Chapter 8

Live Imaging of Chromosome Dynamics

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Abstract

Progression of meiosis has been traditionally reconstructed from microscopic images collected from fixed cells. However, studies conducted in a number of species, including plants, indicate that this approach has clear shortcomings in accurately portraying the dynamic nature of meiotic processes. Here, we describe two methods to study chromosome dynamics in live meiocytes in maize, a protocol to observe chromosomes during meiotic prophase I and a technique to monitor chromosome segregation in anaphase I and anaphase II. The first method relies on culturing intact maize anthers and observing meiocytes embedded in the anthers with multiphoton excitation (MPE) microscopy. This approach circumvents difficulties in culturing isolated prophase I meiocytes in plants. The second technique uses culturing isolated meiocytes, which is possible with anaphase cells. Both methods can be fairly easily adapted for use in other plant species. We also detail the kinds of time-lapse movies that can be captured and analyzed using this technique, and describe software that can be utilized for analysis of movies chromosome dynamic in live meiocytes.

Keywords Meiosis, Chromosomes, Chromosome dynamics, Prophase, Anaphase, Cytology, Live imaging, Microscopy

1 Introduction

Reconstructions of the progression of meiotic prophase have been traditionally conducted using fixed meiocytes. Although these reconstructions provided information on the general patterns of chromosome behavior, live imaging studies conducted in a number of species (1–6) indicate that observations of fixed cells are not able to convey the dynamics and complexity of chromosome behavior in live meiocytes. Extensive live microscopy studies of chromosome dynamics have been conducted in unicellular fungi (5, 7). However, meiocytes in higher plants are more difficult targets for live imaging as they are normally embedded in multicellular reproductive structures. In this chapter, we describe two methods to conduct observations of chromosomes in live meiocytes of maize: a protocol to examine chromosome dynamics during early substages of meiotic prophase I using intact cultured anthers (4)

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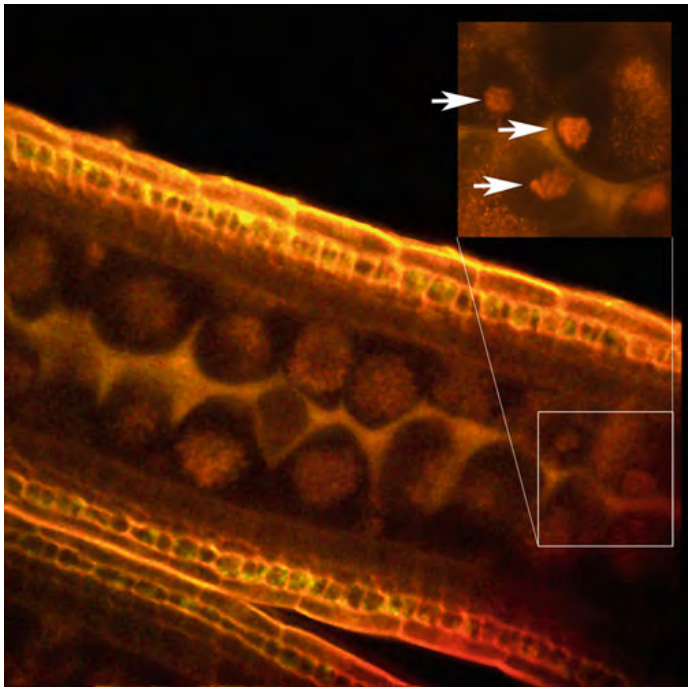

Fig. 1 An optical cross-section through a living maize anther generated with MPE microscopy. The anther was stained with DAPI (*pseudo-colored red*) and Mitotracker Green FM (*green*). A fragment of the anther locule in magnified in *inset*. *Arrows* point to three meiocyte nuclei at the zygotene stage

and a technique to monitor chromosome behavior during their segregation in anaphase I and II in cultured isolated meiocytes that was described by Yu et al. (1).

