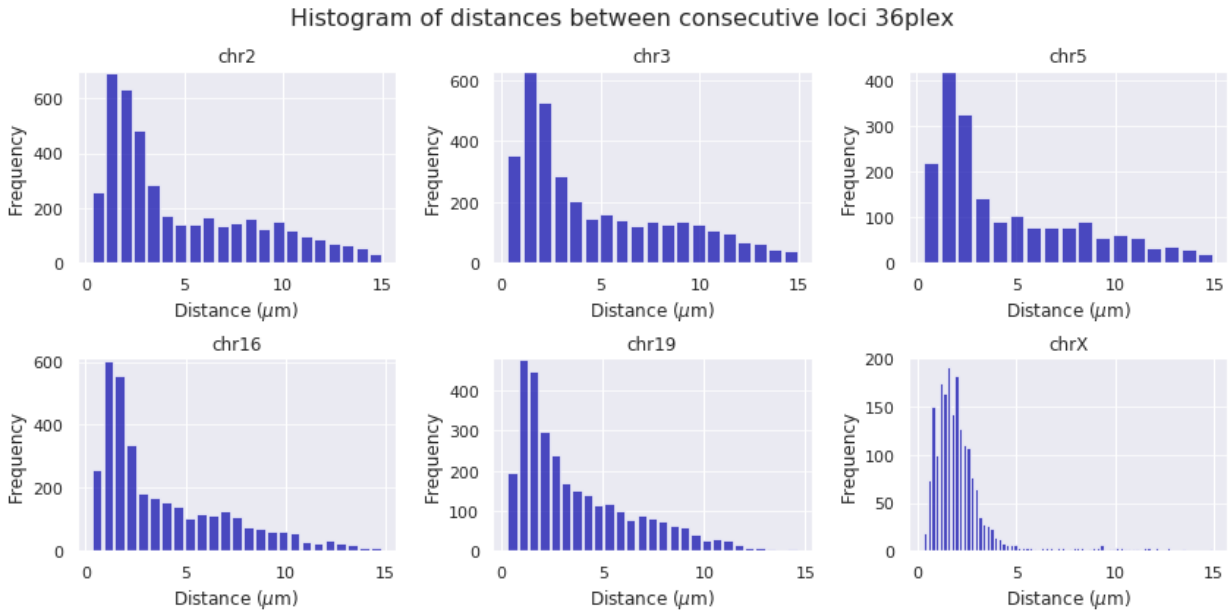
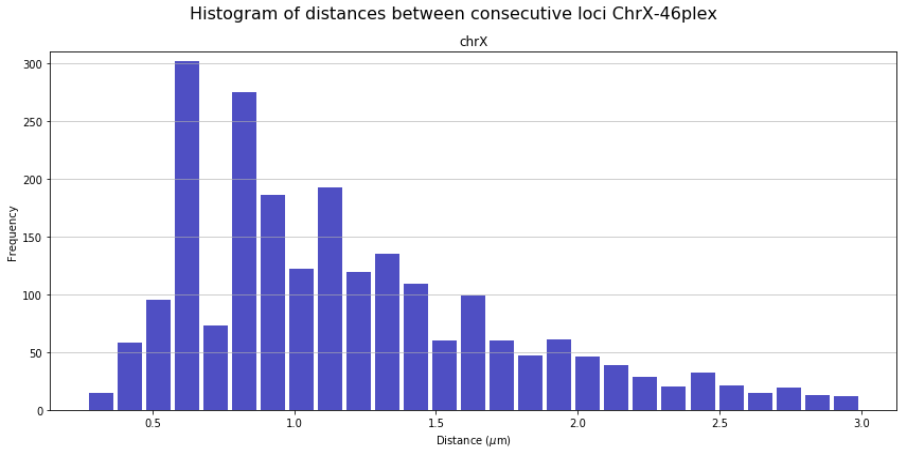
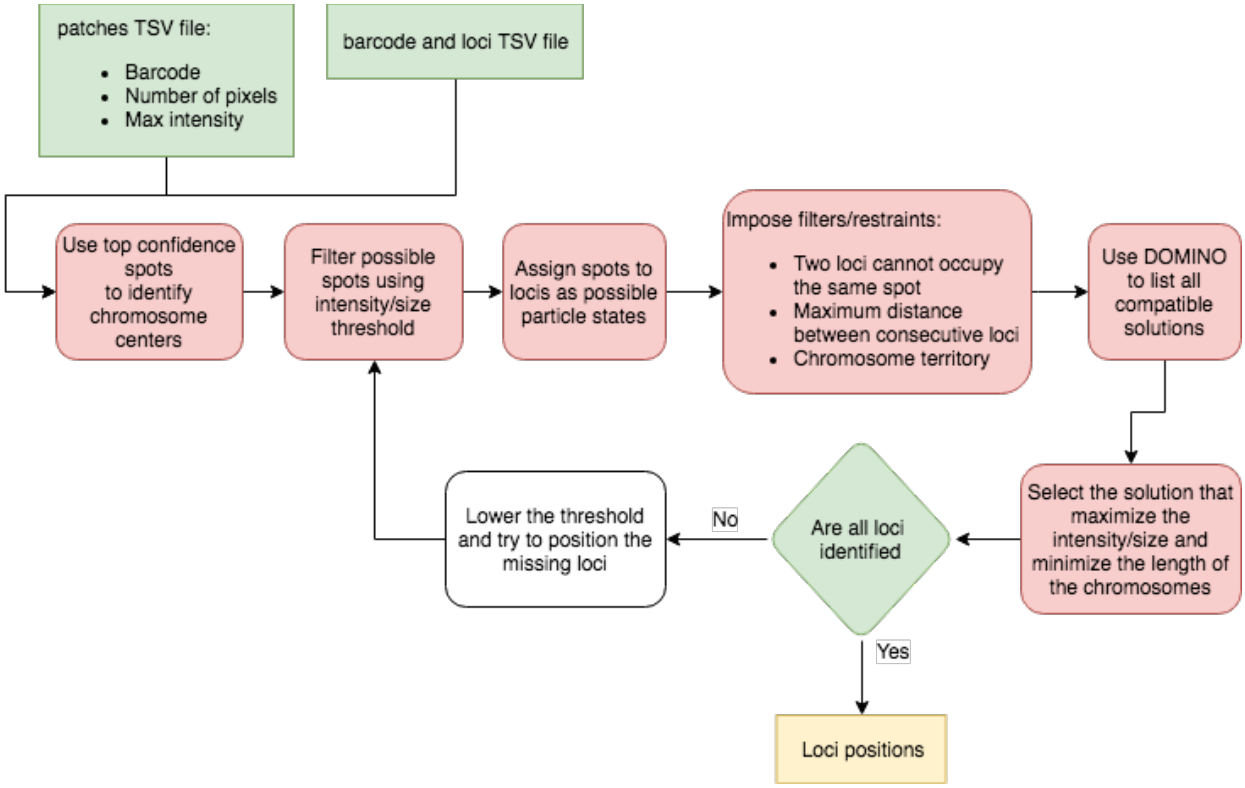


