Superresolution imaging reveals structurally distinct periodic patterns of chromatin along pachytene chromosomes

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During meiosis, homologous chromosomes associate to form the synaptonemal complex (SC), a structure essential for fertility. Information about the epigenetic features of chromatin within this structure at the level of superresolution microscopy is largely lacking. We combined single-molecule localization microscopy (SMLM) with quantitative analytical methods to describe the epigenetic landscape of meiotic chromosomes at the pachytene stage in mouse oocytes. DNA is found to be nonrandomly distributed along the length of the SC in condensed clusters. Periodic clusters of repressive chromatin [trimethylation of histone H3 at Lys3 (H3K9me3)] are found at 500-nm intervals along the SC, whereas one of the ends of the SC displays a large and dense cluster of centromeric histone mark [trimethylation of histone H3 at Lys9 (H3K9me3)]. Chromatin associated with active transcription [trimethylation of histone H3 at Lys4 (H3K4me3)] is arranged in a radial hair-like loop pattern emerging laterally from the SC. These loops seem to be punctuated with small clusters of H3K4me3 with an average spread larger than their periodicity. Our findings indicate that the nanoscale structure of the pachytene chromosomes is constrained by periodic patterns of chromatin marks, whose function in recombination and higher order genome organization is yet to be elucidated.

chromatin organization | epigenetics | meiosis | histone modifications | superresolution microscopy

Meiosis is a specialized cell division essential for the life cycle of sexual organisms and is needed to generate genetic diversity of haploid gametes. This diversity comes from two major chromosomal events, homologous recombination and chromosome segregation. Homologous recombination is initiated by topoisomerase-like enzyme Spo11 in the leptotene stage of prophase I via double-strand breaks (DSBs) (1). Homologous chromosomes search for each other to repair the breaks and coalign or pair through this process. The association of homologous chromosomes is later strengthened during zygotene by formation of the synaptonemal complex (SC). Completion of the SC defines pachytene, when homolog axes are linked along their lengths and reciprocal exchanges of homologous chromosomes result in crossovers. As a consequence, obtaining information on the detailed structure of the pachytene chromosomes could help to understand the mechanistic steps that have to take place in order for recombination to happen (2-4). Although the major recombinase proteins involved in the process of recombination have been localized (5-7), the way the structure is influenced by epigenetic modifications is unclear.

Epigenetic modifications play an important role in regulating gene expression by modifying chromatin accessibility and recruiting factors to allow genes to become active or silenced (8). This chromatin modulation is done by regulation of DNA binding proteins called histones. The amino acid tails of histones (H2A, H2B, H3, and H4) are subjected to various posttranslational modifications, including acetylation and methylation, which can regulate the compaction of chromatin and modulate gene expression (9). Posttranslational modifications of histones have been shown to contribute to gene expression and recombination during meiosis, but if and how the structure of the pachytene chromosome is regulated by them is still elusive. A major regulator of pachytene recombination is the methylation of lysine (Lys) 4 of histone 3 (H3K4) methyltransferase PRDM9 (10). The presence of trimethylation of histone H3 at Lys4 (H3K4me3) is associated with recombination, along with dimethylation of histone H3 at Lys4 (H3K4me2) and acetylation of histone H3 at Lys9 (H3K9ac) (11, 12). Conversely, other histone marks are depleted at recombination sites, such as H3K27me3 (trimethylation of histone H3 at Lys27) and H3K9me3 (trimethylation of histone H3 at Lys9) (12), leaving one with the expectation that histone modifications might mark different locations on the pachytene chromosomes. Therefore, we decided to localize DNA, H3K4me3, H3K9me3, and H3K27me3 along the length of the SC to describe the epigenetic status of the pachytene chromosomes during meiosis.

Conventional light microscopy (LM) has shown the colocalization of H3K27me3 and H3K9me3 with synaptonemal complex protein 3 (SYCP3) (13), but due to the resolution limitations of LM (∼200 nm), the precise distribution of these marks along the pachytene chromosome has not been described yet. With the advent of various superresolution methods (14), particularly single-molecule localization microscopy (SMLM)-based methods (15–17) and recently developed strategies for labeling DNA (18, 19), it is now possible to study DNA structures at the molecular scale. The underlying principle of most SMLM-based methods is to label molecules of interest with fluorescent moieties that can reversibly switch between a fluorescent state and a stable dark state. Using single molecule localization microscopy with DNA staining, we show that chromatin is heavily constrained by defined periodic clusters along the synaptonemal complex (SC). Staining of various posttranslational histone modifications further reveals that the pachytene chromosome is associated with three distinct nanoscale compartments. Whereas chromatin associated with active transcription emanates both axially and radially in hair-like loop structures, the chromatin associated with repressed transcription follows periodic clusters close to the central axis of the SC. These findings suggest a model showing how chromatin and epigenetic modification patterns can be incorporated within the SC to shape the pachytene chromosome.

