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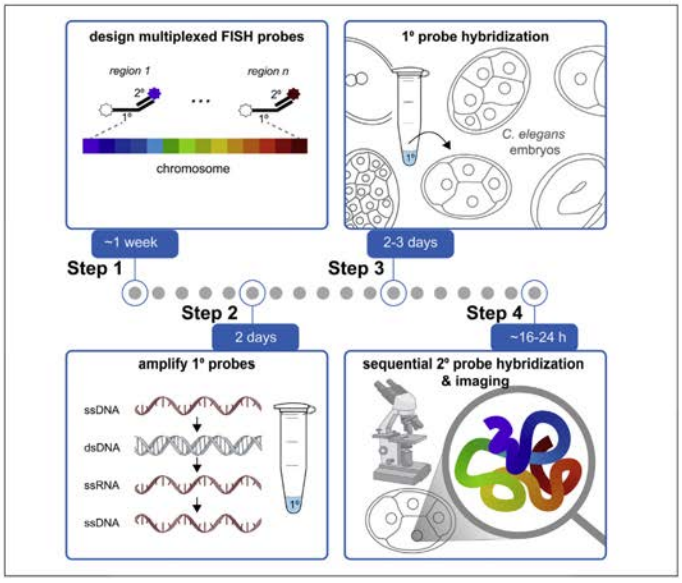
Multiplexed Sequential DNA FISH in *Caenorhabditis elegans* Embryos



This protocol describes a high-throughput and multiplexed DNA fluorescence *in situ* hybridization method to trace chromosome conformation in *Caenorhabditis elegans* embryos. This approach generates single-cell and single-chromosome localization data that can be used to determine chromosome conformation and assess the heterogeneity of structures that exist *in vivo*. This strategy is flexible through modifications to the probe design steps to interrogate chromosome structure at the desired genomic scale (small-scale loops to whole-chromosome organization).

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HIGHLIGHTS

An optimized FISH protocol to map chromosome conformation in *C. elegans* embryos

Removes limitations on the maximum number of targets visualized in a single sample

Customizable to interrogate conformation from the kb to Mb to whole-genome scale

Unsupervised clustering of single chromosomes can define similar populations *in vivo*

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Multiplexed Sequential DNA FISH in *Caenorhabditis elegans* Embryos

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SUMMARY

This protocol describes a high-throughput and multiplexed DNA fluorescence *in situ* hybridization method to trace chromosome conformation in *Caenorhabditis elegans* embryos. This approach generates single-cell and single-chromosome localization data that can be used to determine chromosome conformation and assess the heterogeneity of structures that exist *in vivo*. This strategy is flexible through modifications to the probe design steps to interrogate chromosome structure at the desired genomic scale (small-scale loops to whole-chromosome organization).

For complete details on the use and execution of this protocol, please refer to Sawh et al. (2020).

BEFORE YOU BEGIN

Choose the genomic resolution and scale of the desired chromosome conformational analysis. The ideal resolution and scale are highly dependent on the biological question under investigation and the configuration of your imaging system (see below). For example, if you would like to trace a whole chromosome at 1 Mb resolution (i.e., differentiate regions that are 1 Mb apart in genomic distance), design probes against 30–100 kb target regions along the entire length of the chromosome. See Figure 1 for example strategies on a 20 Mb chromosome. If you would like to visualize a total 5 Mb genomic stretch at 50 kb resolution, this will involve tracing one hundred individual adjacent target regions of interest within the genomic stretch. To achieve higher genomic and spatial resolution, divide the total genomic stretch into a larger number of individual targets. This strategy of probe design is based on early multiplexed error-robust fluorescence *in situ* hybridization (MERFISH) and chromosome tracing methods in cultured cells (Chen et al., 2015; Moffitt and Zhuang, 2016; Wang et al., 2016).

Design Sequential FISH Probes

Timing: 2 days

1. Assemble the genomic coordinates of all the regions of interest (targets).
2. Extract the genomic sequence from the above coordinates for each target from the genome assembly. This can be done using bedtools *getfasta* (Quinlan and Hall, 2010) or similar tools (see below).
3. Design non-overlapping 30 nt (or 42 nt) long sequences homologous to the extracted genomic sequences for each target. A shorter sequence length (i.e., 30 nt) will allow you to design a larger number of individual probes per target for increased signal, while a longer sequence will have greater hybridization stringency.

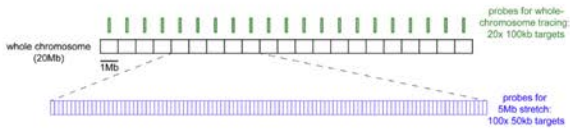

Figure 1. Schematic of Chromosome Tracing Strategies

Two example strategies are presented: whole-chromosome tracing (green, 1 Mb resolution) and sub-chromosomal tracing (blue, 50 kb resolution). The average localization precision is ~50 nm (Wang et al., 2016). Modifications to increase the number of individual targets and/or decrease the spacing between targets will increase the genomic resolution of chromosome tracing.

Use the following parameters:

a. Density of at least 10 sequences/kb genomic DNA (eg. 500 sequences for a 50 kb target).
b. T_m 60°C–100°C (78°C–100°C for 42 nt sequences). We calculate T_m according to the nearest neighbor model using 1 M Na^+ and 1 μM DNA concentrations (Rouillard et al., 2003).
c. GC content 30%–90%.
d. No secondary structure or cross-hybridization with a T_m greater than 70°C (76°C for 42 nt sequences).
e. No stretches of 7 or more identical nucleotides.

