Versatile design and synthesis platform for visualizing genomes with Oligopaint FISH probes

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A host of observations demonstrating the relationship between nuclear architecture and processes such as gene expression have led to a number of new technologies for interrogating chromosome positioning. Whereas some of these technologies reconstruct intermolecular interactions, others have enhanced our ability to visualize chromosomes in situ. Here, we describe an oligonucleotide- and PCR-based strategy for fluorescence in situ hybridization (FISH) and a bioinformatic platform that enables this technology to be extended to any organism whose genome has been sequenced. The oligonucleotide probes are renewable, highly efficient, and able to robustly label chromosomes in cell culture, fixed tissues, and metaphase spreads. Our method gives researchers precise control over the sequences they target and allows for single and multicolor imaging of regions ranging from tens of kilobases to megabases with the same basic protocol. We anticipate this technology will lead to an enhanced ability to visualize interphase and metaphase chromosomes.

The role of chromosome positioning in gene regulation and chromosome stability is fueling a growing interest in technologies that reveal the in situ organization of the genome. Among these technologies are chromosome conformation capture (3C) (1) and its several iterations, such as Hi-C (2), which are applied to populations of nuclei to identify chromosomal regions that are in close proximity to each other (3, 4). Another technology is fluorescence in situ hybridization (FISH), wherein nucleic acids are targeted by fluorescently labeled probes and then visualized via microscopy; this technology is an extension of methods that once used radioactive probes and autoradiography but have since been adapted to use nonradioactive labels (5–11). FISH is a single-cell assay, making it especially powerful for the detection of rare events that might otherwise be lost in mixed or asynchronous populations of cells. In addition, because FISH is applied to fixed cells, it can reveal the positioning of chromosomes relative to nuclear, cytoplasmic, and even tissue structures. FISH can also be used to visualize RNA, permitting the simultaneous assessment of gene expression, chromosome position, and protein localization.

FISH probes are typically derived from cloned genomic regions or flow-sorted chromosomes, which are labeled directly via nick translation or PCR in the presence of fluorophore-conjugated nucleotides or labeled indirectly with nucleotide-conjugated haptens, such as biotin and digoxigenin, and then visualized with secondary detection reagents. Probe DNA is often fragmented into ~150- to 250-bp pieces to facilitate its penetration into fixed cells (12) and, as many genomic clones contain repetitive sequences that occur abundantly in the genome, hybridization is typically performed in the presence of unlabeled repetitive DNA (13). Another limitation to clone-based probes is that the genomic regions that can be visualized with them are restricted by the availability of clones and the size of their genomic inserts, which typically range from 50 to 300 kb. Whereas it is possible to target larger regions and establish banding patterns by combining probes (9, 14–17), this approach is often challenging, as each clone needs to be prepared and optimized for hybridization separately. The efficiency of these probes can also be variable, even among different preparations of the same probe. This variation may sometimes be a consequence of random labeling and fragmentation during probe production.

Many types of custom-synthesized oligonucleotides (oligos) have also been used as FISH probes, including peptide nucleic acid (PNA) and locked nucleic acid (LNA) oligos (18–23). Rather than relying on the isolation of a clone, such probes are designed to target precisely defined sequences. Also, as these probes are typically short (~20–50 bases) (24–26) and single stranded, they diffuse efficiently into fixed cells and tissues and are unhindered by competitive hybridization with complementary probe fragments. Oligo probes have allowed the visualization of single-copy viral DNA as well as individual mRNA molecules using branched DNA signal amplification (27) or a handful to a few dozen short oligo probes (26, 28), and, by targeting blocks of repetitive sequences as a strategy to amplify signal, enabled the first FISH-based genome-wide RNAi screen (29). Oligo probes have also been generated directly from genomic DNA using parallel PCR reactions (30, 31). However, the high cost of synthesizing oligo probes has limited their use.

The availability of complex oligo libraries produced by massively parallel synthesis has enabled a new generation of oligo-based technologies. These libraries are synthesized on a solid substrate, then amplified or chemically cleaved to move the library into solution (32, 33). Two very recent studies have used complex libraries to visualize single-copy regions of mammalian genomes by FISH. One study used long oligos (>150 bases) as templates for PCR, and then labeled the amplification products nonspecifically (34), whereas the other adapted a 75–100 base single-stranded sequence-capture library for FISH by replacing the 5′ biotin with a fluorophore (35).