Significance

The structure of the pachytene chromosome is essential to understand how genetic recombination can happen during meiosis. Using single molecule localization microscopy with DNA staining, we show that chromatin is heavily constrained by defined periodic clusters along the synaptonemal complex (SC). Staining of various posttranslational histone modifications further reveals that the pachytene chromosome is associated with three distinct nanoscale compartments. Whereas chromatin associated with active transcription emanates both axially and radially in hair-like loop structures, the chromatin associated with repressed transcription follows periodic clusters close to the central axis of the SC. These findings suggest a model showing how chromatin and epigenetic modification patterns can be incorporated within the SC to shape the pachytene chromosome.

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state. This process of switching between states is called blinking, and allows for optical isolation of single molecules. Because only a fraction of molecules will actually fluoresce at a given time, their precise location can be determined and a reconstructed super-resolved image can be produced. In this work, we use a variation of SMLM called spectral precision determination microscopy (20, 21) that allows for the use of conventional fluorophores, which enabled us to investigate chromatin organization via localizing DNA and particular histone modifications along the SC.

To describe the general shape of chromatin during meiosis, we applied a recently developed method based on photoconversion of standard DNA dyes (18, 19) that generates high-quality density maps of chromatin by promoting blinking. Using this strategy in conjunction with fluorescently tagged antibodies targeted to particular histone modifications, we studied localization patterns of chromatin and epigenetic modifications in pachytenomas. Chromatin was found to display a characteristic clustering pattern of 550–700 nm along the SC. We then investigated the localization of H3K4me3, H3K27me3, and H3K9me3 along the pachytene chromosomes.

Interestingly, we found that the histone marks form highly identifiable clusters, with defined localization and periodicity. Large clusters of H3K27-trimethylated chromatin are periodically detected at intervals of ~500 nm along the SC, whereas one of the ends of the SC, presumably the centromeric end, displays a large and dense cluster of histone mark H3K9me3. Chromatin associated with potential recombination regions marked by H3K4me3 (12) is arranged both axially and in radial loop-like patterns along the SC. The arrangement of H3K4me3 along these patterns seems to be discontinuous, forming small clusters, similar to the bead-on-the-string model. The average spacing of 200 nm suggests that regions from which these patterns emanate could alternate with H3K27me3 regions. Overall, these findings help to describe the structure of the pachytene chromosomes, which is punctuated by clusters of epigenetically regulated chromatin that may alternatively promote or suppress recombination.

Results

Superresolution Imaging of SC Proteins. To study the chromatin distribution along the pachytene chromosomes in high resolution, we first characterized the structure of the SC that acts as a scaffold for chromatin during pachytene. This framework served as our benchmark for the subsequent analyses. We localized the distribution of two important components of the SC, namely, SYCP1 (the central element of the SC) and SYCP3 (the lateral element of the SC), which are critical for the synopsis of homologous chromosomes. For quantitative analysis, we labeled the SYCP3 and SYCP1 C-terminus with Alexa Fluor 555 and detected, on average, 2,500 photons per cycle. Localization maps of SYCP3 and SYCP1 proteins were generated by integrating roughly 20,000 observations, each of which captured photons emitted during 100 ms of camera integration time. These observations localized individual fluorophores with an average precision of 11 nm and 16 nm and a Fourier ring correlation resolution (22) of 43 nm and 56 nm for the SYCP3 and SYCP1 C-terminus, respectively. For visualization, we used the mean distance to 20 nearest neighbor molecules to blur individual molecules of SYCPs. For SYCP3, the nearest neighbor distance was 14 ± 5 nm, and for SYCP1, this distance was 19 ± 7 nm (Fig. 1E). Comparison with localization precision-based Gaussian blurring and triangulation is shown in SI Appendix, Fig. 1.