Note: Since genomic coverage/number of desired targets is typically large, this step is greatly facilitated by using probe design software like OligoArray (Rouillard et al., 2003), or similar programs, on a high performance computing cluster to process genomic sequences in batch.

4. Ensure the homologous sequences designed in step 3 are unique using NCBI BLAST+.
5. For each of the probes to each target, fuse the homologous sequence obtained in steps 3–4 to three additional unique sequences to produce the primary FISH probe templates (see Figure 2). These sequences should be absent from the genome.
   a. 20 nt Forward amplification sequence (red)
   b. 20 nt Reverse amplification sequence (blue)
   c. 20–30 nt Secondary probe binding sequence, unique for each target region (green)

Note: For users who prefer not to use UNIX and other programming languages, steps 2–5 can be completed by sub-setting from pre-designed Oligopaint probe libraries through a recently developed user-friendly application called PaintSHOP (Hershberg et al., 2020).

Note: Modifications to probe structure can easily be made to accommodate alternative secondary probe removal strategies and/or to increase fluorescent signal per primary probe (Mateo et al., 2019; Moffitt et al., 2016; Sawh et al., 2020; Xia et al., 2019). For example, adding an additional secondary binding sequence (2× green) on each primary probe will increase the signal 2-fold. See the note after step 8 in "Design Sequential FISH Probes" for more information regarding secondary probe alternatives. A list of validated amplification and secondary binding sequences for *Caenorhabditis elegans* samples can be found in (Sawh et al., 2020).

6. Order all of the template oligonucleotides to be synthesized as a pool, where up to ~1 fmol of each oligonucleotide is present. Sub-libraries can be incorporated in a single large oligo pool by specifying different forward and reverse amplification sequences for each sub-library.
7. Order oligonucleotides to amplify the template pool. The final FISH probes will be the reverse complement of the template (see "Amplify Probe Library," Figure 3).

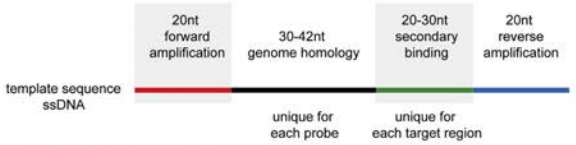

Figure 2. Template for Primary DNA FISH Probes
Template oligonucleotides contain at least four regions: a forward amplification sequence (red), a genomic homologous region (black), a secondary probe binding sequence (green), and a reverse amplification sequence (blue).

a. The forward amplification primer contains the same sequence found in the template (5a, red) and an additional T7 promoter sequence. The following fused to the 5′ of the sequence in 5a has worked well in amplification: GCCGTACGGATAATACGACTCACTATAGGG. This primer will append three additional C nucleotides to the 3′ end of the final FISH probes.
b. The reverse amplification primer is the reverse complement of the sequence at step 5b.
c. The reverse transcription primer is the same sequence as the reverse amplification primer (7b) but contains a 5′ fluorophore label.

8. Order 5′ end fluorescently labeled secondary probes of the same sequence as step 5c. Secondary probes can be additionally 3′ labeled to increase fluorescent signal per target.

Note: Instead of directly fluorophore-labeled secondary probes, alternative strategies can be readily used to detect each target and remove the signal from each round. One option is to use secondary probes where the fluorophore is coupled to the oligo through a disulfide bond (Moffitt et al., 2016). Fluorescent signal is removed by cleavage of this bond to remove the fluorophore. Another economical strategy involves using an unlabeled and target-specific bridging oligo between the primary and secondary probes, where all targets share a common secondary probe (Mateo et al., 2019). The latter strategy allows the user to remove the

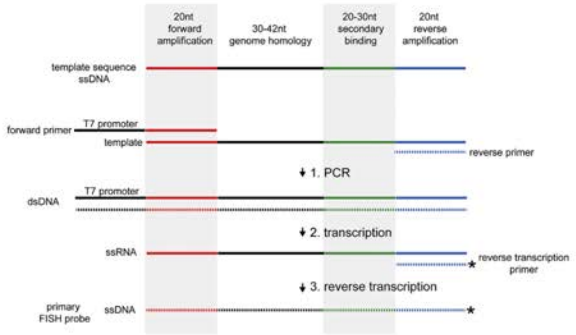

Figure 3. Generation of Primary DNA FISH Probes
Primary probes are generated by enzymatic amplification of the template ssDNA complex oligonucleotide pool. First, a T7 promoter is added and dsDNA is produced. Second, a high yield in vitro T7 transcription reaction generates ssRNA. Third, the ssRNA is converted to fluorophore-labeled ssDNA by reverse transcription and used for FISH. Solid lines indicate the sense strand, dashed lines indicate the antisense strand, and an asterisk indicates the fluorophore.

fluorescent signal from each round using strand-displacement. These alternatives decrease the total experiment time (vs. photo-bleaching) if a large number of fields of view are imaged.

Amplify Probe Library

Timing: 2–3 days

Note: All reagents, consumables, and equipment should be RNase free. Tubes should be low DNA/RNA binding.

9. Perform a limited cycle amplification of the template oligonucleotide pool to add a T7 promoter.
   a. Prepare the PCR reaction mixture, adding reagents in the following order.

Reagent	Volume (μL)
ddH_2O	12.5
Phusion Hot Start Master Mix	25
5 μM primer mix (from step 7a and 7b)	5
20× EvaGreen	2.5
0.6 ng/μL probe template pool	5
Total	**50**

   b. Run the following program on a q-RT PCR machine to monitor the reaction product amounts from cycle to cycle.

