Visualizing Genomes with Oligopaint FISH Probes

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ABSTRACT

Oligopaint probes are fluorescently labeled, single-stranded DNA oligonucleotides that can be used to visualize genomic regions ranging in size from tens of kilobases to many megabases. This unit details how Oligopaint probes can be synthesized using basic molecular biological techniques, and provides protocols for FISH, 3D-FISH, and sample preparation. *Curr. Protoc. Mol. Biol*. 105:14.23.1-14.23.20. © 2014 by John Wiley & Sons, Inc.

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INTRODUCTION

The availability of inexpensive DNA oligonucleotide (oligo) libraries containing hundreds to hundreds of thousands of unique oligo species has enabled technological advances in areas such as cytogenetics and the study of nuclear biology. In particular, these libraries can serve as a renewable source of probes for fluorescent in situ hybridization (FISH) and allow researchers to precisely define the sequences to be targeted by the probes. This unit details a strategy that applies a few standard molecular biological protocols to complex libraries of ssDNA oligos in order to produce short, single-stranded, highly efficient Oligopaint FISH probes that label >90% of nuclei in fixed tissue culture cells (Beliveau et al., 2012). Probe generation can be accomplished in a matter of days and at a cost that is below that of commercially available bacterial artificial chromosome (BAC) -based probes (Beliveau et al., 2012). Oligopaint probes can target regions ranging in size from tens of kilobases to many megabases, can be made strand-specific, and can be bioinformatically designed to produce customizable patterns, such as multicolor banding.

Basic Protocol 1 presents the strategy for synthesizing Oligopaint probes, while Basic Protocol 2 details a streamlined protocol for using Oligopaint probes to label interphase and mitotic chromosomes in tissue culture cells. Alternate Protocol 1 describes a strategy for 3D-FISH (Lanzuolo et al., 2007; Cremer et al., 2008), which avoids high-temperature steps in order to better preserve the three-dimensional morphology of nuclei, while Alternate Protocol 2 describes a strategy for labeling metaphase chromosomes. Finally, the Support Protocol addresses the preparation of sample slides for use with Basic Protocol 2.

GENERATION OF SINGLE-STRANDED OLIGOPAINT FISH PROBES FROM A COMPLEX DNA LIBRARY

BASIC PROTOCOL 1

Oligopaint FISH probes are produced by a series of standard molecular biological techniques, including PCR, DNA precipitation, and gel electrophoresis, and the protocol presented below describes the entire process, starting with the incorporation of fluorescent labels during PCR and finishing with the quantification of the purified ssDNA probe. For a discussion of some of the considerations in probe design, see Critical Parameters. This probe synthesis protocol uses the strategy described by Beliveau et al. (2012), in which

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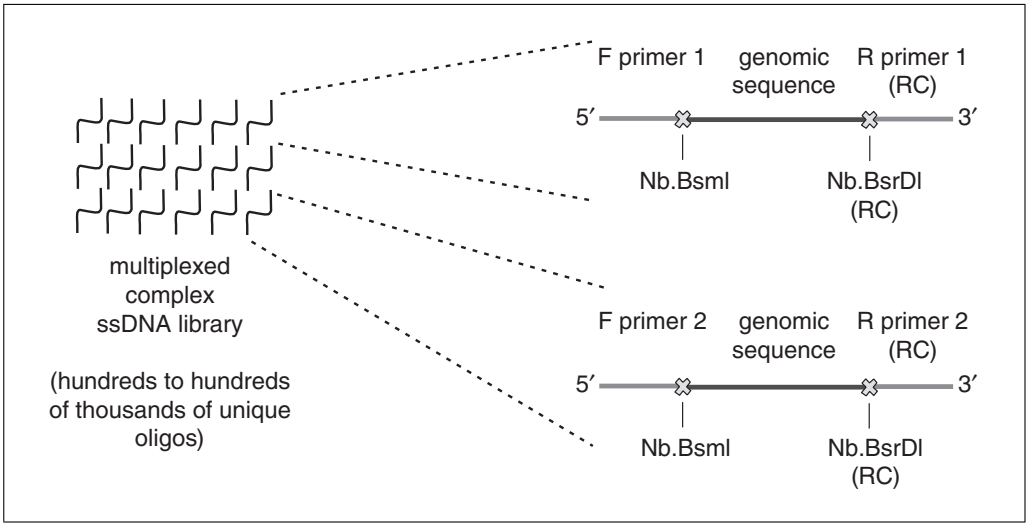

Figure 14.23.1 Library design using the Oligopaints strategy. Each synthetic ssDNA library may contain hundreds to hundreds of thousands or more unique oligo species and can contain multiple distinct Oligopaint probes through the use of multiple primer pairs. This figure diagrams the structure of two oligos from different probe sets in a hypothetical library: an oligo belonging to a probe that can be amplified using primer pair 1 (top left) and an oligo belonging to a probe that can be amplified using primer pair 2 (bottom left). Both oligos contain, in 5′ to 3′ order, the sequence of the forward primer, genomic sequence, and then the reverse complement (RC) of the reverse primer. The sites for two nicking endonucleases, Nb.BsmI and Nb.BsrDI, are placed between the primer sequences and the genomic sequences. The use of two nicking endonuclease sites in the library molecules allows for the production of strand specific probes: amplification with a labeled F primer and digestion with Nb.BsrDI will yield probe targeting the reverse complement of the genomic sequence encoded in the ssDNA molecules of the library, whereas amplification with a labeled R primer and digestion with Nb.BsmI will yield probe targeting the genomic sequence encoded in the ssDNA molecules of the library. The use of two nicking endonuclease sites, instead of one, is an update of the strategy presented in Figure 1 of Beliveau et al. (2012).

a nicking endonuclease recognition sequence (e.g., 5′ . . . GCAATG . . . 3′ for Nb.BsrDI) is included in every molecule of the ssDNA library (see Fig. 14.23.1) to facilitate the isolation of single-stranded probe molecules after the conversion of the library to dsDNA by PCR amplification. Note that while Figure 1 of Beliveau et al. (2012) illustrates the use of a single nicking endonuclease site, it is also possible to include two distinct nicking endonuclease sites (e.g., Nb.BsrDI and Nb.BsmI; Fig. 14.23.1) such that either strand of the dsDNA duplex can be used to generate FISH probe. While this protocol and Beliveau et al. (2012) exclusively focus on the nicking endonuclease plus gel extraction method of converting dsDNA PCR products into ssDNA, other strategies such as biotinylation plus bead capture, asymmetric PCR, or lambda exonuclease treatment could be substituted if desired.

