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Supplementary Materials for

TADs are 3D structural units of higher-order chromosome organization in *Drosophila*

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Supplementary Materials

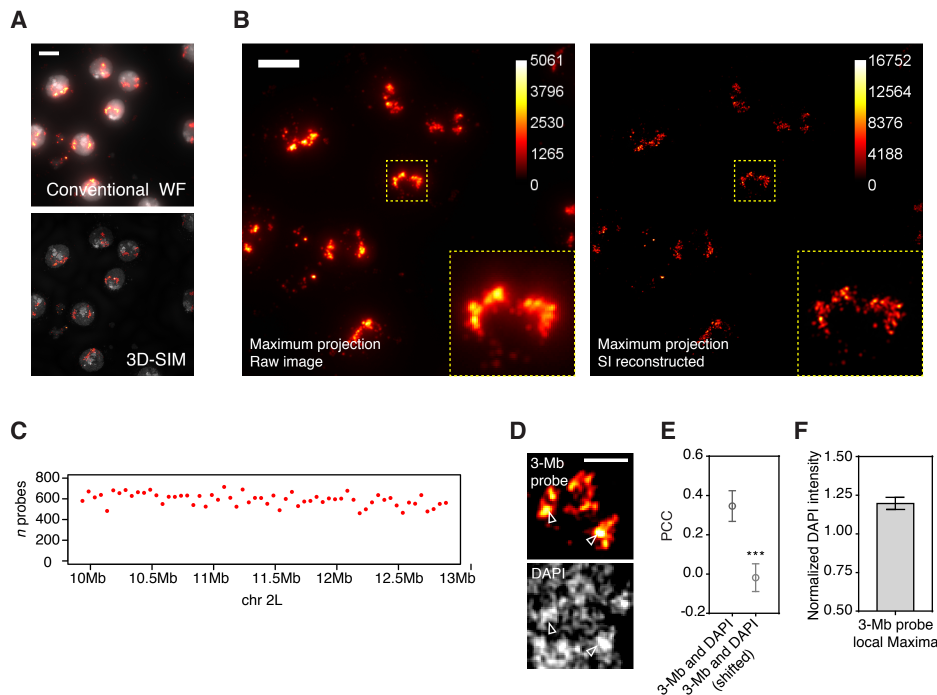

fig. S1. Chromatin nanostructure visualization with 3D-SIM imaging. (**A**) Example of conventional WF and 3D-SIM reconstructed image of DAPI (gray) and 3 Mb probe labeling in S2R+ nuclei. Scale bar: 5 µm. (**B**) Raw image and structured illumination (SI) reconstructed image of the 3 Mb probe shown in (**A**). Scale bars: 5 µm. (**C**) The 3 Mb region is homogenously covered by a mean of 11.85 oligonucleotide probes per kb. Graph shows probe numbers in 50 kb bins along the 3 Mb region. (**D**) 3 Mb probe (top) and DAPI (bottom) 3D-SIM imaging in an S2R+ nucleus (single *z*-slices). Arrowheads indicate dense compartments for both labels. Scale bar: 1 µm. (**E**) Pearson's correlation coefficient (PCC, mean ± SD) between 3 Mb probe signal and DAPI, before or after DAPI channel shift (3 voxels in *x*/*y*/*z*). *** corresponds to *p*-value <0.0001 using Wilcoxon matched-pairs signed rank test, 20 nuclei were analyzed. (**F**) DAPI intensity (mean ± SD) of the 3 Mb probe local maxima, normalized to the mean DAPI intensity of the 3D segmented 3 Mb probe. Ratios were calculated per image (7 images/55 nuclei in total).

