Chapter 19

In Situ Super-Resolution Imaging of Genomic DNA with OligoSTORM and OligoDNA-PAINT

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Abstract

OligoSTORM and OligoDNA-PAINT meld the Oligopaint technology for fluorescent *in situ* hybridization (FISH) with, respectively, Stochastic Optical Reconstruction Microscopy (STORM) and DNA-based Point Accumulation for Imaging in Nanoscale Topography (DNA-PAINT) to enable in situ single-molecule super-resolution imaging of nucleic acids. Both strategies enable $\leq$20 nm resolution and are appropriate for imaging nanoscale features of the genomes of a wide range of species, including human, mouse, and fruit fly (Drosophila).

Key words Single-molecule, Super-resolution, Genome, Chromosomes, Chromatin, FISH, Oligopaint, STORM, DNA-PAINT, OligoSTORM, OligoDNA-PAINT

1 Introduction

Single-molecule-based super-resolution imaging methods can offer resolutions that represent a ten-fold or greater improvement as compared to the diffraction limit, reaching a few tens of nanometers or even below 10 nm [1–4]. These techniques rely on the stochastic switching of individual fluorophores between fluorescent and dark states or the binding and unbinding of individual labeled molecules in order to separate the spatially overlapping images of single molecules in time. This strategy allows the position of numerous individual fluorescent molecules to be determined with high precision from the centroid positions of their images. A super-resolution image is then rendered from a collection of single-molecule localizations. Here, we describe how two forms of single-molecule-based super-resolution imaging can be used for the in situ visualization of chromosomal DNA [5, 6]. These

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technologies, called OligoSTORM and OligoDNA-PAINT, use Oligopaint technology [7, 8] to generate single-stranded oligonucleotide probes for fluorescent in situ hybridization (FISH) and then use the probes in combination with, respectively, Stochastic Optical Reconstruction Microscopy (STORM) [9–11] and DNA-based Point Accumulation for Imaging in Nanoscale Topography (DNA-PAINT) [12–14] to generate high-resolution images of genomic DNA. Below, we provide brief descriptions of Oligopaints, OligoSTORM, and OligoDNA-PAINT, followed by protocols for generating Oligopaint probes from a library of oligonucleotides (oligos) and preparing samples for imaging by OligoSTORM or OligoDNA-PAINT. As for the actual steps of image acquisition, we refer the reader to published literature for STORM and OligoSTORM [5, 6, 9, 10, 11, 15] and DNA-PAINT and OligoDNA-PAINT [5, 13, 14, 16, 17].

1.1 Oligopaints

Oligopaints are short single-stranded oligos which, when labeled directly or indirectly with a fluorophore, can be used to visualize genomic regions as small as a few kilobases (kbs) to as large as tens of megabses (Mbs) or more [7, 8]. The oligos feature a short region of genomic homology (typically, 30–42 bases) flanked by nongenomic sequences, called Mainstreet and Backstreet, and are generated through amplification of oligo libraries that have been computationally designed and then synthesized by traditional column-based or massively parallel array-based methods—in either case, libraries can be readily purchased from commercial vendors (Fig. 1A). The region of genomic homology targets the oligos to genomic loci, wherein the typical number of binding sites in single-copy genomic regions ranges from ~1 to 20 per kb, depending on the parameters used to select the target sites ([7]; unpublished data). Mainstreet and Backstreet are used for a variety for applications, including amplifying, and thus renewing, libraries (e.g., via the incorporation of primer sequences), multiplexing libraries (e.g., through the use of multiple primer pairs), and colocalizing complementary secondary oligos through hybridization [5–7, 18, 19]. Thus, Oligopaint oligos can be made into imaging probes by conjugating them with fluorophores during amplification (Fig. 1B) or targeting them with labeled secondary oligos that are complementary to sequences on Mainstreet or Backstreet (Fig. 1C). Below, we include a protocol for synthesizing Oligopaint probes.

1.2 OligoSTORM

STORM enables super-resolution imaging through the switching and localization of single fluorescent molecules [9–11]. It achieves this by permitting only a stochastic subset of fluorophores to be switched on at any given time, such that their images do not substantially overlap. This strategy allows the positions of fluorophores to be determined with very high precision. A variety of photoswitchable fluorophores can be used to achieve stochastic

