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Technical Note

A ClearSee-Based Clearing Protocol for 3D Visualization of *Arabidopsis thaliana* Embryos

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Abstract: Tissue clearing methods combined with confocal microscopy have been widely used for studying developmental biology. In plants, ClearSee is a reliable clearing method that is applicable to a wide range of tissues and is suitable for gene expression analysis using fluorescent reporters, but its application to the *Arabidopsis thaliana* embryo, a model system to study morphogenesis and pattern formation, has not been described in the original literature. Here we describe a ClearSee-based clearing protocol, which is suitable for obtaining 3D images of *Arabidopsis thaliana* embryos. The method consists of embryo dissection, fixation, washing, clearing, and cell wall staining, and enables high quality 3D imaging of embryo morphology and expression of a fluorescent reporter with the cellular resolution.

Keywords: clearing; 3D imaging; *Arabidopsis thaliana*, embryo, confocal microscopy, cell wall staining, fluorescent reporter, GFP

1. Introduction

In plant development, oriented cell division and expansion play essential roles in morphogenesis and pattern formation[1]. Embryogenesis of *Arabidopsis thaliana*, in which relatively a small number of tissues and organs are arranged in a simple pattern, provides an excellent system to study morphogenesis and pattern formation, and many regulatory factors that affect these processes have been identified and studied extensively[2,3]. Moreover, because patterns of cell division and elongation are significantly regular during *Arabidopsis* embryogenesis[4-6], their possible roles in development and the underlying mechanisms for oriented cell division and elongation have been an important subject[7-10].

Because morphogenesis and pattern formation occur not only in the surfaces of the embryo but also in its internal structures (e.g vascular and ground tissues), a reliable method for visualizing morphological and patterning events that occurs deep inside the embryo is necessary. Tissue clearing is a powerful technique to meet such requirements, and several protocols for clearing plant structures has been reported[11-14]. Among them, TOMEI-II[13] and ClearSee[11] have an advantage for visualizing gene expression patterns, as these methods well preserve fluorescence of various fluorescent proteins. Although both methods can be applicable to a wide range of tissue types and to various plant species, whether they can also work with embryos has not been reported. Here we established a protocol to apply the ClearSee method to the embryo of *Arabidopsis thaliana* and demonstrate that the protocol can visualize cellular arrangement and the signal of GREEN FLUORESCENT PROTEIN (GFP) signals in 3D.

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2. Results and Discussion

2.1. Dissection of Embryos

We first applied the original ClearSee protocol[11] to the whole ovules with expectation of visualizing embryos without dissection. However, ovules processed with this protocol exhibited brown color in the endothelium (Figure 1A), preventing us from imaging internal embryos. We therefore decided to manually dissect embryos before applying the protocol.

For dissecting embryos, ovules were first removed from the fruit under a stereo microscope according to the method described previously[15] except that 7% glucose solution instead of N5T medium was used. Briefly, each of the valves was slit open using a needle and was partly removed from the fruit by using forceps to expose ovules. The half-opened fruit was completely immersed in 7% glucose solution in a 35 mmi dish, and ovules were excised by using forceps.

The excised ovules were then subjected to manual dissection of embryos. Within an ovule, the embryo is located on the micropyle/chalaza side (Figure 1B). To isolate embryos, the other side of the ovule was excised by using forceps and the ovule surface around the micropyle was gently pushed several times with the tips of the forceps until the embryo popped out from the open end (Figure 1C). Each time isolating 5-10 embryos, they were collected using a P20 micropipette, which was adjusted to 1-2 µl, and were assembled within a small area in the same 35 mm dish, where debris produced by dissection were not present. This process was important to avoid losing isolated embryos by mixing them with the debris. Occasionally, small debris may adhere to an embryo, reducing the quality of imaging. Such debris can often be removed by gently scratching it with an eyelash attached to a toothpick.