In the prophase I live imaging protocol, intact anthers are cultured (8) and the meiocytes are viewed using multiphoton excitation (MPE) microscopy (9), which can penetrate through the surrounding support tissue. The center of the anther locule, where maize meiocytes develop, lies roughly 70–100 μm from the anther surface (Fig. 1). While this depth is beyond the capabilities of confocal microscopy, it is within the demonstrated range of ~200 μm of MPE (9). Using this technique, Sheehan and Pawlowski found that meiotic chromosomes in maize at zygotene and pachytene exhibit extremely vigorous motility (4). In zygotene, the chromosomes exhibited short-range movements, whereas in pachytene, there were slower sweeping motions of large chromosome segments. In addition to the motility of individual chromosome segments, the entire chromatin mass within the nucleus was subject to oscillating rotational motions. This method may also be applicable to later stages of meiosis but it has not yet been tested for those applications.

In the later stages of meiosis, beginning at metaphase I and extending through the remaining stages, meiocytes can be extruded

from anthers and observed directly. Separating the cells from their support tissue makes it possible to use simpler microscopy methods since great depth of focus is not required. The method of Yu et al. (1) has been used to measure the rates of anaphase segregation in both meiosis I and II (1, 10). While the isolated meiocyte protocol can be very powerful, cells in prophase I or earlier cannot be extruded without damaging them. Even at later stages when meiocytes can be extruded, few of the isolated cells survive. The methods described here are reliable, but are probably best viewed as starting points, as both protocols can no doubt be improved with further study.

2 Materials

2.1 Plants (See Note 1)

To ensure uninterrupted availability of anthers, plants should be grown at regular intervals in a growth chamber or greenhouse. The time from planting to meiosis varies by genotype and is affected by environmental conditions. In maize, all anthers on the plant usually enter meiosis within less than a week of each other.

2.2 Reagents and Materials for Initial Anther Staging and Male Inflorescence Harvesting

1. Farmer's fixative—used to fix anthers for initial staging: a 3:1 mixture of 100% ethanol and glacial acetic acid.
2. Acetocarmine—used to stain chromosomes for a quick determination of meiosis stage (see Note 2): dissolve 2% acetocarmine powder in 45% acetic acid and boil 6–8 h in a flask with an attached reflux column. Filter through filter paper when the solution is still warm. Store in a dark bottle.
3. Needle-nosed forceps.
4. Dissecting needles.
5. Razor blades or scalpels.
6. Glass scintillation vials.
7. Glass microscope slides and coverslips.
8. Dissecting stereomicroscope.
9. Wide-field light microscope.

2.3 Reagents and Materials for Live Imaging of Intact Anthers at Prophase I

1. Artificial Pond Water (APW)—anther culture medium: 0.1 M NaCl, 0.1 M $CaCl_2$, and 0.1 M KCl, mixed, filter-sterilized, and stored at room temperature (see Notes 3 and 4).
2. DAPI solution—chromatin stain: make a concentrated stock of 50 mg/ml in water. Add to APW on the day of anther harvest for a final concentration of 50 μg/ml (see Notes 5–7). Store stock at −20°C.
3. DMSO—enhances penetration of stains and other reagents through anther tissues: use at final concentration of 1% for

DAPI staining and no more than 5% for any other application (see Note 8). Store stock at −20°C. Add to APW on the day of anther harvest.

