Observation of Chloroplast-actin Filaments in Leaves of *Arabidopsis*

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[Abstract] Chloroplast-actin (cp-actin) filaments play a pivotal role in chloroplast photorelocation movement. This protocol describes observation of cp-actin filaments in intact palisade cells of *Arabidopsis* leaves (Kong *et al*., 2013). The live cell imaging of cp-actin filaments is taken on moving chloroplasts, so that this protocol is useful for analysis of cp-actin dynamics that are induced by blue light.

Materials and Reagents

1. Plant materials
   a. *Arabidopsis thaliana* transgenic plants expressing GFP-TALIN in wild-type or mutant plants (Kong *et al*., 2013). Actin probe lines such as LIFEACT-YFP and GFP-fABD2, THRUMIN1-GFP transgenic lines are also useful.
   b. Young and fully expanded rosette leaves of 3-4-week-old plants grown under 100 µmol/m^2/s white light (16 h)/dark (8 h) cycles at 23 °C.
   Note: Plants grown on soil are better than those in the plate of MS medium. The reason is that the leaves of the plants grown in the plate are more susceptible to wither during sample handling than those grown on soil.
2. Red cellophane film (TokyoButaiShowmei, catalog number: No. 20)
3. (Optional) 2,3-butanedione monoxime (BDM) (Sigma-Aldrich, catalog number: B0753) as an inhibitor of myosin ATPase that inhibits actin dynamics
4. Evacuation solution containing Silwet L-77 (Bristol-Myers Squibb Company, catalog number: BMS-SL7755) (see Recipes)

Equipment

1. Syringe (10 ml)
2. Confocal microscope (Leica Microsystems, model: SP5 equipped with a 63x/1.20 W objective lens and multi-line 100 mW argon laser)
3. Plant growth room or chamber
4. Red safe-light (red LED or fluorescent lamp filtered with red films)

5. Forceps
6. Razor blade
7. Scissors
8. A custom-made cuvette system and ring holder: this system is composed of two steel rings mating each other with complementary threads, two round cover glasses (22 mm in diameter, No. 1 and No. 5), and a silicon ring (100 μm in thickness) (Wada and Kong, 2011; see Video 1)

Software
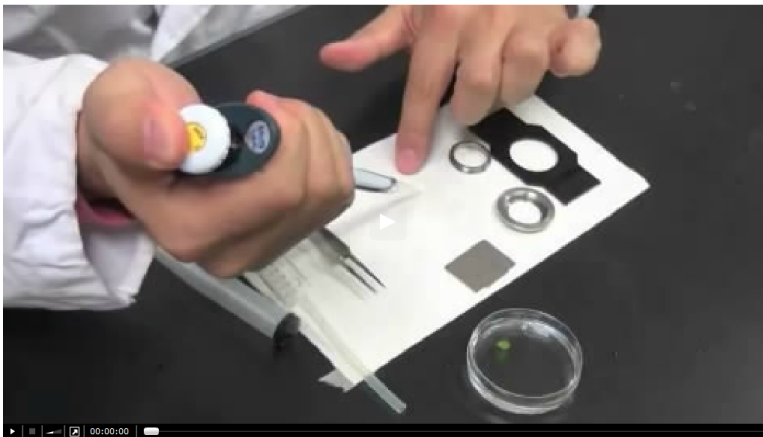
1. Open source software ImageJ (http://rsbweb.nih.gov/ij/)

Procedure

A. Preparation of leaf specimen (see Video 1)

Video 1. Preparation of leaf specimen in the cuvette system

1. Incubate plants under weak light conditions (30 μmol/m^2/s white light) for a couple of hours.
   Notes:
   a. *Under weak light conditions, chloroplasts accumulate along the cell walls that are perpendicular to the incident light (accumulation response). However, they move away from the area under strong light conditions and accumulate to the anticlinal walls that are parallel to the incident light (avoidance response).*
   b. *This step is useful to observe chloroplasts during avoidance response. In addition, this step can be skipped or modified depending on your experimental purposes.*

2. Detach a rosette leaf and eliminate airspaces in the leaf by evacuation in a syringe filled with an evacuation solution (0.01% Silwet L-77).

3. Cut the leaf into small pieces (ca. 4 mm x 4 mm) with a razor blade.

4. (Optional) Inhibitor treatment such as 50 mM 2,3-butanedione monoxime (BDM).

   a. Dilute interesting inhibitors to appropriate concentrations in the evacuation solution. As a mock treatment, carrier solution (DMSO) without the inhibitor is added to the evacuation solution at the same dilution rate.

   b. Eliminate air spaces in the leaf by gentle evacuation using a syringe that is filled with the solution.

   c. Incubate the leaf samples for the appropriate times (0.5 ~ 1 h) under dim red light.

5. Place the excised and cut leaf (treated with inhibitor solution, if necessary) adaxial side up on a round coverslip (No. 5)

6. Cover the leaf specimen carefully with a round coverslip (No. 1) and set it in a cuvette system.

   Note: Remove carefully any bubbles in the space between leaf sample and coverslips not only the air in the air space of a leaf.

B. Confocal microscopy

1. Find an appropriate chloroplast(s) by observing the specimen under dim red light and adjust focus at the vicinity between the plasma membrane and chloroplast(s) in palisade cells using rapid scans at weak laser power.

