Supplemental Figures

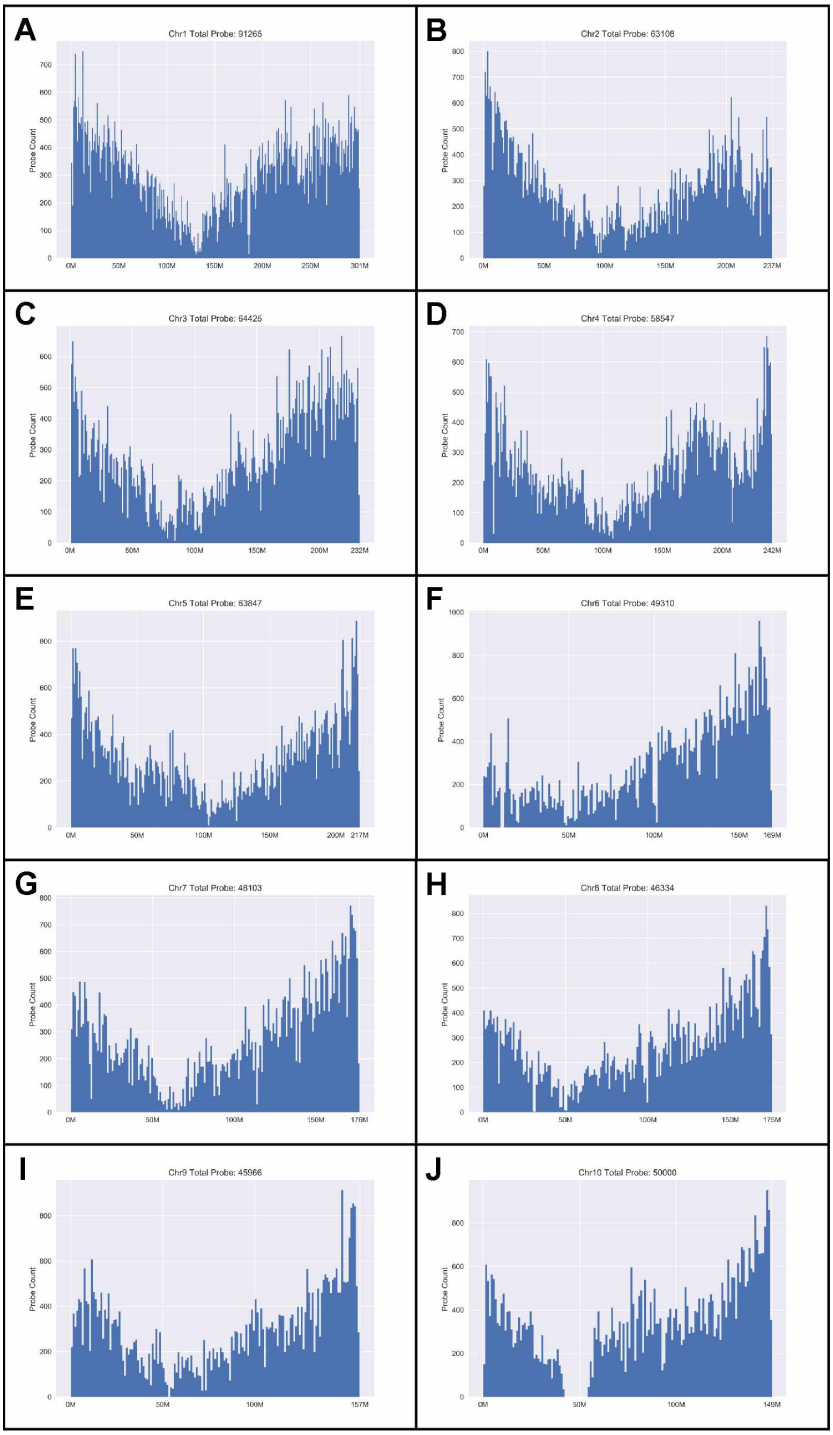

Figure S1. Distribution of all unique oligos on maize chromosomes. The x-axis shows DNA sequence position on each maize chromosome. The y-axis shows the number of oligos in 1 Mb windows. Panels A-J correspond to chromosomes 1-10, respectively.