4. Rhodamine 123 (Invitrogen, Carlsbad, CA, USA)—mitochondrial activity stain used to monitor cell viability: make a stock solution of 20 mM in DMSO, store at −20°C. Use at a final concentration of 20 μM. Rhodamine 123 will generate green fluorescence in actively respiring mitochondria (see Note 9).
5. Paraformaldehyde fixative—used for more precise staging of the third anther in each maize floret from which anthers are collected for live imaging (see Note 2): 4% electron microscopy-grade paraformaldehyde in buffer A (see item 6 below), 50 μg/ml DAPI, and 1% DMSO. To fix anthers, incubate them in the fixative for 1 h with gentle shacking. After incubation, replace with buffer A without paraformaldehyde and wash the anthers for 1 h.
6. Buffer A (11):
   (a) 20% of 10× Buffer A salts (150 mM PIPES, 800 mM KCl, 200 mM NaCl, 20 mM EDTA, 5 mM EGTA in water, pH with 1 M NaOH to 6.8, store at 4°C).
   (b) 0.1% spermine stock (0.4 M spermine tetra HCl; Sigma Aldrich, St. Louis, MO, USA) in 50 mM PIPES, store at −20°C.
   (c) 0.25% spermidine stock (0.4 M spermidine; Calbiochem, San Diego, CA, USA) in 50 mM PIPES, store at −20°C.
   (d) 0.2% DTT stock (1.0 M dithiothreotol; Calbiochem, San Diego, CA, USA) in 0.01 M sodium acetate, pH 5.2, store at −20°C.
   (e) 32% sorbitol stock (2 M solution in water, store at 4°C).
   (f) Water.
   (g) Filter-sterilize and store at 4°C.
7. Culture chamber microscope slides, sterile. We use the 8-chamber culture slides (Fig. 2) (Nunc-155411; Thermo Fisher Scientific, Waltham, MA, USA). One to two anthers can be placed in each chamber filled with ~200 μL of culture medium. Chamber slides are also available with a single chamber, 2, 4, and 16 chambers. Prior to anther collection, the chambers should be pre-filled with the desired culture medium.
8. Disposable transfer pipettes, fine-tipped—useful for applying and removing washes and other solutions to and from the culture chamber. Some examples of the pipettes are models 70960-3, -4, and -5 available at http://www.emsdiasum.com/microscopy/products/preparation/pipette.aspx.
9. Adjustable volume micropipettes: used for mixing culture medium components.

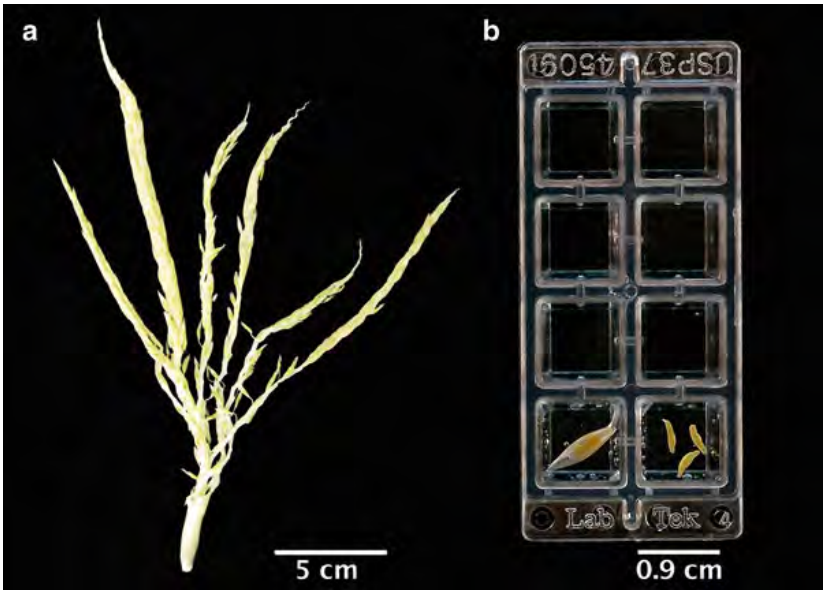

Fig. 2 Culturing maize anthers for live imaging of meiotic prophase I. (**a**) Immature male inflorescence containing anthers with meiocytes at the zygotene stage. (**b**) The 8-chamber culture slide used for live imaging of maize anthers. The upper chamber on the right contains two maize anthers. The lower chamber on the right contains a whole flower

2.4 Reagents and Materials for Live Imaging of Isolated Meiocytes at Anaphase I and II

1. Meiocyte culture medium (see Note 10): 0.3 g/l $Ca(NO_3)_2$, 0.028 g/l $FeSO_3$, 0.08 g/l KNO_3, 0.065 g/l KC1, 0.75 g/l $MgSO_4$, 0.01 mg/l MoO_3, 0.019 g/l NaH_2PO_4, 0.2 g/l $NaSO_4$, 0.050 g/l glutamine, 0.051 g/l glycine, 0.050 g/l L-isoleucine, 0.050 g/l lysine, 0.1 g/l meso-inositol, 0.050 g/l methionine, 0.5 mg/l nicotinic acid, 0.1 g/l pyridoxine, 0.1 mg/l thiamine, 0.050 g/l threonine, 0.050 g/l valine, 0.25 mM *n*-propyl gallate, 0.1 M sucrose, pH 5.8–5.9. Filter-sterilize and store at –20°C.
2. Cell viability stain: 5 mM Calcein AM (Invitrogen, Carlsbad, CA, USA) in meiocyte culture medium (see Note 11).
3. SYTO 12 fluorescent DNA stain (Invitrogen, Carlsbad, CA, USA) (see Note 7).
4. Culture chamber microscope slides (sterile). Any form of deep-well depression slide will probably work as long as an inverted microscope is used. For the published work, we used single-depression slides (catalog no. 12-560A; Thermo Fisher Scientific, Waltham, MA, USA).

