APPLY IMMUNO-FISH IN ZEA MAIZE

A Dissertation

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by

Xin Wen

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ABSTRACT

The process of meiosis, a fundamental mechanism in plant sexual reproduction, orchestrates the creation of germ cells with genetically diverse homologous chromosomes, primarily through the intricate event of crossing-over (CO). CO plays a pivotal role in promoting genetic variation by enabling the exchange of genetic material among chromosomes. Despite DNA Double Strand Breaks (DSBs) initiating chromosome recombination, not all DSB events culminate in COs. This study seeks to revolutionize the CO process in plants to expedite plant breeding and produce novel genotypes harboring traits unattainable through traditional methods.

In pursuit of this goal, transgenic Zea mays lines have been developed, introducing a bespoke recombinant protein, Cas9:SPO11. This engineered protein is adept at guiding DSBs to precise chromosome regions during meiosis, imparting a degree of control over the recombination landscape. Our hypothesis posits that these targeted regions, under the influence of Cas9:SPO11, experience heightened recombination rates compared to their wild-type counterparts. The innovation lies in the potential to channel recombination events towards desired genomic loci, thereby creating new avenues for directed trait incorporation.

The present study is poised to investigate the anticipated increase in CO events at the designated target loci in the transgenic lines when contrasted with the natural recombination pattern of wild-type maize. This investigation hinges on a multimodal approach, combining the power of Immuno-Fluorescent in situ Hybridization (Immuno-FISH) with protein immunolabeling. These techniques afford visualization of chromosome dynamics, elucidating the spatial and temporal aspects of the recombination process.

The research aligns with a significant body of literature emphasizing the indispensability of CO in generating genetic diversity and facilitating adaptive evolution. Moreover, the role of DSBs as initiators of recombination events has been underscored by studies in various organisms, including model plant species. The exploration of CRISPR-Cas9 technology in the context of enhancing recombination

dynamics is an emerging avenue, with diverse applications ranging from basic research to agricultural innovation.

As the scope of plant breeding broadens to address contemporary challenges, such as climate resilience and nutritional enhancement, the ability to strategically modulate recombination events offers an enticing proposition. The outcomes of this study could revolutionize crop improvement strategies, allowing for the accelerated development of plant varieties with desirable traits. Ultimately, this research underscores the potential of leveraging genetic recombination mechanisms to usher in a new era of precision agriculture.

BIOGRAPHICAL SKETCH

Xin Wen grew up in Zhangjiakou, Hebei Province, China. Before entering graduate school, she graduated from China Agricultural University with a Bachelor of Science in Agronomy, where she was an undergraduate student in Prof. Weiwei Jing's lab working on CRISPER-Cas9 applications in maize. She then attended Cornell University for her master's degree, where she was part of Prof. Wojtek Pawlowski's research group, working on research related to maize chromosomes.

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One's exploration of one's life path is a long one. In the year I have been here at Cornell, I have not only gained knowledge as well as progress in my research, but more than that, I have been given the opportunity to explore myself as well as trial and error. It has been a long road to get here.

I am very grateful to Prof. Wojtek Pawlowski for all the help he has given me and for his tolerance of my mistakes, as well as for giving me the opportunity to work in the lab. I would also like to thank Olga Zimina, who has been my constant companion in the lab and has patiently guided me through the writing process, and whose patience has made my research so much clearer. I would also like to thank Tara Reed, who has been a great source of affirmation and encouragement during a time of academic uncertainty. I would like to thank all the people I have met at Cornell for their help and encouragement when I had questions. It made me feel the warmth of being in a foreign country.

Finally, and most importantly, I would like to express my heartfelt gratitude to my mother, my father, and the rest of my family for always encouraging me, supporting me, and making a great effort to help me on my academic path.

