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Chiasma Counting in Maize Male Meiocytes

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[Abstract] This protocol combines a classical chromosome spreading technique with 3-dimensional image reconstruction for visualization of bivalent configurations during meiosis to aid chiasma counting.
Keywords: Chromosomes, Meiosis, Recombination, Chiasma, Maize

[Background] Chiasmata are cytological manifestations of crossovers. Chiasma counting provides a fast and reliable assessment of meiotic recombination rates. Chiasmata are best quantified in maize at diakinesis in meiotic prophase I, as at this stage, they are most conspicuous. Advantages of cytological assessment of crossover rates are that it is fast, inexpensive, and can be performed on genetically homozygous individuals. This protocol improves on the classical chromosome spreading technique by combining it with modern 3-dimensional imaging, which improves the accuracy of chiasma counting, resulting in an error rate of 10% or less (Sidhu *et al*., 2015; Kianian *et al*., 2018).

Materials and reagents

1. 10 or 20 µl pipette tips
2. Acetocarmine solution (VWR, catalog number: 470300-054)
3. 15-ml Falcon tubes (Corning, catalog number: 352096)
4. Coplin jar
5. Dissecting needle (Fisher Scientific, catalog number: 13-820-024)
6. Diamond marking pen
7. Glass microscope slides (Millipore Sigma, catalog number: S8902) and cover slips (Millipore Sigma, catalog number: S1815)
8. Nail polish
9. Plastic Petri dish (Fisher Scientific, catalog number: FB0875713)
10. 95:5 (v/v) acetic acid:methanol solution (Millipore Sigma, catalog numbers: A6283 and 322415)
11. 90% acetic acid (Millipore Sigma, catalog number: A6283)
12. 70% ethanol (Millipore Sigma, catalog number: E7023)
13. Sodium citrate (Millipore Sigma, catalog number: 1613859)
14. EDTA (Millipore Sigma, catalog number: E9884)
15. Onozuka R-10 cellulase (GoldBio, catalog number: C8001)
16. Macerozyme (GoldBio, catalog number: M8002)

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17. Cytohelicase (Millipore Sigma, catalog number: C8274)
18. Citric acid (Millipore Sigma, catalog number: 251275)
19. DAPI powder (4,6-diamidino-2-phenylindole) (Millipore Sigma, catalog number: 10236276001)
20. NaCl (Millipore Sigma, catalog number: S9888)
21. KCl (Millipore Sigma, catalog number: P3911)
22. Na_2HPO_4 (Millipore Sigma, catalog number: NIST2186II)
23. KH_2PO_4 (Millipore Sigma, catalog number: NIST200B)
24. DABCO (1,4 diazabicyclo-[2,2,2] octane) (EMS, catalog number: 17989-50)
25. Tris (Millipore Sigma, catalog number: 10708976001)
26. Glycerol (Millipore Sigma, catalog number: G7893)
27. Citric acid buffer pH 5.5 (see Recipes)
28. Structural weakening buffer (see Recipes)
29. DAPI (see Recipes)
30. 1x PBS (see Recipes)
31. DABCO solution (see Recipes)

Equipment

1. Fine-tip forceps (Fisher Scientific, catalog number: 22-327379)
2. Alcohol lamp (Fisher Scientific, catalog number: S41898B)
3. Dissecting microscope
4. Fluorescence microscope
5. UV crosslinker

Procedure

A. Tissue harvesting

1. Collect entire tassels for fixation. Before harvesting the tassels, analyze anthers from a few flowers to determine the meiosis stage. Meiosis in maize starts in the middle of the tassel and extends bi-directionally. Therefore, flowers in the middle are at later stages of meiosis than flowers above and below.

   Note: It takes approximately 6 weeks for the maize inbred B73 to reach meiosis under the following conditions in a growth chamber: 12 h light, 31 °C day/28 °C night temp. The time to meiosis in other inbreds varies. To ensure that tassels are at the right stage, cut open one of the plants by making a long vertical slit on the stalk to reveal the tassel, and collect a few flowers to determine the meiosis stage (see Step B4). If the flowers are too young, the stalk can be closed back by taping over with laboratory tape.
2. Fix the tassels in ice-cold 90% acetic acid for 45 min to 1 h in a 15-ml Falcon tube (Figure 1).