PCR Cycling Conditions			
Steps	**Temperature**	**Time**	**Cycles**
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	10 s	10–20[b]
Annealing	59°C	10 s	
Extension	72°C	15 s[a]	

[a]This extension time is suitable for 100-nt-long probe templates
[b]End the program when the slope of the amplification curve becomes constant (curve begins to look linear), and at the end of a 72°C extension step.

10. Purify the PCR product using a Zymo Spin IC column according to the manufacturer's instructions, except elute the DNA with 13 μL ddH_2O.
11. Perform a high yield in vitro transcription reaction to amplify the probe amount.
    a. Prepare the reaction mixture using the NEB Quick T7 High Yield kit and incubate at 37°C for 16 h in a PCR machine.

Reagent	Volume (μL)
ddH_2O	7.5
10 mM NTP Buffer Mix	10
T7 mix	2
RNase inhibitor	1
Template DNA (PCR product from step 10)	10
Total	**30.5**

12. Perform a reverse transcription reaction to convert the high yield RNA in step 11 to single-stranded DNA. This product is the primary probe pool used in FISH, and contains a 5′ fluorophore.

⚠ CRITICAL: protect the reaction from light from this step forward to prevent bleaching the fluorophore.

a. Prepare the reaction mixture and incubate at 50°C for 1 h.

Reagent	Volume (μL)
ddH_2O	5
10 mM dNTP Buffer Mix	12
100 μM reverse transcription primer (step 7c)	10
5× Maxima Buffer	15
RNase inhibitor	1.5
Maxima H-	1.5
RNA template (product from step 11)	30
Total	**75**

13. Add 75 μL Alkaline Hydrolysis Buffer (equal parts 0.5 M EDTA and 1 N NaOH) to the reaction and incubate at 95°C for 7–10 min. ATTO565-, Alexa647-, or ATTO647-labeled cDNA is stable to these conditions.

Optional: RNA may also be removed by enzymatic degradation with RNase A treatment.

14. Purify the ssDNA using Zymo Spin V columns and the Oligo Clean and Concentrate protocol according to the manufacturer's instructions. Elute the purified DNA with 90 μL ddH_2O.
15. Quantify the amount of DNA in the above eluate. The expected yield is ~350 ng/μL for 100-nt-long probes.
16. Reserve 1 μL of the probe solution to analyze by UREA PAGE for size and purity (next step), and dry the remaining amount of probe in a speedvac and store at −20°C.
17. Analyze probes by UREA PAGE.
    a. Dilute the 1 μL of probe with 9 μL ddH_2O and mix with 10 μL TBE-Urea sample buffer. Create the same dilution for 1 μL of the GeneRuler UltraLow Range ladder.
    b. Heat the sample and ladder at 95°C for 5 min.
    c. Run the sample and ladder on a 15% TBE/Urea polyacrylamide gel in TBE buffer at ~150 V.
    d. Stain the gel with GelGreen or similar nucleic acid stain and visualize with a transilluminator or laser scanner with a long pass green filter. A successful amplification will generate a strong band at the expected size on the gel. See Figure 7.
18. When ready to use the purified primary probes for FISH, resuspend the dried probe to 100 μM in ddH_2O. Use typically ~1 μM final concentration primary probe per hybridization. Store the resuspended probe at −20°C.

Note: It is important to optimize this concentration for each new amplification reaction or probe set by performing several hybridizations in parallel with a range of probe concentrations (i.e., 0.5–3 μM).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
UltraPure DNase/RNase-Free Distilled Water	Fisher Scientific	10977049
20× EvaGreen	Anawa	31000-T
EDTA (0.5 M), pH 8.0	Fisher Scientific	AM9260G
Sodium hydroxide solution (1.0 N)	VWR	EM-SX0607H-6
2× TBE-Urea sample buffer	Bio-Rad	1610768
GeneRuler UltraLow Range DNA ladder	Fisher Scientific	SM1213
GelGreen	Anawa	41005-T
Dextran Sulfate	Fisher Scientific	bp1585100
Tween-20	Sigma-Aldrich	P9416
20× SSC	Fisher Scientific	AM9763
Poly-L-lysine	Sigma-Aldrich	P8920-100ML
Paraformaldehyde, 16%	EMS	EMS-15710-10X10ML
PureLink™ RNase A (20 mg/mL)	Thermo Fisher Scientific	12091021
Triton-X100	Sigma-Aldrich	T8787
10× PBS pH 7.4 (RNase free)	Life Technologies	AM9625
Methanol, >99.8% purity	Sigma-Aldrich	32213-2.5L
OmniPur® Formamide, Deionized	Merck Millipore	4650-500ML
Ethylene Carbonate 98%	Sigma-Aldrich	E26258
DAPI Solution (1 mg/mL)	ThermoFisher	62248
TetraSpeck™ Microspheres, 0.1 µm, fluorescent blue/green/orange/dark red	Fisher Scientific	T7279
FluoSpheres™ Carboxylate-Modified Microspheres, 0.1 µm, yellow-green fluorescent (505/515), 2% solids	Thermo Fisher	F8803
RiboLock RNase Inhibitor (40 U/µL)	Fisher Scientific	EO0381
Ethanol, 96% purity	Sigma-Aldrich	02890-2.5L-F
GeneRuler Ultra Low Range DNA Ladder	Fisher Scientific	SM1211
Critical Commercial Assays		
Phusion Hot Start Master Mix	Thermo Scientific	F565S
HiScribe T7 Quick High Yield RNA Synthesis Kit	BioConcept	E2050S
Maxima H Minus Reverse Transcriptase (200 U/µL)	Fisher Scientific	EP0751
Zymo DNA Clean & Concentrator-5 with Spin IC Columns	LucernaChem	D4013
Zymo Spin V columns	LucernaChem	C1012-25
Zymo Oligo Binding Buffer	LucernaChem	D4060-1-10
Deposited Data		
Raw imaging data generated in this study	Mendeley Data	https://doi.org/10.17632/dj98d9z2rr.1
Experimental Models: Organisms/Strains		
C. elegans: wild-type:N2	Caenorhabditis Genetics Center (CGC)	N2