Materials

10× *Taq* DNA polymerase buffer (KAPA Biosystems)
10 mM dNTP mix
200 μM fluorophore-labeled "forward" primer (Integrated DNA Technologies)
200 μM unlabeled "reverse" primer (Integrated DNA Technologies)
20 pg/μl complex DNA library (MYcroarray, LC Sciences, CustomArray)
5 U/μl *Taq* DNA polymerase (KAPA Biosystems)
Molecular biology-grade water
20 mg/ml molecular biology-grade glycogen (Thermo Scientific)
4 M ammonium acetate solution in distilled, deionized water (ddH_2O)
100% ethanol

70% (v/v) ethanol solution in ddH_2O
10× Nb.BsrDI enzyme buffer (nicking enzyme buffer; New England Biolabs)
10 U/μl Nb.BsrDI enzyme (New England Biolabs)
1× Tris-Borate-EDTA (TBE) buffer (see recipe)
15% TBE + 7 M Urea denaturing polyacrylamide gel (Bio-Rad)
Low-molecular-weight DNA ladder
2× TBE + urea gel loading buffer containing xylene cyanol FF and bromphenol blue (Bio-Rad)
Ice
10 mg/ml ethidium bromide solution (Calbiochem)
0.4 M ammonium acetate solution in ddH_2O

0.2-ml thin-walled strip tubes *or* thin-walled 96-well plate
Programmable thermal cycler
50-ml conical tubes
Benchtop vortex mixer
2.0-ml microcentrifuge tubes
−80°C *or* −20°C freezer
Refrigerated centrifuge
Vacuum trap *or* micropipets
Adjustable heat block
37°C incubator
Microcentrifuge
15-ml conical tubes
Gel box and power supply
1000-μl micropipets
Ethidium bromide staining dish
Benchtop orbital shaker
UV box
Heated vortex mixer or shaking incubator
Spectrophotometer

NOTE: Add 100-fold more template if using raw ssDNA library (i.e., direct from manufacturer, never amplified via PCR) as many of the raw oligos may not be suitable templates for amplification. Raw libraries can be amplified using unlabeled "F" and "R" primers and purified using a PCR cleanup kit to provide a greater amount of template material for labeling PCRs.

Perform PCR amplification with labeled primers and PCR reaction concentration

1. Set up a PCR master mix using the component ratios listed below and transfer into 0.2-ml thin-walled strip tubes or a thin-walled 96-well plate. Do not add more than 100 μl of master mix per tube/well.

   1× PCR master mix
   10 μl of 10× *Taq* DNA polymerase buffer
   2 μl of 10 mM dNTP mix
   0.5 μl of 200 μM fluorophore-labeled "forward" primer
   0.5 μl of 200 μM unlabeled "reverse" primer
   1 μl of 20 pg/μl complex DNA library
   1 μl of 5 U/μl *Taq* DNA polymerase
   85 μl molecular biology-grade water.

   The scale of the PCR reaction should correlate with the desired number of FISH assays. In an efficient probe preparation, ≥20% of fluorescently labeled primer added to the PCR reaction will be recovered as ssDNA FISH probe. Thus, a 10-ml PCR reaction using

10,000 pmol of labeled primer can be expected to produce 2,000 pmol of FISH probe, or 100 20 pmol FISH assays.

The proofreading 3′ → 5′ exonuclease activity of some high-fidelity thermostable DNA polymerases can lead to DNA degradation in subsequent steps. A phenol-chloroform extraction is recommended after the PCR is completed if the polymerase has strong exonuclease activity. This extraction is not necessary for most standard polymerases.

2. Transfer the PCR reactions into a programmable thermal cycler and run the following program:

1 cycle:	5 min	95°C (initial denaturation)
42 cycles:	30 sec	95°C (denaturation)
	30 sec	60°C (annealing)
	15 sec	72°C (extension)
1 cycle:	5 min	72°C (final extension)
1 cycle:	indefinite	4°C (final hold).

This program assumes a primer T_M of ~60°C for both primers; raise or lower the annealing temperature accordingly if necessary.

3. Pool the cycled PCR reactions into a 50-ml conical tube.

To accelerate this step for large volumes, extract the PCR reaction using a multichannel pipettor and transfer to a disposable reagent reservoir in a PCR or tissue culture hood.

4. Set up a DNA precipitation in the 50-ml conical tube containing the PCR product: for every 600 µl of PCR reaction, add 2 µl of 20 mg/ml glycogen, 65 µl 4 M ammonium acetate, and 1350 µl ice-cold 100% ethanol. Vortex vigorously for 30 sec and divide into aliquots into 2.0-ml microcentrifuge tubes.

5. Incubate the precipitations for 35 min at −80°C or for >2 hr at −20°C.

Precipitations can be left at −20°C overnight or indefinitely; this step represents a convenient stopping point.

6. Centrifuge the precipitation for 1 hr at maximum speed,4°C, in a refrigerated centrifuge.

The precipitation can also been centrifuged at room temperature if a refrigerated centrifuge is not available.

7. Carefully aspirate off the ethanol supernatant using a vacuum trap or micropipet.

A prominent pellet should be visible after the centrifugation. Depending on the fluorophore used, the pellet may appear colored.

8. Add 1350 µl ice-cold 70% ethanol to each tube, taking care not to disturb the pellet.

9. Centrifuge the precipitation for 30 min at maximum speed, 4°C, in a refrigerated centrifuge.

10. Carefully aspirate off the 70% ethanol using a vacuum trap or micropipet.

11. Air dry the pellets by placing the open tubes onto a 42°C heat block for 15 min.

12. Add 60 µl molecular biology-grade water to each pellet. Incubate for 30 min at 37°C to resuspend. Vortex each tube after the 37°C incubation, and then spin briefly in a microcentrifuge.

Shaking at 1400 rpm in a heated vortex mixer will speed the resuspension.

This step represents a convenient stopping point—samples can be left at 4°C for several weeks or frozen at −20°C and left indefinitely.

Nicking endonuclease digestion and digest concentration

13. Set up a nicking endonuclease digestion as follows:

    a. For every 60 µl of precipitated PCR product, add 7.5 µl of 10× nicking enzyme buffer and 7.5 µl 10 U/µl Nb.BsrDI.
    b. Transfer the digestion into 0.2-ml thin-walled strip tubes or a thin-walled 96-well plate. Do not add more than 25 µl of master mix per tube/well.

    Incubating the digestion in a single 0.6-ml thin-walled tube or 1.7-ml microcentrifuge tube may result in reduced nicking efficiency.

14. Incubate the digestion for 4 hr at 65°C in a programmable thermal cycler, and then heat-inactivate the enzyme by incubating for 20 min at 80°C.

    Incubating for >4 hr at 65°C is not recommended as it may result in star activity. While Nb.BsrDI and Nb.BsmI require incubation at 65°C, other nicking enzymes may require incubation at 37°C.

15. Pool the digestion reaction in a 15-ml conical tube and set up a DNA precipitation as follows:

    For every 600 µl of digestion reaction, add 2 µl of 20 mg/ml glycogen, 65 µl of 4 M ammonium acetate, and 1350 µl ice-cold 100% ethanol. Vortex vigorously for 30 sec and transfer into 2.0-ml microcentrifuge tubes.

    Molecular biology-grade water can be used to adjust the volume of the pooled digestion reaction to a balanced number of 2.0-ml microcentrifuge tubes (e.g., a multiple of two or three), if desired. The DNA will be very concentrated at this step; a precipitate may form as soon as the digestion is mixed with 100% ethanol.