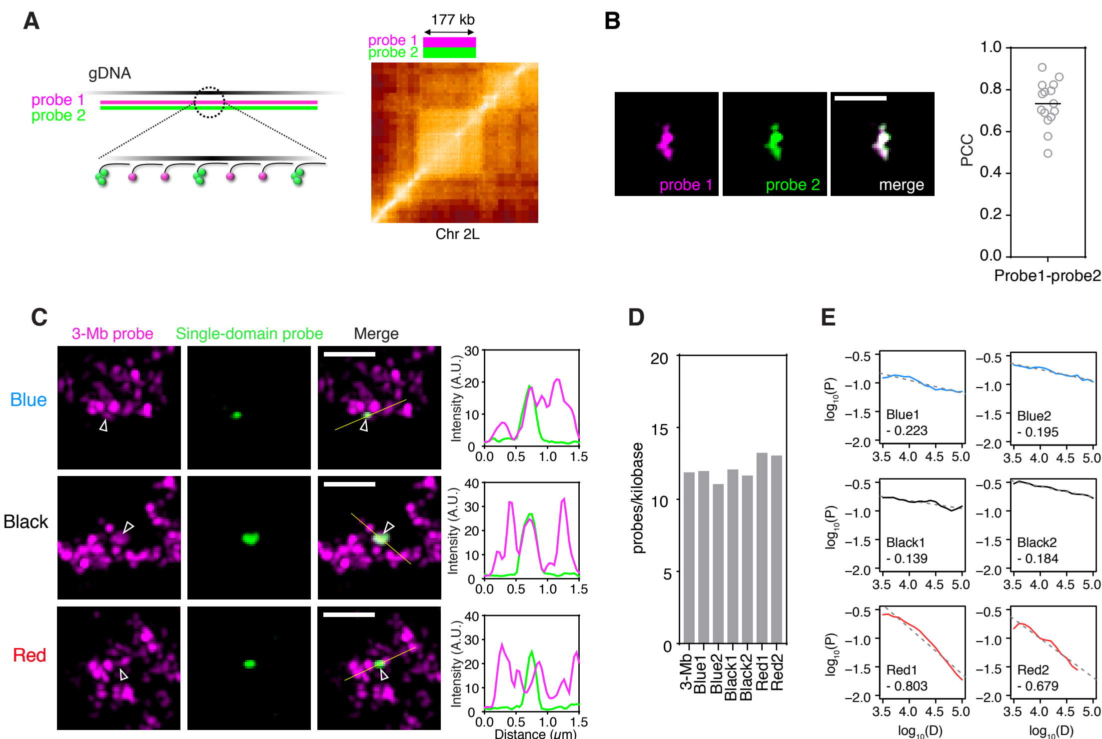

fig. S2. Dual labeling Oligopaint FISH. (**A**) Schematic representation of the dual FISH labeling. Probe 2 (green) has an oligonucleotide coverage of 1/3 of probe 1 (magenta), and is added in molar excess compared to probe 1 (at least 3:1 ratio), allowing efficient co-labeling of the same region. The probe 2 signal is increased using complementary secondary oligonucleotides carrying 2 additional fluorophores. On the right, Hi-C map around a single TAD (Black1), labeled with the two sets of probes. (**B**) Double labeled Black1 TAD (maximum projection). On the right, PCC between probe 1 and probe 2 intensities. 15 nuclei were analyzed, the black line indicates the mean. (**C**) Examples of dual FISH labeling (maximum projections) with the 3 Mb probe and single epigenetic domains (Blue2, Black1 or Red2, indicated with arrowheads). Right panel: intensity distributions of the two probes along the yellow line. (**D**) Average Oligopaint coverage of the 3 Mb probe in the whole region and in specific epigenetic domains targeted with single domain probes. (**E**) Cis-decay curves (contact probability as a function of genomic distance) within the single domains calculated in S2R+ Hi-C. On bottom left of the plots, the slope of fitted linear regression (dashed gray lines) are indicated. Scale bars: 1 μm.