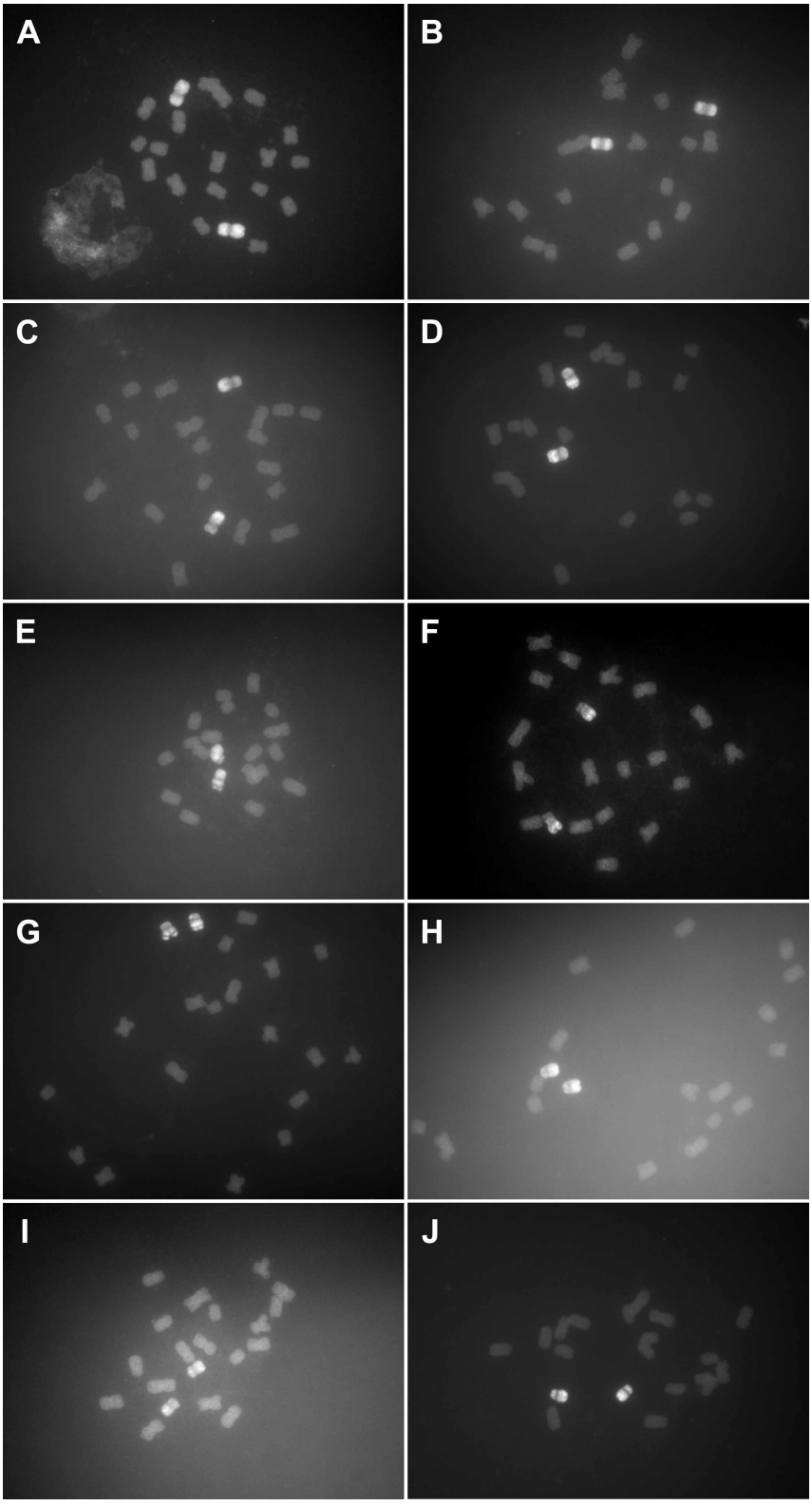

Figure S2. Digital signal enhancement of Figure 1 chromosome-specific probe channels depicted in grayscale as described in *Materials and Methods*.

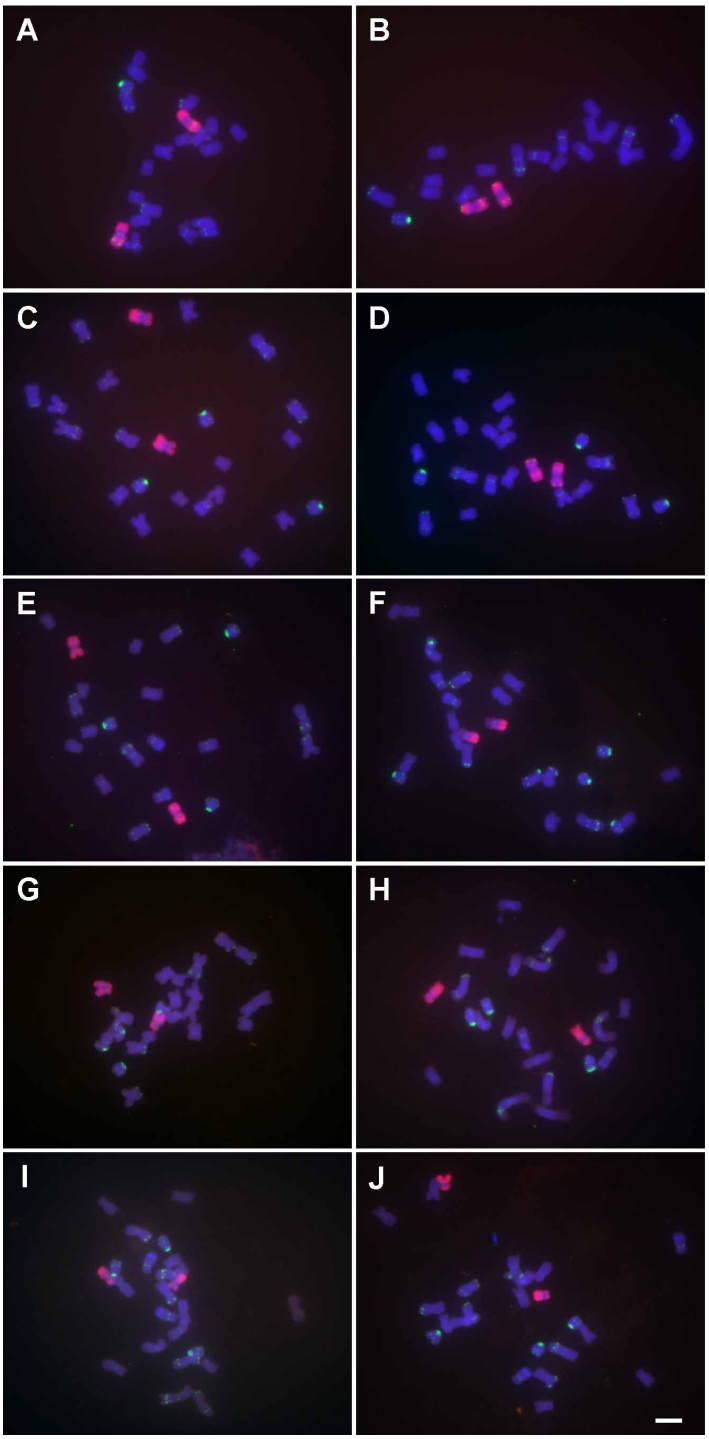

Figure S3. Tests of labeling of each chromosomal paint on the supernumerary B chromosome. Panels A-J correspond to chromosomes 1-10, respectively. Chromosomes are stained with DAPI (blue). Chromosome-specific paints are labeled in red and the B chromosome-specific repeat, in green. (Scale bar, 5 μm).