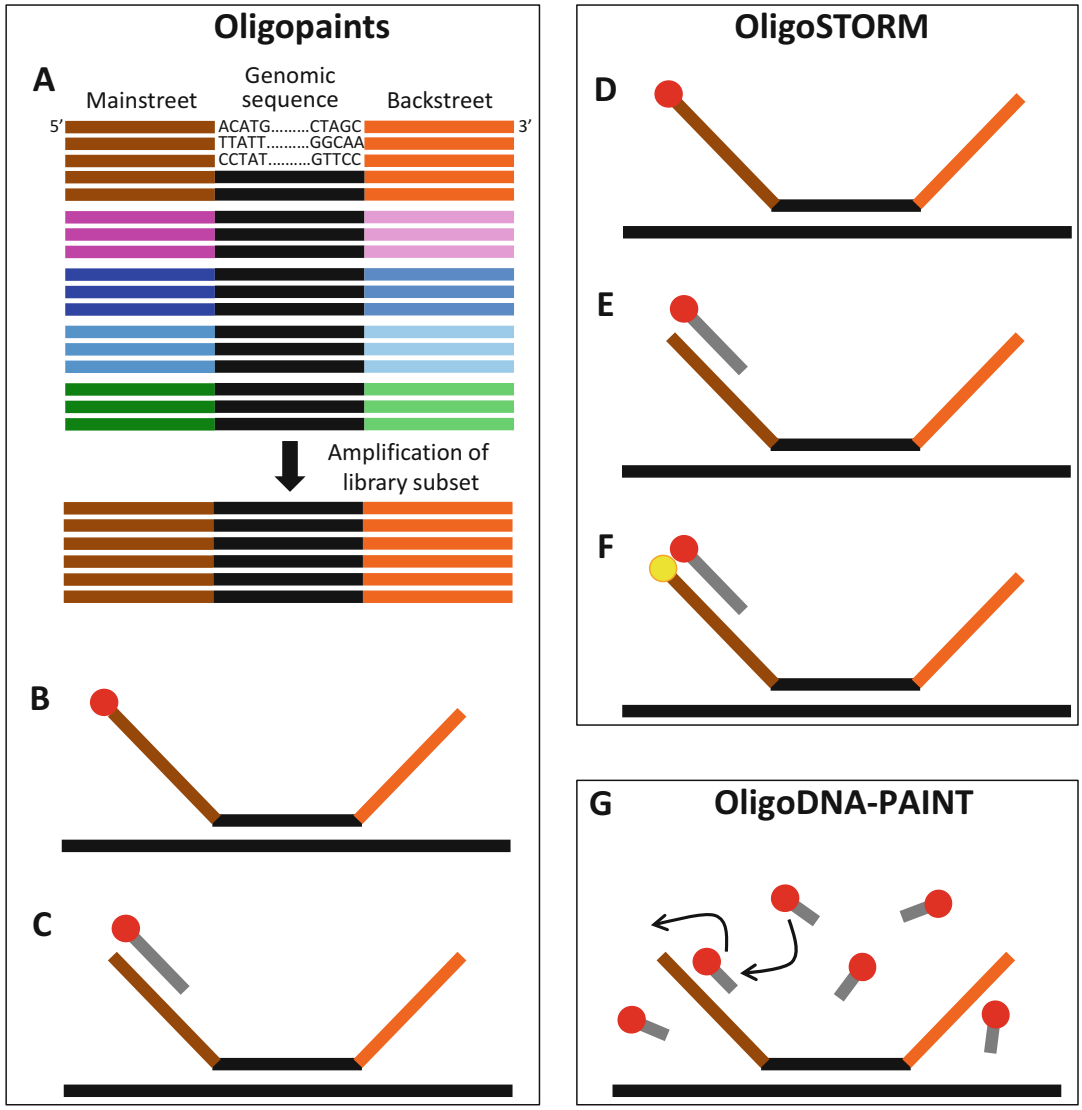

Fig. 1 Implementation of OligoSTORM and OligoDNA-PAINT using Oligopaints. (**A**) The Mainstreets and Backstreets of Oligopaint oligos consist of nongenomic sequences that can be used for a variety of purposes. For example, inclusion of primer sequences enables oligo amplification, including selective amplification of subsets of the oligo library. (**B**) Oligopaint oligos can be directly labeled with a fluorophore (*red circle*) via amplification with primers conjugated with a fluorophore. (**C**) Oligopaint oligos can be indirectly labeled via hybridization of labeled secondary oligos to Mainstreet and/or Backstreet. (**D**–**F**) OligoSTORM can be achieved via a variety of strategies, including labeling Oligopaint oligos (**D**) or secondary oligos (**E**) with a photoswitchable reporter (*red*) or pairing an activator (*yellow*) conjugated to Mainstreet or Backstreet with a reporter (*red*) that is carried by a secondary oligo hybridized to Mainstreet or Backstreet (**F**). (**G**) OligoDNA-PAINT can be achieved via the repeated, transient binding to Mainstreet or Backstreet of short imager strands conjugated with a fluorophore

switching, examples that have been used in OligoSTORM including AlexaFluor 647 and AlexaFluor 750 and their respective analogs, Cy5 and Cy7, all of which can exist in two states: a dark state, which does not fluoresce, and a bright or "on" state, which emits fluorescence upon excitation [11, 20, 21]. While these photoswitchable fluorophores, which we call reporters, can be activated from the dark to the fluorescent state through direct UV or near-UV excitation, the rate and efficiency of activation can be substantially increased when they are placed in close proximity to an activator molecule, such as AlexaFluor 405. In OligoSTORM, a variety of labeling and excitation strategies can be used [5, 6], including (1) conjugating Oligopaint oligos with a reporter (Fig. 1D), (2) hybridizing secondary oligos labeled with a reporter to Mainstreet or Backstreet (Fig. 1E), and (3) using a dual-fluorophore system in which, for example, an activator is conjugated to the 5′ end of Mainstreet and a secondary oligo bearing a reporter is hybridized to Mainstreet such that the reporter and the activator are in close proximity (Fig. 1F). In the first two strategies, a 405 nm laser is used to directly switch the reporter to the fluorescent state, while in the third strategy, the 405 nm laser excites the activator, which facilitates the activation of the reporter. We have found that the third strategy gives a greater number of localizations and have used it to image chromosomal regions ranging in size from 5 kb to more than a megabase ([5, 6]; unpublished data). Below, we provide a protocol for preparing samples for OligoSTORM.

1.3 OligoDNA-PAINT

DNA-PAINT achieves super-resolution imaging through the transient binding of fluorescently labeled oligos, called imager strands, that are freely diffusing in the imaging buffer to complementary strands, called docking strands, present on the target to be imaged [12–14]. Here, one round of binding and dissociation of an imager strand is detected as a single event of fluorescence, wherein the transient spikes in intensity of fluorescence at sites of hybridization permit users to discriminate between the fluorescence of imager strands bound to their target from the fluorescence of unbound imager strands diffusing in the imaging buffer. Imager strands, which are continuously replenished from the buffer, are designed to be short (e.g., 9–10 bases) in order to promote the transient nature of their binding. Additionally, as the concentration of imager strands and thermodynamic stability of the hybridized duplex control the rates of, respectively, binding and dissociation, users can tune the conditions of the imaging buffer to attain a desired blinking rate [13, 14, 16, 17]. Moreover, users can achieve numerous pseudocolors using only one spectral channel via the assignment of orthogonal docking strand sequences to distinct targets and, using a version of DNA-PAINT, called Exchange-PAINT, enable multiple targets to be distinguished via serial

imaging [14]. In OligoDNA-PAINT, docking sites for the imager strands can be included in Mainstreet or Backstreet, such that binding of Oligopaint oligos to a genomic target renders that target amenable to super-resolution imaging when imager strands are introduced (Fig. 1G). Using this approach, we have generated super-resolution images of genomic regions ranging in size from 5 kb to more than a megabase [[5]; unpublished data]. Below, we present a protocol for preparing samples for OligoDNA-PAINT.