MATERIALS AND EQUIPMENT

Fixation Solution	Final Concentration	Volume (μL)
16% Paraformaldehyde (PFA)	1%	12.5
1% Triton X-100	0.05%	10
10× PBS	1×	20
ddH_2O	n/a	157.5
Total	**n/a**	**200**

⚠ CRITICAL: PFA is a hazardous chemical, follow the appropriate guidelines in the safety data sheet. Store in ~50 μL aliquots and avoid multiple freeze-thaw cycles.

PBS	Final Concentration	Volume (mL)
10× PBS	1×	50
ddH_2O	n/a	450
Total	**n/a**	**500**
PBST	**Final Concentration**	**Volume (mL)**
10× PBS	1×	50
100% Triton X-100	0.5%	2.5
ddH_2O	n/a	447.5
Total	**n/a**	**500**
2× Primary Probe Hybridization Buffer	**Final Concentration**	**Volume (mL)**
Dextran sulfate	20%	2g
10% Tween-20	0.2%	0.2
20× SSC	4×	2
ddH_2O	n/a	7.8
Total	**n/a**	**10**

Dissolve the dextran sulfate in the buffer by rotating tube end-over-end for several hours until dissolved, store aliquots at −20°C. Defrost and mix the aliquot well before use.

1× Primary Probe Hybridization Buffer	Final Concentration	Volume (μL)
2× Primary probe hybridization buffer	1×	50
Formamide	50%	50
Total	**n/a**	**100**

⚠ CRITICAL: Formamide is a hazardous chemical, follow the appropriate guidelines in the safety data sheet.

2× SSC/Imaging Buffer	Final Concentration	Volume (mL)
20× SSC	2×	50
ddH_2O	n/a	450
Total	**n/a**	**500**
0.5× SSC	**Final Concentration**	**Volume (mL)**
20× SSC	0.5×	12.5

(Continued on next page)

Continued

0.5× SSC	Final Concentration	Volume (mL)
ddH_2O	n/a	487.5
Total	n/a	500

Secondary Probe Hybridization/Wash Buffer	Final Concentration	Volume (mL)
20× SSC	2×	5
50% Ethylene Carbonate	25%	25
ddH_2O	n/a	20
Total	n/a	50

Equipment:

A computer-controlled microfluidics system similar to one described in (Moffitt and Zhuang, 2016) will greatly facilitate automated buffer exchange steps during the sequential secondary hybridizations (See Figure 6), however, these steps can be performed manually if necessary. The probes described above worked well with a fluidics system allowing at least 20 hybridizations + washes with two color imaging, with a ~16–24 h run time.

An FCS2 sample chamber (Bioptechs, 060319-2-03) is needed to house the sample on the microscope and is compatible with both imaging and fluid-handling.

A microscope capable of smFISH imaging is required for this protocol. We have experienced good results using near-TIRF and wide-field illumination for *C. elegans* embryos. We currently use a Nikon Ti2 microscope, a 100× oil objective (CFI Plan Apochromat Lamda, N.A. 1.45), a Lumencor SpectraIII light source (390 nm, 475 nm, 555 nm, 635 nm) for imaging, Omicron 1 W lasers (561 nm, 647 nm) for photo-bleaching, and a Photometrics Prime 95B CMOS camera. This labeling strategy is also compatible with super-resolution imaging techniques like STORM if the appropriate fluorophores are employed (Bintu et al., 2018).

STEP-BY-STEP METHOD DETAILS

Embryo Preparation

Timing: 2–3 h

The following sample preparation steps were optimized for early *C. elegans* embryos, but may also be applicable to other tissue samples of similar thickness (20–30 μm). The initial simultaneous fixation and sample clearing step is key to obtain low non-specific and cytoplasmic staining. Embryos are isolated from *C. elegans* adults, fixed using paraformaldehyde, permeabilized, and cleared before FISH probe hybridization:

1. Coat a 40 mm round coverslip with poly-L-lysine.
   a. Pre-clean the coverslip with >95% ethanol and dry well with a dust-free wipe.
   b. Pipet a small amount (2–3 μL) of poly-L-lysine onto the center of the cleaned coverslip.
   c. Turn the pipet tip parallel to the coverslip and rub the solution evenly across the glass until it has dried.
   d. Allow the coverslip to dry for at least 2 h at 20°C–23°C before use, protected from light and dust. Use within 2 days.

   Note: It is important that the film dries evenly on the coverslip surface to create a uniform layer for the embryos. Alternative coating procedures can be followed, such as using diluted poly-L-lysine and drying the coverslip in an oven (see Sigma-Aldrich product technical bulletin).

2. Dissect embryos and adhere to coverslip.
   a. Transfer ~50 adult *C. elegans* into a watchglass with 200 µL MQ-H_2O.
   b. Using a needle, dissect embryos from adult animals under a dissecting microscope.
   c. Remove adult bodies and as much MQ-H_2O as possible from the watchglass.
   d. Rinse the embryos with fresh MQ-H_2O twice.
   e. Pipet dissected embryos to the coated coverslip. Let them settle and adhere for 5 min.

