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FOLD-FISH step-by-step protocol

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We use this protocol and it's working

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Abstract

DNA fluorescence in situ hybridization (FISH) methods based on the use of oligonucleotide (oligo) probes have emerged as powerful tools to study genome architecture in single cells. However, their application to formalin-fixed paraffin-embedded (FFPE) tissues—the clinical gold standard for histopathology—remains challenging. Here, we develop and optimize FFPE OLigo-based DNA FISH (FOLD-FISH), a robust protocol for oligo-based DNA FISH on FFPE tissue samples. We demonstrate FOLD-FISH technical performance across diverse human tumor samples and genomic targets and show that it enables robust quantification of selected DNA loci copy number, in both surgical resections and core biopsies, even at low magnification (20× air objectives). To facilitate the adoption of FOLD-FISH in cancer research and diagnostics, we designed probes targeting 1,026 genes included in the OncoKB and COSMIC databases, which can be readily used to detect genes commonly assayed in diagnostics. Finally, we leveraged FOLD-FISH chromosome-spotting probes to visualize and quantitatively assess chromosome 17 territories in two breast cancer tissues, setting the stage for an atlas of chromosomal territories in tumor samples. FOLD-FISH is a versatile protocol for oligo-based DNA FISH in FFPE samples, with broad applications in pathological diagnostics, intratumor heterogeneity characterization, and 3D genome profiling in clinical samples.

Materials

REAGENTS:

Note

Reagents are ordered based on their first appearance in the ‘Procedure’ section.

Pre-hybridization treatment:

- Nuclease-Free Water **Thermo Fisher Scientific Catalog #**AM9932
- Xylenes **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**534056
- Ethanol absolute ≥99.8% **VWR International (Avantor) Catalog #**20821.365
- Ethanol ≥96% (v/v), TechniSolv®, pure **VWR International (Avantor) Catalog #**83804.360
- Ethanol ≥70% (v/v), TechniSolv® **VWR International (Avantor) Catalog #**83801.290
- Tween 20 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9416
- 10% Sodium dodecyl sulfate solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**05030
- EDTA (0.5 M), pH 8.0, RNase-free **Thermo Fisher Catalog #**AM9260G
- SSC (20X), RNase-free **Thermo Fisher Catalog #**AM9765
- Glycerol, 99+% **Fisher Scientific Catalog #**AAA16205AP
- 1M Citrate Buffer, pH 6.0, 10×, Antigen Retriever **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**C9999
- 1M Trizma® hydrochloride solution pH 9.0 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T2819
- 1M DL-Dithiothreitol solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**43816
- PBS - Phosphate-Buffered Saline (10X) pH 7.4, RNase-free **Thermo Fisher Catalog #**AM9625
- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T8787
- Hydrochloric acid (HCl) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**H1758
- Pepsin from porcine gastric mucosa **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P6887
- Sodium phosphate, 0.5 M buffer solution, pH 7.6 (Thermo Fisher Scientific. cat. no. J60158.AP)
- Abnova™ Collagenase Type 3 (C. histolyticum) **Fisher Scientific Catalog #**16111810
- Methanol ≥99.6% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**179957
- Formamide (Deionized) **Thermo Fisher Catalog #**AM9344

- Acetic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A6283
- PARAFORMALDEHYDE 16% Aqueous SOL. EM GRADE **Electron Microscopy Sciences Catalog #**15710
- Glycine **Fisher Scientific Catalog #**BP381-500
- Fixogum (Leica, Cat. no. LK071)

Probe hybridization:

- Denhardt's Solution (50X) **Thermo Fisher Catalog #**750018
- Dextran sulfate sodium salt from Leuconostoc spp. **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D8906
- Ribonucleic acid, transfer from Escherichia coli **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**R1753
- UltraPure™ Salmon Sperm DNA Solution, 10 mg/mL **Thermo Fisher Scientific Catalog #**15632011
- UltraPure 50 mg/mL BSA (bovine serum albumin) **Thermo Fisher Scientific Catalog #**AM2618

Mounting and imaging:

- Hoechst 33342 Solution (20 mM) **Thermo Fisher Catalog #**62249
- ProLong™ Diamond Antifade Mountant **Invitrogen - Thermo Fisher Catalog #**P36961

Consumables:

- Eppendorf® DNA LoBind tubes, 5 ml **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**EP0030108310
- Eppendorf® DNA LoBind tubes, 1.5 ml **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**EP0030108051
- Eppendorf® DNA LoBind tubes, 0.5 ml **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**EP0030108035
- Corning™ Centrifuge Tubes with CentriStar™ Cap **Fisher Scientific Catalog #**430790
- Falcon™ Round Bottom Polystyrene Test Tubes **Fisher Scientific Catalog #**10100151
- VWR®, Colour-Frosted Microscope Slides **VWR International (Avantor) Catalog #**631-0093
- VWR® Cover Glasses, Square **VWR International (Avantor) Catalog #**630-2186
- PAP pen **Abcam Catalog #**ab2601
- Sealing film for manual application, Parafilm M (VWR, Cat. no. 291-0057)

Equipment:

- Tissue staining jars (Electron Microscopy Sciences, Cat. No. 71385-Y)

Equipment

Slide Staining Jar, Yellow	NAME
Staining jar	TYPE
EasyDip™	BRAND
71385-Y	SKU
https://www.emsdiasum.com/easydip-slide-staining-jar-yellow	LINK

- Boekel Scientific 280001 12 Position Programmable Slide Moat 115V (Marshall Scientific, Cat. no. 280001 or equivalent)
- Boekel Inslide Out Hybridization Oven (Boekel Scientific 241000) or equivalent
- Water Bath (Precision General Purpose Water Bath, Precision GP 10)
- Centrifuge (Eppendorf Microcentrifuge 5424 and 5810 or equivalent)
- Sample mixer (Thermo Scientific Tube Revolver Rotator or equivalent)

Preparation of solutions

1 85% (v/v) Ethanol solution

- Mix 170 mL of absolute Ethanol into 30 mL of nuclease-free water.

2 70% (v/v) Ethanol solution

- Mix 140 mL of absolute Ethanol into 70 mL of nuclease-free water.

3 50% (v/v) Ethanol solution

- Mix 100 mL of absolute Ethanol into 100 mL of nuclease-free water.

4 20% (v/v) Tween 20 solution

- Mix 20 mL of Tween 20 solution into 80 mL of nuclease-free water.

5 Pre-hybridization solution A (PHA): 0.1x SSC, 20% glycerol pH 7 , pH 5 .

- Mix 0.75 mL of 20x SCC solution into 119.25 mL of nuclease-free water. Then slowly add 30 mL of 100% glycerol.

6 Pre-hybridization solution B (PHB): [M] 0.01 Mass Percent Sodium citrate buffer, 0.05% Tween 20

- Mix 1.5 mL of [M] 1 Mass Percent sodium citrate buffer and 0.375 mL of 20% Tween 20 solution into 148.125 mL of nuclease-free water.

7 Pre-hybridization solution C (PHC): [M] 100 millimolar (mM) Tris-Cl pH 9 , 0.2% SDS, [M] 1 millimolar (mM) EDTA, [M] 10 millimolar (mM) DTT

- Mix 15 mL of [M] 1 Mass Percent Tris-HCl (pH 9) solution, 3 mL of 10% SDS solution and 0.3 mL of [M] 0.5 Mass Percent EDTA solution into 130.2 mL of nuclease-free water. Add 1.5 mL of [M] 1 Mass Percent DTT solution right before use.

8 20% (v/v) Triton X-100 solution

- Mix 20 mL of Triton X-100 solution into 80 mL of nuclease-free water.

9 1x PBS solution

- Mix 100 mL of 10x PBS solution into 900 mL of nuclease-free water.

10 1x PBS, 0.05% Triton X-100 solution

- Mix 100 mL of 10x PBS solution and 2.5 mL of 20% Triton X-100 solution into 897.5 mL of nuclease-free water.