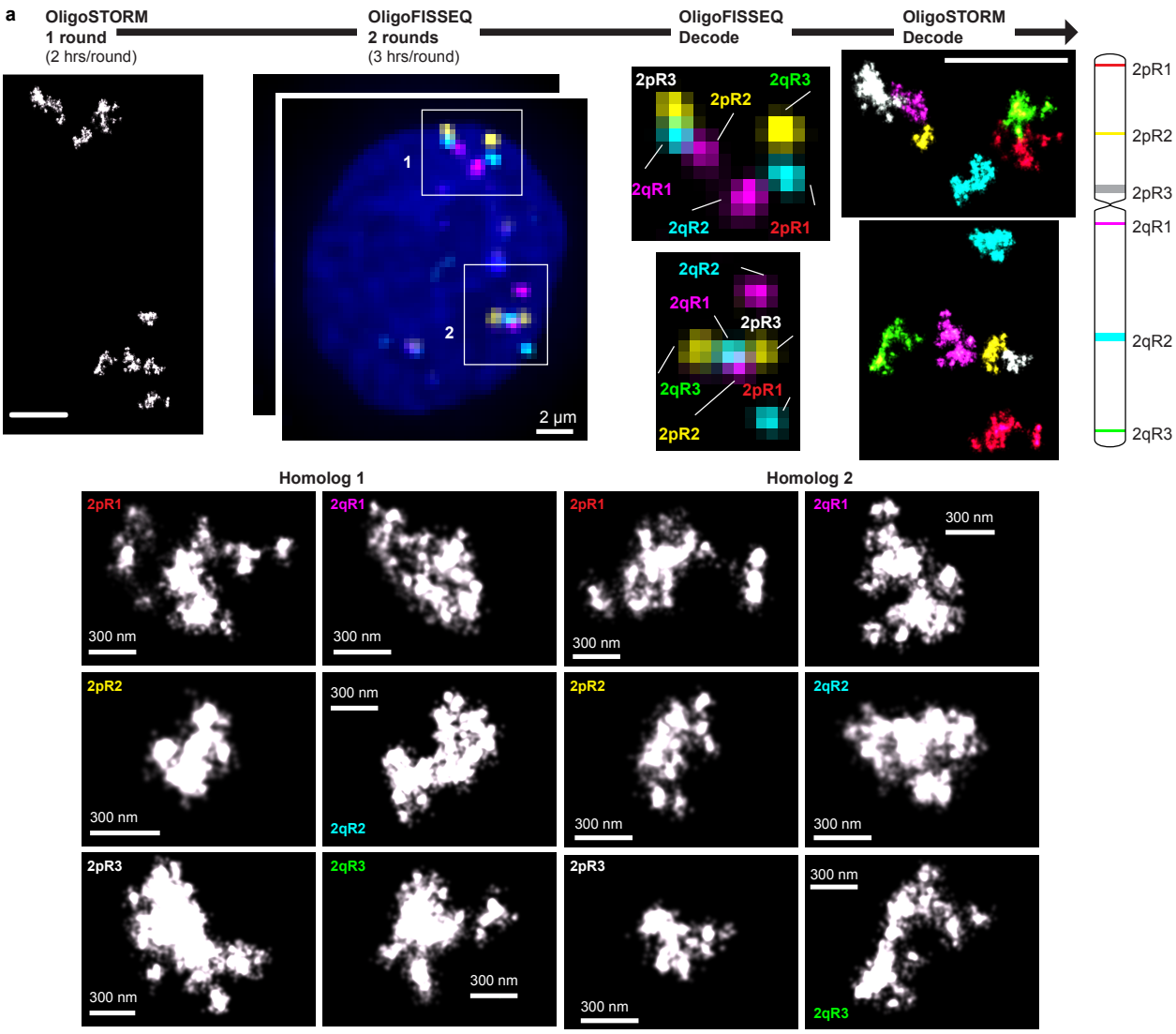
Supplementary Figure 1. Histogram of distances between consecutive loci for 36plex library



Supplementary Figure 2. Histogram of distances between consecutive loci for ChrX-46plex library



Supplementary Figure 3. Workflow chart of the Chromosome tracing process



Supplementary Figure 4. Combining OligoSTORM and OligoFISSEQ to accelerate super-resolution of Chr2-6plex library

a) Chr2-6plex-5K was hybridized to PGP1f cells and imaged with 1 round of OligoSTORM to visualize all targets simultaneously, followed by 2 rounds of O-LIT to decode targets. Left, OligoSTORM image showing 12 clusters, all unidentified at this point. Middle left, micrograph from first round of O-LIT. Image from deconvolved maximum z-projection. Middle right, zoom-in of targets decoded. Right, identified OligoSTORM images. Bottom, OligoSTORM images for each target. All scale bars represent 300 nm. n = 1.