1.4 Organization of the Protocols

In keeping with the format of the journal, the protocols for synthesizing Oligopaint probes and preparing samples for imaging by OligoSTORM or OligoDNA-PAINT are integrated, such that the Materials for the three protocols are presented together in one section (Subheading 2), the Methods for the three protocols are presented together in one section (Subheading 3), and the Notes for the three protocols are presented together in one section (Subheading 4). Thus, the organization of the protocols appears as follows:

Materials

1. Materials for synthesizing Oligopaint probes.
2. Materials for preparing samples for OligoSTORM.
3. Materials for preparing samples for OligoDNA-PAINT.

Methods

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Notes

1. Notes for synthesizing Oligopaint probes.
2. Notes for preparing samples for OligoSTORM.
3. Notes for preparing samples for OligoDNA-PAINT.

2 Materials

2.1 Materials for Synthesizing Oligopaint Probes

Oligopaint probes typically consist of ~100–100,000 or more species of oligos, each carrying either a fluorophore or a binding site for a fluorescently labeled secondary oligo (Figs. 1A–C). In general, they are generated from libraries of oligos that are computationally designed by the user and then synthesized either in-house or by commercial vendors; for libraries with fewer than ~100–200 species of oligos, users might wish to order the oligos individually (that is, have them synthesized through column-based methods), as this strategy allows the user to control the concentration of each oligo

species in the probe and, furthermore, can preclude the need for library amplification. Once a library is obtained, Oligopaint probes can be generated from the library in a variety of ways, all of which produce products that are single-stranded; single-stranded products are preferable to those that can become double-stranded, as the latter can renature after the denaturation step and thus become unavailable to their genomic targets during the hybridization step. There are several strategies for producing Oligopaint probes, including PCR amplification followed by asymmetric nicking and gel purification [5, 7, 8], PCR amplification followed by exonuclease digestion [5], T7 amplification followed by reverse transcription (RT; [5, 6, 18, 19, 22, 23]), and rolling-circle amplification followed by cleavage [5, 24]. Depending on the strategy used, the final oligos may carry both Mainstreet and Backstreet or just one. Below, we outline a protocol using T7 amplification, after which the products are reverse transcribed using labeled primers to produce oligos with both Mainstreet and Backstreet and a fluorophore conjugated at the 5′ end [modified from refs. 5–8, 18, 19, 22, 23].

All solutions should be prepared using distilled, deionized water (ddH_2O). Take care to avoid nuclease contamination, particularly from DNases. The recipes provided here for stock solutions will support many samples, while the rest are intended for a single sample.

2.1.1 Oligopaints: PCR Amplification of Oligopaints Library

1. Phusion Hot-start Master Mix (New England BioLabs).
2. Forward primer.
3. Reverse primer with T7 promoter sequence (e.g., TAATACGACTCACTATAGGG) appended to its 5′ end.
4. Oligopaints library (synthesized either in-house or by a commercial vendor).
5. A fluorescent intercalating dye for real-time PCR, such as EvaGreen Dye (20× in ddH_2O; Biotium) or equivalent.

2.1.2 Oligopaints: PCR Cleanup and Gel Electrophoresis

1. DNA Clean & Concentrator-5 (DCC-5) kit (Zymo Research).
2. 6× DNA loading dye.
3. LE Agarose.
4. Borax solution: 1 g anhydrous sodium tetraborate in 1 l of ddH_2O.

2.1.3 Oligopaints: T7 Reaction

1. HiScribe T7 Quick High Yield RNA Synthesis Kit (New England BioLabs).
2. RNasin Plus RNase Inhibitor (Promega).

2.1.4 Oligopaints: Reverse Transcription (RT)

1. Maxima H Minus RT Transcriptase (ThermoFisher Scientific).
2. dNTP Mix—10 mM each.

3. 15% Mini-PROTEAN TBE-Urea Precast Gels (Bio-Rad).
4. Urea-loading buffer.

2.1.5 Oligopaints: Probe Cleanup

1. DNA Clean & Concentrator-25 (DCC-25) kit (Zymo Research).
2. Oligo binding buffer (Zymo).

2.2 Materials for Preparing Samples for OligoSTORM

As illustrated in Figs. 1D–F, OligoSTORM can be implemented in a variety of ways. The following protocol describes the dual-fluorophore version, in which an activator, conjugated to the 5′ end of an Oligopaint oligo, is paired with a reporter carried by a secondary oligo bound to Mainstreet. It outlines the steps of sample preparation, application of Oligopaint oligos, and then the introduction of labeled secondary oligos [modified from refs. 5, 6].

All solutions should be prepared using distilled, deionized water (ddH_2O). Take care to avoid nuclease contamination, particularly from DNases. The recipes provided here for stock solutions will support many samples, while the rest are intended for a single sample.

2.2.1 OligoSTORM: Sample Fixation

1. Coverslip #1.5 (Electron Microscopy Science).
2. Tissue culture cell suspension.
3. 1× phosphate-buffered solution (PBS) pH 7.4 solution: Dilute a 10× PBS pH 7.4 stock solution (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4) tenfold in ddH_2O.
4. Fixation solution: Combine 4 ml 10× PBS pH 7.4, 10 ml 16% (vol/vol) paraformaldehyde, and 26 ml ddH_2O (*see* **Note 4.2.1**). Vortex vigorously to mix. Always prepare fresh.

2.2.2 OligoSTORM: Sample Permeabilization

1. Borohydride solution: 1 mg/ml sodium borohydride in dH_2O, make fresh.
2. 1× PBS.
3. Permeabilization solution: 1× PBS + 0.5% (vol/vol) Triton X-100 (10 ml 1× PBS + 50 µl Triton X-100). Always prepare fresh.
4. Kimwipes.
5. 0.1 N HCl (425 µl of 36% HCl in 50 ml = 0.1 M).
6. RNaseA solution: 100 µg/ml RNaseA in PBS.
7. 2× SSC solution.
8. Formamide (*see* **Note 4.2.1**).
9. 0.1% Tween-20.