Here we report a method, called "Oligopaints," which uses oligo libraries as a renewable source of FISH probes carrying only 32 bases of homology to the genome. We amplify these libraries with fluorophore-conjugated PCR primers, thereby ensuring one

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Data deposition: The database files listing the genomic locations of all oligonucleotide FISH probe sequences are hosted at the Oligopaints website, http://genetics.med.harvard.edu/oligopaints.

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fluorophore per oligo probe, and are, furthermore, able to process the amplification products enzymatically to produce highly efficient single-stranded, strand-specific probes that can visualize regions ranging from tens of kilobases to megabases. We also describe a set of bioinformatics tools to facilitate the design of these probes, which makes our technology compatible with any sequenced organism.

Results

From Oligo Library to FISH Probe. Our strategy for generating Oligopaints begins with the design and synthesis of libraries of single-stranded 74mers (ss74mers), where each oligo contains 32 bases of genomic sequence flanked by 21-base primer sequences (Fig. 1). As such, more than one probe set can be synthesized from the same library through the use of multiple primer pairs. The oligos are then amplified via PCR, which can be carried out with or without an emulsion (*Materials and Methods*) (36). Importantly, one of the primers contains a 5′-conjugated fluorophore, whereas the other contains the recognition site for a nicking endonuclease (NE) (37), which provides a strategy for making Oligopaints single stranded. As shown in Fig. 1, the NE recognition site is oriented such that the nick occurs immediately 3′ of the 32 bases of genomic sequence on the labeled strand. Upon denaturation, the nicked strand separates into 53- and 21-base fragments, whereas the undigested strand remains at 74 bases. Finally, we use denaturing gel electrophoresis to isolate and extract the labeled ss53mers, which can then be used as a strand-specific FISH probe.

Genome-Scale Probe Design. The Oligopaints approach calls for identifying genomically unique sequences with desirable hybridization properties. To this end, we have created a bioinformatics pipeline that uses the program OligoArray, which simulates the thermodynamics of probe-target hybridizations and allows the user to specify several parameters, including melting temperature (T_M), percent G+C content (GC%), and sequences to avoid (38). Candidate probes are then assessed using the UNAfold package (39) for the propensity to form secondary structures and verified using BLAST to have only a single genomic target (40).

To use OligoArray to design FISH probes, we first assembled a sequence database of tiled 1-kb segments for each genome analyzed. Whereas Oligopaints can theoretically be made to carry any length of homology to the genome, we elected to search for 32mer sequences, as this length is compatible with short array formats and gave us the densest coverage in our pilot searches for probe sequences (*SI Appendix*, Table S1). We searched for unique 32mers with a T_M between 75 and 90 °C, to select probe sequences whose hybridization with their targets would withstand stringent FISH wash conditions, and with a GC% between 35 and 80%, to increase the likelihood of amplification. A minimum spacing of 10 bases between probe sequences was imposed to minimize steric interference by adjacent probes during hybridization, and homopolymeric stretches of five or more A's or T's, or four or more G's or C's were avoided to maximize PCR fidelity and minimize spurious probe–probe interactions. The thermodynamics were simulated at 70 °C, as this temperature mimics the most stringent conditions under which we anticipated performing FISH washes.

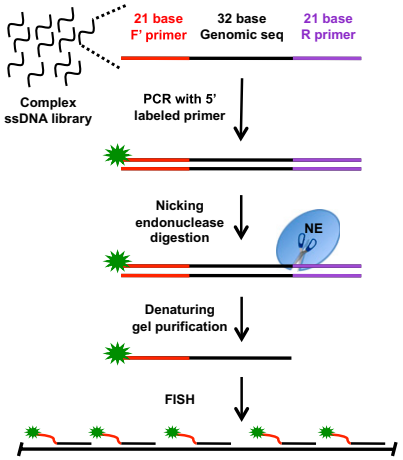

Fig. 1. Each oligonucleotide in the library is composed of 32 bases of genomic sequence flanked by 21-base primer sequences. One of the primers carries a 5′ fluorophore, whereas the other contains a recognition site for a nicking endonuclease (NE) (37). A nicking reaction followed by denaturing gel electrophoresis yields 53-base ssDNAs.

Genomic Target Sites Are Abundant. We have used OligoArray to mine the *Caenorhabditis elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana*, *Mus musculus*, and human genomes for probe sequences and, despite differences in genome size and complexity, found an average density of ~10 per kb in each (Table 1). Furthermore, although there are many 1-kb segments in which we found no appropriate sequences, the overall distribution of probe sequences tends to be fairly uniform (*SI Appendix*, Figs. S1–S3). These observations suggest that most genomic regions will be amenable to Oligopaints. Indeed, our coverage ranges from 90.3% for the human genome to 100% for the *C. elegans* genome (Table 1). Oligopaints is supported by a website (http://genetics.med.harvard.edu/oligopaints) that hosts files detailing the genomic locations of all of the target sites we have discovered as well as a suite of scripts and documentation to assist with the design of probe sets (*SI Appendix*, Fig. S4). The website also provides tools and instructions that will allow researchers to use OligoArray to search for targets using different parameters and to mine the genomes of additional organisms for probe sequences.