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Supplementary Protocol 1

T7 Oligopaint Synthesis – Wu Lab Protocol

(Adapted from MERFISH protocol, Zhuang lab, Chen et al 2015 Science)

Overview of the method:

1) Amplification of the library, low cycle (~25 cycles)
2) Another amplification round from the product from step 1, and using touch-up PCR to add the T7 promoter to the reverse strand
3) T7 RNA synthesis (overnight incubation)
4) Reverse transcriptase for single-stranded DNA probe synthesis
5) Degradation of RNA via alkaline hydrolysis
6) Purification of ssDNA

"Linear" PCR of Library or Sub-library

· Goal: since the amount of "raw" library is limited (ie. CustomArray produces around 80 ul of a library at around 50-100 ng/ul), we regularly preform PCR to produce more template

Materials:

□ DNA Clean and Concentrator Kit -5 (Zymo D4013 or D4014)
□ Library (ie. Customarray 90K Oligo Pool)
□ Taq (Kapa BK1002)
□ dNTPs (10 mM stock)

Reagent	Initial	Final	1 reaction
Kapa Buffer A	10x	1x	10 ul
Forward Primer	200 uM	2 uM	1 ul
Reverse Primer	200 uM	2 uM	1 ul
dNTP mix	10 mM	0.4 mM	4 ul
Kapa Taq*	5 U/ul	5 U	1 ul
Raw library**		10-30 ng	1 ul
Water (**up to 100 ul**)			82 ul

*you can opt to use different Taq if you wish, but we generally use Kapa Taq for our experiments
**if your library has many sub-libraries, you want to scale the amount of library you use to bring up the sub-library amount to approximately 10-30 ng optimally

1) Linear PCR program **-**95C-5min, (95C-30s, **58C**-30s, 72C-15s)X**25**, 72C-5min

2) Purify with Zymo-5 kit, following manufacturer's instructions (2X PCR volume of DNA Binding Buffer + 4X PCR volume of 100% EtOH → then load onto columns)

a. We also add an additional spin step after last wash (max speed, no buffer) to remove residual ethanol from wash buffer

3) resuspend in ~30 ul of water

4) Expected yield: ≥ 20 ng/ul

Bulk touch-up PCR – to add T7 promoter

· Goal: produce PCR product with the T7 promoter, enough to produce 500 ng – 1 ug of product

Materials

□ Reverse Primer with T7 promoter sequence

○ Add TAATACGACTCACTATAGGG to the 5' end of the reverse primer

○ Note that T7 synthesis later adds the last three Gs into the sequence

1) Make the following master mix

a. Here we are doing a 50x reaction so that we have enough template to do multiple T7 reactions in the future – you can scale this according to your expected usage

Reagent	Initial	Final	1 reaction	50 reactions
Kapa Buffer A	10x	1x	10 ul	500 ul
Forward	10uM	0.4 uM	4ul	200ul
Reverse (T7)	10uM	0.4 uM	4ul	200ul
dNTP mix	10mM	0.2 mM	4ul	200ul
Kapa Taq			1ul	50ul
Linear-amplified library	1 ng/ul	1 ng	1ul	50ul
Water (**up to 100 ul**)			76ul	3.8ml
		Total	100 ul	

1) Do new program (based on EH1) T7PCR35 -95C-5min, (95C-30s, 60C-30s, 72C-15s)X35, 72C-5min

2) Purify – using Zymo-100 column for PCR purification, (2X PCR volume of DNA Binding Buffer + 4X PCR volume of 100% EtOH → then load onto columns) elute in 150 ul of water

3) **Expected Result:** you want samples to be around 75 ng/ul or more

T7 Reaction (using manufacturer's suggestions)

· Goal: produce excess RNA in order to maximize the amount of dNTPs

- 1 reaction can produce around 750 to 1500 pmol of probe

Materials

□ T7 HiScribe Kit (NEB E2040S)

□ Zymo-100 DNA Clean and Concentrator Kit (D4029 or D4030)

□ RNAseOUT (Thermo, 10777019)

1) Measure DNA to make sure amplification worked , run on gel
2) Use 1 ug of DNA per reaction

a. While you may opt to do 1 reaction, for large scale probe synthesis you can fit 4x reactions comfortably in a 1.5 ml tube

b. If you decide on the larger format (1.5 ml tube), make sure you have proper equipment to do incubations later on (at 37 ^{o}C, 50 ^{o}C, 85 ^{o}C, and 95 ^{o}C)

Reagent	Initial	Final	1x reaction (manufacturer)	4x reactions
DNA (1.3ug/RXN)		>1.3ug/Reaction	7	28
Water				
ATP (provided by kit)			2	8
CTP (provided by kit)			2	8
GTP (provided by kit)			2	8
UTP (provided by kit)			2	8
T7 buffer			2	8
RNaseOUT			1	4
T7 Pol Mix			2	8
		Total	20	80

RT PCR:

· Goal: convert RNA to DNA

Materials

□ Forward Primer

○ you can opt to use a forward primer that is unlabeled (for labeling in situ with secondary fluor-conjugated oligos) or you can use labeled primers. However, primers that are labeled with NHS-Ester linkages may be sensitive to alkaline hydrolysis in the next step (**see Murgha et al. 2015) ;** Cy3 and Cy5 oligos from IDT seem to survive the process, which may be due to linkage independent of NHS chemistry