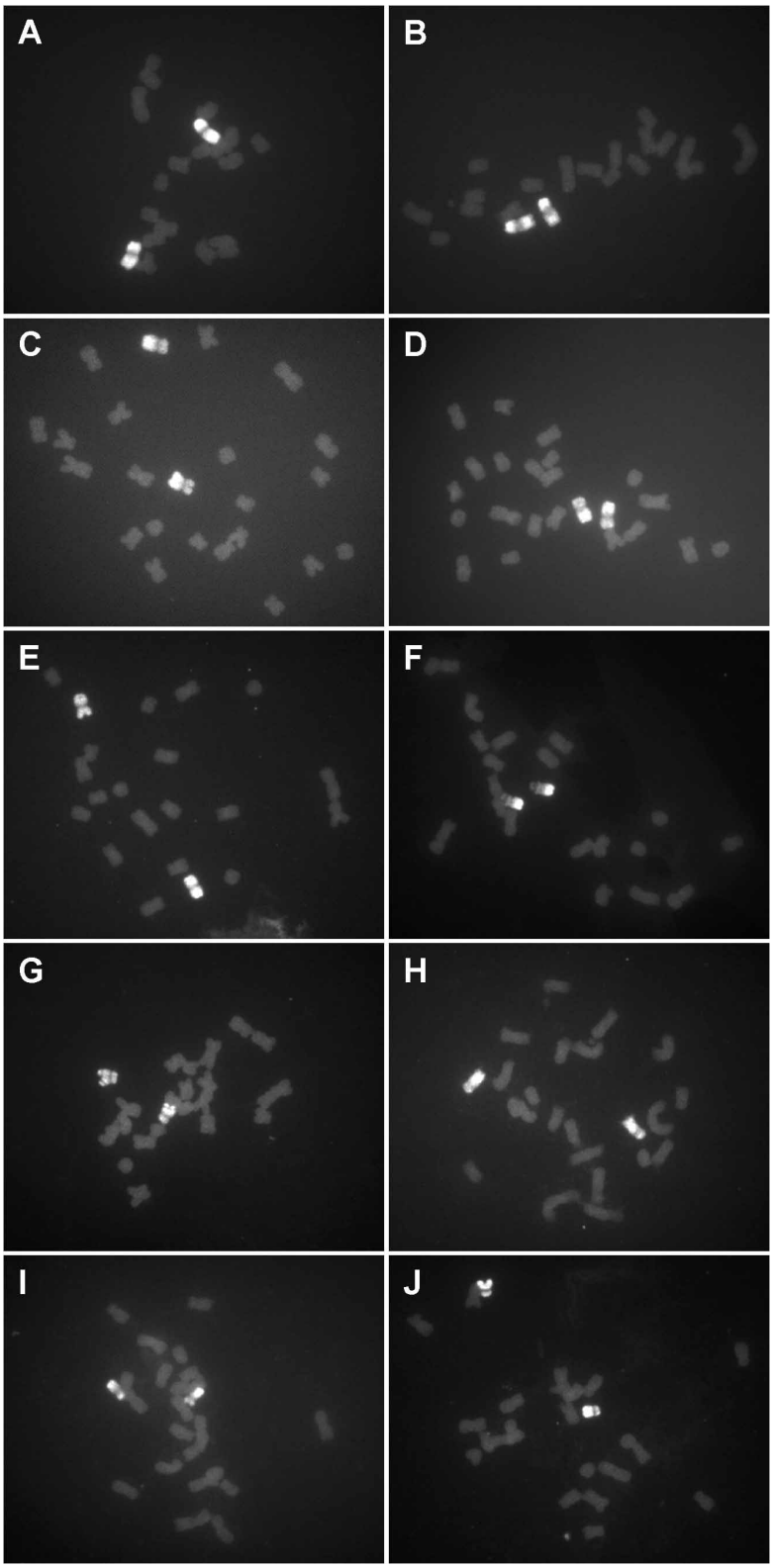

Figure S4. Digital signal enhancement of Figure S3 chromosome-specific probe channels depicted in grayscale as described in *Materials and Methods*.

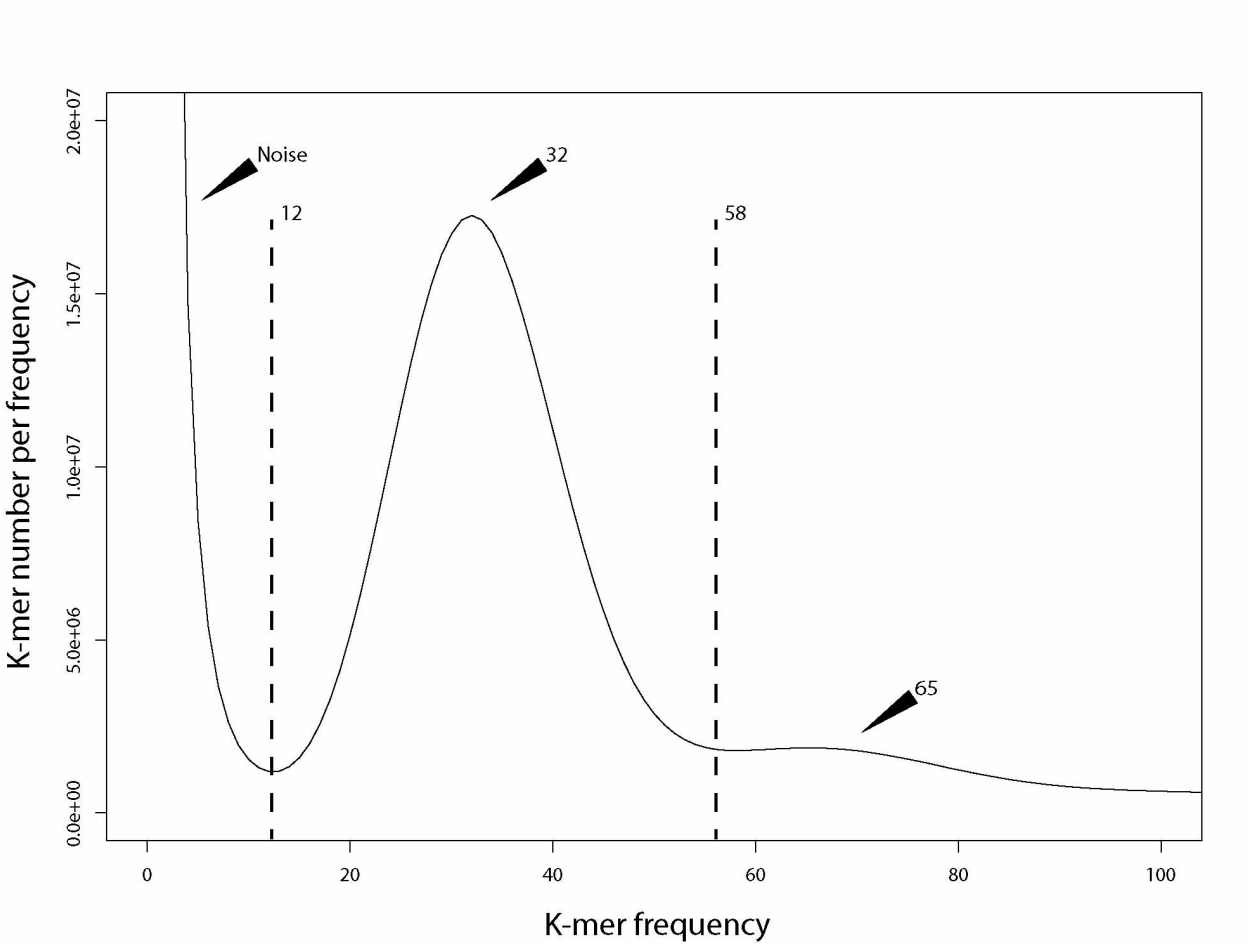

Figure S5. K-mer frequencies and histogram of an Illumina shotgun sequence library (SRA:SRR404240, 479M 100 bp paired-end) from maize inbred B73. The x-axis represents k-mer frequency observed in the Illumina shotgun library. The y-axis represents the total number of k-mer for a given frequency. The noise is likely caused by sequencing error associated with reads. We assume that each unique k-mer presents only once in the maize genome. The unique k-mer frequency in the shotgun library will reflect the coverage of this data set. The first peak in the k-mer histogram is located in 32, indicating that this shotgun library covered 32x of unique sequences of the maize genome.