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INTRODUCTION

Meiosis, an essential process in plant sexual reproduction, is instrumental in generating genetic diversity through the intricate event of crossing-over (CO). CO facilitates the exchange of genetic material between homologous chromosomes, contributing to the creation of germ cells with unique combinations of genetic traits. While the initiation of chromosome recombination is attributed to DNA Double Strand Breaks (DSBs), not all DSBs culminate in CO events. This study seeks to revolutionize the meiotic recombination landscape in plants, using a novel approach that holds promise for expediting plant breeding and producing innovative genotypes with complex traits.

One of the pivotal developments in this study is the utilization of the Cas9:SPO11 recombinant protein. Cas9, a protein derived from the CRISPR-Cas9 system, has gained immense popularity for its ability to precisely target and modify specific DNA sequences. When fused with SPO11, a key player in initiating meiotic recombination by creating DSBs, the resulting Cas9:SPO11 complex presents an innovative strategy for influencing the recombination process. By harnessing the power of this protein, researchers aim to direct DSBs to precise chromosome regions during meiosis, thereby potentially enhancing CO events at these targeted loci. This novel methodology represents a significant departure from traditional approaches to meiotic recombination modulation.

One of the central hypotheses of this study is that the Cas9:SPO11-guided DSBs will lead to an increased frequency of CO events at the designated target regions when compared to the recombination pattern in wild-type organisms. This hypothesis is grounded in the assumption that the precise localization of DSBs, facilitated by the Cas9 component, will channel recombination toward the desired genomic loci. This, in turn, could result in the generation of plant genotypes with tailored combinations of traits that are otherwise challenging to attain through conventional breeding methods. The significance and relevance of this research extend beyond the realm of fundamental plant biology. The potential to manipulate meiotic recombination patterns

through targeted DSB induction has transformative implications for agriculture and plant breeding. With the global population surging, the demand for crops with enhanced traits, such as disease resistance, drought tolerance, and improved nutritional content, has become paramount. Traditional breeding methods often involve protracted timelines and imprecise outcomes. The ability to harness Cas9:SPO11-mediated recombination offers a novel pathway to accelerate the development of crops with desired characteristics, thereby addressing pressing agricultural challenges.
Moreover, the study's focus on Immuno-Fluorescent in situ Hybridization (Immuno-FISH) presents a methodological advancement that underscores the sophisticated nature of this research. Immuno-FISH combines the power of immunolabeling and fluorescence in situ hybridization, enabling researchers to visualize and analyze chromosome dynamics with unprecedented precision. The integration of this technique into the study's investigative framework promises to unravel the spatial and temporal aspects of the recombination process, shedding light on the behavior of recombination events guided by the Cas9:SPO11 complex.
In sum, this study stands at the intersection of cutting-edge molecular biology techniques, fundamental genetic principles, and the practical exigencies of modern agriculture. The convergence of these domains underpins the research's relevance and potential impact, making it a pioneering endeavor with far-reaching implications for crop improvement and genetic innovation.

LITERATURE REVIEW

The phenomenon of meiosis is a cornerstone of sexual reproduction, underpinning genetic diversity in organisms. During meiosis, germ cells are produced with recombined homologous chromosomes, a process driven by crossing-over (CO). CO enables the reciprocal exchange of genetic material between chromatids, fostering genetic diversity within a population. This genetic diversity is fundamental for species adaptation, evolution, and survival in changing environments.

Central to CO initiation are DNA Double Strand Breaks (DSBs), which are introduced by the SPO11 protein. These DSBs serve as the substrates for CO events, where homologous chromatids exchange genetic material. The precise control of DSB formation and subsequent recombination is crucial for maintaining genomic integrity and balanced genetic variation.

The advent of CRISPR-Cas9 technology revolutionized genome editing by enabling targeted modifications to DNA sequences. The fusion of Cas9 with SPO11 presents a novel approach to steer DSBs to specific chromosomal regions during meiosis, offering a potential means to manipulate recombination dynamics. This innovation could lead to the creation of genotypes with desired traits, circumventing the limitations of conventional breeding methods.

The strategic modulation of recombination patterns holds immense promise for agriculture. Crop improvement through conventional breeding methods is often time-consuming and unpredictable. The ability to enhance CO events at targeted loci could accelerate the development of crops with improved traits. This concept aligns with the goals of precision agriculture, enabling the tailored design of plant varieties for specific environments and challenges.

