nature portfolio

Peer Review File

Stepwise pairing and fast transition regulated by recombination drive meiotic chromosome interactions

Corresponding Author: Dr Roberto Pezza

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors developed an elegant system to monitor the juxta-positioning of homologous chromosomes in a mammalian meiotic model organism by introducing the lac operator-lac repressor system at two distinct chromosome loci. The dynamics of those were video-recorded in 3D in in vitro-cultured seminiferous tubules using microfluidic chambers (an impressive optimization compared to previous reports about movement in mammals). First, the authors validated the system using FISH and compared the movements of Lac repressor-marked loci with those assessed by tracking Hoechst-enriched structures. The characteristics of movements are the same, regardless of the chromosome position of the LacO insertion. Furthermore, the authors showed that the pairing process occurs in rapid sequential steps of juxta-positioning. Analysis of 3D pairing in recombination mutants revealed that the strand invasion step mediates the intermediate stage of pairing. Absence of sequential pairing was also confirmed by FISH in both DMC1 and SPO11 mutants. In mutants required at a later step of pairing, the authors state that full pairing is achieved before the stage of double-holiday junction resolution. The assessment of the pairing stage via chromosome axis stainings in HFM1 and SHOC1 mutants showed an intermediate phenotype, supporting a model in which full pairing is achieved through the maturation of crossover recombination products.

Overall, this study represents a major technological step forward. It is the first study to show meiotic chromosome movements at specific chromosomal loci in mammals. The authors provide evidence that the models for homolog alignment developed in yeasts likely also hold true in mammals. This is really a major achievement! The FROS system can be used for other research questions and thus might become a resource for many other projects, even in unrelated fields. Overall, the authors presented strong arguments that chromosome juxta-positioning in mammals operates in a manner like that observed in yeasts. Below, find some comments that would benefit from clarification.

Perhaps it would be helpful to briefly discuss whether the repetitive nature of the LacO sites could influence pairing; Can the employed GFP dimerize?

I missed the filming data for at least one late recombination mutant (MSH4/5?). However, I am aware that strain construction is expensive and likely very time-consuming with this model system.

Line 83-91: Please add a citation to this statement.

Line 136: Please indicate with an image what defines centromeric heterochromatin.

Fig. 1D: It remains somewhat enigmatic how the authors can tell chromosome 8 from 9 in that figure. Please mention in the results text or legend.

Line 144: Ref 35 is about a non-meiotic function of the LINC complex, and reference 41 refers to fixed samples (unless the presence of a bouquet implies movements).

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Line 159: Is that nm/s? Please add.

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Line 179: It is hidden in the legend that this value represents an average of recordings of different stages. Please mention this in the text.

Line 187-188: I missed a clear explanation of how the inter-chromosome distances were assessed. How were chromosomes 8 and 9 told apart?

Line 202ff: What do you mean by “initial engagement”?

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It would also be good to provide plots for all the different measurements (n=12), as the authors did in Figs. S1 and S2.

Line 227ff: It is not clear how this was defined. Is it arbitrary or based on the results of Fig. 3, taking the average±SD for the early and late meiosis?

Line 253: The Authors showed that the transition from pre-aligned to paired lasts for 1h. Should there not be a longer recording period for DMC1 mutants (longer than 30 minutes)?

Line 269: Which type?

Line 273: Where can this be found in the methods?
It is also not clear which FISH data is being used to measure these 2D distances.

Line 293: Add references and explain which yeast homologs these proteins likely represent.

Line 309: Has the appropriate test been used here?

Line 324: Please, add a citation to the Mer3 homolog; I missed that explanation earlier on as well.

Line 330: Where in the methods part can one find how these measurements were done?

Line 364ff: Please explain better.

The model in Fig. 5G is not cited in the text.

Fig. 5F: Please bring the content of this figure to the main text. Can these bridges also be seen with MSH4/5 stainings?

Line 478: Is there a list of used antibodies provided?

Line 535: Will this script be provided to the community?

Line 553: The Method could be described in a supplemental figure. Will the code be shared?

Line 634: Are the sequences of the probes provided?

Fig. 3: Consider using colour-blind friendly colouring.

Fig. 4A: Scale bar is missing.

Fig. 5: Scale bars are missing.

Reviewer #2

(Remarks to the Author)
Review of the manuscript entitled “Stepwise pairing and fast transition regulated by recombination drive meiotic chromosome interactions” by de Almeida et al.

This manuscript addresses the long-standing question of how homologous chromosomes recognize and pair during meiosis. The authors develop an elegant fluorescence reporter–operator system that allows live imaging of defined genomic loci in mouse meiotic cells, ex vivo, within intact seminiferous tubules. This approach represents a genuine technical tour de force and, to my knowledge, enables for the first time the direct analysis of homologous chromosome pairing dynamics in living mammalian tissue.
Using this system, the authors convincingly demonstrate that homologous chromosomes pair through a stepwise process, rather than via a single continuous transition. They identify distinct distance regimes corresponding to widely separated homologs, an intermediate juxtaposed state, and close pairing, with rapid transitions between some of these states. Importantly, this process is abolished in Dmc1 mutants, highlighting the role of SEI in pairing. Complementary analyses using FISH and immunofluorescence in fixed cells further confirms that the initial transition toward juxtaposition depends on early recombination events, and further demonstrate that final stable pairing requires crossover maturation intermediates and the synaptonemal complex protein SYCP1.

The manuscript is carefully and pedagogically illustrated, and the imaging data are of high quality. However, several points require clarification or additional information to make the study fully accessible and quantitatively robust.

Major points
1. Validation of the lacO-lacR reporter lines (FROS lines)
While Figure 1 convincingly demonstrates that the lacO/lacR system works technically, I could not find clear evidence that meiosis proceeds normally in these transgenic animals. Specifically:
- Have meiotic progression, synapsis, and fertility been assessed in the FROS lines?
- Is there evidence that insertion of the lacO repeats does not perturb the local chromosomal environment or alter pairing behaviour compared to the native locus?
Even if these aspects are difficult to assess comprehensively, they should be discussed explicitly.

2. Definitions and staging of meiotic progression
Several key concepts require clearer definition:
- The classification of cells into early / mid / late meiotic stages is not sufficiently explained. The Methods refer to a previous publication, but this is not sufficient for readers unfamiliar with the system.
What criteria are used for staging : time after RA treatment, SYCP3/SYCP1 patterns, nuclear morphology, else?
- The terms “widely separated,” “juxtaposed,” and “paired” are used throughout, but objective distance thresholds are not clearly defined. Please provide explicit quantitative criteria (e.g. distance ranges) for each category. According to figure 3C, there is partial overlap between the average distances calculated at each stage (early / mid / late). Therefore, I don’t think it is appropriate to say (for example) that “In early meiotic stages, homologs were widely separated” (l178), since some acquisitions from early meiosis will fall in the range of the mid meiosis.
Another example of this confusion is given l185, when the authors write “all cells analyzed for the 90 minute period analyzed moved within one of these three pairing categories (Fig. S1).” What defined these pairing categories? It cannot be the average distance since there is this overlap I already mentioned. This way to present the data should be reconsidered, or better explained and defined.

3. RPMs characteristics
- Only two parameters are discussed: the average speed and the displacement length.
Concerning the displacement length metric. Its releveance depends on all acquisitions having the same duration, which is not specified. Moreover, this descriptor appears to be largely redundant with the average speed. As such, it is unclear how much additional information it provides.
- Instead, I wonder whether the authors could analyze alternative descriptors of chromosomal motion that would better characterize the nature of the trajectories. This seems particularly important given the surprising observation that all investigated chromosomal loci exhibit similar behavior. For instance, since the K9 probe is located close to the telomere, was it expected to behave similarly to K8, which is interstitial? A more detailed characterization of displacement patterns might reveal distinct behaviors between telomeric and non-telomeric loci that are not captured by the current analysis.
- Is K9 signal always close to the nuclear envelope/nuclear periphery or can it be detected everywhere in the nucleoplasm?