11 1x PBS, 0.5% Triton X-100 solution

- Mix 100 mL of 10x PBS solution and 25 mL of 20% Triton X-100 solution into 875 mL of nuclease-free water.

12 [M] 0.1 Mass Percent HCl solution

- Mix 50 mL of 10x PBS solution and 4.1 mL of 38% HCL into 445.9 mL of nuclease-free water.

13 Pepsin stock solution (2 µL)

- Dissolved 2 mg of pepsin into 1 mL of nuclease-free water.

14 Wash solution A (WSA): 0.1x SSC

- Mix 2.5 mL of 20x SSC solution into 497.5 mL of nuclease-free water.

15 Collagenase III stock solution (10 µL)

- Dilute 100 mg of collagenase III into 10 mL of nuclease-free water.

16 FA50 solution: 2x SSC, [M] 50 millimolar (mM) sodium phosphate buffer (pH 7.6), 50% formamide

- Mix 15 mL of 20x SSC solution and 15 mL of [M] 0.5 Mass Percent sodium phosphate buffer (pH 7.6) into 45 mL of nuclease-free water. Add 75 mL of 100% formamide solution under a chemical hood.

17 Pre-hybridization solution D (PHD): 1x PBS, [M] 100 millimolar (mM) Tris-Cl (pH 9)

- Mix 15 mL of 10x PBS solution and 15 mL of [M] 1 Mass Percent Tris-Cl (pH 9) solution into 120 mL of nuclease-free water.

18 Methanol/acetic acid (MAA) solution

- Mix 112.5 mL of Methanol and 37.5 mL of Acetic Acid.

19 Post-fixation solution (PFS): 1x PBS, 1% paraformaldehyde

- Mix 15 mL of 10x PBS into 125.625 mL of nuclease-free water, then add 9.375 mL of 16% PFA solution under a chemical hood.

20 [M] 1.25 Mass Percent glycine solution

- Dissolved 93 g , 73 g of glycine into 1 L of nuclease-free water and sterilize the solution with 0.22 µm pore size membrane filter.

21 PFA quenching solution: 1x PBS, [M] 125 millimolar (mM) glycine

- Mix 15 mL of 10x PBS solution and 15 mL of [M] 1.25 Mass Percent glycine solution into 120 mL of nuclease-free water.

22 Primary hybridization solution 1 (PHS-1): 5x Denhardt's solution, 2x SSC, [M] 50 millimolar (mM) sodium phosphate buffer, [M] 1 millimolar (mM) EDTA, 100 µL UltraPure Salmon Sperm DNA Solution, 50% formamide

- Mix the following reagents in the indicated order:

1. 1 mL of 50x Denhardt's solution
2. 1 mL of 20x SSC solution
3. 1 mL of the [M] 0.5 Mass Percent sodium phosphate buffer
4. 20 µL of [M] 0.5 Mass Percent EDTA solution
5. 100 µL of 10 µL UltraPure Salmon Sperm DNA Solution
6. 5 mL of 100% formamide solution
7. Adjust the pH to pH 8.0 – pH 8.5 and bring the volume up to 10 mL with nuclease-free water.

23 Primary hybridization solution 2 (PHS-2): 5.55x Denhardt's solution, 2.22x SSC, [M] 55.5 millimolar (mM) sodium phosphate buffer, [M] 1.11 millimolar (mM) EDTA, 111 µL UltraPure Salmon Sperm DNA Solution, 11.1% (w/v) dextran sulfate, 55.5% formamide

- Mix the following reagents in the indicated order:

1. 1.11 mL of 50x Denhardt's solution
2. 1.11 mL of 20x SSC solution
3. 1.11 mL of [M] 0.5 Mass Percent sodium phosphate buffer
4. 22.2 µL of [M] 0.5 Mass Percent EDTA solution
5. 111 µL of 10 µL UltraPure Salmon Sperm DNA Solution
6. 1.1 g of dextran sulfate
7. 5.5 mL of 100% formamide solution
8. Adjust the pH to pH 8.0 – pH 8.5 and bring the volume up to 10 mL with nuclease-free water.