2.2.3 OligoSTORM: Sample Denaturation and Hybridization

1. Hybridization buffer: 50% formamide, 2× SSC, 0.1% Tween-20, 10% Dextran Sulfate (*see* **Notes 4.2.1** and **4.2.2**).
2. Oligopaint oligos (assuming a single sample): ~100 pmol of Oligopaint oligos per 100 kb of genomic target (*see* **Note 4.2.3**).
3. Reporter-labeled secondary probe: 100 μM solution (*see* **Note 4.2.4**).
4. Heat source for denaturation (*see* **Note 4.2.5**).
5. Hybridization incubator (*see* **Note 4.2.6**).
6. Humidified chamber (*see* **Note 4.2.7**).
7. Glass slide.

2.2.4 OligoSTORM: Post-hybridization Washes

1. 2× SSCT solution: 2× SSC + 0.1% vol/vol Tween-20.
2. 2× SSC.

2.2.5 OligoSTORM: Mounting Sample

1. Standard glass slide.
2. STORM imaging buffer:

   400 μl of 2× SSC.

   100 μl of 50% glucose.

   10 μl of GLOX buffer: 175 μl 2× SSC, 10 mg glucose oxidase powder, and 25 μl catalase at 16 mg/ml. Can be stored up to 1 week at 4 °C.

   5 μl β-mercaptoethanol.
3. Nail polish.

2.3 Materials for Preparing Samples for OligoDNA-PAINT

OligoDNA-PAINT entails repeated, transient binding of labeled imager strands to complementary docking sites within Mainstreet and/or Backstreet (Fig. 1G). Here, we describe sample preparation using either coverglass chamber slides or coverslips, application of Oligopaint oligos, and then the introduction of imager strands [modified from refs. 5, 12, 13]. Although not described here, sequential application of different species (sequences) of imager strands in the context of Exchange-PAINT [14] will enable pseudocolors.

All solutions should be prepared using distilled, deionized water (ddH_2O). Take care to avoid nuclease contamination, particularly from DNases. The recipes provided here for stock solutions will support many samples, while the rest are intended for a single sample.

2.3.1 OligoDNA-PAINT: Sample Fixation

1. Coverglass chamber slide or #1.5 coverslip (*see* **Note 4.3.1**).
2. Tissue culture cell suspension (*see* **Note 4.3.2**).

3. 1× PBS pH 7.4 solution: Dilute a 10× PBS pH 7.4 stock solution (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4) ten-fold in ddH_2O.
4. Fixation solution: Combine 4 ml 10× PBS pH 7.4, 10 ml 16% (vol/vol) paraformaldehyde, and 26 ml ddH_2O (*see* **Note 4.3.3**). Vortex vigorously to mix. Always prepare fresh.

2.3.2 OligoDNA-PAINT: Sample Permeabilization

1. Permeabilization solution: 1× PBS + 0.5% (vol/vol) Triton X-100 (10 ml 1× PBS + 50 μl Triton X-100). Always prepare fresh.
2. PBT solution: Combine 100 ml 10× PBS pH 7.4, 899 ml ddH_2O, and 1 ml Tween-20.
3. 2× SSCT solution: Combine 100 ml 20× SSC stock solution (3 M NaCl, 300 mM sodium citrate), 899 ml ddH_2O, and 1 ml Tween-20.
4. 0.1 N HCl (425 μl of 36% HCl in 50 ml = 0.1 N).
5. 4× SSCT solution: Combine 200 ml 20× SSC stock solution (3 M NaCl, 300 mM sodium citrate), 798 ml ddH_2O, and 2 ml Tween-20.
6. Deionized formamide (*see* **Note 4.3.3**). Store at 4 °C.
7. 2× SSCT +50% (vol/vol) formamide solution: combine 4× SSCT and deonized formamide in equal proportions. Always prepare fresh (*see* **Note 4.3.3**).

2.3.3 OligoDNA-PAINT: Sample Denaturation and Hybridization

1. 4× hybridization buffer: 40% (wt/vol) dextran sulfate, 8× SSC, 0.8% (vol/vol), Tween-20 (*see* **Note 4.3.4**).
2. Oligopaint oligos (assuming a single sample): enough to reach a concentration of ~1.6 μM in the hybridization mix (*see* **Note 4.3.5**).
3. 10 μg/μl RNase A.
4. Heat source for denaturation (*see* **Note 4.3.6**).
5. Hybridization incubator (*see* **Note 4.3.7**).
6. Humidified chamber (*see* **Note 4.3.8**).
7. Rubber cement if using a coverslip in place of a coverglass chamber slide.
8. A glass microscope slide if using a coverslip in place of a coverglass chamber slide.

2.3.4 OligoDNA-PAINT: Post-hybridization Washes and Preparation for Imaging

1. 2× SSCT, as described above.
2. DAPI staining solution: 998 μl 2× SSCT +2 μl 10 μg/μl 4′,6-diamidino-2-phenylindole.
3. DNA-PAINT Buffer C: Combine 3 ml 10× PBS, 3 ml 5 M NaCl, and 24 ml ddH_2O.

4. Au particle solution: Combine 20 μl 40 nm Au nanoparticle stock solution (Sigma) and 480 μl DNA-PAINT Buffer C.
5. Centrifuge with swinging-bucket rotor.
6. Imager strands (*see* **Note 4.3.9**): dilute from concentrated stocks directly into Buffer C to a final concentration of ~0.1–10 nM.
7. A glass microscope slide if using a coverslip in place of a coverglass chamber slide.

3 Methods

3.1 Methods for Synthesizing Oligopaint Probes

All steps are carried out at room temperature, unless indicated otherwise.

3.1.1 Oligopaints: Limited Cycle PCR (50 μl Reaction; See Notes 4.1.1 and 4.1.2)

1. Prepare on ice:

   15 μl ddH_2O.

   25 μl Phusion Hot-start Master Mix.

   2.5 μl 10 μM Forward primer.

   2.5 μl 10 μM Reverse primer with T7 promoter sequence.

   2.5 μl 1:50 dilution of Oligopaints library (*see* **Note 4.1.3**).

