

Using Live-Cell Markers in Maize to Analyze Cell Division Orientation and Timing

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4

Abstract

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Recently developed live-cell markers provide an opportunity to explore the dynamics and localization of proteins in maize, an important crop and model for monocot development. A step-by-step method is outlined for observing and analyzing the process of division in maize cells. The steps include plant growth conditions, sample preparation, time-lapse setup, and calculation of division rates.

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Key words Maize, Mitosis, Plant, Live-cell imaging, Microtubules, Cell division, Fluorescent proteins, Confocal, Microscopy

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1 Introduction

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Live-cell imaging in plant cells is used to study many dynamic processes including meristem growth [1–3], development [4], root hair growth [5], organelle movement [6], and microtubule and actin dynamics [7–11]. Live-cell imaging has also provided insight into the mechanisms of cell division in plants [12–16]. The recent development of stably transformed maize lines expressing fluorescently tagged proteins makes it possible to answer questions regarding the dynamics of cell division and other processes [17, 18]. Maize is an ideal model system for this type of study because its relatively large cells divide within the framework of other cells while growing in a precisely defined developmental gradient.

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Live plant cell imaging performed to understand the process of division has primarily used *Arabidopsis thaliana*, *Tradescantia virginiana*, and tobacco-cultured cells. Reliable, stable transformation of *A. thaliana* [19] has resulted in the fusion of many proteins of interest to fluorescent proteins with subsequent examination of protein dynamics. A more challenging technique that has also yielded dynamic information is microinjection of fluorescently labeled proteins and subsequent live-cell imaging in the spiderwort

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32 *T. virginiana* [20–22]. Another powerful model used to answer
33 questions about cell division is the cultured tobacco cell line bright
34 yellow 2 (BY-2). BY-2 cells are easy to transform [23], synchronize
35 [24], and observe in vitro on the microscope [25–29]. *Physcomitrella*
36 *patens* recently emerged as a model for live cell imaging [8, 30, 31]
37 which will likely soon be expanded to explore the dynamics of
38 division.

39 Maize has lagged as a model system for live plant cell imaging
40 because there were very few available maize lines expressing pro-
41 teins fused to fluorescent proteins. A notable exception to the lack
42 of live cell imaging is the analysis of chromosome movement dur-
43 ing meiosis, which does not require fluorescent proteins [32].
44 Although bombardment of maize leaf cells has been used to tran-
45 siently express fluorescent proteins, bombardment has the disad-
46 vantage of transforming a small number of cells. Moreover, it is a
47 damaging process that requires lengthy in vitro culture of isolated
48 tissues, which may alter protein dynamics or localization [33].
49 Stable transgenic lines expressing a variety of proteins fused to flu-
50 orescent proteins circumvent some of the problems with transient
51 maize transformation, allowing live-cell imaging of dynamic
52 processes.

53 Maize is an excellent model system for development and cell
54 biology in monocots because its leaves develop progressively and
55 reproducibly. This reproducible developmental gradient in the
56 leaves is referred to as the “base to tip gradient” [34, 35]. Near the
57 base of the leaf, cells divide symmetrically. Further from the base,
58 some cells divide asymmetrically to differentiate and to establish
59 specialized cell types [36]. Finally, towards the tip of the leaf, cells
60 expand rapidly. This reproducibility allows direct comparisons to
61 be made regarding distinct developmental stages, including a
62 recent explosion in large-scale “-omics” analysis [37–43]. The
63 recent influx of genomics resources together with the developmen-
64 tal gradient and live-cell imaging tools will synergize to improve
65 our understanding of monocot biology, potentially impacting
66 next-generation crop production.

67 To explore the subcellular localization and dynamics of many
68 processes, including cell division, stable transformed maize lines
69 expressing live-cell markers have been created [17, 18]. Like many
70 other land plant cells, maize cells form typical dividing structures
71 in symmetric and asymmetric divisions [44–47]. A preprophase
72 band is formed before mitosis and is thought to predict the future
73 site of the new cell wall [48]. The preprophase band is a ring of
74 microtubules, microfilaments and a large number of other proteins
75 that usually forms at the cell cortex around the nucleus [12]. The
76 preprophase band disassembles during metaphase while the spindle
77 forms [49]. The spindle is an organized antiparallel array of micro-
78 tubules that moves chromosomes during anaphase. During
79 telophase, the plant-specific structure called the phragmoplast

forms. The phragmoplast is composed of microfilaments and an antiparallel array of microtubules that serve as tracks for the transport of cell-wall components to the newly forming cell plate. The cell plate is the nascent cell wall, and eventually it expands outwards to the cortex to complete cytokinesis [50, 51].