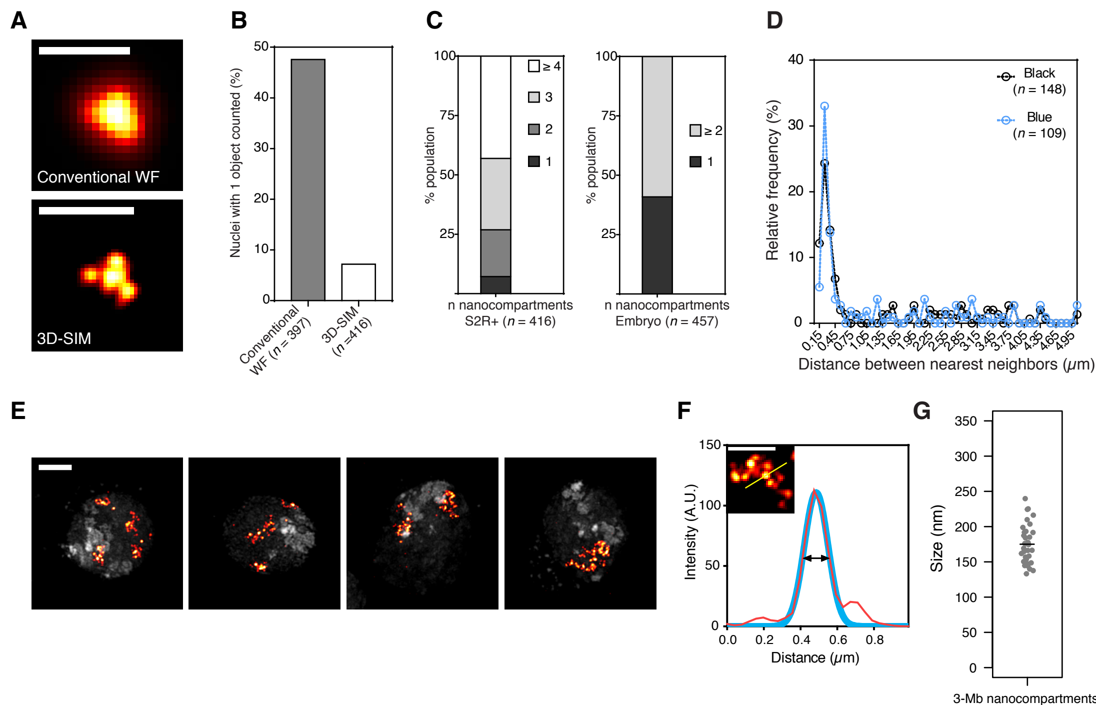

fig. S3. Resolution of homologous TADs with 3D-SIM. (**A**) Images of a single TAD (Blue1) in conventional microscopy (top) and after 3D-SIM reconstruction (bottom) in an S2R+ cell. Maximum projections, scale bars: 1 µm. (**B**) Percentages of nuclei with only one foci resolved in conventional WF microscopy or 3D-SIM (merge of Blue1 and Black1 FISH experiments). *n* represents the number of nuclei analyzed in each condition. (**C**) Distributions of resolved nano-compartment counts per nucleus in S2R+ and embryonic cells (merge of Blue1 and Black1 FISH experiments) with 3D-SIM. *n* represents the number of nuclei analyzed in each condition. (**D**) Histogram of distance distribution between nearest neighbor nano-compartments observed in Black1 and Blue1 single-TAD FISH experiments. The *X*-axis is split into 50 nm bins (last bin represents distances > 5 µm); *n* represents the number of distances measured from G1-staged S2R+ cell populations (37 and 58 nuclei for Blue1 and Black1, respectively; see fig. S5 for cell-cycle staging), to avoid measuring distances between sister chromatids. (**E**) Examples of S2R+ nuclei (DAPI in gray) labelled with the 3 Mb probe displaying different pairing states. From left to right: the four chromosomes unpaired, two chromosomes paired and two unpaired, two chromosomes paired twice, all chromosomes paired. Maximum projections, scale bar: 2 µm. (**F**) Intensity distribution (in red) along the line drawn in the image (single copy of the 3 Mb probe, maximum projection), with a Gaussian fitted curve (in blue). Arrow represents the full width at half maximum (FWHM). Scale bar: 1 µm. (**G**) FWHM-calculated diameters (black line indicates mean) of the nano-compartments observed in the 3 Mb probe (*n*=35).

