethanol** **Overnight** at **4 °C** (recommended) in a Pyrex 60 mm petri dish (Fisher 08-747A).

> For faster results, sample can be incubated in ethanol for 1 hour at room temperatur.

13  10m

Alternatively, pipette **100 µL permeabilization buffer (PBS-t)** to each well and incubate at **Room temperature** for **00:10:00** with gentle rocking.

14 Aspirate rinse from the wells and let dry.

Part 1 - Step 3: Hybridize Linker (optional; use when gel embedding and digesting sample) 1d 12h 30m

15 Wash & equilibrate sample by immersing slip-mounted sample in 37°C pre-heated 200µL Wash Buffer A for 30 min.

16 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).

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

18 Dispense **200 µL Encoding Hybridization Buffer** containing **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.

19 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

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

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

22

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

23

Wash two times in **Room temperature** 1x PBS.

Part 1 - Step 5: Gel Embed Sample (optional - gel embed when clearing and digesting) 1h 34m

24 2m

Wash sample for **00:02:00** with de-gassed PA Solution.

25 2m

Wash sample for **00:02:00** with PA Gel Solution and then remove.

26 Cast a thin PA film by adding **50 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. You may need to adjust the volume depending on the sample thickness. Aim to cast a gel that is approximately the same thickness of your sample.

27 1h 30m

Allow casting for **01:30:00** at **Room temperature** .

28 After casting, carefully remove the smaller coverslip from your sample. If the coverslip is stuck, you can loosen the coverslip by immersing in Wash C or SDS-CS at **37 °C** , depending on the next step for your experiment.

Part 1 - Step 6: Clear & Digest Sample (optional) (buffer and enzyme may vary depending on sample type)
15h 55m

29 5m

Wash the gel-casted sample on the coverslip twice with **1 mL SDS Clearing Solution (SDS-CS)** for **00:05:00** each wash.

30 12h

Incubate with **3 mL SDS Clearing Solution** with **1 % Proteinase K** in a humidified chamber for a minimum **12:00:00** at **37 °C** .

31 5m

Wash the sample by immersing it in Wash Buffer B four times for **00:05:00** .

32 Wash in PBS 3 times for 5 min each at **Room temperature** .

33 3h

Immerse sample in **3 mL PBS** with **10 % collagenase/elastase** at **20.000 U/mL** and incubate for **03:00:00** at **37 °C** .

34 Wash the sample in 1x PBS 3 times for 5 min each at **Room temperature** .

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

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

36 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).

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

38 Dispense **200 µL Encoding Hybridization Buffer** containing **5 Micromolar (µM)** - **200 Micromolar (µM) encoding probes** (depending on the number of unique encoding probes in the probe set) to your sample, replace the petri dish lid, parafilm the dish and place the dish with the sample in the humidified chamber.

39 1d 12h

Incubate at **41 °C** in a humidified chamber for **18:00:00** - **24:00:00** up to **36:00:00** .

Part 1 - Step 8: Wash Away Residual Encoding Probes 1h 10m

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

41 Immerse the slip in pre-heated **41 °C** Wash Buffer A for 30 min, two times.

42 Wash two times in **41 °C** pre-heated Encoding Wash Buffer (SSC-tw) for 5 mins.

43 Wash two times in **Room temperature** 1x PBS.

Part 1 - Step 9: Hybridize amplifiers (optional) 1h 25m

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

45 30m

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

46 Remove from Wash Buffer C and blot off extra buffer.

47 15m

Hybridize primary amplifier. Invert sample onto a **50 µL droplet** of **5 Nanomolar (nM) primary amplifier** in amplifier hybridization buffer for **00:15:00** in humidity-controlled **37 °C** incubator. Adjust amplifier hybridization volume and hybridization time according to sample size and thickness.

48 Wash 3 times with Wash C for 5 min each at **Room temperature** .

49 15m

Hybridize secondary amplifier. Invert sample onto a **50 µL droplet** of **5 Nanomolar (nM) secondary amplifier** in amplifier hybridization buffer for **00:15:00** in humidity-controlled **37 °C** incubator. Adjust amplifier hybridization volume and hybridization time according to sample size and thickness.

50 15m

Wash twice in Wash C for 5 min followed by a **00:15:00** wash in Wash C.

51 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 Fluidics System

52

The following steps are used for the Full MULTIPLEXED ITERATIVE FISH protocol. For a trial that doesn´t use the fluidics system, move to Part 2B (optional) below.

53 The basic imaging process for MULTIPLEXED ITERATIVE FISH involves iterative fluid exchange to apply dye-conjugated readout probes, washes, anti-photobleaching imaging buffer, reductive cleavage buffer and rinse. A single round of fluidics takes approximately 45 minutes with an automated fluidics system not including imaging times. We strongly suggest using an automated fluidics system to improve the consistency of each round as well as efficiency. While manually controlling the fluidics system is possible, this would require inconvenient around the clock staffing to run a single multiple round experiment of 16 genes with two color readouts.

Part 2a - Step 1: Prepare Solutions for Imaging

54 Prepare the following solutions with the corresponding volumes:

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

Part 2a - Step 2: Assemble Fluidics System

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

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

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

58 Load the sample to the fluidics chamber.

59 Carefully load all solutions to the proper reservoirs.

Part 2a - Step 3: MULTIPLEXED ITERATIVE FISH Imaging Protocol 1h 5m

60 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 (example pump speeds listed in bold below).

61 11m

(9) Readout hybridization buffer (with probes)

a. Wash **2 mL** over **00:05:00** to flush.

b. Wash additional **2 mL** over sample in **00:06:00** .

62 9m

(4) Readout Wash Buffer (Wash D) - **2 mL** in **00:09:00** .

63 6m

(7) Imaging Buffer - **2 mL** in **00:06:00** then halt flow.

64 Imaging. Proceed with Imaging.

65 15m

(3) TCEP Cleavage Buffer - **3 mL** in **00:15:00** .

66 4m

(10) 2x SSC Wash Buffer (Wash B) - **2 mL** in **00:04:00** .

67 Repeat steps 63-68 for each probe set.

68 When all readout rounds are complete proceed with steps 8-11.

69 10m

DAPI Stain – Wash **2 mL** DAPI in 2xSSC (Wash B) for **00:10:00** .

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

b. Use [M]**1 µg/mL** -[M]**10 µg/mL** for 10 µm samples.

This step may need to be longer incubation for thicker tissue samples. Moffitt uses only 10µm thick samples.

70 2xSSC (Wash B) - **2 mL** for **00:04:00** . 4m

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

72 

Imaging. Image at 405 nm.

Part 2b - Alternate MULTIPLEXED ITERATIVE FISH Imaging without Fluidics

73

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).

74 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 Protocol - Single Hybridization using coverslip mounted sample.

1h

75  10m

Readout Probe Hybridization.

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.

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.

30m

77 

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** .

78 

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

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

80 

Proceed with imaging.

Part 2b - Step 1b: MULTIPLEXED ITERATIVE FISH Imaging - Multiple Hybridizations Using Chamber-slip

35m

10m

81 Readout Probe Hybridization.

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.

82 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.

83 

Add **100 µL** - **200 µL Imaging buffer** to sample. Proceed with imaging of the round.

15m

84 TCEP Cleavage Buffer – **100 µL** for **00:15:00** .

85 2x SSC Wash Buffer (Wash B) – 250µL each well, three times.

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

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

Part 2b - Step 2: Dapi Stain the Sample 40m

88 30m

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

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

Part 2b - Step 3:

90 Proceed to Imaging of the Sample.