□ dNTPs (**25 mM stock**)

○ take individual dNTPs at 100 mM concentration, and mix together to at 1:1:1:1 volume to produce 25 mM stock

□ maxima RT –H (Thermo, EP0751)

□ RNAseOUT (Thermo, 10777019)

No need to purify the T7 sample; add RT reagents right into tube and incubate

Reagent	Initial	Final	1 reaction	4 reactions	Huy's 4X
T7 sample			20	80	80
Water			79.4	317.6	77.6
Forward primer	200 uM	1500 pmol	7.5 ul	30	30
dNTP mix	**25 mM**	1.6 mM	9.6 ul	38.4	38.4
RT buffer	5x	1x	30 uL	120	60
RNaseOUT			1.5 uL	6	6
Maxima RT -H			2 uL	8	8
		Total	150 uL	600	300

1) Incubate for **two hours** at 50 °C (this is longer than manufacturer suggestion)
2) Inactivate RT at 85 C for 5 min

RNA degradation via alkaline hydrolysis:

· Goal: removal of RNA from the RNA:DNA hybrids, and removal of excess RNA not converted to cDNA

Materials

□ EDTA (0.5 M)
□ NaOH (1 M)

1) Digest remaining RNA:

a. by mixing 0.5 EDTA and 1 M NaOH, 1:1 to each other, and then add to RT reaction at equal volume (final conc: 0.25 M NaOH)
   i. 1x reaction: add 150 ul of EDTA-NaOH mixture to each sample
   ii. 4x reaction: add 600 ul
b. Heat at 95 °C for 10 min
   i. Alternative alkaline hydrolysis temperatures and times may also work
c. Put on ice and proceed right away with purification
d. Total rxn vol:
   i. 300 ul for 1x reaction
   ii. 1200 ul for 4x reaction

Purification with Zymo-100 kit

Materials

□ Zymo-100 DNA Clean and Concentrator Kit (D4029 or D4030)
○ We use this kit because of the scalability to purify 100 ug of DNA, but because the oligos are single stranded and short, we DO NOT use DNA binding buffer
○ Instead, we use Oligo binding buffer (purchased separately)
□ Oligo Binding Buffer, 40 ml (D4060-1-40)
□ Wash Buffer (additional bottles - D4003-2-48)

1) Add Oligo binding buffer (ordered separately from Zymo) (2x rxn vol = 2400 ul)
2) Add 100% Ethanol (4x oligo binding buffer = 9600 ul)
3) Purify on column and follow manufacturer instructions

a. Because the column has a 100 ug capacity, split your samples accordingly (ie. if you are doing a 4x reaction, split your mixture into 4, and run it on the column 4 times)

b. To concentrate the sample, use the eluate from the 1st purification to elute the 2nd purification, and continue this

Calculate Yield

1) ng/ul to pmol/ul conversion:
   - Formula: (ng/ul concentration) * (1E3 pg/ ng) * (1 pmol nucleotides / 330 pg) * (1 /length in nucleotides)
   - [conc] * 3.03 * (1/length) = ____ pmol/ul
2) Total pmol amount - Multiply concentration to elution volume
3) Calculate yield by: probe (pmol)/RT primer (pmol) * 100
   - We generally get 50% yield or more

Supplementary Protocol 2

Oligopaint FISH on Slides

DAY 1 (2-3hrs):

- Plate cells onto 1mm glass microscope slides; score/etch cell area with diamond pen
- 100uL of cells per spot

FISH 1o (2-3hrs):

- Perform in glass coplin jars (35-40mL)

1) Rinse: 1X PBS wash; **5 min @ RT**
2) Fix Cells; 4% Formaldehyde/PBS; (40mL = 10mL 16% formaldehyde + 4mL 10X PBS + 26mL H2O); **10 min @ RT**

2a) 1XPBS rinse

3) Permeabalize; 0.5% Triton/PBS; (40mL = 200uL Triton X-100 + 40mL 1X PBS); **15 min @ RT**
4) PBT (PBS + 0.1% Triton) wash; **5 min @ RT**
5) 0.1N HCl; 0.1N HCl/H2O; (40mL = 500uL 8N HCl + 39.5mL H2O); **5min @ RT**
6) 2XSSCT washes; **2x 5min @ RT**
7) Pre-hybe RT; 50% Formamide/2XSSCT; **10min @ RT**
8) Pre-hybe 60oC; 50% Formamide/2XSSCT; **20min @ 60oC**
9) 1o Oligopaint Hybridization;

***Prepare while sample is pre-hybing
***Air dry slides and wipe around cell area
***Generally, 100pmol per slide
***8X Hybe Buffer: 27-40% Crowding Agent (Dextran sulfate OR Polyacrylic Acid ~1200MW) + 8XSSCT
***Add to cells → cover with 22x22mm coverslip → seal edges with rubber cement → allow to dry before denaturing

Reagent	Stock	Final	Amount
Formamide	1	0.5	12.5
Hybe Buffer	8X	2X	6.25
RNAseA	10mg/uL	20mg	2
1o Oligopaint	100pmol/uL	100pmol (4uM)	1
H2O	n/a	n/a	3.25

10) Denature sample; **3min @ 80oC**

***Perform on hot block

11) Hybridization; transfer samples to humid chamber and hybridize O/N @ (**42oC**)

Day 2:

FISH 2o (~2hrs):

1) 2XSSCT hot wash; **20min @ 60oC**
2) 2XSSCT washes @ RT; **2x 7min @ RT**
3) 30% formamide/2XSSCT wash; **7min @ RT**
4) 2o Oligo; **>1hr @ RT**

***25uL per slide ***Protect from light

Reagent	Stock	Final	Amount
Formamide	100%	30%	7.5
SSCT	4X	2X	12.5
2o Probe	10pmol/uL	30pmol (1.2uM)	3

H2O	n/a	n/a	2

5) 30% Formamide/2XSSCT washes; **20min @ RT**
6) 2XSSCT washes; **2x 10min @ RT**
7) 1XPBS wash; **1x 10min @ RT**
8) Mount with No1.5 coverslip using Antifade with DAPI and seal edges with nail polish
9) Party.

