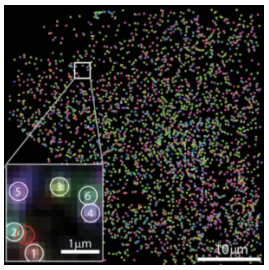
Mar 29, 2018

RNA Imaging with MERFISH - Imaging



In 1 collection

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Human Cell Atlas Metho... Neurodegeneration Met...

Max Brown

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We use this protocol and it's working

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Abstract

The basic imaging process for MERFISH involves several fluid handling steps, e.g. introduction of hybridization buffers and wash and imaging buffers, in conjunction with the collection of smFISH images and the photobleaching of the sample. While it would be possible to perform these fluid exchange and imaging steps manually, we strongly recommend the use of an integrated and automated fluid exchange and imaging approach. Here we provide protocols for how to construct a fluid handling system that should be compatible with a variety of microscopes. We also discuss the basic protocols for hybridizing readout probes to samples on the microscope, imaging the sample, and bleaching residual signal.

Attachments

PDF

merFISH.pdf

1.4MB

Guidelines

The protocol requires the following reagents:

1. Readout probe hybridization buffer
- 25 mL 20% w/v dextran sulfate (Section 5.1)
- 5 mL 20X SSC
- 5 mL 100% deionized formamide
- 14.5 mL nuclease-free water
- 0.5 mL 200 mM VRC
- Make fresh each day

2. Imaging buffer master mix
- In an RNAse-free bottle (such as an empty nuclease-free water bottle) add the following
- 50 mL 20X SSC
- 25 mL 1 M TrisHCl pH 8 (Ambion; AM9856)
- 325 mL nuclease-free water
- 50 g glucose (Sigma; G8270)
- A D/RNaseFree-treated stir bar
- Stir until glucose is dissolved
- Add nuclease-free water to 500 mL
- Store at room temperature for up to a few weeks

3. Trolox solution
- 20 mg (±)-6-hydrozy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; Sigma; 238813)
- 200 µL methanol (Sigma; 322415)
- Make fresh each day

4. Imaging buffer
- 40 mL imaging buffer master mix
- 200 µL Trolox solution
- 200 µL catalase (Sigma; C30)
- 42 mg glucose oxidase (Sigma; G2133)
- Make fresh and use immediately

5. Photobleaching buffer
- 5 mL 20X SSC
- 44.5 mL nuclease-free water
- 0.5 mL 200 mM VRC
- Make fresh each day

6. Readout probe wash buffer
- 5 mL 2X SSC

- 10 mL 100% deionized formamide
- 34.5 mL nuclease-free water
- 0.5 mL 200 mM VRC
- Make fresh each day

7. Light mineral oil (Sigma; M5904)

Components for an automated fluidics system:

1. Flow/imaging chamber (Bioptechs; FCS2)
2. 5″-long 20-gauge needles (Hamilton; 7750-11)
3. Luer to ¼-28 female fitting adaptor (IDEX; P-655)
4. 1/16″ barb to .-28 female fitting adapter (IDEX; P-646)
5.¼-28 fitting (IDEX; XP-202)
6. Clear ETFE tubing, 0.02″ inner diameter (McMasterCarr; 5583K52)
7. Clear PVC tubing, 1/16″ inner diameter (McMasterCarr; 5233K51)
8. 0.38-mm inner-diameter peristaltic tubing (Pulse Instrumentation; 116-0549-04)
9. Peristaltic pump (Gilson; Minipuls 3)
10. Computer-controlled valve (Hamilton; MVP; 36798)
11. 8-way valve (Hamilton; HVXM 8-5; 36766)
12. 5-minute epoxy (VWR; 300050-778)
13. 25-gauge needles (VWR; BD305125)
14. 18-gauge needles (VWR; BD305196)
15. USB to RS-232 converter (Keyspan; USA-19HS)

The protocol requires the following equipment:

1. FCS2 flow system (Bioptechs; FCS2)
2. Objective heater (Bioptechs; 150803)
3. Automated flow system (see Section 6.2)
4. Microscope capable of smFISH imaging (see Section 6.3)