3 Methods

3.1 Initial Anther Staging and Male Inflorescence Harvest (See Note 1)

In maize and other grasses, immature flowers containing cells undergoing meiosis are not visible without dissection, and determination of meiotic stage is required prior to plant harvesting. Determining the developmental stage of male meiocytes in maize

is not difficult but requires some practice. To stage and harvest anthers for live imaging of meiosis, we use the following protocol:

1. At the time of meiosis, the immature tassel (the male inflorescence) is still inside the stalk. The presence of the tassel can be felt just below the top node of the plant by gently squeezing the leaf whorl.
2. After establishing that the tassel is large enough to be felt at the first node, make a small incision with a razor blade through the leaves to the tassel, just below the top node.
3. Remove several flowers with needle-nosed forceps into a glass scintillation vial containing Farmer's fixative.
4. Dissect anthers from the collected flowers on a microscope slide under a stereo dissecting microscope.
5. Add a drop of acetocarmine solution for staining (see Note 2). Mix anthers with the stain using a dissecting needle over gentle heat until the color of the stain turns from deep red to purple. Place a coverslip over the anthers and gently press on to break the anthers and release meiocytes. Determine the stage of meiosis under a wide-field light microscope as described by Golubovskaya et al. (12).
6. If the anthers are not yet at the desired meiosis stage, tape over the incision with masking tape and repeat the staging procedure in a day or two.
7. Harvest plants with tassels containing anthers at the desired stage of meiosis at several nodes below the tassel and take to the laboratory for anther dissection.
8. Gently remove leaves surrounding the tassel. To prevent the tassel from drying out, place the tassel on wet paper towels in a tray and put more wet paper towels on top of it.

3.2 Live Imaging of Meiotic Prophase I in Intact Maize Anthers

3.2.1 Preparing Maize Anthers for Live Imaging (See Note 1)

1. After locating florets that contain anthers at the desired stage of meiosis, collect several anthers for the live imaging experiment. Be careful not to puncture or damage the anthers, as it may cause the meiocytes to die. In maize, each floret has three large anthers, which develop synchronously. We always use two of the three anthers for live imaging. Precise meiosis stage determination is more difficult in live anthers than in fixed anthers, and therefore, the third anther from each floret is placed in a paraformaldehyde fixative for more accurate staging (see Note 2).
2. Place the dissected anthers in a microscope chamber slide containing ~200 μl of anther culture medium (Fig. 2) (see Notes 3 and 4).
3. Stain the anthers with a solution of DAPI in the APW medium for 1 h to allow good stain penetration of the inside of the anther (see Notes 5–7).

4. Replace the stain solution with DAPI-free APW (see Notes 9 and 12).
5. The chamber slide should be protected from light whenever possible to minimize photobleaching of fluorophores. Imaging can begin immediately.

3.2.2 Image Capture Using Multiphoton Excitation Microscopy

The exact multiphoton excitation (MPE) settings and acquisition method will vary depending on the microscope workstation used. In our MPE system, we used a TI:Sapphire laser tuned to 780–800 nm to detect both DAPI and Rhodamine 123.