The method described here provides a protocol for live-cell imaging of actively dividing maize cells using YFP-TUBULIN as an example. In it, plant growth conditions are outlined, as well as sample preparation for microscopy, setting up the time lapse, and finally analyzing the results. Recognition of common problems, such as sample damage and movement, is discussed and solutions presented.

2 Materials

2.1 Greenhouse Materials

1. Maize seeds that produce plants expressing one or more live-cell markers such as YFP-TUBULIN [18]. Full list available at (<http://maize.jcvi.org/cellgenomics/index.php>).
2. Soil.
3. Slow release fertilizer.
4. Calcium-magnesium supplementary fertilizer (e.g., Peters Excel Cal-Mag Special 15-5-15 water-soluble fertilizer).
5. 10 cm square pots in 15 pot flats.
6. 4 g/L glufosinate-ammonium in 0.1 % Tween 20.
7. Large 7.5 L (“2 gallon”) pots.
8. Cotton applicators.
9. LED lights or other supplemental lighting. Light intensity should be $\sim 230 \mu\text{E}/\text{m}^2/\text{s}$ at a height of 1 m. A detailed protocol for greenhouse growing conditions can be found at www.agron.iastate.edu/ptf/protocol/Greenhouse%20Protocol.pdf.

2.2 Microscopy Materials

1. Confocal microscope system. Either a spinning disk or a point scanning confocal system can be used together with an inverted microscope, an EM-CCD camera, and appropriate lasers and filters for imaging various fluorescently labeled proteins. Various microscopes have benefits and drawbacks (*see Note 1*).
2. Airstream incubator or thermostat.
3. Mid-range infrared thermometer.
4. Glass cover slips.
5. Rose chamber for holding the sample still during long time lapse. Rose chambers or alternatives can be used to stabilize the sample within the field of view (*see Note 2*).

- 120 6. Vacuum grease loaded into a 10 cm³ needle-less syringe.
 121 7. Water.
 122 8. 200 µL pipette and tips.
 123 9. Digital calipers.
 124 10. Straight scalpel blade.
 125 11. Forceps.
 126 12. Software for running the microscope (e.g., Micromanager 1.4
 127 <https://www.micro-manager.org/>).

128 **2.3 Data Analysis**
 129 **Materials**

1. ImageJ or FIJI (a regularly updated and modified version of ImageJ available at <http://fiji.sc/Fiji>).
 2. Statistical package to import and analyze data.

131 **3 Methods**

132 **3.1 Plant Growth**

133 Temperature and lighting conditions will affect the growth rate
 134 and cell division rate of maize. Consistent growth conditions, con-
 135 sistent plant age, and tissue type are required for any comparative
 136 quantitative analyses.

137 The following steps list a reproducible method of growing
 138 plants.

- 138 1. Plant seeds ~2 cm deep in pre-wetted, pre-fertilized soil in
 139 10 cm square pots loaded into a 15-pot flat for easy transport.
 140 2. Germinate seedlings in standard long-day greenhouse condi-
 141 tions. The conditions are 16 h light at 25 °C, 8 h dark at 21 °C
 142 with supplemental lighting provided by LED or high-pressure
 143 sodium and metal halide lamps.
 144 3. Water plants when the soil is dry, likely three times a week.
 145 4. Fertilize with the Cal-Mag fertilizer once a week.
 146 5. After 1 week of growth, use a permanent marker to mark the
 147 second or third leaf, and then apply 4 g/L glufosinate-
 148 ammonium in 0.1 % Tween to the marked location using a
 149 cotton-tipped applicator.
 150 6. Two to three days after herbicide application, score resistant
 151 and sensitive plants (*see Note 3*).
 152 7. Transplant resistant plants into 2-gallon pots.
 153 8. Grow for a total of 3–5 weeks after planting. Plants should
 154 have at least seven visible leaves at this stage of growth. Do not
 155 use diseased or poorly growing plants. Figure 1a shows accept-
 156 able growth for a 4-week-old plant and describes the parts of
 157 the plants discussed in this paper (*see Note 4*).

9. Figure 1b indicates the part of the plant dissected to observe many dividing cells (described in more detail below in sample preparation for microscopy and *see Note 4*).

3.2 Sample Preparation for Microscopy

1. Set the thermostat. Alternatively, turn on the airstream incubator and point it onto the stage for at least 10 min prior to placing the sample (Fig. 2a). Determine what setting will provide the correct temperature before use. I used 21 °C (*see Note 5*).
2. Use a mid-range-infrared thermometer to measure the temperature using the laser to guide the thermometer to stage right next to the sample (Fig. 2b).
3. Place a clean glass cover slip on the lower half of the Rose chamber (Fig. 3a).
4. Prepare a glass cover slip for the sample by applying a thin film of vacuum grease in a circle with ~1 cm diameter (*see Note 6*).
5. Add a ~100 μ L drop of water inside the vacuum grease circle with a pipette (*see Note 7*).