The images obtained (Fig. 1A and B) show a clear separation of the two lateral elements of the SC and highlight the spatial distribution of individual SYCP3 and SYCP1 proteins along the two strands. Comparison of SMLM images with wide-field images shows that the separation between the two strands of SYCP3 is not resolvable with wide-field microscopy (Fig. 1C). By computing the distance from the maxima of each individual fluorophore of SYCP3, we estimated the distance between the two strands of SYCP3 to be around 181 nm and the width at half maximum of an individual strand to be 60 nm (Fig. 1D). Regarding the C terminus of SYCP1 protein, the distance between the strands was 88 nm and the width was 47 nm. Our results are in good agreement with previously reported results based on EM and high-resolution LM. EM studies indicate that when fully synapsed, the lateral elements of the SC are roughly 100–200 nm apart, although other extreme values have been observed (23–25). Much more recently, using SMLM, the width of SYCP3 was reported to be around 56 nm and the distance between strands to be 165 nm (26). Schücker et al. (26) found the width of SYCP1 C-terminus to be 45 nm, with the overall width of the central region [composed of transverse filaments and the central element] to be around 148 nm, which is in line with our observations.

Fig. 1. Superresolution imaging of SYCPs. SMLM images of the SYCP3 (A) and SYCP1 C terminus (B) labeled with Alexa Fluor 555. (Insets: A1 and B1) Wide-field equivalent of the underlying localization image. [Scale bar: (1 μm) is the same for A, Inset A1, B, and Inset B1.] (C) Plot profile of the transversal distribution of SYCP3 in wide-field imaging (blue plot) and in localization microscopy (red plot). (D) Plot profile of the bimodal distribution of proteins SYCP1 C-terminus (red plot) and SYCP3 (blue plot). We estimate the interstrand distance of SYCP3 to be around 180.7 nm and the interstrand distance for the C terminus of the SYCP1 protein to be around 87.9 nm. (E) Mean distance to 20 nearest neighbor molecules was used to blur individual molecules of SYCPs. For SYCP3, the nearest neighbor distance was 14.31 ± 8.74 nm, and for SYCP1, this distance was 18.81 ± 7.40 nm.
Nonrandom Distribution of Condensed Chromatin Structures Along the Pachytene Chromosomes. We next analyzed, at enhanced resolution, the distribution of chromatin along the pachytene spreads. We immunostained pachytene spreads with anti-SYCP3 (Alexa Fluor 555) and then stained chromatin with Vybrant DyeCycle Violet (Life Technologies) (19) (details are provided in Materials and Methods). Pictures obtained revealed highly condensed chromatin along the SC, with many radial projections (Fig. 2 A–C). The highest density of chromatin is found around the central axis of the SC, but chromatin distribution can expand as far as 500 nm away from the center of the SC, although with decreasing density (Fig. 2D).

The general shape of chromatin is reproducible (SI Appendix, Fig. 2) and presents characteristic clusters whose diameter is found to be in the range of 170–225 nm and whose periodicity is found to be between 550 nm and 700 nm (Fig. 2E and SI Appendix, Fig. 2). To validate these clusters, we tested random distributions of chromatin (SI Appendix, Fig. 3). The local condensation (clustering) in the SMLM image was characterized by calculating the distance to 20–500 nearest neighbors and using the same method on the simulated data. The mean distance of 500 nearest neighbors was 86 nm for chromatin and 110 nm for the random data (SI Appendix, Fig. 3), thereby indicating that the clustering seen in the real SMLM images of DNA arises from structural packing of DNA in the SC.

Repressive Histone Mark H3K27me3 Shows Characteristic Periodic Clusters Along the SC. Because histone modifications are known to regulate DNA participation in recombination and transcription, we tested if SMLM could be used to localize histone modifications in the context of chromatin. As was previously shown with confocal microscopy (13), the signals of repressive histone mark (H3K27me3) colocalized with SYCP3 at several points in our images as well (Fig. 3A). However, instead of colocalization with SYCP3 over the whole length, we find large periodic clusters (Fig. 3B) of H3K27me3 occurring at an average lateral distance of 40–50 nm from the strands of SYCP3, with occasional overlap (Fig. 3C). When comparing these results with previous results with confocal microscopy (13) (and viewing our own wide-field results), it is clear how colocalization of SYCP3 and H3K27me3 would be assumed because of the ~200-nm resolution of the imaging process. To estimate the periodicity and the size of the clusters, we computed an autocorrelation measure along the tangential direction to the SYCP3. We observe that the clusters appear at an average distance of 450–650 nm (Fig. 3D and SI Appendix, Fig. 4), with the average cluster diameter between 90 nm and 130 nm (SI Appendix, Fig. 4). Staining of different chromosomes shows reproducibility of shape and hints at possible looping of chromatin around helical patterns of the SC (SI Appendix, Fig. 4). Given the H3K27me3 cluster spacing, it is possible that the H3K27me3 clusters may correspond to the chromatin clusters observed along the length of the SC (Fig. 2C). Furthermore, due to the occasional symmetry of H3K27me3 clusters on both sides of the SC, we hypothesize that this histone mark might be associated with nonrandom sites along the genome.