24 Wash solution B (WSB): 2x SSC, 0.2% Tween 20

- Mix 50 mL of 20x SSC solution and 5 mL of 20% Tween 20 solution into 445 mL of nuclease-free water.

25 Wash solution C (WSC): 0.2x SSC, 0.2% Tween 20

- Mix 5 mL of 20x SSC solution and 5 mL of 20% Tween 20 solution into 490 mL of nuclease-free water.

26 Wash solution D (WSD): 4x SSC, 0.2% Tween 20

- Mix 100 mL of 20x SSC solution and 5 mL of 20% Tween 20 solution into 395 mL of nuclease-free water.

27 Wash solution E (WSE): 2x SSC, 25% formamide

- Mix 15 mL of 20x SSC solution into 97.5 mL of nuclease-free water, then add 37.5 mL of 100% FA under a chemical hood.

28 Detection hybridization solution (DHS): 2x SSC, 1 µL *E.coli* tRNA, 0.02% BSA, 25% formamide and 10% (w/v) dextran sulfate

- Mix the following reagents in the indicated order:

1. 1 mL of 20x SSC solution
2. 500 µL of 20 µL *E.coli* tRNA
3. 40 µL of 50 µL UltraPure BSA
4. 1 g of dextran sulfate
5. 2.5 mL of 100% formamide solution
6. Adjust the pH to 8.0 – 8.5 and bring the volume up to 10 mL with nuclease-free water.

DAY 1: Tissue deparaffinization

1h 17m

29

Note

Unless otherwise specified, solutions are prepared and used at room temperature. Use maximum 6 slides per staining jar.

Note

CRITICAL: We recommend performing all the following steps in a fume hood, as xylene is classified as a hazardous chemical that causes health hazards. Except for step 30, all the other steps are performed using tissue staining jars (for example, Electron Microscopy Sciences, Cat. No. 71385-Y).

30 Bake the FFPE slides at 60 °C for 01:00:00 in an incubator (for example, Boekel Scientific 241000).

1h

31 Transfer the slides into a jar pre-filled with xylene solution.

32 Incubate the slides in xylene solution for 00:03:00 at Room temperature .

3m

33 Repeat steps 31–32, by transferring the slides into a new jar pre-filled with fresh xylene solution.

34 Transfer the slides into a jar pre-filled with 100% ethanol solution.

35 Incubate the slides in 100% ethanol solution for 00:03:00 at Room temperature .

3m

Note

CRITICAL: Before transferring the slides into the 100% ethanol solution, gently absorb any remaining xylene solution by gently placing the shortest side of each slide (opposite to the white, slide-handling side) against a piece of tissue paper. Do not touch the tissue section with the tissue.

36 Transfer the slides into a new jar pre-filled with fresh 100% ethanol solution.

37 Incubate the slides in 100% ethanol solution for 00:03:00 at Room temperature . 3m

38 Transfer the slides into a jar pre-filled with 85% ethanol solution.

39 Incubate the slides in 85% ethanol solution for 00:02:00 at Room temperature . 2m

40 Transfer the slides into a jar pre-filled with 70% ethanol solution.

41 Incubate the slides in 70% ethanol solution for 00:02:00 at Room temperature . 2m

42 Transfer the slides into a jar pre-filled with 50% ethanol solution.

43 Incubate the slides in 50% ethanol solution for 00:02:00 at Room temperature . 2m

44 Transfer the slides into a jar pre-filled with nuclease-free water.

45 Incubate the slides in nuclease-free water for 00:02:00 at Room temperature . 2m

DAY 1: Pre-hybridization treatment

10h 58m

46

Note

CRITICAL: Unless otherwise specified, the following steps are performed in tissue staining jars. Do not use more than 6 slides per jar.

47 Fill in three tissue staining jars with PHA, PHB, and PHC solution, respectively, and place them in a water bath at 90 °C (for PHA) and 80 °C (for PHB and PHC).