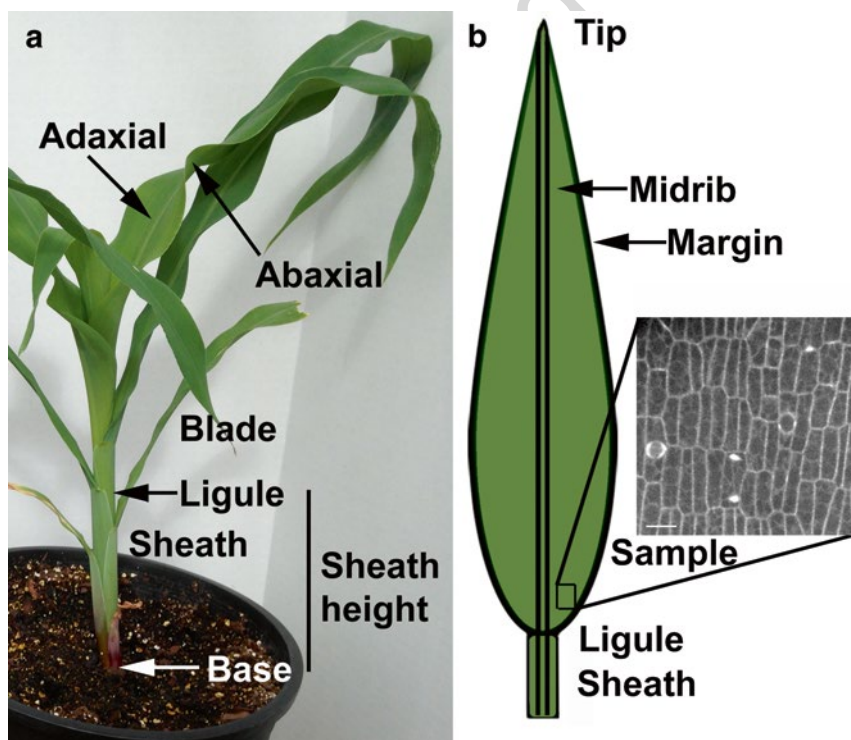


Fig. 1 Representative example of a maize plant and descriptions of the parts of the leaf used in this method. (a) A 28-day-old plant grown in standard greenhouse conditions with relevant parts and descriptors of the plant indicated. (b) Schematic of a plant leaf, with more descriptors, as well as a micrograph of a young maize leaf expressing YFP-TUBULIN. Bar is 50 μ m

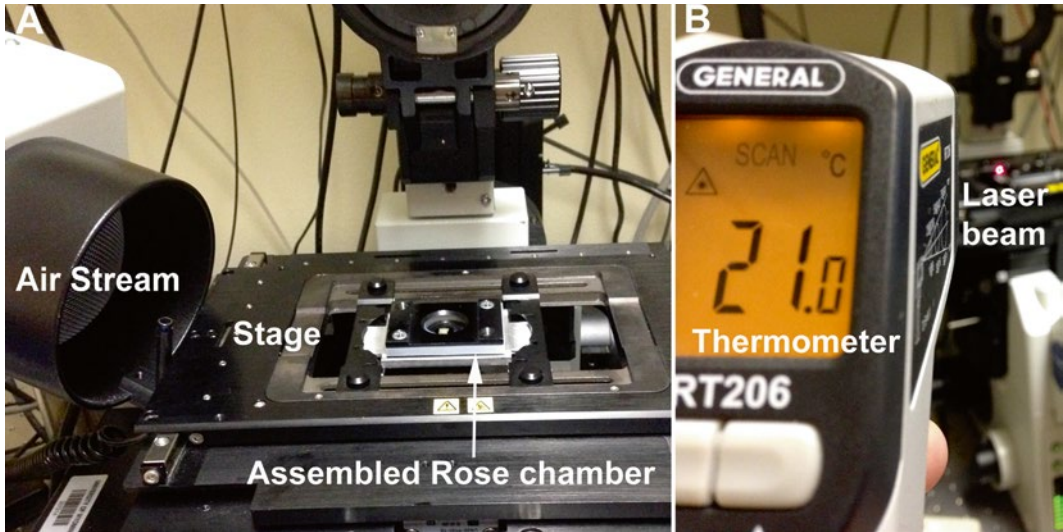


Fig. 2 Temperature control. (a) Photograph of the inverted stage with the airstream pointing toward the sample. The Rose chamber is assembled on the microscope. (b) The infrared thermometer is shown with the laser readout pointed toward the sample on the microscope