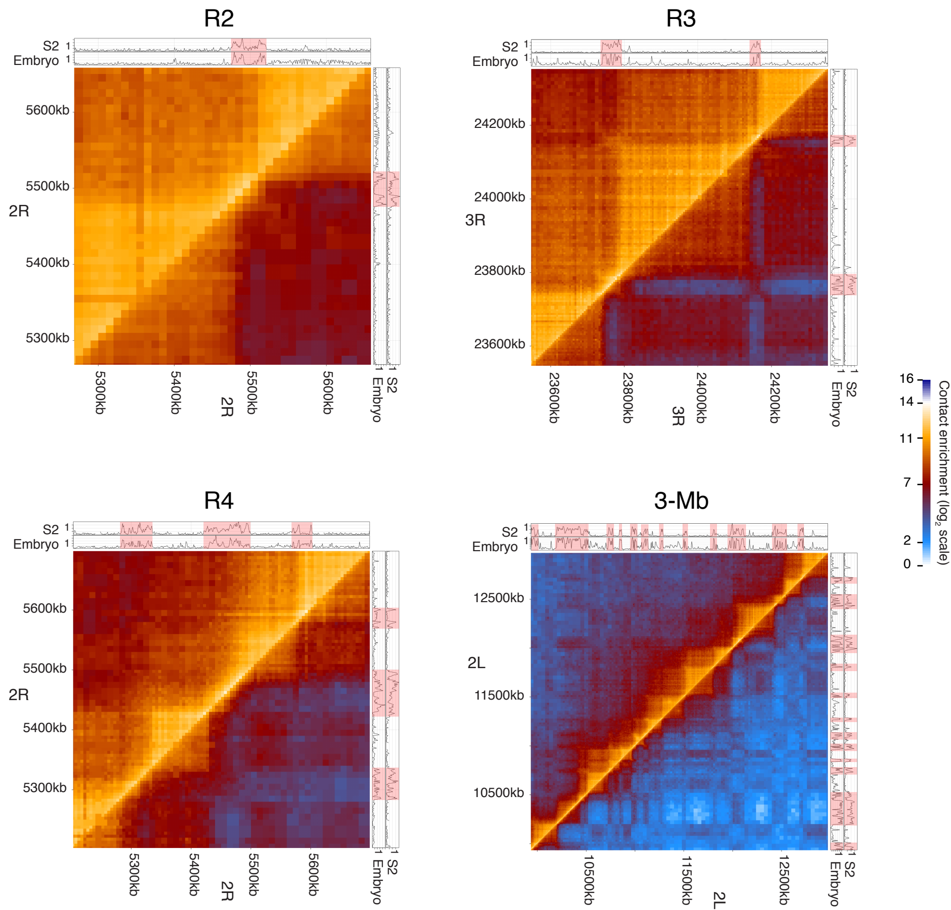

fig. S4. Oligopaint probe–targeted regions in S2R+ cells and embryos. Hi-C maps of Oligopaint labeled regions in S2R+ cells (top of the diagonal) and 16-18 hr embryos (bottom), showing conservation of the TADs. H3K4me3 ChIP profiles in both S2 cells and 14-16 hr embryos are also shown, allowing identification of repressed TADs. We counted 2, 3, 4 and 12 repressed TADs for R2, R3, R4 and the 3 Mb region, respectively.