16. Incubate the precipitations for 35 min at −80°C or for >2 hr at −20°C.

    Precipitations can be left at −20°C overnight or indefinitely; this step represents a convenient stopping point.

17. Centrifuge the precipitation for 1 hr at maximum speed, 4°C, in a refrigerated centrifuge.

    The precipitation can also be centrifuged at room temperature if a refrigerated centrifuge is not available.

18. Carefully aspirate off the ethanol using a vacuum trap or micropipet.

    A very large pellet should be visible after the centrifugation. Depending on the fluorophore used, the pellet may appear colored.

19. Add 1350 µl ice-cold 70% ethanol to each tube, taking care not to disturb the pellet.

20. Centrifuge the precipitation for 30 min at maximum speed for 30 min, 4°C, in a refrigerated centrifuge.

21. Carefully aspirate off the 70% ethanol using a vacuum trap or micropipet.

22. Air dry the pellets by placing the open tubes onto a 42°C heat block for 15 min.

23. Add 40 µl of molecular biology-grade water to each pellet. Incubate for 30 min at 37°C to resuspend. Vortex each tube for 5 sec after the 37°C incubation, and then spin briefly in a microcentrifuge.

    Shaking at 1400 rpm in a heated vortex mixer will speed the resuspension.

    Step 24 can be started during the 37°C incubation.

Gel electrophoresis, extraction, and ssDNA concentration

24. Pre-run a 15% TBE-Urea polyacrylamide gel in prewarmed (to ~60°C) 1× TBE at constant wattage for at least 30 min. Use a wattage that maintains a buffer temperature of 55° to 60°C.

    CAUTION: *The gel casing may crack if the temperature is too high.*

    It is essential that the gel is sufficiently hot to prevent the renaturation of the nicked DNA. Renaturation will result in the appearance of slow-migrating smears in the gel.

25. While the gel is pre-running, mix the resuspended precipitation products 1:1 with 2× TBE-urea gel loading buffer and transfer into 0.2-ml thin-walled strip tubes. Load one gel well volume (e.g., 40 µl) per tube. In addition, mix an aliquot of low-molecular-weight DNA ladder 1:1 with 2× TBE-urea gel loading buffer and load into a 0.2-ml thin-walled strip tube.

26. Denature the samples for 5 min at 95°C in a programmable thermal cycler, and then transfer directly to ice.

27. Rinse each gel well with 1 ml of 1× TBE (from the gel box) using a 1000-µl micropipet to blast out any urea that may have settled in the well prior to loading the samples.

28. Load the samples and run at the same constant wattage as the pre-run (again maintaining temperature of 55° to 60°C) until the bromphenol blue marker dye is near the bottom of the gel (~15 to 20 min for an 8.7 cm gel).

    The labeled ssDNA product of interest will essentially co-migrate with the xylene cyanol FF marker dye, running slightly slower. Prominent-colored bands may be visible depending on the fluorophore used.

29. Carefully remove the gel from its casing and add to a staining dish containing 50 ml of 0.6 µg/ml ethidium bromide solution (3 µl of 10 mg/ml ethidium bromine solution in 50 ml ddH_2O).

30. Stain the gel for 5 min at room temperature with gentle mixing on a benchtop orbital shaker.

31. Pour off the ethidium bromide stain and add 50 ml of ddH_2O. De-stain for 5 min with gentle mixing on a benchtop orbital shaker.

32. Pour off the de-stain ddH_2O and add a small amount of fresh ddH_2O to the dish.

    The fresh ddH_2O makes it easier to remove the gel from the dish.

    At this point, the gel can be imaged on a gel-doc or UV scanner prior to the gel extraction step.

33. Prepare a 2.0-ml microcentrifuge tube containing 600 µl of 0.4 M ammonium acetate for each gel lane loaded.

34. Image the gel on a UV box—the ssDNA product of interest is 59 bases long. Excise the bands of interest and transfer to them into the microcentrifuge tubes prepared in step 33 (one gel slice/tube).

    Some fluorophores may slightly alter the migration of the ssDNA relative to the DNA ladder. Three prominent bands are expected: one containing unlabeled strand and any un-nicked labeled DNA resulting from incomplete digestion; a faster-migrating band containing the labeled probe DNA; and the fastest-migrating band, containing the un-incorporated primer sequences and the unlabeled fragment released by the nicking reaction.

The final labeled ssDNA fragment of interest reported in Beliveau et al. (2012) is 53 bases long, while the fragment described here is 59 bases. The difference in length is the result of the inclusion of an additional 6-base nicking endonuclease recognition site that allows the isolation of either strand of the duplex produced by PCR (see Fig. 14.23.1).

35. Incubate the gel slices overnight at 55°C in a heated vortex mixer at maximum speed (if available) or a shaking incubator at ≥300 rpm.

36. The next day, centrifuge the tubes containing the gel slices for 5 min at maximum speed, room temperature, in a microcentrifuge.

37. Collect the supernatant from each tube, leaving the gel slice behind, and transfer into a fresh 2.0-ml microcentrifuge tube.

    If desired, the gel slices can be incubated at 55°C with shaking in an additional 600 µl of 0.4 M ammonium acetate to recover any product not eluted by the first overnight incubation.

38. Set up a DNA precipitation as follows:

    a. Add 13.5 µl of 20 mg/ml glycogen and 1350 µl ice-cold 100% ethanol to each 2.0-ml tube.
    b. Vortex vigorously for 15 sec to mix.

39. Incubate the precipitations for 35 min at −80°C or for >2 hr at −20°C.

    Precipitations can be left at −20°C overnight or indefinitely; this step represents a convenient stopping point.

40. Centrifuge the precipitation for 1 hr at maximum speed, 4°C, in a refrigerated centrifuge.

    The precipitation can also be centrifuged at room temperature if a refrigerated centrifuge is not available.

41. Carefully aspirate off the ethanol using a vacuum trap or micropipettor.

    A prominent pellet should be visible after the centrifugation. Depending on the fluorophore used, the pellet may appear colored.

42. Add 1350 µl ice-cold 70% ethanol to each tube, taking care not to disturb the pellet.

43. Centrifuge the precipitation for 30 min at maximum speed, 4°C, in a refrigerated centrifuge.

44. Carefully aspirate off the 70% ethanol using a vacuum trap or micropipettor.

45. Air dry the pellets by placing the open tubes onto a 42°C heat block for 15 min.

46. Add 10 µl molecular biology-grade water to each pellet. Incubate for 60 min at 37°C to resuspend. Vortex each tube for 15 sec after the 37°C incubation, and then spin briefly in a microcentrifuge.

    Shaking at 1400 rpm in a heated vortex mixer will speed the resuspension.