Oligopaints Robustly Label Interphase and Metaphase Chromosomes. We have used Oligopaints to visualize single-copy DNA in a variety of cell lines, including *Drosophila* Kc_{167} (XXXX 4N) and human WI-38 (XX 2N) and MRC-5 (XY 2N) cells, and have found it to be applicable for a range of target sizes (*SI Appendix*, Tables S2 and S3). At the lower end, we have found that a 10-kb interval on human chromosome 4 (4p16.1) with very dense coverage (>18 probe sequences/kb) could be visualized with 200 oligos, producing at least one FISH focus in 90% of WI-38 nuclei, with 88% displaying two foci (n = 137; Fig. 2*A*). This same interval could also be visualized in human metaphase spreads (*SI Appendix*, Fig. S5). We obtained similar success when we extended coverage in this region to 52 kb using an additional 650 probes, with 98% of nuclei displaying at least one FISH focus and 86% displaying two foci (n = 126; Fig. 2*B*). We have also visualized larger regions, such as a 2.1-Mb interval on the human X chromosome. Here, we used 20,020 probes and observed 100% of nuclei with one focus and 97% with two in WI-38 (XX) cells (n = 119) and 98% of nuclei with at least one focus in MRC-5 (XY) cells (n = 124; Fig. 2 *C* and *D*). Comparable efficiency (95%; n = 136) was observed for a probe set composed of 25,000 oligos targeting a 2.7-Mb region (50D1–53C7) of the right arm of the second chromosome of *Drosophila* (2R) (Fig. 2*E*).

Because our bioinformatics platform allows us to specify custom hybridization patterns, such as multicolor banding, the effectiveness of Oligopaints can be extended to chromosomal regions on the order of tens of megabases or more. For example, we have used three-color FISH to visualize 7.6 Mb on the human X (Xq13.1–q21.1; *SI Appendix*, Table S3) in both WI-38 interphase nuclei (Fig. 3*A*) and human primary metaphase spreads (Abbott Molecular) (Fig. 3*B*) using 60,060 oligos, and 19.5 Mb of *Drosophila* 2R (41E3–60D14; *SI Appendix*, Table S3) using 180,000 oligos (Fig. 3*C*). The same 180,000-oligo pool has been used to reveal chromosome packaging in a polytenized salivary gland nucleus (Fig. 3*D*), whereas a 75,000-oligo subset of that pool has been combined with fluorescently labeled wheat germ agglutinin

Table 1. Occurrence of probe sequences in the genomes of five eukaryotic organisms

Organism	Assembly	Size, Mbp	No. probes × 10^6	Probes/kb	Coverage, %
C. elegans	ce6	100	1.10	10.9	100
D. melanogaster	dm3	140	1.79	12.8	95.4
A. thaliana	tair10	119	1.45	12.2	98.1
M. musculus	mm9	2,655	30.1	11.4	94.1
H. sapiens	hg19	3,096	29.9	9.7	90.3

For each, we present the genome assembly version, the haploid genome size in megabase pairs, the number of probes found in millions, the mean density of probe sequences per kilobase, and our estimated percent coverage. The estimation of percent coverage is the percentage of 250-kb windows in a given genome in which at least 500 probe sequences occur. Genome size corresponds to size of the genome assembly used (*Materials and Methods*).

to provide an in situ rendering of the positioning of *Drosophila* chromosome 2R relative to the nuclear envelope (41) (Fig. 3*E*).

