# Chapter 10

# Quantitative Analysis of Photobodies

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## Abstract

Photobodies are membraneless subnuclear organelles that contain the red and far-red photoreceptors, phytochromes. Photobody biogenesis has been postulated to play important roles in early light signaling events. The size and number of photobodies are highly dynamic in response to the quality and quantity of light and correlated tightly with phytochrome-mediated seedling morphogenesis. Here, we provide a detailed protocol for characterization of the three-dimensional morphology of photobodies, including sample preparation, fluorescence microscopy, and image analysis. Although this method was developed initially for characterizing photobodies, it can be adopted to analyze other membraneless or membrane-bound subcellular organelles.

**Key words** Photobody, Phytochrome, Nuclear body, Photomorphogenesis, Subnuclear organization

## 1 Introduction

Photobodies are membraneless photoreceptor-containing, light-sensory subnuclear domains in plants [1]. In Arabidopsis, photobodies contain the red and far-red light photoreceptors phytochrome A to E (phyA-E) [2, 3], the blue light photoreceptor CRY2 (cryptochrome 2) and possibly CRY1 [4–9], the UV-B receptor UVR8 (UV RESISTANCE LOCUS 8) [10], and a battery of light signaling components [11]. Although the interactions among the photoreceptors on photobodies have not been systematically investigated, the dynamic changes in photobody morphology have been well studied using Arabidopsis transgenic lines expressing fluorescent protein-tagged phytochrome B (phyB)—a photostable and the most prominent phytochrome in the light [2, 12, 13]. Phytochromes are bilin-containing proteins that exist in two relatively stable and photoconvertible forms, a red light-absorbing biologically inactive Pr form and a far-red light-absorbing biologically active Pfr form [14, 15]. Besides photoconversion, Pfr can also thermodynamically convert back to Pr in a process called dark reversion or thermoreversion; because the rate of

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thermoreversion of phyB is accelerated with an increase in temperature, phyB is also considered a thermosensor [16, 17]. One of the earliest light responses at the cellular level is the translocation of photoactivated phytochromes from the cytoplasm to the nucleus and their subsequent localization to photobodies [1, 2, 12]. The biogenesis of photobodies is promoted by the Pfr form of phyB and therefore is highly dynamic in response to changes in light quality and quantity as well as temperature [13, 17, 18].

The steady-state patterns of phyB-FP photobodies correlate with early light signaling events as well as downstream phytochrome-mediated morphological responses. Formation of a few large phyB-FP photobodies with diameters between 1 and 2 μm under high intensity of red light correlates with degradation of the hypocotyl growth-promoting transcription factor PIF3 (PHYTOCHROME-INTERACTING FACTOR 3) and phyB-mediated inhibition of hypocotyl growth [13, 19–21]. By contrast, disassembly of large phyB-FP photobodies into tens to hundreds of small photobodies by inactivation of phyB or in mutants of phyB signaling correlates with the accumulation of PIF3 and promotion of hypocotyl growth [18, 19, 22–24]. These results, combined with the data that PIF3 is localized to photobodies before its degradation during the dark-to-light transition [25, 26], support the model that photobodies play important roles in PIF3 degradation and PIF3-dependent regulation of light-responsive genes [23]. Similarly, the dynamics of phyB photobodies is also associated with the hypocotyl response to changes in temperature [17]. Taken together, although the precise function of photobodies is still poorly understood, the steady-state pattern of photobodies can be used as a readout for early light signaling events and even genetic screens to identify components involved in early steps of phytochrome signaling [13, 19, 21].

The dynamic nature of photobodies presents two major challenges in characterization of photobody morphology. The first challenge is that exposure of living plant cells to the excitation light for FPs in fluorescence or confocal microscopy will alter the steady-state pattern of photobodies, and therefore, introduce experimental errors into photobody characterization. Based on our experience, this is particularly true for plant materials grown in the dark or dim light, as the strong excitation light (e.g., 488 nm excitation light for GFP) will promote rapid formation of small photobodies. The second challenge lies in the difficulty to quantitatively measure the number and volume of various sized photobodies. Here we describe an improved photobody characterization protocol that circumvents these obstacles by fixing plant materials without denaturing the fluorescent protein and analyzing the three-dimensional morphology of photobodies using Huygens Essential software. This method has been applied to characterize photobodies under a variety of light conditions in both wild-type and mutant backgrounds [21, 22, 24].