47. Quantify the amount of ssDNA FISH probe using a spectrophotometer.

    The concentration of the ssDNA FISH probe can be inferred by measuring the absorbance of light at 260 nm (A_{260}). Remember to use the ssDNA absorbance conversion factor of 37 µg per 1 A_{260} unit, not the 50 µg per 1 A_{260} unit conversion factor for dsDNA. It is also important to note that some fluorophores, particularly those in the blue to green portion of the visible spectrum, absorb light at 260 nm. Thus, to determine an accurate DNA concentration, a correction factor (CF) may need to be used. CF is defined as $A_{260\ free\ dye}/A_{max\ free\ dye}$, or the fluorophore's absorbance as a free dye at 260 nm divided by the fluorophore's absorbance as a free dye at its absorbance maximum (i.e., excitation

Table 14.23.1 CF_{260} Values of Commonly Used Flurophores

Dye	λ excitation (nm)	λ emission (nm)	CF_{260}
Alexa Fluor 488[a]	495	519	0.30
6-FAM[b]	495	520	0.28
ATTO 488[c]	501	523	0.25
TYE563[b]	549	563	0.07
Alexa Fluor 555[a]	555	565	0.08
Cy3[b]	550	564	0.04
ATTO 550[c]	554	576	0.24
ATTO 565[c]	563	592	0.34
ATTO 590[c]	594	624	0.42
ATTO 633[c]	629	657	0.05
TYE665[b]	645	665	0.12
Alexa Fluor 647[a]	650	665	0.00
ATTO 647N[c]	644	669	0.06
Cy5[b]	648	668	0.04
Cy5.5[b]	685	706	0.10
ATTO 740[c]	740	764	0.11
Alexa Fluor 750[a]	749	775	0.00

[a]Can be purchased from Life Technologies.
[b]Can be purchased from Integrated DNA Technologies.
[c]Can be purchased from ATTO-TEC.

wavelength). See Table 14.23.1 for the CF_{260} values of commonly used fluorophores. Therefore, the corrected DNA absorbance is expressed as $A_{DNA} = A_{260} - (A_{max} * CF)$, where A_{260} is the absorbance of the solution at 260 nm and A_{max} is the absorbance of the solution at the absorbance maximum of the fluorophore.

Probe preparations can be stored for several weeks at 4°C or indefinitely −20°C indefinitely. Divide large preparations into aliquots before freezing to minimize the number of times the probe is freeze-thawed.

BASIC PROTOCOL 2

INTERPHASE FISH USING OLIGOPAINT PROBES

Oligopaint probes label interphase nuclei extremely efficiently. Described here is a fast FISH protocol optimized for labeling chromosomal DNA in fixed tissue culture cells. Alternate Protocol 1 describes a modified 3D-FISH (Lanzuolo et al., 2007; Cremer et al., 2008) version of this protocol in which the temperature is never raised above 78°C, and Alternate Protocol 2 presents a modified protocol for applying FISH with Oligopaint probes to metaphase chromosome preparations.

Materials

4× SSCT (see recipe)
Formamide (store at 4°C, away from light)
Fixed interphase cells adhered to a glass microscope slide (see Support Protocol)
2× hybridization cocktail (see recipe)
10 mg/ml RNase A
Oligopaint probe (see Basic Protocol 1)
2× SSCT (see recipe)
0.2× SSC (see recipe)

Anti-fade mounting medium with DAPI
Nail polish
100-ml graduated cylinders
Plastic paraffin film
Glass Coplin slide staining jars
Adjustable temperature water baths
Anodized aluminum heat block
Forceps
1.7-ml microcentrifuge tubes
Benchtop vortex mixer
Benchtop microcentrifuge
Aluminum foil
Paper towels
22 × 22–mm no. 1.5 coverslips
Pipets
Rubber cement
Hybridization chamber (see step 15)
Heated incubator
22 × 30–mm no. 1.5 coverslips
Epifluorescent *or* confocal microscope

Prehybridization treatment and denaturation

1. Prepare 100 ml of 2× SSCT + 50% formamide by adding 50 ml of 4× SSCT and 50 ml of formamide to a 100-ml graduated cylinder. Seal the top of the cylinder with plastic paraffin film and invert several times to mix.

2. Add 50 ml of 2× SSCT + 50% formamide per jar to two Coplin glass slide staining jars. Place each Coplin jar in a separate temperature-adjustable water bath at room temperature.

   CAUTION*: Do not place a room temperature Coplin jar directly into a hot water bath, as this may weaken the Coplin jar or cause it to break instantly.*

3. Heat one water bath to 60°C and the other to 92°C. In addition, place an anodized aluminum block into the bath to be set to 92°C and adjust the water level such that all but about 1 cm of the block is submerged. Allow the sample slides to warm from 4°C to room temperature while the water baths heat up.

4. Transfer the sample slides into the Coplin jar containing 2× SSCT + 50% formamide at 92°C using forceps and incubate for 2.5 min.

5. Transfer the slides into the Coplin jar containing 2× SSCT + 50% formamide at 60°C using forceps and incubate for 20 min.

6. While the slides are incubating at 60°C, prepare a hybridization master mix. For each sample, add 12.5 μl of 2× hybridization cocktail, 12.5 μl of formamide, and 1 μl of 10 mg/ml RNase. For each sample, add 26 μl of hybridization master mix into a 1.7-ml microcentrifuge tube.

   The 2× hybridization cocktail is very viscous and can be difficult to pipet. Removing 1 to 2 cm from the end of a plastic pipet tip with scissors or a razor blade will make this step easier. If available, a positive-displacement pipet simplifies the pipetting.

7. Add the Oligopaint probe to each microcentrifuge tube containing hybridization master mix.

   A total of 20 to 30 pmol of Oligopaint probe is typically sufficient to produce strong staining in fixed tissue culture cells; 10-fold more probe is recommended for tissue sections and whole-mount tissues.

The amount of probe needed to produce strong signal may need to be determined empirically for each type of sample. See Critical Parameters for further discussion of this issue.

Try to keep the volume of probe added as small as possible –(i.e., use concentrated stocks of probe). Dilute probes can be concentrated by lyophilization or salt-ethanol precipitation (UNIT 2.1A) followed by resuspension in a reduced volume. Adding ≤ ~4 μl of probe per sample is recommended.

8. Mix the contents of each microcentrifuge tube by vortexing, and microcentrifuge briefly to collect the hybridization mix. Protect the hybridization mixes from light by covering the tubes with foil or placing them in a drawer until the slides are ready.

9. After the 20-min incubation at 60°C, use forceps to carefully remove the slides from the Coplin jar in the water bath. Partially dry the sample slides by tapping the thin edge of the slides against a paper towel, and then place the slides into an empty Coplin jar.

   Take care that the slide surface containing the sample never comes into direct contact with the paper towel, as the paper towel can introduce unwelcome debris.

10. Place a 22 × 22–mm coverslip onto a dry paper towel and pipet the hybridization mix directly onto the coverslip.

    The final hybridization mix will be less viscous than the 2× hybridization cocktail and can be pipetted using a standard micropipet with standard tips. Take care to avoid creating air bubbles when pipetting.

11. Gently invert the sample slide onto the coverslip such that the sample (typically visible as a cloudy circle) is placed in direct contact with the hybridization mix. Do not press the slide down against the coverslip as this will result in the ejection of hybridization mix onto the paper towel.

12. Turn the slide over so the coverslip is on the upward-facing side of the slide. Seal the coverslip onto the slide by adding a layer of rubber cement around the edges of the coverslip.