Supplementary Protocol 3

OligoFISSEQ-LIT on Ibidi Slides

DAY 1 (2-3hrs):

- Plate cells into Ibidi Sticky cell chambers (6 channels)
- 130-150uL per channel of reagent

FISH 1o (3-4hrs):

1) Aspirate media: 1X PBS wash; **5 min @ RT**
2) Fix Cells; 4% Formaldehyde/PBS; (1mL = 250uL 16% formaldehyde + 100uL 10X PBS + 650uL H2O); **10 min @ RT**
3) Permeabalize; 0.5% Triton/PBS; (1mL = 5uL Triton X-100 + 995uL 1X PBS); **15 min @ RT**
4) PBT (PBS + 0.1% Triton) wash; **5 min @ RT**
5) 0.1N HCl; 0.1N HCl/H2O; (1mL = 12.5uL 8N HCl + 987.5uL H2O); **8min @ RT**
6) 2XSSCT wash; **1x 5min @ RT**

6a) RNAse treat; 2uL/50uL 2XSSCT; **1hr @ 37oC**

6b) 2XSSCT wash in, asp, then; **2min @ RT**

7) Pre-hybe RT; 50% Formamide/2XSSCT; >**30min @ RT**
8) Pre-hybe 60oC; 50% Formamide/2XSSCT; >**30min @ 60oC**

***Prepare 1o while sample is pre-hybing

9) 1o Oligopaint Hybridization;

***Generally, 100pmol per channel of each probe

***8X Hybe Buffer: 40% Crowding Agent (Dextran sulfate) + 8XSSCT

Reagent	**Stock**	**Final**	**Amount**
Formamide	1	0.5	25
Hybe Buffer	8X	2X	12.5
RNAseA	10mg/uL	20mg	2
1o Oligopaint	100pmol/uL	100pmol (2uM)	1
H2O	n/a	n/a	9.5

10) Denature sample on hot block under weight; **3min @ 80oC**
11) Hybridization; transfer samples to humid chamber and hybridize O/N @ (**44.5oC**)

Day 2 (8+hrs, or can split up; 4hrs):

3) 2XSSCT washes; **2x 15min @ 60oC, then wash in 60oC at RT**

*******Add 100uL pre-heated 2XSSCT into channels with 1o Oligopaint; **aspirate, then wash more in**

*******On hot block as before

4) 2XSSCT washes @ RT; **2x 7min @ RT**

*******wash in 60oC 2XSSCT then do 1st at RT

4a) CutSmart Buffer wash; 1X (50uL) in H2O; **1x 10min @ RT**

4b) Shrimp Phosphatase (NEB) treatment; 7.5U/50uL RXN; **1hr @ 37oC humid**

4c) Inactivate Phosphatase; transfer slide to 65oC heatblock; **5min**

4d) 2XSSCT washes @ 65oC, wash in, asp, then; **2x 7min @ 65oC**

4e) 2XSSCT washes @ RT; **2x 7min @ RT**

FISH 2o + Seq Primer (~2hrs):

5a) 30% formamide/2XSSCT (1.5mL Formamide + 1mL UPW + 2.5mL 4XSSCT); **1x 7min @ RT**

5) 2o Oligo + Sequencing Oligo Hybridization in 30% formamide; **>30min @ RT**

***50uL volume per channel; make up with UPW

***~40pmol of seq primer, ~30pmol of bridge/2o oligo

***Protect from light

6) 30% Formamide/2XSSCT wash in, asp, then; **2x 20min @ RT humid**
7) 2XSSCT washes; **1x 10min @ RT**

7a) DAPI; 1:1000 in PBS; **10min @ RT**
8a) 1XPBS washes; **1x 10min @ RT**
8b) 2XSSCT wash; **1x 10min @ RT**
*****Can pause here and continue the next day**
Sequencing by Ligation (3hrs for SBL; 1.5hrs for QC; 2hrs for Cleave, excluding imaging):
3) Pre-Ligation wash; 1X *Quick Ligation Buffer (5X)*/H2O; **100uL/ch; 10min @ RT**
4) Sequence by ligation; **100uL/ch; 1hr @ 25oC**

Reagent	**Stock**	**Amount**
Quick Ligase Buffer	NEB; 2X	50
Quick Ligase (T4)	NEB; 2KU/uL	5
T3 Ligase	NEB; 3KU/uL	5
SOLiD Oligos	5500 W SOLiD FWD75; ?	2
UPW		38

***Put in 25oC Drosophila incubator
5) 2XSSCT rinse; **3x @ RT**
6) 1M Guanadine Hydrochloride (GHCL) ; **2x 20min @ RT**
a7) 1XPBS washes; **1x 10min @ RT**
7) PBS wash; **7min RT**
8) TrueBlack treat?; 1:20 in 70% EtOH (700uL EtOH + 250uL UPW + 50uL Trueblack); **1x 2min @ RT**
***If using TB, do not use detergent (tween/triton) in any further steps until after imaging
9) PBS rinse x3 then **10min @ RT**
10) Image in imaging buffer: to make 1mL:

Reagent	**Stock**	**Amount**			**Recipe**
Trolox	"100X"	15		**Trolox**	100mg Trolox + 430uL MeOH + 345uL 1M NaOH + 3.2mL UPW
PCD	"100X"	15		**PCD**	6.3mg PCD + 9mL buffer [50% glycerol + 50mM KCl + 1mM EDTA + 100mM Tris HCl 8.0
PCA	"40X"	40		**PCA**	154mg PCA in 10mL UPW; pH 9 with NaOH
PBS	1X	930			

11) PBS wash; **1x 5min @ RT**
12) 2XSSCT wash: **3x 5min @ RT**
***Wash until liquid is clear
13) CutSmart Buffer wash; 1X (50uL) in H2O; **1x 7min @ RT**
14) Quick CIPhosphtase (NEB); 2.5uL/50uL RXN; **35min @ 37oC humid**
15) 2XSSC/T rinses then wash; **3x, then 2x 20min @ RT**
16) PBS wash; **1x 10min @ RT**
17) 0.1XPBS rinse; **1x @ RT**
18) Cleave SBL: Shyam Cleave Buffer 1; **100uL/well; rinse then 15min @ RT**
***Cleave Buffer 1: 50mM AgNO3 (1:1 [0.1N AgNO3 + UPW])
19) Shyam Cleave Buffer 2; **100uL/well; 2x; rinse then 15min @ RT**

Cleave 2		
Reagent	**Final Conc**	**Amnt**
MESNA (2-mercaptoethanolsulfate)	50mM	0.082g
NaCL	150mM	0.876g
TRIS HCl 1M pH 8.0	20mM	200uL

UPW		Up to 10mL

20) 2XSSCT rinse **x3**
21) 1M GHCL ; **2x 20min @ RT**
22) 2XSSCT washes; **2x 10min @ RT**
23) Repeat SBL (step 3 under Sequencing by Ligation) until BC is read
24) After imaging last bit of BC, skip QC and cleave; after imaging > 11, 12 > 16-22
25) Hybridize all hybe secondary; 30% form wash 7min → 2o (step 5a-7) and image in imaging buffer

Immunofluorescence:

26) Wash off Oligopaints 80% formamide/2XSSCT x2 10min RT > 2XSSCT 10min > 1XPBS 10min > 4% formaldehyde fix; 10min
27) 2XSSCT wash; **1x 10min @ RT**
28) PBS wash: **1x 10min @ RT**
29) 4% foramldehyde fix; **10min @ RT**
30) PBS rinse and wash; **5min @ RT**
31) PBTriton; **7min @ RT**
32) Block in 3% BSA/PBT; >**45min @ RT**
33) 1o Antibody in 1% BSA/PBT; **O/N @ 4oC**
34) PBTriton washes; **3x 5min @ RT**
35) 2o Antibody in 1% BSA/PBT (1:500 generally); **1hr @ RT**
36) PBTriton wash; **3x 5min @ RT**
37) Image in PBS
24) Profit **$$$$**

Reagents:

Reagent	**stock**	**Brand**	**Product #**	**Location**
Formaldehyde	16%	EMS	15710	Chem Shelf
RNAseA	10mg/mL	Thermo	EN0531	-20oC
Formamide	100%	EMD	344206	4oC
Dextran Sulfate	100%	Sigma	D8906	4oC
Shrimp Phosphatase	1,000U/mL	NEB	M0371L	-20oC
Quick Ligation Buffer	5X	NEB	B6058S	-20oC
Quick Ligase		NEB	M2200L	-20oC
T3 DNA Ligase		NEB	M0317L	-20oC
Guanadine HCL	100%	Sigma	G3273	Dessication chamber
Tetraspeck Beads		Thermo	T7279	4oC
True Black	20X	Biotium	23007	RT
Trolox	100%	Sigma	238813	Chem Shelf
PCD (Protocatechuate 3,4-Dioxygenase)	100%	Sigma	P8279	-20oC
PCA (Protocatechuic acid)	100%	Sigma	37580	Chem Shelf
Quick CIP		NEB	M0508L	-20oC
AgNO3	0.1N	EMD	SX0206A-2	4oC walk

MESNA	100%	Sigma	M1511	Chem Shelf
TRIS HCL 8.0	1M	Thermo	AM9856	Chem Shelf
Protein Free Blocking Buffer	1X	Fisher	PI37570	4oC walk
Digitonin	5%	Fisher	BN2006	4oC
SYBR FITC	10,000X	Invitrogen	S11494	-20oC

Supplementary Protocol 4

OligoFISSEQ on Hydrogels (PolyAcrylamide)

DAY 1 (2-3hrs):

- Plate cells onto 1mm glass microscope slides; score/etch cell area with diamond pen
- 100uL of cells per spot

FISH 1o (2-3hrs):

1) Rinse: 1X PBS wash; **5 min @ RT**
2) Fix Cells; 4% Formaldehyde/PBS; (40mL = 10mL 16% formaldehyde + 4mL 10X PBS + 26mL H2O); **10 min @ RT**

2a) 1XPBS rinse

3) Permeabalize; 0.5% Triton/PBS; (40mL = 200uL Triton X-100 + 40mL 1X PBS); **15 min @ RT**
4) PBT (PBS + 0.1% Triton) wash; **5 min @ RT**
5) 0.1N HCl; 0.1N HCl/H2O; (40mL = 500uL 8N HCl + 39.5mL H2O); **5min @ RT**
6) 2XSSCT washes; **2x 5min @ RT**
7) Pre-hybe RT; 50% Formamide/2XSSCT; **10min @ RT**
8) Pre-hybe 60oC; 50% Formamide/2XSSCT; **20min @ 60oC**
9) 1o Oligopaint Hybridization;