The integration of Immuno-FISH into this research marks a significant advancement in methodology. Immuno-FISH allows the visualization of specific DNA sequences within the context of chromosome dynamics. This technique, when coupled with immunolabeling for protein visualization, enables researchers to scrutinize recombination events and their outcomes with high spatial and temporal resolution.

In conclusion, the synthesis of molecular techniques, genetic principles, and agricultural imperatives has set the stage for a transformative research endeavor. By capitalizing on the potential of the Cas9:SPO11 complex and leveraging Immuno-FISH, this study strives to unravel the intricacies of recombination dynamics during meiosis. The outcome of this research could reshape the landscape of crop improvement, enabling the creation of plant genotypes optimized for diverse challenges and conditions. The intersection of fundamental biology and applied agricultural innovation positions this study at the vanguard of genetic research with broad-reaching implications.

RESULTS AND DISCUSSION

1. The efficiency of the short probes

In this part, we used Labeling Protocol of myTags and obtained a high concentration short probe 1 and short probe 2, which will help to increase the rate of molecular hybridization in Immuno- FiSH.

To determine dye labeling efficiency, convert the ssDNA in ng/ul to pmol/ul and compare with dye pmol/ul.

labeled ssDNA size = 66 nt, assuming a 45mer variable region. 1 ug = 47 pmol(47pmol/1000ng=0.047 pmol/ng)

For immortal libraries made with the indexing services the ssDNA is 82 bases long and 1 ug = 38 pmol(38pmol/1000ng=0.038 pmol/ng)

	short probe 1	short probe 2
concentration of ssDNA(ng/ul)	472.6	18.9
concentration of dye(pmol/ul)	596.1	22.86

FIG 1 The concentration of the short probes.

According to FIG1, the effeciency of short probe 1 is:

472.6 ng/ul ssDNA × 0.038 pmol/ng=17.96 pmol/ul ssDNA

17.96 pmol/ul ssDNA ÷ 18.9 pmol/ul dye = 95 %

The efficiency of short probe 2 is:

596.1 ng/ul ssDNA × 0.038 pmol/ng=22.65 pmol/ul ssDNA

22.65 pmol/ul ssDNA ÷ 22.86 pmol/ul dye = 99 %

The efficiency of short probe 1 and short probe 2 are all above 90%.

2. Microscopic images of the slides

Electron microscope micrographs of maize pollen cells demonstrate that the appropriate meiotic period for doing Immuno-FiSH is late pachytene and diplotene stages and that the distribution of appropriate cells is good.

While performing the maize slides, we found differences in the degree of anther development in the three strains of maize. Transgenic Line spo-11 have a suitable selection range of anther between 20-22 mm. For the wild type, there were more pollen grains in the tetrad period in the 20-22mm anther, so through subsequent observation and adjustment, we chose the 19-20mm anther.

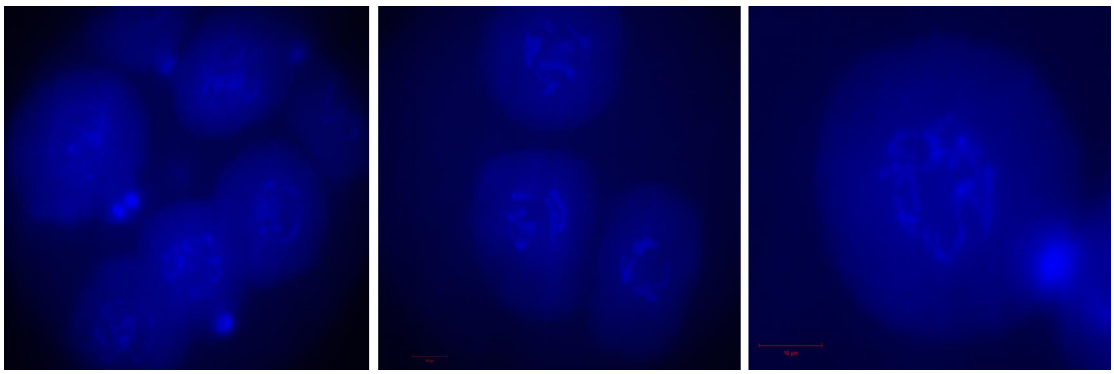

FIG 2 The first from the left shows the transgenic maize line with pollen cells at meiotic late pachytene and diplotene stages. The second from the left shows the wild type maize line with pollen cells at meiotic late pachytene and diplotene stages. The right one shows the late pachytene stages of pollen cells of a single transgenic maize strain.

