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Immunolabeling of Maize Meiocytes

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[Abstract] This protocol describes a quick method for immunolocalization of meiotic proteins in maize. It includes a fixation step that allows for long-term storage of material and provides good preservation of chromatin structure.
Keywords: Immunolocalization, Meiosis, Meiocytes, Chromosomes, Maize

[Background] Meiocytes are generative cells, in which specialized processes of homologous chromosomes pairing, synapsis, and recombination take place, followed by chromosome segregation to create haploid nuclei. Elucidating localization of meiotic proteins is crucial for understanding these processes. Although many variations of this protocol are possible, we found that the microwave heating step is critical for it to work reliably for immunolocalization in maize.

Materials and reagents

1. 15-ml Falcon tubes (VWR, catalog number: 62406-200)
2. 22 x 22 mm glass coverslips (VWR, catalog number: 48366-227)
3. Parafilm (Bemis, catalog number: PM-999)
4. Small Petri dishes (VWR, catalog number: 25384-302)
5. Coplin jar
6. Diamond marking pen
7. Pasteur pipette
8. Humid chamber
   Note: To make a humid chamber, place wet paper towels on the bottom of a large plastic dish with a lid.
9. Metal rod (for macerating anthers)
10. Microscopic glass slides (Thermo Fisher Scientific, catalog number: 12-550D)
11. Acetic acid, 60% (Thermo Fisher Scientific, catalog number: A38-212)
12. BSA (bovine serum albumin, Sigma, catalog number: A9418)
13. Distilled H_2O
14. Vectashield (Vector Laboratories, catalog number: H-1200)
15. Ethanol
16. Tween-20 (Sigma, catalog number: P9416)
17. Sodium citrate dihydrate (Sigma, catalog number: W302600)

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18. Citric acid powder (Sigma, catalog number: W230618)
19. DAPI powder (4,6-diamidino-2-phenylindole, Vector Laboratories, catalog number: H-1200)
20. Onozuka R10 Cellulase (Grainger, catalog number: 31FX29)
21. Cytohelicase (Sigma, catalog number: 45-C8274)
22. Pectolyase from *Aspergillus japonicus* (Sigma, catalog number: P5936)
23. Sodium chloride (NaCl) (Sigma, catalog number: S7653)
24. Disodium phosphate (Na_2HPO_4) (Sigma, CAS number: 7558-79-4)
25. Monopotassium phosphate (KH_2PO_4) (Sigma, CAS number: 7778-77-0)
26. Potassium chloride (KCl) (Fisher Scientific, catalog number: p333-500)
27. Triton X-100 (Fisher Scientific, catalog number: 9002-93-1)
28. Blocking solution (see Recipes)
29. Carnoy's fixative (see Recipes)
30. Citric buffer 10 mM pH 4.6 (see Recipes)
31. Citric buffer 10 mM pH 6.0 (see Recipes)
32. DAPI (see Recipes)
33. Enzyme digestion mix (see Recipes)
34. PBS (Phosphate-buffered saline) buffer (see Recipes)
35. Phosphate-buffered saline + Triton X-100 (PBST) buffer (see Recipes)
36. Tris-buffered saline buffer + Tween-20 (TBST) (see Recipes)

Equipment

1. 37 °C incubator
2. Forceps
3. Dissecting microscope
4. Fluorescence microscope
5. Microwave oven
6. Rocking platform shaker
7. Warming plate (Premiere, model: XH-201)

Procedure

A. Material fixation

1. Collect meiotic tassels and place them in 15-ml Falcon tubes containing 13 ml of Carnoy's fixative.
   Note: We normally put one tassel per tube.
2. Incubate the tassels overnight at room temperature, then replace the fixative solution.
3. Incubate at room temperature for at least 5 days (Figure 1). Fixed tassels may be preserved in Carnoy's fixative at 4 °C for several months.