    Do not worry about adding too much rubber cement, as it is easily removed after the denaturation and hybridization steps. It is essential that the perimeter of the coverslip be completely covered by rubber cement, or the hybridization mix may leak or evaporate during the denaturation or hybridization step.

13. Allow the rubber cement to dry for 5 min.

    The slides can be placed inside a box or in a drawer during this incubation to protect them from light.

14. Denature each slide by placing it coverslip-side up on the top of the submerged anodized aluminum block in the 92°C water bath for 2.5 min.

    Take care not to let the slides sit on the block for longer than 2.5 min, as the sample may begin to be destroyed by extended high-temperature treatment. If the size of the water bath and the number of aluminum blocks available permits, several samples can be denatured in parallel.

Hybridization

15. Transfer the denatured slide to a humidified hybridization chamber and allow the hybridization reaction to occur overnight (>14 hr) at 42°C in a heated incubator.

    A humidified hybridization chamber can be assembled from a plastic chamber with a lid (e.g., a plastic Tupperware container or an empty pipet tips box), damp paper towels,

and a plastic rest for the slides (to keep them from sitting directly on the damp paper towels), such as a reagent reservoir or few pieces of a cut serological pipet. The chamber does not need to be airtight and preferably will have the lid only loosely attached, to allow for air exchange when placed in the heated incubator.

Perform washes and mounting

16. The next day, add 50 ml of 2× SSCT to a Coplin jar. Place the Coplin jar in a water bath and heat to 60°C.

    If the anti-fade mounting medium is stored at −20°C, remove it from the freezer at this point and allow it to warm to room temperature.

17. Carefully remove the rubber cement and coverslips from the sample slides and transfer to the Coplin jar containing 2× SSCT in the 60°C water bath. Incubate for 15 min at 60°C.

    The coverslip can often by removed by gently sliding it along the surface of the slide towards the edge using a gloved finger. If the coverslip does not move readily, remove the rubber cement with fine forceps while applying gentle pressure to the center of the coverslip. In both methods, it is essential to avoid violently prying the coverslip upwards (this often occurs when just one edge is freed from rubber cement but its parallel edge is still covered), as this will result in the stretching/shearing of chromosomal DNA.

18. Transfer the slides to a Coplin jar containing 50 ml of 2× SSCT and incubate for 10 min at room temperature.

    The Coplin jar can be covered with foil or placed in a drawer to protect the samples from light.

19. Transfer the slides to a Coplin jar containing 50 ml of 0.2× SSC and incubate for 10 min at room temperature.

    The Coplin jar can be covered with foil or placed in a drawer to protect the samples from light.

20. Remove the slides from the 0.2× SSC Coplin jar and partially dry the slides by tapping the thin edge of the slides against a paper towel, and then placing the slides into an empty Coplin jar.

21. Place a 30 × 22–mm coverslip onto a dry paper towel and pipet 12.5 μl of anti-fade mounting medium containing DAPI onto the center of the coverslip. Take care to avoid creating air bubbles.

22. Gently invert the sample slide onto the coverslip such that the sample (typically visible as a cloudy circle) is placed in direct contact with the mounting medium. Press down gently but firmly to eject excess mounting medium.

    The coverslip should be immobile to the touch, being held in place by a thin monolayer of mounting medium. If the coverslip is still loose, move the inverted slide + coverslip to another spot on the paper towel and repeat. Take care not to press too firmly, as the slide may break.

23. Turn the slide over so the coverslip is on the upward-facing side of the slide. Seal the coverslip onto the slide by adding a layer of nail polish around the edges of the coverslip.

24. Allow at least 30 min for the nail polish to dry before imaging the slides.

    The FISH can be visualized using an epifluorescent or confocal microscope.

ALTERNATE PROTOCOL 1

FAST 3D-FISH USING OLIGOPAINT PROBES

This section presents a relatively quick 3D-FISH (Lanzuolo et al., 2007; Cremer et al., 2008) protocol that avoids using high-temperature treatments in order to better preserve the nuclear morphology of samples. This protocol is similar to Basic Protocol 2, but contains additional prehybridization steps to improve sample permeability.

Additional Materials *(also see Basic Protocol 2)*

1× PBS (see recipe)
1× PBST (see recipe)
1× PBS + 0.5% (v/v) Triton-X100
0.1 N HCl (625 μl of 8 N HCl in 50 ml ddH_2O)

Prehybridization treatment

1. Heat one water bath to 60°C and the other to 78°C. Place a Coplin jar containing 50 ml of 2× SSCT + 50% formamide (Basic Protocol 2, steps 1 and 2) into the 60°C bath, place an anodized aluminum block into the 78°C bath, and adjust the water level such that all but ~1 cm of the block is submerged. Allow the sample slides to warm from 4°C to room temperature while the water baths heat up.

   Unless otherwise indicated, perform all of the following incubations at room temperature.

2. Transfer the sample slides using forceps to a Coplin jar containing 50 ml of 1× PBS and incubate for 1 min.
3. Transfer the sample slides to a Coplin jar containing 50 ml of 1× PBST and incubate for 1 min.
4. Transfer the sample slides to a Coplin jar containing 50 ml of 1× PBS + 0.5% (v/v) Triton-X100 (250 μl in 50 ml) and incubate for 10 min.
5. Transfer the sample slides to a Coplin jar containing 50 ml of 1× PBST and incubate for 2 min.
6. Transfer the sample slides to a Coplin jar containing 50 ml of 0.1 N HCl and incubate for 5 min.
7. Transfer the sample slides to a Coplin jar containing 50 ml of 2× SSCT and incubate for 1 min.
8. Transfer the sample slides to a Coplin jar containing 50 ml of 2× SSCT and incubate for 2 min.
9. Transfer the sample slides to a Coplin jar containing 50 ml of 2× SSCT and incubate for 2 min.
10. Transfer the sample slides to a Coplin jar containing 50 ml of 2× SSCT + 50% formamide and incubate for 5 min.
11. Transfer the sample slides to the Coplin jar containing 50 ml of 2× SSCT + 50% formamide in the 60°C water bath and incubate for 20 min.

Denature, hybridize, perform washes, and mount

12. Follow steps 6 to 24 of Basic Protocol 2 exactly as written, except denaturing at 78°C for 2.5 min in step 14.

METAPHASE FISH WITH OLIGOPAINT PROBES

ALTERNATE PROTOCOL 2

Metaphase FISH with Oligopaint probes calls for a slightly modified protocol that uses different denaturation conditions and includes a progressive ethanol dehydration prior to probe addition.

Additional Materials *(also see Basic Protocols 1 and 2)*

Sample slide containing spread mitotic chromosomes
70% (v/v) ethanol in ddH_2O
90% (v/v) ethanol in ddH_2O
100% (v/v) ethanol

1. Place a Coplin jar containing 50 ml of 2× SSCT + 70% (v/v) formamide in a water bath and warm to 70°C.

   50 ml of 2× SSCT + 70% (v/v) formamide can be made by combining 35 ml formamide, 12.5 ml 8× SSCT (see recipe), and 2.5 ml ddH_2O.