3. Immuno-FiSH

In Figure 3 and Figure 4 from the combination of MLH3, probe and DAPI merge we can find the location of chromosome 3 of two maize strains by the location of short probe. Although we can find antibody MLH3 on the chromosome, there are many non-specific background of the antibodies outside the chromosome group that we still need to improve Immuno-FiSH.

For improving the Immuno-FiSH, we try to change the concentration of the antibodies. The concentration of the antibodies we used are:

guinea pig MLH3 : 1:300

secondary Cy5 anti-guinea pig 1:400

The non-specific background of the antibodies indicates the secondary Cy5 anti-guinea pig did not work well. So the next time we will use a higer dilution of the seondary antibodies or the different kind of antibodies.

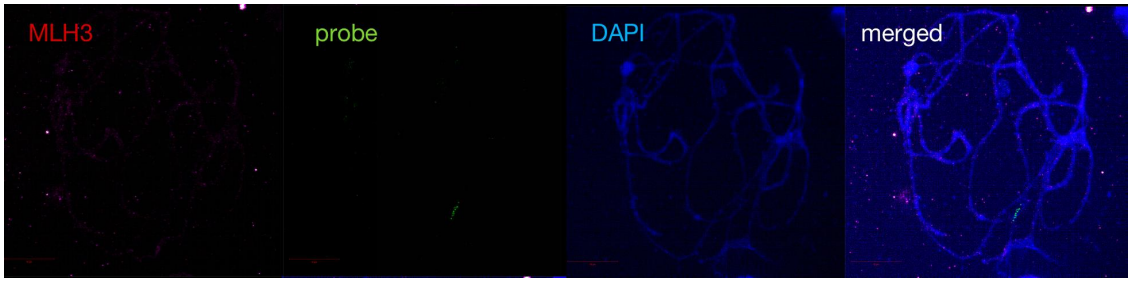

FIG 3 From left to right are the distribution of antibody MLH3, the green fluorescence short probe map, the distribution of maize chromosome groups, and the results of doing Immuno-FiSH of the transgenic line.

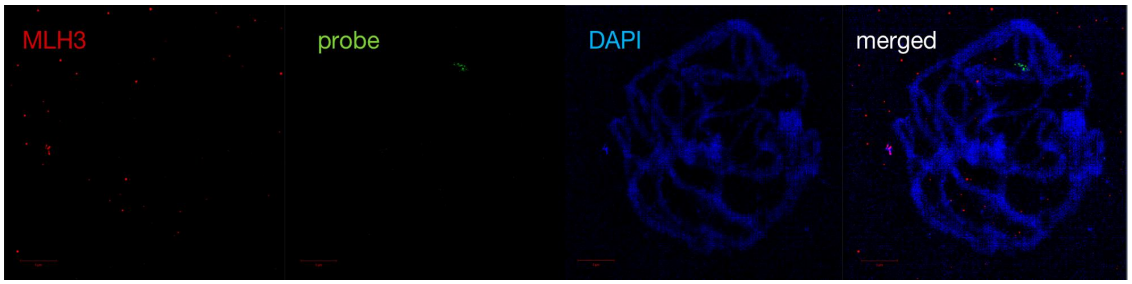

FIG 4 From left to right are the distribution of antibody MLH3, the green fluorescence short probe map, the distribution of maize chromosome groups, and the results of doing Immuno-FiSH of the wild type.