1. Firmly place the micro-chamber slide on the microscope stage with clips. Leave the chamber lid on to minimize dust contamination. Prior to imaging, it may be useful to first locate the anthers inside the culture chamber using the eyepieces rather than the image acquisition software.
2. MPE Images can be collected at one or more optical sections through the 3D object using one or more wavelengths. 2D images taken at a single Z section and at a single time point are useful for measuring objects (lengths, areas, diameters, etc.), and for acquiring wide views of tissue structures (Fig. 1). To capture and analyze chromosome dynamics, we found that the most informative movies contained 20–30 time-lapse images collected at 10-, 20-, and 30-s intervals at a single Z plane. These image numbers and imaging intervals guaranteed no photobleaching or tissue damage, which might occur with more numerous or frequent exposures. Movies covering longer time spans are valuable sources of information on longer-term chromosome dynamics but are often difficult to analyze as cells often move in the microscope field of view after several minutes of imaging. If this happens, post-image analysis may help obtaining useful data from these movies.
3. Test several different combinations of image numbers, image capture intervals, Z section numbers, etc. for every different imaging goal, MPE workstation type, meiosis stage, species, etc.

3.3 Live Imaging of Anaphase I and II in Isolated Meiocytes

3.3.1 Preparing Isolated Meiocytes for Live Imaging

1. Locate florets that contain anthers 1–2 mm in length and place them into a small Petri dish filled with meiocyte culture medium. Pre-staging of anthers by fixation and DAPI staining can be used to determine the sizes of anthers that contain anaphase I and later stages (although with practice it may not be necessary). Very gently cut the end of the anther with a fresh number 15 scalpel, and, as gently as possible, press on the back of the anther with the forceps. Meiocytes will invariably come out from the cut end but they are often damaged and appear as a "string of mush." The youngest meiocytes that can be extruded as separated, undamaged cells are usually in anaphase II.

However, in some genetic backgrounds and on some days, separated and intact meiocytes in anaphase I can be also extruded. Cells should be monitored using Calcein AM since, even in ideal conditions, more than half of the cells will be dead upon extrusion (10).

2. Draw extruded meiocytes into a standard 200 µl pipette tip. Transfer approximately 65 µl of meiocyte suspension into the Fisher 12-560A depression slide. Add SYTO 12 to the medium at a final concentration of 2 mM (see Notes 7 and 8). Place a coverslip over the concavity, leaving a ~2 mm air bubble. The edges of the coverslip can be sealed using rubber cement.
3. Imaging can begin immediately. An FITC filter set can be used to visualize SYTO 12. Since many of the cells will not proceed past the stage observed at extrusion, and because it can take hours for a cell to progress from metaphase to anaphase, it is best to monitor multiple cells at once (but this is only possible with advanced software and a motorized stage). The amount of light a cell is exposed to should be kept to an absolute minimum. In our hands, when total light exposure (from a mercury lamp) for any cell exceeds 1 min, cell viability drops and cells fail to proceed through meiosis.

3.3.2 Image Capture by Standard Epifluorescence Microscopy

Maize chromosomes are large, and isolated meiocytes have little autofluorescence in the FITC channel. A standard fluorescence microscope will probably yield good data, although further processing by deconvolution will sharpen the images. A confocal microscope would further improve image quality, although we have not tested the effects of the laser on isolated cells.

3.4 Analyzing Chromosome Movies Using ImageJ

We use ImageJ (available from: http://rsbweb.nih.gov/ij/), a freeware imaging analysis package, for processing and analyzing live imaging movies.

1. All files are imported to ImageJ as grayscale image stacks. In multiwavelength imaging, each image is a composite of all wavelength channels, which can be pseudo-colored.
2. Prior to analysis, images may be despeckled to improve the signal-to-noise ratio.
3. To improve the ability to identify cellular objects in the images, use the Find Edges algorithm of ImageJ (available under the Process menu). This algorithm analyzes the intensity (brightness) of adjacent pixels to define boundaries of objects, which are placed where the pixel intensity values change. This feature can be used to delineate the nuclear boundaries and identify the position of the nuclear envelope, utilizing the relatively small difference in brightness between the cytoplasm and the nucleoplasm.