Assembly of and Operation of Flow System

Each round of MERFISH hybridization and imaging requires the controlled exchange of four different buffers at precise intervals. For this reason, integration of a fully automated fluid handling system with the microscope control software can significantly facilitate the collection of MERFISH data. Here we describe the construction and operation of the automated fluid system that we have used. This system is constructed around a peristaltic pump (Gilson; Minipuls 3) which controls the flow rate of different buffers through the flow system and a series of valves (Hamilton; MVP) that control which buffer is being pulled across the sample at any given time. The

components required for the flow system are listed in Before Starting Section, and a schematic diagram of the flow system required for 16-rounds of hybridization is included in Figure 4. Once assembled, the components of this flow system do not need to be replaced between measurements, unless otherwise specified.

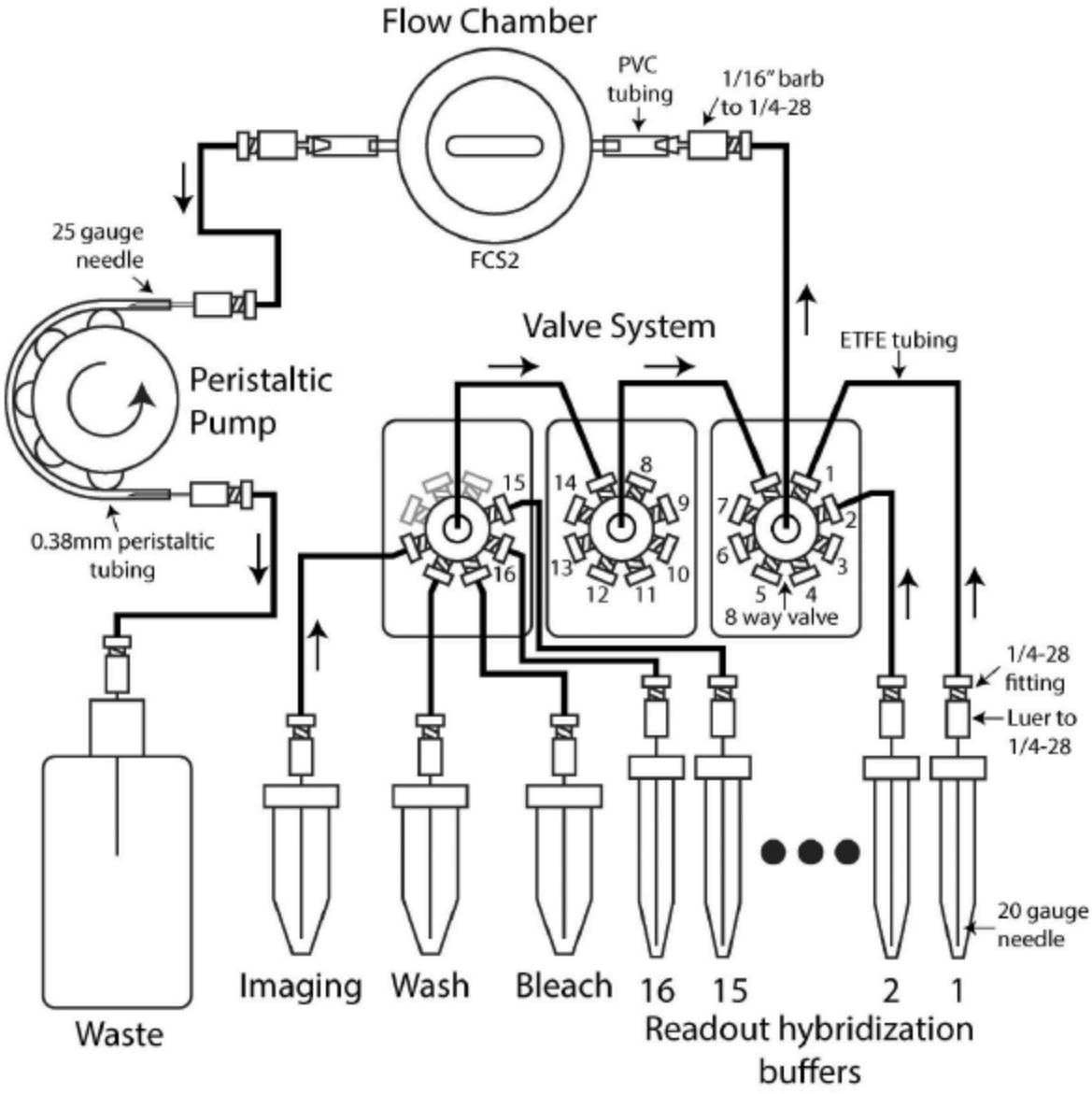

Figure 4.