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MATERIALS AND METHODS

The basic steps of this experimental design are labeling the probes, preparing slides of transgenic lines and wild-type lines, immuno-FiSH, and microscopic observation.

1. labing the probes

Part 1. PCR Amplification

1) Assemble the master mix and debubbling mix in separate 0.2 ml PCR reaction tubes.
2) Set the debubbling mix aside on ice or at 4°C. Pipet 5 µl of the master mix into a third PCR tube and set aside as a negative control.
3) Add 2.5 µl of the working stock myTags immortal library (0.07 ng/µl) to 45 µl of the master mix and mix by pipetting.
4) Perform PCR Cycling Conditions on both the negative control and template master mix in thermo cycler.
5) Leave the negative control in the thermocycler at 24°C and remove the template master mix. Add 20 µl debubbling mix to the template master mix and mix by pipetting. Return the template master mix + debubbling mix to the thermocycler and continue with Program 2 for both the template master mix + debubbling mix and the negative control.
6) After PCR cycling is complete, do the Agarose Gel Electrophoresis and use the QIAquick PCR Purification Kit to purify the DNA.
7) Quantify the purified dsDNA using Nanodrop or other method. A minimum yield of 480 ng is required for in vitro transcription. Store at-20°C.

Part 2. In vitro Transcription – MEGAshortscriptTM T7 Kit

1) In a 0.2 ml tube, assemble the in vitro transcription (IVT) mix on ice.
2) Vortex for 5 sec and quick spin down in a mini-centrifuge for 5 sec.
3) Incubate in thermocycler at 37°C for 4 hr (with hot lid on, set to 42°C).

4) Use the RNeasy Mini Kit to purify the RNA.

Part 3. Reverse Transcription- SuperScript II Reverse Transcriptase

1) In a 0.2 ml tube, assemble mix 1 on ice. Vortex for 5 seconds, quick spin down for 5 seconds.
2) Incubate in a thermocycler at 65°C (with hot lid on, set to 75°C) for 5 minutes, then chill on ice for 5 min. While waiting, proceed to step 4.
3) In a 0.2 ml tube, assemble mix 2 on ice.
4) Vortex for 5 sec, quick spin down for 5 sec, and store on ice while waiting to complete step 3.
5) Following the completion of steps 3-5, add Mix 2 to Mix 1 to assemble the reverse transcription (RT) mix. Vortex for 5 sec, quick spin down for 5 sec.
6) Incubate the reaction at 42°C (with hot lid on, set to 52°C) for 5 min.
7) Add 2.5 µl SuperScript II Reverse Transcriptase to RT mix, vortex for 5 sec, quick spin down for 5 sec. Incubate the reaction at 42°C (with hot lid on, set to 52°C) for 2 hr. Repeat this step. The total incubation time required to convert the RT mix into the RT sample is 4 hours at 42°C.
8) Use Exonuclease I to digest unincorporated primer. And this step is time-sensitive. Add 11 µl of Exonuclease I Buffer to RT sample. Add 2 µl of Exonuclease I to RT sample. Vortex for 5 sec and quick spin down for 5 sec. Incubate in thermo cycler at 37°C for 15 min. Remove RT sample from the thermo cycler, then preheat thermo cycler to 80°C. Add 12 µl 0.5 M EDTA to RT sample, vortex for 5 sec and quick spin down for 5 sec. Incubate at 80°C in pre-heated thermo cycler for 20 min. Store on ice to cool. Then use the Zymo Quick-RNA to purify ssDNA.

Part 4: RNA Digestion - RNase H and RNase A

1) In a 0.2 ml tube, assemble the RNA digestion mix on ice.
2) Vortex for 5 sec and quick spin down for 5 sec.
3) In a 0.2 ml tube, combine 18 µl of RNase digestion mix with eluted RNA:DNA (~82-84 µl). Vortex 5 sec and quick spin down for 5 sec.

4) Incubate in thermo cycler using the RNA Digestion Program.

5) Use Zymo Quick - RNA to purify ssDNA.

