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Hi-M: a Multiplex oligopaint FISH method to capture chromatin conformations *in situ* and accompanying open-source acquisition software

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Abstract

The simultaneous observation of 3D chromatin arrangement and transcription in single cells is critical to understand how DNA is organized inside cells and how this organization influences or affects other processes, such as transcriptional regulation. We have recently introduced an innovative technology known as Hi-M, which enables the sequential tagging, three-dimensional (3D) visualization, and precise localization of multiple genomic DNA regions alongside RNA expression within individual cells. In this document, we present a comprehensive guide outlining the creation of probes, as well as sample preparation and labeling. Finally, we provide a step-by-step guide to conduct a complete Hi-M acquisition using our open-source software package, Qudi-HiM, which controls the robotic microscope handling the entire acquisition procedure.

Key words

fluorescence microscopy, fluorescent in situ hybridization, 3D genome architecture, transcription.

Introduction

Genomes are folded in a hierarchical organization that reflects and contributes to regulating transcription and other processes [1,2]. In the last decade, chromosome conformation capture (3C and derivatives) have revolutionized our understanding of chromatin architecture, and have provided important inklinks into the molecular mechanisms involved [3,4]. Microscopy-based strategies based on fluorescence in situ hybridization (FISH) have more recently been developed to image chromatin organization in single cells, unveiling a large degree of heterogeneity [5,6]. These approaches, however, could only detect a small number of genomic locations at once (typically 3-4), limiting their ability to dissect structure-function relationships or to detect levels of chromatin organization involving multiple loci. We and others have recently overcome this limitation by combining DNA-FISH with robotized microfluidics devices. This new technology, that we termed Hi-M (high-throughput, high-resolution, high-coverage microscopy-based technology, see below), employs high-throughput synthesis of short oligonucleotide (oligo) probes combined with RNA labeling and multiple rounds of hybridization in a sequential imaging scheme to enable the localization of tens of different genomic loci alongside the transcriptional state of the cell [7] (**Fig. 1**). Similar approaches have also been employed in concurrent work [8–11], and builds upon pioneering work from Wang and colleagues [12]. More recently, combinatorial encoding was used to acquire highly multiplexed datasets [13,14].

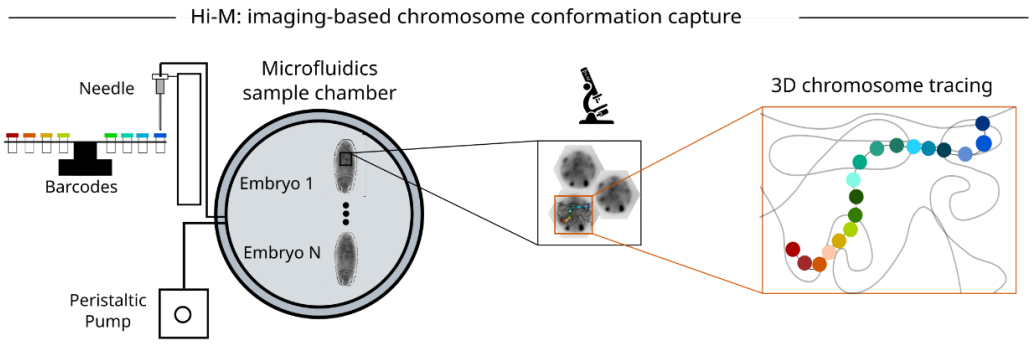

Figure 1. Outline of Hi-M experiment.

Schematic description of the main steps in the Hi-M protocol. Samples are immobilized in a microfluidics chamber (gray circle). A pump is used to deliver the FISH probes and buffers into the chamber. Barcode solutions (tubes with colored caps) are sequentially injected using a needle mounted on a XYZ translation stage. Images are acquired using a widefield or a confocal microscope (microscope icon). DNA-FISH spots (dots of different colors on the chromosome) are imaged and sequentially bleached to enable 3D chromosome tracing in single cells.

Hi-M is built upon two fundamental concepts: the recent advancement in FISH probe design known as Oligopaints, which utilizes high-throughput microarray oligo synthesis [15,16], and the groundbreaking development of multiplexing strategies for imaging numerous RNA species [17,18]. In the Hi-M technique, a microarray library containing thousands of bioinformatically designed and commercially synthesized oligonucleotides (referred to as an oligopool) is employed to target multiple genomic locations. These genomic loci, typically spanning 3-25 kb, are labeled using unique sets of ~40-250 tiled oligos, forming what is termed 'barcodes'. Each oligo within a barcode comprises three segments: a genomic homology region, a barcode-specific readout sequence, and a priming region for PCR amplification (**Fig. 2a**). The labeling process encompasses four steps. Initially, the oligopool library undergoes enzymatic amplification and purification (**Fig. 2b-e**). Subsequently, samples are harvested and fixed. Finally, the oligopool is hybridized to the genomic DNA.

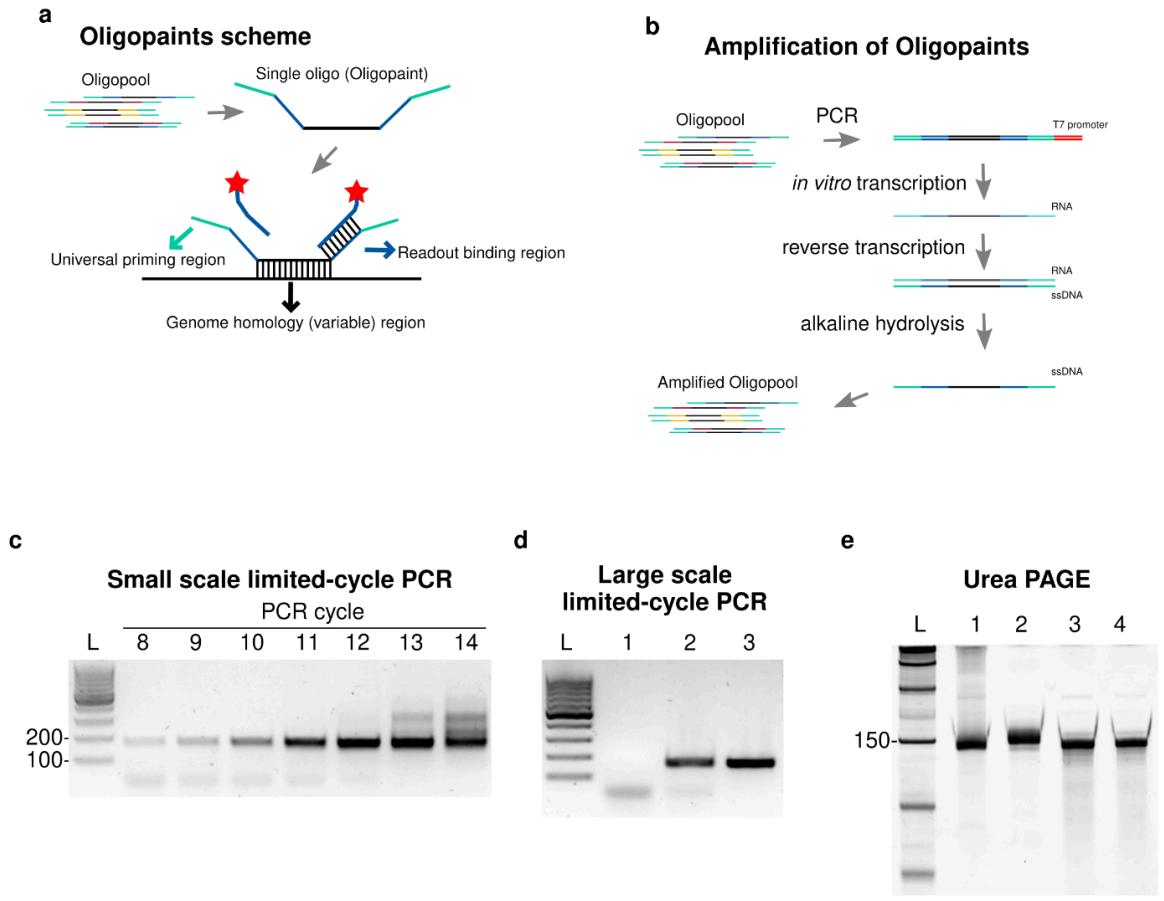

Figure 2. Design and amplification of Oligopaints

a *Upper panel, schematic structure of oligos: (1) a forward and reverse 20-nt universal priming region in blue-green, (2) a 32-nt readout sequence in blue, (3) a 42-nt genome homology variable region in black. Lower panel, schematic description of the steps to design an oligopool.*

b *Upper panel, oligopool amplification scheme. Blue-green represents the universal priming region common to all barcodes whereas blue, amber and burgundy represent barcode-specific readout sequences. Lower panel, schematic description of main steps involved.*

c *Example of an agarose gel electrophoresis result for the small scale limited-cycle PCR step. "L" is the DNA ladder or molecular weight size marker. A band of the expected size (166-nt in this case) is observed between the 100- and 200-nt bands of the ladder. In cycles 13 and 14 a second, non-specific band of ~300-nt begins to appear. Therefore, 11 amplification cycles (at PCR cycle 12) were chosen for this specific amplification reaction.*

d *Example of an agarose gel electrophoresis result for the large scale limited-cycle PCR step. "L" is the ladder as in panel Lane 1 and 2 correspond to a PCR performed without or with a template, respectively. dNTPs are observed at the bottom. Lane 3 corresponds to column-purified PCR products.* ***e*** *Example of an Urea PAGE result. "L" is the low range ssRNA ladder. Bands from lanes 1-4 appear close to the height of the 150-nt band from the ladder. Lane 1 corresponds to 200 ng of emulsion PCR break, lane 2 to 200 ng of RNA product from in vitro transcription (note the higher size due to the presence of the T7-promoter region), lanes 3 and 4 to 200 ng of ssDNA before and after precipitation, respectively.*

Figure panels were adapted from Ref [19].