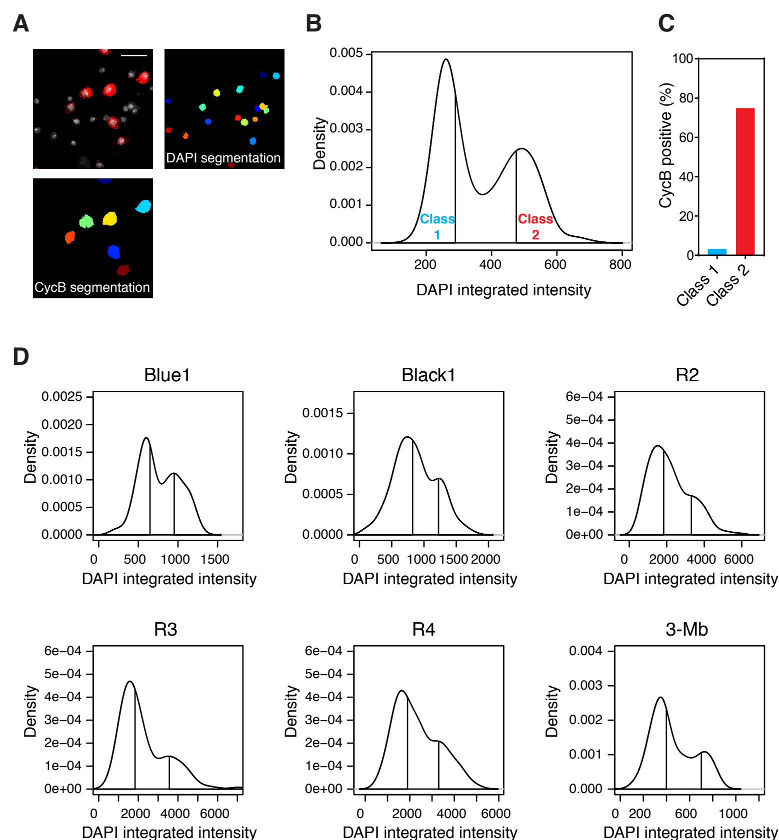

fig. S5. Cell cycle staging of S2R+ cells. (**A**) Confocal image S2R+ cells labeled with DAPI (in gray) and Cyclin-B (CycB, in red), and their 2D segmentation (individual segmented objects are represented with colored surfaces). Average projection, scale bar: 20 µm. (**B**) DAPI integrated intensity distribution of the S2R+ nuclei population labeled with CycB (*n* = 414). The DAPI integrated intensity reflecting the amount of DNA per nucleus (*29*), we obtained a profile of the cell cycle with G1 (class 1 of the distribution) and G2 (class 2 of the distribution) cells. (**C**) Percentage of nuclei positive for CycB segmentation in class 1 and class 2 of the distribution in (**B**), showing the efficiency of the cell cycle staging method. (**D**) DAPI integrated intensity distributions obtained in FISH experiments for Black1, Blue1, R2, R3, R4 and the 3 Mb probes (254, 213, 180, 136, 166 and 156 nuclei, respectively). Based on the DAPI integrated intensity distribution, we considered as G1 nuclei in left parts of the plots and as G2 nuclei in right part of the plots (delimitated by vertical lines).

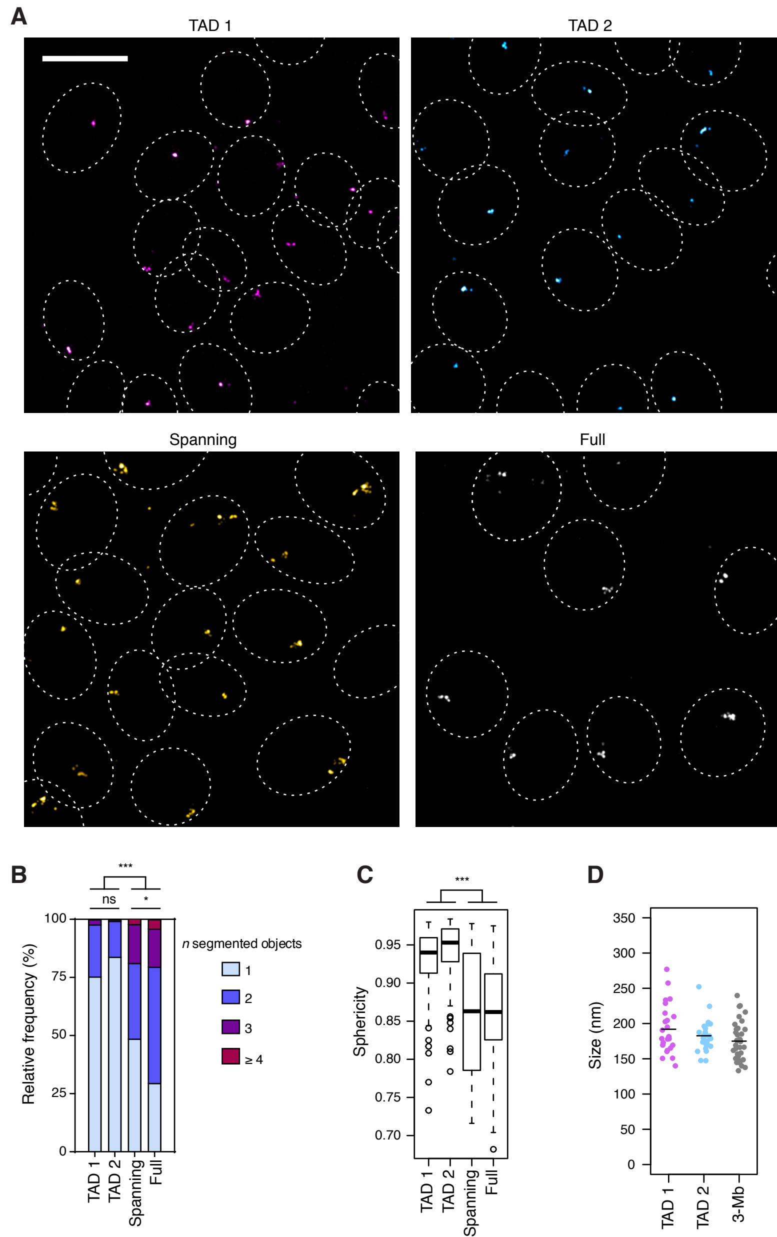

fig. S6. Super-resolution imaging of haploid chromatin folding. (**A**) 3D-SIM images of the TAD 1, TAD 2, Spanning and Full probes. Dashed lines indicate DAPI contours. Maximum projections, scale bar: 5µm. (**B**) Quantification of the number of segmented objects per nucleus (at least 122 nuclei per condition, analysis in maximum *z*-projected images). (**C**) Sphericity scores of the 3D-segmented probes (at least 68 nuclei per condition). (**D**) FWHM-calculated diameters (black lines indicate means) of TAD 1 (n=25), TAD 2 (n=25), and of nano-compartments observed in the 3 Mb probe (n=35). Statistics were performed with Kruskal-Wallis and Dunn's multiple comparisons tests, *** indicates p-value < 0.0001, * indicates p-value < 0.05).