Schematic diagram of the setup of an automated flow system for a 16-round MERFISH measurement. Arrows mark the local flow direction. For clarity, only 4 of the 16 tubes and flow lines required for the different hybridization buffers are depicted. The sample is contained within the FCS2 flow chamber.

Microscope Requirements

The physical requirements for a microscope for MERFISH measurements are essentially identical to the requirements for smFISH measurements. Even with many probes per RNA, the signal from individual RNAs is often relatively dim; thus, high numerical aperture objectives and sensitive cameras such as electron-multiplying CCD (EMCCD) or scientific CMOS are often needed. MERFISH requires relatively high laser powers to bleach the sample after each round of imaging; thus, the microscope should be able to illuminate the sample with at least 100 mW of light in the wavelength used to image the labeled RNAs, as measured at the back focal plane of the objective. Only a few mW of light is needed for illumination of the fluorescent fiducial beads. If less illumination light is available in the color channel used for smFISH, it should still be possible to perform MERFISH measurements. The user will simply need to devote more time to photobleaching each region of the sample. Finally, to achieve any reasonable level of automation and throughput with MERFISH, a motorized sample stage will be required as well as some form of automatic focus system.

For published MERFISH measurements, we used a 1.45 NA, 100X oil immersion Olympus objective, and illuminated our sample with ~200 mW of 641-nm light and ~20 mW of 561-nm light, as measured at the back focal plane of the objective, using solid state lasers (MPB communications; VFL-P500-642; and Coherent, 561-200CWCDRH). The 641-nm laser was used to excite our Cy5-labeled readout probes while the 561-nm laser was used to excite the fiducial beads. A custom dichroic (Chroma, zy405/488/561/647/752RP-UF1) and a custom notch filter (Chroma, ZET405/488/561/647-656/752m) were used to couple this light into the objective and filter the emission. These custom optics were used so that our home-built microscope had the capability of imaging at 750-nm. This color band was not used in our published MERFISH work (Chen et al., 2015), and thus stock dichroics and emission filters would also have worked. For example, the Chroma 89016bs dichroic in combination with the Chroma ZET561/10x and ZET642NF notch filters would also work. The fluorescent signal from the sample was imaged onto an EMCDD camera (Andor; iXon-897) through a QuadView (Photometrics), which used several stock dichroics (Chroma, T560lpxr; T650lpxr, and 750dcxxr) and emission filters (Chroma; ET525/50m, WT59550m-2f, ET700/75m, HQ770lp) to image several different color channels onto different quadrants of the camera. The 640-nm channel, corresponding to our RNA signal, and the 561-nm channel, corresponding to our fiducial beads were excited and imaged simultaneously with this setup. The 750-nm channel allows future work to include probes of this color but again was not used in our published MERFISH work (Chen et al., 2015). However, it would also be possible to forego the use of a QuadView and image each color channel one at a time, using the selective excitation of each laser to discriminate the color channels corresponding to Cy5 and the fiducial beads. That being said, it is crucial that no offset be introduced between the image of the smFISH signal and the image of the fiducial beads; thus, the same dichroic filter cube should be used for both color channels. We collected images corresponding to a 40×40 μm field-of-view with a pixel size of 167 nm in the sample plane. Our sample position was controlled with a motorized stage (Marzhauser) and our focus was maintained with a home-built autofocus system consisting of an objective nanopositioner (Mad City Labs, Nano-F) whose position was locked to the position of a IR laser spot (940-nm) reflected off of the sample-coverslip interface and imaged with a CMOS camera (Thorlabs, uc480).