4. Stage specific inter-homolog distances
- It is not clear to me if early/midle/late stages can come from the same tubules or not.
- It is not mentioned on how many acquisitions the measures have been obtained. Please provide the numbers in the text. Also specify the number of repeats/independent samples analysed.
- In early meiosis, when neither homolog pairing nor juxtaposition is observed, how do the author explain that the inter-locus distances are so constant over time (Figure 4B)? if the two loci are independent one from the other, I would have expected that their inter-distance should vary considerably from one time frame to the other. I don’t think this observation is discussed anywhere.

5. Abstract

Only 3D time-lapse imaging is mentioned as experimental set up. This is misleading since FISH and IF on fixed samples are also equally important in terms of results shown. The inter-homologues distances given in the abstract for example are not calculated from the time lapse. This should be clarified.

6. Figure legends should be more detailed in many places. And also think to refer to corresponding sup data in the legends when relevant.
For example:
- Figure 3A: The legend should explain the difference between the upper (false-colored) and lower panels. The panel showing the trajectory reconstructions is not mentioned in the legend and should be described.
- Figure 3B:
Is the reported average speed calculated for both loci together? Are there differences between them?
The number of biological replicates and total number of trajectories should be clearly stated in the legend or supplementary data.
I could not find statistical analyses comparing K8, K9, and heterochromatic loci. Do they behave similarly or not? Please provide statistics and n values.
The term “global” for heterochromatin is unclear and should be revised.
Please specify the duration of the acquisitions (are all 18 min as in panel A?). If not, provide the details in supplementary data.
- Figure 3C: I don’t understand what is being shown. The legend refers to nm/sec, while the figure uses µm.
- Figure 4A: Is the lower panel derived from the same cells as the upper panel? If not, this should be clarified, as the current presentation is potentially misleading.
- Figure 4B and 4D: Please refer explicitly to supplementary data for the additional kymographs.
- Figure 4C: Specify the time window over which the averages were calculated.
- Video S1: Acquisition parameters (time interval, duration, projection) should be included in the legend.

Other points
- Clarify the number of LacO insertions (256x in figure1, vs 300 line 119)
- Give more details on the developed system for culture of seminiferous tubule explants: what quality controls were performed to be sure that this ex vivo system recapitulates in vivo meiosis?
- The text lacks precisions about the parameters of the acquisitions (notably the duration). Are they variable or always the same? Maybe the authors could provide in Sup data a table recapitulating the data set.
- It is mentioned l175 that the acquisitions last “approximately” 90 min. what % of the prophase does it represents?
- The sample sizes must be added in the text when measures are given.
- When comparing average numbers (speeds, distances etc) please provide the relevant statistics

Reviewer #3

(Remarks to the Author)
The manuscript by Pezza and colleagues addresses a major question in meiosis (and chromosome biology more broadly): how homologous chromosomes come together during meiosis. The authors develop a new LacI/lacO-based system for tracking specific chromosomes in mice, together with a microfluidic setup to image seminiferous tubules. This important technological advance is used to gain insight into the mechanisms and kinetics of chromosome pairing and alignment. The work is significant both for the mechanistic insight it provides into meiotic chromosome dynamics and for the development of mouse models and imaging conditions that allow one to follow individual chromosomes in live cells and tissues. However, on both fronts, the manuscript would benefit from substantial editing and reorganization to clarify the mechanistic claims and facilitate broader adoption of the methodology. Key points are outlined below.

Major:
Foci tracking: I appreciate the inclusion of the movie as a supplementary file for clarifying how the raw data looks and how it was interpreted. One point that it reveals is the rotational movement of the signal (the Imaris tracks around 0:40). Nuclear rotation was also documented in previous live imaging, as referenced by the authors. However, it is not clear if any correction for these nuclear (as opposed to chromosomal) movements was performed. This is crucial, since a major finding of this work is that different positions along the chromosomes move at similar rates. That would be expected if nuclear rotation contributes significantly to foci movement. If the measurements were not corrected for nuclear rotation, it would be crucial to point that out. If a correction was performed, that should be clearly explained.

Figure 3: The visualization and data analysis relating to foci speed & displacement should be improved. The graphs in panel B & C are challenging to interpret; smaller symbols for each datapoint would be helpful. Even with the current visualization, it seems that there are sample-to-sample differences (e.g., chr 9, early). It seems that in some cases, this variation exceeds stage-to-stage variation; e.g., the green dots in chr 9 early seem to overlap with the green dots in chr 8 mid. Statistical analysis of such potential experimental variation should be performed and the results should be discussed.

The analysis in Figure 4 is well done and is of high quality. In fact, given the generous figure allowance, inclusion of some of the galleries from Figures S1 & S2 would help convey some of the important findings of this work. One issue that should be clarified is the reason the analysis of 'widely-separated' to 'juxtaposed' was not repeated for transitions between 'juxtaposed' and 'close'. Are these events more rare? Are they harder to define given the distances? Is the transition slower?

Figure 5B: The dmc1- live analysis data should be compared to wild type (Fig. 4B-C). Cursory analysis suggests that in dmc1- there is a population of lower distances (<2um) that does not exist in early-meiosis wild type, and that there is a general a shift to shorter inter-homolog distances. The two distributions should be compared statistically, and any identified difference should be discussed in the text.

Figure 5D: All data should be presented, ideally in the main figure. Right now, missing are the histograms for wt-mitotic-chr8, wt-meiotic-chr 1, spo11-chr8, and msh4-chr1. It is impossible to analyze the claims in the text without this information. Additionally, statistical analysis is either lacking or unclear, making the claims related to this data impossible to assess. Some examples: line 269 - what is the statistical analysis that backs the claim of bimodal distribution? What are alternative hypotheses? Downstream of this calculation is the claim in lines 278-9, which describes 'strong bimodality', and defines 'stages' of pairing, which are then (presumably) used below for comparing mutants (e.g. line 309). This analysis is opaque, and in many cases unsubstantiated. Finally, the use of 'density' in the y-axis is insufficiently described, and is inconsistent with other histograms in the manuscript.

Figure 5E: The analysis performed to quantify the images in Fig. 5E is not explained. Indeed, it is impossible to correlate the images with the quantification. E.g., spreads from sycp1- and shoc1- appear very different, yet the distribution of '2D distances' is essentially the same. No statistical analysis is performed, although claims of difference and similarity are being made. This section must be revised before any assessment of its validity could be made. A minor point: the fact that SYCP1 is required for synapsis is made in a separate section of the results and presented as a novel finding, when it is really a control; the manuscript would benefit from presenting it as such.

The authors justifiably stress the technological advance their work represents, and its potential future use. However, the manuscript lacks any data or discussion about how the technique was validated. e.g., comparisons to previous measurements of meiotic chromosome dynamics. Discussion of the shortcomings of the technique are also necessary. For instance, as with many ex vivo live analysis approaches, it is impossible to know whether the imaging perturbs meiosis. This and other potential limitations should be clearly discussed.

Minor:
One of the important conclusions of this work is that intermediate, pre-synapsis juxtaposition is stable. However, the usage of 'stable' in the text is quite confusing. First, as noted above, the stability of synapsis (referred to as 'close pairing') is confirmatory, and matches previous data. The text would benefit from clarifying this point. For instance, the section heading in line 290 is misleading, since the role of CO maturation in SC assembly in mice is well documented. A more minor case is in lines 411-2: "stable synapsis"; was any case of 'unstable synapsis' documented here?

Line 189: "Given the lacO-lacR-GFP fluorescent tag is likely located outside the chromosome axis" - what is the evidence for this claim? In fact, Figure 1D suggests that the GFP signal is on the axis.

line 523: the seminiferous tubules are referred to as 'ovaries'.

The LacO/lacI system is referred to in various places as a paradigm, framework, platform and technology. The acronym 'FROS' is also used in some places. A uniform and accurate description is necessary. Also, the name of the repressor is LacI, not LacR.

line 119 & Fig. 1B: please clarify inconsistency in lacO repeat number.