We have also found Oligopaints to be quite robust. They are compatible with a range of hybridization and wash conditions (*SI Appendix*, Figs. S6–S8), work in the 40- to 800-nM range (*SI Appendix*, Table S2), and are amenable to repeated rounds of hybridization (Fig. 4*A* and *SI Appendix*, Fig. S9), indicating that they will enable researchers to "walk" along the lengths of chromosomes, especially if each step, or hybridization, were itself to involve multicolor FISH targeting several contiguous or even noncontiguous regions. In addition, our bioinformatics platform yielded probe sets that permitted a single hybridization to visualize a 2.5-Mb region centered on the X-inactivation center (XIC) as well as the Xist RNA produced by this region (42) (Fig. 4*B* and *SI Appendix*, Fig. S10); while simultaneous visualization of both genomic regions and transcripts has been achieved previously (43), visualization of both DNA and RNA typically requires sequential hybridizations to avoid cross-talk between the probe sets targeting DNA and those targeting RNA (44). Oligopaints are also suitable for conducting high-throughput FISH in 384-well plates (29) (Fig. 4*C*; *SI Appendix*, Fig. S11), opening up the possibility of Oligopaints-based whole-genome RNAi and small molecule screens. Finally, we anticipate that Oligopaints may be useful for discerning chromatin structure; our probes covering megabase-sized regions have occasionally produced foci that display substructures, with some appearing spherical or "ball-like" and others more linear or "thread-like" (Fig. 4*D*).

Chromosome Painting in Whole-Mounted *Drosophila* Ovaries. To assess the efficacy of Oligopaints in vivo, we turned to the *Drosophila* ovary and, using a probe set targeting the 2.7-Mb 50D1–53C7 region, demonstrated robust labeling of three cell types: oocytes containing pachytene chromosomes undergoing homolog pairing, polytene nurse cells, and somatic follicle cells (Fig. 5*A* and *SI Appendix*, Table S2). For example, 100% of pachytene nuclei ($n = 28$) identified using an antibody against the nuclear synaptonemal complex protein C(3)G (45) were labeled, with 89% containing a single focus and the remaining 11% displaying two closely positioned foci (≤1.0 μm apart), as would be expected for cells containing paired homologs. This result is especially notable, as condensed, pachytene chromosomes are often difficult to label. Our probes also labeled 100% of the polytene nurse cells ($n = 24$) and follicle cells ($n = 110$) (*SI Appendix*, Table S2). Moreover, we attained 99–100% efficient multicolor FISH in all three cell types by introducing a second probe set targeting the 3.1- and 2.6-Mb regions of 41E3–44C4 and 58D2–60D14, respectively, flanking 50D1–53C7 (Fig. 5 *B–D* and *SI Appendix*, Table S2). These results demonstrate that Oligopaints are capable of labeling chromosomes from whole-mounted tissue preparations with high efficiency regardless of copy number or level of compaction.

Discussion

Oligopaints are renewable and highly efficient probes that are amenable to studies of any sequenced organism. In addition to

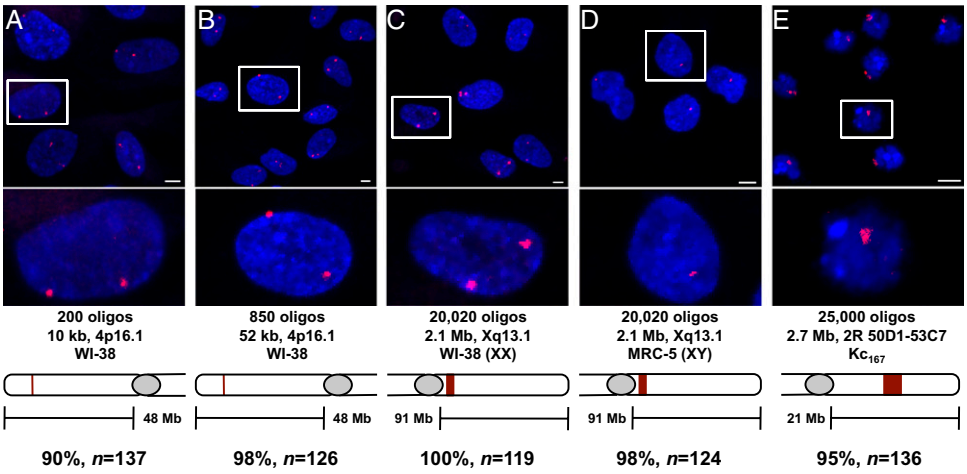

Fig. 2. Oligopaints efficiently label interphase human and *Drosophila* nuclei. (*A*) Probe set of 200 oligos targeting 10 kb at human 4p16.1 was hybridized to WI-38 (2N) cells. (*B*) Probe set targeting the region shown in *A*, but extended to 850 oligos targeting 52 kb was hybridized to WI-38 cells. (*C* and *D*) Probe set of 20,020 oligos targeting 2.1 Mb at human Xq13.1 was hybridized to WI-38 cells (XX) (*C*) or MRC-5 (XY 2N) cells (*D*). (*E*) Probe set of 25,000 oligos targeting 2.7 Mb at 50D1-53C7 on *Drosophila* 2R was hybridized to Kc_{167} (4N) cells. Enlarged image of the *Inset* is shown beneath each micrograph. (*Bottom*) Labeling efficiencies presented as the percentage of cells that displayed at least one FISH focus (*SI Appendix*, Table S2). All probe sets were labeled with TYE563 (Cy3 mimic; red); DNA was identified with DAPI (blue). (Scale bars, 5 μM.) Images are maximum Z projections. Each micrograph was acquired using parameters optimized for entire fields of cells; thus, the sizes of the foci do not necessarily correlate with the sizes of the targeted regions.