MERFISH Imaging Protocol

The general MERFISH imaging protocol involves the hybridization of each readout probe, a brief wash step to remove some of the non-specifically bound probe, imaging of the sample, and then photobleaching of the sample, before hybridization of the next readout probe.

Materials

MATERIALS

Please see Before Starting section for required materials.

Assembly of and Operation of Flow System

1 Assemble buffer reservoirs. Using an 18-gauge needle, create a hole in the cap of one 15 mL falcon tube for each hybridization round. In the example depicted in Figure 4, sixteen such tubes would be needed. Insert a 5″-long 20 gauge needle into each hole. Slide the needle so that it is ~ 1 mm from the bottom of the 15 mL falcon tube and use 5-minute epoxy to secure it. Using the same protocol, insert and secure a needle into the cap of three 50 mL falcon tubes (one each for the imaging buffer, wash buffer, and bleaching buffer). Connect a luer to ¼-28 fitting adapter to the end of each needle.

Note

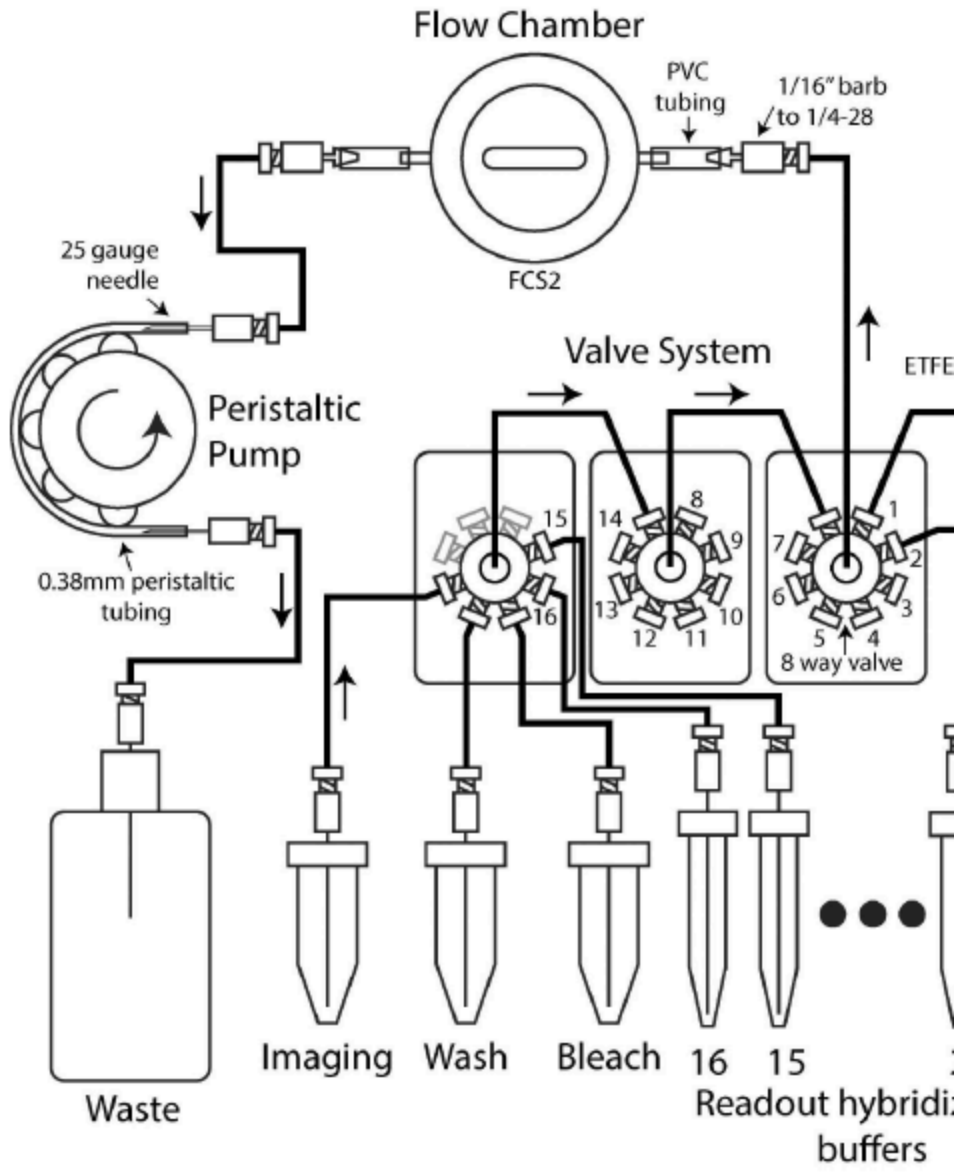

Figure 4.