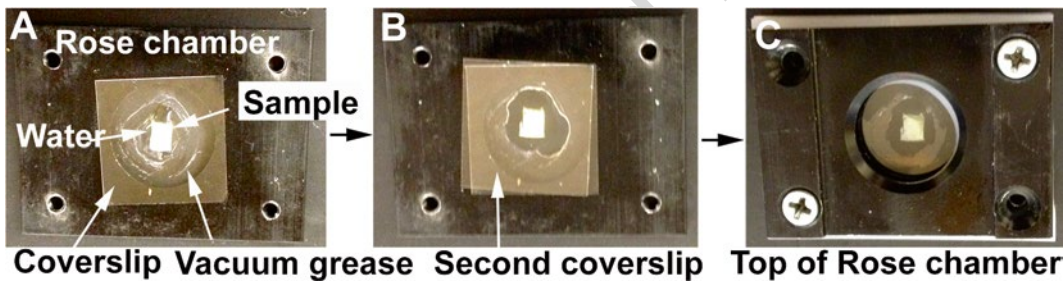


Fig. 3 The Rose chamber holds the sample steady during time-lapse imaging. This figure shows the steps needed to assemble it. (a) The bottom part of the rose chamber is shown with the cover slip, vacuum grease, water and sample mounted. (b) The second cover slip is carefully mounted on top of the sample, spreading the water evenly. (c) The top of the Rose chamber is screwed into place, and the sample is ready to be loaded onto the holder and the stage

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6. After 3–5 weeks of growth, harvest plants for microscopy. Refer to Subheading 3.1 for growth conditions and Subheading 3.1, step 8 for acceptable plants to harvest.
7. Cut off the above-ground portion of the maize plant, at the base of the sheath, leaving behind the roots (Fig. 1b).
8. Sequentially peel away the outer leaves until the sheath height is less than 3 mm. Sheath height can be measured with digital calipers or a ruler.
9. Excise a ~0.2–1.0 cm² leaf piece just above the ligule and between the midrib and the margin (Fig. 1b) using a straight scalpel blade.

10. Carefully peel the excised portion from the rest of the leaf, holding the sample by the edge with forceps. Avoid touching the tissue directly. 185
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11. Mount the adaxial side down in the water droplet towards the objective when the rose chamber is fully assembled (*see* Figs. 1a and 4 and Note 4). 188
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12. If there are air bubbles (*see* Fig. 5d), carefully remove your sample from the water and set it down again (*see* Note 8). 191
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13. Gently place another cover slip on top of the sample and bottom cover slip (Fig. 3b). 193
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14. Adjust the sample so that it is in the middle of the Rose chamber (*see* Note 9). 195
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15. Place the top half of the Rose chamber, including the silicone sandwich, onto the top cover slip, and carefully screw it down (Fig. 3c). 197
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16. Load the rose chamber into the holder and mount it on the microscope stage after adding the correct immersion liquid for the objective (*see* Notes 10 and 11). 200
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17. Figure 2a shows a Rose chamber loaded onto the stage and Fig. 4 shows the schematics of the Rose chamber and its final orientation relative to the objective. 203
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Rose Chamber Schematic

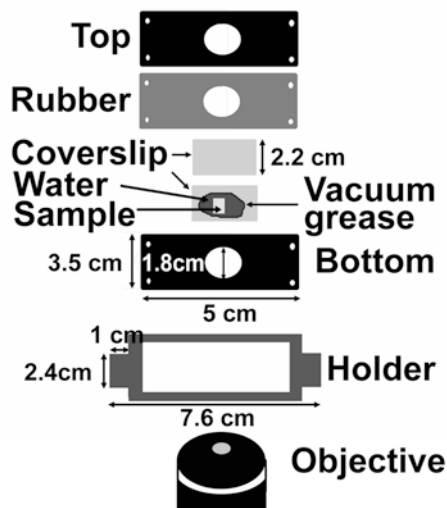


Fig. 4 Schematic of the Rose chamber with parts and measurements labeled. All pieces but the metal holder will eventually be screwed together to gently sandwich the sample and stabilize it during time-lapse imaging. The cover slip at the bottom of the Rose chamber will come into contact with the immersion liquid and the objective