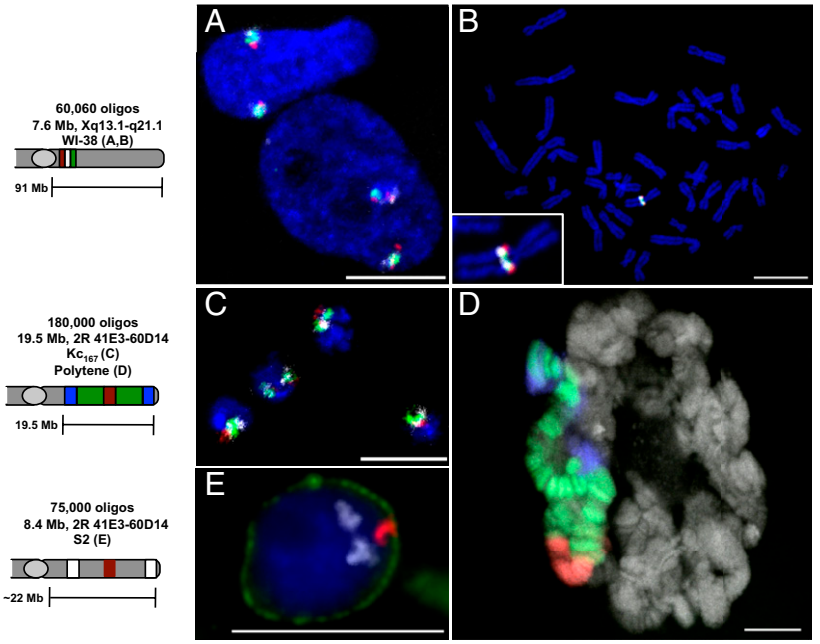

Fig. 3. Multicolor FISH with Oligopaints. (*A* and *B*) Three 20,020 oligo probe sets targeting adjacent regions at human Xq13.1, Xq13.2, and Xq13.3-q21.1 (*SI Appendix*, Fig. S1*A* and Table S3) were used to produce 3-color FISH images from WI-38 (XX) interphase (*A*) and primary metaphase (Abbott Molecular) (*B*) chromosomes. Probe sets were labeled with TYE563 (Cy3 mimic; red), TYE665 (Cy5 mimic; white), or 6-FAM (green), respectively. (*C* and *D*) Probe set of 180,000 oligos was used to paint a multicolor banding pattern from 41E3 to 60D14 (*SI Appendix*, Fig. S1*B* and Table S3) on *Drosophila* 2R in interphase Kc_{167} (4N) nuclei (*C*) and salivary gland polytene chromosomes (*D*) with the following pattern: 41E3–44C4 [white (*C*) or blue (*D*); 25,000 TYE665-labeled oligos], 44C4–50C9 (green; 52,500 6-FAM–labeled oligos), 50D1–53C7 (red; 25,000 TYE563-labeled oligos), 53C9–58B6 (green, 52,500 6-FAM–labeled oligos), and 58D2–60D14 [white (*C*) or blue (*D*); 25,000 TYE665-labeled oligos]. (*E*) Two probe sets were combined to span *Drosophila* 2R. One probe set was composed of 25,000 TYE563 (red)-labeled oligos targeting 50D1–53C7, whereas the second was composed of 25,000 TYE665 (white)-labeled oligos targeting 41E3–44C4 and 25,000 TYE665 (white)-labeled oligos targeting 58D2–60D14. The nuclear envelope was stained with wheat germ agglutinin conjugated to Alexa Fluor 488 (green). *A*, *C*, and *E* are maximum Z projections, whereas *B* and *D* are single Z slices. DNA was identified with DAPI (blue for *A*–*C* and *E*; gray for *D*). (Scale bars, 10 μM.)

revealing chromosome positioning, the probes should facilitate the detection of chromosomal aberrations, especially in conjunction with combinatorial labeling technologies (9, 14–16) and therefore contribute also in the clinical setting. In terms of expense, discounting the one-time investment for an oligo library, the cost of consumables (*SI Appendix*, Table S4) ranges from ~$0.10 to $1.50 per assay and is significantly below the cost of commercial probes. Even so, we are continuing to work toward improving the yield and reproducibility of our probe preparations and hence reducing cost. Importantly, probe sets targeting several megabases work at the same concentrations as do probe sets targeting tens of kilobases (*SI Appendix*, Table S2), making the cost of painting large stretches of the genome extremely low.