Oligo	CTAAACTCTGAAGCACAATTAGCATAATTCCCACATGTAACTCTT	K-mer Frequency
17-mer	CTAAACTCTGAAGCACA----------	26
	-TAAACTCTGAAGCACAA----------	28
	--AAACTCTGAAGCACAAT----------	27
	---AACTCTGAAGCACAATT----------	27
	----ACTCTGAAGCACAATTA----------	27
	-----CTCTGAAGCACAATTAG----------	29
	------TCTGAAGCACAATTAGC----------	29
	-------CTGAAGCACAATTAGCA----------	63
	--------TGAAGCACAATTAGCAT----------	27
	---------GAAGCACAATTAGCATA----------	27
	----------AAGCACAATTAGCATAA----------	27
	-----------AGCACAATTAGCATAAT----------	29
	------------GCACAATTAGCATAATT----------	28
	-------------CACAATTAGCATAATTC----------	29
	--------------ACAATTAGCATAATTCC----------	29
	---------------CAATTAGCATAATTCCC----------	30
	----------------AATTAGCATAATTCCCA----------	28
	-----------------ATTAGCATAATTCCCAC----------	27
	------------------TTAGCATAATTCCCACA----------	27
	-------------------TAGCATAATTCCCACAT----------	27
	--------------------AGCATAATTCCCACATG----------	27
	---------------------GCATAATTCCCACATGT----------	28
	----------------------CATAATTCCCACATGTA----------	27
	-----------------------ATAATTCCCACATGTAA----------	27
	------------------------TAATTCCCACATGTAAC----------	27
	-------------------------AATTCCCACATGTAACT----------	30
	--------------------------ATTCCCACATGTAACTC----------	53
	---------------------------TTCCCACATGTAACTCT----------	30
	----------------------------TCCCACATGTAACTCTT----------	30
		K-mer Score: 870

Figure S6. K-mer score of a representative 45-nt oligo, CTAAACTCTGAAGCACAATTAGCATAATTCCCACATGTAACTCTT. This figure illustrates the algorithm of how the k-mer score of an oligo is calculated. The oligo is split into 17-mers; the frequency of each 17-mer is calculated within the shotgun library. The sum of the frequencies from all 29 17-mers is the k-mer score of this oligo.

SI Text S1
Library Amplification and Labeling

From ‘‘Preparing Single-Stranded Labeled Probes from a myTags Immortal Library Version 1.4” May 6th, 2014, adapted from Murgha et al., 2014:

> Murgha YE, Rouillard J-M, Gulari E (2014) Methods for the Preparation of Large Quantities of Complex Single-Stranded Oligonucleotide Libraries. PLoS ONE 9(4): e94752.

Updates to the myTags labeling protocol can be found at http://www.arborbiosci.com/ .

Library Amplification

I. PCR – First Stage Amplification

A. Prepare Library Working Stock

1. Mix 2 µL of the primary stock of the myTags oligonucleotide library (1 ng/µL, Arbor Biosciences custom synthesis) with 26.6 µL nuclease-free water to make a working stock of 0.07 ng/µL.

2. Store at -20˚C.

B. PCR

1. For each PCR amplification, prepare the following master mix and debubbling mix in separate 0.2 mL PCR tubes.

Components	***Master Mix***	***Debubbling Mix***
KAPA HiFi Hotstart Readymix (Kapa Biosystems, #KK2601)	25 µL	6 µL
myTags primer mix (100 µM each, F and R; provided)	0.25 µL	1.2 µL
nuclease-free water (Ambion #AM9937)	24.75 µL	2.8 µL
Reaction Volume	**50 µL**	**10 µL**

2. Vortex gently for 5 sec and centrifuge briefly (mini-centrifuge, 2200 rpm).

3. Set debubbling mix tube aside (on ice or at 4˚C).

4. Transfer 5 µL of the master mix to a new PCR tube (negative control).

5. Add 1 µL of the 0.07 ng/µL working stock library to the remaining 45 µL and mix by pipetting.

6. Perform amplification on both the negative control and template-containing reaction using the following program (hot lid on):

Step	Temperature	Time
1	95°C	3 min
2	98°C	20 sec
3	54°C	15 sec
4	72°C	30 sec
5	Repeat Steps 2-4, 4 more times	
6	98°C	20 sec
7	56°C	15 sec
8	72°C	30 sec
9	Repeat Steps 6-8, 14 more times	
10	Hold at 24°C	

7. During the 24˚C hold, remove the template tube and add to it the 10-µL debubbling mix (none for negative control). Mix by pipetting. Centrifuge briefly. Return tube to cycler and complete the following program for both tubes.

Step	Temperature	Time
11	95°C	3 min
12	98°C	20 sec
13	56°C	15 sec
14	72°C	30 sec
15	Repeat Steps 12-14, once more	
16	Hold at 24°C	

8. Upon completion, store both tubes at -20˚C or proceed to Electrophoresis.

C. Agarose Gel Electrophoresis

1. Prepare a 2.5% agarose gel made with tris-acetate-EDTA buffer (standard molecular protocol). Thinner gels will result in better resolution of the bands. The gel may include a DNA stain or can be post-stained with ethidium bromide.

2. Prepare samples for loading: Add 1 µL of 6x gel loading dye to a 5-µL aliquot of the template reaction and add 1 µL of loading dye to the entire 5-µL negative control tube.

3. Load the two samples and a lane containing a low molecular weight DNA ladder into separate wells. The expected product size for the template is 99 bp. No product should be seen in the negative control lane, although a primer band (40-50 bp) might be visible.

> **Note.** If a faint 120-bp band is visible, either decrease the amount of template DNA by 15-20% and repeat the amplification steps, or reamplify and "debubble" with 10 µL Readymix, 1.2 µL primer mix, and 8.8 µL nuclease-free water (Arbor Biosciences). Proceed with purification when template and negative control lanes show appropriate band patterns.

D. DNA Purification using the Qiagen QIAquick PCR Purification Kit (#28104)

1. Place a QIAquick spin column in a 2-mL collection tube.

2. Transfer the template PCR product to a 1.7 mL tube and add nuclease-free water to bring the volume to 100 µL.

3. Add 5 µL of 3 M sodium acetate, pH 5.2.

4. Add 500 µL of Qiagen Buffer PB and vortex for 5 seconds.

5. Apply entire sample to the QIAquick column. Centrifuge 1 minute (13,000 rpm; 17,900 x g).

6. Discard the flow-through. Place spin column back into the same tube.

7. Add 700 µL of Qiagen Buffer PE to the spin column and centrifuge for 1 minute at 17,900 x g.

8. Discard the flow-through and place spin column back into the same tube.

9. Centrifuge the column for an additional 2 minutes at 17,900 x g.

10. Place the QIAquick column in a clean 1.7 mL centrifuge tube.

11. To elute DNA, add 30 µL of Qiagen Buffer EB to the center of the column membrane. Let the column stand for 1-2 minutes and centrifuge for 1 minute at 17,900 x g.