Line 113: Recommend removing the use of 'universal'. 'Unifying' or 'conserved' are more appropriate.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors did a great job in addressing the points I had raised in my review.
I think the manuscript is now ready for publication.

Reviewer #2

(Remarks to the Author)
I am satisfied with the revisions made by the authors. The changes adequately address my previous comments and significantly improve the quality of the manuscript.

Reviewer #3

(Remarks to the Author)
I appreciate the many improvements the authors introduced to the manuscript. Specifically, the analysis of the bimodal distribution, the inclusion of some of the missing data, and many additions to the text and improvements to the figures. That

said, several key issues raised in the previous review cycle remain insufficiently addressed.

Major points:
There is still a lack of clarity regarding the use of the various kinds of pairing, juxtaposition, alignment terms. The term 'intermediate' seems to be used with regard to pairing and to distance, and the term 'pairing' appears to refer both to a discrete (final) state and a range of distances. Currently, they are not clearly defined and are used interchangeably (e.g., lines 277-278, 327-328, 370-371). Using uniform, clearly defined terms would be critical to make the manuscript accessible to the wide readership of Nature Communications (this issue has also been raised by Reviewer 2).

While I appreciate the clarification in the text regarding the nuclear rotation (lines 209-212), it is unfortunate that the authors did not control for it in the calculations of RPMs. As it stands, it is hard to conclude much from the measurements. A very plausible hypothesis is that nuclear rotations are responsible for most of the measured movements of the FROS foci and heterochromatin. If that were the case, it is impossible to conclude that "RPM characteristics may be uniform across chromosomes" (lines 213-214 and 232-235). Ideally, rotational movements should be controlled for. If that is not done, the conclusions must be significantly toned down, and alternative explanations for the similarity must be presented.

In the "Preparation and imaging of live mouse seminiferous tubules" section in the Methods, the claims regarding the non-perturbative nature of the ex vivo culturing and imaging are not quantified. Specifically, quantification is required for the duration over which RPMs were imaged, as well as the similarity between RPMs in the beginning and end of the experiments.

Line 311-312: That "pairing occurs ... with rapid transitions among individual pairing stages"
is only shown for the first transition ("widely-spaced" --> "intermediate pairing") but not for the transition between the two latter stages. The sentence should be edited to reflect this.

Minor points:
It is unfortunate that the authors chose to not split Fig. 5 into multiple independent figures, each with a different conceptual and experimental focus; as it stands, it is easy to confuse the different experimental modalities and findings. However, that is ultimately an editorial decision.

Fig. S8B: The y-axis seems to be wrongly labeled. Adding some information in the legend about what is shown in the graph is necessary (even if the legend refers to the Methods).

Lines 293-294: The use of the word 'apparently' is unclear. As it stands, it is not obvious what is the relevance of this sentence to the rest of the paragraph.

Line 368: What does "strong bimodality" mean? Suggest remove or clarify.

Fig. 5E: The inclusion of Dmc1-/- and Hop2-/- in main figure is confusing, since they are not quantified or referred to in the main text.

Fig. 5F: The panel is only referred to in the discussion. Perhaps more importantly, the data is not quantified, so the claims associated with it ("HFM1 and SHOC1 are located on DNA bridges") should be edited. Ideally, this panel is either quantified or removed.

Line 273-274: Stating such precise distances for 'early' and 'mid' would best be avoided. If it is mentioned, it should be given with a range. On a more conceptual level, the difference 0.44um makes on these distances is minor, unlike for the 'late' group, where the resulting distance is within the size scale of synapsed chromosome.

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Dear Editor,
We thank you and the reviewers for the positive review of our manuscript. We found the reviewer's comments constructive, and our response to them has improved the manuscript.

We have carefully considered the reviewer's comments, and below we present a point-by-point response. We have added new data and figures, redesigned previous figures, and rewritten several sections of the manuscript.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors developed an elegant system to monitor the juxta-positioning of homologous chromosomes in a mammalian meiotic model organism by introducing the lac operator-lac repressor system at two distinct chromosome loci. The dynamics of those were video-recorded in 3D in in vitro-cultured seminiferous tubules using microfluidic chambers (an impressive optimization compared to previous reports about movement in mammals). First, the authors validated the system using FISH and compared the movements of Lac repressor-marked loci with those assessed by tracking Hoechst-enriched structures. The characteristics of movements are the same, regardless of the chromosome position of the LacO insertion. Furthermore, the authors showed that the pairing process occurs in rapid sequential steps of juxta-positioning. Analysis of 3D pairing in recombination mutants revealed that the strand invasion step mediates the intermediate stage of pairing. Absence of sequential pairing was also confirmed by FISH in both DMC1 and SPO11 mutants. In mutants required at a later step of pairing, the authors state that full pairing is achieved before the stage of double-holiday junction resolution. The assessment of the pairing stage via chromosome axis stainings in HFM1 and SHOC1 mutants showed an intermediate phenotype, supporting a model in which full pairing is achieved through the maturation of crossover recombination products.

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Perhaps it would be helpful to briefly discuss whether the repetitive nature of the LacO sites could influence pairing; Can the employed GFP dimerize?

We have examined the potential effects of the lacO arrays, alone or in combination with lacI–GFP. We have analyzed potential effects on pairing and/or synapsis of the homologous chromosomes locally (at lacO insertions on chromosome 9) and globally using spermatocyte chromosome spreads immunostained with SYCP3 and SYCP1 synaptonemal complex elements (Fig. 1D, and Fig. S1E). Additionally, the effect of the lacO-GFP-lacI insertion on local chromatin condensation was monitored by staining with Hoechst. The analysis shows that neither lacO repeats alone or lacO-lacI-GFP affect the assembly of the synaptonemal complex, homologous chromosome pairing and synapsis locally or globally in our system.

LacI-GFP was constructed using a monomeric green fluorescent protein, mNeon Green GFP, derived from *Branchiostoma lanceolatum* (published in PMID: 23524392**)**, so we do not think homologous chromosome pairing in our system may be affected by dimerization of GFP.

I missed the filming data for at least one late recombination mutant (MSH4/5?). However, I am aware that strain construction is expensive and likely very time-consuming with this model system.

We did not monitor chromosome pairing in MSH4- or MSH5-mutant mice using this lacO-lacI-GFP system and live imaging. Doing so would require generating transgenic animals carrying both the lacO array and lacI reporter together with knockout alleles of either MSH4 or MSH5. Because MSH4 and MSH5 homozygous mutants are infertile, establishing and maintaining the necessary breeding scheme would be technically challenging and time-consuming. Instead, we assessed homolog pairing using FISH. These experiments showed that MSH4-deficient spermatocytes display a significantly reduced proportion of paired homologs at mid-meiotic stages, suggesting that recombination intermediates generated in the crossover pathway contribute to the establishment and/or stabilization of full synapsis.

Line 83-91: Please add a citation to this statement.

This statement already cites two referenced publications (42 and 43). Line 82: “However, recent observations in budding yeast have challenged this view. Live imaging has revealed that colocalization between any two loci is very dynamic, consistent with random diffusive movement of most chromosome loci. Relatively few linkages between chromosomes (e.g., COs) are apparently sufficient to establish an association between homologs[42]. Furthermore, recent work has shown that homologs juxtapose at defined stages characterized by characteristic distances, and that transitions between alignment stages occur rapidly. Homologous loci at an average distance of 1.39 μm move together to an intermediate stage with average distances around 0.79 μm and finally to a distance of 0.45 μm corresponding to close pairing. This model of meiotic chromosome pairing proposes that pairing transitions are regulated by recombination and the establishment of the synaptonemal complex[43].”

References:

42 - Newman, T. A. C. *et al.* Diffusion and distal linkages govern interchromosomal dynamics during meiotic prophase. *Proc Natl Acad Sci U S A* 119, e2115883119, doi:10.1073/pnas.2115883119 (2022).