6) Count the efficiency of the probes. If the ratio for pmol/ul dye : pmol/ul ssDNA is not 0.9 or higher, repeat RNase digestion.

2. Preparing the slides

1) According to the need of Immuno-FiSH, we need pollen mother cells of late pachytene and diplotene stages. Based on comprehensive literature analysis, it was established that the ideal length of maize anthers falls within the range of 20-22mm. The selected maize anthers were dissected under microscopic observation and were preserved in a solution of 70% alcohol.

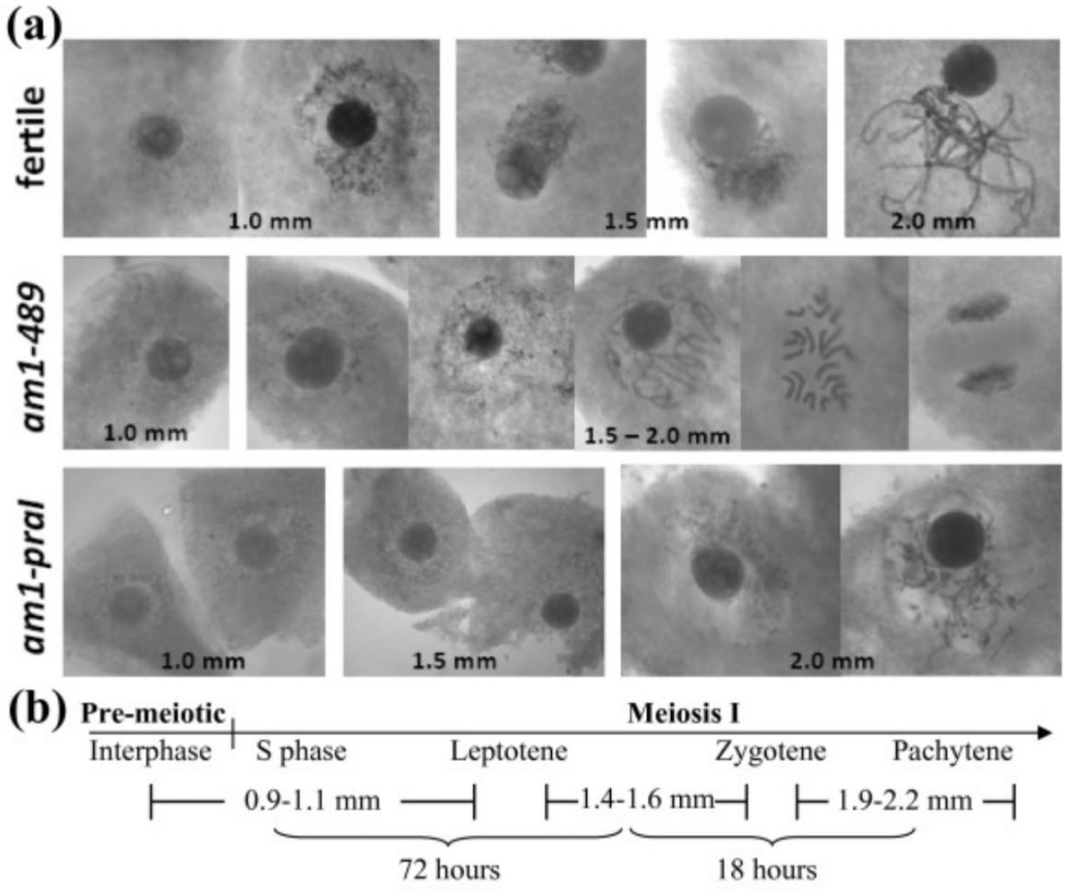

FIG 5 Microelectron microscopy of maize pollen mother cells at different stages of meiosis and corresponding anther lengths.

2) Making enzyme solution. Fixed anthers (ethanol-glacial acetic acid (3:1), or fixed in PFA 4%, or in FAA) containing leptotene-pachytene-stage meiocytes were washed in 70% Ethanol, then in PBS, and treated with 2% (w/v) Onozuka R10 cellulase (Yakult Honsha), 1% (w/v) Macerozyme R10 (Yakult Honsha), and 1% (w/v) cytohelicase (Sigma-Aldrich) in 4 mM citric acid/6 mM sodium citrate buffer, pH 4.8, at 37°C for 10 to 15 min.