48 Transfer the slides from step 45 into the jar containing the PHA solution.

49 Incubate the slides in PHA solution for 00:10:00 at 90 °C.

10m

50 Transfer the slides into the jar containing the PHB solution.

51 Incubate the slides in PHB solution for 00:45:00 at 80 °C.

45m

52 Transfer the slides into the jar containing the PHC solution.

53 Incubate the slides in PHC solution for 00:20:00 at 80 °C.

20m

54 Transfer the jar containing the slides in PHC solution to Room temperature.

55 Let the PHC solution cool down over a period of 00:30:00 until it reaches Room temperature.

30m

56 Transfer the slides into a jar pre-filled with 1x PBS solution.

57 Quickly wash the slides in 1x PBS solution at Room temperature .

58 Transfer the slides into a jar pre-filled with 1x PBS, 0.05% Triton X-100 solution.

59 Place the jar with the slides in 1x PBS, 0.05% Triton X-100 solution on a shaking platform, gently shaking.

60 Incubate the slides in 1x PBS, 0.05% Triton X-100 solution for 00:01:00 at Room temperature . 1m

61 Repeat steps 58–60 twice, by transferring the slides into a new jar containing fresh 1x PBS, 0.05% Triton X-100 solution.

62 Transfer the slides into a jar pre-filled with 1x PBS, 0.5% Triton X-100 solution.

63 Incubate the slides in 1x PBS, 0.5% Triton X-100 solution for 00:20:00 at Room temperature . 20m

Note

CRITICAL: Do not shake the jar in this step.

64 Transfer the slides into a jar pre-filled with 1x PBS, 0.05% Triton X-100 solution.

65 Place the jar with the slides in 1x PBS, 0.05% Triton X-100 solution on a shaking platform, gently shaking.

66 Repeat steps 64–65 twice, each time transferring the slides into a new jar containing fresh 1x PBS, 0.5% Triton X-100 solution.

67 Transfer the slides into a jar pre-filled with [M] 0.1 Mass Percent HCl solution.

68 Quickly wash the slides in [M] 0.1 Mass Percent HCl solution at Room temperature .

69 Transfer the slides into a new jar pre-filled with fresh [M] 0.1 Mass Percent HCl solution.

70 Incubate the slides in [M] 0.1 Mass Percent HCl solution up to 00:05:00 at Room temperature . 5m

> Note
>
> **CRITICAL:** The total incubation time in HCl solution starting from step 68 until step 70 should not exceed 5 min in total.

71 Transfer the slides into a new jar pre-filled with 1x PBS, 0.05% Triton X-100 solution.

72 Quickly wash the slides in 1x PBS, 0.05% Triton X-100 solution at Room temperature .

73 Repeat steps 71–72 twice, each time transferring the slides into a new jar containing fresh 1x PBS, 0.05% Triton X-100 solution.

74 Transfer the slides into a jar pre-filled with 1x PBS solution.

75 Incubate the slides in 1x PBS solution for 00:05:00 at Room temperature . 5m

76 Transfer the slides from the jar containing 1x PBS solution onto the tray provided with the Boekel Inslide Out Hybridization Oven.

Note

CRITICAL: Avoid drying the slides by covering them with a sufficient volume of 1x PBS solution using a 3 mL Pasteur pipette.

77 Using a hydrophobic pen, mark the boundary of each tissue section and let the mark completely dry at Room temperature .

78 Prepare the pepsin working solution by diluting the pepsin stock solution in [M] 0.01 Mass Percent HCl solution at a final concentration of 0.05 µL . Prepare the following mix by adjusting the volumes depending on the number of slides:

	A	B
	Pepsin working solution	**Volume for 1 slide**
	Pepsin stock solution (2 mg/ml)	5 µL
	0.1 M HCL	20 µL
	Nuclease-free water	175 µL
	Total volume	**200 µL**

79 Pre-warm the pepsin working solution at 37 °C for 00:10:00 . 10m

80 Immediately dispense 200 µL of pre-warmed pepsin working solution onto each tissue section.

Note

CRITICAL: Make sure to completely cover the tissue sections.