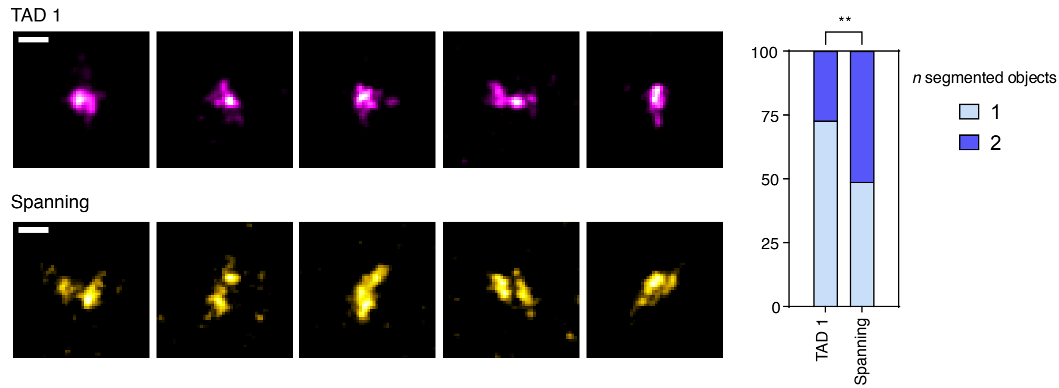

fig. S7. 2D dSTORM imaging of TAD 1 and spanning probes. On the left, representative examples of dSTORM imaging of TAD 1 and Spanning probes, both having the same genomic size. Scale bars: 250 nm. On the right, quantification of the number of segmented objects per nucleus (at least 55 nuclei per condition). ** indicates *p*-value < 0.01 using two-tailed Fisher's exact test.