Following labeling, the samples are affixed to a microfluidic chamber connected to a microfluidics pump system and inserted into an automated widefield fluorescence microscope. In the initial stage, DAPI and RNA signals are captured in various fields of view (FOVs) (also referred to as regions of interest (ROIs) in the acquisition software Qudi-HiM described below) (**Fig. 3**). The subsequent imaging of each barcode involves: (1) labeling the sample with fluorescently-labeled readout oligonucleotides specific to the barcode; (2) washing away unbound readout oligos; (3) capturing 3D, two-color images of all FOVs; and (4) photobleaching. This sequence is iterated for each barcode (**Fig. 3**). Throughout the sequential rounds, an additional fluorescent oligo with a distinct spectrum serves as the fiducial barcode to correct for any drift during post-acquisition analysis.

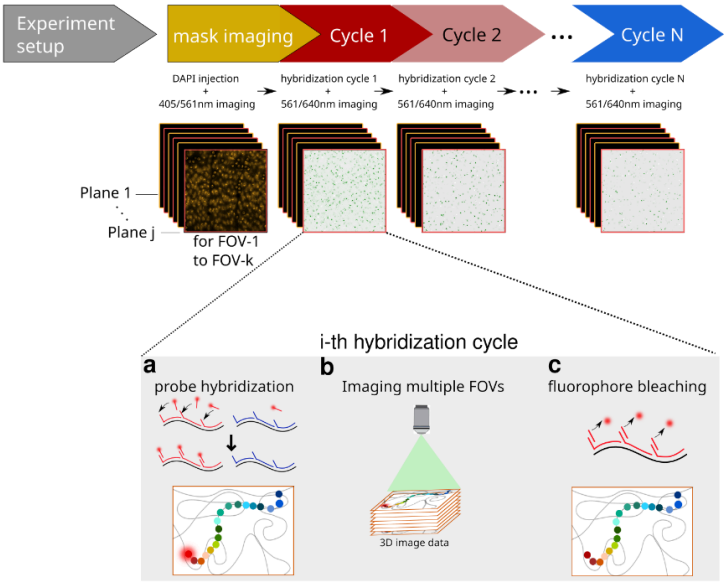

Figure 3. Pipeline for Hi-M experiment.

a *A primary oligopool library (blue) is hybridized in the bench to genomic DNA prior to mounting to the fluidics device. A readout oligo (red), bearing a fluorophore (red star), specifically binds to the primary oligopool library.*

b *3D, multi-color images are taken for a user-specified number of FOVs.*

c *The fluorophore on the readout oligo is chemically cleaved or alternatively, photobleached.*

Figure adapted from Ref [22].

Since a typical Hi-M experiment requires several days of automated acquisition, we developed Qudi-HiM, an open-source Python software suite that allows unsupervised and robust data acquisition and handling. Qudi-HiM controls and coordinates all hardware components needed for fluid handling (e.g. pumps, valves, etc.) and fluorescence 3D imaging (e.g. camera, piezo stage, acousto-optic tunable filter, *etc*). Therefore, human intervention is only required to define the acquisition parameters (e.g. number of barcodes to inject, injection routines for barcode labeling and washing, laser powers, FOV positions, etc.) and Qudi-HiM automatically performs the Hi-M experiment for efficient and reproducible data acquisition.

In this chapter, we provide a complete list of materials and step-by-step protocols for designing and amplifying Hi-M libraries using oligopaints. We explain the procedures for sample preparation and

DNA-FISH labeling in detail and provide examples of expected outcomes. Additionally, we outline the setup of a Hi-M experiment using our homemade widefield microscope, covering equipment and setup instructions. Lastly, we introduce Qudi-HiM and describe how to use it to acquire a full Hi-M dataset.

Materials

Reagents

- 20X Saline-sodium citrate buffer (SSC) containing 3 M NaCl in 0.3 M sodium citrate (Thermo Fisher Scientific, cat. no. AM 9770)
- 30 % (w/w) hydrogen peroxide solution (Sigma-Aldrich, cat. no. H1009)
- 16 % Formaldehyde solution (w/v) (ThermoFisher Scientific, cat. no. 28906)
- 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI; Roche, cat. no. 10236276001)
- Acetone (Merck, cat. no. 1000122500)
- Agarose Standard DNA Grade (Euromedex, cat. no. D5-E)
- Alexa Fluor 488 Tyramide Reagent (Invitrogen, cat. no. B40953)
- Atto-550 readout probes (IDT DNA) used for the fiducials.
- Alexa-647 readout probes (IDT DNA).
- Ammonium Persulfate (APS) (Fisher Scientific, cat. no. 17874)
- Ammonium acetate 5 M (Fisher Scientific, cat. no. 10534645)
- Anti-Digoxigenin-POD, Fab fragments (Roche, cat. no. 11207733910, RRID:AB_514500)
- BSA (Roche, cat. no. 10711454001)
- CHAPS (Sigma, cat. no. 226947)
- Cetyl PEG/PPG-10/1 dimethicone (ABIL EM-90, Evonik)
- Clorox Ultra Germicidal Liquid Bleach (Fisher Scientific, cat. no. 50371500)
- D(+) Glucose anhydrous (Euromedex, cat. no. UG3050)
- DNA Clean & Concentrator kit - 100 µg capacity (Zymo, cat. no. D4029)
- DNA Clean & Concentrator kit - 25 µg capacity (Zymo, cat. no. D4033)
- Dextran sulfate (Sigma-Aldrich, cat. no. D8906)
- Diethyl ether (Sigma-Aldrich, cat. no. 296082)
- Dulbecco's phosphate-buffered saline (PBS) (Gibco, cat. no. 14190169)
- Dry fine yeast (Lab Scientific, cat. no. FLY-8040-20F)
- Ethyl acetate (Sigma-Aldrich, cat. no. 270989)
- GeneRuler 100 bp DNA Ladder (Fisher Scientific, cat. no. SM0243)
- Glycogen 5 mg/mL (Ambion, cat. no. AM9510)
- Glucose oxidase (Sigma-Aldrich, cat. no. G2133)
- Heparin (Sigma-Aldrich, cat. no. H4784)
- HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, cat. no. E2040S)
- KAPA Taq Kit with dNTPs (CliniSciences, cat. no. BK1003)
- Low Range ssRNA Ladder (New England Biolabs, cat. no. N0364S)
- Maxima H Minus Reverse transcriptase kit (Fisher Scientific, cat. no. 13243159)
- Methanol (Fisher Chemical, cat. no. A412-4)
- Mineral oil (500 mL; Sigma-Aldrich, cat. no. M5904)
- Oligo clean & concentrator kit (Zymo, cat. no. D4060)
- Poly-L-lysine solution (Sigma-Aldrich, cat. no. P8920)
- RNase A (Sigma-Aldrich, cat. no. R6513)
- RNA Loading Dye 2X (New England Biolabs, cat.no. B0363S)
- Rnasin Ribonuclease Inhibitors (Promega, cat. no. N2515)

- SYBR Gold Nucleic Acid Gel Stain (Fisher Scientific, cat. no. S11494)
- SYBR Safe nucleic acid gel stain (Invitrogen, cat. no. S33102)
- Sodium chloride (99,5%) (Euromedex, cat. no. 1112-A)
- Sodium dihydrogen phosphate, dihydrate (Euromedex, cat. no. T879)
- TAE Buffer 50X (Tris-acetate-EDTA) (Thermo Scientific, cat. no. 10399519)
- TCEP (tris(2-carboxyethyl)phosphine) (UBP Bio, cat. no. P1021)
- TEMED (Thermo Scientific, cat. no. 17919)
- Tris Base, BM grade (Euromedex, cat. no. 200923-A)
- Triton X-100 (250 mL; Sigma-Aldrich, cat. no. T8787)
- Tween-20 (500 mL; Sigma-Aldrich, cat. no. P2287)
- dNTP Set 100 mM Solution (Fisher Scientific, cat. no. 10083252)
- deionized formamide (100 ml; Amresco, cat. no. 0606)
- dry fine yeast (Lab Scientific, cat. no. FLY-8040-20F)

Equipment list

- PCR Machine (T100 Thermal cycler, Bio-Rad)
- Positive displacement micropipette Gilson M250 (Fisher Scientific, cat. no. F148505)
- 1.8 mL plastic cryotube () (ThermoFisher Scientific, cat. no 377267)
- Magnetic stirring bar (BelArt, cat. no. 371191083)
- Magnetic stirrer (10 mm)
- NanoDrop spectrophotometer (Thermo Scientific, model no. ND-1000UV/Vis)
- Vortex, standard mini vortex (VWR)
- Falcon 15-mL Conical Centrifuge Tubes (Fisher Scientific, cat. no. 14-959-53A)
- Falcon 50-mL Conical Centrifuge Tubes (Fisher Scientific, cat. no. 14-959-49A)
- Tabletop centrifuge (Eppendorf, cat. no. 5424)
- Syringe 30 mL (Terumo, cat. no. SS-30S)
- Syringe 20 mL (Terumo, cat. no. SS-20S2)
- Embryo collection cage (8.75 cm × 14.8 cm; Flystuff.com, cat. no. 59-101)
- Nylon Filter (BD Falcon, cat. no. 352350)
- Water bath Grant Instruments JBN5 (Fisher Scientific, cat. no. 15177015).
- Thermomixer-AccuTherm Microtube Shaking Incubator (Labnet, cat. no. I-4001-HCS)
- Gas burner
- Disposable scalpel (Swann-Morton, cat. no. 0516)
- Plastic petri dishes (Greiner Bio-One; 60 mm diameter petri dish, cat. no.628163)
- Disposable glass Pasteur pipette (VWR, cat. no. 612-1702)
- Glass vial for embryo collection (DWK Life Sciences, cat. no. 986562)
- Rotating wheel
- Wide-field epifluorescence microscope (see the Wide-field epifluorescence microscope section)
- Laser protective google (Thorlabs, cat. No. LG9)
- Microscope coverslips (Bioptechs Inc., cat. no. 40-1313-0319)
- Microfluidics FCS2 chamber, no heat and low dead volume (Bioptechs Inc., cat. no. 060319-2-03)
- PEEK tubing 1/16” x 0.75mm green (CIL, cat. no. 1533L)
- TEFLON tubing 1/16” x 1mm (CIL, cat. no. 1507L)
- MFCS-EZ negative pressure pump (Fluigent, cat. no. EZ-80345001)
- Micro-perfusion peristaltic pump (Instech, cat. no. P720)

- Flow unit L (Fluigent, ref. FLU_L_D)
- HVXM8-5 & HVMXM2-5 injection valves and controllers (Hamilton, cat. no. 36766 & 36798)
- Huygens deconvolution software (Scientific Volume Imaging, https://svi.nl/HuygensSoftware)
- Currently, we run image analysis using a server running Linux with 256 threads, a GeForce GTX 2080ti GPU card, and 512GB of RAM.

