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Tracking intercellular movement of fluorescent proteins in bryophytes

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Running title: Intercellular protein movement in bryophytes

Abstract

An important approach to investigate intercellular connectivity *via* plasmodesmata is to visualize and track the movement of fluorescent proteins between cells. The intercellular connectivity is largely controlled by the size exclusion limit of the pores. Over the past few decades, the technique to observe and analyze intercellular movement of a fluorescent protein has been developed mainly in angiosperms such as *Arabidopsis thaliana*. We recently applied the corresponding system to track the intercellular movement of the fluorescent protein Dendra2 in the moss *Physcomitrium (Physcomitrella) patens*. The protonemal tissues are

particularly suited for observation of the intercellular movement due to the simple organization. Here, we describe a protocol suitable for the analysis of Dendra2 movement between cells in *P. patens*.

Keywords

Confocal microscopy, Dendra2, ImageJ, photoconversion, *Physcomitrium patens*, plasmodesmata

1. Introduction

Fluorescent proteins (FPs) are widely used as reporters of intercellular movement through plasmodesmata (PD). Unless FPs are fused with endogenous proteins, FP movement between cells is non-selective and determined by the molecular size of the FP and the given PD aperture [1–5]. Some endogenous proteins fused with FPs can migrate to adjacent cells in a mode of selective transport, where an additional component likely alters PD aperture and/or the endogenous molecules to modulate PD permeability [6–10].

To determine intercellular transport, stably fluorescent FPs such as green fluorescent protein (GFP) must be expressed in single cells to observe their transport into adjacent cells. Thus, introduction by microinjection, microparticle bombardment, or expression under a tissue-specific promoter have been used for tracking intercellular movement [1–5, 7, 11–13]. Such analyses provided evidence that developmental and environmental cues alter intercellular connectivity through PD [2–5, 11–13]. Additional techniques, such as fluorescence recovery after photobleaching (FRAP) enabled to quantitatively describe the mode of GFP movement, for example, with a mathematical model [14]. In addition, photoactivatable FPs (PAFPs), photoconvertible FPs (PCFPs) or photoswitchable FPs (PSFPs) became available that allow to determine directional movement of a pool of the FPs by time-lapse-imaging without influence of *de novo* FP synthesis [15–18]. Moreover, the observation of intercellular movement of FPs was further advanced by new microscopic techniques. For example, while conventional confocal microscopy limits the observations of intercellular transport to surface cell layers such as the epidermis, the use of two-photon microscopy in combination with photoswitching of

PSFPs [17] or potentially with FRAP allows visualization of intercellular movement also in deeper tissue layers. Furthermore, photoconversion of PCFP through two stages with dual-wavelength (blue and red/near-infrared lasers) illumination, called primed conversion, may allow spatially restricted visualization and tracking of the intercellular movement of PCFP in deeper tissue layers of plants [19, 20].

We previously established an experimental system to track PD-mediated transport of the PCFP Dendra2 (\approx 26 kDa) in the moss *Physcomitrium* (*Physcomitrella*) *patens* [16, 21]. *P. patens* forms filamentous tissues (protonemata, consisting of two types of tissues, chloronemata and caulonemata) and a leaf-like structure (phyllid) composed of a single cell layer [22]. This simple structure makes *P. patens* highly suitable for observation of intercellular movement of FPs. Here, we describe a protocol to track intercellular movement of Dendra2 between protonemal cells, and give an account of how to measure photoconverted Dendra2 intensity using the open-source imaging analysis software Fiji (ImageJ) [16, 23, 24].

Analyses of intercellular movement through plasmodesmata in bryophytes have a relatively short history. We hope that this protocol applied to bryophytes will help researchers to investigate the regulatory mechanisms and function of intercellular movement of endogenous proteins and gain a better understanding of cell-cell communication *via* PD in plants.