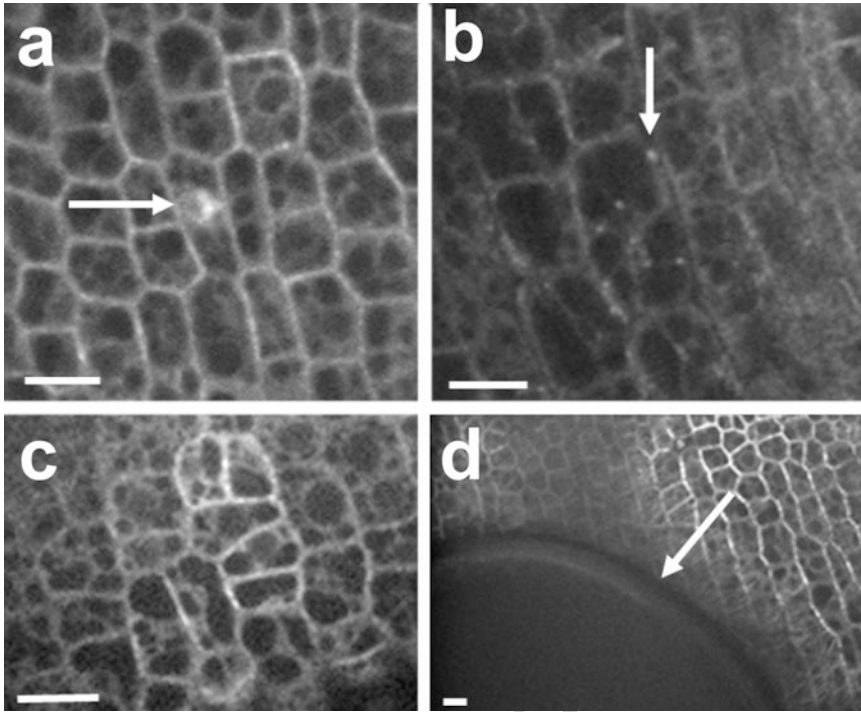


Fig. 5 Examples of cells expressing YFP-TUBULIN that are damaged and an air bubble. **(a)** Aberrant division structure indicated by an *arrow*. **(b)** Abnormal microtubule structure that looks like a dot indicated by an *arrow*. **(c)** Example of cells with a lot of small vacuoles. **(d)** Air bubble indicated by an *arrow*. Bar is 20 μm

206 3.3 Microscopy

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1. Start the computer, microscope, camera, focus and filter controller, laser launch, etc. Consult a microscope manual or knowledgeable person for details on its operation.
2. Find the epidermal cell layer using bright field. Avoid taking images near the cut sites.
3. Switch to the camera view to start observing the fluorescent proteins in the cells. If cells are damaged or there is an air bubble, prepare a new sample. Examples of damaged cells are shown in Fig. 5 (*see Note 8*).
4. Adjust the exposure time, EM gain, and laser attenuation (if necessary) so that the illuminated sample has high dynamic range (*see Note 12*).
5. If the goal of the time lapse is to capture the entire process of division, prioritize capturing cells in late prophase at the start of the time lapse. Figure 6, time 0, shows an example of a cell in late prophase with a disassembling preprophase band. More examples of cells in late prophase are shown in Fig. 7 (*see Note 13*).
6. Use the multi-acquisition tool to set up your time lapse. Specify Z stack positions, exposure time, EM gain, and amount of

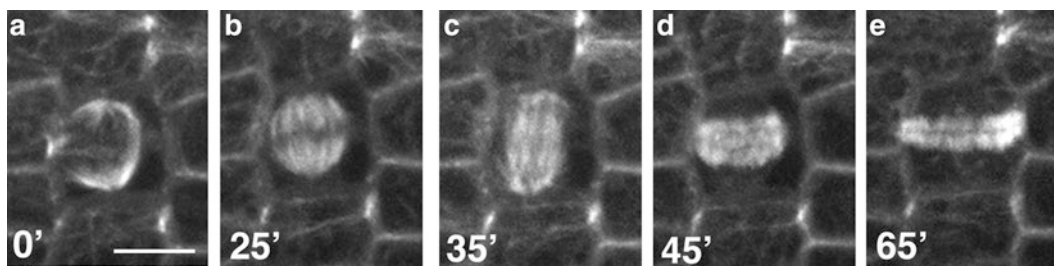


Fig. 6 Time lapse of a cell progressing from late prophase to the end of telophase and examples of cells in late prophase. Microtubules are labeled with YFP-TUBULIN. Time is indicated in the *bottom left-hand side* in minutes. **(a)** Late prophase. Note that the preprophase band has almost fully disassembled. **(b)** The bipolar spindle is formed in metaphase. **(c)** The anaphase spindle elongates to separate the chromosomes. This is the shortest stage in the cell cycle and generally takes 10–15 min. **(d)** A phragmoplast, an antiparallel set of microtubule arrays, forms in telophase. The cell wall materials are transported along the microtubule tracks towards the phragmoplast midline. **(e)** The phragmoplast expands outwards towards the cortex to complete cytokinesis. Bar is 10 μm