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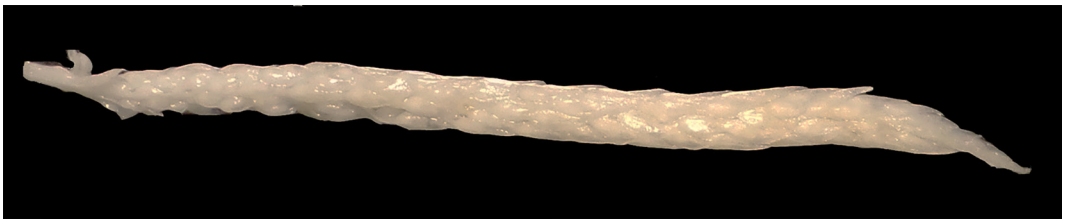

Figure 1. Maize tassel after 5 days of fixation in Carnoy's fixative

4. Determine the meiosis stage of pollen mother cells following Dukowic-Schulze *et al*. (2014).

B. Anther preparation

1. Dissect flowers from inflorescences into a small Petri dish containing 3 ml of ice-cold Carnoy's fixative (Figure 2, Video 1: 0 min 11 s).

Figure 2. Fixed maize flower buds

2. Dissect anthers from flowers by opening each flower and removing the 3 larger anthers. Place the anthers in a small Petri dish containing 3 ml of ice-cold Carnoy’s fixative (Figure 3).

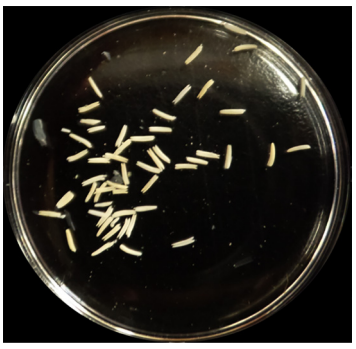

Figure 3. Fixed maize anthers

3. Wash the anthers by replacing buffer with fresh Carnoy's fixative. To ensure proper fixation, submerge anthers floating on the top of the solution by pushing them down with a metal rod.
4. Replace the fixative with ice-cold 10 mM citric buffer pH 4.6 (Video 1: 0 min 31 s).

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5. Wash anthers by replacing the citric buffer three times with fresh ice-cold citric buffer, incubate for 5 min each time. Ensure that all anthers are submerged.
6. Discard citric buffer and apply 1x enzyme digestion mix. Make sure that all anthers are submerged (Video 1: 0 min 44 s).
7. Wrap the Petri dish in parafilm and incubate at 37 °C for 2 h (Video 1: 1 min 50 s).
8. Stop the digestion reaction by adding 2 ml of ice-cold 10 mM citric buffer pH 4.6.
   Note: Following enzymatic digestion, anthers may be stored overnight at 4 °C.

C. Slide preparation

1. Under the dissecting microscope, use forceps to transfer 3 to 5 anthers onto a glass slide (Figure 4). Pipette out most of the liquid, leaving only a tiny amount (~3-5 µl) (Video 1: 2 min 16 s).

Figure 4. Slide with dissected anthers

2. Macerate anthers with the metal rod to release meiocytes. Continue until anthers are thoroughly mashed and the cell suspension has milky appearance (Figure 5). Do not let dry, add 5 µl of ice-cold 10 mM citric buffer pH 4.6, if needed (Video 1: 2 min 27 s).