43 - Nozaki, T., Weiner, B. & Kleckner, N. Rapid homologue juxtaposition during meiotic chromosome pairing. *Nature* 634, 1221-1228, doi:10.1038/s41586-024-07999-5 (2024).

Line 136: Please indicate with an image what defines centromeric heterochromatin.

Supplementary Figure 2 now shows examples of primary spermatocytes (at the zygonema and pachynema stages) within a whole seminiferous tubule, with Hoechst staining highlighting pericentromeric heterochromatin.

Fig. 1D: It remains somewhat enigmatic how the authors can tell chromosome 8 from 9 in that figure. Please mention in the results text or legend.

Identification of chromosomes carrying lacO insertions was performed by combining lacO FISH with individual chromosome painting using mouse fibroblast cells obtained from ear samples corresponding to the two lacO mouse lines we have (Fig. 1B). This analysis revealed that lacO insertions were close to the centromere in chromosome 9 and the second near the center of chromosome 8.

Testes from these two mouse lines were used to prepare spermatocyte chromosome spreads, which were then immunostained with SYCP3 (to reveal the chromosome core) and lacI antibodies (to mark sites of GFP-LacI accumulation) (Fig. 1D). In agreement with the observation in Figure 1B, Figure

1D shows that lacI-GFP is located near the end of one chromosome for one of the strains and near the center of chromosome 8 in the second strain.

Line 144: Ref 35 is about a non-meiotic function of the LINC complex, and reference 41 refers to fixed samples (unless the presence of a bouquet implies movements).

Thank you for noting these references. Referencing manuscript 35 here is inappropriate, and 41 studies bouquet formation, which is related to RPMs. To avoid any confusion, we have removed these references in the statement.

Line 157 ff: Are there statistical tests for the average speed and displacements?

Details of statistical test results for both average speed and track displacement length are now in Supplementary Figure 3 and in the legend of Figure 3.

Line 159: Is that nm/s? Please add.

The measurement corresponds to the track displacement length. We added the unit µm for distance to the measurement.

Line 160ff: Please add a statistical test to this statement.

We added a statement comparing the average speed at the mid meiosis stage for chr. 9 and chr. 8. P-value is not significant. Details in supplementary Fig. 3 and line 203-209, main text.

Line 164ff: Please add the statistical test.

The statistics associated with this statement are now on lines 227-232. Details in Fig. S3.

Line 1275ff: You are talking about 90 minutes, but in fact, you filmed in a time window between 50-150 minutes.

That is correct. We changed “90 min” to 45-250 minutes (average 135.9 ± 55.9 minutes) (line 240).

Line 179: It is hidden in the legend that this value represents an average of recordings of different stages. Please mention this in the text.

This is now in lines 257-260.

Line 187-188: I missed a clear explanation of how the inter-chromosome distances were assessed. How were chromosomes 8 and 9 told apart?

Average inter homolog distances were calculated over the entire time-lapse acquisition, as indicated by the kymographs (this is indicated in lines 702-703).

Line 202ff: What do you mean by “initial engagement”?

Thank you for the observation. Perhaps this terminology is confusing because, likely at this stage, any transaction between homologous chromosomes is minimal.

We have changed the statement (please see lines 275-278).

Line 222ff: Is the code provided?

The R code we used to generate kymographs and analyze stages and transitions in homologous chromosome interactions is available in GitHub (see Methods section under "Inter-homolog distance analysis", line 709).

It would also be good to provide plots for all the different measurements (n=12), as the authors did in Figs.
S1 and S2.

Plots are now shown in Figure 4D, Figure S8, and Figure S9.

Line 227ff: It is not clear how this was defined. Is it arbitrary or based on the results of Fig. 3, taking the average±SD for the early and late meiosis?

Specific stages within a pairing event were defined by the range of distances observed in early (2.6-9 μm) and mid (1.0-2.5 μm) meiosis. This is confirmed by detection of relatively stable stages of homologous chromosome inter-distance, calculated using the mean and/or average function in the changepoint package of R.

Line 253: The Authors showed that the transition from pre-aligned to paired lasts for 1h. Should there not be a longer recording period for DMC1 mutants (longer than 30 minutes)?

That is correct. However, our intention with live imaging in *Dmc1*$^{-/-}$ cells was to determine typical interhomolog distances. Indeed, all *Dmc1*$^{-/-}$ cells analyzed appear to be within the range of widely separated distances, indicating that DMC1 is likely required to transition from a widely separated state to an intermediate stage of homolog pairing.

We attempted to register inter homolog dynamics for longer time periods using *Dmc1*$^{-/-}$ cells. However, we observed that chromosome movement stopped on average 30 minutes into the 3D time-lapse recording, accompanied by a rapid loss of GFP signal, all of which, we speculate, may be is associated with poor health of spermatocytes in the mutant condition.

Line 269: Which type?

Primary spermatocytes at mid prophase show a typical bimodal distribution (Fig. 5D) (starting in line 349).

Line 273: Where can this be found in the methods?

Thank you for identifying this oversight. We have added information about this modeling to the methods, which now reads: "Bimodal distributions were modeled as a mixture of two normal distributions, fitted with the mixtools package in R using the normalmixEM2comp function. The significance of bimodality was calculated using boot.comp function to perform 100 realizations of a parametric bootstrap to determine the likelihood ratios of a 1-component (null hypothesis) versus a 2-component (alternative

hypothesis) fit of normal distributions. Bimodality was assigned if the p-value for this comparison was below 0.01." (starting at line 749)

It is also not clear which FISH data is being used to measure these 2D distances.

All probes were pooled to measure the 2D distances described. To make this clearer, we have added the following text to the main body: "Visual analysis (Fig. S11A) and Kolmogorov-Smirnov test analysis show that inter-spot distance distributions between probes on chromosomes 1 and 8 in mouse spermatocytes in mid prophase, Sertoli cells, and Leydig cells are not significantly different ($P > 0.01$ after correction for multiple hypothesis testing for all cell types analyzed). Therefore, for subsequent analyses, we pooled data from all probes." (line 344).

Line 293: Add references and explain which yeast homologs these proteins likely represent.

We added the budding yeast equivalent to the indicated mammalian proteins and added some key references in yeast ("and their budding yeast counterpart Mer3[18,19], Zip2[18,20], Msh4[21], Msh5[18] and Pms1[22,23], respectively"). Other references regarding protein function can be found below in the Results section (lines 386-395).

Line 309: Has the appropriate test been used here?

We chose a binomial test as the most appropriate for comparing relative proportions between samples. However, as the argument we are trying to make is more accurately that the overall distance distributions have changed, we also performed a Kolmogorov-Smirnov test in each case and have included it in the revised text (lines 398-406).

Line 324: Please, add a citation to the Mer3 homolog; I missed that explanation earlier on as well.

Done.

Line 330: Where in the methods part can one find how these measurements were done?

Explanation can be found in lines 591-600.

Line 364ff: Please explain better.

The indicated section is intended only to highlight the potential of the lacI-lacO technology we adapted for the first time in a mammal, which marks individual genomic loci and can be used to eventually direct any protein to lacO insertion sites.

The model in Fig. 5G is not cited in the text.

Fig. 5G is now cited in the text (line 550).

Fig. 5F: Please bring the content of this figure to the main text. Can these bridges also be seen with MSH4/5 stainings?

We introduced functional and experimental context relevant to Fig. 5F in the main text (lines 536-542).

The quality of antibody/imaging for MSH4 immunostaining at the relevant resolution impaired the assessment of MSH4 location at DNA bridges.

Line 478: Is there a list of used antibodies provided?

Yes, Table S2.

Line 535: Will this script be provided to the community?

Yes. A direct link to R code for kymograph creation, change-point analysis, and Chow index analysis is available on GitHub (https://github.com/OMRF/pezza-lab-stepwise-pairing-and-fast-transition-regulated-by-recomb-drive-meiotic-chrom-interactions)

Line 553: The Method could be described in a supplemental figure. Will the code be shared?