4. The ImageStablizer plugin (13) can be used to stabilize images of objects exhibiting jittery movements due to stage vibrations, cellular motions, or for any other reasons. It can also be used to electronically immobilize objects that move laterally across the field of view. Using this plugin, the object is "pinned" down in one place. Doing this allows for analysis of smaller objects moving inside larger objects that also move, for example, fine movements of individual chromosome segments inside nuclei that exhibit rotational movements of the whole chromatin. Relative velocities of the small chromosome segments can be determined without confounding them with the movement of the entire nucleus.
5. For files containing multi-Z image stacks, flat projections can be generated by using a built-in algorithm Z Project and a plugin, Grouped_ZProject (14). Both algorithms generate flat projections from several Z sections in still 3D images or in 3D movies. Flat Z projections are helpful in simplifying 3D images and can be generated based on the average intensity, the maximum intensity, or by a sum of slices.
6. To obtain basic measurements of cellular structures (such as nuclei, nucleoli, or the whole chromatin mass in the nucleus) in still images or in single frames of time-lapse movies, we use the Drawing Tools and the Region-of-Interest (ROI) manager of ImageJ. The drawing tools can also be used to select and straighten curved chromosomes to measure their length.
7. To track and analyze movements of small, single point objects moving in time in time-lapse movies, we use two particle tracking plugins that essentially perform the same task: MTrackJ (15) and ParticleTracker (16). MTrackJ is easier to use and provides the ability to produce publication-quality outputs as it offers more editing options. It also has a more user-friendly interface. The object to be tracked must be manually selected in each time frame. Several objects can be tracked independently in parallel by using differently colored tracks. To do this, one must first track one object, and then do the same for each additional object. The object tracks can be saved as separate movies or can be overlaid over the objects in the originals movies (Fig. 3). Still images with cumulative tracks can also be plugin. Finally, statistics on object velocity and movement directionality through time can be generated.
8. To trace behavior of larger objects, for example, the movements of the entire chromatin mass in the nucleus or shape changes of the nuclear envelope, we utilize the Segmented Line feature. The object is delineated in each time frame and each of the object outlines is saved in the ROI manager. Finally, a time-lapse movie of the outlines is generated, which can be saved separately or overlaid over the originals movie (Fig. 3).