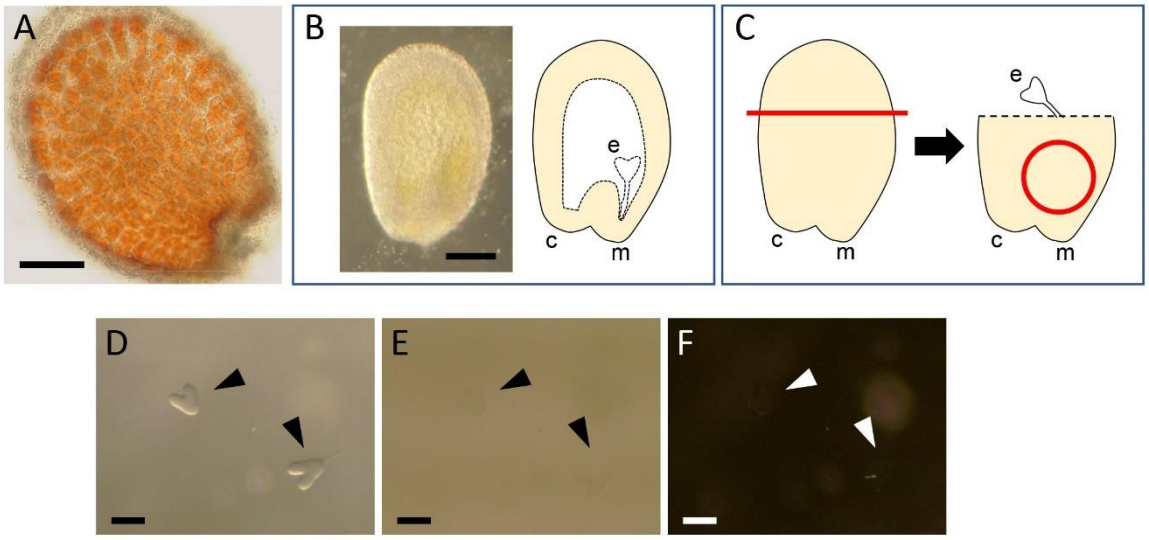

Figure 1. Dissection of *Arabidopsis thaliana* embryos. (**A**) Ovule processed with the original ClearSee protocol[11] is not transparent and exhibits brown color. (**B**) Ovule excised from a fruit in 7% glucose solution (**left**) and schematic diagram of its internal structure (**right**). (**C**) Procedure of embryo isolation. Half of the ovule is excised along the red line (**left**) and the region around the micropyle marked with red circle (**right**) is pushed several times until the embryo pops out. (**D–F**) Effects of illumination of stereo microscope. In ClearSee solution, embryos are clearly visible with oblique transmitted illumination (**D**) whereas they are almost invisible with bright-field (**E**) or dark-field (**F**) illumination. Arrowheads indicate the positions of embryos. c, chalaza; e, embryo; and m, micropyle. Bars = 100 µm. Diagram in **C** is modified from Hughes, 2009[16].

2.2. Fixation and Washing

After tens of embryos were isolated and assembled, they were subjected to fixation and washing by transferring embryos from one solution to another by a P20 micropipette. To minimize the risk of

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losing embryos during this process, we put a drop of solution (100 to 200 µl) at the center of a dish instead of filling up the dish with a large amount of solution. Moreover, embryos often adhered to the bottom of the dish or the inner surface of the pipette tips when they were in the fixative or the washing buffer, further increasing the risk of losing them. To avoid this, we added a very small amount of ClearSee to each solution (e.g. 0.5 µl per 1 ml) prior to use, because ClearSee contained the detergent sodium deoxycholate, which prevented embryos from adhering. When transferring embryos, the volume of the micropipette should be adjusted to 1-2 µl to minimize the carry over of solution.

The isolated embryos were collected from 7% glucose by using P20 micropipette under stereomicroscope and were transferred to a drop of the fixative. The embryos were incubated in the fixative for 10 min at room temperature. The fixed embryos were then washed twice by sequentially transferring them to the first and second drops of the washing buffer that were placed in separate 35 mm dishes and incubating them for 1 min each. Vacuum infiltration, which was described in the original ClearSee protocol [11] was not necessary.

2.3. Clearing and Staining

Clearing was carried out by transferring the fixed and washed embryos to ClearSee solution in a 35 mm dish. When the embryos were released from the P20 micropipette, they initially floated at the surface of the solution and then gradually sank until they reached to the bottom as infiltration proceeded. The dish was then sealed with parafilm and was kept dark for 1-7 days at room temperature. Embryos at late stages required longer incubation time compared to those at early stages.

From this step on, the embryos became difficult to see as the progression of clearing. To visualize the embryos for subsequent processing, illumination settings of the stereo microscope was critical. Off-axis (oblique) illumination[17] gave significantly higher contrast than bright- or dark-field illumination, facilitating monitoring and collection of the embryo samples (Figure 1D–F).

The cleared embryos were then transferred to the staining solution containing Calcofluor White and were kept for 1 hr at room temperature. Again, a 100-200 µl drop of the staining solution was used to avoid loss of embryo samples. After staining, the embryos were transferred to ClearSee and kept for 1 hr to remove excess of the dye.