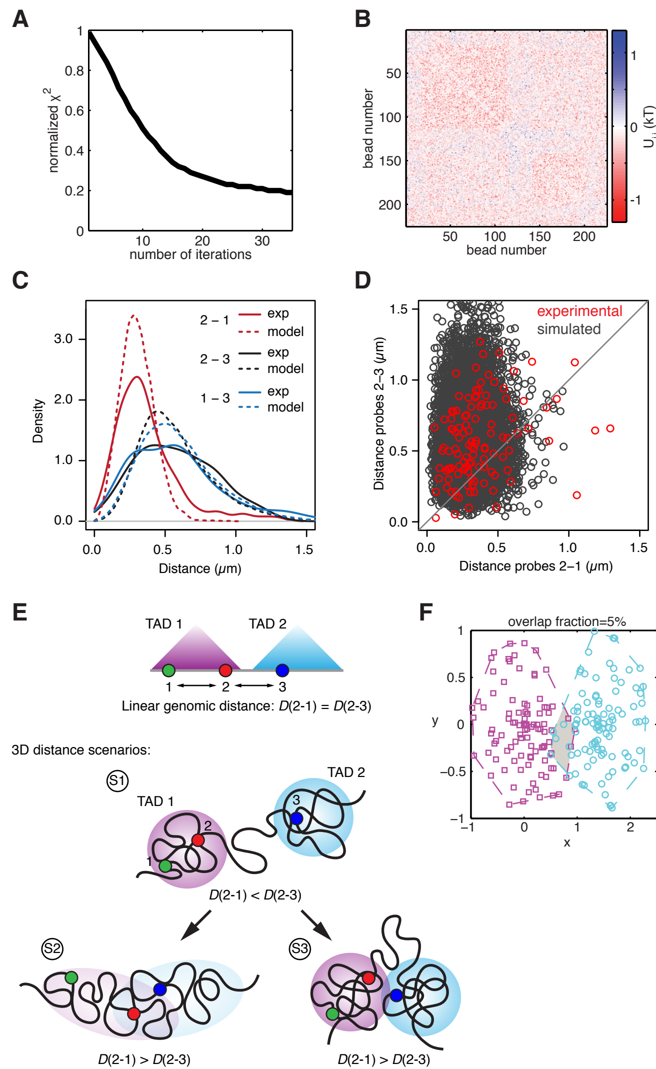

fig. S8. Modeling the X chromatin region. (**A**) Evolution of the normalized score χ^2 as a function of the number of iterations of the inference scheme, for an interaction radius *R=143* nm. (**B**) Corresponding inferred monomer-monomer interactions $\{U_{i,j}\}$. (**C**) 3D distances distributions between the probes 1, 2 and 3 from FISH experiment (full lines) and inferred model (dashed lines). (**D**) Scatter plot of paired distances between probe 2 and probe 1 (*X*-axis) versus probe 2 and probe 3 (*Y*-axis) from FISH experiments (red) and inferred model (gray). (**E**) Schematic representations of 3D chromatin structure scenarios that might explain FISH experiments with equidistant probes used here. Scenario 1 (S1) represents conformations where the intra-TAD (2-1) physical distance is shorter than the inter-TAD (2-3), consistent with the formation of an individual nano-compartment for each TAD. When the intra-TAD distance is larger than the inter-TAD one, we envisaged two different scenarios: in scenario 2 (S2) the shorter inter-TAD distance is due to intermingling between TADs and loss of individual TAD nano-compartments, while in scenario 3 (S3), this is due to a change of relative spatial positioning of the TADs with maintenance of two separated nano-compartments corresponding to each of the TADs. (**F**) Illustration of the computation of the overlap fraction in 2D between two ensembles of points (symbols). Dashed lines represents the convex envelopes of each ensemble (purple and blue for TAD 1 and TAD 2, respectively). The overlap fraction quantifies the proportion of points that belongs to the intersection between the two envelopes (gray area).

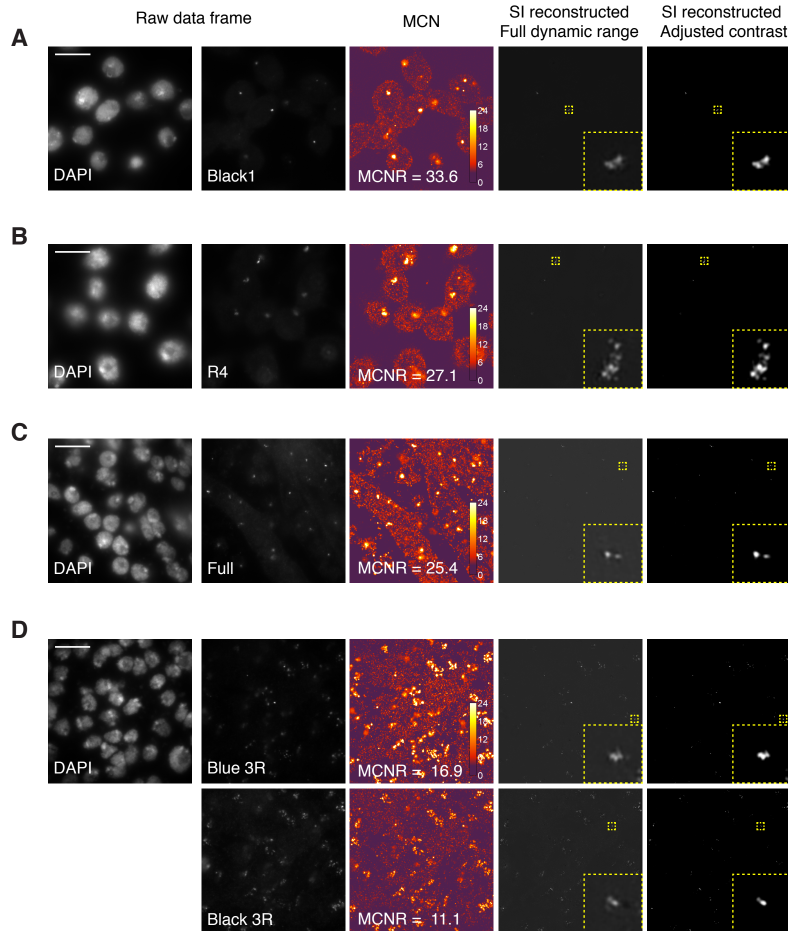

fig. S9. Quality control of SI acquisitions. (A-D) Examples of images and SI reconstruction outputs of FISH signals: Black1 FISH (Cy3 dye) in S2R+ cells; R4 (Atto-565 dye) FISH in S2R+ cells; Full (Atto-565 dye) FISH in embryonic cells, Blue 3R (Cy3 dye) and Black 3R (Alexa-488 dye) FISH in embryonic cells. From left to right: single frame of raw image; modulation contrast to noise (MCN) output from SIMcheck (*39*) plugin of ImageJ with average modulation contrast to noise ratio (MCNR) value (4-8 low to moderate, 8-12 good, > 12 good-excellent); reconstructed images with full dynamic range (from 32-bit data); reconstructed images with adjusted contrast after *Threshold and 16-bit Conversion* (SIMcheck) processing. Single *z*-slices, scale bars: 10 µm.

table S1. Libraries for Oligopaint probes. Coordinates, genomic sizes, number of probes and coverage of the Oligopaint probes. Library code refers to the primers used to amplify libraries (see Material and Methods). Probe names in italic refer to the epigenetic state specific probes used in Fig. 5: B for Blue; Bk for Black; R for Red.