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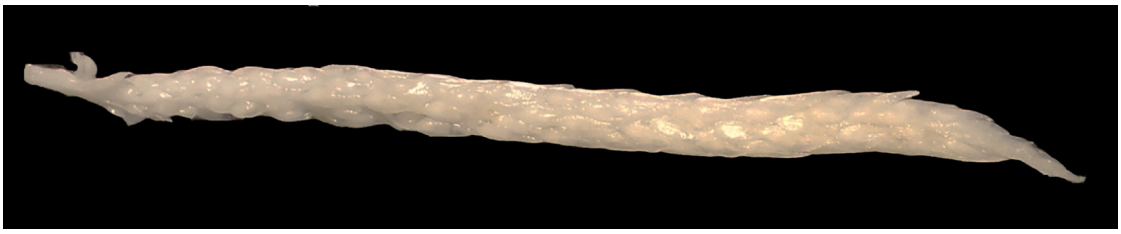

Figure 1. Fixed maize tassel

3. After fixation, remove the acetic acid solution and wash the tassels three times with 70% ethanol.
   Note: Use enough ethanol to completely submerge the tassel.
4. The tassels can be stored in 70% ethanol at -20 °C in a 15-ml Falcon tube for several weeks.
   Note: Use enough ethanol to completely submerge the tassel.

B. Chromosome spreading
   1. Take tassel out of the 15-ml Falcon tube and place it in a Petri dish (Video 1: 0 min 01 s).
   2. Remove flowers using fine-tip forceps and put them in a dish with enough 70% ethanol so that they are completely submerged (Video 1: 0 min 35 s) (Figure 2)

Video 1. Main steps of sample and slide preparation

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Figure 2. Dissected maize flowers

3. Dissect anther from flowers using fine-tip forceps and a dissecting needle (Video 1: 1 min 03 s) (Figure 3).

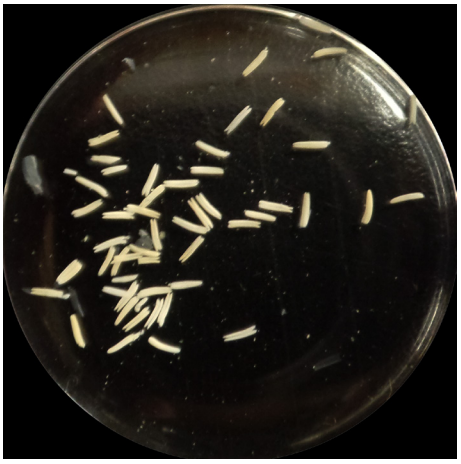

Figure 3. Dissected anthers

4. To determine the meiosis stage, place an anther on a glass microscope slide (Video 1: 1 min 12 s) and add a drop of acetocarmine solution (Video 1: 1 min 25 s). Gently smash the anther with a dissecting needle (Video 1: 1 min 36 s). Use a dissecting needle to mix the solution over gentle heat (Video 1: 1 min 58 s) until the color of the stain turns from red to purple. Place a cover slip over the anther and gently press to release meiocytes (Video 1: 2 min 16 s). Use a wide-field light microscope (Video 1: 2 min 36 s) to determine the stage of meiosis (Golubovskaya *et al.*, 2002).
5. Transfer the dissected anthers into a new dish and wash them in citric acid buffer (Video 1: 2 min 42 s). Use enough buffer so that the anthers are completely submerged (Video 1: 3 min 27 s). Incubate for 10 min at room temperature (Video 1: 3 min 57 s).

6. Incubate the anthers in structural weakening buffer containing cell wall degrading enzymes (Video 1: 4 min 17 s). Use enough buffer so that the anthers are completely submerged (Video 1: 4 min 56 s). Incubate at room temperature for 60 min (Video 1: 5 min 23 s).
7. Transfer the enzyme-digested anthers to a microscope slide with forceps (Video 1: 5 min 32 s), add a small drop of 95:5 (v/v) acetic acid: methanol solution (Video 1: 5 min 54 s) and macerate them by gently squeezing out meiocytes with a needle (Video 1: 6 min 14 s).
8. Label slides with a diamond marking pen and drop the meiocyte-containing solution onto a glass slide from a height of approximately one foot for effective spreading. Use cells from about 3 anthers per slide, and drop ~8 µl of the suspension volume on each slide. Use a 10 or 20 µl pipette tip to perform this step.
   Note: It is helpful to practice this step with acetic acid: methanol solution alone.
9. Air-dry the slides and cross-link the chromosomes to the glass using a UV crosslinker set at 1,200 J. It is helpful to outline the area where meiocytes were dropped, which will be clearly visible at this stage, with a diamond marking pen.
10. Stain the slides with 500 µl of DAPI (4,6-diamidino-2-phenylindole) solution (1 µg/ml). For this step, add the DAPI solution to the slide and leave the slide in the dark (*e.g.*, in a drawer) for 30 min. A coverslip is not needed for this step.
11. Wash the slides in a Coplin jar with 1x PBS three times, 10 min each wash.
12. Use DABCO solution to mount a cover slip.
13. Seal the cover slip on the slide using nail polish.
    Note: Make sure to remove excess DABCO.
14. Slides can be stored for up to 6 months at -20 °C.