## 2 Materials

### 2.1 Plant Materials, Plant Growth Supplies, and Equipment

1. *PBG* (*phyB-GFP*), Arabidopsis transgenic line expressing phyB-GFP in *phyB-5* background [2].
2. ½ Murashige and Skoog (½ MS) with Gamborg's vitamins (Caisson Labs, MSP06) and 0.8% phyto agar.
3. LED chamber (Percival Scientific, Perry, IA).
4. Spectroradiometer (model PS-200, Apogee Instruments Inc., Logan, UT).

### 2.2 Reagents

1. 1% (v/v) paraformaldehyde (Electron Microscopy Sciences, 15710) (*see* **Note 1**).
2. 50 mM $NH_4Cl$ in PBS.
3. 0.2% (v/v) Triton X-100 in PBS.
4. 300 nM DAPI in PBS.
5. ProLong Diamond Antifade Mountant (Thermo Fisher Scientific, P36965).
6. Superfrost Plus Micro Slide.
7. Micro cover glasses, rectangular, 22 × 40 mm, No. 1.5.

### 2.3 Imaging and Image Analysis

1. Confocal microscope or fluorescence microscope with deconvolution software (*see* **Note 2**).
2. Huygens Essential software (Scientific Volume Imaging, The Netherlands).

## 3 Methods

### 3.1 Preparation of Plant Materials

1. Sterilize *PBG* seeds and sow on ½ MS plate.
2. Stratify the seeds in the dark at 4 °C for 5 days.
3. Grow *PBG* seedlings under monochromatic 10 μmol $m^{-2}$ $s^{-1}$ red light at 21 °C for 4 days (*see* **Note 3**) or adjust the light/temperature conditions according to your specific experiments.

### 3.2 Fixation and Mounting

1. Fix seedlings with 1% paraformaldehyde in PBS under vacuum for 10 min under the respective growth conditions (*see* **Note 4**).
2. Wash seedlings 3× with 50 mM $NH_4Cl$ in PBS for 5 min each to quench the residual amount of fixative.
3. Permeabilize seedlings with 0.2% Triton X-100 in PBS for 5 min.
4. Stain nuclei with 300 nM DAPI in PBS for 10 min.
5. Wash seedlings 3× with PBS for 5 min each (*see* **Note 5**).

6. Mount a few seedlings in ProLong Diamond Antifade on a slide with a coverslip and incubate overnight in the dark to cure the sample (*see* **Note 6**).
7. Seal the edges of the coverslip with nail polish. The slide can be imaged immediately or stored at 4 °C in the dark (*see* **Note 7**).

### *3.3 Fluorescence Imaging and Photobody Quantification*

1. Select a specific cell type to characterize photobody morphology. We found that the morphology of photobodies varies dramatically in different cell types and even in the same cell type under different modes of growth. To demonstrate a correlation between photobody biogenesis and the regulation of hypocotyl growth by light, In our previous studies we have focused on the epidermal cells in the upper one third of the hypocotyl [13, 19, 21, 22], because these cells are in the growing zone along the hypocotyl on the fourth day after germination when the seedlings are imaged [27, 28].
2. PhyB-GFP photobodies can be imaged with either a confocal microscope or a fluorescence microscope with a 63× or 100× oil immersion objective (*see* **Note 8**). Three-dimensional image stacks of individual nuclei containing photobodies are acquired. For confocal microscopy, we take optical sections with a Z step of 0.70 μm. For fluorescence microscopy, we take optical sections with a Z step of 0.24 μm. For statistical analysis, we collect photobody images from at least 50 nuclei from three biological replicates.
3. Load image stacks into Huygens Essential. Three-dimensional stacks taken by a fluorescence microscope can be deconvolved by Huygens Essential software or other deconvolution software before imported into Huygens Essential.
4. Analyze the number and volume of photobodies using Object Analyzer (Fig. 1). The threshold and seeds are set as default (*see* **Note 9**).
5. Export the data and calculate the number of large or small photobodies for each nuclei using Excel (*see* **Note 10**).