2. While the water bath is heating up, allow the sample slides to warm to room temperature.
3. Denature the samples by incubating them in the Coplin jar containing 2× SSCT + 70% formamide for 1.5 min at 70°C.
4. Transfer the samples to a Coplin jar containing ice-cold 70% ethanol and incubate for 5 min at room temperature.
5. Transfer the samples to a Coplin jar containing ice-cold 90% ethanol and incubate for 5 min at room temperature.
6. Transfer the samples to a Coplin jar containing ice-cold 100% ethanol and incubate for 5 min at room temperature.
7. Air dry the samples face up on a paper towel at room temperature (1 to 2 min).

   The sample slides can be placed inside a box or drawer to protect them from floating dust.

8. Prepare hybridization mix as directed in steps 6 to 8 of Basic Protocol 2.
9. Add the hybridization mix to the sample slides and seal with rubber cement as directed in steps 10 to 12 of Basic Protocol 2.
10. Transfer the sample slides to a humidified hybridization chamber and allow the hybridization reaction to occur overnight (>14 hr) at 37°C in a heated incubator.
11. Follow steps 16 to 24 of Basic Protocol 2.

PREPARING TISSUE CULTURE CELLS FOR INTERPHASE FISH

SUPPORT PROTOCOL

Tissue culture cells provide a convenient substrate for interphase FISH. This section describes a standard protocol for creating FISH sample slides from a suspension of adherent or semi-adherent cells.

Additional Materials *(also see Basic Protocols 1 and 2)*

0.01% (v/v) poly-L-lysine solution in ddH_2O (5 ml of 0.1% stock + 45 ml ddH_2O)
1×10^5–1×10^6 cells/ml cell suspension
Complete growth medium
1× PBS + 4% (v/v) paraformaldehyde (see recipe)

Lint-free paper towels (e.g., Kimwipes)

25 × 75 × 1–mm glass microscope slides
Plastic Coplin staining jar
Cell culture incubator
Micropipets

NOTE: All steps are performed at room temperature unless otherwise indicated.

Pretreat the slides

1. Use a lint-free paper towel soaked in 100% ethanol to clean each slide.

   This step is recommended for all slides, even those advertised as "precleaned" by their manufacturer, and is intended to remove microscopic dust and debris from the slides.

2. Allow the slides to air dry.

3. Prepare 50 ml of 0.01% (v/v) poly-L-lysine solution in ddH_2O and add to a plastic Coplin jar.

   A glass Coplin jar can be used here, but its inside will be coated by the poly-L-lysine solution as well.

4. Allow the slides to air dry.

   This step can be sped up using the airflow of a tissue culture hood.

Attach the cells to the slides

5. Prepare a suspension of 1×10^5–1×10^6 cells/ml in complete growth medium.

   This density works for most cell lines but can be adjusted if necessary.

6. Use a micropipet to place 100 µl of cell suspension roughly in the center of a poly-L-lysine-treated slide.

7. Allow the cells to adhere for 1.5 to 3 hr in a cell culture incubator.

   Large petri dishes and metal trays covered with aluminum foil are convenient vessels to hold the slides. If working with mammalian cells, be sure to allow for gas exchange between the slides and the atmosphere of the incubator.

Perform fixation and washes

8. Transfer the slides to a Coplin jar containing 50 ml of 1× PBS and incubate for 1 min.

9. Transfer the slides to a Coplin jar containing 40 ml of 1× PBS + 4% (v/v) paraformaldehyde and incubate for 10 min.

10. Transfer the slides to a Coplin jar containing 50 ml of 1× PBS and incubate for 1 min.

11. Transfer the slides to a Coplin jar containing 50 ml of 2× SSCT and incubate for 5 min.

12. Transfer the slides to a Coplin jar containing 50 ml of 2× SSCT + 50% formamide (25 ml 4× SSCT + 25 ml formamide) and incubate for 5 min.

13. Transfer the slides to a Coplin jar containing 50 ml of 2× SSCT + 50% formamide and transfer to 4°C for storage.

    Slides should be used within 2 weeks of creation. Screw-top Coplin jars are convenient for storing slides; paraffin film can be used to seal the top of standard Coplin jars if screw-top jars are not available.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****. Note ddH_2O refers to deionized, distilled water.*

Hybridization cocktail, 2×

For 10 ml, combine the following:
4 ml of 50% (w/v) dextran sulfate solution (in ddH_2O)
2 ml 20× SSC
4 ml ddH_2O
Store up to 1 year at room temperature

PBS, 10×

To prepare 1 liter stock solution, dissolve 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, and 2.4 g KH_2PO_4 in 800 ml ddH_2O. Adjust the pH to 7.4 using HCl, and then add ddH_2O to 1 liter. Store up to 1 year at room temperature.

PBS (1×) + 4% (v/v) paraformaldehyde

For 40 ml, combine the following:
4 ml of 10× PBS (see recipe)
26 ml ddH_2O
10 ml of 16% (w/v) paraformaldehyde solution (made with ddH_2O)
Prepare fresh

PBST, 1×

For 1 liter, combine the following:
100 ml of 10× PBS (see recipe)
899 ml ddH_2O
1 ml Tween-20
Store up to 1 year at room temperature

Phosphate-buffered saline (PBS), 1×

Dilute 10× PBS (see recipe) 1:10 in ddH_2O
Store up to 1 year at room temperature

SSC, 0.2×

For 1 liter, combine the following:
20 ml of 20× SSC (see recipe)
980 ml ddH_2O
Store up to 1 year at room temperature

SSC, 20×

For 1 liter, dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of ddH_2O. Adjust the pH to 7.0 with 1 M HCl, and then add ddH_2O to 1 liter. Store up to 1 year at room temperature.

SSCT, 2×

For 1 liter, combine the following:
200 ml 20× SSC (see recipe)
799 ml ddH_2O
1 ml Tween 20
Store up to 1 year at room temperature

SSCT, 4×

For 1 liter, combine the following:
400 ml of 20× SSC (see recipe)
598 ml ddH_2O
2 ml Tween 20
Store up to 1 year at room temperature

SSCT, 8×

For 1 liter, combine the following:
800 ml of 20× SSC (see recipe)
196 ml ddH_2O
4 ml Tween 20
Store up to 1 year at room temperature

TBE, 1×

To prepare 1 liter of a 5× stock solution, dissolve 54 g Tris base, 27.5 g boric acid, and 20 ml 0.5 M ethylenediaminetetraacetic acid (EDTA), pH 8.0, in ddH_2O. Dilute the 5× stock 1:5 in ddH_2O to make 1×. Store up to 1 year at room temperature.

COMMENTARY

Background Information

FISH is an adaptation of a nucleic acid in situ hybridization method that was developed by Pardue and Gall (1969) and used radiolabeled probes (Bauman et al., 1980; Pinkel et al., 1986; reviewed in Levsky and Singer, 2003; Volpi and Bridger, 2008; Itzkovitz and van Oudenaarden, 2011). It is a powerful tool for studying chromosome structure and position, as well as gene expression, as it can be used to interrogate both DNA and RNA molecules. It is informative at the single-cell level and can be combined with other single-cell imaging methods, such as the immunofluorescent detection of proteins.