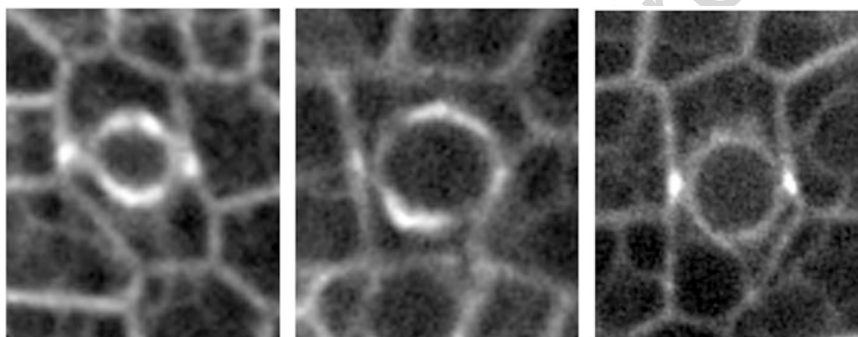


Fig. 7 Three examples of cells transitioning between prophase and metaphase. Note the prominent microtubule accumulations around the nuclear envelope

- time between image collection. For observing mitosis in maize, 225
 5-min time lapse is a good compromise between photo- 226
 bleaching and loss of temporal resolution (*see Note 14*). 227
7. Choose the “save as function” to save time-lapse images as 228
 they are produced into a single folder so that images are not 229
 lost in case of power outages (*see Note 15*). 230
 8. Start the time lapse. 231
 9. Adjust for sample movement, especially in the first 20 min. 232
 The most frequent trouble is slow drift in one direction. If 233
 there is a lot of drift, the sample is incorrectly positioned on 234
 the cover slip and a new sample should be loaded. 235
 10. Check the time lapse for sample movement every hour. 236
 Compare the start of the time lapse with the new positions, 237
 and readjust your sample if the x , y , or z planes shift during the 238
 time-lapse. 239

240 11. Stop the time lapse after the cells are finished dividing or 5 h,
241 whichever occurs first. Empirically, 5 h was the maximum
242 amount of time that division progressed with this sample type
243 at this temperature.

244 3.4 Data Analysis

- 245 1. Open stacks in the image analysis program Fiji or ImageJ.
- 246 2. Make maximum projections of the Z stacks: Process:
247 Batch:Macro. Then specify input (the folder with the Z stacks)
248 and output files (a new folder to put the max projections) with
249 output format [Tiff]. Use this macro: run (“Z Project...”,
“start=1 stop=10 projection=[Max intensity]”) (*see Note 16*).
- 250 3. Open the maximum projections as an image sequence
251 (File:Import:Image sequence) and scroll through the file.
- 252 4. If the sample drifts a lot during the time-lapse, perform a cor-
253 rection by loading Plugin:Registration:StackReg or
254 Plugin:Registration:Register Virtual Stack Slices and choose
255 “Translation” (*see Note 17*).
- 256 5. Use the timestamps on original Z stacks to calculate the timing
257 of division stages. Figure 5 illustrates the microtubule struc-
258 tures to look for at various stages of the cell cycle, and illus-
259 trates one potential way of indicating time.
- 260 6. Measure the amount of time needed to progress from one
261 stage to another. These transitions are very clear when time-
262 lapse images are viewed as a movie. The time can be analyzed
263 in by calculating (the time between frames) × (number of
264 frames cells are in a particular stage) = total time cells are in that
265 state. An alternative method, if the stacks are not generated in
266 5-min intervals, is to compare the time-stamps on the images
267 as they were originally saved.
- 268 7. Scroll through the image sequence focused on one mitotic
269 structure. The spindle microtubules will start accumulating
270 before the preprophase band disassembles. Preprophase band
271 disassembly is observed as a loss of fluorescence at the cortex.
- 272 8. Next, the spindle, a football-shaped structure, coalesces per-
273 pendicular to the final division site and then expands during
274 anaphase.
- 275 9. A sharp transition occurs from the orientation of the spindle to
276 the orientation of the phragmoplast. The spindle is generally
277 perpendicular to the final division site, while the phragmoplast
278 is generally aligned with the final division site. This visually
279 striking morphological change is usually obvious from one
280 frame to the next.
- 281 10. The phragmoplast expands towards the cortex, sometimes
282 contacting one side before the other [52]. Viewed from the
283 side, it looks like two microtubule-containing disks with the

midline containing very few microtubules. As the phragmo- 284
plast expands, the interior microtubules may disassemble. 285
Viewed from the top the phragmoplast looks like an expanding 286
torus. 287

11. Finally, the phragmoplast reaches the cortex and starts to disas- 288
semble. Once it is completely disassembled, stop the time lapse 289
and measure the complete time of all the steps. 290
12. Import the time values into Microsoft Excel or another spread- 291
sheet program. Usually, the long and unpredictable amount of 292
time that cells spend in preprophase/prophase means that 293
gathering this type of data is more difficult. If cells do not 294
progress, they may be damaged (Fig. 5 and *see Note 8*). 295
13. Other measurements can also be made such as the rate of 296
phragmoplast expansion or the rotation and movement of the 297
spindle. These are not discussed further in this method. 298