2. Materials

1. Plant material: *P. patens* constitutively expressing *Dendra2* under the promoter of *ELONGATION FACTOR 1 α* (Pp3c2_6690V3) (*ProEF1 α :D2*) [16] (see **Notes 1-3**). Grow *P. patens* on solid BCDAT medium at 25°C under continuous light [25].
2. Glass bottom dish. We used four-well chamber slides (Iwaki).
3. Stock B: 100 mM MgSO₄·7H₂O in water (autoclaved).
4. Stock C: 184 mM K₂PO₄ (adjusted to pH6.5 with KOH) in water (autoclaved).
5. Stock D: 1 M KNO₃ and 4.5 mM FeSO₄·7H₂O in water (not autoclaved).
6. 500 mM ammonium tartrate (dibasic) in water (autoclaved).
7. Alternative TES: 0.22 mM CuSO₄·5H₂O, 10 mM H₃BO₃, 0.23 mM CoCl₂·6H₂O, 0.1 mM Na₂MoO₄·2H₂O, 0.19 mM ZnSO₄·7H₂O, 2 mM MnCl₂·4H₂O, and 0.17 mM KI.

8. 50 mM CaCl₂·2H₂O in water (autoclaved).
9. BCD medium: 10% (v/v) of stock B, 10% (v/v) of stock C, 10% (v/v) of stock D, 1% (v/v) of alternative TES, 20% (v/v) of 50 mM CaCl₂, and filled up with water to the specific total volume (autoclaved).
10. BCDAT medium: BCD medium also containing 10% (v/v) of 500 mM ammonium tartrate.
11. BCDG: BCD medium containing 0.5% (w/v) glucose solidified with 0.5% (w/v) gellan gum (autoclaved) [25] (*see Notes 4 and 5*).
12. BCTATG: BCDAT medium containing 0.5% (w/v) glucose solidified with 0.5% (w/v) gellan gum (autoclaved) [25] (*see Notes 4 and 5*).
13. Pipette.
14. Thin forceps.
15. Parafilm.
16. Black container with no lid.
17. Red acrylic filter (Mitsubishi Rayon). This filter is needed to culture of *P. patens* under red light. Use of a red light-emitting diode (LED) may be an alternative.
18. Confocal microscope. To observe Dendra2 movement between cells, use 405-nm and 561-nm lasers for the photoconversion and excitation of the photoconverted (red) Dendra2, respectively. A confocal microscope Nikon A1 (Nikon) equipped with a 405-nm diode laser (36 mW) and a 561-nm solid laser (10 mW, 2% output), and emission filter set to 570-620 nm for the detection of photoconverted Dendra2 was used for this protocol (*see Note 6*).
19. Image analysis software. We use the open-source software Fiji (ImageJ; <https://imagej.net/Fiji>).

3. Methods

3.1. Sample preparation

1. Melt the solidified BCDATG or BCDG medium in a microwave.

2. Allow the medium to cool so that the glass of a four-well chamber slide is not broken in the subsequent step.
3. Pour 500 μ l of the medium into each well of a four-well chamber slide and let it solidify.
4. Pick up and embed a small amount of 3 to 5 days old protonemal tissues into the medium of each well with thin forceps (*see Note 7*).
5. Overlay the embedded tissue with additional 250 μ l of the medium. The medium should be warm and liquid but not too hot to avoid any damage to protonemal tissues by heat.
6. Put on the lid and seal the chamber slide with parafilm (*see Note 8*).
7. Cultivate the protonemal tissues for 10 to 15 days under continuous red light with the glass surface of the chamber slide oriented upward (*see Note 9*). For red light conditions, place the four-well chamber slide into a black container with no lid, and place the lid of the red acrylic filter on the box for a red-light culture (Fig. 1). We use a fluorescent lamp as the light source (*see Notes 10 and 11*).

3.2. Photoconversion

Perform photoconversion of Dendra2 with the 405-nm diode laser (10.0% laser output, 36 mW) with 3 iterations (8 seconds/frame, zoom 4 \times) (*see Notes 12-16*).

3.3. Time-lapse imaging

Apply the time-lapse settings to image intercellular movement of Dendra2 (Fig. 2A) (*see Note 17-19*).