Schematic diagram of the setup of an automated flow system for a 16 measurement. Arrows mark the local flow direction. For clarity, only flow lines required for the different hybridization buffers are depicted contained within the FCS2 flow chamber.

2 Create a waste bottle. Using the same approach as described in Step 1, insert a needle into the top of a 1 L plastic bottle which will serve as waste collection. The collected

waste from a MERFISH measurement will contain formamide and, thus, must be discarded as toxic waste. Choose a plastic bottle appropriate for this requirement.

3 Assemble tubing for flow lines. Cut the ETFE tubing for each desired flow line using a fresh razor blade. Take care to make the end as flat as possible. The length of each tubing section should be as short as possible to reduce the dead volume within each flow line but long enough to allow easy manipulation of the component to which it is attached, such as a Falcon tube. Typical lengths are ~12". Assemble a ¼-28 fitting on each end of each tubing section.

4 Assemble the flow lines. Insert the 8-way valve into each MVP valve system following the manufacturer's instructions. The valve system is going to be run as a daisy chain, so connect fluidics lines such that the first valve addresses the first seven hybridization buffers and the contents of the second valve system, the second valve system addresses the next seven hybridization buffers and the contents of the third valve, and the third valve addresses the remaining two hybridization buffers (assuming 16 rounds of hybridization) and the imaging, wash, and bleaching buffers. See Figure 4 for a flow diagram. This layout ensures that when common buffers are flown across the sample, i.e. imaging, wash or bleach buffers, residual hybridization buffer from other rounds are not accidentally introduced into the sample.