FISH probes have typically been derived from flow-sorted chromosomes or genomic inserts subcloned into vectors, such as plasmids, cosmids, and BACs. These chromosomes and inserts can be labeled via the incorporation of fluorophore- or hapten-conjugated dNTPs in enzymatic nick-translation or PCR reactions, producing ~100 bp dsDNA (Lichter et al., 1988). Because the starting material can contain sequences that are highly repeated in the genome, the resulting probes often need to be used in the presence of unlabeled repetitive DNA so as to minimize background signal due to off-target hybridization (Landegent et al., 1987).

Synthetic nucleic acid oligos, including DNA, RNA, peptide nucleic acid (PNA), and locked nucleic acid (LNA) oligos, have also been used as FISH probes (Larsson et al., 1988; Landsdorp et al., 1996; O'Keefe et al., 1996; Silahtaroglu et al., 2003). Such oligo probes have typically been used to detect RNA molecules (Femino et al., 1998; Player et al., 2001; Raj et al., 2008) or to visualize DNA targets that facilitate signal detection because they are internally repetitive (Dernburg et al., 1996). In these situations, the number of oligo species in a probe has hovered in the range of ~1 to 50.

Excitingly, recent technological advances permitting the parallel synthesis of hundreds to hundreds of thousands of oligo species have enabled several new methods for generating oligo-based FISH probes. One family of these methods uses synthetic oligos containing genomic sequence as PCR primers to amplify fragments of genomic sequence in parallel reactions (Martinez et al., 2006; Navin et al., 2006; Lamb et al., 2007; Bienko et al., 2013). Other methods encode the entirety of the genomic region to be targeted in a library of oligos and, therefore, permit the pool of oligos to be used directly as a FISH probe, if fluorescent label is added during synthesis of the library (Boyle et al., 2011), or, if the genomic sequences are flanked by primers, enable the pool to become a renewable resource that can be amplified in the presence of fluorescent label to generate dsDNA (Yamada et al., 2011; Beliveau et al., 2012) or ssDNA (Beliveau et al., 2012) probes. Note that the use of primers that have been synthesized to contain precisely positioned fluorophores (e.g., 5′ labeled primers) will produce probes with uniform specific activities of fluorescence (Beliveau et al., 2012).

The strategy of Beliveau et al. (2012), which produces Oligopaints, is compatible with many standard molecular biological protocols and can be used to generate a variety of probe structures (see Fig. S12 in Beliveau et al., 2012). In the current unit, we detail the version that produces probes that are short (e.g., 59 bases) and single-stranded, features that likely enhance the diffusion of probes into samples, as well as increase the efficiency of hybridization. The strand-specific nature of Oligopaints will also aid in the design of experiments that target RNA molecules or must distinguish one strand of DNA from the other.

Critical Parameters

Probe design for DNA FISH

In general, targeting genomic regions with >500 to 1000 probes, where each oligo carries one fluorophore, is sufficient to produce a strong signal. The number of oligo species required to produce a robust FISH signal may, however, vary depending on the genomic target and the type of sample (e.g., cell culture or tissue sample) being interrogated. If the region of interest (e.g., a gene body) is too small to accommodate the desired number of probe oligos, it may be useful to extend the target into flanking sequences. In cases where extending the target is not desirable, it may be necessary to consider methods for amplifying signals, such as placing a hapten (e.g., biotin or digoxigenin) on the primer and then visualizing the FISH using antibodies that target the hapten.

Probe design for RNA FISH

The design of probes for RNA FISH may vary greatly depending on the target. In general, 20 to 50 oligos targeting exons have been sufficient to illuminate mRNA molecules (Raj et al., 2008), while ∽20 oligos targeting introns have been used to illuminate nascent mRNA molecules at their site of transcription (Levesque and Raj, 2013). In either case, particular attention should be paid to the strand-specificity of the probe sequences to ensure that the final ssDNA probe molecules are antisense to the target RNA. Additionally, if the RNA FISH is to be combined with DNA FISH, the probe for DNA FISH can be designed such that there is a gap where the targeted RNA is transcribed, thus allowing the RNA and DNA FISH probes to be used simultaneously (Beliveau et al., 2012). Readers interested in probe design for RNA FISH are encouraged to read the discussion of this topic in Itzkovitz and van Oudenaarden (2011).

Reagent quality

The generation of Oligopaint probes using the method outlined in this unit relies heavily on two enzymatic steps—PCR and digestion with a nicking endonuclease. It is important to monitor the quality of the reagents used in these steps, as one bad tube of enzyme, buffer, or stock of molecular biology-grade water can destroy an entire preparation. Some general guidelines for handling these reagents are listed below:

- Take care to store enzymes at the temperature recommended by their manufacturer (typically −20°C) and always store the enzymes in a cooler/caddy when they are not in a freezer.
- Avoid repeated freeze/thaw cycles. Using a laboratory-grade marker, place a dot on the top of the tube each time it has been thawed to keep track of the number of times a tube has been frozen and thawed. Divide into larger stocks if necessary.
- Only use molecular biology-grade water in enzymatic reactions; make 15-ml aliquots by decanting from the stock bottle into conical tubes in a tissue culture or PCR hood to avoid having debris fall into the stock bottle. Date each aliquot and use within 2 weeks of opening for the first time.

Physical mixing of reactions

Our protocol for generating Oligopaints requires that commonly used molecular biological techniques be performed at scales that may be significantly larger than is customary for many users. As such, we emphasize the importance of vortexing large master mixes (e.g., for PCR, DNA precipitation, nicking endonuclease digestion) sufficiently to ensure homogeneity. In our hands, master mixes containing thermostable DNA polymerase and the Nb.BsrDI and Nb.BsmI nicking enzymes are robust to repeated (e.g., 3 to 5 times) 10 to 15 second full-speed vortex pulses.

Running gels under denaturing conditions

It is critical to run the denaturing gel at a temperature that will maintain separation of the desired labeled ssDNA probe molecules from their complement (∼55° to 60°C, for the conditions described above). Note that a given probe molecule will have partial complementarity to many DNA molecules after denaturation by virtue of the retained primer sequence; thus, partial reannealing may occur even between molecules that have heterologous genomic sequence inserts. If the gel is not