Centromeric Histone Mark H3K9me3 Labels One End of the SC. Mouse pachytene chromosomes are known to be telocentric, meaning that the centromeres are typically very close to one of the two telomeres of the chromosome, and these centromeric regions are associated with H3K9me3. Seeing nanoscale spatial structure in H3K27me3, we next probed whether the large, condensed chromatin structures found at one end of the SC might be marked by H3K9me3, with unique nanoscale patterns. We communostained SYCP3 and H3K9me3 to study the localization of the H3K9me3 mark with respect to SYCP3. Large, dense clusters of H3K9me3 could be seen at one of the ends of the pachytene chromosomes (Fig. 4), which we speculate to be the centromeric end. We hypothesize that the high density of H3K9me3 observed at the presumed centromeric end of SYCP3 (Fig. 4C) may be concomitant with the high chromatin density observed at the top axial end of the chromosomes (Fig. 2B), because the average axial spreads of the large cluster in axial chromatin (Fig. 2B) and H3K9me3 (Fig. 4A) and H3K9me3 (Fig. 4A) are both in range of 1–1.5 μm. Moreover, we note that in some images, the two SC strands seem to move apart at the presumed noncentromeric end, as observed previously (27). We have not observed such splitting at the presumed centromeric ends (Fig. 4 and SI Appendix, Fig. 5).

Histone Mark H3K4me3 Associated with Active Transcription Emanates Radially from the Axis of the SC. Finally, we studied the distribution of H3K4me3, a histone modification associated with active transcription, along the pachytene chromosome. Surprisingly, we found the mark to be distributed both axially and radially, forming protrusions as long as 500 nm. Like H3K27me3, H3K4me3 appears discontinuous for the most part and tends to form tiny clusters (Fig. 5A and B and SI Appendix, Fig. 6). The average spread of the radial emanations of H3K4me3 ranged from 300 to 500 nm, with the overall distribution of H3K4me3 qualitatively similar to the chromatin distribution (Figs. 2D and 5D and SI Appendix, Fig. 6). By autocorrelating the tangential distances from the central axis of SYCP3 (Fig. 5E), we estimated the average spread (~500 nm) of the protrusions to be larger than their spacing (~200 nm). Parts of our images show a possible looping structure of H3K4me3-stained processes (Fig. 5C), compatible with data from EM of pachytene chromosomes observed outside of the mammalian class (28). H3K4me3 labeling enabled us to infer loop structures because we could

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**Fig. 2.** Superresolution microscopy reveals high-order clusters of chromatin patterns along the pachytene chromosome. Pachytene spreads were immunostained with anti-SYCP3 (Alexa Fluor 555) and then stained with Vybrant DyeCycle Violet. (A) Two-color SMLM was performed. (Inset: A1) For comparison, the wide-field counterpart of the underlying SMLM image. (B and C) Differential chromatin clusters are highlighted. [Scale bar: (1 μm) is the same for A, inset A1, and B (250 nm) for C] (D) In the representative image of pachytene chromosomes stained with Vybrant DyeCycle Violet, DNA is normally distributed around the two strands of SYCP3 proteins, with a maximum spread around 500 nm. (E) Auto-correlation plot (chromosome domain in the yellow box of B) shows a periodicity of 550–700 nm tangentially along the central axis of SYCP3. The estimated cluster diameter varied from 170–225 nm (SI Appendix, Fig. 2). The blue lines in the plot correspond to 95% confidence bounds (± 0.08),
connect distinct clusters of signals that were not observable in the case of DNA staining due to higher signal density [DNA staining: 5,000 single-molecule signals per square micrometer (19), histone staining: 100 single-molecule signals per square micrometer (29)]. Although the loops were not observed directly using DNA staining, the size of lateral extensions (500 nm) found for DNA staining (Fig. 2D) matches the size found for H3K4me3 staining (Fig. 5D).