Reagent setup

- For the PCR oil phase, create a mixture with a final composition of 95.95% mineral oil, 4% ABIL EM-90, and 0.05% Triton X-100 (v/v/v). If you have access to a positive displacement pipette, you can conveniently pipette 2 mL of ABIL EM90, 65 µL of Triton X-100, and 47.975 mL of mineral oil into a 50-mL Falcon tube. When adding the mineral oil, do so in two steps, vortexing in between. In case a positive displacement pipette is not available, you can accurately measure the volume by weight. To prepare 50 mL of the PCR oil phase, weigh 20.3 g of mineral oil (approximately 24 mL) directly into a 50 mL Falcon tube. Then, add 2 mL of ABIL EM90 and 65 µL of Triton X-100, vortex the mixture thoroughly, and allow it to sit for 5 minutes. Next, add 20 g of mineral oil and homogenize the mixture by gently inverting the tube. Finally, create 20 mL aliquots of the PCR oil phase and store them at 4 °C indefinitely.
- 1 M NaOH solution. Weigh 2 g of NaOH and mix it with 30 mL ddH_2O in a 50-mL Falcon tube and dissolve. Complete to 50 mL and pass it through a 0.22 µm filter. The solution can be stored at room temperature (RT, ~23 °C) for several months.
- 0.5 M EDTA solution. To prepare 200 mL, weigh 37.23 g of EDTA and mix it with 150 mL of ddH_2O in a glass beaker. Add a magnetic stirring bar and stir while adjusting to pH 8 with NaOH 10 M. Add ddH_2O up to 200 mL. Filter solution with a 0.22 µm filter. The solution can be stored at 4 °C for several months.
- Water-saturated diethyl ether. Mix 3 mL of diethyl ether with 3 mL of ddH_2O and vortex for 30 sec. Allow the mixture to settle and use the organic upper phase. Prepare freshly.
- Water-saturated ethyl acetate. Mix 2 mL of ethyl acetate with 2 mL of ddH_2O and vortex for 30 sec. Allow the mixture to settle and use the organic upper phase. Prepare freshly.
- 10% Tween 20 (v/v) solution. Mix 50 µL of Tween 20 with 450 µL of ddH_2O and vortex until the solution becomes homogeneous. Store at 4 °C for up to two weeks.
- PBS–Tween 20 (PBT) solution. Combine 49.5 mL of PBS with 500 µL of 10% Tween 20 (v/v).
- PBS-Triton X-100 (PBS-Tr) solution. Add 50 µL of Triton X-100 to 10 mL of PBS and vortex until the solution becomes homogeneous.
- 4% (w/v) formaldehyde in PBS. To prepare 8 mL, mix 2 mL of 16% formaldehyde (w/v) -methanol free with 6 mL of PBS. The solution can be stored at -20 °C for several months.
- 5% (w/v) formaldehyde in PBT. Mix 3.1 mL of 16 % formaldehyde solution with 6.9 mL of PBT.
- TBE 10X solution. Dissolve 60.55 g of Tris base, 30.9 g Boric acid and 3.7 g of EDTA. Adjust volume to 500 mL with ddH_2O. Store at RT indefinitely.
- Gel for denaturing urea polyacrylamide gel electrophoresis (Urea PAGE). Mix 6 g urea, 1.25 mL TBE 10X and 3.5 mL of ddH_2O. Heat the solution at 60 °C in a water bath until the urea is dissolved. Add 3.125 mL of acrylamide/bisacrylamide, 75 µL of APS 10% and 15 µL of TEMED. Cast the polyacrylamide gel in 0.75 mm thick spacers. Prepare freshly.
- RNA Hybridization solution (RHS). To prepare 250 mL of RHS, mix 125 mL of formamide, 62.5 mL of 20X SSC, 1.25 mL of 10 mg/mL heparin, 2.5 mL 10% Tween-20 (v/v), 2.5 mL of

10 mg/mL of salmon sperm and 56.5 mL ddH_2O. Prepare 50 mL aliquots and stock at -20 °C for several months.

- RNA probe preparation. Put 2 µL of RNA probe in 250 µL of RHS, incubate at 85 °C on a dry bath incubator for 2.5 min. Then incubate on ice for at least two minutes before adding it to the embryos. Freshly prepare the RNA probe, keeping it on ice no more than an hour before its use. Check the temperature of the dry bath to avoid probe degradation. Do not exceed 2.5 min incubation as high temperature might degrade the probe.
- Maleic acid buffer. To make 200 mL of buffer, add 2.3 g of maleic acid, 1.7 g of NaCl, 1.2 g of NaOH and 100 mL of ddH_2O. Measure pH and adjust to pH=7.5 with 5 M NaOH. Make up the volume to 200 mL with ddH_2O and filter. Solution can be stored at RT for up to six months.
- 5X blocking solution. Combine 10 g of blocking reagent with 50 mL of maleic acid buffer, agitate and heat until complete dissolution. Complete to 100 mL with a maleic acid buffer. Autoclave and make 10 mL aliquots. The 5X blocking solution can be stored at -20 °C for up to several months. Once defrozen, keep it on ice at all times.
- 1X blocking solution. Prepare the solution by diluting to one fifth the 5X Blocking solution with PBT.
- RNAse A solution. Dissolve the 10 mg vial in 1 mL of ddH_2O (100X). Make small aliquots and store at -20 °C for up to a year.
- 50% (w/v) Dextran sulfate. To prepare the solution combine 25 g of dextran sulfate with 40 mL of ddH_2O, heat to 37 °C until it fully dissolves and then add ddH_2O to a final volume of 50 mL. The solution can be stored at 4 °C for several months.
- 100 mM NaH_2PO_4 pH=7. To prepare 50 mL of this solution, mix 0.78 g of NaH_2PO_4 in 30 mL of ddH_2O. Adjust to pH=7, and complete with ddH_2O to 50 mL. Pass it through a 0.22 µm filter. The solution can be stored at 4 °C for several months.
- Pre-Hybridization Mixture (pHM) 50 % formamide, 4× SSC, 100 mM NaH_2PO_4, pH=7, 0.1 % Tween 20. Prepare freshly.
- DNA hybridization solution (DHS). Combine 5 mL of formamide, 2 mL of 50% (v/v) Dextran sulfate, 1 mL of 20X SSC, 500 µL of Salmon Sperm (10 mg/mL) and 1.5 mL of ddH_2O. Store at -20 °C for up to several months. Prewarm at 37 °C before use.
- 50% (w/v) Glucose. To prepare 40 mL of 50% (w/v) glucose, combine 20 g of glucose with 30 mL ddH_2O, heat to 60 °C until it dissolves and then add ddH_2O to 40 mL. The solution can be stored at RT for several months.
- DAPI solution. Prepare a 0.5 µg/mL DAPI solution in 1× PBS by diluting the stock solution. Store the solution with an aluminum fold at 4 °C. The solution can be employed for several weeks.
- 1 M NaCl solution. To prepare 50 mL of this solution, mix 2.92 g of NaCl with 30 mL of ddH_2O in a 50-mL Falcon tube and dissolve. Complete to 50 mL with ddH_2O and pass it through a 0.22 µm filter. The solution can be stored at RT for several months.
- 1 M Tris-HCl pH=8 solution. To prepare 50 mL of this solution, mix 6 g of Tris base with 30 mL of ddH_2O. Using a pH meter, slowly add HCl using a glass pasteur pipette to reach the desired pH. Complete to 50 mL with ddH_2O, and pass it through a 0.22 µm filter. The solution can be stored at RT for several months.
- 55 mM NaCl in 11 mM Tris-HCl pH=8 solution. To prepare 50 mL of this solution, mix 2.75 mL of 1 M NaCl solution, 0.55 mL of 1M Tris-HCl pH=8 solution, and 46.7 mL of ddH_2O. Prepare freshly.
- Gloxy solution. To prepare 1 mL of Gloxy solution, mix 50 mg Glucose oxidase, 100 µL catalase and 900 µL of 55 mM NaCl in 11 mM Tris-HCl pH=8. Make 60 µL aliquots and store

at -20 °C. Solution is stable for several months. Defrost on the day of the experiment and keep it on ice until use. If there is a precipitate, spin it down and employ the supernatant. Once defrozen, the aliquot should be used within one week.

- Hi-M wash buffer. To prepare 100 mL, combine 10 mL of 20X SSC, 40 mL of formamide and make up to 100 mL with ddH_2O (Final concentration of 40% v/v formamide) and filter.
- Imaging buffer. Combine 1.1 mL of 50% (w/v) glucose with 9.9 mL of PBS and 110 µL of Gloxy. Add Gloxy solution just before using the solution and mix. Once the tubing is introduced, add a layer of mineral oil to prevent contact with oxygen from the ambient. Replace after 12-15 h.
- Readout probe solution. The final composition of this solution is 25 nM of the corresponding readout oligo in 40% (v/v) formamide 2X SSC. Readout oligos sequences can be found in Supplementary Table 1. Prepare freshly and keep it protected from light.
- Chemical bleaching solution. To prepare 10 mL of chemical bleaching solution, mix 0.5 mL of TCEP with 9.5 mL of 2X SSC. Prepare the solution right before its use and discard any remaining solution.