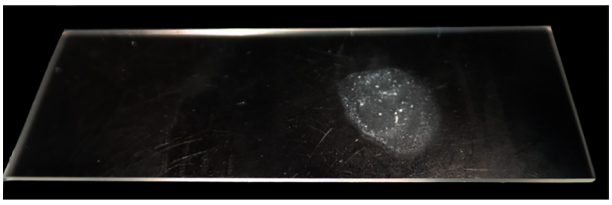

Figure 5. Slide with released meiocytes

3. Circle with a diamond pen the slide area containing meiocytes.
4. Add 10 µl of 60% ice-cold acetic acid to the meiocyte suspension (Video 1: 2 min 45 s).
5. Place the slide on a warming plate set at 43 °C for 50 s (Video 1: 3 min 28 s).
6. Immediately add 10 µl of ice-cold 60% acetic acid (Video 1: 3 min 36 s).
7. Pipette 40 µl of ice-cold Carnoy's fixative to make a ring around the meiocyte drop (Video 1: 3 min 49 s).
8. Rinse slide by adding 200 µl of Carnoy's fixative to the meiocyte drop (Video 1: 4 min 5 s).
9. Air-dry the slide for 10 min (Video 1: 4 min 24 s).
10. Place the slide in a Coplin jar containing 100 ml of 10 mM citric acid buffer pH 6.0.
11. Warm the slide for 35 s in a microwave set at 1,250 W.

Note: Do not let the liquid boil during microwaving. End the treatment when boiling starts.

12. Immediately transfer the slide in to a Coplin jar with PBST.

Video 1. Main steps of microscope slide preparation

D. Immunolabeling

1. Wash the slide in PBST in a Coplin jar, three times, 5 min each time, replacing buffer with fresh PBST.
2. Place the slide in a humid chamber.
3. Apply 50 µl of blocking solution. Cover the slide with a piece of parafilm to prevent drying (Figure 6).

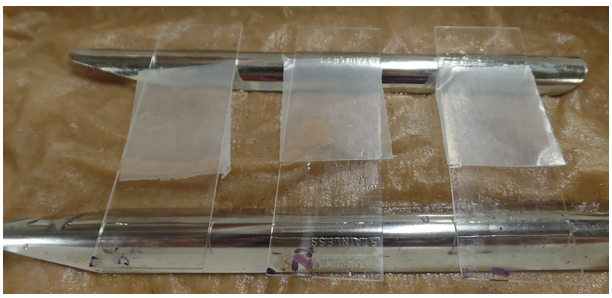

Figure 6. Slides in a humid chamber with blocking solutions covered with parafilm

4. Incubate the slide in a humid chamber with blocking solution for 30 min at room temperature.
5. Wash the slide in PBST in a Coplin jar, three times, 5 min each time, replacing buffer with fresh PBST.
6. Dilute primary antibody in 50 µl of PBST and apply by pipetting onto the center of the slide. Cover with parafilm to prevent drying.
   Note: Primary antibody dilution should be determined experimentally and usually varies from 1:50 to 1:2,000.
7. Incubate overnight at 4 °C in a humid chamber.

Note: The primary antibody incubation time might be prolonged up to 48 h. The length and temperature of incubation may need to be optimized for each antibody to ensure best results.

8. Wash the slide three times in PBST, 5 min each time, in a dish placed on a rocking platform shaker.
9. Dilute secondary antibody in 50 µl of PBST and apply by pipetting onto the center of the slide. Cover with parafilm to prevent drying.
   Note: Secondary antibody dilution should be determined experimentally and usually varies from 1:200 to 1:1,000.
10. Incubate the slide for 90 min at room temperature followed by 30 min at 37 °C.
    Note: After applying the secondary antibody, protect the slide from intense light exposure.
11. Wash the slide three times in PBST, 5 min each time, in a dish placed on a rocking platform shaker.
12. Place the slide in the vertical position and air-dry for 20-40 s. Do not over-dry; the slide should still be moist.
13. Apply 6 µl of DAPI in Vectashield. Place a glass cover slip on top of the slide and press lightly.
14. Leave for 1 h before analyzing under the fluorescent microscope (Figure 7).
    Note: This protocol was successfully tested using the anti-HEI10 (Wang et al., 2012) and anti-ZYP1 (Ronceret et al., 2009) antibodies.