3.4. Analysis

Analyze the Dendra2 movement between protonemal cells. Here, we limit the protocol to the analysis to Dendra2 intensity measurement using Fiji (Image J) software.

1. Select ROIs for photoconverted cells (ROI_P) and for neighboring cells on both sides of the photoconverted cell, i.e., in apical (ROI_{Na}) and basal (ROI_{Nb}) direction, and for the medium (ROI_M) (Fig. 2B). ROI_M is used to determine the background, which may be different between samples and observation times.

2. Measure mean intensity of the ROIs (*Analyze > Tools > ROI Manager... > More >> Multi Measure*) to obtain it as “*Results*” (see **Note 20**).
3. Pick up the max intensity of the mean values among the 5 z-stack images at the observation time from the data of “*Results*” (see **Note 21**).
4. Make the graph from the extracted data (Fig. 2C).

4. Notes

1. The *Dendra2* sequence in *ProEF1 α :D2* is optimized to the human codon usage (Evrogen). We could not obtain transgenic lines possessing *Dendra2* fluorescence with the *Dendra2* sequence optimized to *A. thaliana* codon usage (Evrogen).
2. Check beforehand whether cells of your interest show fluorescence levels sufficient for imaging analysis. In this regard, you may need to consider to test a choice of promoters. We observed *Dendra2* fluorescence in different tissues such as protonemata and gametophores with the *EF1 α* promoter (Fig. 3).
3. Instead of *Dendra2* for visual tracking of the intercellular movement [16], other monomeric PCFPs (mClavGR2, mKikGR) may be used. The use of monomeric PAFPs (PA-GFP, PA-mCherry, etc.) may provide data equivalent to the data obtained by using *Dendra2*. However, unlike PCFPs such as *Dendra2*, PAFPs have the disadvantage that they require photoactivation for visualization. Thus, it is not possible to verify the PAFP expression level just before the mobility analysis experiment. For the same reason, the use of monomeric PSFPs that are initially non-fluorescent (“off”-state; e.g., Padron), can be also tricky. Furthermore, PSFPs which are initially in “on”-state such as *Dronpa*, may not be suitable to analyze the intercellular mobility. During observation of intercellular movement of *Dronpa*-type PSFPs, newly synthesized “on”-state *Dronpa* adds to the background. Moreover, in almost all of PSFPs, the excitation wavelength to image the “on”-state PSFP and the wavelength for “on” to “off” photoswitching are quite similar (e.g., there is only a 23-nm interval between the wavelength for excitation with the 488-nm laser and the wavelengths required for “on” to “off” photoswitching), resulting in decrease in fluorescence intensity of the tracked PSFPs. Such characteristics

of Dronpa could be problematic especially in protonemal tissues of *P. patens* because intercellular mobility is low and excitations are repeatedly carried out to obtain images during a long period of time-laps observation. Dronpa is, however, applicable for the analysis in, for example, root tissues of *A. thaliana* due to the relatively higher intercellular mobility compared to the mobility in protonemal tissues of *P. patens* and the absence of a requirement for many times of fluorescence excitation of Dronpa [17, 24].

4. Protonemal growth is reduced under red-light conditions. Thus, glucose is supplemented to improve the growth. Instead of agar, gellan gum is used for solidification of medium to increase the translucence of medium which facilitates observation and imaging.
5. This protocol is also applicable to gametophores [21]. In this case, the BCDG medium is more suitable than BCDATG medium because the BCDG medium allows for relatively less growth of protonemal tissues. Although *P. patens* produces gametophores also in BCDATG medium, the crowded protonemal tissues seen in this medium can disturb the observation of gametophores.
6. A 488-nm laser with emission filter 500-550 nm can be used for imaging of non-photoconverted (green) Dendra2. However, we usually do not observe green Dendra2 during time-lapse imaging in order to avoid inefficient but cumulative photoconversion of Dendra2 by repeated excitations with the 488-nm laser [26].
7. We embedded protonemal tissues at the bottom of the gellan gum medium, onto the glass surface of the chamber slides. This allows them to grow along the glass surface soon after the inoculation. When protonemal tissues are embedded at multiple places in the same well of a four-well chamber slide, leave a space between the places to avoid overlapping growth during the 10 days long culture (Fig. 3A, B).
8. We wrap parafilm around the chamber slide once and half. In this case, gas exchange is maintained for protonemal growth while the medium is kept from drying out.
9. Culture under red light suppresses Dendra2 photoconversion as well as formation of protonemal side branches.