   Note: Embryos may also be isolated by standard *C. elegans* bleaching protocols (Stiernagle, 2006).

   f. Remove as much MQ-H_2O as possible, but do not allow the embryos to dry out.
3. Simultaneous fixation and first permeabilization.

   ⚠ CRITICAL: The presence of detergent at the fixation step improves cytoplasmic clearance in the embryo. Prepare the fixation solution immediately prior to this step.

   a. Pipet 50 µL of fixation solution onto embryos.
   b. Place a 20 × 20 mm square coverslip over this drop to create a sandwich. See Figure 4.
   c. Wick away fixation solution with a dust-free wipe until the square coverslip touches the embryo shell. Monitor the embryos during this step using a dissection microscope. Do not over-compress the embryos.
   d. Incubate at 20°C–23°C for 5 min.
   e. Place sandwich on a metal block pre-cooled with dry ice for 30 min minimum, with the square coverslip facing up.

   ⏸ **Pause Point:** Sandwiches can be stored at least a few months at −80°C at this point in a frost-free container.

4. With a razor blade, quickly flick off the square coverslip from the frozen sandwich while it is still on the metal block. This step removes the top layer of the embryo shell.

   ⚠ CRITICAL: Keep the sandwich on the dry ice-cooled block and do not allow the sample to thaw.

5. Immediately submerge the round coverslip with embryos in ice-cold methanol for 5 min.
6. Wash in 1× PBS for 5 min. A volume of 10 mL is sufficient for each washing step.
7. Wash 3× in PBST (PBS/0.5% Triton) for 15 min each.

   ⚠ CRITICAL: Use freshly prepared PBST.

8. Wash in 1× PBS for 5 min.
9. Wipe away excess liquid.
10. RNase A treatment. Prepare 2 µL 10 mg/mL RNase A in 400 µL 2× SSC. Apply 100 µL per coverslip and incubate at 37°C for 30 min in a humid chamber.
11. Wipe away excess liquid.
12. Block the sample for 1 h at 37°C with 100 µL Hybridization buffer/50% formamide in a humid chamber.

    ⚠ CRITICAL: Ensure the blocking and hybridization buffers are well mixed.

    Note: This temperature is used for probes with 30 nt homology to genomic sequence. For probes with 42 nt homology to genome target, increase all 37°C temp steps beginning

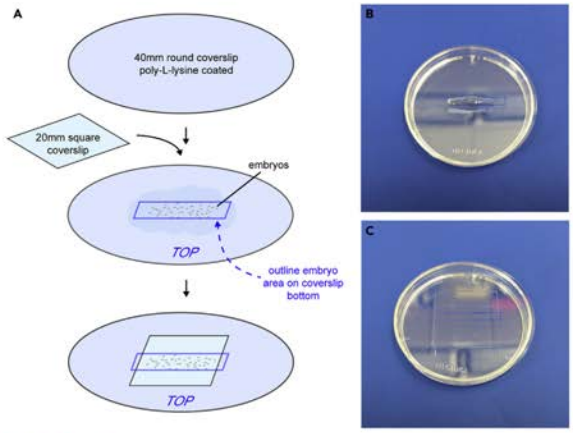

Figure 4. Sample Preparation

(A) Schematic of initial sample preparation. Embryos are adhered to a coverslip suitable for the flow chamber and sandwiched with a second coverslip.

(B) Image of a round coverslip with embryos in a drop near the center.

(C) Image of the coverslip-embryo-coverslip sandwich.

with blocking to 47°C. Optimal temperatures for other probe lengths should be empirically determined.

Primary Probe Hybridization

Timing: 2–3 days

Primary FISH probes are hybridized to their genomic targets.

13. Prepare the primary probe at ~1 μM final concentration in Hybridization buffer/50% formamide. Mix well.

Note: Optimal probe concentrations should be empirically determined for each newly prepared library amplification product.

14. Remove as much blocking solution as possible from the coverslip with the sample by wiping carefully around the sample with a dust-free wipe.
15. Apply 30 μL probe solution to a cleaned glass slide and invert sample coverslip onto the slide to create another sandwich (embryos facing the middle). Avoid creating bubbles. See Figure 5.

16. Incubate at 20°C–23°C for 10 min in a light-protected humid chamber.
17. Transfer sandwich onto the surface of an 80°C humid heat block, on top of a water droplet, and cover with a light-protected box for 10 min.

Note: A simple humid heat block can be created using a dry bath that holds two removable heating blocks. Invert one block to create a smooth surface to place the sample, and fill the