   2.5 μl 20× EvaGreen.
2. Run reactions on a real-time PCR machine, stopping the reaction for each tube while it is still in the exponential growth phase (i.e., before it reaches saturation). A calibration run may be necessary to identify when this will happen (*see* **Notes 4.1.4** and **4.1.5**).
   (a) Incubate at 98 °C for 3 minutes (min).
   (b) Incubate at 98 °C for 5 seconds (s).
   (c) Incubate at 72 °C for 20 s.
   (d) Cycle through **steps b** and **c** approximately 15–25 times (*see* **Note 4.1.4** regarding number of cycles).
3. Recommended: validate PCR reaction by running 2 μl out on a 2% agarose borax gel.
   (a) Dissolve 2 g agarose in 100 ml borax solution by boiling for 30 s.
   (b) Cast the hot solution and let it sit until gelled.
   (c) A satisfying result will feature a single band, which will be more likely to happen if the PCR reaction does not saturate.

3.1.2 Oligopaints: PCR Cleanup (50 µl Reaction)

1. In a 1.5 ml tube, add 350 µl Zymo DNA binding buffer to ~50 µl of PCR product.
2. Transfer to a Zymo DCC-5 column and spin at >10,000 × *g* for 30 s.
3. Add 200 µl DNA Wash Buffer to column and spin at >10,000 × *g* for 30 s.
4. Repeat the wash step.
5. Discard flow-through by aspirating the collection tube dry.
6. Spin at >10,000 × *g* for 1 min to remove residual buffer.
7. Transfer column to clean 1.5 ml tube.
8. Incubate at room temperature for 1 min.
9. Elute DNA in 11 µl ddH_2O.

3.1.3 Oligopaints: T7 Reaction (30 µl Reaction)

1. Prepare on ice in RNase-free environment:
   10 µl PCR product.
   10 µl NTP buffer mix (from HiScribe kit).
   2 µl T7 Pol Mix (from HiScribe kit).
   1 µl RNase inhibitor.
   7 µl ddH_2O.
2. Incubate at 37 °C for 4–16 h (*see* **Note 4.1.6**).

3.1.4 Oligopaints: RT Reaction (70 µl Reaction) (See Note 4.1.7)

1. Prepare on ice in RNase-free environment:
   7 µl dNTP (10 mM each).
   14 µl Maxima H Minus RT buffer.
   10 µl 100 µM unlabeled or labeled primer (*see* **Note 4.1.8**).
   5 µl or less Maxima H Minus RT enzyme.
   4 µl RNase inhibitor.
   30 µl RNA from T7 reaction.
2. Incubate at 50 °C for 1–2 hours (h) (no pre-denaturing step required).
3. Digest remaining RNA:
   (a) Mix 1:1 0.5 M EDTA and 1 M NaOH.
   (b) Add 50 µl of the NaOH-EDTA solution.
   (c) Heat at 95 °C for 10 min.
4. Recommended: Test reaction product by running 2 µl out on a 15% urea TBE gel. Use urea-loading buffer.
   (a) Run gel in 60 °C water bath for ~40 min to ensure probe is denatured. This allows for clear separation between the RT primer and the desired ssDNA probe.
   (b) 80% or more incorporation of the RT primer would be a satisfying outcome.

3.1.5 Oligopaints: Probe Cleanup

1. Add 240 µl Oligo binding buffer and mix. (The volume of Oligo binding buffer added should be 2× the volume of the sample after **step 3.1.4.3**; 2 × 120 µl = 240 µl).
2. Split volume evenly into two 1.5 ml tubes (180 µl per tube). To each tube add 480 µl of 100% ethanol and mix. (The total volume of ethanol added should be 8× the volume of the sample after **step 3.1.4.3**; 8 × 120 µl = 960 µl = 2 × 480 µl).
3. Transfer the solution of one tube to a Zymo DCC-25 column and spin at ≥10,000 × *g* for 30 s. (Note, the maximum capacity of the column is 750 µl.) Dispose the flow-through, add contents of the second tube to the column, and spin again at ≥10,000 × *g* for 30 s (*see* **Note 4.1.9**).
4. Add 750 µl DNA Wash Buffer.
5. Spin at >10,000 × *g* for 30 s and discard flow-through.
6. Spin at ≥10,000 × *g* for 30 s to remove excess buffer.
7. Transfer column to a clean 1.5 ml tube.
8. Elute in 30 µl ddH_2O.
9. Measure final probe concentration.

3.2 Methods for Preparing Samples for OligoSTORM

All steps are carried out at room temperature, unless indicated otherwise. Conduct all RNaseA work such that RNase A does not contaminate work areas and equipment.

3.2.1 OligoSTORM: Sample Fixation

1. Prepare a sample of tissue culture cells adhered at desired confluency to a coverslip.
2. Gently rinse cells with 1× PBS by briefly immersing coverslip in a small petri dish containing the buffer and then placing into a fresh, dry petri dish.
3. Immediately add enough freshly prepared fixation solution to cover the cells and let it stand for 10 min (*see* **Notes 4.2.1** and **4.2.8**).
4. Remove fixation solution and rinse the sample once or twice with 1× PBS (*see* **Note 4.2.1**).

3.2.2 OligoSTORM: Sample Permeabilization (See Note 4.2.9)

1. Incubate fixed cells for 10 min in permeabilization solution.
2. Incubate for 2 min in 1× PBS.
3. Incubate for 5 min in 0.1 M HCl.
4. Wash 3× for 1 min in PBS.
5. For DNA FISH only: incubate for 1 h at 37 °C in RNaseA solution.
6. Wash 3× for 1 min in 2× SSC.
7. Incubate for 35 min in 2× SSC + 50% vol/vol formamide and 0.1% Tween-20 (*see* **Note 4.2.1**).

8. Use sample directly in **step 3.2.3** or store at 4 °C. Coverslips may be stored at 4 °C for several days.

3.2.3 OligoSTORM: Sample Denaturation and Hybridization (Assuming One Sample)

1. Prepare hybridization mix on ice (*see* **Note 4.2.1**):

   ~100 pmol of Oligopaint oligos (*see* **Note 4.2.3**).

   1 μl of 100 μM Reporter-labeled secondary oligo.