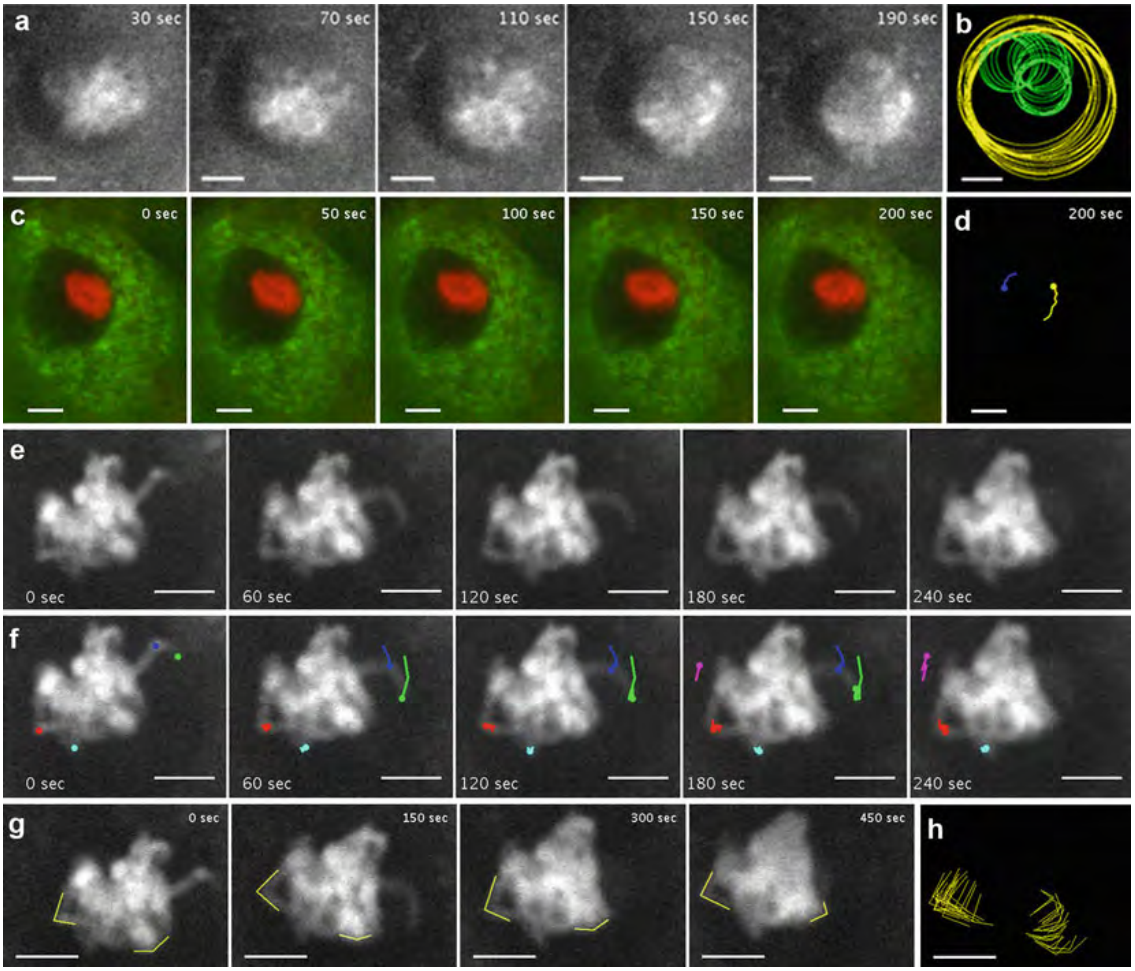

Fig. 3 Patterns of chromosome movements during meiotic prophase I in maize. (**a**) A zygotene nucleus exhibiting rotational motions. (**b**) Cumulative tracks of the nuclear envelope (*yellow*) and nucleolus (*green*) in the nucleus in (**a**) after 190 s. (**c**) A zygotene nucleus showing sliding rotation of the entire chromatin (*red*) along the nuclear envelope. *Green* = cytoplasm stained with Rhodamine 123. (**d**) Cumulative tracks of two anonymous chromosome marks located at the nuclear periphery in the nucleus shown in (**c**) after 200 s. (**e**) Rotational movements of the entire chromatin in a pachytene nucleus. (**f**) The nucleus shown in (**e**) overlaid with trajectories of chromosome marks shown every 60 s for 240 s. *Green* and *blue* mark the chromosome end and an interstitial knob, respectively, of a chromosome arm, which exhibits long-distance sweeping movements. *Red* = a chromosome loop whose both ends are embedded in the chromatin mass. *Cyan* = a stationary chromosome region on the periphery of the chromatin mass. *Magenta* = a free, fast moving chromosome end. (**g**) The nucleus shown in (**e**) with *yellow lines* marking chromatin mass edges. (**h**) Cumulative tracks from (**g**) after 570 s but without chromatin shown. Bars = 5 μm. Adapted from Sheehan and Pawlowski (4)

4 Notes

1. For most meiosis studies in maize, male flowers are used because (a) there are many more male meiocytes than female meiocytes on a plant, (b) the timing of male meiocytes development in maize is synchronized within anthers and florets

and is less variable that that of female meiocytes, and (c) the dissection of male flowers is much easier than female flowers and carries less potential for damage to the meiocytes.

2. The Pawlowski lab uses acetocarmine for quick and rough determination of meiocyte stage, as described in Subheading 3.1, step 5. For precise staging of anthers used for live imaging of prophase I, we fix anthers using paraformaldehyde and conduct 3D microscopy, as described in Chapter 6. The latter method allows very exact determination of meiosis stage, for example, establishing whether meiocytes are at early, mid or late zygotene. Paraformaldehyde fixation is preferred for precise staging as it preserves chromatin and chromosome structure better than the Farmer's fixative. We found live (unfixed) anthers at prophase I difficult to stage directly.
3. Culturing organs for live imaging requires isotonic solutions to maintain tissue viability without altering the speed of growth and development. Furthermore, the medium should be close to optically clear to reduce light scattering. We also tested Murashige and Skoog (MS) medium. However, we found that it had inferior optical clarity compared to APW. Additionally, as APW is a minimal medium (i.e., lacks a carbon source), it is less likely than MS medium to become contaminated by bacteria over the course of experiments.
4. To maintain normal anther growth rate, APW is not supplemented with any growth regulators. Although APW should not be used for long-term culturing (several days or longer), we found that anthers had excellent viability for over 30 h, as evidenced by mitochondrial viability staining (17–19). For example, we observed zygotene meiocytes that progressed to pachytene and pachytene cells that progressed to dyads. Both progression patterns were consistent with the timing of meiosis observed *in planta* (20).
5. Staining live tissues is generally more difficult and often requires higher stain concentrations than staining fixed cells.
6. For MPE microscopy, the fluorophore emission wavelength is an important consideration. Stains with emission in blue or green are the easiest to use in MPE. Stains with yellow and red emissions may also be used but require an MPE system with a laser that can be tuned to an excitation wavelength of 900–950 nm.
7. DAPI was an excellent vital stain for DNA in the anther culture system, but was not effective as a live-cell stain for isolated meiocytes. In the anther culture system, DAPI was more efficient than several SYTO dyes (SYTO 11, SYTO 12, SYTO 13, SYTO 14, SYTO 15, and SYTO 16; Invitrogen, Carlsbad, CA, USA) in penetrating anther walls. For isolated anaphase