10. Fluorescent lamp intensity and the distance between the fluorescent lamp and sample determine the intensity of light and heat, affecting the growth of protonemal tissues. Thus, some optimization of culture conditions is required.
11. The amount of medium in the wells is adjustable for any purpose. Conditions using a small amount of medium described here are optimized to facilitate treatment with hormones [24]. However, while a small amount of medium allows more efficient treatment with hormones, this may also cause desiccation stress to the plants during a longer culture period. Using a larger amount of medium enables longer culture without desiccation. Thus, without pharmacological experiments, the use of more medium is advised.
12. We cannot rule out the possibility that 405-nm laser irradiation for photoconversion confers cellular damage. We tested the application of 0 to 100% laser power with 3 times irradiation at 8 seconds/frame (24 seconds in total) and the laser power applied in the protocol (10% output) did not arrest cellular growth following irradiation under our conditions (Table 1). One should pay attention to the laser power in Watt (mW) in addition to the laser power in % as well as to the duration of laser irradiation when checking the cellular growth. Under our conditions, 50% laser power clearly induced cellular damage (Table 1).
13. We checked the efficiency of photoconversion upon varying the number of scan iterations applied within a constant period of total irradiation time and dependent on laser power (10, 25 and 50%) (Fig. 4). The number of scan iterations did not significantly change the efficiency of photoconversion (Fig. 4A). However, when photoconversion was performed at higher laser power, the effect of photobleaching of Dendra2 also increased, resulting in the lower efficiency of photoconversion (Fig. 4B).
14. Despite the absence of photobleaching, proteolytic degradation of Dendra2 can possibly take place particularly in Dendra2 fused with an endogenous protein like ANGUSTIFOLIA3 [27]. Since this leads to decrease in red Dendra2 intensity in photoconverted cells which can perturb the analysis of intercellular movement (a decrease in red Dendra2 intensity due to degradation can be falsely interpreted as

indicative of molecules that moved from the photoactivated cells to neighboring cells), the contribution in the loss of red Dendra2 intensity due to proteolytic degradation should be quantified in isolated protoplasts using a time-lapse experiment. We tested proteolytic degradation of Dendra2 in protoplasts with the settings also used for photoconversion during the imaging of Dendra2 in protonemata [24] and found no significant proteolysis under these conditions.

15. Fluorescence intensity of FPs including Dendra2 is influenced by pH [28]. Thus, when applying hormones or other compounds that may alter pH, the effect on Dendra2 fluorescence and photoconversion must be monitored.
16. We found that photoconverted Dendra2 intensity continues to increase until 5 to 10 minutes after applying photoconversion conditions (Fig. 5). Thus, the time-lapse imaging to score the protein mobility should be started after around 10 min after the photoconversion.
17. Note that light conditions affect protonemal growth. Thus, the absence or presence of light during the time-lapse imaging can influence Dendra2 mobility [16, 24].
18. We performed the time-lapse imaging with the objective lens PlanApo 10×/0.45 NA and 561-nm solid laser (2.0% laser output, 10 mW) at 20-min intervals for 720 min, and set the pinhole size to 16.60 μm in diameter (one airy unit), resulting in a 7.29- μm optical slice.
19. It is also advisable to acquire multiple z-stack images with optical sections of around 6 μm in each time interval for each ROI position to ensure the gaining of focused images of observed cells during the time-lapse experiment. In each time interval, we acquired 5 z-stack images (1,024 \times 1,024 pixels in size) using 6 μm steps between optical sections.
20. The chamber slide and/or protonemal tissues can sometimes move during time-lapse imaging. In such a case, ROIs can be manually adjusted in the images of each time point.