Discussion

We report the first single-molecule resolution images, to our knowledge, of chromatin associated with pachytene chromosomes using LM based on 14 individual chromosomes from 12 oocytes generated from eight different mice. Interestingly, chromatin shows identifiable large clusters that we speculatively associate with potential turns of the SC as modeled in other studies (26, 30). We also present the first high-resolution images, to our knowledge, of histone modifications associated with the pachytene chromosome. Unexpectedly, histones show clusters possibly related to a level of chromatin organization that has not been described before. Wide-field and SMLM images of H3K4me3 (Fig. 5A and B) show patterns that are morphologically compatible with characteristic loops associated with pachytene chromosomes. The function of these loops is still elusive, although one may speculate that they could participate to regulate recombination (4). We imaged chromosome spreads here, and the situation might differ in structurally preserved nuclei. Several publications have shown lateral extensions of meiotic chromosomes that are commonly believed to be loops (31, 32). The validity of measuring loop sizes using our newly developed DNA labeling technique could be tested on cohesin mutant Smc1b−/− (structural maintenance of chromosomes protein 1B) meiocytes, in which loops are known to be larger (32).

H3K9me3 was found to localize at the presumptive centromeric regions. On closer examination, we note that some images hint at spiralization of H3K9me3-marked DNA at the extremity of synapsed chromosomes (Fig. 4D, 1–3). Previously, it was reported that H3K27me3 is present exclusively at the pachytene stage and colocalizes with the SC (13). We find periodic clusters of this histone mark near the axis of the SC. The pattern of H3K27me3 is especially interesting because it is occasionally symmetrical on each side of the SC, which hints at an association with defined regions of the genome (33). These clusters may be selected to escape recombination, similar to how DNA methylation was suggested to prevent recombination in transposon sequences (34). Another possible hypothesis is that H3K27me3 clusters may be involved in the formation of the SC itself (35).

Our findings suggest a model for spatial distribution of chromatin at the pachytene stage of meiosis prophase I (Fig. 6). Based on localization maps of posttranslational histone modifications, we can dissect the pachytene chromosome structure into three distinct spatial morphologies highlighted by the differential nanoscale organization: (i) radial chromatin identified by H3K4me3 indicative of actively transcribed chromatin, (ii) polar chromatin identified by H3K9me3 indicative of centromeric chromatin, and (iii) tangential chromatin identified by H3K27me3 indicative of repressed chromatin. The overall view of the epigenetic makeup of the pachytene chromosome is consistent with increased chromatin accessibility away from the SC, and compaction of regions close to the SC. Relating this type of microscopy data to genomic data will be necessary to understand the biological function of the structural patterns we observe.

Fig. 4. Centromeric distribution of H3K9me3 around SYCP3 molecules. (A) Two-color SMLM image immunostained with anti-SYCP3 (Alexa Fluor 555) and anti-H3K9me3 (Alexa Fluor 488). (Inset: A1) Underlying wide-field image. Large dense clusters of repressive centromere mark H3K9me3 can be seen at the presumptive centromeric end of the pachytene chromosomes. (C) High H3K9me3 density at the centromeric ends of SYCP3 is concomitant with high chromatin density at the top axial end in (Fig. 2B). (D) We observed that the strands of SYCP3 at the end where H3K9me3 colocalizes (presumptive centromeric end) were closer (~135 nm) than at the presumptive noncentromeric end (~180 nm, yellow box of A) (SI Appendix, Fig. 5). The point of representation of single molecules of SYCP3 and H3K9me3 underneath the binary mask (described in Material and Methods) is shown in C. (D, 1–3) Different visualization strategies are compared. [Scale bar: (1 μm) is the same for A, Inset A1, C, and D; (250 nm) is the same for D1, D2, and D3.]
Our aim here was to demonstrate the power of SMLM to describe the epigenetic landscape of pachytene chromosomes. Clearly, experimental evidence will be required to ascribe functions to the observed patterns of epigenetically marked chromosomal subdomains. In a first step, it will be interesting to determine at which stage of prophase I the patterns of H3K4me3 and H3K27me3 are established (i.e., are they exclusive features of pachytene chromosomes, or does a certain periodicity precede synopsis?). By combining DNA, histone marks, and markers of crossovers, it will be possible to localize crossover sites to epigenetic landmarks in single pachytene chromosomes. Beyond descriptive studies, the toolbox of genetic mouse mutants will help to decipher how the radial and tangential chromatin domains are influenced by DSB formation and crossover resolution, and vice versa. It is also conceivable to modify DNA in one locus and to observe the local consequences on chromatin structure, whereas the overall chromosome remains intact. Lastly, we cannot exclude the possibility that some of our observations are due to the spreading of pachytene chromosomes. Further investigations are needed to determine whether our findings hold true for in situ fixed pachytene cells. Because the SMLM technique used here requires high laser power resulting in fast photobleaching and labeling intensity is restricted, imaging of whole nuclei will require technical advances that go beyond the current setup.