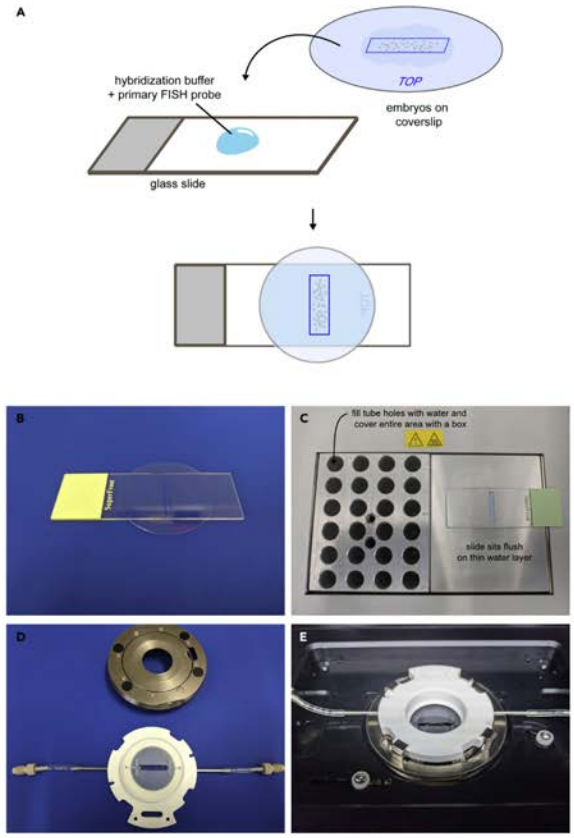

Figure 5. Primary Probe Hybridization

(A) Schematic of primary probe hybridization sandwich. Embryos on the round coverslip are inverted onto a droplet of probe in hybridization buffer.

(B) Image of inverted coverslip on glass slide.

(C) Image of humid heat block with slide-embryo-coverslip sandwich.

(D and E) Image of sample coverslip assembled in the Bioptechs chamber and mounted on the microscope for imaging.

tube holes of the second block with water to humidify the environment when the entire two-block area is covered with a light-protected box.

18. Transfer the sample to a light-protected humid chamber and incubate at 37°C for 16 h.

Note: This hybridization time can be extended to at least 48 h to improve signal strength.

19. Dislodge the coverslip from the slide carefully by submerging the sandwich in 2× SSC and allowing the coverslip to float off.
20. Wash for 1 h in pre-warmed 2× SSC/50% formamide at 37°C.
21. Wash 2 × 30 min in pre-warmed 2× SSC at 37°C.
22. Wash 2 × 30 min in pre-warmed 0.5× SSC at 37°C.

Optional: Post-fix the sample in 1% PFA/PBS/0.05% Triton X-100 for 10 min at 20°C–23°C. This additional fixation step could be helpful if the primary probes have shorter homology lengths, or to preserve the sample if you observe some degradation after numerous secondary hybridization rounds. We have not observed any degradation of *C. elegans* embryos with up to 20 rounds.

23. Store the sample coverslip in 2× SSC at 4°C for as little time as possible before imaging. Fluorescent signal is strongest if the washes are performed the same day as imaging.

Sequential FISH

Primary FISH probes are imaged followed by secondary probe hybridizations and imaging. See Figure 6.

Note: Use multiple spectrally distinct fluorophores in a single secondary hybridization round to detect more than one target in a round. This will increase the multiplexing and decrease the total experiment time, but requires correction for chromatic aberration.

24. Incubate sample with nuclear stain.
    a. Dilute the DAPI Stock Solution 1:1000 in 2× SSC.
    b. Filter the stain to remove dye aggregates using a 0.2 μm syringe filter.
    c. Add stain to completely cover the sample. Protect the sample from light and incubate at 20°C–23°C for 2–10 min.
    d. Wash 2 × 1 min with 2× SSC.
25. Assemble the Bioptechs sample chamber according to the manufacturer's instructions and add 100 μL fiducial beads solution to the flow channel.

Note: Since the beads will begin to stick to the plastic tube surface, prepare the fiducial bead dilution at this point, not earlier. The optimal dilution should be determined empirically to result in 5–10 beads adhered to the coverslip per field of view. Vortex the bead stock and dilution thoroughly immediately prior to pipetting.

26. Invert the sample onto the channel (embryos facing beads solution in the channel) and seal the chamber as tightly as possible. Ensure there are no leaks in the chamber and clean the coverslip surface with isopropanol.
27. Prepare a glass microscope slide with well-separated 100 nm TetraSpeck beads to calibrate the microscope and computationally register the channels if more than one channel is used for imaging secondary probes.
28. Capture a ~10 μm z-stack of the TetraSpeck beads in the channels used for FISH probes. A field of view containing ~20 beads is suitable.
29. Prepare ~2.5 mL of each secondary hybridization solution with the appropriate fluorescent probe(s) at 8 nM final concentration. Multiple targets can be visualized in a single hybridization round if unique fluorophores are combined.
30. Flush all tubes of the flow system with freshly prepared 2× SSC. Ensure there are no bubbles in the fluid-handling tubing and that the flow rate is constant (0.6 mL/min).
31. Replace the storage buffers for each flow system position with the appropriate secondary hybridization solution, imaging buffer, or washing buffer.
32. Attach the sample chamber to the flow system and wash with imaging buffer for 2 min.