***Air dry slides and wipe around cell area
***25uL per slide
***Generally, 100pmol per slide
***8X Hybe Buffer: 27-40% Crowding Agent (Dextran sulfate OR Polyacrylic Acid ~1200MW) + 8XSSCT
***Add to cells → cover with 22x22mm coverslip → seal edges with rubber cement → allow to dry before denaturing

Reagent	Stock	Final	Amount
Formamide	1	0.5	12.5
Hybe Buffer	8X	2X	6.25
RNAseA	10mg/uL	20mg	2
1o Oligopaint	100pmol/uL	100pmol (2uM)	1
H2O	n/a	n/a	3.25

10) Denature sample; **3min @ 80oC**

***Perform on hot block

2) Hybridization; transfer samples to humid chamber and hybridize O/N @ (**42oC**)

Day 2 (8-9hrs):

3) 2XSSCT hot wash; **20min @ 60oC**
4) 2XSSCT washes @ RT; **2x 7min @ RT**
5) 30% formamide/2XSSCT (40mL = 12mL Formamide + 8mL H2O + 20mL 4XSSCT); **20min @ RT**
6) 2XSSCT washes; **2x 7min @ RT**
7) 0.2XSSC wash; **15min**

Gel Casting (~2-3hrs-O/N):

8) Cast hydrogel; **~22uL/slide; 90min @ RT**

***Air dry slides and wipe around cell area

Reagent	Manufacturer	Concentration	Final	Amount for 200uL	Amount for 3mL
40% Acrylamide/Bis 19:1	Biorad	40%	4%	20	300

1M Tris HCl pH 8.0	Invitrogen	1M	60mM	12	180
5M NaCl	Active Motif	5M	300mM	12	180
10% APS	Biorad	10%	0.20%	4	0
10% TEMED	Sigma	10%	0.20%	4	0
UPW				148	2340

***Use gelation chamber: 1mm slide wrapped in parafilm; using 2-22x22mm coverslips as spacers

***Spot ~22uL hydrogel solution onto parafilm → wait ~10sec → drop slide with cells face down onto hydrogel solution → want a distinct disc/island to form → gel with parafilm slide on the bottom

9) Remove gelation chamber, cut, and rinse gels with **200uL** Zhuang digestion buffer:

***Gently remove slide from gelation chamber; gel should stay stuck to cell area

***Gently trim outer edges of gel with razor blade and discard

Zhuang digestion buffer:

(0.8M Guanadine-HCl, 50mM Tris-HCL pH8.0, 1mM EDTA, 0.5% Triton X-100, UPW)

10) Digest hydrogel; **~2mL/slide; O/N @ 37oC**

→ break non-cell area of glass slide with diamond pen → add gel/cell/slide sample to 35mm dish with 1:100 Proteinase K (NEB; 10mg/mL) in Zhuang digestion buffer → digest in humid chamber

Day 3 (8-9hrs):

FISH 2o + Seq Primer (~2hrs):

1) Aspirate digestion solution and remove glass slide
2) 2XSSC washes; **1.5mL/dish; shaker; 20min x3 @ RT**

***Cut off smaller pieces to proceed with 2o and sequencing

***Move all pieces into 1.5mL Eppendorf tubes

3) 2XSSCT wash; **200uL/gel; 2x 7min @ RT nutator**
4) 2o Oligo + Sequencing Oligo Hybridization; **>1hr @ RT**

***50uL per gel

***Protect from light

Reagent	**Stock**	**Final**	**Amount**
Formamide	100%	30%	15
SSCT	4X	2X	25
2o Probe	10pmol/uL	30pmol	3
Seq Probe	10pmol/uL	40pmol	4
H2O	n/a	n/a	3

1) 30% Formamide/2XSSCT washes; **2x 10min @ RT**
2) 2XSSCT washes; **3x 20min @ RT**

***Perform on rocker/nutator

Sequencing by Ligation (5-6hrs):

3) Pre-Ligation wash; 1X *Quick Ligation Buffer*/H2O; **100uL/ch; 10min @ RT**

***Use Quick Ligation buffer (stock = 5X)

4) Sequence by ligation; **100uL/ch; 1.5hr @ 25oC**

Reagent	**Stock**	**Amount**

Quick Ligase Buffer	NEB; 2X	50
Quick Ligase	NEB; 2KU/uL	5
T3 Ligase	NEB; 3KU/uL	5
SOLiD Oligos	5500 W SOLiD FWD75; ?	2
UPW		38
Total		100

***Put in 25oC Drosophila incubator

5) 2XSSCT rinses; **3x @ RT**
6) 6XSSC 60oC washes; **2x 12min @ 60oC**
7) 2XSSCT washes on nutator; **3x 10min @ RT**
8) DAP (1:500 in 1XPBS); **10min @ RT**
9) 1XPBS wash; **10min @ RT**
10) Image in 1XPBS or without PBS; coat coverslip with gel slick beforehand
11) Cleave

9) Profit $$$$

Supplementary Protocol 5

OligoFISSEQ PadLock RCA on Ibidi Slides

***Modifications from BaristaSeq. Chen, Lee, and Zador. BioRXIV. 2017.

DAY 1 (2-3hrs):

- Plate cells into Ibidi Sticky cell chambers (6 wells)
- 130-150uL per channel of reagent

FISH 1o (2-3hrs):