To conclude, SMLM imaging provides the first nanoscale localization map, to our knowledge, of three histone marks and chromatin on mouse pachytene chromosomes undergoing meiotic recombination. Observing histone modifications at the single-molecule level will help define hypotheses for the functions of epigenetic modifications in meiotic recombination and in shaping higher order genome architecture.

Materials and Methods

All mice were bred and maintained at the Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA), Vienna, in accordance with ethical animal license protocols approved by the Federal Ministry of Science, Research and Economy.

Sample Preparation of Pachytene Spreads. Pachytene spreads were prepared as described previously (36). Briefly, ovaries were dissected from embryonic day 18.5 embryos and treated with hypotonic buffer (17 mM trisodium citrate-dihydrate, 50 mM sucrose, 5 mM EDTA, 0.5 mM DTT, 30 mM Tris (pH 8.2)) for 25 min. Ovaries were dispersed in 100 mM sucrose solution using 21-gauge needles. The cell suspension was applied to poly-L-lysine-covered slides and fixed in 1% paraformaldehyde and 0.2% Triton X-100 solution overnight in humidified chambers. Spreads were air-dried, washed in 0.1% Triton X-100 for 10 min, and washed three times in PBS. Slides were blocked in 5% (v/v) BSA in PBS for 30 min at room temperature and stained with SYCP1 (ab51509; Abcam) and SYCP3 (ab97672; Abcam) antibodies (1:800 dilution) at 37 °C for 1.5 h. For double-staining of SYCP3 and histone modifications, anti-H3K9me3 (ab8898; Abcam), anti-H3K27me3 (no. 07-449; Merck Millipore), and anti-H3K4me3 (ab8580; Abcam) at a concentration of 1:1,000 were used. Slides were washed three times for 10 min in PBS, incubated with Alexa Fluor 488 and Alexa Fluor 555 secondary antibodies (Life Technologies), and washed again. Slides were embedded in ProLong Gold mounting reagent (Life Technologies). The DNA staining protocol has been described elsewhere (18, 19). Briefly, the slides were incubated with Vybrant DyeCycle Violet (VdcV) (0.2 μg/mL) for 20 min and washed with PBS briefly. In these cases, slides were embedded after modified switching buffer consisting of glycerol with 10% (v/v) imaging buffer (stock comprising 0.25 mg/mL glucose oxidase, 0.02 mg/mL catalase, 0.05 g/mL glucose in PBS). Ten microliters of the imaging buffer was placed on a glass slide, after which the coverslip with the sample was placed upside-down on the buffer droplet. The coverslip was attached to the slide with either nail polish or biologically inert dental paste (Picodent Twinill) before imaging.