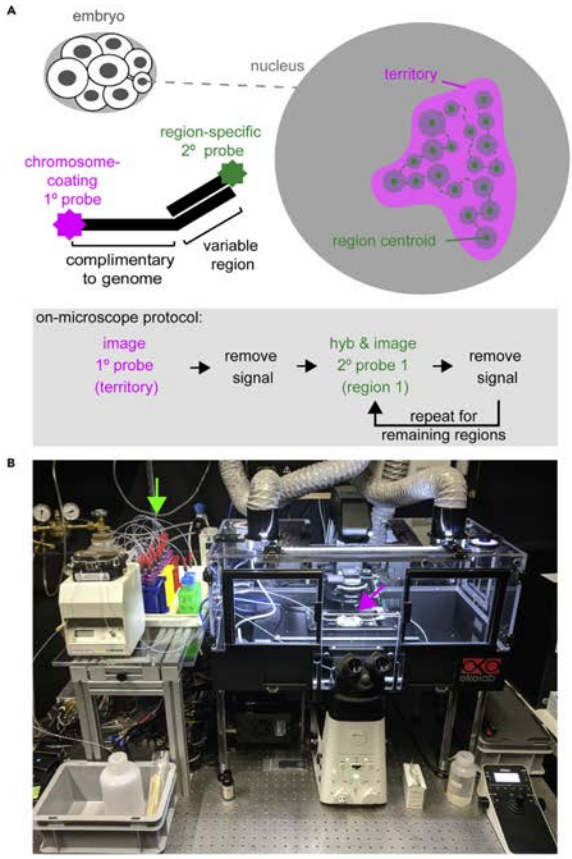

Figure 6. Overview of Chromosome Tracing

(A) Schematic of sequential FISH protocol. First, primary probes are imaged to define the volume occupied by the entire genomic stretch (e.g., chromosome territory, magenta). Second, primary probe fluorescent signal is removed by photo-bleaching or other means. Third, secondary hybridization and imaging commences. Secondary rounds are separated with signal removal steps.

(B) Image of a microfluidics system (green arrow) coupled to the sample chamber (magenta arrow) mounted on a wide-field inverted microscope (Nikon Ti2).

33. Choose multiple fields of view (FOVs) containing embryos of interest with strong primary probe signal and 5–10 well-separated fiducial beads.
34. In the first round of imaging the FOVs, capture z-stacks of the entire embryo in the DAPI, bead, and primary probe channels.

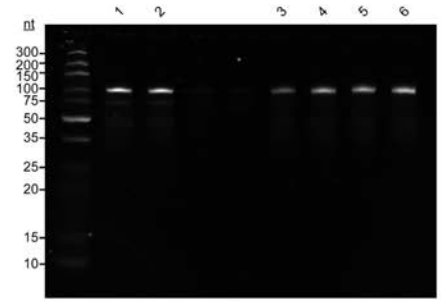

Figure 7. UREA PAGE Verification of Primary Probe Pool

ATTO565-labeled primary probe pools were run on gel to confirm their size and purity after amplification and purification. Samples in lanes 1–6 were amplified from the same 102 nt oligo pool template in independent reactions.

Optional: If the fluorescent signal is weak, and/or fewer than ~100 probes are used per target, consider alternative imaging buffers to boost the probe signal (Moffitt et al., 2016).

35. Photo-bleach each FOV in the probe channel until FISH signal is undetectable.

Note: The time of bleaching is dependent on the strength of the illumination source. This parameter should be empirically determined on each microscopy system. For example, photo-bleaching with a 1 W laser at full power is typically complete in 30–40 s, while an LED may take 2–3 times longer.

36. Exchange the imaging chamber buffer for the first secondary hybridization solution by flowing this solution through the chamber for 3 min. Let the sample incubate for 10–30 min to allow the secondary probes to anneal to their targets.
37. Wash the sample in the chamber by flowing washing buffer for 3 min.
38. Exchange the wash buffer for imaging buffer for 2 min.
39. Capture z-stacks of all FOVs in the bead and probe channels.
40. Photo-bleach each FOV in the probe channels until FISH signal is undetectable.

Optional: The secondary bleaching steps may be replaced with alternative chemical cleavage (Moffitt et al., 2016) or strand-displacement methods (Mateo et al., 2019) to remove probe signal.

41. Repeat steps 35–39 for all remaining secondary hybridization rounds.

EXPECTED OUTCOMES

The primary probe signal detected by wide-field fluorescence imaging appears as a sub-nuclear volume. If the primary probes label a continuous genomic stretch, the signal will be continuous. If individual targets are separated by unlabeled genomic regions (Figure 8), the signal will appear more punctate. Individual targets detected by secondary probe hybridization and imaging appear as diffraction-limited spots (Figure 9). The degree of chromatin compaction will influence the diffusiveness of the fluorescent signal for both primary and secondary probes. The 3D locations of the DNA targets can be determined using spot finder algorithms, corrected for stage drift during the

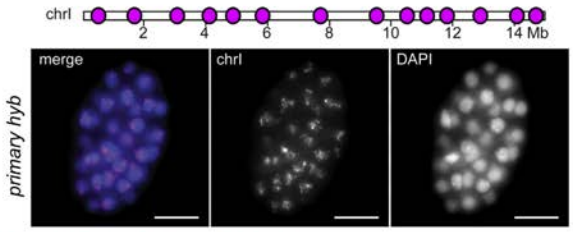

Figure 8. Primary Probes Detect Chromosome Territories

(Top) ChrI FISH probe locations along the chromosome. The probes detect 100 kb regions at the indicated locations, separated by unprobed genomic stretches. All regions were detected simultaneously using the primary probe fluorophore (magenta) and overlayed with the DAPI nuclear stain (blue). The images are z-projections of a central 6 μm z-stack, taken on a Nikon Ti2 under 100× magnification with a Photometrics Prime 95B CMOS camera. The entire image was de-noised automatically in NIS Elements. Scale bar, 10 μm.

experiment (Bintu et al., 2018; Cardozo Gizzi et al., 2019; Mateo et al., 2019; Nir et al., 2018; Sawh et al., 2020; Wang et al., 2016).

LIMITATIONS

In all FISH-based techniques, increasing the number of probes will increase the signal to noise ratios per target. In chromosome tracing we aspire to roughly equivalent signal strengths for all the targets in a sample, so an equivalent number of probes should be designed for each location. This method will also be limited by the sensitivity and resolution of the microscopy system at hand. In practice we have achieved easily detectable foci using a wide-field system on targets using 10 probes/kb density. It is important to note that if one target region is more compactly folded than another, its FISH signal focus will appear brighter and less diffuse. In this strategy we probe genomic regions that are unique, thus as a result repetitive sequences are avoided because the genomic location of any foci detected would be ambiguous. Our protocol is optimized for early *C. elegans* embryos (up to ~100 cells), but should generate similar results in older embryos and larvae if more stringent washes and cytoplasmic clearing are performed to reduce background signal.