12. Quantify the purified dsDNA using a NanoDrop or other method. A minimum of 480 ng is required for the next step.

13. Store at -20˚C or proceed to *In vitro* Transcription.

II. *In vitro* Transcription (IVT) – Second Stage Amplification

The MEGAshortscript T7 kit (Invitrogen, #AM1354) is used to make microgram quantities of RNA using the dsDNA PCR product as a template. Before starting, pool 52 µL of each stock rNTP into a single tube.

A. IVT Reaction

1. Assemble the *in vitro* transcription reaction mix in a 0.2 mL PCR tube on ice as follows:

Components	***Volume***
Nuclease-free water	___ µL
Template DNA (480 ng)	___ µL
10x T7 reaction buffer	4 µL
Four rNTP pool	16 µL
T7 enzyme mix	4 µL
Reaction Volume	**40 µL**

2. Vortex gently for 5 seconds, quick-spin for 5 seconds in a mini-centrifuge.

3. Incubate the reaction at 37˚C for 4 hours (hot lid at 42˚C). If necessary, store at -80˚C overnight prior to purification.

B. RNA Purification using the Qiagen RNeasy Mini Kit (#74104)

1. Two RNeasy spin column assemblies and four 1.7-mL centrifuge tubes are needed per 40-µL IVT reaction.

2. Add 160 µL of nuclease-free water to the IVT reaction, mix, and split into two user-supplied, 1.7-mL tubes (100 µL each). Proceed with RNeasy purification steps for each aliquot.

3. Add 350 µL of Qiagen Buffer RLT and vortex gently for 5 seconds.

4. Add 250 µL of 100% ethanol and vortex gently for 5 seconds. Do not centrifuge. Proceed immediately to next step.

5. Transfer the sample (700 µL) to an RNeasy spin column in a 2-mL collection tube (supplied). Centrifuge for 1 minute at 12,000 rpm (13,523 x g). Discard the flow-through.

6. Add 500 µL of Qiagen Buffer RPE to the spin column. Centrifuge for 1 minute at 13,523 x g to wash the spin column membrane. Discard the flow-through.

7. Repeat Step 6 once.

8. Place the RNeasy spin column in a new 2-mL collection tube (supplied) and discard the old collection tube with the flow-through. Centrifuge for 3 minutes at 13,523 x g.

9. Primary eluate: Place the spin column in a new 1.5-mL collection tube (supplied). Add 50 µL nuclease-free water directly to the spin column membrane. Wait 1 minute. Centrifuge for 1 minute at 13,523 x g to elute the RNA.

10. Secondary eluate: Transfer the column to a new 1.7-mL tube (user supplied). Add 20 µL nuclease-free water directly to the spin column membrane. Wait for 1 minute. Centrifuge for 1 minute at 13,523 x g to elute more RNA.

11. For each IVT reaction, pool the two primary eluates and, separately, the two secondary eluates.

12. Measure RNA concentrations of each eluate pool using a NanoDrop.

> **Note**: The amount of RNA required for a 100-µL labeling reaction (next step) is about 42 µg; therefore, a minimum concentration of ~ 1010 ng/µL is required to meet the volume requirements of the labeling reaction. All eluates are combined if the overall concentration exceeds the minimum, or vacuum centrifugation without added heat can be used to concentrate the sample.

13. Store at -80˚C or proceed to Probe Labeling.

> **Note**: Long-term storage of RNA is not recommended. It is better to convert the RNA into more stable single-stranded DNA probes as soon as possible. Although next day is best, good probes have been made with RNA properly stored for about one year.

Probe Labeling via Reverse Transcription

I. Labeling the Probe Sequences

A. Reverse Transcription using SuperScript II (Invitrogen, #18064014)

1. Prepare the following reaction on ice (0.2 ml PCR tube):

Components	*Volume*
Add nuclease-free water up to reaction volume	___ µL
RNA (42 µg)	___ µL
1 mM (1 nmol/µL) dye-labeled RT primer*	2.4 µL
10 mM dNTP (New England Biolabs, #N0447S)	15 µL
20 U/µL SUPERase-In (Invitrogen, #AM2696)	1 µL
Reaction Volume	**60 µL**

*Dye-labeled RT primer (5' Dye/ CGTGGTCGCGTCTCA) resuspended at 1 mM in nuclease-free water. Order from commercial oligo companies (IDT-DNA, Bio-synthesis, Operon, etc.) and inquire about dye quenching. Although dual-HPLC purification is recommended, single-HPLC purification from IDT-DNA works for primers labeled with ATTO-550.

2. Vortex gently for 5 seconds, centrifuge briefly (2200 rpm), and incubate the tube in a thermocycler at 65°C with hot lid set to 75°C for 5 minutes. Quick chill on ice for 5 minutes.

3. Prepare the following components on ice and add the 35 µL to the tube containing the RNA and primer:

Components	*Volume*
nuclease-free water	4 µL
5 X first-strand buffer (provided)	20 µL
0.1 M DTT (provided)	10 µL
20 U/µL SUPERase-In	1 µL
Total Volume	**35 µL**

4. Vortex gently for 5 seconds, centrifuge briefly, and incubate the reaction at 42°C (hot lid: 52°C) for 5 minutes.

5. Add 2.5 µL 200 U/µL SuperScript II Reverse Transcriptase.

6. Vortex gently for 5 seconds, centrifuge briefly, and incubate the reaction at 42°C (hot lid: 52°C) for 2 hours.

7. Add an additional 2.5 µL 200 U/µL SuperScript II Reverse Transcriptase and repeat Step 6.

8. Add 11 µL of exonuclease I buffer (enzyme and buffer, NEB #M0293S).

9. Add 2 µL of exonuclease I enzyme. Vortex gently for 5 seconds and centrifuge briefly.

10. Incubate at 37°C for 15 min. This step will degrade unincorporated reverse-transcription primers.

11. Add 12 µL of 0.5 M EDTA pH 8.0. Vortex gently for 5 seconds and centrifuge briefly.

12. Put the tube directly in the thermocycler at 80°C for 20 min (no temperature ramp-up). Transfer tube to ice immediately to stop enzyme inactivation step.

B. Clean-up (125 µL; RNA/cDNA hybrids) using Zymo Quick-RNA MiniPrep Kit (#R1054S)

Before starting, put spin column in collection tube; also need two 1.7-mL tubes – one to hold lysis reaction and other for final elution from column.

1. Transfer RNA/cDNA hybrids to 1.7-mL tube and add 500 µL of Zymo RNA lysis buffer. Vortex for 5 seconds.

2. Add 625 µL of 100% ethanol. Vortex for 5 seconds.

3. Transfer 650 µL of the lysis solution to a Zymo-Spin IIICG Column in a collection tube and centrifuge for 30 seconds at 13,000 rpm (17,900 x g). Discard the flow-through.

> **Note.** The flow-through will have dye color which indicates removal of excess primers. This is expected in the first Zymo Quick-RNA cleanup.