81 Incubate the tissue sections covered by pepsin working solution for 00:10:00 at 37 °C in the Boekel Inslide Out Hybridization Oven. 10m

Note

CRITICAL: The incubation time should be optimized when working with a new tissue type.

82 Take the slides out of the Boekel Inslide Out Hybridization Oven and tap each slide on a piece of absorbent paper to remove any remaining traces of pepsin working solution.

83 Immediately place the slides in a staining jar pre-filled with WSA solution.

84 Incubate the slides in WSA solution for 00:01:00 at Room temperature . 1m

85 Repeat steps 83–84, by transferring the slides to a new jar containing fresh WSA solution.

86 Prepare the collagenase III working solution by diluting the collagenase III stock solution (10 µL) to a final concentration of 0.5 µL with nuclease-free water. Prepare the following mix by adjusting the volumes depending on the number of slides:

	A	B
	Collagenase III working solution	**Volume for 1 slide**
	Collagenase III stock solution (10 mg/mL)	10 µL
	Nuclease-free water	190 µL
	Total volume	**200 µL**

87 Transfer the slides from the jar containing the WSA solution (step 85) onto the tray provided with the Boekel Inslide Out Hybridization Oven..

88 Dispense 200 µL of collagenase III working solution onto each tissue section.

Note

CRITICAL: Use enough solution so that each tissue section is completely covered by liquid.

89 Incubate the tissue sections covered by collagenase III working solution for 00:20:00 at 37 °C in the Boekel Inslide Out Hybridization Oven. 20m

90 Take the slides out of the Boekel Inslide Out Hybridization Oven and tap each slide on a piece of absorbent paper to remove any remaining traces of collagenase III working solution.

91 Immediately place the slides in a staining jar pre-filled with WSA solution.

92 Incubate the slides in WSA solution for 00:01:00 at Room temperature. 1m

93 Repeat steps 91–92, by transferring the slides to a new jar containing fresh WSA solution.

94 Transfer the slides into a jar pre-filled with FA50 solution.

95 Quickly wash the slides in FA50 solution at Room temperature.

96 Transfer the slides into a new jar pre-filled with fresh FA50 solution and cover the jar with its glass lid.

97 Incubate the slides in FA50 solution with Overnight at Room temperature protected from light, by wrapping a piece of aluminum foil around the staining jar and placing it in a drawer or cabinet. 8h

Note

CRITICAL: We recommend keeping the jar containing the FA50 solution in a fume hood as formamide is classified as hazardous chemical causing carcinogenicity, reproductive toxicity and target organ toxicity following repeated exposure.

DAY 2

57m

98 Remove the slides from the FA50 solution (step 97) and transfer them into a jar pre-filled with PHD solution.

99 Incubate the slides in PHD solution for 00:10:00 at Room temperature . 10m

100 Transfer the slides into a jar pre-filled with fresh WSA solution.

101 Incubate the slides in WSA solution for 00:01:00 at Room temperature . 1m

102 Repeat steps 100–101, by transferring the slides to a new jar containing fresh WSA solution.

103 Transfer the slides into a jar pre-filled with MAA solution pre-chilled at -20 °C .

104 Incubate the slides in MAA solution for 00:30:00 at -20 °C . 30m

105 Transfer the slides into a jar pre-filled with fresh WSA solution.

106 Incubate the slides in WSA solution for 00:01:00 at Room temperature . 1m

107 Repeat steps 105–106, by transferring the slides to a new jar containing fresh WSA solution.

108 Transfer the slides into a jar pre-filled with freshly prepared PFS solution.

109 Incubate the slides in PFS solution for 00:05:00 at Room temperature . 5m

110 Transfer the slides into a jar pre-filled with PFA quenching solution.

111 Incubate the slides in PFA quenching solution for 00:05:00 at Room temperature . 5m

112 Transfer the slides into a jar pre-filled with 1x PBS solution.

113 Incubate the slides in 1x PBS solution for 00:05:00 at Room temperature . 5m

114 Repeat steps 112–113, by transferring the slides to a new jar containing fresh 1x PBS solution.

DAY 2: Tissue dehydration and drying 36m

115 Fill in three tissue staining jars with 70%, 85%, and 100% Ethanol solution, respectively.

116 Remove the slides from the 1x PBS solution (step 114) and transfer them into the jar pre-filled with 70% Ethanol solution.