Wide-field epifluorescence microscope setup

We have implemented Hi-M in both a widefield and a confocal microscope with very similar hardware configurations. Both setups are controlled by the same software interface (Qudi-HiM) with minor adaptations. For simplicity, we will describe in this chapter our home-made widefield microscope system (**Fig. 4a-c**), built around a RAMM chassis (Applied Scientific Instrument), equipped with a 60x water-immersion objective (Plan-achromat NA=1.2, Nikon) and a sCMOS camera (Orca Flash 4.0v3, Hamamatsu). Our setup uses a standard 200 mm Nikon tube lens (C60-TUBE-B, Applied Scientific Instrument) to achieve a 106 nm effective pixel size, leading to a ~217x217 µm² field of view. To avoid water evaporation during Hi-M experiments, we use immersion oil for imaging (Zeiss, Immersol™ W).

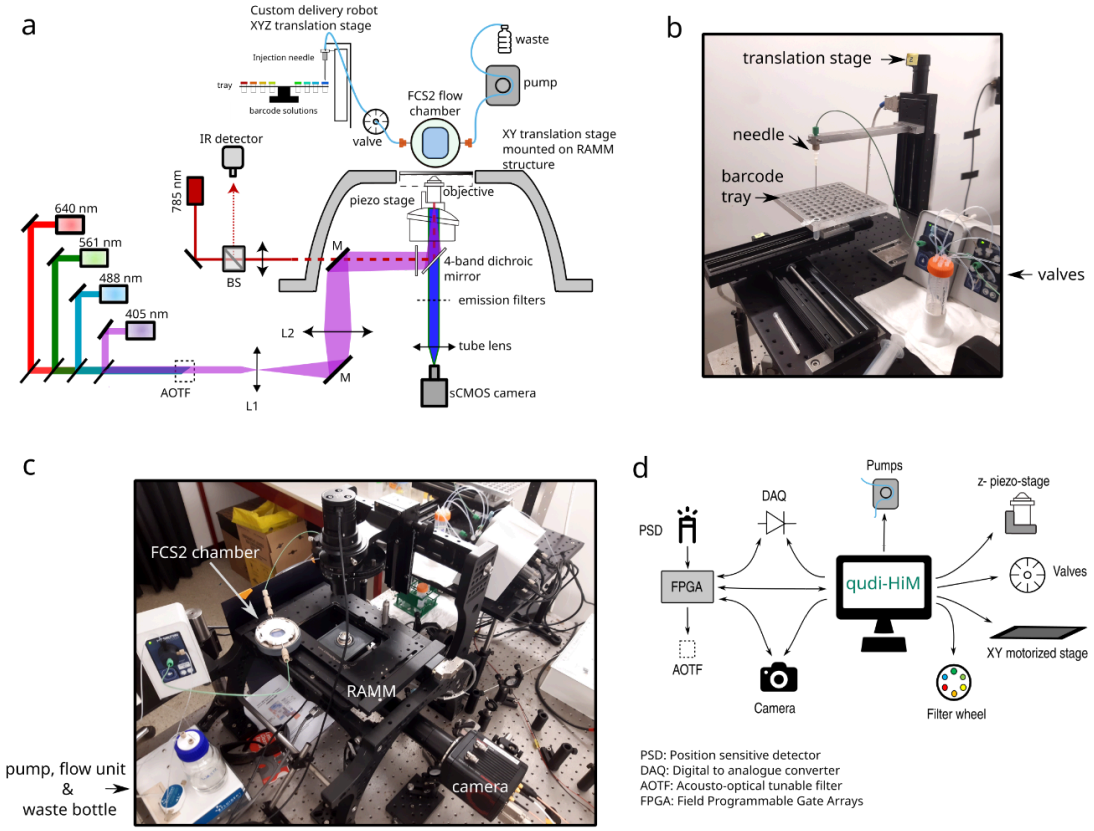

Figure 4. The microscope design.

a *Our homemade Hi-M setup was constructed using a RAMM microscope (gray curved boxes). The lasers are combined by dichroic mirrors (DM) and redirected to the microscope by mirrors (M). The beams are expanded by a telescope and focused at the back focal plane of a 60X objective by lenses L1 and L2. An acousto-optic filter (AOTF) is used to switch on/off the different laser lines and to change their intensity. The emitted light is filtered and focused on the sCMOS camera using a tube lens. The sample is placed in an FCS2 fluidic chamber and the buffer flow is controlled by a pump (vacuum or peristaltic) and an online flow unit that constantly monitors the flow rate at the chamber outlet. Valves are used to select the type of liquid to be injected into the chamber (e.g. buffer, fluorescent probes, etc.). Liquid is drawn from the inlet of a valve, passes through the FCS2 flow chamber, the online flow unit, and is discarded into a waste bottle. A custom-built delivery robot with an XYZ translation stage is used to guide a needle into different tubes (see rainbow tubes organized on a tray), allowing selection of the liquid to be injected. The microfluidic tube is shown in light blue.*

b *Illustrative picture of the custom-made delivery robot for readout probe injections.*

c *Picture of our home-made epi-fluorescence setup.*

d *Overview of hardware devices that are controlled and synchronized by Qudi-HiM. The software controls all hardware required for imaging (e.g. camera, filters wheel), sample positioning (motorized and piezo stages) and fluidic injection (pumps, valves and motorized stages). Acquisition boards (DAQ and FPGA) are used to optimize the speed and reproducibility of specific tasks, such as the acquisition of large 3D image stacks (Figure adapted from Ref [22]).*

Wide-field epifluorescence illumination is achieved using 405/488/561 nm (OBIS-405/488, Sapphire 561, Coherent) and 641 nm (VFL-P-1000-642-OEM1-B1, MPB) lasers combined with an acousto-optic tunable filter (AOTFnC-400.650, AA opto-electronics). To avoid the use of a mechanical filter-wheel, separation between laser excitation and fluorescence emission is achieved

using a four-band dichroic mirror (zt405/488/561/640rpc-UF2, Chroma) combined with a four-band emission filter (ZET405/488/561/640m, Chroma).

Sample displacement and FOVs/ROIs selection are performed using a 3-axis motorized stage (MS2000, Applied Scientific Instrumentation). Finally, the objective lens is mounted on a single-axis piezo-stage (Nano-F100, Mad City Labs Inc.), allowing for a nm-precision control of the objective axial position during z-scan and focus stabilization.

A home-made focus stabilization system is used to compensate for the axial drift in real-time. A 785 nm laser beam (OBIS-785, Coherent) is focused on the back-focal plane of the objective, reaching the coverslip/sample interface in near-TIRF illumination conditions. The position of the reflected beam is then measured on a position-sensitive detector (OBP-A-4H, Newport) and any variation in the objective-sample distance above 100 nm is automatically compensated by repositioning the objective lens using the single-axis piezo stage.

User/microscope interface

Image acquisition, sample positioning and liquid handling are controlled by a custom-made software package (Qudi-HiM) described in a dedicated section below.

Hi-M sequential hybridization

The fluid handling circuit design was implemented as described in Cardozo Gizzi et al. [7,19], and typical sequences are shown in **Tables 8-10**. The sample is mounted in an FCS2 chamber and flow is generated using a pump (either negative pressure or peristaltic). An online flow unit is used to continuously monitor the flow rate. This allows precise control of the injected volumes and maintains a steady flow in the chamber.

The complete injection sequence (including buffer type, volume, flow-rate, incubation time, etc.) are defined by the user using Qudi-HiM (**Fig. 5**). For each sequence, readout probes (see **Table 9** for an example) are first selected using a custom-built delivery device consisting of three single-axis motorized stages (VT-80, PI) that allow X-Y selection of individual Eppendorf tubes and Z translation of a needle to pump the barcode solution into the FCS2 chamber (**Figs. 4-a, b**). Up to 100 different readout probes can be sequentially injected during a Hi-M experiment using this system. All other buffers are then injected sequentially by selecting a specific outlet from the 8-way HVMX8-5 valves.

Methods

Design of Oligopaint libraries

1. Clone the repository from https://github.com/HiM-public-resources/oligopaint-design, and download the *.bed* files containing the sequences of previously mined oligos covering the *Drosophila* non-repetitive genome (https://oligopaints.hms.harvard.edu/genome-files) [15,20,21].
2. In the *input_parameters.json* file, enter the various parameters required, such as the chromosome of interest, the location of the *.bed* files, the start-end coordinates of the target region, the desired number of loci, their genomic size, and the minimum number of primary probes per locus.

3. The *Library_Design.py* script will: i) calculate the coordinates for each barcode, ii) select primary probe sequences for each locus, iii) concatenate primary sequences with readout sequences and with universal primers, iiii) check the homogeneity of the size of the differents barcodes.
4. Several output text files are created after running *Library_Design.py*.
    a. *Library_Summary.csv* contains a table summarizing the information for each barcode (number, start-end position, readout probe, primer forward, primer reverse, number of probes).
    b. *outputParameters.json* is a JSON dictionary file containing the parameters used to generate the library.
    c. *Full_sequence_only.txt* contains the raw primary probe sequences for the oligos used to order the microarray.
5. It is possible to embed multiple libraries within one oligopool by using different sets of universal primers.
6. Order the microarray from an oligopool synthesizer company.