4. Repeat Step 3 once such that the entire sample has been loaded into the column.

5. Add 400 µL of Zymo RNA Prep Buffer to the column and centrifuge (17,900 x g) for 30 seconds. Discard the flow-through.

6. Add 700 µL of Zymo RNA Wash Buffer to the column and centrifuge (17,900 x g) for 30 seconds. Discard the flow-through.

7. Add 400 µL of Zymo RNA Wash Buffer to the column and centrifuge (17,900 x g) for 30 seconds. Discard the flow-through.

8. Place the Zymo spin column back into collection tube and centrifuge at (17,900 x g) for 3 minutes to ensure complete removal of the wash buffer. Discard the collection tube with the flow-through.

9. Place the column into a new RNase-free tube. Add 42 µL of room temperature nuclease-free water to the column matrix, wait 1 minute, and then centrifuge (17,900 x g) for 1 minute.

10. Repeat Step 9 using 42 µL of room temperature nuclease-free water. Reuse the collection tube from Step 9.

11. Make sure all of probe has been washed from the column by briefly examining at the low setting on a UV transilluminator. If probe is visible, transfer all liquid to the column matrix and re-centrifuge as above.

12. Store a -20°C or proceed to RNA Removal.

II. Removal of RNA

Note. Certain 5' end labels [especially N-hydroxysuccinimide (NHS) ester dyes and moieties (e.g., Digoxygenin NHS ester, ATTO NHS ester, AlexaFluor NHS ester)] are susceptible to alkaline hydrolytic damage. For these dyes, use the enzymatic RNA removal protocol below. For alkaline-resistant labels, such as biotin, either enzymatic or a chemical RNA removal protocol can be used. For the latter, contact Arbor Biosciences.

A. Enzymatic Removal of RNA

1. Add the following components to a 0.2 mL PCR tube on ice (enzyme mix):

Components	***Volume***
10x RNase H buffer* (New England Biolabs, M0297S)	10 µL
5 U/µL RNase H (see above)	4 µL
10 mg/mL RNase A (Thermo Scientific, EN0531)	4 µL
Reaction Volume	**18 µL**

*If precipitate is visible, bring to room temperature before use.
Small amount is acceptable according to New England Biolabs technical representative.

2. Add RNA/cDNA hybrids (~82 µL) to the 18 µL of enzyme mix. Vortex gently for 5 seconds and centrifuge briefly.

3. Put tube in thermal cycler programmed as follows:

Temperature	**Time**
37°C	120 min
70°C	20 min
50°C	60 min
95°C	5 min
ramp down 95°C to 37°C	0.1°C/sec
50°C	60 min
4°C	hold

Note: Program provides optimal activation of RNases and inactivation of RNase H but not RNase A. Therefore, probe is not suitable for RNA FISH unless extracted with phenol chloroform.

B. Clean-up Reaction (100 µL) with the Zymo Quick-RNA MiniPrep Kit (#R5410S)

Before starting, put spin column in collection tube; also need four 1.7-mL tubes – one to hold lysis reaction, one for warming the elution water, one for final elution from column, and one to hold half of elution volume for concentration step.

Put ~110 µL of nuclease-free water at 65°C; needed for step 9.

1. Transfer enzyme digest mixture to 1.7-mL tube and add 400 µL of Zymo RNA lysis buffer. Vortex for 5 seconds.

2. Add 500 µL of 100% ethanol. Vortex for 5 seconds.

3. Transfer 650 µL of the lysis solution to a Zymo-Spin™ IIICG Column in a collection tube and centrifuge for 30 seconds at 13,000 rpm (17,900 x g). Discard the flow-through.

4. Repeat Step 3 once such that the entire sample has been loaded into the column.

5. Add 400 µL of Zymo RNA Prep Buffer to the column and centrifuge (17,900 x g) for 30 seconds. Discard the flow-through.

6. Add 700 µL of Zymo RNA Wash Buffer to the column and centrifuge (17,900 x g) for 30 seconds. Discard the flow-through.

7. Add 400 µL of Zymo RNA Wash Buffer to the column and centrifuge (17,900 x g) for 30 seconds. Discard the flow-through.

8. Place the Zymo spin column back into collection tube and centrifuge (17,900 x g) for 3 minutes to ensure complete removal of the wash buffer. Discard the collection tube with the flow-through.

9. Place the column into a new RNase-free tube. Add 50 µL of warm (65°C) nuclease-free water to the column matrix, wait 1 minute, and then centrifuge (17,900 x g) for 1 minute.

10. Repeat Step 9 using an additional 50 µL of 65°C nuclease-free water, for a total of 100 µL. Reuse the collection tube from Step 9.

Note: Use remaining water, at room temperature, to later obtain "blank" spectrophotometer measurement.

11. Make sure all of probe has been washed from the column by briefly examining at the low setting on a UV transilluminator. If probe is visible, transfer all liquid to the column matrix and re-centrifuge as above.

III. Finishing the Probe

1. Measure concentration of dye (pmol/µL) and single-stranded nucleic acid (ng/µL) using the 'microarray setting' on a nanodrop type spectrophotometer. Use 1.5 µL for the measurements.

2. Record or print the results.

3. Separate the total volume into two 1.7 mL tubes (~49 µL each) and place in pre-chilled Speed Vac evaporator for concentration. Fully dried pellets may not resuspend well.

4. Calculate the efficiency of dye incorporation.

   a. First calculate the expected dye concentration.
   If the NanoDrop reading is X ng/µL ssDNA, the pmol/µL dye expected =

   $$X \text{ ng}/\mu\text{L} \;/\; 10^9 \text{ ng/g} \;/\; (330 \text{ g/mol} \times 66 \text{ nt}) \;\times\; 10^{12} \text{ pmol/mol}$$

   Note. cDNA size: 66 bases. 1 µg = 50 pmol; calculations are performed from left to right.

   b. Efficiency = (Observed NanoDrop pmol/µL)/(Expected concentration).
   A value close to 1.00 (or 100% efficiency) is best; 0.85 is usually okay.

5. Calculate the volume of nuclease-free water needed to bring the probe to the desired concentration. For 25 pmol/µL:

   a. First determine the pmole yield. If assume 98 µL were concentrated,

   $$\text{yield} = 98\ \mu\text{L} \times X \text{ pmol}/\mu\text{L}.$$

   b. Final volume required for desired concentration = $\text{pmole yield} / 25 \text{ pmol}/\mu\text{L}$.

   c. Determine volume of combined concentrated probe tubes and add nuclease-free water to desired final volume. Vortex gently to mix.

6. Store at -20°C in the dark. If making large volumes of probe, freeze aliquots.

SI Text S2
FISH Procedures for Maize Whole Chromosome Oligo-Paints

Modified from Kato *et al.* (2011) Chromosome painting for plant biotechnology. *Methods Mol Biol* 701:67-96. See also https://birchler.biology.missouri.edu/wp-content/uploads/2015/07/Maize-Karyotyping-and-FISH-Manual_2015.pdf.