Note

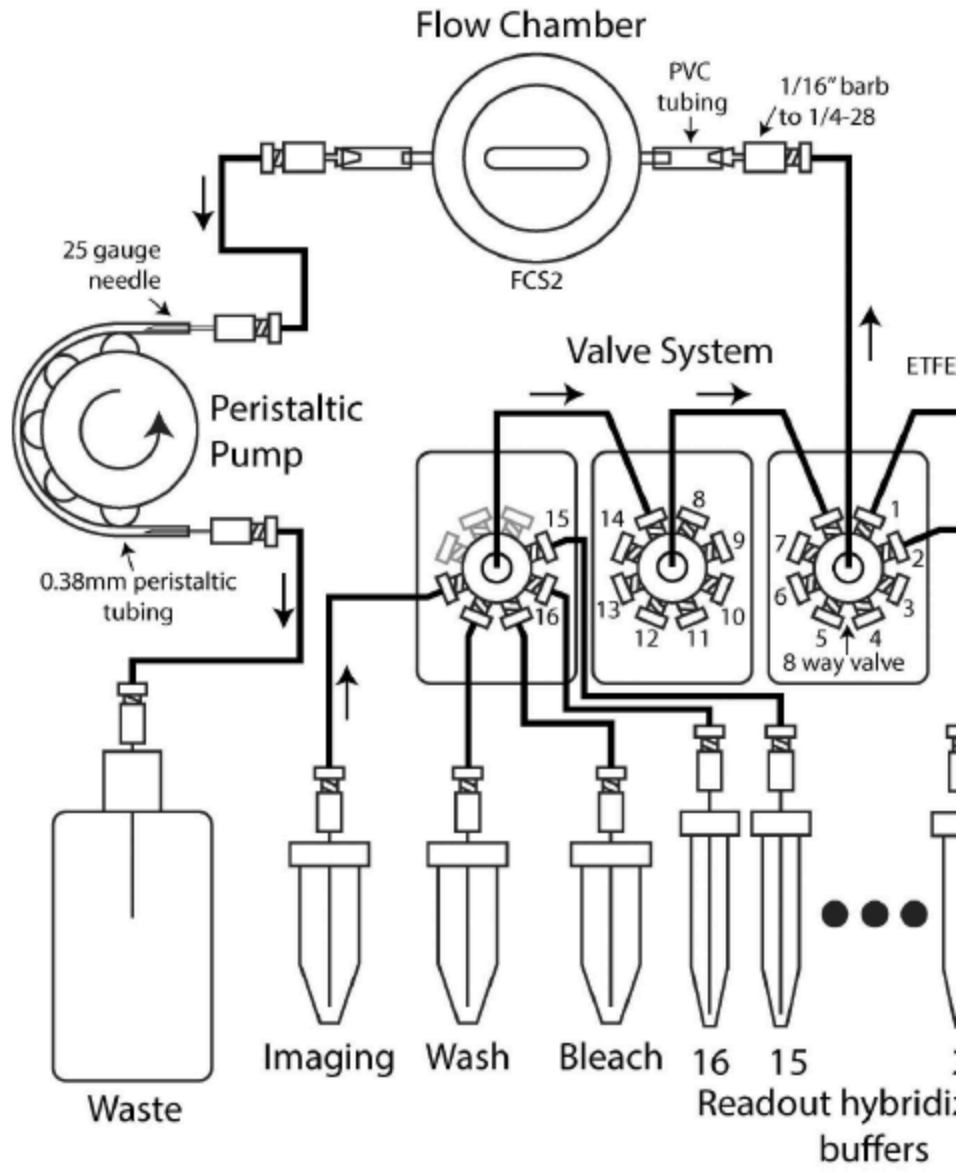

Figure 4.

Schematic diagram of the setup of an automated flow system for a 16 measurement. Arrows mark the local flow direction. For clarity, only flow lines required for the different hybridization buffers are depicted contained within the FCS2 flow chamber.

5 Connect flow lines to the FCS2 chamber. Flow connections are made to the FCS2 chamber via two metal tubes cast into this chamber. Cut two short sections of PVC

tubing and gently slide these onto the metal tubes. Insert a 1/16" barb to ¼-28 female adapter into each PVC tube to allow flow lines to be connected to the flow chamber.

6 Connect flow lines to the peristaltic tubing. Insert a 25-gauge needle into each end of the peristaltic tubing. Connect these needles to the ETFE tubing flow lines using luer to ¼-28 adapters.

7 Setup computer control. Both the peristaltic pump and the valve units can be controlled via serial communication. Most computers no longer contain serial ports, so we recommend using USB to serial converters such as a USB to RS-232 adapter as well as a RS-422 to RS-232 converter for the peristaltic pump. Custom software can be written to control these pumps via serial command; however, we have written a python-based graphical-user-interface program to control a pump and several valves. This software—named Kilroy in honor of the World-War-II-era character—is open source and can be found here: https://github.com/zhuanglab/storm-control . It can communicate with other software using TCP/IP, and, thus, it should be possible to integrate this fluidics control software with a wide variety of scripting languages available on many commercial microscopes.

MERFISH Imaging Protocol

8 Prepare the microscope and the flow system. Preheat the objective using the Bioptechs objective heater to 37.0 °C for at least two hours before the start of the measurement. This increased temperature is required to favor the proper hybridization of readout probes to the readout sequences. Room temperature is also suitable if the concentration of formamide is increased to 30% v/v and 40% v/v in the readout probe hybridization buffer and the readout probe wash buffer, respectively.

02:00:00 Preheating the objective

37 °C Preheating the objective

9 Prepare a 5 mL aliquot of Readout Hybridization Buffer as described in Before Starting Section for each round of hybridization and dilute a single readout probe (designed in **Design of Readout Probes**) into each buffer to a final concentration of 10 nM.

10 Prepare the imaging, wash, and bleaching buffers as described in Before Starting Section.

11 Load all buffers into the flow system. The imaging buffer is O_2 sensitive, so add ~5-10 mL of light mineral oil to the top of this buffer by decanting it into the 50 mL falcon tube holding this buffer. This oil layer prevents the diffusion of O_2 into this buffer during the course of the measurement.