C. Analysis of chiasma numbers

1. To analyze chiasmata, first examine the meiocytes on the slide and identify those in which all bivalents can be visualized clearly.
2. To improve the ability to recognize chiasmata, use 3-dimensional microscopy. Acquire stacks of Z sections, taken approximately every 0.15 µm across the whole nucleus and generate 3-D projections to visualize bivalents oriented along the ZY plane.
   Notes: Deconvolve the 3-D stacks before analysis, if your microscope software allows it. We used the SoftWoRx (Applied Precision, Issaquah, WA) for 3-D rendering. In this software, 3-D reconstructions of image stacks can be generated using the Volume Viewer tool.
3. Bivalents have generally two distinct configurations at diakinesis: rod (Figure 4A), which has a single chiasma, and ring, which has two chiasmata, one at each end (Figure 4B). Unequivocal identification of chiasmata may occasionally be challenging, as chromosome twists may be mistaken for chiasmata and nearby chiasmata may be difficult to distinguish from each other. 3-D image reconstruction allows for differentiating between chromosome twists and *bona fide* chiasmata, as homologous chromosome pairs can be clearly visualized at all different angles (Figures 4C-4D). To distinguish multiple chiasmata located very close together, the size of an

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individual chiasma can be estimated (Figure 4E). The chiasma length should be consistent in any given cell and among cells at the same meiosis stage in any given species. Longer chiasma structures are thought to represent multiple chiasmata located next to each other. Occasionally, chiasmata may be thin and difficult to spot (Figure 4F).

4. After the selection of cells that show clear bivalent morphology, chiasma numbers can be counted manually by carefully analyzing each bivalent. Perform blind tests to validate the reliability of the results.

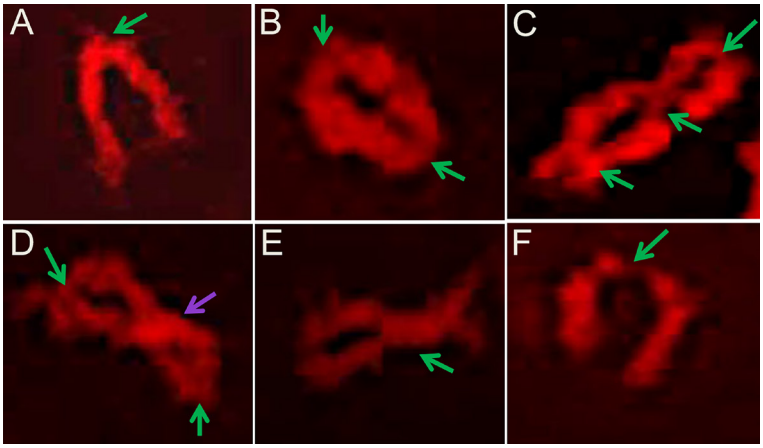

Figure 4. Quantification of chiasmata numbers in maize during meiosis. A-C. Typical chromosome morphology for bivalents that have one, two, or three chiasmata. D-F. Ambiguous bivalent morphologies that appear as three, one, and no chiasma. A. A rod bivalent that has one chiasma at one end. B. A ring bivalent, with one chiasma at each end. C. A bivalent with three chiasmata, two terminal and one interstitial. D. A bivalent with two terminal chiasmata and one chromosome twist in the middle, which appears as a chiasma. E. A bivalent with two chiasmata located next to each other. F. A bivalent with one terminal chiasma, appearing as two univalents. Chiasmata are indicated by green arrows. A chromosome twist is indicated by a purple arrow. Reprinted from Sidhu *et al.* (2015).

Recipes

1. Citric buffer 10 mM pH 5.5
   50 mM Na Citrate
   50 mM EDTA
   Adjust pH to 5.5 with citric acid
2. Structural weakening buffer
   2% Onozuka R-10 cellulase
   1% macerozyme
   1% cytohelicase
   4 mM citric acid

6 mM sodium citrate

Adjust pH to 4.8 with citric acid

3. DAPI

   Dissolve 1 µg of DAPI powder (4,6-diamidino-2-phenylindole) in 1 ml of water

4. 1x PBS (Phosphate-Buffered Saline) buffer
   a. Dissolve in 800 ml distilled H_2O

      8.0 g NaCl

      0.2 g KCl

      1.4 g of Na_2HPO_4

      2.4 g of KH_2PO_4

   b. Adjust pH to 7.4 with HCl or NaOH

   c. Adjust volume to 1 L with additional distilled H_2O

5. DABCO solution

   2.5% w/v DABCO (1,4 diazabicyclo-[2,2,2] octane)

   50 mM Tris pH 8.0

   90% glycerol

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