Microscope Setup. The configuration of the microscope setup used here has recently been described (18). Briefly, for the present experiments, we used two diode-pumped solid-state lasers: a 491-nm laser (Calypso 05 series; Cobolt) and a 561-nm laser (Calypso 05 series; Cobolt). The laser beam enters the microscope via mirrors, and a collimator arrangement (5x expansion of beam diameter). The beam is focused by a lens into the back focal plane of an oil immersion objective lens (1.4 N.A., 63x oil immersion with a refractive index of 1.518; Leica Microsystems). The sample is illuminated by a piezoelectrical stage for focusing. To achieve the high laser intensity for the localization mode, a lens is inserted in the optical pathway, leading to a smaller illuminated area (higher intensity) in the optical pathway, leading to a smaller illuminated area (higher intensity) in the object region of interest, allowing for the appropriate conditions for the reversible photobleaching. The emitted fluorescent light passes through the dichroic mirror and is focused by a tube lens (1.0x, f = 200 mm) onto the CCD chip of a highly sensitive 12-bit black and white CCD chip (SensiCam QE; PCO Imaging). With a 102-μm effective pixel size in the optical pathway, leading to a smaller illuminated area (higher intensity) in the object region of interest, allowing for the appropriate conditions for the reversible photobleaching. The emitted fluorescent light passes through the dichroic mirror and is focused by a tube lens (1.0x, f = 200 mm) onto the CCD chip of a highly sensitive 12-bit black and white CCD chip (SensiCam QE; PCO Imaging). With a 102-μm effective pixel size in the optical pathway, leading to a smaller illuminated area (higher intensity) in the object region of interest, allowing for the appropriate conditions for the reversible photobleaching. The emitted fluorescent light passes through the dichroic mirror and is focused by a tube lens (1.0x, f = 200 mm) onto the CCD chip of a highly sensitive 12-bit black and white CCD chip (SensiCam QE; PCO Imaging). With a 102-μm effective pixel size in the optical pathway, leading to a smaller illuminated area (higher intensity) in the object region of interest, allowing for the appropriate conditions for the reversible photobleaching. The emitted fluorescent light passes through the dichroic mirror and is focused by a tube lens (1.0x, f = 200 mm) onto the CCD chip of a highly sensitive 12-bit black and white CCD chip (SensiCam QE; PCO Imaging). With a 102-μm effective pixel size in the optical pathway, leading to a smaller illuminated area (higher intensity) in the object region of interest, allowing for the appropriate conditions for the reversible photobleaching. The emitted fluorescent light passes through the dichroic mirror and is focused by a tube lens (1.0x, f = 200 mm) onto the CCD chip of a highly sensitive 12-bit black and white CCD chip (SensiCam QE; PCO Imaging). With a 102-μm effective pixel size in the optical pathway, leading to a smaller illuminated area (higher intensity) in the object region of interest, allowing for the appropriate conditions for the reversible photobleaching. The emitted fluorescent light passes through the dichroic mirror and is focused by a tube lens (1.0x, f = 200 mm) onto the CCD chip of a highly sensitive 12-bit black and white CCD chip (SensiCam QE; PCO Imaging). With a 102-μm effective pixel size in the optical pathway, leading to a smaller illuminated area (higher intensity) in the object region of interest, allowing for the appropriate conditions for the reversible photobleaching. The emitted fluorescent light passes through the dichroic mirror and is focused by a tube lens (1.0x, f = 200 mm) onto the CCD chip of a highly sensitive 12-bit black and white CCD chip (SensiCam QE; PCO Imaging).
Data Acquisition and Evaluation. For the dual-color experiments, we sequentially imaged Alexa Fluor 555 (in the case of SYCP3), followed by Alexa Fluor 488 (in the case of H3K27me3), and H3K4me3 or by VYbrant DyeCycle Violet (DvD) stain (Life Technologies), in the case of DNA staining. For imaging of Alexa Fluor 555, 4,000 frames with a camera integration time of 90 ms were acquired with 90 mW of 561-nm excitation, whereas imaging of Alexa Fluor 488 was performed by acquiring 3,000 frames with a camera integration time of 100 ms at 90 mW with 491-nm excitation in the sample plane. The single-fluorophore signal positions were determined by calculating the center of mass with custom-written software in MATLAB (MathWorks) (37). Temperature changes or mechanical relaxations can cause sample-instrument drift. A cross-correlation algorithm was implemented to detect drift and extract shift vectors between reconstructed subsets (typically 100 frames). The extracted shift values were interpolated and used to correct the list of positions if an overall drift larger than 20 nm was detected. Measurements in dual-color microscopy usually suffer from chromatic aberrations. The chromatic shift between the two-color channel images was extracted by reconstructing the individual color channels and calculating the cross-correlation between the reconstructions.

Visualization and Data Analysis. For visualization, we blurred each molecule position with a Gaussian distribution of SD equal to the mean value of the distance to the next 20 nearest neighbor molecule positions. In the generated image, the intensity corresponds to the local density of fluorophores, resulting in an image equivalent to the one in conventional LM (38). For high-density single-molecule reconstructions, structural resolution is dominated by localisation precision. Nearest neighbor visualization is independent of localisation precision and helps in bringing out the underlying structure where the final reconstructions suffer from poor labeling density. A comparison with Gaussian blurring based on localisation precision, triangulation, and point representation is shown in SI Appendix, Figs. 2, 4, and 6. Due to the curvilinear shape of the SC, we first manually fitted the contours of the SC to best fit the single-fluorophore signal positions that span the width of the SC, (i) lateral to indicate positions of molecules that are perpendicular to the central axis of the SC, and (ii) tangential to indicate positions that are parallel to the central axis of the SC (a description is provided in SI Appendix, Fig. 7). We refrain from using the term “axial” other than in reference to the central axis of the SC because this term is already associated with description of the single strands of the SC before synopsis.