   Enough hybridization buffer to achieve a final volume of ~20–70 μl (*see* **Notes 4.2.3** and **4.2.10**).
2. Add ~20–70 μl hybridization mix to a clean glass slide. It is important that enough solution is added such that, after the following step (3.2.3.3), all cells are exposed to the solution; too little solution results in air bubbles, where cells will not be exposed to hybridization mix.
3. Gently tap coverslip of cells dry on a Kimwipe. Invert coverslip carrying the cells onto the solution on the glass slide.
4. Seal edges of coverslip with rubber cement and allow the rubber cement to dry for 5 min (*see* **Note 4.2.11**).
5. Denature the slides at 78 °C for 3 min (*see* **Notes 4.2.5** and **4.2.12**).
6. Hybridize overnight (*see* **Note 4.2.13**) at the hybridization temperature appropriate for the Oligopaint probe(s) being used (*see* **Notes 4.2.6** and **4.2.14**). If an air incubator is being used, place the sample in a humidified chamber (*see* **Note 4.2.7**).

3.2.4 OligoSTORM: Post-hybridization Washes

1. Pre-warm 2× SSCT solution to 60 °C.
2. Remove the coverslip and wash 2× for 10 min each in 2× SSCT at 60 °C.
3. Incubate in 2× SSCT for 10 min.
4. Wash in 2× SSC.
5. Optional: Store in 2× SSC at 4 °C for up to 2 weeks.

3.2.5 OligoSTORM: Mounting Sample (See Notes 4.2.15 and 4.2.16)

1. Make chambered slide:
   (a) Adhere two strips of double stick tape along both long edges of a standard slide.
   (b) Gently tap off excess fluid from coverslip and mount it on the doublestick tape (cells facing the slide).
2. Add STORM imaging buffer to side of coverslip and allow channel to fill by capillary action (*see* **Note 4.2.17**).
3. Seal coverslip to slide with nail polish.

3.3 Methods for Preparing Samples for OligoDNA-PAINT

Carry out all procedures at room temperature unless indicated otherwise. Conduct all RNaseA work such that RNase A does not contaminate work areas and equipment. If using a coverglass chamber slide, ~75% maximum well volume can be used for all steps unless otherwise indicated; if using a coverslip, incubations and washes can be performed in a mini-staining jar or in a well of a 6-well tissue culture plate using a volume sufficient to fully immerse the coverslip unless otherwise indicated.

3.3.1 OligoDNA-PAINT: Sample Fixation

1. Prepare a sample of tissue culture cells adhered at desired confluency to a coverglass chamber slide or a coverslip (*see* **Notes 4.3.1** and **4.3.2**).
2. Add enough 1× PBS to cover the cells and let it stand for 1 min.
3. Aspirate the 1× PBS and then add enough freshly prepared fixation solution to cover the cells and let it stand for 10 min (*see* **Notes 4.3.3** and **4.3.10**).
4. Remove the fixation solution and rinse the sample once or twice with 1× PBS (*see* **Note 4.3.3**).

3.3.2 OligoDNA-PAINT: Sample Permeabilization (See Note 4.3.11)

1. Add freshly prepared permeabilization solution and incubate for 10 min.
2. Remove the Triton solution and incubate the sample with PBT for 2 min.
3. Remove the PBT and incubate the sample in 0.1 N HCl for 5 min.
4. Remove the HCl and rinse the sample twice with 2× SSCT.
5. Add freshly prepared 2× SSCT + 50% (vol/vol) formamide solution and incubate for 2 min (*see* **Note 4.3.3**).
6. Remove the solution from **step 3.3.2.5**, then add pre-warmed 2× SSCT +50% (vol/vol) formamide and incubate at 60 °C for 20 min (*see* **Notes 4.3.3** and **4.3.12**).

3.3.3 OligoDNA-PAINT: Sample Denaturation and Hybridization

1. During 3.3.2.6, prepare a 100 μl hybridization mix (assuming one sample; if using a coverslip in place of a coverglass chamber slide, the reaction can be scaled down four-fold to a final volume of 25 μl.

   50 μl deionized formamide (*see* **Note 4.3.3**).

   25 μl 4× hybridization buffer.

   4 μl 10 μg/μl RNase A.

   Enough Oligopaint oligos to reach a concentration of ~1.6 μM (*see* **Note 4.3.5**)

   ddH_2O needed to raise the total volume to 100 μl.

2. When 3.3.2.6 is complete, remove the 2× SSCT +50% (vol/vol) formamide solution and add the hybridization mix (*see* **Note 4.3.3**). If using a coverslip in place of a coverglass chamber slide, pipet the hybridization mix directly onto the coverslip, place a glass microscope slide on the top of the coverslip, carefully invert the slide, and seal the coverslip to the slide with rubber cement.
3. Denature the sample at 78 °C for 3 min (*see* **Notes 4.3.6** and **4.3.13**).
4. Hybridize overnight (*see* **Note 4.3.14**) at the hybridization temperature appropriate for the Oligopaint probe(s) being used (*see* **Notes 4.3.7** and **4.3.15**). If an air incubator is being used, place the sample in a humidified chamber (*see* **Note 4.3.8**).

3.3.4 OligoDNA-PAINT: Post-Hybridization Washes and Preparation for Imaging

1. Pre-warm 2× SSCT solution to 60 °C.
2. Remove coverslip and then remove the hybridization mix (*see* **Note 4.3.16**).
3. Add pre-warmed 2× SSCT, incubate at 60 °C for 5 min, and then remove 2× SSCT.
4. Repeat **step 3.3.4.3** three additional times (for a total of four 5 min washes).
5. Add 2× SSCT, incubate for 2 min at room temperature, and then remove 2× SSCT.
6. Repeat **step 3.3.4.5** one additional time.
7. Add DAPI solution and incubate for 5 min.
8. Remove the DAPI solution and incubate the sample in DNA-PAINT Buffer C for 2 min.
9. Remove the DNA-PAINT Buffer C and add Au particle solution (*see* **Note 4.3.17**).
10. Spin the sample at 500 RCF in a swinging bucket centrifuge for 3 min (*see* **Note 4.3.18**).
11. Remove the Au particle solution and incubate the sample in DNA-PAINT Buffer C for 2 min.
12. Remove the DNA-PAINT Buffer C and add DNA-PAINT Buffer C containing DNA-PAINT imager strand. Optionally, the solution can include an oxygen-scavenging system (*see* **Notes 4.3.9** and **4.3.19**).
13. Place the sample on a microscope stage for imaging or store at 4 °C for up to 2 weeks. Coverslips will need to be mounted onto a glass microscope slide prior to imaging (*see* **Note 4.3.20**). Take care to avoid adding antifade mounting media at this stage (*see* **Note 4.3.21**).