4 Notes

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1. *Microscope selection*

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A confocal microscope must be used to avoid out of plane 301
fluorescence with intact plant tissues. Either a laser scanning 302
microscope or a spinning disk microscope can be used. The 303
benefits of using a spinning disk microscope are speed and 304
reduced photo-damage to the specimen. For fluorescent pro- 305
teins that photo-bleach rapidly, a spinning disk microscope 306
with a sensitive camera is essential. If the fluorescently tagged 307
protein is abundant and does not photo-bleach easily, such as 308
YFP-TUBULIN, it is possible to use a point-scanning micro- 309
scope for time-lapse imaging. If necessary, the images can also 310
be binned to reduce both photo-damage and the time required 311
to gather each individual image. 312

2. *The Rose chamber*

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The Rose chamber as it is used here prevents movement of 314
the sample by applying enough pressure to flatten a curved 315
sample, but not too much to damage the cells. There is no 316
commercially available option for purchase of a Rose chamber, 317
but it is straightforward for a machine shop to make one. It 318
consists of a metal holder, two pieces of metal and one piece of 319
silicone or rubber with a width of 5 cm × 3.5 cm with a hole 320
cut in the middle of both 1.8 cm diameter, four aligned screw 321
holes, illustrated with dimensions shown in Fig. 4. An inex- 322
pensive alternative to the Rose Chamber is a glass slide sealed 323
with VALAP (This is a 1:1:1 mixture by weight of paraffin, 324
lanolin and Vaseline, doi:10.1101/pdb.rec12380 *Cold Spring* 325
Harb Protoc 2010.). VALAP, a solid at room temperature, is 326

327 gently heated on a hot plate to liquefy. VALAP provides a
328 strong and biologically inert attachment between the slide and
329 the cover slip. Do not use Vaseline or vacuum grease unless
330 the sample is thin because the cover slip may slip during
331 imaging.

332 3. *PCR to distinguish segregating transgenes*

333 When multiple transgenes are segregating, it is helpful to
334 confirm the genotype using a PCR based method, particularly
335 when the plants express fluorescent proteins only in dividing
336 tissue. A general method for maize DNA extraction and PCR
337 can be found at <http://rasmussenlab.weebly.com/protocols.html>.
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339 4. *Age and type of maize material suitable for imaging cell division*
340 *by time lapse.*

341 Overall, it is important to observe similar samples from one
342 experiment to the next so that the data can be combined in the
343 quantitative analyses. This is also why temperature control (*see*
344 **Note 5**) and growth conditions are vital to the success of the
345 experiment. Several parts of the plant are actively dividing
346 including the base of the leaf, the meristem, the young sheath
347 material and the root tips. Maize primary roots are thick, and
348 cutting them appropriately for stable time-lapse imaging can
349 be difficult. Young blade or sheath tissue is preferred because
350 it is flat, has mostly undeveloped chloroplasts (and therefore
351 little to no autofluorescence), and has a high proportion of
352 dividing cells. If asymmetric divisions are preferred, older leaf
353 tissue should be used. Either the adaxial or abaxial side of the
354 leaf can be used. If juvenile leaves are preferred, either because
355 the fluorescent protein is better expressed or the developmen-
356 tal stage is more ideal for the experiment, plants ~2 weeks old
357 can be harvested.

358 5. *Temperature control*

359 The temperature must be the same between experiments if
360 comparisons are going to be made between samples. The main
361 reason to keep the temperature then same is that microtubule
362 dynamics (and other protein dynamics) are different at differ-
363 ent temperatures [53]. Other methods that can be used to
364 control temperature are a thermostat-regulated room or a
365 heated chamber for microscopy.

366 6. *Vacuum grease*

367 Vacuum grease is recommended to form the small well
368 between the two cover slips because it has the correct viscosity
369 and is chemically inert. It is important that a thin, consistent
370 layer is applied for optimal time-lapse microscopy. If the layer
371 is too thin or if it is spread unevenly, the sample will slip during
372 time lapse (*see Note 8* on sample slipping). Other materials

that can be used are Vaseline and VALAP. Vaseline will work in a pinch, but it may not adequately protect the sample during mounting because it is much softer than vacuum grease. VALAP is tricky to apply in a thin layer because it solidifies very quickly.

7. *Water*

Use water to mount maize samples. Other materials, such as mineral oil or phosphate buffered saline, will damage the cells and prevent timely progression of mitosis. Other materials, such as perfluorocarbons [54], may be better for imaging, but I have not had an opportunity to use them.