2.4. Confocal Microscopy

For mounting embryo samples, two pieces of double-sided tape were pasted with an appropriate interval onto a glass slide as spacers. The cleared embryos were mounted in an area between the spacers and covered with a coverslip. Marking the positions of the mounted embryos with a felt-tip pen on the coverslip helps to locate embryos under a confocal microscope.

Figure 2 shows a set of images obtained from an embryo carrying the *DR5rev::GFP* reporter[18]. Z-stack images of 157 serial optical sections with the 0.3 µm interval was acquired (Supplementary Movie 1) and was used for observing a single focal plane (Figure 2A) or for reconstructing 3D image (Figures 2B,C). Both cell walls labelled by Calcofluor White and auxin response marked by accumulation of endoplasmic reticulum-localized GFP are clearly visible, and both patterns of cellular configuration and distribution of the *DR5rev* activity are confirmatory with previous observations[8,19], showing that the cell wall structure and GFP fluorescence are well preserved after the processing with our protocol. Moreover, 3D reconstruction allows for identifying geometrical features of cell morphology and gene expression patterns. The image would also be suitable for quantitative analyses using imaging software such as ImageJ[20] or MorphographX[21].

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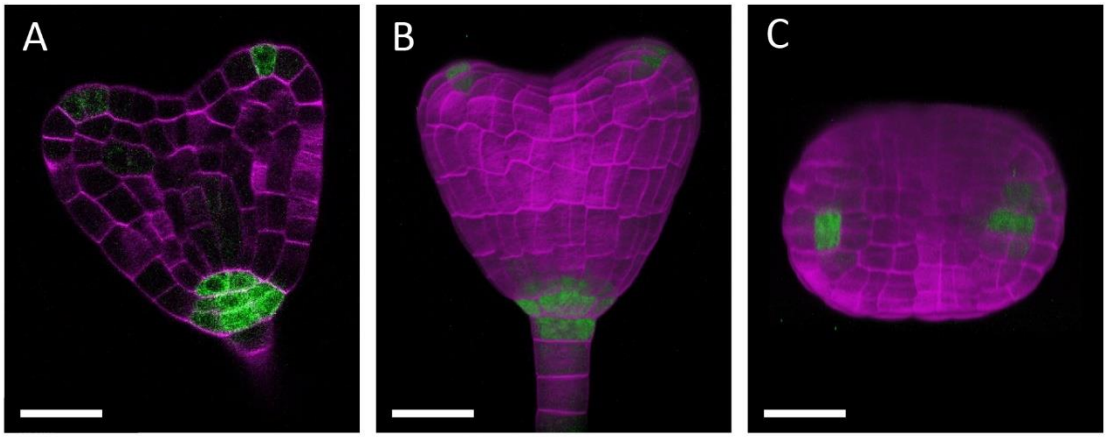

Figure 2. Confocal microscopic images of a ClearSee-processed embryo. (**A**) Frontal optical section of heart stage embryo carrying *DR5rev::GFP*[18]. (**B,C**) 3D reconstruction of 157 serial optical sections obtained from the same embryo as in **A** in frontal (**B**) and top (**C**) views. Signals of Calcofluor White and GFP are represented with magenta and green, respectively. Bars = 20 μm.

3. Materials and Equipment

3.1 Plant materials

The *Arabidopsis thaliana DRrev::GFP* reporter line was used as plant material and was obtained from the Arabidopsis Biological Resource Center (ABRC stock number CS9361). Plants were grown as described previously[22].

3.2 Solutions

7% glucose in water (w/v), fixative (4% paraformaldehyde in phosphate buffered saline (PBS, w/v)), washing buffer (PBS; 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH7.0). ClearSee and staining solution was prepared as described previously[11].

3.3 Equipment

Stereo microscopes equipped with a transmitted light unit capable of oblique illumination (Stemi 2000-C, Carl Zeiss, Oberkochen, Germany), Confocal microscope (Leica TCS-SPE, Leica microsystems GmbH, Wetzlar, Germany), forceps (Dumont #5, Manufactures D'Outils Dumont SA, Montignez, Switzerland), dishes (IWAKI non-treated 35mm culture dishes 1000-035, IWAKI, Shizuoka, Japan). For Z-stack image acquisition of confocal microscopy, a 63x oil-immersion objective lens was used. Calcofluor White and GFP were excited by the 405 nm and 488 nm laser lines, respectively, and were detected using 410-480 nm and 490-540 nm filter settings, respectively, with the sequential line scan mode. Images were processed using Leica LAS-X software (Leica microsystems GmbH, Wetzlar, Germany) and ImageJ[20].

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Movie S1: Z-stack images of 157 serial optical sections used for Figure 2.

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Conflicts of Interest: The authors declare no conflict of interest.

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