   Note: Eye observation under dim red light conditions is recommended to reduce any light effect on cp-actin filament dynamics. The dim red light condition is made using a piece of red cellophane film that is put in the light path of halogen lamp. If necessary to adjust focus, set output laser at the lowest power (1-5% of 488 nm) as much as chlorophyll fluorescence is detectable.

2. Incubate the specimen in darkness for several minutes on the microscope stage.

   Note: Disappearance (depolymerization) of cp-actin filaments is induced by several scans of optical blue laser. However, dark incubation induces polymerization of cp-actin filaments following the blue laser scanning.

3. Set optical parameters to optimize signal to noise ratio using SP5 software (live data mode).

   a. Excitation lasers: the multi-line argon laser (100 mW of laser power) is set at 20% output power, and further at 10% of 488 nm for GFP excitation and 5% of 458 nm for the induction of chloroplast avoidance response.

Note: Laser powers are empirically adjustable to obtain high signal to noise images depending on experimental conditions and individual systems' performances that may vary.

b. Emission bands for the specific fluorescent signals: 500 to 550 nm for GFP and 640 to 740 nm for chlorophyll.

c. Capture the images (three to four images in 0.5-μm steps, if necessary) with a lens (63x/1.20 W) at an appropriate resolution (for example, 256 x 256, or 512 x 256) with 4x digital zooming for 20 or 30 time-lapse cycles with at least 20-sec time-interval (see below). Scope is in XYZT mode.

Note: Scanning speed of confocal microscopy is limited and cp-actin filaments are dynamically regulated. Hence, temporal and spatial resolutions can be controlled at some ranges by changing scan speed and y-axes resolution. Bidirectional mode can also be used to shorten acquisition time.
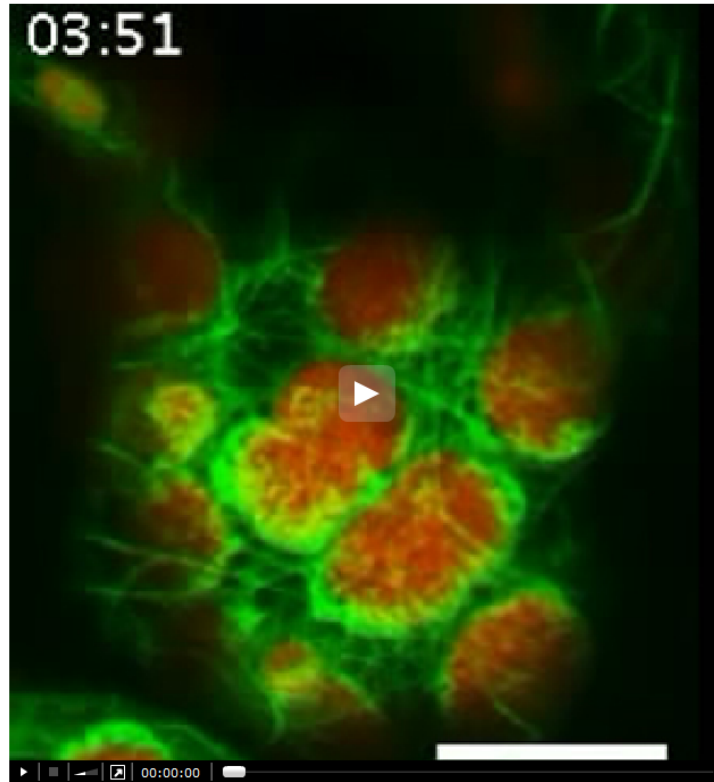
d. To induce chloroplast avoidance response, scan an appropriate rectangular or circular regions using region of interest (ROI) with the stimulating laser (458 nm, 2.8 μW) during intervals for the appropriate times.

Note: To induce avoidance response, blue light is given directly on a half side of chloroplast. The width of ROI is usually set around 10-20 μm to observe all processes (depolymerization, polymerization, asymmetric distribution) of cp-actin filament dynamics on moving chloroplasts (see Video 2).

e. (Optional) The sequential images of cp-actin filament disappearance could be captured at the higher optical and temporal resolutions (for example, a resolution of 128 x 68 with 6x digital zooming).

4. The other microscopic conditions are adjusted to optimize image quality.

Note: The optical set should be variable depending on experimental conditions. The imaging quality is mostly dependent on the fluorescent intensity that is variable on the expression level of actin probes and the property of fluorescent proteins. Hence, the best optical set should be empirically determined on a case-by-case basis.

Video 2. Cp-actin filament dynamics during the blue light-induced chloroplast avoidance response. Time-lapse images (maximized with four images in a 1.5-µm depth) were collected at approximately 33-sec intervals and played back at 5 frames per second (fps). The total elapsed time is 11:33 (mm:ss). The images are false-colored to indicate GFP (green) and chlorophyll (red) fluorescence. The region indicated with the blue circle (15 µm in diameter) was irradiated using 458-nm laser scans during the intervals between the image acquisitions to induce the avoidance response. Scale bar = 10 µm.

C. Quantitative Analysis
   1. Obtain enough time-lapse images from independent experiments for quantitative analysis.
   2. Analyze the time-lapse images using the ImageJ software program (http://rsbweb.nih.gov/ij/), which is populated with the appropriate plugins such as kymograph, intensity and length of actin filaments (Kong *et al*., 2013).

Recipes

   1. Evacuation solution
       0.01% (v/v) Silwet in deionized water

Acknowledgments

References

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2. Wada, M. and Kong, S. G. (2011). Analysis of chloroplast movement and relocation in *Arabidopsis*. *Methods Mol Biol* 774: 87-102.