I. Preparation of Metaphase Spreads from Root Tissue

A. Supplemental Information

The nitrous oxide - enzymatic maceration method (Kato, 1999) was used to prepare slides for FISH from plant tissue. Root tips can be obtained from newly germinated seeds, from young plants, or from new growth on root-pruned plants. Root tips to be nitrous oxide treated are placed in 0.6 mL or 1.7 mL tubes, each with a hole punched in the lid to facilitate gas exposure and lightly misted with water to prevent desiccation. Longer treatment times (2.5-3 hr) generally result in chromosomes that are more condensed.

The length of time the root meristematic tissue is digested depends on the number and size of the tissue pieces, and the degree of chromosome spread desired. The tissue can be left intact, cut transversely into several pieces or cut both longitudinally and transversely. Whatever the size, quickly transfer the pieces into the enzyme solution. Ideal conditions for different lines will vary. For smaller diameter roots, two or more roots per enzyme tube might be needed to produce adequate cell density.

To make ~10 mL of the enzyme solution, mix together (on ice):

0.1 g of pectolyase Y-23 (1% w/w),
0.2 g cellulase Onozuka R-10 (2% w/w)
9.7 g of 1x citric buffer

Quickly dispense as 20-μL aliquots into thin-walled 0.5-mL PCR tubes, quick freeze on dry ice, and store at -20°C.

Note: Concentrations might need to be adjusted when changing an enzyme source or lot number. Currently 3% cellulase, 1.5% pectolyase is used. If background nucleoplasm is a problem, as is with some maize lines, increase the cellulase concentration to 4%.

The humid chamber consists of a shallow, open-top cardboard box, subdivided into sections that will easily accommodate the width and number of microscope slides to be processed. All surfaces are lined with multiple layers of paper towels (stapled into position), which are moistened with reverse osmosis (RO) purified water just prior to use. Slides placed in the chamber are elevated on a dry plant stake. Covering the chamber with a large Kimwipe (either damp or dry) is optional. The chamber is reused.

Prepare slides using the suspension dropping method, which is better for FISH than the smear-scraping technique. Signal intensity is stronger, background level is lower, and the

incidence of non-overlapping chromosomes is higher with the dropping method.

When hybridizing with whole chromosome paint probes, the goal in terms of slide preparation is to have a sufficient number of metaphase spreads on the slide but not so many nuclei and cell clumps that excessive competition for the probe occurs.

B. Preparation of Root Tips

1. Germinate kernels in moist vermiculite or on nearly wet Kimwipes for 2-3 days at 28-30°C, or harvest young roots from plants. Choose roots 1-5 cm in length (3-4 cm ideal; longer end of range if plant to be kept). Keep roots moist.

2. Cut about 1 cm from each root tip and transfer using forceps to a prepared 0.6 mL tube (labeled, hole in cap, spritzed with fine layer of RO water). Multiple roots can be treated in a single tube if there's no reason to keep them separate, e.g. different genotypes.

3. Transfer tubes to a metal pressure chamber. Treat with N_20 at 10 atm for 1-3 hr.

4. Fix roots in ice cold 90% acetic acid for 10-12 min (longer times not tested).

5. Remove acid (non-filtered P200 tip on end of plastic transfer pipette). Add ~400 µL 70% ethanol. Remove 70% and add ~400 µL fresh 70% ethanol. Transfer roots to a new tube containing 70% ethanol.

6. Continue to Step 7 or store at -20°C.

Note: Signal quality after storage for longer than ~5 months has not been determined.

7. Transfer roots selected for digestion from the storage ethanol to a 0.6 mL tube filled with RO water. Place on ice.

8. After 1-2 minutes, remove the RO water and replace with fresh. Soak recently prepared roots for a total 5-8 minutes to remove ethanol (20-30 minutes, with several changes of water, if stored for about a week or longer).

9. Remove enzyme tubes from -20°C (one tube per root), label, and place on ice.

10. Perform Steps 11-13 for each root before proceeding to Step 14.

11. Remove the sticky substance on the root tip by rolling the root on dry filter paper.

12. Cut off a very small portion of the tip of the root cap. Discard. This step is optional and should not be done on small, thin roots.

13. Excise 0.75 - 1 mm of the distal part of the meristem (opaque area, about 2 mm) and transfer the tissue to a tube containing 20 μL cold enzyme solution. Return tube to ice.

14. Put tubes in foam floater placed on ice. Place floater in 37°C water bath.

15. Incubate for 30-60 minutes (species-, line-, size-, and age-dependent). Plunge tube into ice and fill with cold 70% ethanol (~350 μL) to stop digest. Invert to mix well. Return to ice.

16. Remove ethanol/enzyme mixture using a plastic transfer pipette fitted with a non-filtered P200 tip. Fill tube with 70% ethanol.

17. Rinse tissue once more with 70% ethanol, this time removing as much ethanol as possible (invert tube, blot edge, and use non-filtered P200 tip to tease out remaining volume). Tissue will most likely stick to interior of tube.

18. Immediately add 15-30 μL of freshly prepared 90% acetic acid - 10% methanol.

> **Note:** Volume used is related to tissue size. Start with ~22 μL. The goal is to have a sufficient number of metaphase spreads without having too many probe-competing nuclei present or negatively affecting the ability of the cells to disperse and dry properly.

19. Carefully break the root section with a rounded-off dissecting needle by trapping tissue between needle and tube; roll needle back and forth over the tissue. Stir with needle and tap the tube with your finger several times to suspend the cells.

> **Note:** Upon addition of acetic acid-methanol, keep cells on ice and only for as long as is necessary to drop the cell suspension onto a slide, observe the spreads, and either drop the rest of the suspension, or first change the density by adjusting the volume of acetic acid-methanol solution.

C. Drop and Cross-link Cells

1. Label slides onto which cells will be dropped (Frosted slide or Tough Tag at one end).

2. Drop 5-6 μL of the cell suspension onto a microscope slide already placed in a humid chamber. (High humidity is required for solvent spreading and cell dispersion.)

3. When dry (10-15 minutes), view using a compound microscope fitted with 10X and 40X objective lenses to select slides with the best spreads (quality and number) for hybridization.

4. Cross-link chromatin to slides by exposure to UV light. Set cross-linker to "optimum." A total energy of 120-125 mJ per square cm is delivered.

5. Cross-linked slides can be used immediately for hybridization or stored for 1-2 days at 4˚C.

> **Note**: Storage for longer periods of time has not been tested with chromosome paint probes. To maximize the signal quality, store root tips in 70% ethanol until ready to proceed with the FISH procedure.