We are especially interested in the capacity of Oligopaints to reveal the telomere-to-telomere positioning of interphase chromosomes and believe that our short, single-stranded oligo probes are particularly suited for this task. For instance, clone-based FISH probe fragments are typically much longer and thus may bind to their genomic targets without being fully hybridized; this may place the label further from the chromosome as well as promote the formation of networks of interacting probe fragments that could extend well beyond their genomic target. In contrast, the short, strand-specific nature of Oligopaints argues that they are more likely to fully hybridize to their target and less likely to network, ensuring that they "hug" the chromosome and thereby enhance their ability to reveal chromosome structure. These features may also enhance entry of Oligopaints into the nucleus and their maneuverability through fixed chromatin. The advantages over conventional probes may be particularly relevant at the <100-nm resolution of superresolution fluorescence microscopy (46–49), wherein precision in probe placement and an exact number of fluorophores per probe could augment image interpretation.

Finally, we note that our technology is versatile and able to interface with many other technologies (*SI Appendix*, Fig. S12).

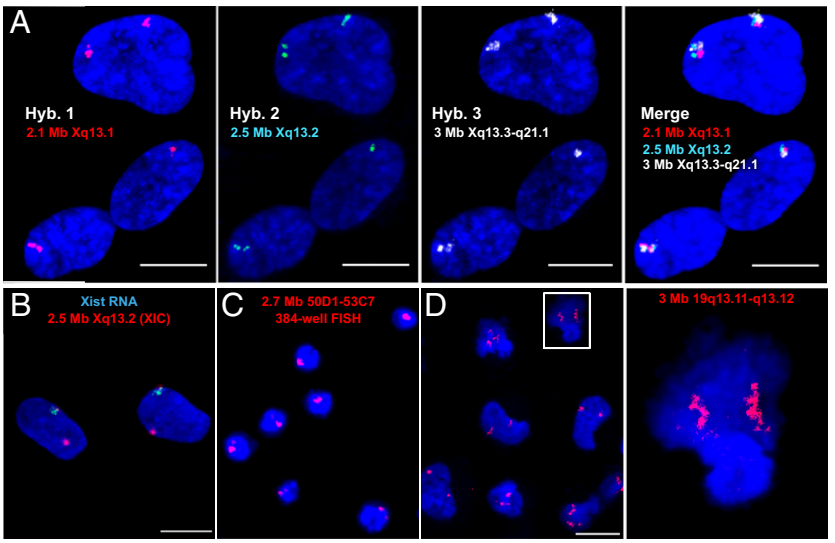

Fig. 4. Multiple applications for Oligopaints. (*A*) Repeated rounds of hybridization in WI-38 (XX 2N) cells using a probe set composed of 20,020 TYE563 (Cy3 mimic; red)-labeled oligos targeting 2.1 Mb at Xq13.1, a probe set composed of 20,020 TYE665 (Cy5 mimic; green)-labeled oligos targeting 2.5 Mb at Xq13.2, and a probe set composed of 20,020 6-FAM (white)-labeled oligos targeting 3.0 Mb at Xq13.3–q21.1. Once a slide was hybridized with a probe set labeled with a given fluorophore (e.g., Tye563 for Hyb 1), the slide was then scanned for the presence of that fluorophore after all successive hybridizations. For all panels, DNA was identified by DAPI (blue). Also see *SI Appendix*, Fig. S9. (*B*) Simultaneous RNA/DNA FISH using a probe set composed of 20,020 TYE563 (red)-labeled oligos targeting the XIC at Xq13.2 and spanning 2.5 Mb and a probe set composed of 96 6-FAM (green)-labeled oligos targeting the Xist RNA in WI-38 cells (42). Also see *SI Appendix*, Fig. S10. (*C*) Automated imaging of a probe set composed of 25,000 TYE563-labeled oligos targeting a 2.7-Mb region at 50D1–53C7 on *Drosophila* 2R in Kc_{167} cells (4N; single focus reflects pairing of homologous chromosomes in *Drosophila*) seeded in a 384-well plate. Also see *SI Appendix*, Fig. S11. (*D*) Examples of morphologies produced by a probe set composed of 20,020 Cy3-labeled oligos targeting 3 Mb at human chr19q13.11–q13.12 in WI-38 cells. (Right) Enlarged image of *Inset*. All images are maximum Z projections. (Scale bars, 10 μM.)