The method and the code with examples for use will be available on GitHub (https://github.com/OMRF/pezza-lab-stepwise-pairing-and-fast-transition-regulated-by-recomb-drive-meiotic-chrom-interactions).

Line 634: Are the sequences of the probes provided?

Yes, MetaSystem probes XMP 8 D-1408-050-OR and XMP 9 D-1409-050-OR, respectively. Line 135.

Fig. 3: Consider using colour-blind friendly colouring.

Done.

Fig. 4A: Scale bar is missing.

A scale bar was added to the representative image.

Fig. 5: Scale bars are missing.

A scale bar was added to the representative image.

Reviewer #2 (Remarks to the Author):

Review of the manuscript entitled "Stepwise pairing and fast transition regulated by recombination drive meiotic chromosome interactions" by de Almeida et al.

This manuscript addresses the long-standing question of how homologous chromosomes recognize and pair during meiosis. The authors develop an elegant fluorescence reporter–operator system that allows live imaging of defined genomic loci in mouse meiotic cells, ex vivo, within intact seminiferous tubules. This approach represents a genuine technical tour de force

and, to my knowledge, enables for the first time the direct analysis of homologous chromosome pairing dynamics in living mammalian tissue.
Using this system, the authors convincingly demonstrate that homologous chromosomes pair through a stepwise process, rather than via a single continuous transition. They identify distinct distance regimes corresponding to widely separated homologs, an intermediate juxtaposed state, and close pairing, with rapid transitions between some of these states. Importantly, this process is abolished in Dmc1 mutants, highlighting the role of SEI in pairing. Complementary analyses using FISH and immunofluorescence in fixed cells further confirms that the initial transition toward juxtaposition depends on early recombination events, and further demonstrate that final stable pairing requires crossover maturation intermediates and the synaptonemal complex protein SYCP1.

The manuscript is carefully and pedagogically illustrated, and the imaging data are of high quality. However, several points require clarification or additional information to make the study fully accessible and quantitatively robust.

Major points

1. Validation of the lacO-lacR reporter lines (FROS lines)

While Figure 1 convincingly demonstrates that the lacO/lacR system works technically, I could not find clear evidence that meiosis proceeds normally in these transgenic animals. Specifically:

- Have meiotic progression, synapsis, and fertility been assessed in the FROS lines?

As a response, we have added new data showing normal meiotic progression and normal testis development (testis weight, fertility assessment, morphological characterization - H&E-stained testis sections and apoptosis - TUNEL assay, Fig. S1A-D).

Additionally, we use immunocytochemistry on chromosome spreads to assess the effect of the lacO insertion and lacO-lacI-GFP in sister chromatid cohesion, homologous chromosome synapsis, and distribution of chromatin around insertion sites (Fig. 1D and S1E).

- Is there evidence that insertion of the lacO repeats does not perturb the local chromosomal environment or alter pairing behaviour compared to the native locus?

Even if these aspects are difficult to assess comprehensively, they should be discussed explicitly.

Our results indicate that chromosome pairing and synapsis appear normal around lacO insertions (Fig. 1D and Fig. S1E). Additionally, Hoechst staining, which can serve as a marker of chromatin condensation/heterochromatinization, shows normal staining at and around lacO insertion sites (Fig. S1E).

2. Definitions and staging of meiotic progression
Several key concepts require clearer definition:
- The classification of cells into early / mid / late meiotic stages is not sufficiently explained. The Methods refer to a previous publication, but this is not sufficient for readers unfamiliar with the system.
What criteria are used for staging : time after RA treatment, SYCP3/SYCP1 patterns, nuclear morphology, else?

Thank you for your comment. We have added additional information in the Methods section to clarify this point. Starting at line 655.

- The terms "widely separated," "juxtaposed," and "paired" are used throughout, but objective distance thresholds are not clearly defined. Please provide explicit quantitative criteria (e.g. distance ranges) for each category. According to figure 3C, there is partial overlap between the average distances calculated at each stage (early / mid / late). Therefore, I don't think it is appropriate to say (for example) that "In early meiotic stages, homologs were widely separated" (l178), since some acquisitions from early meiosis will fall in the range of the mid meiosis.

Another example of this confusion is given l185, when the authors write "all cells analyzed for the 90 minute period analyzed moved within one of these three pairing categories (Fig. S1)." What defined these pairing categories? It cannot be the average distance since there is this overlap I already mentioned. This way to present the data should be reconsidered, or better explained and defined.

We apologize for the confusion and have now clarified how we define these three stages of homologous chromosome distances. Briefly, these three stages are defined by typical ranges of homologous-chromosome distances for specific meiotic stages. In early meiotic stages, homologs were widely separated, with inter-spot distances ranging from 2.6 - 9 μm. As cells progressed through meiosis, homologs exhibited a marked reduction in inter-spot distance, with mid-meiotic cells showing an apparent intermediate stage with juxtaposed chromosomes ranging from 1.0 – 2.5 μm. Spermatocytes in late meiosis (late zygotene, early pachytene) show further increased proximity to a close pairing state with homolog distances ranging 0.36 - 0.96 μm (starting at line 242).

The frequency graph in Fig. 4C was slightly adjusted to more accurately reflect the distribution of cells across meiotic stages and homologous chromosome distances. We corrected an error in the original version of the frequency/bin-size we chose to show the results.

3. RPMs characteristics

- Only two parameters are discussed: the average speed and the displacement length.

Concerning the displacement length metric. Its relevance depends on all acquisitions having the same duration, which is not specified. Moreover, this descriptor appears to be largely redundant with the average speed. As such, it is unclear how much additional information it provides.
- Instead, I wonder whether the authors could analyze alternative descriptors of chromosomal motion that would better characterize the nature of the trajectories. This seems particularly important given the surprising observation that all investigated chromosomal loci exhibit similar behavior. For instance, since the K9 probe is located close to the telomere, was it expected to behave similarly to K8, which is interstitial? A more detailed characterization of displacement patterns might reveal distinct behaviors between telomeric and non-telomeric loci that are not captured by the current analysis.

We thank the reviewer for this comment. All acquisitions were performed with identical duration (10 minutes), which ensures that all chromosome movement parameters and statistics are comparable across conditions.

Average Speed and displacement length (now we report track displacement length) are two classical parameters that reflect typical characteristics of particle motion. While average speed reflects the total path length traveled per unit time, track displacement length captures the net movement from the origin to the endpoint and therefore provides complementary information about processivity and the nature of trajectories, such as movement directionality.

Based on single-particle tracking theory and current practices, we have included additional parameters in our analysis. We believe the most informative descriptors are those that capture directionality, confinement, and motion type. We have included mean squared displacement, track displacement length, track speed maximum, track speed standard deviation, and track length (Fig. S4). Text starting at line 216.

- Is K9 signal always close to the nuclear envelope/nuclear periphery or can it be detected everywhere in the nucleoplasm?

Across most analyzed cells at mid meiosis, both live-cell imaging and fixed-sample analysis show that the lacO–lacI–GFP array inserted into chromosome 9 localizes at or near the nuclear periphery.

4. Stage specific inter-homolog distances.

- It is not clear to me if early/midle/late stages can come from the same tubules or not.

Most cells analyzed in the group of cells at early, mid, and late stages come from different tubule explants. This is expected, as most primary spermatocytes in a microscope field are within a syncytium. Occasionally, we find two stages in the same tubule/movie (possibly corresponding to a developmental transition).

- It is not mentioned on how many acquisitions the measures have been obtained. Please provide the numbers in the text. Also specify the number of repeats/independent samples analysed.

This information has been incorporated in the legend of Fig. 3.

- In early meiosis, when neither homolog pairing nor juxtaposition is observed, how do the author explain that the inter-locus distances are so constant over time (Figure 4B)? if the two loci are independent one from the other, I would have expected that their inter-distance should vary considerably from one time frame to the other. I don't think this observation is discussed anywhere.