TROUBLESHOOTING

Problem 1

Little or no probe amplification in limited cycle PCR.

Potential Solution 1

The first PCR reaction is highly dependent on the concentration of the template used. Ensure that the template is close to 0.6 ng/μL and that the template DNA is fully dissolved (if it was ordered lyophilized) prior to the PCR step. For the most consistent results, have the template dissolved in TE buffer by the company used for synthesis.

Low apparent yield at this step could also be caused by inefficient detection by the dye. EvaGreen dye can sometimes precipitate or stick to the inner surface of its tube, so it is important to vortex the dye vigorously prior to use and to replace the stock regularly.

Problem 2

Multiple strong species of primary probe on gel.

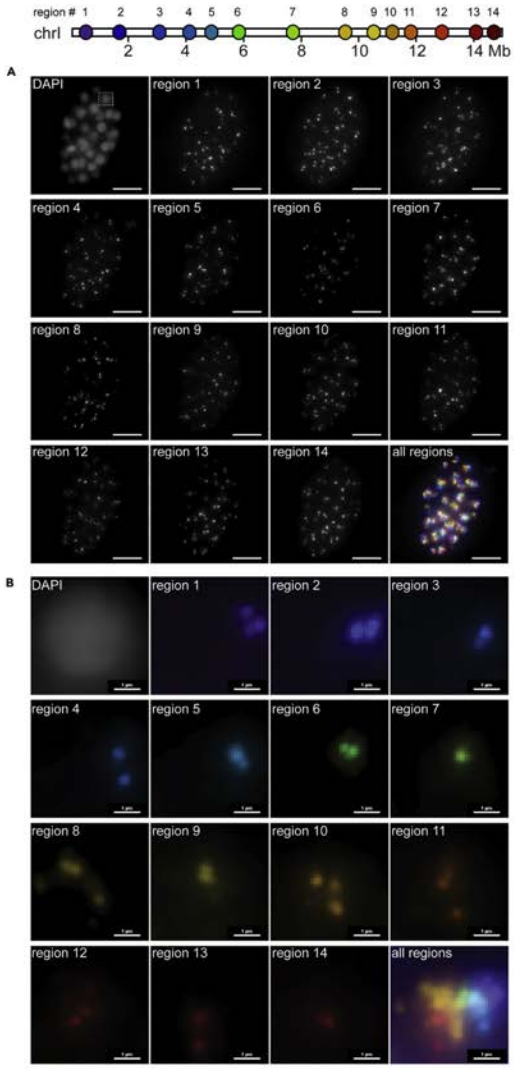
region # 1 2 3 4 5 6 7 8 9 10 11 12 13 14
chrI
2 4 6 8 10 12 14 Mb
A
DAPI
region 1
region 2
region 3
region 4
region 5
region 6
region 7
region 8
region 9
region 10
region 11
region 12
region 13
region 14
all regions
B
DAPI
region 1
region 2
region 3
region 4
region 5
region 6
region 7
region 8
region 9
region 10
region 11
region 12
region 13
region 14
all regions

Figure 9. Example of Representative Data

(A) Sequential secondary probe hybridizations detect individual targets. The 14 individual targets were imaged in seven sequential hybridization rounds using their unique secondary probes (two targets per round). Different targets in the same round used spectrally distinct fluorophores (ATTO565 for even-numbered targets and Alexa647 for odd-numbered targets), and their genomic positions are indicated in the top schematic. The expected number of foci per nucleus per target is 2–4, since embryonic cells are diploid and replicating. The images are central 6 μm z-projections of the same embryo in Figure 8, with identical processing. All targets are overlayed in pseudocolors according to the top schematic. Scale bar, 10 μm.

(B) Zoom-in of the nucleus in dashed outline in (A) with each target pseudo-colored according to the top schematic. Scale bar, 1 μm.

Potential Solution 2

The primary probe should appear as a strong band at the expected size on gel. Lower molecular weight species may be a result of over-amplification during the first limited cycle PCR reaction, which could lead to mis-priming. A lower number of cycles should prevent this problem.

A smear of smaller species may also indicate some degradation of the product at the RNA level. Ensure all reagents, equipment, and handling techniques are RNase free and to avoid this issue.

Problem 3

Little or no FISH signal in embryos or high background in images.

Potential Solution 3

If embryos are not sufficiently permeabilized and cleared, it will be more difficult for the DNA FISH probes to find their targets. This likely occurs if the embryo shell is not properly freeze-cracked or the buffers containing detergent are not freshly and accurately prepared.

If genomic DNA is not sufficiently denatured at the local scale prior to probe hybridization, this will also cause low signal:noise in the FISH images. Ensure that the temperature of the heat block is accurate and that the slide sits flush on the heat block surface on a thin layer of water during this step.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Susan Mango (susan.mango@unibas.ch).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

Raw imaging data from this study has been deposited to Mendeley Data (https://doi.org/10.17632/dj98d9z2rr.1). This study did not generate any additional unique datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.N.S. and S.E.M.; Investigation, A.N.S; Writing, A.N.S. and S.E.M.; Funding Acquisition, A.N.S. and S.E.M.; Supervision, S.E.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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