## 4 Notes

1. This paraformaldehyde solution is methanol-free, which is important to prevent the fluorescent protein from denaturation during fixation [29].
2. We used a Zeiss LSM510 inverted confocal microscope equipped with a Plan-Apochromat 100×/1.4 Oil DIC objective and a Zeiss Axio Observer Z1 inverted fluorescence microscope equipped with a Plan-Apochromat 100×/1.4 Oil

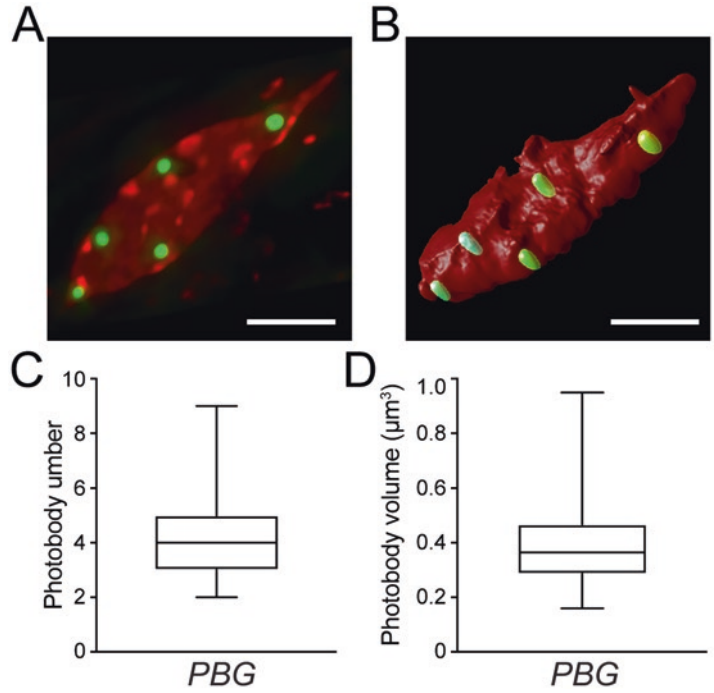


**Fig. 1** Quantitative analysis of photobody morphology. Photobodies were analyzed in nuclei of epidermal cells in the upper 1/3 region of hypocotyls of 4-day-old ***PBG*** seedlings grown under 10 μmol $m^{-2}$ $s^{-1}$ red light. Three-dimensional image stacks of individual nuclei were acquired at room temperature with a Zeiss Axio Observer Z1 fluorescence microscope equipped with a Plan-Apochromat 100×/1.4 Oil DIC objective and an Axiocam 506 mono CCD camera (Carl Zeiss, Thornwood, NY). Filters used for GFP were exciter, 470/40 nm/nm; emitter, 525/50 nm/nm; and FT 495 nm beamsplitter (Zeiss Filter Set 38 HE). Filters used for DAPI were exciter, G 365 nm; emitter, 445/50 nm/nm; FT 395 beamsplitter (Zeiss Filter Set 49). The image stacks of nuclei with a ***Z*** step size of 0.24 μm were subjected to Quick Maximum Likelihood Estimation deconvolution by using Huygens Essential software (Scientific Volume Imaging) on a HP Z840 Workstation (HP, Palo Alto, CA) (**a**) Representative maximum projection image of a DAPI-stained (red) nucleus with photobodies (green). Scale bar equals to 5 μm (**b**) Three-dimensional image of the nucleus shown in (**a**) reconstructed by using Huygens Essential Object Analyzer (**c**, **d**) Box and whisker plots showing the number (**c**) and volume (**d**) of the photobodies described above. The boxes represent from 25th to 75th percentile; the bars equal to the median values. The data were from total 66 nuclei

DIC objective and an Axiocam 506 mono CCD camera (Carl Zeiss, Thornwood, NY).

3. This is the standard condition where phyB-GFP is localized to only large photobodies [13]. When characterizing a new light signaling mutant in the *PBG* background, we look at its steady-state photobody morphology under this condition first, and then alter either the light intensity or the temperature.

4. We fix seedlings in their growth condition to minimize potential changes in photobody morphology during fixation. Do not fix samples on ice because temperature may affect the morphology of photobodies.
5. Seedlings can be stored in PBS at 4 °C. We normally mount and image the seedlings within a week.
6. Seedlings can also be mounted with PBS for imaging. However, ProLong Diamond Antifade is recommended to reduce photobleaching.
7. The fluorescent signal may decrease slowly at 4 °C over time. Therefore, we usually image photobodies within a week after fixation.
8. Either a confocal microscope or a fluorescence microscope can be used for imaging nuclei of epidermal cells. However, a confocal microscope is recommended for imaging nuclei of inner cells.
9. Threshold and seeds are automatically calculated from the image. Do not change threshold and seeds, which will affect the size of photobodies.
10. PhyB-GFP is localized only to large photobodies under 10 μmol $m^{-2}$ $s^{-1}$ red light. We therefore define large photobodies based on the volumes of photobodies under 10 μmol $m^{-2}$ $s^{-1}$ red light.

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