4 Notes

4.1 Notes for Synthesizing Oligopaint Probes

1. This step also serves as an early check on the quality of the Oligopaint library.
2. We recommend designing oligos with the minimum length that can satisfy the labeling requirements. This may improve the overall success of probe generation, including the step of amplification.
3. Oligo libraries vary in concentration of full product, which is not perfectly correlated with the reported concentration. However, these minor variations in concentration, which are due to library-synthesis and uncertainties in dilution, are generally not critical at this step.
4. For best results, users may wish to optimize the PCR conditions (e.g., template and primer concentrations), including determining at which rounds of amplification the exponential and saturation phases begin. In our experience, halting the reaction after 15–25 rounds has generally given satisfactory results.
5. You can increase the quantity of your stock of the Oligopaints library via PCR amplification of your raw library (e.g., the initial library received from a commercial source), using as few cycles as necessary (with $\leq$25 cycles as a guideline).
6. The T7 reaction saturates after approximately 4–6 h. The RNA product remains stable for 16 h. Thus, the suggested 4–16 h reaction time allows for convenient time management of the protocol.
7. We did not include an RNA purification step between **steps 3.1.3** and **3.1.4** as we have not found such a step to improve the outcome of reverse transcription. However, if RNA degradation is observed, we recommend purifying the RNA followed by polyacrylamide gel electrophoresis. Otherwise, users may wish to proceed immediately to the RT reaction after the in vitro transcription step has been completed, as RNA is more prone than ssDNA to degradation.
8. Use unlabeled primer when generating Oligopaint oligos for use in OligoDNA-PAINT (Fig. 1G) and labeled primer when generating Oligopaint oligos that carry a 5′ fluorophore, such as would be case for the dual-fluorophore variation of Oligo-STORM (Fig. 1F). Fluorophores that we have found to be compatible with the RT step include AlexaFluor 405, AlexaFluor 488, AlexaFluor 647, Cy3, and Cy5.
9. For large-scale probe cleanup, use the Zymo DCC-100 columns (1–10 nmol of primer). Scale up washes as directed by the manufacturer and elute probe in 100 µl of ddH_2O. Concentrate sample using a SpeedVac concentrator or vacuum lyophilizer or by ethanol precipitation.

4.2 Notes for Preparing Samples for OligoSTORM

1. Paraformaldehyde and formamide are toxic and should be disposed of using appropriate hazardous waste streams.
2. Dextran sulfate can be difficult to get into solution, and thus users may wish to prepare the hybridization buffer with commercially available aqueous solutions of 50% dextran sulfate. (*See* **Note 4.3.4** for another approach, which we have found useful when the desired final concentration of dextran sulfate is relatively high.)
3. The concentration of Oligopaint probe in the hybridization mix may need to be adjusted in order to optimize the ratio of signal to noise (SNR). Thus, while we have achieved good SNR using ~100 pmol of probe per sample per ~100 kb of genomic target (=5 μM Oligopaint oligos when dissolved in 20 μl hybridization mix), users may wish to adjust probe concentrations, keeping in mind that higher concentrations for any particular oligo species (targeting a single 30–42 base genomic target) may increase target occupancy, but overall higher concentration of all oligos, together, could increase background. (*See* **Note 4.3.5** for our experience with respect to OligoDNA-PAINT.)
4. With regard to choice of reporter and activator fluorophores, we recommend Alexa647 as the reporter and Alexa405 as the activator for single color experiments and, for multicolor imaging, we recommend Alexa647 and Alexa750 as reporters and Alexa405 as the activator for both.
5. The heat source for denaturation can simply be an anodized aluminum heat block placed in a benchtop heating unit or immersed in a water bath. For more precise control, a flat-block thermocycler can be used.
6. A flat-block thermocycler can be used for the hybridization step and is the instrument of choice, as its heated lid prevents the evaporation of the hybridization mix.
7. A humidified chamber can help reduce sample drying. A simple humidified chamber can be prepared by placing 3–5 damp lab tissues in the bottom of a 10 cm diameter tissue culture dish. The coverglass chamber slide or microscope slide can get wet, provided the sample side does not come into contact with the lab tissues.
8. While the protocol includes a standard fixation time, users may wish to empirically determine optimal fixation times.
9. We recommend being gentle with the washes, as cells tend to lose adherence to the coverslip under flow even when the coverslips have been pretreated with poly-D-lysine. Note that some cells adhere well on their own and, in such cases, it is not necessary to pretreat the coverslips.

10. The final volume will depend on the size of the coverslip. It is important that enough solution is added such that, after **step 3.2.3.3**, all cells are exposed to the solution; too little solution results in air bubbles, where cells will not be exposed to hybridization mix.
11. Sealing the edges of the coverslip with rubber cement is not an essential step and can be skipped, if desired.
12. While users may consider varying the temperature of denaturation, care should be taken to avoid temperatures that might excessively perturb chromatin structure.
13. Although not carefully documented, there is a general sense that long hybridization times, even up to 24 or 48 h, may be beneficial when targeting large (on the order of Mbs) single copy regions.
14. The optimal hybridization temperature will vary from probe to probe and typically will range from 42 °C for shorter probes (e.g., with $\leq$32 bases of genomic homology) to 52 °C (e.g., with $\geq$40 bases of genomic homology).
15. Particularly when imaging large genomic targets, we recommend adding fiducial beads (e.g., 200 nm 540/560 FluorSpheres, ThermoFisher) to the samples prior to adding STORM imaging buffer in order to correct for stage drift during image acquisition. This can be achieved by rinsing the coverslip with a 2× SSC solution containing 100 nm beads labeled with a fluorophore that is spectrally distinct from the reporter.
16. For brighter and thus more accurate localization, we recommend adding 3 μl of 2 M cyclooctatetraene in DMSO to the STORM imaging buffer [25] prior to adding the buffer to the sample. Note, this buffer ages very quickly.
17. Use the buffer that is appropriate for your fluorophore. For example, when using fluorophores that require thiol for switching, use an oxygen-scavenging buffer with a thiol [21].