meiocytes, SYTO 12 (Invitrogen, Carlsbad, CA, USA) was the most effective chromatin stain. It rapidly penetrates live maize meiocytes to reveal the chromosomes when used at a final concentration of 2 mM. None of the other SYTO stains worked for maize anaphase meiocytes (10), nor did DAPI, Hoechst stains, or propidium iodide (they stained chromosomes but only in dead cells, as assessed using Calcein AM).

8. DMSO is known to polymerize microtubules in vitro. However, it does so when used at concentrations between 8 and 12% (21, 22), which are much higher than the concentrations that we used in live imaging. Consistently, we did not see any difference in either chromosome or cytoplasmic (organellar) motility between DMSO concentrations ranging from 0.1% (which is frequently used in live confocal microscopy experiments (23)) to 5%. It seems likely that DMSO would also facilitate the uptake of DAPI into isolated meiocytes but this has not been tested.
9. In a live imaging system, there is often a need to monitor cell viability in order to distinguish dead cells from cells whose dynamics are disrupted for other reasons. To monitor cell viability, we visualize mitochondrial activity because many live mitochondrial stains are available and can be tried. We tested three dyes that can only fluoresce when within actively respiring mitochondria. Rhodamine 123 provided the most consistent mitochondrial staining of meiocytes of the three stains. We also tried $DiOC_7(3)$ at a final concentration of 200 μM and Mitotracker Green FM, which was tested at a final concentration of 200 nM. $DiOC_7(3)$ stained well the anther epidermal layers but was not visible in meiocytes, suggesting that the stain could not sufficiently penetrate to the inside of the anther. Mitotracker Green FM provided sufficient mitochondrial staining in leptotene meiocytes but staining was not reliably found in zygotene or pachytene meiocytes.
10. Isolated maize meiocytes survive for at least 8 h in a rye meiocyte culture medium described by Pena (24). We use this medium without micronutrients and with a reduced sucrose concentration. In addition, we add 0.25 mM *n*-propyl gallate, which was shown to increase the longevity of maize protoplasts in culture (25) and showed a measurable increase in viability when cells were exposed to light (10).
11. Calcein AM is converted into a fluorescent product in living cells but does not stain dead cells.
12. The anther culturing system can be easily used to test the effects of various drugs on meiocyte development and chromosome dynamics. Drugs are added to the DAPI-containing anther culture medium for incubation simultaneous with

chromosome staining. After staining, the culture medium should be replaced with medium free of DAPI but with the same concentration of the drug. We used this approach to test the effects of cytoskeleton-disrupting drugs:

(a) Latrunculin B (Lat B): disrupts actin filament polymerization. 100 μM latrunculin B (Sigma Aldrich, St. Louis, MO, USA) stock solution should be stored at –20°C. We used final concentrations of 500 nM and 1 μM.

(b) Colchicine: disrupts microtubule polymerization. 100 mM colchicine (Sigma Aldrich, St. Louis, MO, USA) stock solution should be stored at –20°C. We used final concentrations of 1 mM and 5 mM. For the 1 mM colchicine concentration, we used DMSO at a final concentration of 1% but for the 5 mM colchicine treatment, we used 5% as the final DMSO concentration.

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