5 mL Light mineral oil

12 Connect the input flow line to the FCS2 channel directly to the exit line, bypassing the FCS2 system, and prime the flow system by flowing enough of each buffer to completely fill its specific flow line.

13 Flush the system with bleaching buffer to ensure that no hybridization buffers will flow onto the sample once it is added.

14 Inspect each connection to see if bubbles are forming.

Note

The presence of bubbles indicates a loose or faulty connection. Tighten the connector or replace the tubing. If flow rates appear to be lower than expected, replace the peristaltic tubing. We recommend replacing the peristaltic tubing every few measurements.

15 Load the sample. Assemble the 40-mm coverslip containing the sample as prepared in **Sample Preparation and Staining** into the FCS2 flow system. Flow bleaching buffer through the tubes to fill the chamber with liquid. Inspect the flow chamber to insure that no air bubbles remain in the chamber. If bubbles are present, gentle agitation of the flow chamber, via tapping on the coverslip, can often dislodge them.

16 Hybridize the first readout probe. Flow ~2 mL of the first readout hybridization buffer across the sample at a flow rate of 0.5 mL/min. This volume should be sufficient to fill the dead volume of the flow system and chamber as well as to exchange the volume of the chamber many times over.

2 mL First readout hybridization buffer

17 Stop the flow and incubate the sample in the first hybridization buffer for 15 minutes. If the quality of individual stains is low, increasing this hybridization time can sometimes improve the quality of the stain.

00:15:00 Incubation

18 Wash the sample. Flow ~2 mL of the readout wash buffer across the sample, again at a flow rate of 0.5 mL/min. Incubate the sample for 5 minutes.

2 mL Readout wash buffer

00:05:00 Incubation

19 (Optional): Inspect the quality of the sample and select regions of interest. After the first readout probe is hybridized to the sample, it is often useful to pause the automated imaging and fluid handling program and visually inspect the quality of the sample. For new samples, it is often necessary to adjust the excitation light intensity so as to produce bright signal but not saturate the camera. Similarly, it can be convenient to examine the sample at this point to identify specific regions or cells to image. Before examining the sample, be sure to flow imaging buffer across the sample as described in Step 20 below.

The brief exposure to light at this stage typically is insufficient to produce any significant photobleaching as long as the sample is immersed in imaging buffer.

20 Image the sample. Flow ~2 mL of imaging buffer across the sample at 0.5 mL/min. When flow has stopped, collect an image in both the color channel corresponding to the labeled readout probes (e.g. 641-nm) and the color channel corresponding to the fiducial beads (e.g. 561-nm) for all desired fields of view.

2 mL Imaging buffer

Note

We utilize ~50 mW at the microscope back port to excite our Cy5-labeled readout probes, which corresponds to an average power density of ~1kW/cm^2 , and we use ~5 mW at the microscope back port to excite the fiducial beads, which corresponds to an average power density of 100W/cm^2 .

21 Bleach the signal from each region. Flow ~2 mL of the bleaching buffer across the sample at 0.5 mL/min. When flow has stopped, return to each field of view, turn up the illumination to the maximum value for the color channel corresponding to the labeled readout probes. Illuminate each sample for a sufficient time to completely photobleach the signal.

2 mL Bleaching buffer

Note

For our measurements, this time was typically 3 s. In our measurements, we utilize 200 mW of 641-nm laser power at the microscope back port, which corresponds to a power density of ~4kW/cm^2 . However, the bleaching rate will depend strongly on the illumination properties of each microscope, and the optimal bleaching time should be determined empirically for each microscope.

22 Repeat steps 16–21 for the remaining hybridization rounds.

go to step #16 Repeat steps 16-21

23 Cleanup the system. When the experiment is complete, replace all buffers with nuclease-free water and flow 5 mL through each flow line. Store the flow lines and buffer reservoirs filled with deionized water to prevent the crystallization of salts within the flow system. If the mineral oil used to protect the imaging buffer has been accidentally introduced into the flow system, wash the affected lines with 5-10 mL of isopropanol and then nuclease-free water.

5 µL Nuclease-free water for each flow line

10 mL Isopropanol (when mineral oil has been introduced into the flow system)