8. *Avoid sample damage and air bubbles*

Damage and air bubbles both cause significant trouble for time-lapse imaging. Avoid smashing, squashing, or otherwise disturbing the sample. If the sample is damaged, take a new slice from the plant, which should be wrapped in moist paper towels to preserve it for a few hours if necessary. The most obvious signs that cells are damaged are a lack of organized cortical interphase microtubule array in nondividing cells, cells with many small vacuoles, cells that fail to progress in the cell cycle or cells with abnormal mitotic arrays (*see* Fig. 5). Air bubbles will cause your sample to slip out of focus and may alter the rate of division. If the sample has an air bubble, carefully take the sample out, and set up a fresh cover slip, vacuum grease, and water to place the sample.

9. *Sample placement*

Placing the sample in the middle of the Rose chamber is very important because if it is not correctly centered, the objective may run into the side of the chamber. This has the potential to scratch the objective, disturb correct Z stack imaging, and make the sample slip.

10. *Objective selection*

For semi-high-throughput imaging, the lowest objective that still allows unambiguous identification of mitotic structures should be used. I used a 20 \times objective for this purpose. For producing a time-lapse figure, a higher objective (such as 40 or 60 \times) should be used to more clearly illustrate the mitotic figures. An alternative, should it be feasible with your microscope setup, is to use x,y motorization to move between multiple different samples during time-lapse.

11. *Immersion liquid*

The correct immersion liquid must be used on your objective, but what type of objective is best for time-lapse imaging? Some objectives do not require any immersion liquid but use air instead. Unfortunately, the refractive index of air is very different than plant cells. The benefit of using an oil objective is

419 that the immersion oil will not evaporate during imaging.
420 However, it reduces the quality of imaging because the refrac-
421 tive index of immersion oil is not very similar to plant cells.
422 Water has a closer refractive index to your sample, and will
423 produce a more beautiful micrograph. However, water objec-
424 tives are not ideal for long time lapse because the water can
425 evaporate within 1 h of time-lapse imaging. Glycerin or silicone
426 oil may provide the best compromise between a good refractive
427 index and slower evaporation but I do not have experience
428 with either of these materials. Perfluorocarbon immersion liq-
429 uids can also be used.

430 12. *Laser power*

431 Keep laser power low to minimize photo-damage to the
432 cells. Instead of increasing laser power, consider increasing
433 EM gain or exposure times. Empirical testing may be done to
434 confirm that the settings do not cause photo-damage before
435 long time lapses are performed. Use a sample to test for photo-
436 damage by collecting the total number of images the time
437 lapse will acquire during one session. This can be done in
438 micromanager using the “burst” function. After imaging,
439 measure fluorescence loss and observe whether damage occurs.

440 13. *Cells in lateprophase*

441 Since the amount of time a cell spends in preprophase can
442 be quite long (>3 h), it is best to focus the sample such that a
443 cell in late prophase is in the middle of the field of view. These
444 cells are characterized by an obvious accumulation of microtu-
445 bules at both poles of the imminent spindle, but still have a
446 preprophase band (Fig. 7).

447 14. *Selecting appropriate image collection intervals*

448 One goal of time-lapse imaging is good temporal resolution
449 but another more important goal is prevention of photo-
450 damage. Short time intervals (e.g., 2 min) can increase the risk
451 of photo-damage to the cells, which impedes division. Long
452 time intervals (e.g., 15 min) can result in data loss. For exam-
453 ple, anaphase, the shortest stage of the cell cycle (~10 min)
454 will not be observed within 15-min intervals. A decent time
455 compromise is 5-min intervals for dividing cells, but this will
456 need to be optimized depending on the type of sample and
457 temperature (*see Note 5*). An alternative way to minimize
458 photo-damage is to capture a single image or a short Z stack,
459 such as 4–1 μm intervals at each time point instead of a full Z
460 stack (covering the top through the bottom of the cells usually
461 at 1 or 1.5 μm stacks for 10–20 separate Z stacks).

462 15. *Automatic saves*

463 The “save as” function saves images as they are produced,
464 showing an accurate timestamp on each Z stack. The save as

- function also protects against loss of already acquired images during power failures or other malfunctions that can occur during time-lapse imaging.
16. *Set the Z stack so that the maximum projection will provide a clear image*
- Maximum projection, a post-acquisition method of visualizing the Z stacks compressed into one image, can be used to more clearly distinguish structure types and changes in mitotic structures. Sometimes the maximum projection to produce the clearest image will not be the entire Z stack, particularly if the sample is thick.
17. *Automatic corrections in ImageJ or FIJI*
- Two plug-in programs in ImageJ/FIJI can be used to adjust for sample movement during imaging: Stack Reg and Registration: Register Virtual Stack Slices. Both can automatically correct the slow drift caused by plant growth or movement in one direction. However, if large manual adjustments are performed during time-lapse imaging, these programs may not be able to correct the image. Manually correct large shifts in ImageJ, and then run StackReg or Register Virtual Stack Slices.

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