The kymograph in Fig. 4B represents only one cell. We observed that inter-homolog distances in cells at early stages of meiosis are substantially more variable than those in mid- and late-stage meiosis. This is reflected in the wide distribution of distances at this stage (2.6 to 9 μm, 6.4 μm range) compared to 1.5 μm and 0.6 μm range of variation for mid and late stages, respectively (see frequency distribution Fig. 4C).

The wide range of distances in the early stages is also comparable to the wide range obtained from mice carrying lacO-lacI-GFP insertions on two different chromosomes (Fig. 4C)

5. Abstract

Only 3D time-lapse imaging is mentioned as experimental set up. This is misleading since FISH and IF on fixed samples are also equally important in terms of results shown. The inter-homologues distances given in the abstract for example are not calculated from the time lapse. This should be clarified.

We rewrote the abstract to expressly state the use and results obtained with fixed samples: "Complementary studies of recombination mutants using 3D DNA fluorescence *in situ* hybridization (FISH) on whole mounted tubules and immunofluorescence on chromosome spreads reveal that double-strand breaks and early recombination events trigger the transition from a widely separated to an

intermediate inter-homolog distance. Subsequently, close pairing requires crossover maturation intermediates and SYCP1/synaptonemal complex."

Typical inter homolog distances were calculated from time lapse movies (Fig 4B and C and Fig. S5 and 6).

6. Figure legends should be more detailed in many places. And also think to refer to corresponding sup data in the legends when relevant.

For example:

- Figure 3A: The legend should explain the difference between the upper (false-colored) and lower panels. The panel showing the trajectory reconstructions is not mentioned in the legend and should be described.

Thank you for the comment. We have reviewed figure legends, including Fig. 3A, and added necessary information.

- Figure 3B: Is the reported average speed calculated for both loci together? Are there differences between them? The number of biological replicates and total number of trajectories should be clearly stated in the legend or supplementary data.

The number of biological replicates and number of cells analyzed can be found in the corresponding Figure 3 legend.

RPMs measurements were done in spermatocytes with one or two homologs marked by lacO-lacI-GFP. Most instances in which both homologs could be monitored in spermatocytes occurred at mid- or late meiotic stages, where both alleles move at similar speeds. In this case, we report the average speed of both homologs per cell. In this way, the number of trajectories quantified is equal to the number of cells reported.

I could not find statistical analyses comparing K8, K9, and heterochromatic loci. Do they behave similarly or not? Please provide statistics and n values.

This data is now available in Supplementary Figure 3. The number of cells quantified, n, has been reported in the legend of Figure 3.

The term "global" for heterochromatin is unclear and should be revised.

In mouse spermatocytes, for all chromosomes, the centromere is very close to one end of the chromosome, and the centromeres are associated with long blocks of pericentric heterochromatin. These heterochromatin blobs are readily visible in our movies and indicate the positions of individual and grouped chromosomes simultaneously, and they can be tracked over time-lapse images. We typically monitor 3 or more of these heterochromatic blobs per cells, providing an estimate of several telomeres - "global", averaged movements representative of the entire chromosome complement.

Please specify the duration of the acquisitions (are all 18 min as in panel A?). If not, provide the details in supplementary data.

Quantification of RPM characteristics were done over a period of 10 minutes. Under these conditions, the RPMs measured at the beginning of this time period are not different from those measured at the end.

- Figure 3C: I don't understand what is being shown. The legend refers to nm/sec, while the figure uses µm.

We apologize for the mistake. Track displacement length (previously displacement length) is reported in µm. We changed the measurement units in the figure legend accordingly.

- Figure 4A: Is the lower panel derived from the same cells as the upper panel? If not, this should be clarified, as the current presentation is potentially misleading.

The cells depicted in the lower and upper panels of Figure 4A are different. We now clarify this in the figure legend.

- Figure 4B and 4D: Please refer explicitly to supplementary data for the additional kymographs.

Done.

- Figure 4C: Specify the time window over which the averages were calculated.

The time window is 45 - 250 minutes. This information can now be found in the text (line 240).

- Video S1: Acquisition parameters (time interval, duration, projection) should be included in the legend.

Done.

Other points

- Clarify the number of LacO insertions (256x in figure1, vs 300 line 119)

The insert contains 256 repeats. This is now clarified in the main text (lines 121-130).

- Give more details on the developed system for culture of seminiferous tubule explants: what quality controls were performed to be sure that this ex vivo system recapitulates in vivo meiosis?

RPMs are very sensitive to changes in oxygen levels and nutrient levels, providing a measure of quality. We observed that, when not irradiated, seminiferous tubule explants that entered the Ibidy microfluidics system showed active RPMs after 16 hours or culture.

The conditions we use for the seminiferous tubule explant culture are similar (with modifications for imaging) to those described in PMID: 21430778, in which cultured seminiferous tubules undergo spermatogenesis.

Details of microfluidics culture conditions can be found under "Preparation and imaging of live mouse seminiferous tubules" in the Methods section.

- The text lacks precisions about the parameters of the acquisitions (notably the duration). Are they variable or always the same? Maybe the authors could provide in Sup data a table recapitulating the data set.

Time-lapse acquisition times vary. For movies providing data on the frequency of inter-homolog distribution (Figure 4B and C), the time-lapse ranged from 45 minutes to 250 minutes. For the results reported in Figure 4D time laps were approximately 375-480 min.

- It is mentioned l175 that the acquisitions last "approximately" 90 min. what % of the prophase does it represents?

This is now clarified in the main text (e.g., line 239). "we initially tracked homologous loci for periods of 45-250 minutes (average 135.9 ± 55.9 minutes)".

- The sample sizes must be added in the text when measures are given.

The number of cells analyzed is provided in the main text and text or figure legends.

- When comparing average numbers (speeds, distances etc) please provide the relevant statistics.

For RPMs, see Supplementary Figure 3 and legend of Figure 3.

Others can be found in Results and legends of Figures and Supplementary Figures.

Reviewer #3 (Remarks to the Author):

The manuscript by Pezza and colleagues addresses a major question in meiosis (and chromosome biology more broadly): how homologous chromosomes come together during meiosis. The authors develop a new LacI/lacO-based system for tracking specific chromosomes in mice, together with a microfluidic setup to image seminiferous tubules. This important technological advance is used to gain insight into the mechanisms and kinetics of chromosome pairing and alignment.

The work is significant both for the mechanistic insight it provides into meiotic chromosome dynamics and for the development of mouse models and imaging conditions that allow one to follow individual chromosomes in live cells and tissues. However, on both fronts, the manuscript would benefit from substantial editing and reorganization to clarify the mechanistic claims and facilitate broader adoption of the methodology. Key points are outlined below.

Major:
Foci tracking: I appreciate the inclusion of the movie as a supplementary file for clarifying how the raw data looks and how it was interpreted. One point that it reveals is the rotational movement of the signal (the Imaris tracks around 0:40). Nuclear rotation was also documented in previous live imaging, as referenced by the authors. However, it is not clear if any correction for these nuclear (as opposed to chromosomal) movements was performed. This is crucial, since a major finding of this work is that different positions along the chromosomes move at similar rates. That

would be expected if nuclear rotation contributes significantly to foci movement. If the measurements were not corrected for nuclear rotation, it would be crucial to point that out. If a correction was performed, that should be clearly explained.

Thank you for the comment. The reviewer is correct that nuclear rotation and telomere-independent movements are part of the chromosome movements observed in mouse spermatocytes. We have not corrected for nuclear rotation in our measurements. We clarify this in the text (line 210).

Figure 3: The visualization and data analysis relating to foci speed & displacement should be improved. The graphs in panel B & C are challenging to interpret; smaller symbols for each datapoint would be helpful. Even with the current visualization, it seems than there are sample-to-sample differences (e.g., chr 9, early). It seems than in some cases, this variation exceeds stage-to-stage variation; e.g., the green dots in chr 9 early seem to overlap with the green dots in chr 8 mid. Statistical analysis of such potential experimental variation should be performed and the results should be discussed.

In response to this comment, we have changed the symbol style in the graphs in Figure 3.

Statistical analysis (including experimental variation, SD) and comments can be found now in the main text, legend of Figure 3 and in Supplementary Figure 3.