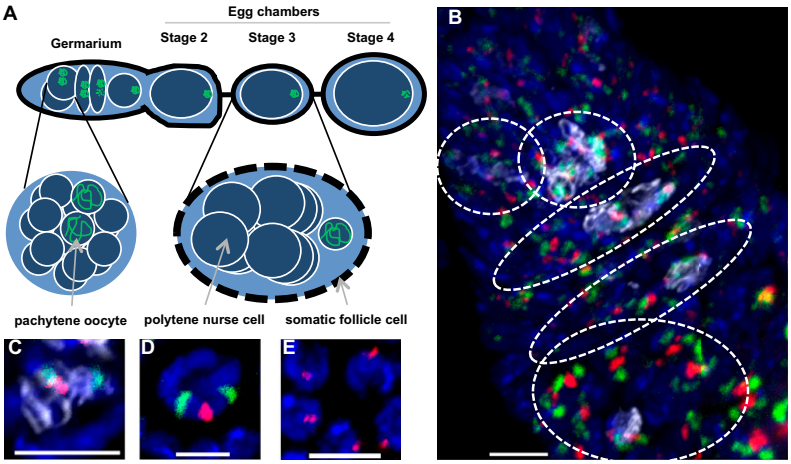

Fig. 5. Oligopaints efficiently label nuclei from whole-mounted *Drosophila* ovaries. (*A*) Cartoon of a *Drosophila* ovariole displaying three cell types: the pachytene oocytes and polytene nurse cells within the meiotic cysts and the somatic follicle cells that encase them. (*B*) *Drosophila* germarium labeled with two probe sets composed of 25,000 TYE563 (Cy3 mimic; red)-labeled oligos targeting a 2.7-Mb region at 50D1–53C7 and an additional set composed of two pools of 25,000 TYE665 (Cy5 mimic; green)-labeled oligos targeting 41E3–44C4 and 58D2–60D14 (green), all regions located on *Drosophila* 2R. An antibody to the synaptonemal complex component C(3)G (white) was used to identify oocytes. Hashed circles demarcate the meiotic cysts. (*C* and *D*) Same probe set as described for *B* in a magnified view of a single oocyte (*C*) and a polytene nurse cell (*D*). (*E*) The 50D1–53C7 probe set in a magnified view of follicle cells. (Scale bars, 5 μM.) For all panels, DNA was identified by DAPI (blue).

Importantly, the primer sequences retained in the probe provide general strategies for coupling a wide variety of functionalities to bioinformatically designed oligo libraries, thereby extending the potential usefulness of Oligopaints. Functionalities can be attached directly to primers and incorporated into the probe during amplification, or they can be brought in by the hybridization of a secondary oligo (48, 50, 51) that is homologous to the primer sequence; either way, significant cost savings can be achieved by bulk orders of modified oligos that can then be applied to any number of libraries. The availability of primer sequences further opens opportunities for bringing in functionalities via DNA binding factors or assembling DNA structures, such as those used in branched signal amplification (11, 27). Thus, we believe that Oligopaints has the potential to become a reagent not only for visualization, but also a broader spectrum of methods that require the targeting of biochemical modifications and functional chemistries to nucleic acids in a sequence-specific fashion.

Materials and Methods

Genome Sequences. The ce6, dm3, mm9, and hg19 genomic sequences were obtained from the University of California Santa Cruz genome bioinformatics website. The tair10 sequence was obtained from the Joint Genome Institute and Center for Integrative Genomics Phytozome website.

Probe Discovery. Genomes were inputted into OligoArray2.1 (38), which was run on the Harvard Medical School Research Information Technology Group Orchestra UNIX cluster with the following parameters: -n 22 -l 32 -L 32 -D 1000 -t 75 -T 90 -s 70 -x 70 -p 35 -P 80 -m "GGGG;CCCC;TTTTT;AAAAA" -g 42.

PCR Primers. For descriptions, please see *SI Appendix, SI Methods*.

Oligonucleotide Libraries. Oligonucleotide libraries were synthesized by MYcroarray (Ann Arbor, MI). Libraries were either ordered as ssDNA 74mers or ssDNA 60mers. The 60mer libraries were extended to 74mers using the emulsion PCR protocol detailed in ref. 36 and using the "touch-up" cycle described for the generation of probe set for the Xist RNA.