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Supplementary Material:
Superresolution imaging reveals structurally distinct periodic patterns of chromatin along pachytene chromosomes

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Figure 1. Comparison of various visualization algorithms: We compared nearest neighbor blurring to Gaussian blurring based on localization precision and to triangulation of individual molecules. We found that when enough nearest neighbor points are taken into account, the underlying structures can be better observed. Nearest neighbor blurring can help to bring out the underlying structure in images lacking proper labelling density. Moreover, the points that are not part of a structure are suppressed.
Figure 2. Reproducible patterns of chromatin organization along different chromosomes: Two different meiotic chromosomes are shown (A,B). All characteristics were found to be similar: spread, tangential distribution and periodicity. The average spread (A1 and B1) is around 500 nm. The cluster diameter in (A) was found to be 223 nm while in (B), it was 184 nm. (A2 and B2) show the histogram of the tangential distances taken along the central axis of SYCP3 in the yellow boxes indicated in (A and B). Chromatin clusters were found to occur at a periodicity of 685 nm (A3) and 558 nm (B3). The blue lines in the plot correspond to 95 percent confidence bounds (±0.08).
Figure 3. Comparison of nearest neighbor distances in SMLM and random data: In order to validate the clusters found in the DNA SMLM image (A), we compared the extracted list of molecule signal localizations to a randomly simulated dataset (B). We generated a binary mask of the nearest neighbor blurred image and then randomly generated the same number of points as in SMLM image. The local condensation in SMLM and random images was characterized by calculating the mean distance to nearest neighbor molecule signals (in nanometer) as a function of the number of nearest neighbor molecule signals considered (from 20 to 500 nearest neighbors) (C); in each case the mean nearest neighbor distance was significantly shorter than in the random simulated dataset. The mean distance to 500 nearest neighbors was 86 nm for chromatin and 110 nm for the random data.
Figure 4. Reproducible patterns of periodic clusters of H3K27me3: Clusters in two different acquisitions of H3K27me3 are shown (A, B). The cluster diameter in (A) was found to be 111 nm while in (B) cluster diameter was 128 nm. (A1 and B1) show the frequency (number of molecule signals) along the central axis of SYCP3 in the yellow boxes indicated in (A and B). Autocorrelation analyses of the histograms in A1 and B1 underline the periodic distribution of the clusters of H3K27me3, which were found to occur at intervals of 640 nm (A2) and 480 nm (B2). The blue lines in the plot correspond to the 95 percent confidence bounds (A2, ±0.1265) and (B2, ±0.1414).
Figure 5. Reproducible patterns of H3K9me3 distribution and organization of SYCP3 at the presumptive telomeric and telocentric ends: The telomeric end of SYCP3 seems to be more apart than the telocentric end in (A and B). Overall, H3K9me3 seems to be normally distributed along the axis of SYCP3. (A1 and B1) show the lateral frequency distribution (number of molecule signals) perpendicular to the central axis of SYCP3. The average distance at the telocentric end in A and B was 142 nm and 130 nm respectively, while the average distance at the telomeric end was 171 nm and 190 nm respectively (A1 and B1).
Figure 6. Reproducible patterns of distribution and periodicity of radial loops of H3K4me3: Two examples of radial loops of H3K4me3 are shown (A, B). (A1 and B1) show the lateral frequency distribution (number of molecule signals) perpendicular to the central axis of SYCP3, while (A2 and B2) show the frequency (number of molecule signals) along the central axis of SYCP3 in the white boxes indicated in (A and B). The average spread of H3K4me3 suggests periodicity along the direction normal to the central axis of SYCP3, possibly indicating individual H3K4me3 clusters (A1 and B1). (A3 and B3) The autocorrelation of the histograms (A2 and B2) show the periodic clusters of H3K4me3 loops. The periodicity in A was found to be around 216 nm while in B loops occurred at intervals of 209 nm. The blue lines in the A3 and B3 correspond to 95 percent confidence bounds (A3, ±0.1414) and (B2, ±0.1613). The average cluster diameter in (A) was found to be 47 nm while in (B) the cluster diameter was 45 nm.
Figure 7. Coordinate system referring to the orientation around the synaptonemal complex (SC): We used the Frenet frame to describe the geometric positions of the single molecules relative to the central axis of SC.