4.3 Notes for Preparing Samples for OligoDNA-PAINT

1. With respect to sample preparation, we recommend using 8-well coverglass chambers, such as Lab-Tek II Chambered Coverglass (ThermoFisher) or iBiDi μ-slide (iBiDi), as these permit easy fluid exchange. Alternatively, a #1.5 coverslip (17 mm optimal thickness) can be used—in this case, a fine-tipped diamond scribe can be used for marking which side of the coverslip contains cells and the identity of the sample. In either case, the critical issue is simply that the FISH must be performed on a glass support that is very thin (17 mm optimal thickness). For those using coverslips, note that some microscope objectives include correction collars to account for different thicknesses of the glass support, in which case

experienced users can opt to use a different thickness. Cells should not be placed on the surface of a slide and then mounted with a coverslip, as this configuration will place the cells too far from the microscope objective to observe single-molecule fluorescence events.

2. Tissue cultures of adherent cell lines can be distributed into 8-well coverglass chamber slides and further cultured. Alternatively, ~100–200 µl of a 1×10^5 cells/ml suspension of cells can be deposited into the well of a coverglass chamber slide or onto the surface of a coverslip, after which cells should be allowed to adhere for 1–3 h as described [5, 7, 8]. For loosely adherent and suspension cell lines, pretreatment of the glass surface with poly-D-lysine [5, 7] may improve cell adherence.
3. Paraformaldehyde and formamide are toxic and should be disposed of using appropriate hazardous waste streams.
4. Dextran sulfate adds a considerable amount of powder volume upon solvation, so care needs to be taken when preparing this solution. First, add the appropriate volume of 20× SSC stock to reach a final dilution of 8× SSC (e.g., 8 ml of 20× SSC for 20 ml total volume of 4× hybridization buffer). Next, add ~70% of the ddH_2O that would be needed (e.g., 8.5 ml for 20 ml total volume). Then, add the dextran sulfate powder to this solution and mix overnight by inversion on a rotary shaker (e.g., 8 g dextran sulfate powder for 20 ml total volume). Once the dextran powder is fully dissolved, add the appropriate amount of Tween-20 to reach 0.8% (vol/vol) in the total volume (e.g., 160 µl for 20 ml) and enough ddH_2O until the final volume (e.g., 20 ml) of hybridization buffer is reached. Mix again overnight by inversion on a rotary shaker.
5. The optimal hybridization mix will likely vary, as the optimal probe concentration may vary from probe to probe and target to target. If 1.6 µM does not lead to optimal results, concentrations as high as 32 µM can be tried. For example, dense samples and tissues often need ~10× more probe than typical tissue culture samples. (*See* **Note 4.2.3** for our experience with respect to OligoSTORM.)
6. The heat source for denaturation can simply be an anodized aluminum heat block placed in a benchtop heating unit or immersed in a water bath. For more precise control, a flat-block thermocycler can be used.
7. A flat-block thermocycler can be used for the hybridization step and is the instrument of choice, as its heated lid prevents the evaporation of the hybridization mix. If using an air incubator, larger hybridization volumes may be required to offset the evaporation that occurs during extended high-temperature incubation when using coverglass chamber slides. A 50% increase in hybridization volume is generally sufficient in our hands.

8. A humidified chamber can help reduce sample drying. A simple humidified chamber can be prepared by placing 3–5 damp lab tissues in the bottom of a 10 cm diameter tissue culture dish. The coverglass chamber slide or microscope slide can get wet, provided the sample side does not come into contact with the lab tissues.
9. Imager strands, added in DNA-PAINT Buffer C, are typically 9–10 base ssDNA oligos containing a 3′ Cy3B or ATTO655 fluorophore and are used at concentrations ranging from 0.1 to 10 nM. A detailed discussion of imager strands is provided elsewhere [12–14].
10. While the protocol includes a standard fixation time, users may wish to empirically determine optimal fixation times.
11. We recommend being gentle with the washes, as cells tend to lose adherence to the coverslip under flow even when the coverslips have been pretreated with poly-D-lysine.
12. If using a coverslip in combination with a glass staining jar, place the staining jar containing 2× SSCT + 50% (vol/vol) formamide in the water bath at room temperature and allow the jar and water bath to heat up together. Likewise, after the step is complete, allow the jar and water bath to cool down together. Glass staining jars typically are not made of borosilicate glass and thus are heat labile and sometimes break upon large shifts in temperature.
13. While users may consider varying the temperature of denaturation, care should be taken to avoid temperatures that might excessively perturb chromatin structure.
14. Although not carefully documented, there is a general sense that long hybridization times, even up to 24 or 48 h, may be beneficial when targeting large (on the order of Mbs) single copy regions.
15. The optimal hybridization temperature will vary from probe to probe and typically will range from 42 °C for shorter probes (e.g., with $\leq$32 bases of genomic homology) to 52 °C (e.g., with $\geq$40 bases of genomic homology).
16. If using coverglass chamber slides, adding ~100–200 μl (space-allowing) of pre-warmed 2× SSCT makes it easier to remove the viscous hybridization mix.
17. The inclusion of fiducial markers, such as Au nanoparticles, enables the user to correct for drift during image acquisition.
18. Coverglass chamber slides can be taped to bucket attachments for multi-well plates. Coverslips can be placed in the well of a 6-well tissue culture plate and spun in the same bucket attachment.

19. An oxygen-scavenging system such as glucose oxidase/catalase or protocatechuic acid/protocatechuate-3,4-dioxygenase [26] may increase imaging performance.
20. A mounting chamber can be prepared by placing two strips of double-sided tape roughly 75% of the width of the coverslip apart on a microscope slide, such that the coverslip can sit between them while still leaving the majority of the surface untouched by the tape (cells should face downward toward the microscope slide). Buffer can be pipetted into this channel, which can eventually be sealed on the open edges (i.e., the ones not contacting the tape) with nail polish or epoxy.
21. Note that OligoDNA-PAINT is typically performed in aqueous buffers such as 1× PBS, and that binding of the imager strand to the docking site may be impeded in the high viscosity antifade mounting media commonly added to diffraction-limited FISH samples being imaged with diffraction-limited light microscopy. Note, also, that some microscope autofocus systems (e.g., those found on the Nikon N-STORM) are incompatible with antifade mounting media due to its high refractive index.

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