Emulsion PCR Amplification. Our strategies for PCR have evolved with the development of the technology, involving changes in template and primer concentrations and ratios as well as the use of emulsion. Although we conducted all earlier PCR reactions for the preparation of probe with emulsion (Figs. 2 *A–E*; 3 *A*, *C*, and *D*; 4 *A*, *C*, and *D*; and 5 *B–E* and *SI Appendix*, Fig. S6 *A* and *B*, S7–S9, and S11) we now use this protocol primarily for renewing the library (as opposed to the generation of FISH probe). For more information about the protocol, please see *SI Appendix, SI Methods*.

Nonemulsion PCR Amplification. DNA FISH probe sets amplified without emulsion used the following cycle: 95 °C for 5 min, 3 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 15 s, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 15 s, with a final extension step at 72 °C for 5 min. One hundred picomoles of each primer and 1 ng of template were used per 100 μL of PCR.

FISH Probe Set Targeting the Xist RNA. The probe set for the Xist RNA was made from 96 ssDNA 60mers (Integrated DNA Technologies, IDT), each containing 32 bases of exonic sequence from Xist exon 1 flanked by 14 base primer sequences. The 60mers were then amplified in a pool using 21 base primers and the same cycle and parameters listed above for nonemulsion PCR amplification except that 10 fmol of template were used per 100 μL of PCR.

Extraction and Purification of ssDNA. For descriptions, please see *SI Appendix, SI Methods*.

Slide Preparation for Interphase FISH. Glass slides (Thermo Scientific; 4951–001) were treated with a 0.01% (vol/vol) poly-L-lysine solution (Sigma; P8920) for 5 min, then air-dried. A total of 100 μL of a 1–2 × 10^6 cells/mL solution was spotted on each slide and allowed to adhere for 1–2 h at 23 °C (*Drosophila*) or 37 °C (mammalian). Slides were rinsed in 1× PBS at room temperature (RT), then fixed at RT for 5 min or 15 min in 1× PBS + 4% (vol/vol) paraformaldehyde (Electron Microscopy Sciences; 15710). Postfixation, slides were rinsed in 1× PBS, washed for 5 min in 2× SSCT (0.3 M NaCl, 0.03 M NaCitrate, 0.1% Tween-20), then washed for 5 min in 2× SSCT + 50% formamide (vol/vol), all at RT. Slides were then transferred to fresh 2× SSCT + 50% formamide for storage at 4 °C. For information on cell culture, see *SI Appendix, SI Methods*.

Interphase FISH. Slides were warmed to RT, then incubated for 2.5 min or 3 min in 2× SSCT + 50% formamide (vol/vol) at 92 °C, then incubated for 20 min at 60 °C in 2× SSCT + 50% formamide, then cooled to RT. A total of 1–20 pmol of each probe was then added to each slide as part of a 25-μL hybridization mixture composed of 2× SSCT, 50% formamide, 10% dextran sulfate (wt/vol), and 10 μg of RNase A (Fermentas; EN0531) and sealed beneath a 22 × 22-cm #1.5 coverslip using rubber cement. Slides were denatured for 2.5 min at 92 °C on a water-immersed heat block and then allowed to hybridize overnight at 37 °C or 42 °C in a humidified chamber. Slides were washed for 15 min in 2× SSCT at 60 °C, then for 10 min 2× SSCT at RT, and then for 10 min in 0.2× SSC at RT. Slides were then mounted in SlowFade Gold + 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen; S36938) and sealed with a 22 × 30-cm #1.5 coverslip using nail polish.

Simultaneous RNA/DNA FISH. Simultaneous RNA/DNA FISH was performed using a "3D-FISH" protocol adapted from ref. 52. For more information, please see *SI Appendix, SI Methods*.

Metaphase Spreads. (46, XY) metaphase spreads were obtained from Abbott Molecular (30-806010).

Metaphase FISH Protocol. For descriptions, please see *SI Appendix, SI Methods*.

Rehybridization Protocol. The same FISH protocol was used as for single-round interphase FISH, with probe being stripped off between hybridizations by a 40-s wash in 2× SSCT + 50% formamide (vol/vol) at 65 °C.

Modified 384-Well FISH Protocol for Oligopaints. For descriptions, please see *SI Appendix, SI Methods*.

Hybridization to *Drosophila* Ovarioles and Polytene Chromosome Squashes. For descriptions, please see *SI Appendix, SI Methods*.

Microscopy and Image Processing. For descriptions, please see *SI Appendix, SI Methods*.

Note Added in Proof. We encourage interested readers to also view Bienko et al. (53), which was accepted by *Nature Methods* while galley proofs were being prepared for our article. This study describes a related strategy for making FISH probes.

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