3) Alcohol or PBS was aspirated from the stored anthers and the enzymatic reaction was carried out with the enzyme solution prepared in the previous step. Incubate in vacuum for 5 mins later at 37 degrees Celsius for ten minutes.

4) After completion of incubation rinse three times with PBS and recover the enzyme solution.

5) A drop of 30 microliters of PBS solution was placed on the slide and 18-20 anthers were taken and infiltrated into the solution. Anthers were squeezed to release meiocytes, wall debris was removed, meiocyte suspension (~10uL) transferred to the SuperFrost slide (ThermoFisherSci), and then a cover slip was added. The slide was placed on filter paper, and the chromosomes were squashed by applying gentle pressure to the slide. After freezing the slide in liquid nitrogen, the cover slip was removed with a razor blade. Observe the cell stage and the concentration under the microscope.

3. Immuno-FiSH

1) preheat water bath to ~60C, preheat 10m HCl in incubator to 37C. Slides were then dehydrated in 50%, 70%, and 100% ethanol.

2) Check slides under inverted light microscope. Slides with good chromosome

spreads were pretreated with 100 µg/mL DNase-free RNase A (Sigma-Aldrich) in 2× SSC (dilute RNase A in 2× SSC, apply 500ul on slide, cover with parafilm) at 37°C for 30 min and then washed three times for 5 min in 2× SSC.

3) For removing the cytoplasm, the slides were treated with 100 µg/mL pepsin (Sigma-Aldrich) in 10 mM HCl for 20-30 min at 37°C (time duration should be optimized; preheat 50 ml of 10 mM HCl at 37C for 30 min ahead, add 50 ul of pepsin before use) and then placed in distilled water for 1 min to stop the reaction and washed three times in 2× SSC for 5 min.

4) Subsequently, slides were treated in 4% formaldehyde for 10 min at room temperature, washed three times in 2× SSC for 5 min.

5) For better epitope accessibility slides were microwaved in a preheated 10 mM citric buffer pH=6 for 3s-5s-5s and immediately transferred to the permeabilization solution.

6) Dehydrated in 70, 90, and 100% ethanol.

7) The chromosomal slides were denatured in 70% formamide in 2× SSC at 72°C for 3 min and dehydrated in prechilled 70, 90, and 100% ethanol.

8) A 30-µL hybridization mixture consisting of 50% formamide, 10% dextran sulfate, 2× SSC, 0.2% SDS, 100 ng/µL salmon sperm DNA, and 100-pmol chromosome-specific probe labeled with ATTO-488 was denatured at 85 °C for 3 min, put on ice for 3-5 min, and then applied on the slide. (30uL+0,3uL salmon sperm DNA+ ~3-5ul DNAprobe)

9) Denature slides together with probe at 73°C for 3min on PCR machine. The slide should be covered with parafilm. Transfer slides to hybridization box filled with water of moist tissues, seal the slides with Fixogum.

10) Hybridization was performed at 37°C for ~20 h (overnight).

11) Next day, Remove Fixogum and parafilm. Wash slides in 1xTNT two times for 5 min.

12) Blocking: Add 200uL of 1xTNB to the slides and cover with a parafilm. Block in 37℃ humid chamber for 1 hour.

13) Discard the parafilm. Apply 200uL of 1st antibody diluted in 1xTNB. Cover it with

a parafilm and incubate in a humid chamber at 37℃ for 2hours. Discard the parafilm.

Wash slides in a coplin jar with fresh 1xTNT three times for 10 min.

Apply 200uL of 2nd antibody diluted in 1xTNB and cover with a parafilm. Incubate for 1 hour a 37℃.

Discard the parafilm and wash slides in a coplin jar filled with fresh 1xTNT three times for 10 min.

After washing, slides were mounted with Prolong Gold mountant with DAPI.