Amplification of Oligopaints libraries

1. Emulsion PCR. This step is performed to amplify the starting oligopool (which can be limiting) in a non-biased manner. Set up a PCR Master Mix for each library as indicated in Table 1 and keep it on ice until needed. Pre-chill a 1.8 ml cryotube in the freezer, place it on the center of a controlled stirring plate and then add a pre-cooled stirring bar to the cryotube. Transfer 600 µL of PCR oil phase to the with a positive displacement pipette. Stir at 1000 rpm for at least 1 minute. While the stirring bar is still spinning, add 100 µL of PCR master mix in steps of 20 µL increments using a P20 pipette (i.e. dispense 20 µL 5 times). Stir at 1000 rpm for 10 minutes, the emulsion should appear milky white and foamy. Transfer the emulsion to a PCR strip tube (~8 x 75 µL) with a positive displacement pipette. Forward primer is the 5' => 3' whereas reverse primer is the reverse complement of the reverse universal priming sequence. Emulsion preparation must be performed in a cold room at 4°C. All the equipment must be put there in advance to cool it down before use. It will not be possible to transfer the whole emulsion volume to the PCR strip tube, quality over quantity here.
2. Perform the PCR using the following cycling conditions (Table 2). PCR products can be stored at 4 °C for a few days.
3. Small scale emulsion PCR breaking. Pool the emulsion PCR reactions in a 1.5 mL eppendorf tube. Add 1 µL of gel loading buffer to visualize the aqueous phase. Add 200 µL of mineral oil and vortex for 30 sec. Centrifuge at maximum speed for 10 min and remove the upper organic phase.
4. Add 1 mL of water-saturated diethyl ether and vortex for 1 min. Centrifuge at maximum speed for 1 min and remove the diethyl ether upper phase.
5. Add 1 mL of water-saturated ethyl acetate and vortex 1 for min. Centrifuge at maximum speed for 1 min and remove the ethyl acetate upper phase.
6. Repeat step 4. Evaporate the residual diethyl ether by incubating the tube at 37 °C for 5 min with the cap open. The final volume should be around 80 µL. PCR products can be stored at 4 °C for a few days.
7. Purify the DNA by using Zymo Oligo Clean & Concentrator kit. Mix 80 µL of DNA from the emulsion PCR breaking, 160 µL of oligo binding buffer and 320 µL of ethanol. Homogenize the solution by pipetting up and down 10 times. Follow the manufacturer's instructions up to the DNA elution. Repeat elution with an extra 15 µL of water.

8. Quantify DNA concentration using a NanoDrop by directly taking 2 µL of purified PCR product. Concentration should be between 20-40 ng/µL.
9. Run a gel electrophoresis to check for a single band amplification with 200 ng of PCR product in a 1.5 % agarose TAE 0.5x gel with 0.01% SYBR Safe at 100 V for 45 min. Purified products can be frozen at -20 °C for several months.
10. Perform the small scale limited-cycle PCR by setting up the following reaction mix for 8 tubes as indicated in Table 3. The limited number of cycles is performed to find the cycle number where the PCR is still at its exponential phase (Fig. 2c). Perform this step before proceeding to the large-scale PCR. The T7 promoter sequence (5'-TAATACGACTCACTATAGGGT-3') should be added to the reverse primer used for the emulsion PCR step to allow for the reverse transcription step.
11. Run the following PCR program indicated in Table 4. Pick up the corresponding tube after each of the cycles 8-15 just after the extension phase. To do so, quickly open the PCR machine, remove the corresponding tube, close the lid and resume the program. PCR products can be left overnight (ON) at 4°C or frozen for up to a month at -20°C.
12. Run 20 µL of the PCR product in a 1.5 % agarose gel with 0.01% SYBR Safe at 100 V for 45 min. Find the cycle with a single band of the expected size and the maximum intensity (Fig. 2c).
13. Perform a large scale limited-cycle PCR by running a reaction mix for 16 tubes as indicated in Table 3. This step will generate a big quantity of Oligopaints. The T7 promoter sequence should be added to the reverse primer to allow for the reverse transcription step.
14. Split the volume of the mix in Table 3 into 16 x 50 µL PCR tubes and run the PCR program from step 11 using the optimized number of cycles determined in step 12. Add a last extension cycle of 5 min at 72 °C. PCR product can be safely stored for months at -20°C.
15. Run 20 µL of the PCR product in an agarose gel as in step 12 to check that the PCR was successful.
16. Collect the 50 µL-aliquots from the previous step in a 15 mL falcon tube and proceed to DNA column purification according to the manufacturer's instructions. Use Zymo DNA purification kit with 25 µg capacity. Elute using 30 µL of DNAse- and RNAse-free water.
17. Quantify product concentration with a NanoDrop using double-stranded DNA parameters. This typically requires a 1/10 dilution of a 2 µL aliquot of the purified product. Concentration should be between 30-50 ng/µL.
18. Run the remainder of the 1/10 stock dilution in a 1.5 % agarose gel as in step 12 (Fig. 2d). Check for a single band of the expected size.
19. Perform in vitro transcription by setting up the reaction mix as indicated in Table 6. This step is a high-yield reaction that further amplifies the template molecules as well as converts them into RNA. It is necessary to keep RNAse-free conditions at all times.
20. Split the volume from the in vitro transcription solution into 3x20 µL PCR tubes and incubate at 37 °C for 12-16 h in a thermocycler. In vitro transcription products can be frozen for months at -80°C.
21. Take 5 µL and purify with a Zymo Oligo Clean & Concentrator kit according to manufacturer's instructions, using 15 µL of DNAse and RNAse-free water to elute purified product. The purification is only performed with a small aliquot to control if the in vitro transcription was successful and to estimate the RNA concentration in the non-purified RNA solution. Use Zymo DNA purification kit with 10 µg capacity.
22. Make a 1/10 dilution to perform a quantification of the purified RNA on NanoDrop using RNA parameters. Concentration should be between 0.5-2 µg/µL. The concentration obtained allows us to estimate concentration in non-purified RNA. For example, a 2 µg/µL

concentration in the purified RNA can be translated to an estimated concentration of 6 µg/µL in the non-purified RNA considering a factor 3 dilution (from a 5 µL aliquot to a final volume of 15 µL). The total yield of the in vitro transcription step should be around 150-450 µg from a single transcription step (60 µL in total).

23. Check for the RNA quality by Urea PAGE (**Fig. 2e**). Perform a pre-run for 30 min in 1X TBE at 190 V to eliminate the excess of persulfate. When finished, wash the wells with the running buffer. Load 100 ng of purified RNA per lane. Heat the samples at 95 °C for 5 min and put it immediately on ice for 2 min. Perform the PAGE for 1 h at 190 V. For gel staining, incubate protected from light for 20 min in 30 mL of TBE 1X and 3 µL of SyBR Gold.
24. Perform the reverse transcription reaction according to Maxima H Reverse Transcriptase kit by setting up the reaction mix indicated in Table 7. In this step, the non-purified RNA from step 20 is directly used. RNA should always be kept in ice to prevent degradation. Primer sequence is the same as for forward primer used in emulsion PCR or limited-cycle PCR.
25. Split the volume obtained in the previous step into two 1.5 mL tubes and incubate for 3 h at 50 °C in a water bath. Reverse transcription products can be frozen for months at -20°C.
26. Perform the RNA degradation by adding into each tube 300 µL of 0.5 M EDTA and 300 µL of 1 M NaOH and incubating at 95 °C for 15 min in a water bath. This step allows to selectively degrade the RNA while keeping single stranded DNA.
27. Take a 10 µL aliquot to control for DNA concentration and to perform a gel electrophoresis as in step 12.
28. DNA probe purification. Mix the 2 aliquots in a sterile 50-mL Falcon tube. Add 4.8 mL of oligo binding buffer and 19.2 mL of ethanol. Homogenize and evenly distribute across two columns. Follow the manufacturer's instructions from this point on. Use the Zymo DNA purification kit with 100 µg capacity.
29. Take a 10 µL aliquot to measure DNA concentration and to perform a gel electrophoresis as in step 12.
30. Ethanol precipitation. Directly add to the 150 µL DNA elution, 24 µL of 5 M ammonium acetate, 6 µL of glycogen and 750 µL of 100% (v/v) ethanol at -20 °C. Vortex and incubate 1 h at -80 °C or overnight at -20 °C. Centrifuge at 13,000G for 1 h at 4 °C. Discard the supernatant and wash the pellet with 1 mL of ice-cold 70% ethanol (v/v). Centrifuge at 13,000G for 15 min at 4 °C. Discard the supernatant and add 20 µL of DNase- and RNase-free water. Let the single stranded DNA (ssDNA) resuspend for 10 min at 37°C with agitation. Keep on ice.
31. Quantify oligo concentration with a NanoDrop using ssDNA parameters. Total quantity of ssDNA should be in the order of 80-120 µg.
32. Control the quality of ssDNA by Urea PAGE as in step 23 (Fig. 2e). This step allows to verify RNA degradation and the efficacy of reverse transcription step. Probes can be stored at -20 °C for months.

Sample preparation and fixation

1. Place 200–400 flies with a 2:1 female/male ratio into an egg-collection cage mounted with an apple juice plate containing a dollop of yeast paste and prewarmed to 25 °C (or the temperature required for the specific experiment). Perform an ON pre-laying step.
2. Replace the plate with a new one containing a dollop of yeast paste and prewarmed to 25 °C. Perform a laying step during 1.5 h at 25 °C.
3. Remove the plate, put the cover and incubate 1 h (or the time required to obtain embryos in the desired developmental stage) at 25 °C.