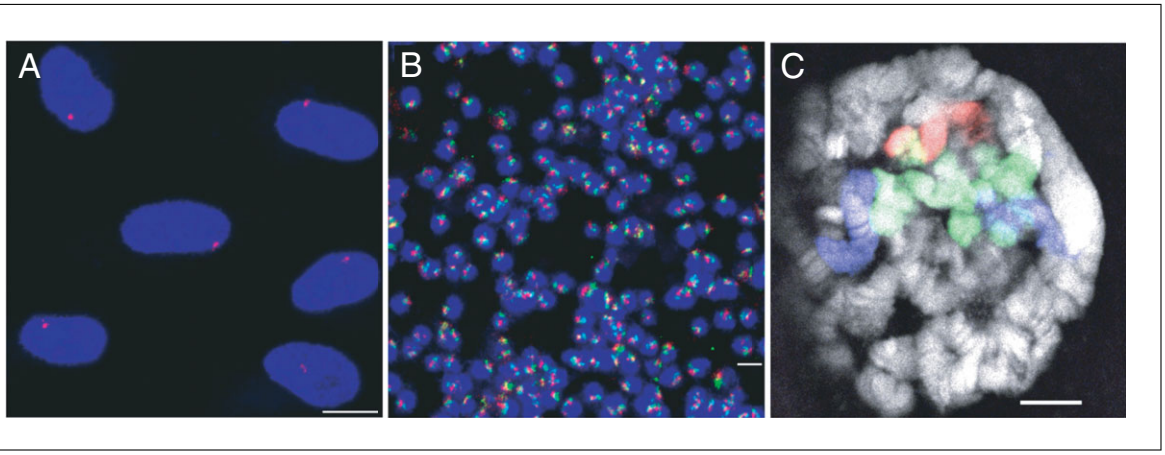

Figure 14.23.2 (**A**) FISH using an Oligopaint probe composed of 20,020 oligos targeting a 2.5-Mb region on human chromosome X (Xq13.1) in diploid (XY) MRC-5 cells. Hybridization was carried out according to Basic Protocol 2 in a 25 μl volume using 35 pmol of the probe (Cy3, red) produced using Basic Protocol 1. DNA is stained with DAPI (blue). (**B**) Two-color FISH on a field of tetraploid Drosophila Kc_{167} cells using two Oligopaint probes targeting the right arm of chromosome 2: one composed of 25,000 oligos targeting a 2.7-Mb region (50D1-53D7), and another composed of 50,000 oligos—half targeting a 3-Mb region (41E3-44C4) and the other half targeting a 2.6-Mb region (58D2-60D14). The hybridization was carried out according to Basic Protocol 2 in a 25 μl volume using 5 pmol of the first probe (Cy3, red) and 2.5 pmol of the second probe (Cy5, green), both produced using Basic Protocol 1. (**C**) Three-color FISH performed on *Drosophila* salivary polytene chromosomes with the two Oligopaint probes used in (B) plus an additional Oligopaint probe that also targets the right arm of chromosome 2 and is composed of 105,000 oligos—half targeting a 5.6-Mb region (44C4-50C9) and the other half targeting a 5.5-Mb region (53C9-58B6). The hybridization was carried out according to Beliveau et al. (2012) in a 25 μl volume using 20 pmol of each of the three probes labeled with TYE563 (red), TYE665 (blue), and 6-FAM (green), respectively, all produced using Basic Protocol 1. DNA is stained with DAPI (gray). Images represent confocal maximum *Z* projections (A, B) or a single confocal *XY* plane (C). Scale bars: 10 μM. For the color version of this figure, go to *http://www.currentprotocols.com/protocol/mb1423*.

sufficiently hot, slow-migrating smears may be present above both the band of the desired ssDNA product and the band of the full length uncut strands. Note that a labeled ssDNA fragment can still be successfully purified if it has migrated far enough from the smeared DNA to allow for its unambiguous isolation.

Probe concentration

While Oligopaint probes tend to work robustly at oligo concentrations of 800 nM or higher (20+ pmol per 25-μl hybridization reaction) for tissue culture cells, users are encouraged to determine the optimal concentration for their specific needs via a concentration curve. For example, increasing the concentration of probe in the hybridization reaction may be necessary should an 800 nM concentration of Oligopaint probes be insufficient to produce a strong signal. As for tissue samples, increasing the concentration of probe by up to 10-fold or more may be necessary, with the optimal concentration likely to vary depending on the sample type and target.

Conditions for hybridization and washes

The hybridization and wash conditions can be altered to optimize the performance of the FISH probes. Both hybridization temperature and wash conditions can impact the signal-to-noise ratio of the FISH (see Figs. S6 and S8 in Beliveau et al., 2012). For example, higher hybridization temperatures or higher temperatures in the first wash may reduce the background signal. The length of the hybridization step can also be important; >14 hr is recommended, and >24 hr can be tried for probes that do not produce strong signals after 14 hr. The signal-to-noise ratio of the FISH can also be adjusted by increasing the number of high-temperature wash steps (e.g., 2× 15 min, 60°C 2× SSCT), increasing the temperature of the first wash (e.g., 70°C SSCT), or adding formamide to the first wash step (e.g., instead 15 min, 37°C 2× SSCT + 50% (v/v) formamide instead of 15 min, 60°C 2× SSCT).

Sample permeability for FISH

Some samples may require permeabilization steps beyond the prehybridization treatments included in Basic Protocol 2. Common options include incubation in detergents such as Tween-20 or Triton X-100, treatment with proteases such as pepsin and proteinase K, treatment with HCl, flash-freezes in liquid nitrogen, and fixation in 3:1 (v/v)

methanol:acetic acid instead of paraformaldehyde (Lanzuolo et al., 2007; Cremer et al., 2008). For samples that do not work readily in Basic Protocol 2, users can try performing steps 1 to 11 of Alternate Protocol 1 and then following steps 6 to 24 of Basic Protocol 2.

Anticipated Results

A successful Oligopaint probe preparation typically converts 20% to 30% of the labeled primer into purified ssDNA FISH probe. This yield should be reasonably consistent across different preparations of the same Oligopaint probe, as well as preparations of distinct Oligopaint probes, and, in our hands, appears to be largely insensitive to the specific fluorophores we have used (Alexa405, 6-FAM, Alexa488, atto550, Cy3, TYE563, atto565, Cy5, atto647N, Alexa647, TYE665, Cy5.5).

FISH performed according to Basic Protocol 2 with a probe complexity of 1000 oligos or more and a total oligo concentration of 800 nM should produce a signal in the nucleus of >90% and often close to 100% of tissue culture cells. FISH efficiency may vary depending on the genomic target, the complexity and concentration of the probe, and the nature of the sample.

Figure 14.23.2 shows some examples of FISH using Oligopaint probes. Interphase FISH is depicted in panels A and B, in human and *Drosophila* cells, respectively. In both cases, the FISH probe produces strong, focal fluorescent signals with little to no background. The specificity of Oligopaint probes is highlighted in panel C, where three probes are used to paint an arm of a poltytenized *Drosophila* chromosome in a blue-green-red-green-blue pattern.

Time Considerations

Oligopaint probe preparations can be completed in as few as 3 days or spread over the course of a week.

Preparing sample slides according to Alternate Protocol 1 takes ~3 to 4 hr.

FISH with Oligopaint probes takes ~4 hr spread over 2 days: 2 hr on the first day to perform the prehybridization and denaturation steps and set up the overnight hybridization, and 2 hr on the following day to perform the wash steps, mount the slides, and perform microscopy.

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Key Reference

Beliveau et al., 2012. See above.

Introduces the Oligopaint FISH method and demonstrates its efficacy in tissue culture cells and tissue specimens.

Internet Resources

http://genetics.med.harvard.edu/oligopaints/

The Oligopaints Web site contains additional protocols, information about the location of probe sequences in several eukaryotic organisms, and scripts + documentation to assist users in the computational design of Oligopaint probes.