RPM variation between cells, both within and among tested conditions, agrees with our previous observations (PMID: 25892231).

The analysis in Figure 4 is well done and is of high quality. In fact, given the generous figure allowance, inclusion of some of the galleries from Figures S1 & S2 would help convey some of the important findings of this work. One issue that should be clarified is the reason the analysis of 'widely-separated' to 'juxtaposed' was not repeated for transitions between 'juxtaposed' and 'close'. Are these events more rare? Are they harder to define given the distances? Is the transition slower?

This is an important question. We focused on the first transition occurring at early meiotic cells (widely separated to juxtaposed) mainly for two reasons. First, prioritization of quality control. We believe this is particularly important given the possible limitations of our cultured seminiferous tubule explant analysis (e.g., cell arrest). Sustained RPMs are a sensitive parameter indicative of normal cell activity and meiotic stability, and early meiotic cells are the most abundant cell type exhibiting robust RPMs. Second, in budding yeast, RPMs are required for efficient progression between chromosome pairing stages [34]. Again, early meiotic cells exhibit by far the most robust RPMs.

We expect that, in future work, improvements to our tissue culture and imaging system may enable monitoring of both transitions in the same cell samples.

Figure 5B: The dmc1- live analysis data should be compared to wild type (Fig. 4B-C). Cursory analysis suggests that in dmc1- there is a population of lower distances (<2um) that does not exist in early-meiosis wild type, and that there is a general a shift to shorter inter-homolog distances. The two distributions should be compared statistically, and any identified difference should be discussed in the text.

This is an interesting point, and we thank the reviewer for raising it. In response, we reanalyzed the 3D time-lapse movies and included only datasets that passed stringent quality-control criteria, including robust chromosome movement and strong GFP signal intensity. The average inter-homolog distance

observed is 2.9 ± 1.0 μm (Fig. 5B). This distance falls within the range defined for widely separated homologs, and we observed several cells in which homologs remained separated by more than 3 μm.

Importantly, the distribution of inter-homolog distances in *Dmc1*$^{-/-}$ spermatocytes was statistically distinct from that of wild-type cells during mid- and late-meiotic prophase (Kolmogorov–Smirnov test; P = 0.0001 and P < 0.0001, respectively). In contrast, the distribution in *Dmc1*$^{-/-}$ cells differed only modestly, although still significantly, from that observed in early meiotic cells (P = 0.0203). One possible explanation is that the distances observed in 3D movies of *Dmc1*$^{-/-}$ spermatocytes are influenced by the extensive heterologous chromosome interactions present in these cells. This clarification has now been added to the manuscript (lines 324–333).

Figure 5D: All data should be presented, ideally in the main figure. Right now, missing are the histograms for wt-mitotic-chr8, wt-meiotic-chr 1, spo11-chr8, and msh4-chr1. It is impossible to analyze the claims in the text without this information. Additionally, statistical analysis is either lacking or unclear, making the claims related to this data impossible to assess. Some examples: line 269 - what is the statistical analysis that backs the claim of bimodal distribution? What are alternative hypotheses? Downstream of this calculation is the claim in lines 278-9, which describes 'strong bimodality', and defines 'stages' of pairing, which are then (presumably) used below for comparing mutants (e.g. line 309). This analysis is opaque, and in many cases unsubstantiated. Finally, the use of 'density' in the y-axis is insufficiently described, and is inconsistent with other histograms in the manuscript.

We have completely rewritten the analysis of this figure to be clearer and more robust in response to this comment. In particular:

- We justify our decision to pool data from all probes by first demonstrating, using a Kolmogorov-Smirnov test, that there are no significant differences in distance distributions across probes.
- We describe the changes over the course of prophase in wild-type spermatocytes and include these in a figure; their significance is verified with an ANOVA test.
- We demonstrate the robustness of the observed bimodality in mid-prophase samples using a parametric bootstrap, as described in the text and methods sections.
- For differences between mutants and wild-type, we determine differences in centers of distributions with a Wilcoxon-Mann-Whitney test and differences in overall distributions with a Kolmogorov-Smirnov test and describe this in the text.
- We have converted “density” into a raw percentage of cells in the relevant figures.

The revised portions of the main text can be found on lines 344-380, and in Methods.

Figure 5E: The analysis performed to quantify the images in Fig. 5E is not explained. Indeed, it is impossible to correlate the images with the quantification. E.g., spreads from sycp1- and shoc1- appear very different, yet the distribution of '2D distances' is essentially the same. No statistical analysis is performed, although claims of difference and similarity are being made. This section must be revised before any assessment of its validity could be made.

An extended explanation of the analysis and quantification performed for this experiment can now be found in the Method section (lines 591-600).

Statistical analysis can be found in lines 427-442.

A minor point: the fact that SYCP1 is required for synapsis is made in a separate section of the results and presented as a novel finding, when it is really a control; the manuscript would benefit from presenting it as such.

We agree, we have incorporated *Sycp1*$^{-/-}$ cells data in the previous section as suggested.

The authors justifiably stress the technological advance their work represents, and its potential future use. However, the manuscript lacks any data or discussion about how the technique was validated. e.g., comparisons to previous measurements of meiotic chromosome dynamics. Discussion of the shortcomings of the technique are also necessary. For instance, as with many ex vivo live analysis approaches, it is impossible to know whether the imaging perturbs meiosis. This and other potential limitations should be clearly discussed.

We believe our work provides validation at the organismal (e.g., meiosis in lacO-lacI-GFP mice progresses normally and animals are fertile, supplementary Figure 1), tissue (e.g., sustained RPMs indicate that seminiferous tubules explant can be used for ex vivo experiments to some extent), and cellular (e.g., high resolution imaging of chromosome movements in a confided nuclear space) levels, so that the transgenic line we generated could be used in other applications beyond meiotic chromosome dynamics.

Results in Figure 3 provide a direct comparison between previous measurements using relatively lower-resolution Hoechst staining (our own work, PMID: 39126474 and PMID: 25892231) and the newly developed lacO-lacI-GFP locus-specific approach. Unfortunately, comparison of our results with those obtained by other labs using different approaches, such as those described in PMID: 22826121, is not possible due to insufficient data in other works.

Certainly, our lacO-lacI-GFP approach has several shortcomings, and we now discuss some of them in the Discussion section (see lines 464-471).

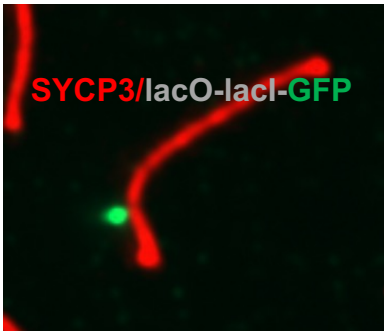
Minor: One of the important conclusions of this work is that intermediate, pre-synapsis juxtaposition is stable. However, the usage of 'stable' in the text is quite confusing. First, as noted above, the stability of synapsis (referred to as 'close pairing') is confirmatory, and matches previous data. The text would benefit from clarifying this point. For instance, the section heading in line 290 is misleading, since the role of CO maturation in SC assembly in mice is well documented. A more minor case is in lines 411-2: "stable synapsis"; was any case of 'unstable synapsis' documented here?

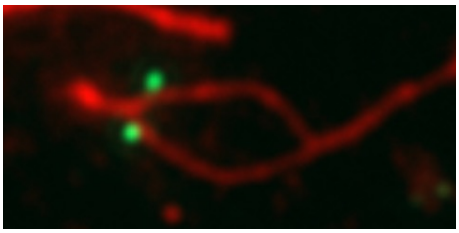
We agree. “Stable” in the context of “close pairing” (synapsis) refers to a known characteristic of homologous chromosome interaction when the synaptonemal complex is established all along the homologs’ axis. “Stable” in the context of the “juxtaposed” intermediate is used to describe the fact that, once they reach this stage, fluctuations in distance are reduced.