4. Rinse the plates with ddH_2O and carefully detach embryos using a paintbrush. Filter the liquid using a nylon filter. Embryos will remain on it. Rinse the nylon filter with water to remove yeast, and remove excess water filter by blotting dry on a paper towel.
5. Prepare one well plate containing bleach at 2.6% active chlorine in water. Put the filter with the embryos in the bleach containing well and incubate for 5 min. Use distilled water to thoroughly rinse the embryos. Ensure all embryos are in the medium by rinsing the walls of the nylon filter.
6. In a 20 mL glass vial, add 5 mL of 4% (v/v) formaldehyde in PBS and 5 mL of heptane.
   Formaldehyde is toxic and should be handled with protective gloves under a fume hood and discarded according to the relevant environmental and safety instructions.
7. Transfer the embryos to the vial using a paintbrush.
8. Close the vial and vigorously shake it manually during 30 sec. You may cover the cap of the vial with parafilm to avoid leakage of the formaldehyde and heptane solution inside. Incubate the embryos during 20 minutes at RT. Embryos dechorionated will float between the two phases.
9. Using a glass Pasteur pipette, aspirate the lower aqueous phase from the bottom of the vial together with the embryos contained therein (non-dechorionated embryos).
10. Add 5 mL of methanol and vortex the glass vial for 15 seconds. Using a glass Pasteur pipet, transfer the embryos at the bottom of the glass vial to a 1.5 mL tube.
    Methanol is toxic and highly volatile and should be handled with protective gloves under a fume hood and discarded according to the relevant environmental and safety instructions. Glass pipet is used to avoid embryos attaching to the walls. Avoid using plastic tips.
11. Wash the embryos three times with 1 mL of methanol. Fixed embryos can be stored in methanol at -20 °C for months.

DNA in situ hybridization

1. Transfer 100-200 embryos from step 11 to a 1.5 mL tube. Use a glass Pasteur pipette to prevent embryos sticking to the plastic tips.
2. Rehydrate the fixed embryos by incubating them with 1 mL of the following solutions (1) 90 % methanol, 10 % PBT; (2) 70 % methanol, 30 % PBT; (3) 50 % methanol, 50 % PBT; (4) 30 % methanol, 70 % PBT; (5) 100 % PBT. Incubate 3–5 min at RT on a rotating wheel for each step. If the embryos are RNA-labeled, omit this step.
3. Incubate the embryos with 1 mL of PBT, 100 µg/mL of RNAse for 2 h at RT or ON at 4 °C in a rotating wheel.
4. Permeabilize the embryos by incubating them with PBS-Tr for 1 h at RT in a rotating wheel.
5. Transfer tissues into pHM by passing embryos through 1 mL of the following freshly made solutions: (1) 80 % PBS-Tr, 20 % pHM; (2) 50 % PBS-Tr, 50 % pHM; (3) 20 % PBS-Tr, 80 % pHM; (4) 100 % pHM. Incubate 20 min at RT on a rotating wheel for each step. Before exchanging solutions, allow the embryos to settle down 2-3 min.
6. Prepare a primary DNA probe by adding 45-225 pmol of Oligopaint probe to 25 µL of DHS. Keep the mix on ice. Denature primary DNA probe by incubating for 15 min at 80 °C in the Thermomixer. The amount of probe employed should be tested by quantifying the efficiency of labeling vs. increasing concentrations of DNA probe .
7. Carefully remove the pHM solution from the embryos tube and add 1 mL of fresh pHM. Denature embryonic DNA by incubating for 15 min at 80 °C in a water bath.
8. Carefully remove the pHM solution from the embryos tube and add 25 µL of the probes. Mix by gently flicking the tube with a finger. Carefully add 40 µL of mineral oil. Change the water bath temperature to 37 °C and incubate the embryos ON at 37 °C in the water bath. Mineral oil

layer is added on top to prevent evaporation. Allowing the embryos to slowly cool down from 80 °C to 37 °C in the water bath greatly increases efficiency of labeling.

9. Carefully remove as much mineral oil as possible from the tube with a P20 pipette. Remaining oil dramatically affects embryo attachment to coverslips, as well as interfere with image acquisition.
10. Add 500 µl of 50 % formamide, 2× SSC, 0.3 % CHAPS and remove supernatant. This helps to immediately remove the mineral oil after hybridization. If the quantity of remaining oil is too big, this step will not be enough to prevent posterior issues of attachment and image acquisition.
11. Perform post-hybridization washes by passing embryos through 1mL of the following freshly made solutions : (1) 50 % formamide, 2× SSC, 0.3 % CHAPS; repeat this wash once; (2) 40 % formamide, 2× SSC, 0.3 % CHAPS; (3) 30 % formamide, 70 % PBT; (4) 20 % formamide, 80 % PBT; (5) 10 % formamide, 90 % PBT; (6) 100 % PBT; (7) 100 % PBS-Tr. Perform washes (1)–(4) 20 min at 37 °C in a thermomixer with agitation (800 to 900 rpm), perform washes (5)–(7) 20 min at RT on a rotating wheel.
12. (Optional) Rinse the embryos with 1 mL of PBT. Crosslink primary library by incubating the embryos with 1 mL of 4% (w/v) paraformaldehyde in PBT for 10 min at RT in a rotating wheel. Although optional, in our hands cross linking the primary library improved the labeling efficiency.
13. Rinse the embryos with 1 mL of PBT. Incubate the embryos with Alexa488-labeled readout probe in 1 mL hybridization buffer (25 nM readout probe, 2X SSC, 40% v/v formamide) for 30 min at RT in a rotating wheel. Alexa488-labeled probe is used for the fiducial barcode, protecting samples from light exposure.
14. (Optional) Perform an additional crosslinking step as in step 12.
15. Rinse the embryos with 1 mL of PBT. Incubate the embryos with DAPI for 20 min at RT in a rotating wheel. Afterward, remove the DAPI and rinse the embryos three times with 1 mL of PBS. Detergent-containing PBT can prevent embryos from attaching to the coverslip. DNA-labeled embryos can be stored for weeks at 4 °C before proceeding to mounting and imaging.

Hi-M experiment preparation and data acquisition with Qudi-HiM

Qudi-HiM [22] (https://github.com/NollmannLab/qudi-HiM) is a user/microscope interface written in Python 3 for automated Hi-M data acquisition. It is based on a software package called Qudi [23], a modular experiment management suite specifically designed for the development of custom user interfaces. Qudi provides a user-friendly framework with all the core functionality needed to build robust acquisition software, including error handling, hardware interfacing, and design of custom acquisition pipelines. Qudi-HiM was built on top of this framework by adding all the functionality needed to set up and run a Hi-M experiment.

Qudi-HiM was designed as an open source and flexible acquisition software that can be transferred to different Hi-M setups with a minimum of work. A list of all hardware already supported by Qudi-HiM can be found here (**Fig. 4-d**). It includes hardware used for image acquisition (lasers, filters, detectors, etc.), sample positioning (motorized and piezo stages), and sequential labeling (valves, pumps, and the injection robot). This list is not restrictive and any new device can be used as long as it is interfaced in Qudi-HiM.

The Hi-M preparation procedure is decomposed in five modules, each associated to a specific operation and a custom user interface (see **Fig. 5**):

- The “Basic Imaging” module is dedicated to image acquisition. When setting up an experiment, this module is used to control the quality of the sample and tuning the acquisition parameters (laser power, acquisition time, etc.)
- The “ROI selector”, together with the “Basic imaging”, module is used to select and save the ROIs on the sample (also referred as FOVs in the text)
- A typical Hi-M experiment can last several days. In order to avoid any loss of focus due to mechanical and thermal drift during the acquisition, a “Focus” module was implemented to define an axial reference for the focus.
- The “Fluidics Control” module is handling all the functionalities related to buffer injection and rinsing. It also controls the custom-built delivery device for injecting the readout probes (**Fig. 4b)**.
- Finally, the injection procedures (hybridization, photobleaching, number of probes, etc.) are defined in the “Injection configurator” module.

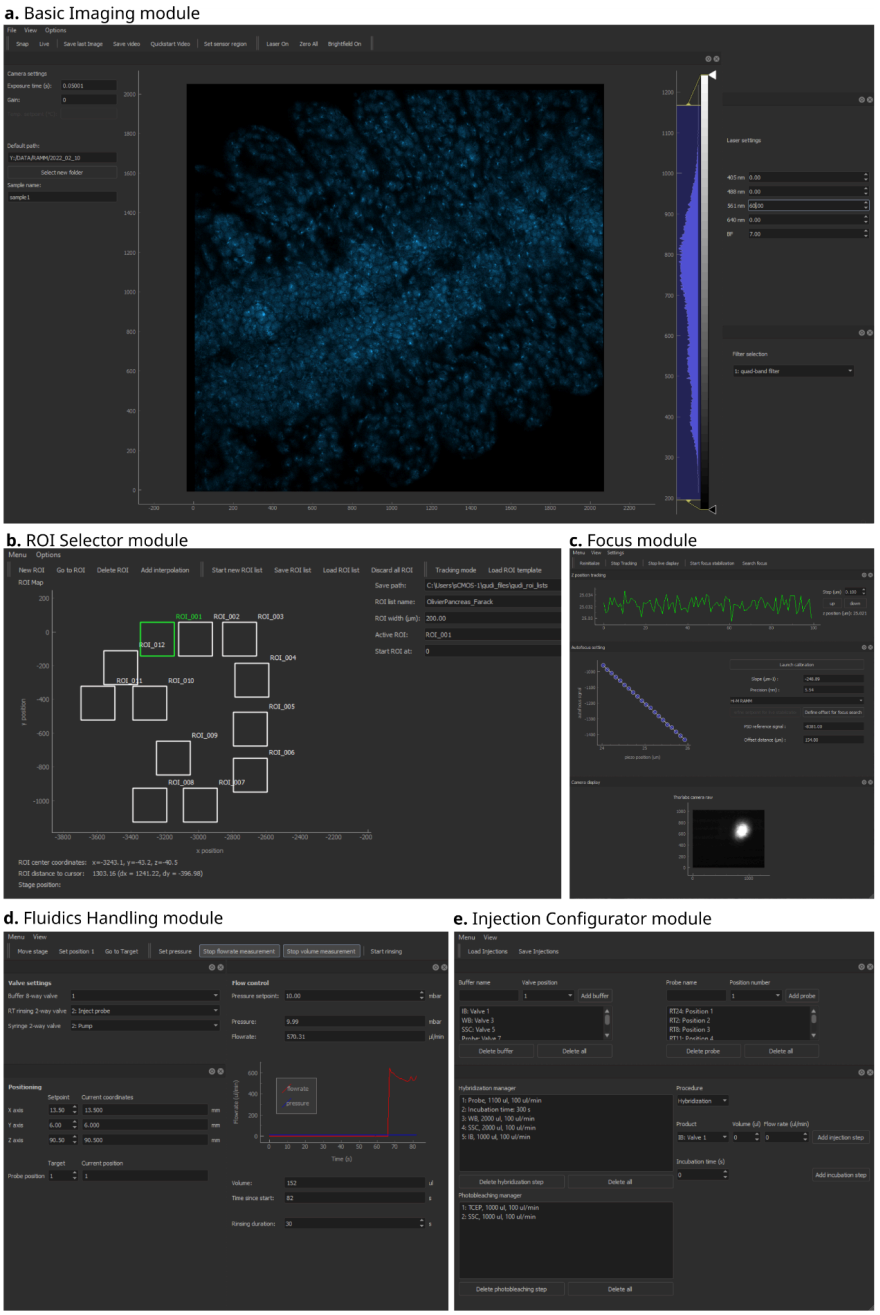

Figure 5. Modules in Qudi-HiM.