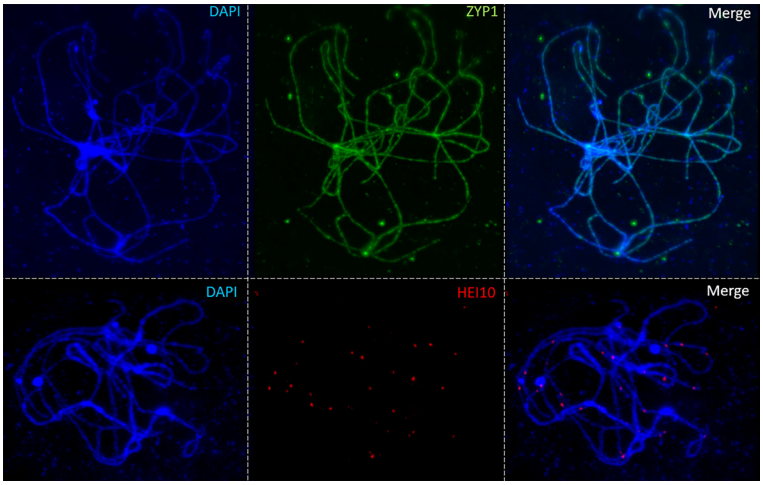

Figure 7. Examples of images: maize pachytene cells immunolabelled with the anti-ZYP1 (green) and anti-HEI10 (red) antibodies. Chromatin (blue) was stained with DAPI.

Recipes

1. Blocking solution
   40 ml of TBST
   0.5 g bovine serum albumin (BSA)
   Mix well
   Adjust volume to 50 ml with TBST

2. Carnoy's fixative
   150 ml of absolute ethanol
   50 ml of glacial acetic acid
3. Citric buffer 10 mM, pH 4.6
   160 ml of distilled H_2O
   0.28 g of sodium citrate dihydrate powder
   0.2 g of citric acid powder
   a. Mix well
   b. Adjust pH to 4.6 with hydrochloric acid or sodium hydroxide
   c. Adjust volume to 200 ml with distilled H_2O
4. Citric buffer 10 mM pH 6.0
   160 ml of distilled H_2O
   0.48 g of sodium citrate dihydrate powder
   0.07 g of citric acid powder
   a. Mix well
   b. Adjust pH to 6.0 with hydrochloric acid or sodium hydroxide
   c. Adjust volume to 200 ml with distilled H_2O
5. DAPI
   Dissolve 2 µg of DAPI powder (4,6-diamidino-2-phenylindole, Vector Laboratories) in 1 ml distilled water
6. Enzyme digestion mix
   9 ml of citric buffer 10 mM, pH 4.6
   0.1 g Onozuka R10 Cellulase (Grainger)
   0.1 g cytohelicase (Sigma)
   0.1 g pectolyase from *Aspergillus japonicus* (Sigma)
   a. Vortex
   b. Adjust volume to 10 ml with citric buffer 10 mM pH 4.6
   c. Aliquot in 0.33 ml aliquots in Eppendorf tubes and store at -20 °C
   d. Before use, dilute the enzyme mixture to a 1x working solution by adding 0.66 ml of citric buffer 10 mM pH 4.6 to a 0.33-ml aliquot
7. PBS (Phosphate-buffered saline) buffer
   800 ml distillated H_2O
   8.0 g sodium chloride
   1.44 g disodium phosphate
   0.24 g monopotassium phosphate
   0.2 g potassium chloride
   a. Mix well
   b. Adjust pH to 7.4 with hydrochloric acid or sodium hydroxide
   c. Adjust volume to 1,000 ml with distilled H_2O

8. Phosphate-buffered saline + Triton X-100 (PBST) buffer
   800 ml distillated H_2O
   8.0 g sodium chloride
   1.44 g disodium phosphate
   0.24 g monopotassium phosphate
   0.2 g potassium chloride
   0.1 ml Triton X-100
   a. Mix well
   b. Adjust pH to 7.4 with hydrochloric acid or sodium hydroxide
   c. Adjust volume to 1,000 ml with distilled H_2O
9. Tris-buffered saline buffer + Tween-20 (TBST)
   2.41 Tris
   8.7 g NaCl
   1 ml Tween-20
   a. Mix well
   b. Adjust pH to 7.4 with hydrochloric acid or sodium hydroxide
   c. Adjust volume to 1,000 ml with distilled H_2O

Acknowledgments

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Competing interests

Authors declare no competing interests.

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