We believe the term "stable" in the context of close pairing is unnecessary and have removed it from the manuscript.

Line 189: "Given the lacO-lacR-GFP fluorescent tag is likely located outside the chromosome axis" - what is the evidence for this claim? In fact, Figure 1D suggests that the GFP signal is on the axis.

We often observed that the GFP signal does not exactly overlap with the SYCP3 signal (see examples below), suggesting that lacO-lacI-GFP is located off the chromosome axis.





line 523: the seminiferous tubules are referred to as 'ovaries'.

Thank you for spotting this error. We changed accordingly.

The LacO/lacI system is referred to in various places as a paradigm, framework, platform and technology. The acronym 'FROS' is also used in some places. A uniform and accurate description is necessary. Also, the name of the repressor is LacI, not LacR.

Across the text, we now use FROS to describe the approach we developed and “lacO-lacI system” to describe FROS.

We now use lacI throughout the manuscript.

line 119 & Fig. 1B: please clarify inconsistency in the lacO repeat number.

We corrected the inconsistency. We used a lacO construct with 254 lacO repeats.

Line 113: Recommend removing the use of 'universal'. 'Unifying' or 'conserved' are more appropriate.

Done. We now use “unifying”.

Reviewer #3 (Remarks to the Author):

I appreciate the many improvements the authors introduced to the manuscript. Specifically, the analysis of the bimodal distribution, the inclusion of some of the missing data, and many additions to the text and improvements to the figures. That said, several key issues raised in the previous review cycle remain insufficiently addressed.

Major points:

There is still a lack of clarity regarding the use of the various kinds of pairing, juxtaposition, alignment terms. The term 'intermediate' seems to be used with regard to pairing and to distance, and the term 'pairing' appears to refer both to a discrete (final) state and a range of distances. Currently, they are not clearly defined and are used interchangeably (e.g., lines 277-278, 327-328, 370-371). Using uniform, clearly defined terms would be critical to make the manuscript accessible to the wide readership of Nature Communications (this issue has also been raised by Reviewer 2).

The terms *pairing* (including unpaired states), *juxtaposition*, and *alignment* (or *co-alignment*) are often used interchangeably to describe the process by which homologous chromosomes recognize one another and progressively align along their axes. During this process, the distance between homologs gradually decreases, ultimately resulting in their close association, which correlates with synapsis and the formation of a mature synaptonemal complex. In some contexts, however, *juxtaposition* and *alignment* may be more appropriate terms because they more explicitly describe the relative positioning of homologous chromosomes along their axes at varying distances. For simplicity and consistency, we now use the term *pairing* throughout the manuscript, as it is widely adopted in the field. Although this choice may, in some instances, reduce terminological precision, it improves accessibility for a broader readership and ensures consistent language throughout the text.

The terms *widely separated*, *intermediate*, and *close pairing* refer to the distances between homologous chromosomes and, in our view, provide appropriate descriptors for the three discrete stages of homologous chromosome pairing observed in this study. These categories are defined and described in lines 307-316.

While I appreciate the clarification in the text regarding the nuclear rotation (lines 209-212), it is unfortunate that the authors did not control for it in the calculations of RPMs. As it stands, it is hard to conclude much from the measurements. A very plausible hypothesis is that nuclear rotations are responsible for most of the measured movements of the FROS foci and heterochromatin. If that were the case, it is impossible to conclude that "RPM characteristics may be uniform across chromosomes" (lines 213-214 and 232-235). Ideally, rotational movements should be controlled for. If that is not done, the conclusions must be significantly toned down, and alternative explanations for the similarity must be presented.

We used two complementary approaches to compare RPM in chromosomes 9 and 8 without the influence of nuclear rotation. In one approach, we compared RPM characteristics in mouse spermatocytes carrying two lacO-lacI-GFP spots that mark chromosomes 8 and 9 within a single nucleus. In the second method, we employed an in silico approach to subtract chromosome movements attributable to nuclear rotation in individual nuclei, and then compared residual RPM movements obtained from spermatocytes carrying a lacO-lacI-GFP insertion on chromosome 8 versus chromosome 9. Using both approaches, we observed a small but statistically significant difference, suggesting that the RPMs of chromosomes 8 and 9 differ independently of nuclear

rotation (Fig. 3D and E and Fig. S4F and G). We have modified the text accordingly (lines 225-285 and 712-751).

In the "Preparation and imaging of live mouse seminiferous tubules" section in the Methods, the claims regarding the non-perturbative nature of the ex vivo culturing and imaging are not quantified. Specifically, quantification is required for the duration over which RPMs were imaged, as well as the similarity between RPMs in the beginning and end of the experiments.

The quantification of the RPM characteristics presented in the manuscript was performed over 10 minutes.

Regarding the similarity of RPM behavior at the beginning and at the end of the quantified interval, we measured the average speed at both the start and the end of the 10-minute period. These data are now presented in Fig. S4E. Statistical analysis revealed no significant difference between the initial and final measured time points, and the standard deviation distributions were also comparable.

Line 311-312: That "pairing occurs ... with rapid transitions among individual pairing stages" is only shown for the first transition ("widely-spaced" --> "intermediate pairing") but not for the transition between the two latter stages. The sentence should be edited to reflect this.

Thank you for noticing this. We corrected the sentence. Now it reads: “We conclude that homologous chromosome pairing occurs in a stepwise manner, with a rapid transition between widely separated to intermediate pairing stages.” (line 313).

Minor points:

It is unfortunate that the authors chose to not split Fig. 5 into multiple independent figures, each with a different conceptual and experimental focus; as it stands, it is easy to confuse the different experimental modalities and findings. However, that is ultimately an editorial decision.

We have separated the data in Fig. 5 into two separate figures, making it easier to discern the data by experimental modality. In Figure 5, we now show data showing live imaging and FISH on whole-mounted seminiferous tubules. Figure 6 shows data obtained from spermatocyte chromosome spreads.

Fig. S8B: The y-axis seems to be wrongly labeled. Adding some information in the legend about what is shown in the graph is necessary (even if the legend refers to the Methods).

Thank you for noticing this; the y-axis was mislabeled. Indeed, the y-axis of the plots is the p-value of the F statistic from the Chow test. The y-axis has been relabeled to “-log10(p-value)”.

We added additional information in the figure legend as follows: “Example of software detection of pairing stages and detection of transitions (Chow method) between pairing stages, supporting a rapid and discrete pairing mechanism. This method detects whether a significant "structural break" or shift occurs in the relationship between variables. The plot displays p-values at -log10(p-value) versus time. Because some p-values were p=0 (and log(0) is undefined), we shifted all p-values by the smallest non-zero p-value for each data set.”

Lines 293-294: The use of the word 'apparently' is unclear. As it stands, it is not obvious what is the relevance of this sentence to the rest of the paragraph.

We have eliminated the word “apparently” from this sentence.

Line 368: What does "strong bimodality" mean? Suggest remove or clarify.

We removed “strong” from the sentence.

Fig. 5E: The inclusion of Dmc1-/- and Hop2-/- in main figure is confusing, since they are not quantified or referred to in the main text.

We believe it is important to show them as mutants representative of key intermediates in the recombination pathway.

We added a reference to these mutants in (line 1021).

Fig. 5F: The panel is only referred to in the discussion. Perhaps more importantly, the data is not quantified, so the claims associated with it ("HFM1 and SHOC1 are located on DNA bridges") should be edited. Ideally, this panel is either quantified or removed.

Quantification for each protein associated with DNA chromosome bridges (average number of chromosomes per nucleus with marked bridges and number of marked bridges per chromosome) is now shown in Fig. 6B.

Line 273-274: Stating such precise distances for 'early' and 'mid' would best be avoided. If it is mentioned, it should be given with a range. On a more conceptual level, the difference 0.44um makes on these distances is minor, unlike for the 'late' group, where the resulting distance is within the size scale of synapsed chromosome.

As indicated in the text, this is an “estimated” value, with relative precision determined by the context of the paragraph. In this way, we believe that providing ranges may lead to confusion.