a *Basic imaging module interface that allows real-time imaging of the sample. The user can adjust camera, illumination (laser & brightfield), and filter settings to optimize imaging conditions. Images can also be saved as needed.*

b *The ROI selector module interface is used to manually or automatically select the ROIs/FOVs that will be acquired during a Hi-M experiment.*

c *The Focus module is used to initialize and calibrate the autofocus procedure. Any axial drift is compensated during a Hi-M experiment to ensure reproducibility of data acquisition.*

d *The Fluidics Handling Module controls all valves and pumps for buffer/probe injections. It also controls the custom-built delivery robot for the readout probes. The Z-positions of the injection needle and the X-Y-positions of the tubes are specified by the user.*

e *The Injection Configurator interface is used to define all injection parameters. In particular, the lists of available buffers and readout probe names. And the injection sequences for the hybridization and photobleaching steps of a Hi-M experiment.*
Figure adapted from Ref [22].

Two other modules are available:

- A module called "Task Manager" is used to launch custom acquisition scripts for Hi-M experiments. For example, we use slightly different Hi-M acquisition conditions depending on sample types or microscopy setups.
- For simplicity, each experiment is associated with a unique list of user-defined parameters such as laser wavelengths and powers, folder name, acquisition time, ROIs/FOVs list, etc. In the "Experiment Configurator" module, the user specifies all the acquisition parameters after selecting the type of experiment to be performed.

The main steps needed for a full Hi-M acquisition are highlighted in **Fig. 3** and described in detail below. Bear in mind that adaptations may be needed depending on your setup and sample.

Experiment Setup

1. The first step requires setting up the microscope and Qudi-HiM (**Fig. 3**, *Experiment setup*). Before switching on the lasers, check that all shutters are closed. When laser 785 nm emits, wear protection goggles. For reproducibility purposes, make sure laser powers are always the same at the beginning of each experiment and that the lasers are properly aligned.
2. Open Qudi-HiM and click on Load all modules. Wait for all modules to be loaded and check there are no errors (they will appear in red in the main Qudi-HiM window). In the event an error might occur, check that all hardwares is properly connected and switched ON. Repeat the previous step.
3. Between experiments, microfluidics tubing should remain filled with 50% ethanol to prevent bacterial growth and facilitate air bubble removal. Therefore, the first step for setting up an experiment consists in rinsing all the microfluidics tubing with ddH_2O and then with filtered 2XSCC. This part can be done manually using a syringe or using the Fluidics handling module from Qudi-HiM. Similarly, at the end of the experiment, rinsing steps need to be repeated twice, once with ddH_2O first, then once with 50% ethanol.
4. Place the sample coverslip in the FCS2 chamber. Ensure that all valves are closed during the process to avoid leaks and air bubbles:
    a. open the chamber and dry the Teflon spacer with a clean tissue. Dry the microfluidic chamber and sample coverslip as much as possible.
    b. replace the dried spacer and mount the coverslip with the attached sample on the inside.
    c. slowly fill the chamber with 2x SSC. Hold the chamber upright to avoid air bubbles.

    Before placing the sample on the microscope, check that the chamber is properly sealed by flowing 2x SSC at 150 µL/min for 10 minutes and make sure that no leakage is detected and no air bubble enters the chamber.
5. Using the Fluidics module of Qudi-HiM:
    a. double check that all valves and pumps are working by checking the flow rate stability (no blockage or air bubble).
    b. for sequential barcode injections, initialize the custom-made delivery device by defining the reference position of the first barcode tube. Make sure the needle is

properly centered in the tube and ~1mm from the bottom. Using the motorized stages, adjust the XYZ positions of the needle if necessary. To avoid the formation of an air bubble in the needle, make sure it is always immersed in liquid.

6. Place a drop of immersion oil on the objective and focus on the coverslip surface using the Basic Imaging Module. We use *Live Mode* with *Brightfield* ON to locate the sample. By changing the flow in the FCS2 chamber (e.g. manually with a syringe), check that the sample is stable and there is no risk of detachment during the experiment.
7. Using the ROI selector module, initialize a new list of FOVs/ROIs by clicking *Start new ROI list*. ROIs are selected using a motorized X-Y stage (with a joystick for ease of use), and the stage position can be displayed in real time with tracking mode ON. In our case, we select ROIs by imaging DAPI-stained nuclei and/or RNA expression patterns. By clicking on *New ROI*, the position of the X-Y stage is recorded in the ROI list and its position is displayed in the main window. When finished, change the file name and click *Save ROI list*.
8. If necessary, an optional photobleaching step can be performed to reduce the fluorescence background of the sample. In the experiment configurator module, select *Photobleaching*, and put lasers 488 nm, 561 nm, 640 nm at 100% (unless you have some labeling you don't wish to bleach). In *Load ROI list*, indicate the appropriate ROI file , set the bleaching time (e.g. 2 min per ROI) and press *Save configuration*. Go to Task runner in Qudi-HiM manager, click *Photobleaching* and press *Run*.

Mask imaging

9. The second step in a Hi-M experiment is acquiring DAPI staining and their corresponding fiducial images (**Fig. 3**, *mask imaging*). In the Injection Configurator module, indicate to which valves the different buffers are assigned by clicking on *Add buffer*. For example:
    i. Valve #7: Probe
    ii. Valve #1: SSC (for the 2x SSC)
    iii. Valve #2: WB (for the Hi-M wash buffer)
    iv. Valve #3: IB (for the imaging buffer)

    In our setup, valve #7 is connected to the custom injection robot with a tray that allows us to organize and inject up to 100 different fluorescent probes/reagents (**Fig. 4b**).
    b. Prepare the fiducial in a 2 mL tube (see **Reagent setup,** Readout probe solution). Place the tube on the tray, on position #1 (reference position). Using the Fluidics module, move the injection needle by clicking on *Go to target*. Make sure the needle goes to the first tube.
    c. Prepare a DAPI staining solution (see **Reagent setup,** DAPI solution). Place the tube on the tray, on position #2.
    d. In the *Hybridization manager* of the Injection Configurator module, set up the injection protocol (see **Table 8**, steps 1 to 6). It should appears as follows :
        i. Probe, 1500 µL, 150 µL/min = injection of tube in position #1 (Fiducials)
        ii. Incubation time 900 s
        iii. Hi-M WB, 1500 µ, 150 µ/min
        iv. 2X SSC, 1000 µl, 150 µL/min
        v. Probe, 1500 µL, 150 µL/min = injection of tube in position #2 (DAPI)
        vi. Incubation time 1800 s

    Save the fluidics settings by clicking on *Save Injections.*
10. Run the DAPI/Fiducial staining procedure. Open the Experiment Configurator and select the experiment handling only injections (e.g. *Fluidics RAMM*). This experiment requires only one

parameter, the path to the fluidics settings saved in step 9. Click on *Save configuration* and launch the experiment from the Task runner module.

11. Using the Basic Imaging module, briefly check the fluorescence intensity of the DAPI and fiducial markers, without photobleaching the signal. If the signal is too low, repeat step 9. Otherwise, perform the following injections manually using the Fluidics Control module (**Table 8**, steps 7 and 8):
    a. 1000 µL of 2x SSC.
    b. 900 µL of imaging buffer.

    This step is critical because the fiducial signal is used to correct for sample drift during post-processing. The imaging buffer is used to minimize photobleaching and ensure that the fluorescence intensity of the fiducial probe remains stable throughout the experiment.
12. The autofocus must be calibrated before the acquisition can begin. Using the Basic Imaging module in *live mode*, select the plane where the acquisition will start. As a rule of thumb, we usually define this reference plane ~1µm below the z-position where the first fluorescence signal is detected. This is to avoid any variability in sample position between ROIs and to ensure that we acquire enough planes to perform accurate 3D localization of the fluorescent spots during post-processing.

    Open the shutter for the 785 nm laser and put on protective goggles. In the Focus Module, click on *Start Live Display* and verify that a clean and bright reflection of the 785 nm laser is detected. Perform the autofocus calibration by clicking *Start Calibration*. The calibration curve should appear as a straight line and the *Precision* should indicate a value lower than 50 nm. Finally, click *Define Offset for Focus Search* to complete the autofocus calibration procedure.
13. Image acquisition of fiducial and DAPI. Open the Experiment Configurator and select the *ROI multicolor scan* experiment. This experiment was designed to acquire a 3D stack of images for each ROIs selected by the user.

    Tick *Dapi* to automatically add "DAPI" to the names of the saved images. Indicate the name of the experiment as well as the exposure time (e.g. 0.05 s). Compose the imaging sequence to be performed. For example, for DAPI and Fiducial imaging, we use the following settings :
    a. 405 nm to 10%
    b. 561 nm to 60%

    This means that two images are acquired sequentially for all planes of the 3D stack. The first with the 405 nm laser (DAPI) and the second with the 561 nm laser (Fiducial). Specify the number of planes and the spacing required for the 3D stack (e.g. 65 planes separated by 0.25 µm), as well as the path to the file where the ROI positions were saved (see step 7). Example DAPI-stained nuclei are shown in Fig. 6a.