Name	Chr	5' coordinates	3' coordinates	Genomic size (bp)	Number of probes	Density probes/kb	Library Code
3-Mb	2L	9935314	12973080	3037766	36001	11,85	-
Blue1	2L	11317986	11468388	150402	1797	11,95	B
Blue2	2L	12564906	12685026	120120	1326	11,04	C
Black1	2L	10540472	10717954	177482	2140	12,06	B
Black2	2L	11547196	11783436	236240	2748	11,63	C
Red1	2L	10203092	10436611	233519	3085	13,21	B
Red2	2L	12690712	12739196	48484	631	13,01	D
Blue1 1:3	2L	11317986	11468388	150402	599	3,98	B
Blue2 1:3	2L	12564906	12685026	120120	442	3,68	C
Black1 1:3	2L	10540472	10717954	177482	713	4,02	C
Black2 1:3	2L	11547196	11783436	236240	916	3,88	D
Red1 1:3	2L	10203092	10436611	233519	1028	4,40	D
Red2 1:3	2L	12690712	12739196	48484	210	4,34	E
R2	2R	10534240	10729120	194880	2353	12,07	A
R3	3R	23547420	24352723	805303	9681	12,02	A
R4	2R	5203174	5698315	495141	6124	12,37	B
Full	X	4602500	4961200	358700	3905	10,89	A
TAD1	X	4602500	4800500	198000	2211	11,17	B
TAD2	X	4851500	4961200	109700	1174	10,70	C
Spanning	X	4727000	4925000	198000	2164	10,93	D
B1	3R	652785	713391	60606	780	12,87	A
B2	3R	2496062	2870013	373951	4226	11,30	A
B3	3R	3960876	4035090	74214	851	11,47	A
B4	3R	6342956	6500513	157557	1815	11,52	A
B5	3R	7155430	7204894	49464	599	12,11	A
B6	3R	8066650	8143013	76363	1006	13,17	A
B7	3R	9675674	9769418	93744	1163	12,41	A
B8	3R	12199694	12252379	52685	605	11,48	A
B9	3R	12488250	12800730	312480	3526	11,28	A
Bk1	3R	2295136	2461552	166416	2002	12,03	A
Bk2	3R	3388064	3520312	132248	1620	12,25	A
Bk3	3R	4204574	4332372	127798	1512	11,83	A
Bk4	3R	6006587	6076245	69658	770	11,05	A
Bk5	3R	6734612	6947878	213266	2425	11,37	A
Bk6	3R	7950439	8030369	79930	1012	12,66	A
Bk7	3R	8556860	8764131	207271	2337	11,28	A
Bk8	3R	9304896	9449352	144456	1646	11,39	A
Bk9	3R	9966695	10042715	76020	798	10,50	A
Bk10	3R	10740256	10895936	155680	1848	11,87	A
Bk11	3R	11523771	11601926	78155	865	11,07	A
Bk12	3R	12989300	13190808	201508	2434	12,08	A
R1	3R	2176766	2217786	41020	547	13,33	A
R2	3R	2890321	2929421	39100	487	12,46	B
R3	3R	3825350	3863180	37830	545	14,41	A
R4	3R	4832892	4891933	59041	794	13,45	B
R5	3R	5567252	5631767	64515	955	14,80	A
R6	3R	6675854	6719646	43792	589	13,45	B
R7	3R	7027809	7070005	42196	588	13,93	A
R8	3R	7407477	7481767	74290	1005	13,53	B
R9	3R	8222335	8291151	68816	928	13,49	A
R10	3R	8809823	8857134	47311	643	13,59	B
R11	3R	9464906	9524338	59432	758	12,75	A
R12	3R	10112285	10152845	40560	525	12,94	B
R13	3R	11144574	11199314	54740	830	15,16	A
R14	3R	12257868	12296968	39100	547	13,99	B
R15	3R	12862169	12906743	44574	544	12,20	A