II. Signal Detection of Directly Labeled Probes

A. Overview

1. Denature chromosomal DNA, 100°C, 5 minutes
2. Hybridization, 55°C, overnight
3. Wash, room temperature 2X SSC, briefly
4. Stringent wash, 2X SSC, 55°C, 5 minutes
5. Apply Vectashield (Vector Laboratories, #H-1200 diluted 1/20 with #H-1000)
6. Observation

B. Preparation

1. Preheat foil-covered water bath, but not insert tray, to boiling point (can use electric skillet).

Water bath insert consists of a small aluminum tray, the bottom of which is covered with several layers of wet Kimwipes (use RO purified water) and a plastic lid large enough to cover the slides.

2. Prepare the probe solution. Per slide, use 7-10 μL total volume (sample recipe shown). Mix in a 0.2-mL PCR tube. Mix by gently pipetting. Centrifuge briefly. Keep on ice.

NOTE: IF NICK-TRANSLATED, dsDNA PROBES WILL ALSO BE INCLUDED IN THE PROBE MIXTURE, SEE 'SECTION D' AT END OF PROTOCOL.

Components	***Volume***
Nuclease-free water (to total volume)	___ μL
ATTO-550 red probe	___ μL (use 50 pmoles/slide)
ATTO-488 green probe	___ μL (use 75 pmoles/slide)
20X SSC	0.8 μL
Probe Volume	**8 μL**

3. Fill insulated bucket to the top with ice. Gather other necessary items and equipment:

Timer, forceps (small and large), P20 and tips, 22 x 22 mm plastic cover slips (Fisher Scientific, 12547), metal tray for chilling slides, pair of square wooden rods, salmon sperm blocking agent (Sigma, D1626; see below), humidified hybridization box

Needed next day after hybridization: two Coplin jars (one preheated overnight to 55°C). Also preheat 30-35 mL 2X SSC in capped 50-mL tube; same volume needed at room temperature.

C. Hybridization of Chromosome Paint Probes

The chromosome paint probes are 66-nt, fluorescently labeled oligonucleotides (ssDNA), which do not require denaturation, and can be negatively affected by exposure to 100°C.

1. Place slides on square wooden dowel rods (allows room for cover slip manipulation).

2. Pipet 7-8 μL of denatured salmon sperm DNA (850 ng/μL 2x SSC, 1x TE made from autoclaved 10 mg/mL DNA in TE stock) onto each slide in the area containing the metaphase spreads. Using forceps, apply a 22 x 22 mm plastic cover slip; one of the corners or an edge needs to hang over the edge of the slide (facilitates removal for probe application).

3. Place slides on moistened Kimwipes in water bath insert. Press slides firmly to establish good contact, then cover lightly with a large, plastic pipette tip box lid. Do not press lid into Kimwipes; loose contact allows steam to vent.

4. Carefully set the insert tray into the boiling water bath. Cover with foil.

5. Denature chromosomal DNA (on slide) in the boiling water bath for 5 minutes (100°C). While waiting, prepare to chill slides – turn metal slide tray upside down and push against ice such that good contact is made.

Note: It takes 2-3 minutes for the water bath to return to rolling boil.

6. Remove insert tray. Take care not to get burned when removing foil. Transfer tray to benchtop using tongs or a large pair of forceps. Keep the plastic lid parallel to the benchtop as it is being removed to avoid getting condensation on the slides.

7. Quickly place each slide on the pre-chilled metal. Allow to cool for 2-5 minutes (keeps dsDNA from reannealing before the probe is added).

8. Fill P20 tip with 8 μL of probe mixture.

9. With fine-tipped forceps, lift plastic cover slip; slide should not be dry. Pipette probe onto the slide. Replace the cover slip in same orientation.

10. Transfer slides to a humid storage container (reusable airtight 'food' container lined with Kimwipes moistened with RO water) for 15-20 hours (overnight) at 55°C.

11. Place slides in a Coplin jar containing room-temperature 2x SSC briefly to remove the cover slip and excess probe. Slides can be placed back-to-back to increase the processing capacity of the jar.

12. Using forceps, transfer slides to a Coplin jar containing 55°C 2x SSC and wash for about 5 minutes at 55°C. The temperature and SSC concentration can be varied for different stringency requirements:

Stringent wash: By changing the temperature and salt concentration, one can change the stringency of DNA binding, optimally reducing background without losing signal. The addition of 50% formamide further increases the stringency, but is not necessary in most cases. Wash for 5-30 min.

42°C, 2x SSC low stringency
55°C, 2x SSC medium stringency
60°C, 2x SSC high stringency
65°C, 2x SSC very high stringency
50°C, 0.1x SSC medium stringency
55°C, 0.1x SSC high stringency
60°C, 0.1x SSC very high stringency (most signals will disappear)

13. Remove slides. Flick to remove excess SSC from the top of the slide and blot to remove it from the bottom and edges of the slide (SSC remaining on slide surface will dilute Vectashield).

14. Apply one drop of pre-mixed Vectashield mounting medium containing 1/20 DAPI and carefully apply a 24 x 50 mm glass cover slip (Fisher Scientific, 12544E). Vectashield without DAPI is used if detecting blue fluorescent probes.

15. Acquire images. Slides may be stored at 4°C (before or after image acquisition). If stored vertically, line box with foil and dry Kimwipe to absorb excess mounting medium and immersion oil.

D. Prehybridization steps for probes containing ssDNA and dsDNA components

The dsDNA probe, usually made via nick-translation, must be denatured prior to hybridization, and the total volume of the probe mixture should be kept about the same. This can be accomplished in one of two ways:

1. Denature with slides.

a. Pipette the required amount of ds-probe, along with some of the allotted 2x SSC (together about 3 μL, minimum), into a 0.2-mL PCR tube.

b. Mix together, in a separate PCR tube, the remaining components of the probe mixture and place on ice. (Alternatively, have the components available to add individually to the chilled, denatured probe from Step “e” below; mix well).

c. Lay the tube from Step “a” against the moist Kimwipes, near the slides, in the water bath inset tray. The probe DNA will be denatured during exposure to heat from the boilng water.

d. When the 5 minutes has elapsed, and after the inset cover has been removed, Immediately place the probe tube in a beaker of slushy ice to keep the DNA from reannealing (also quickly put the slides on the ice cold metal).

e. Quick-spin the now chilled tube and immediately return to ice.

f. Transfer the larger volume into the tube with the smaller volume. Keep on ice.

g. Mix well and continue the protocol from Hybridization Step 8.

2. Denature in a thermal cycler for 2-3 minutes at 95˚C.

a. Denature about 10 μL of the nick-translated probe (no added 2x SSC).
b. Immmediately transfer to ice.
c. Quick-spin and immediately return to ice.
d. Pipette the required amount of probe into the tube containing the library probe components.
e. Mix well and continue the protocol from Hybridization Step 8.

In either case, reduce the amount of the nick-translated probe if one of the chromosome paint probes is labeled with the same color; otherwise, the exposure time might not be optimal for both.

Reference:

Kato A (1999) Air drying method using nitrous oxide for chromosome counting in maize. *Biotechnic & Histochemistry* 74:160-166.