    Finally, save these parameters and launch the acquisition from the Task runner module. Before moving to the next part, check the quality of the DAPI and fiducial signals. Make sure the quality of both images is high enough to enable mask segmentation and correct fiducial registration.

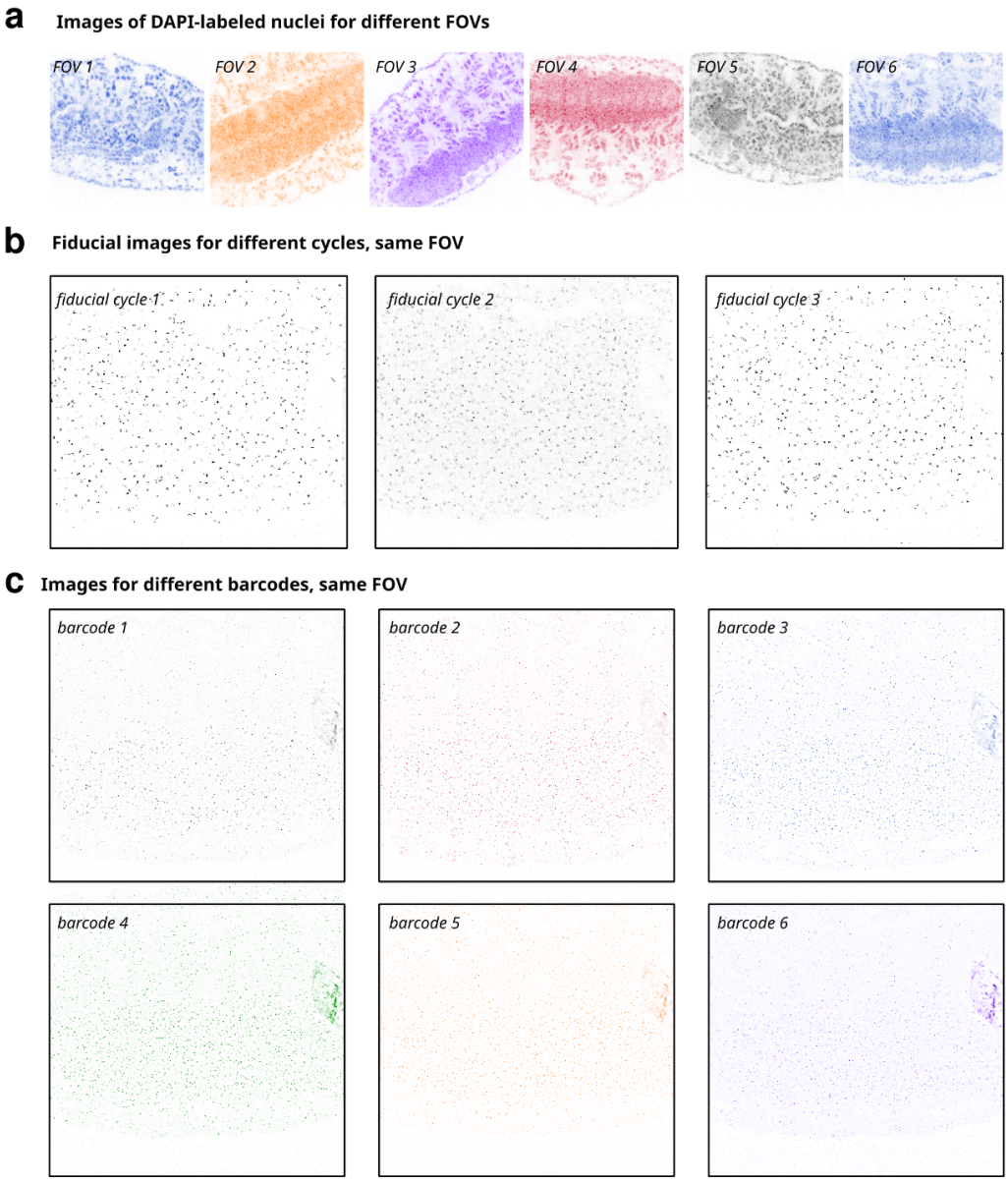

Figure 6. Typical raw images of DAPI, barcodes, fiducials. Image of the outcome of a typical Hi-M experiment.

a *Raw images of DAPI-labeled nuclei from different embryos in six FOVs. Images correspond to single z-planes. Image size: 217 x 217 µm.*

b *Representative images of fiducial labeling in the same FOV, acquired in three different cycles. Images correspond to single z-planes. Image size: 217 x 217 µm.*

c *Representative images of DNA-FISH spots for different barcodes in the same FOV, acquired in different cycles. Images correspond to single z-planes. Image size: 217 x 217 µm.*

Cycle imaging

14. Make sure there is enough buffer for the entire experiment, depending on the number of cycles.
15. Make sure there is enough space on the local computer disk to store the data. In our case, a typical experiment is 0.5-1 To.
16. Place the 2 mL tubes with the different readout probes on the injection robot tray.

17. In the Injection Configurator module, in the *Probe Name* section, enter the names of each readout probe (RT) (e.g. RT01: position 1, RT02: position 2) in the same order as the tubes are organized on the tray. In the *Hybridization Manager* section, enter the injection sequence as described in **Table 9**. In the *Photobleaching Manager* section, indicate the injection sequence used to photobleach the readout probes (see **Table 10**).
    Save these parameters in a separate file by clicking on *Save Injections*.
18. In the Experiment Configurator module, select *Hi-M experiment* and fill in all the required parameters:
    a. the number of planes and the spacing should be the same as those used for DAPI/Fiducial imaging. The ROI list should also be the same.
    b. the laser imaging sequence should be adapted according to the fluorescent dyes used.
    c. indicate the name of the injection parameters file saved in step 17
    d. check the *Transfer data automatically* option if you also want the data to be stored on a remote server. If selected, the transfer will be done as a background task between each cycle, during the photobleaching and hybridization injections. In our case, we use this option to store the data on a server and start the deconvolution while the experiment is still running. This allows us to save time on post-processing.
19. From the Task Manager module, launch the Hi-M experiment.
20. At the end of the experiment, proceed to cleaning as described in step 3, first in ddH2O and then in 50% ethanol for storage.

Typical examples of fiducial and DNA-FISH images for different hybridization cycles are provided in **Figs. 6b-c**.

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Tables

Table 1. Emulsion PCR master mix.

Reagent	Quantity (µL)
ddH_2O	79
10X Taq buffer	10
BSA (10 µg/µl)	5
dNTPs (10 mM)	2
Forward primer (100 µM)	1
Reverse primer (100 µM)	1
Kapa polymerase enzyme (5 U/µL)	1
ssDNA library (10-30 ng/µl)	1

Table 2. PCR amplification conditions

Cycle number	Denature	Anneal	Extend
1	95 °C, 2 min		
2-31	95 °C, 15 sec	60 °C, 15 sec	72 °C, 20 sec
32			72 °C, 5 min

Table 3. Small scale limited-cycle PCR set-up.

Product	Quantity per tube (µL)
Kapa buffer A	5
dNTP (10 mM)	1
Forward primer (100 uM)	0.5
Reverse primer + T7 promoter (100 uM)	0.5
Template emulsion PCR (1 ng/ µL) obtained in step 7	2.5

Kapa polymerase enzyme (5 U/µL)	0.5
ddH_2O	Make up to a final volume of 50 µL

Table 4. PCR programme.

Cycle number	Denature	Anneal	Extend
1	95 °C, 5 min		
2-15	95 °C, 30 sec	60 °C, 45 sec	72 °C, 30 sec

Table 5. Large scale limited-cycle PCR mix.

Product	Quantity (µL)
Kapa buffer A	80
DNTP (10mM)	16
Forward primer (100 µM)	8
Reverse primer + T7 promoter (100 µM)	8
Kapa enzyme (5U/µL)	8
ddH_2O	600
Template Emulsion PCR (1 ng/µl)	40

Table 6. In vitro transcription solution mix.

Product	Quantity (µL)
Purified PCR product	6 µg template DNA
ATP (100 mM)	6
UTP (100 mM)	6
CTP (100 mM)	6
GTP (100 mM)	6
10X T7 buffer	6
Rnase inhibitor (40 U/µL)	2.25

HiScribe T7 polymerase	6
ddH_2O	Make up to a final volume of 60 µL

Table 7. Reverse transcription mix.

Product	Quantity (µL)
Non purified transcription product	150 µg
dNTP mix 100mM	12
Forward Primer (100 µM)	50
5X Maxima buffer	240
RNAsin Plus (40 U/µL)	30
Maxima H reverse transcriptase (200 U/µL)	30
ddH_2O	Make up to a final volume of 1200 µL

Table 8. DAPI - Fiducial labeling.

Step	Solution	Volume (µL)	Flow Rate (µL/min)
1	Fiducial probe	1500	150
2	Incubation time 900s		
3	Hi-M wash buffer	1500	150
4	2X SSC	1000	150
5	DAPI solution	1500	150
6	Incubation time 1800s		
7	2X SSC	1000	150
8	Imaging buffer	900	150

Table 9. Readout probe labeling for Hi-M experiment.

Step	Solution	Volume (µL)	Flow Rate (µL/min)
1	Readout probe solution (RT)	1500	150
2	Incubation time 900s		
3	Hi-M wash buffer	1500	150
4	2X SSC	1000	150
5	Imaging buffer	900	150

Table 10. Photobleaching for Hi-M experiment.

Step	Solution	Volume (µL)	Flow Rate (µL/min)
1	TCEP	1000	150
2	2X SSC	1000	150

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