117 Incubate the slides in 70% Ethanol solution for 00:02:00 at Room temperature . 2m

118 Transfer the slides into the jar pre-filled with 85% Ethanol solution

119 Incubate the slides in 85% Ethanol solution for 00:02:00 at Room temperature .

2m

120 Transfer the slides into the jar pre-filled with 100% Ethanol solution

121 Incubate the slides in 100% Ethanol solution for 00:02:00 at Room temperature .

2m

122 Repeat steps 120–121, by transferring the slides to a new jar containing fresh 100% Ethanol solution.

123 Transfer the slides onto a slide rack and place the rack onto the Boekel Inslide Out Hybridization Oven tray without closing the lid.

124 Place the tray inside the Boekel Inslide Out Hybridization Oven and dry the slides for 00:30:00 at 60 °C .

30m

DAY 2: Hybridization of primary oligos

8h 53m

125 Take the slides out from the oven and cover each tissue section with PHS-1 solution, while keeping the slides onto the Boekel Inslide Out Hybridization Oven tray.

126 Transfer the slides back into the Boekel Inslide Out Hybridization Oven.

127 Incubate the slides for 00:45:00 at 37 °C inside the Boekel Inslide Out Hybridization Oven.

45m

128 Meanwhile, prepare the Hybridization Mix by mixing the following reagents and adjusting the volumes based on the number of tissue sections:

	A	B
	Hybridization Mix	**Volume for 1 slide**

	A	B
	PHS-2 solution	18 µL
	Primary probe (pre-diluted to 0.5 nM)	2 µL

Note

CRITICAL: The concentration of the primary probe should be optimized when working with a new tissue type.

129 Add 20 µL of Hybridization Mix onto each tissue section.

130 Cover the section with a glass coverslip, then seal the coverslip's margins with Fixogum.

Note

CRITICAL: Be careful while pipetting the Hybridization Mix onto the tissue section and while lowering the coverslip onto the solution, to avoid bubble formation.

131 Transfer the slides to the ThermoBrite denaturization system.

132 Perform DNA denaturation for 00:08:00 at 88 °C. 8m

Note

CRITICAL: The denaturation time should be optimized when working with a new tissue type.

133 While denaturation is ongoing, prepare a humidity chamber by placing several stripes of tissue paper pre-wet in water all around the edges of the Boekel Inslide Out Hybridization Oven tray.

134 Quickly take the slides out of the ThermoBrite machine and transfer them onto the tray.

135 Incubate the slides Overnight at 37 °C inside the Boekel Inslide Out Hybridization Oven. 8h

DAY 3: Washes and hybridization of detection oligos 8h 9m

136 Fill in two staining jars with WSC solution and place them in a water bath pre-warmed at 65 °C .

137 Remove the slides from the Boekel Inslide Out Hybridization Oven tray (Step 135) and gently remove the Fixogum and the coverslips by placing each slide in a 10 cm dish pre-filled with WSB solution.

138 Transfer the slides into a staining jar pre-filled with fresh WSB solution.

139 Incubate the slides in WSB solution for 00:01:00 at Room temperature . 1m

140 Repeat steps 138-139, by transferring the slides to a new jar containing fresh WSB solution.

141 Transfer the slides into the jar pre-filled with WSC solution pre-warmed at 65 °C .

142 Incubate the slides in WSC solution for 00:07:00 at 65 °C . 7m

143 Repeat steps 141–142, by transferring the slides to the second jar containing WSC solution pre-warmed at 65 °C .

144 Transfer the slides into a new jar pre-filled with WSD solution.

145 Incubate the slides in WSD solution for 00:01:00 at Room temperature . 1m

146 Transfer the slides into a jar pre-filled with 2x SSC solution.

147 Quickly wash the slides in 2x SSC solution.

148 Transfer the slides into a jar pre-filled with WSE solution.

149 Quickly wash the slides in WSE solution.

150 Repeat steps 148–149, by transferring the slides into a new jar pre-filled with fresh WSE solution.

151 While keeping the slides in WSE solution, prepare the Detection Hybridization Mix by mixing the following reagents and adjusting the volumes based on the number of tissue sections:

152

	A	B
	Detection Mix	**Volume for 1 slide**
	DHS solution	98 µL
	Signal-amplification oligo (10 µM)	1 µL
	Detection oligo (2 µM)	1 µL

Note

CRITICAL: The concentration of the signal-amplification and detection oligos should be optimized when working with a new tissue type.

153 Add 100 µL of Detection Hybridization Mix onto each tissue section.

154 Cover the section with a small piece of Parafilm to prevent evaporation.

Note

CRITICAL: Be careful while pipetting the Hybridization Detection Mix onto the tissue section and while lowering the piece of Parafilm onto the solution, to avoid bubble formation.

155 Transfer the slides into a humidity chamber prepared using the Boekel Inslide Out Hybridization Oven tray (see step 133).

156 Incubate the slides Overnight at 30 °C inside the Boekel Inslide Out Hybridization Oven. 8h

DAY 4: DNA staining and slide mounting 8h 29m

157

Note

CRITICAL: Throughout the following steps, minimize the exposure of the slides to light.

158 Fill in two staining jars with WSC solution and place them in a water bath pre-warmed at 45 °C .

159 Remove the slides from the Boekel Inslide Out Hybridization Oven tray (Step 156) and transfer the slides into a staining jar pre-filled with fresh WSB solution.

160 Gently remove the pieces of Parafilm that detached from the slides.

161 Incubate the slides in WSB solution for 00:01:00 at Room temperature . 1m

162 Transfer the slides to a new jar containing fresh WSB solution.

163 Quickly wash the slides in WSB solution.

164 Transfer the slides into the jar pre-filled with WSC solution pre-warmed at 45 °C.

165 Incubate the slides in WSC solution for 00:07:00 at 45 °C. 7m

166 Repeat steps 164–165, by transferring the slides to the second jar containing WSC solution pre-warmed at 45 °C.

167 Transfer the slides into a new jar pre-filled with WSD solution.

168 Incubate the slides in WSD solution for 00:01:00 at Room temperature. 1m

169 Transfer the slides into a jar pre-filled with 2x SSC solution.

170 While keeping the slides in 2x SSC solution, prepare the DNA Staining Mix by mixing the following reagents and adjusting the volumes based on the number of tissue sections:

	A	B
	Detection Mix	**Volume for 6 slides (1 staining jar)**
	1x PBS	150 mL
	Hoechst 33322 (12.3 mg/mL)	15 µL

171 Fill in a staining jar with the DNA Staining Mix prepared in the previous step. If more than 6 slides need to be processed, fill in more jars.

172 Transfer the slides from 2x SSC solution (step 169) into the jar containing the DNA Staining Mix.

173 Incubate the slides in DNA Staining Mix for 00:20:00 at Room temperature . 20m

174 Transfer the slides into a new jar pre-filled with fresh 1X PBS solution.

175 Quickly wash the slides in 1X PBS solution.

176 Repeat steps 174–175 twice, each time transferring the slides into a new jar pre-filled with fresh 1X PBS solution.

177 Remove one slide from the 1X PBS solution and tap it on a piece of absorbent paper to remove any remaining traces of 1X PBS solution.

178 Add 10 µL of ProLong Diamond Antifade Mountant onto the tissue section (10 mm x 10 mm).

179 Cover the section with a glass coverslip.

Note

CRITICAL: Be careful while pipetting the ProLong Diamond Antifade Mountant onto the tissue section and while lowering the coverslip onto the solution, to avoid bubble formation.

180 Incubate the slides Overnight at Room temperature , protected from light. If the slides are not imaged on the next day, they can be stored for few months at -20 °C . 8h