21. We use Excel (Microsoft) to paste the “*Result*” into a table to extract the intensity of the image in focus, which empirically represents the maximum value in this setting, among the 5 z-stack images.

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Table

	The power of 405-nm diode laser (%)				
	0	10	25	50	100
Growth arrest	1.4% (2/147)	0% (0/12)	0% (0/12)	41.7% (5/12)	91.7% (11/12)

Table 1. Cellular damage by 405-nm laser irradiation. The growth of apical cells without and after 405-nm laser irradiation at different laser powers for 24 seconds (with 3 iterations of 8 seconds/frame). The absence or presence of cell division and cell elongation was monitored throughout time-lapse imaging at 20-min intervals for 12 hours. Growth arrest was determined when the apical cells stopped growing during the time-lapse imaging.

Figure legends

Figure 1. A Black box with red filter used to culture the transgenic *P. patens* line *ProEF1 α :D2*. A four-well chamber slide is placed into a box below the red acrylic filter. The left and right panels show the box without and with the filter, respectively.

Figure 2. An example of measurement of red Dendra2 intensity. (A) Red Dendra2 fluorescence (magenta) images of protonemal cells of the *ProEF1 α :D2* line are shown. Times after the onset of the time-lapse imaging are indicated. The images on the right are enhancements of the images on the left. Scale bars = 100 μ m. Bars shown below the images indicate ranges of displayed pixel value. (B) A differential interference contrast (DIC) image of *ProEF1 α :D2* protonemal cells. The upper image shows the cells analyzed by fluorescence in A and this image has been cropped from the bottom image. The ROI_P, ROI_{Na}, ROI_{Nb} and ROI_M are indicated by green, yellow, orange and blue lines, respectively. Scale bars = 100 μ m (upper) and 200 μ m (bottom), respectively. (C) Normalized red Dendra2 intensity of the protonemal cells in A and B. The exponential decrease in the intensity of red Dendra2 at the

photoconverted cell indicates a simple diffusion phenomenon [24]. Thus, the intensity change is seemingly obvious until around 240 min, while less-visible after 240 min from the pictures (A). In contrast, the slight intensity change is graphically illustrated even after 240 min of the onset of the time-lapse imaging (C).

Figure 3. The growth conditions of *ProEF1α:D2*. (A and B) Representative differential interference contrast (DIC) and green Dendra2 fluorescence images of *ProEF1α:D2* protonemal tissues after 10 days of culture in BCDATG (A) and after 15-day culture in BCDG (B). (C-E) Representative DIC and green Dendra2 fluorescence images in a chloronema in BCDATG (C), and in a caulonema with a bud (D) and a young gametophore (E) cultured in BCDG. Scale bars = 1 mm (A and B), 200 μm (C and D), and 100 μm (E). An arrow head indicates a bud.

Figure 4. Efficiency of Dendra2 photoconversion. (A and B) Red Dendra2 intensity measured (A) after applying different numbers of scan iterations during the 24 seconds long total laser irradiation time and (B) after applying different laser powers with 8 seconds long iterations during the 24 seconds long total irradiation time. Each value of red Dendra2 intensity was normalized in (A) to the average intensity obtained upon applying 3 iterations of irradiation for 8 seconds (normalized intensity set to 1.0) and in (B) to the value obtained with 10% laser power (normalized intensity set to 1.0). $N = 10$ (A) and $N = 6$ (B). The P -values were determined by the Welch's t -test and give the statistical significance between the values obtained for the different conditions and the value for the condition to which the data was normalized and set to 1.0. n.s., non-significant ($P \geq 0.05$); *, $P < 0.05$

Figure 5. Time to completion of Dendra2 photoconversion. Mean fluorescence intensity of red Dendra2 at 30-second intervals for 15 minutes of irradiation with the 561-nm laser in 18 protonemal cells. The standard deviation is indicated by the shaded area.