1) Aspirate media: 1X PBS wash; **5 min @ RT**
2) Fix Cells; 4% Formaldehyde/PBS; (1mL = 250uL 16% formaldehyde + 100uL 10X PBS + 650uL H2O); **10 min @ RT**
3) Permeabalize; 0.5% Triton/PBS; (1mL = 5uL Triton X-100 + 995uL 1X PBS); **15 min @ RT**
4) PBT (PBS + 0.1% Triton) wash; **5 min @ RT**
5) 0.1N HCl; 0.1N HCl/H2O; (1mL = 12.5uL 8N HCl + 987.5uL H2O); **5min @ RT**
6) 2XSSCT washes; **2x 5min @ RT**

6a) RNAse treat; 2uL/50uL 2XSSCT; **1hr @ 37oC**

7) Pre-hybe RT; 50% Formamide/2XSSCT; **10min @ RT**
8) Pre-hybe 60oC; 50% Formamide/2XSSCT; **20min @ 60oC**

***Perform on hot block in pre-heated water bath
***Use weight on top of slide to press against block

9) 1o Oligopaint Hybridization;

***Prepare while sample is pre-hybing
***50uL per channel
***Generally, 100pmol per channel of each probe
***8X Hybe Buffer: 27-40% Crowding Agent (Dextran sulfate OR Polyacrylic Acid ~1200MW) + 8XSSCT

Reagent	**Stock**	**Final**	**Amount**
Formamide	1	0.5	25
Hybe Buffer	8X	2X	12.5
RNAseA	10mg/uL	20mg	2
1o Oligopaint	100pmol/uL	100pmol (2uM)	1
H2O	n/a	n/a	9.5

10) Denature sample; **3min @ 80oC**

***Perform on hot block

2) Hybridization; transfer samples to humid chamber and hybridize O/N @ (**42oC**)

Day 2 (4-5hrs):

3) 2XSSCT washes; **2x 15,20min @ 60oC**
*******Add 100uL pre-heated 2XSSCT into channels with 1o Oligopaint; **10min @ 60oC**
*******Aspirate and add 130uL pre-heated 2XSSCT; **10min @ 60oC**
*******On hot block as before
4) 2XSSCT washes @ RT; **2x 2min @ RT**
4a) CutSmart Buffer wash; 1X (50uL) in H2O; **1x 10min @ RT**
4b) Shrimp Phosphatase (NEB) treatment; 7.5U/50uL RXN; **1hr @ 37oC**
4c) Inactivate Phosphatase; transfer slide to 65oC heatblock; **5min**
4d) 2XSSCT washes @ 65oC; **2x 7min @ 65oC**
4e) 2XSSCT washes @ RT; **2x 5min @ RT**

FISH/Circularize MIP (control) (~2 hrs):

5) MIP Hybridization; **>30min @ RT**
***50uL per channel
***Protect from light

Reagent	Stock	Final	Amount
Formamide	100%	30%	15
SSCT	4X	2X	25
MIP Probe	10pmol/uL	40pmol	4
H2O	n/a	n/a	6

6) 30% Formamide/2XSSCT washes; **2x 10min @ RT**
***Add first wash directly to channel
7) 2XSSCT washes; **3x 7min @ RT**
*******110uL per channel
***Perform on rocker/nutator
8) Pre-Ligation wash; 1X *Quick Ligation Buffer*/H2O; **100uL/ch; 10min @ RT**
*******Use Quick Ligation buffer (stock = 5X)
9) Circularize by ligation; 5uL of T3 + 5uL of Quick Ligase/100uL Quick Ligase buffer; **1hr @ RT**
10) 2XSSCT rinse then washes; **3x 7min @ RT**

Rolling Circle Amplifcation (RCA) (~3-O/N hrs):

11) RCA buffer wash; **100uL/well; 10min @ RT**
12) Rolling Circle Amplification (RCA); **100uL/well; 90min @ 37oC or O/N @ RT**

Reagent	Stock	Final	Amount
Phi29 Buffer	10X	1X	10
dNTP	10mM	0.25mM	2.5
AminoAllyl dUTP	4mM	0.08mM	2
Phi29 Polymerase	10U/uL	100U	10
H2O	n/a	n/a	75.5

13) PBS wash; **130uL/well; 1x 3min @ RT**
14) Cross link Rolonies with BS(Peg)900 (1mL = 20uL BS(PEG) + 980uL 1XPBS); **1hr @ RT**
15) PBS rinse
16) Quench BS(PEG) with 1M Tris HCl pH 8.0; **40min @ RT**

17) 2XSSCT washes; **3x 7min @ RT**

Sequencing Primer Hybe and Sequencing By Ligation (3hrs+):

18) Hybe Seq primer; **50uL/well; 1hr @ RT**

Reagent	**Stock**	**Final**	**Amount**
Formamide	100%	30%	15
SSCT	4X	2X	25
Seq Primer	100uM	2.5uM	1.25
H2O	n/a	n/a	8.75

18a) 30% formamide/2XSSCT wash; **3x 10min @ RT**

18b) 2XSSCT wash; **3x 7min @ RT**

19) Pre-Ligation wash; 1X *Quick Ligation Buffer*/H2O; **100uL/well; 10min @ RT**

*******Use Quick Ligation buffer (stock = 5X)

20) Sequence by ligation; **100uL/well; 1hr @ 25oC**

Reagent	**Stock**	**Amount**
Quick Ligase Buffer	NEB; 2X	50
Quick Ligase	NEB; 2KU/uL	5
T3 Ligase	NEB; 3KU/uL	5
SOLiD Oligos	5500 W SOLiD FWD75; ?	2
UPW		38
Total		100

***Put in 25oC Drosophila incubator

21) 2XSSCT rinses; **3x @ RT**

22) 6XSSC 60oC washes; **2x 15min @ 60oC**

23) 2XSSCT washes on nutator; **3x 10min @ RT**

24) Image in 2XSSCT

25) Cleave

26) Profit $$$$